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Upregulation of $CD4^+CD8^+$ memory cells in the piglet intestine following oral administration of *Bacillus subtilis* spores combined with PEDV whole inactivated virus

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ABSTRACT

Oral immunization is a commonly employed route for inducing local immunity. However, the application of oral immunization is limited by the short-term persistence of immunity, particularly for inactivated viruses. The ultimate goal for mucosal vaccination is to stimulate protective immunological memory. In the intestine, long-term persistence of immunity is related to $CD4^+CD8^+$ memory T-cells. In this study, piglets were orally immunized with *Bacillus subtilis* spores (B.s) plus whole inactivated porcine epidemic diarrhea virus (PEDV WIV), followed by booster oral immunization. Initially, the results showed that B.s plus PEDV WIV enhanced the anti-PEDV capability on mucosal surfaces, as evidenced by plaque reduction neutralization tests in serum and intestinal fluid. Elevated antigen-specific IgG titers in the serum and IgA titers in saliva, feces and nasal washing liquid were also observed. Meanwhile, B.s plus PEDV WIV increased the area of Peyer's patches and the number of intraepithelial lymphocytes in the ileum of piglets. Similarly, the percentage of $CD4^+CD8^+$ memory T-cells were upregulated and proliferation ability of antigen-specific memory T-cell was strengthened in intestinal mucosal-associated lymphocytes, which was accompanied with increased expression of CCR9 after oral immunization with B.s plus PEDV WIV. In addition, the activation of memory T-cells is correlated with the increased mRNA expression of Toll-like receptor 2 and 4, as well as interleukin-6 and induced by B.s. Collectively, the study provided further insight into the potential immunopotentiator ability of B.s to assist PEDV WIV in the potentiation of immunity by upregulating memory $CD4^+CD8^+$ T cells *via* oral immunization.

1. Introduction

It is known that most pathogens initially infect mucosal surfaces including the oropharyngeal, respiratory, gastrointestinal and vaginal tracts (Nikou et al., 2019). The development of induced protective immune responses at mucosal sites is a prime objective of vaccine strategy for the prevention of infection (Rynda-Apple et al., 2014). However, mucosal immunization by oral delivery with inactivated viruses alone is often poorly effective (Kim and Jang, 2017). In addition, these antigens posse poor immunogenicity and must be administered with supplemental materials to enhance their vaccine potency (Gutjahr et al., 2016). Thus, an urgent goal in mucosal vaccine design is to assure the induction of protective and lasting immune responses against potential pathogens on mucosal surfaces.

Numerous researchers have used mucoadhesive agents, such as thermally sensitive hydrogel (Bachmann and Jennings, 2010) and polyethyleneimine (Wegmann et al., 2012), to form complexes with antigens; or employed various immunopotentiators such as CpG DNA (Geeraedts et al., 2008) and cholera toxin (Vajdy and Lycke, 1992), to target the immune system. However, information on the effects of mucosal immunopotentiators on the development of memory responses remains scarce (Harandi and Medaglini, 2010). Previous studies have shown that probiotics can effectively elicit the production of secretory immunoglobulin A (SIgA), activate innate cells and regulate the balance between T cell subset responses, as well as improve the immunogenicity of some vaccines for the mucosal delivery of antigens (Huang et al., 2010; Macpherson et al., 2015; Yu et al., 2017). Administration of probiotics including *Bacillus subtilis* (B.s) spores can be probiotic by virtue of their health benefits. B.s is also non-invasive, highly thermostable, safe, and low-cost (Duc le et al., 2004; Neutra and Kozlowski, 2006; Song et al., 2012). Moreover, studies have shown that the immunization of mice with B.s spores coated with the influenza antigen

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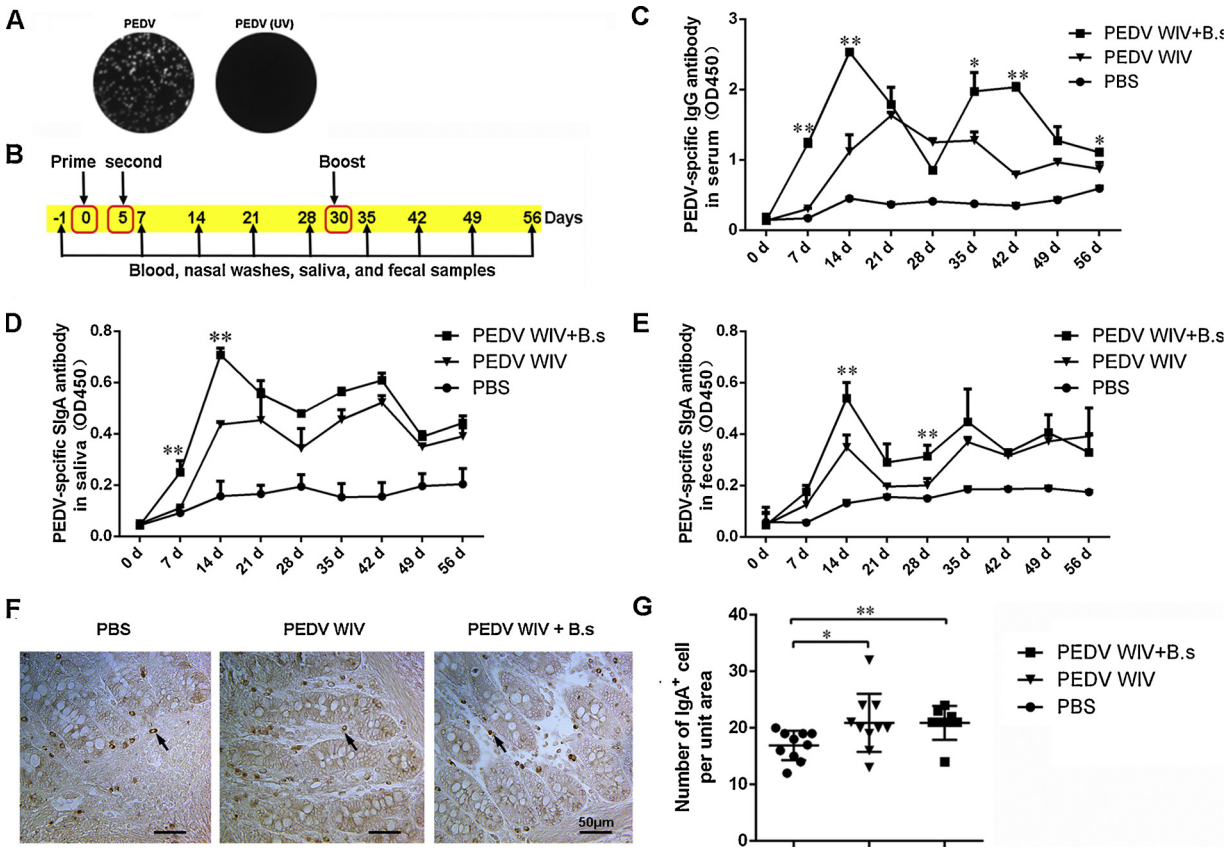

Fig. 1. B.s assists PEDV in enhancing both local and systemic immune responses.
(A) Viruses were inactivated by ultraviolet radiation (UV) for overnight to achieve a complete loss of infectivity. The PEDV or UV inactivated PEDV was cultured in Vero cells for 72 h. Purified PEDV (200 μg/test) was mixed with B.s (10^9 CFU/test). **(B)** Details of the immunization schedule for B.s and sampling design post-immunization. The yellow timeline indicates the immunologic process. Black arrows above the yellow timeline indicate the time points of primary, secondary and booster immunization. Besides, black arrows below the yellow timeline indicate the time points for the collection of secretions from the respiratory tract, the digestive tract and serum. **(C)** Antigen-specific IgG in the serum was detected by ELISA. **(D, E)** Antigen-specific IgA in saliva and feces washing fluid was detected by ELISA. **(F)** Ileal sections were stained for IgA^+ cells by IHC, which were mainly present in the lamina propria of the ileum. **(G)** Quantification of IgA^+ cell number at ten different fields in the ileum. Arrowheads represent positive cells. Data represent the mean ± S.E. of four samples. $^*P < 0.05$; $^{**}P < 0.01$. Scale bars: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

can induce strong humoral immune responses and protect against lethal challenge (Zhao et al., 2014).

Administering vaccines through traditional injectable methods often leads to poor protection against mucosal pathogens, presumably because the vaccines fail to generate memory lymphocytes (Stary et al., 2015). In addition, considerably fewer porcine than human or mouse differentiation markers of immune cells are available (Saalmuller, 1996); and the immune system of swine differs considerably from that of humans and mice. The pigs are also known to contain a substantial number of resting extrathymic $CD4^+CD8^+$ T lymphocytes (Saalmuller et al., 1987). Furthermore, it has been demonstrated that $CD4^+CD8^+$ T lymphocytes comprise memory T cells that proliferate upon stimulation with recall antigens (Zuckermann and Husmann, 1996). Recent studies have shown antiviral T cell memory can be maintained for decades and repeated boosting can drive memory T cells (Bae et al., 2003; Sallusto et al., 2010). However, few studies have addressed whether the choice of immune-potentiators *Bacillus subtilis* spores can impact the development of mucosal memory cells through oral vaccination.

Porcine epidemic diarrhea virus (PEDV) mainly damages intestinal epithelial cells, resulting in severe diarrhea and dehydration in neonatal suckling pigs, with up to 100% mortality (Jung and Saif, 2015). The major transmission mode of PEDV is the fecal-oral route through direct or indirect contact with infected pigs or contaminated feces (Gallien et al., 2018). Many infectious viruses can be prevented by vaccine-induced immunity at the mucosal surfaces (Rose, 2014).

In this study, we assessed the ability of B.s to assist PEDV WIV in the production of IgA and IgG following booster immunization. To gain a better understanding of the immune effector mechanisms conferring protection from B.s assistance with PEDV WIV, we examined the function of $CD4^+CD8^+$ memory lymphocyte subsets. Our findings led us to hypothesize B.s could promote intestinal memory cells capable of providing long-term immunologic memory, with accelerated antigen removal upon response to recalled antigens or virus.

2. Materials and methods

2.1. Probiotics, virus and reagents

Vero E6 cells (ATCC CRL-1586) and the PEDV Zhejiang08 strain were provided by the Veterinary Medicine Research Centre of the Da Bei Nong Group (Li et al., 2017). IPEC-J2 (Intestinal Porcine Epithelial Cell line-J2) cell was a gift from Professor Wei. It is the cell lines originally isolated from jejunal epithelia of a neonatal unsuckled piglet (Zhu et al., 2016). Viruses were purified using a discontinuous sucrose density gradient, and inactivated with ultraviolet radiation overnight to achieve a complete loss of infectivity. Purified virus concentrations were measured by BCA assays (Thermo Fisher, MA, USA). The B.s SQR9 strain was kindly supplied by Professor Shen of Nanjing Agricultural University (Xu et al., 2014).

2.2. Immunogenicity studies and sample collection

A total of 24, DLY (Duroc × Landrace × Yorkshire) piglets with 5 days of age from the same birth day were divided into three groups at birth: PBS, PEDV WIV, and B.s plus PEDV WIV. Pigs were raised in a unique highly sanitary state provided by Jiangsu Academy of Agricultural Sciences (Nanjing, China) characterized by freedom from a wide range of porcine pathogens including Mycoplasma hyopneumoniae, porcine reproductive and respiratory syndrome virus, porcine circovirus, porcine deltacoronavirus, transmissible gastroenteritis virus and porcine epidemic diarrhea virus. They were born in captivity without maternally derived antibodies, and fed artificial milk from the Jiangsu Academy of Agricultural Sciences. All animal studies were approved in accordance with the regulations and guidelines of the animal care committee of Nanjing Agricultural University (Nanjing, China). Immunization was administered orally to the piglets on days 0, 5 and 30, with PBS, PEDV WIV (100 μg/dose), or PEDV WIV together with B.s (10^9 CFU) (Fig. 1B). Serum, feces and saliva were collected at weekly intervals before and after the first immunization at day 7. Piglets were sacrificed on day 35, and ileum tissues were fixed with Bonn's liquid. Serum was collected from the jugular vein of the piglets, and then wash buffers were collected from the nasal cavity, the oral cavity and feces at 7-day intervals.

2.3. Isolation of intestinal mucosal-associated lymphocytes

Isolation of intestinal mucosal-associated lymphocytes (IMALs) was performed as previously described (Ivanov et al., 2006). Briefly, 3-cm segments including Peyer's patches were obtained from the terminal 5–15 cm of the ileum. The intestine was then opened longitudinally after removal of residual mesenteric fat tissue. The intestine was thoroughly washed with ice-cold PBS and cut into 1.5-cm pieces. Intestine sections were then incubated in digestive buffer containing 30 mM EDTA, 4% fetal calf serum, 100 μg/mL penicillin/streptomycin, 0.5 mg/mL Collagenase D (Roche Applied Science, Penzberg, Germany) and DNase I (Sigma Aldrich, St. Louis, Canada), and 50 U/mL Dispase (Sigma Aldrich, St. Louis, Canada) in DPBS for 20 min at 37°C with slow rotation (100 rpm). After incubation, single cell suspensions were collected by intensive vortexing and passing through a 40 μm cell strainer. Single cell suspension was collected and washed twice in PBS. The remaining tissue pieces were re-incubated with digestive buffer for 20 min at 37°C with slow rotation (100 rpm). A third digestion step was repeated as above. Supernatants from the three digestive buffers from a single small intestine were combined and washed in cold RPMI-1640. Cells were then re-suspended in 5 mL of the 40% fraction percoll (Solarbio, Beijing, China) gradient, and overlaid on 5 mL of the 80% fraction in 15 mL Falcon tubes. Percoll gradient separation was performed by centrifugation at 300 *g* for 20 min. IMALs were collected at the interphase of the percoll gradient, washed and resuspended in cold RPMI-1640 with 5% FBS (Gibco, NY, USA). Cells were immediately used for all experiments.

2.4. Specific antibody assay by ELISA

For the collection of saliva, piglets were not allowed to ingest anything for 1 h previous to sample collection. Saliva was collected with a cotton swab, which was bitten by piglets four times. Fecal samples were collected using a cotton swab introduced 4 cm into the rectum. These samples were transported on dry ice to the research lab. Saliva and fecal samples were prepared by adding 800 μl PBS, vortexed at least 30 s, centrifuged at 3000 × g for 10 min at 4 °C and stored at −70 °C until further evaluation. The PEDV-specific IgG in serum and IgA in mucosal wash (oral, feces) was measured by ELISA as described in previous research (Qin et al., 2015). Briefly, ELISA plates were coated with 2 μg of purified PEDV/well at 4 °C overnight. Following virus removal, plates were blocked with 3% bovine serum albumin (BSA) in PBS containing 0.05% Tween (PBST) at 37 °C for 2 h. Next, 100-fold dilutions of serum samples or 2-fold dilutions of lavage fluid from pigs were applied to the plates and incubated at 37 °C for 1.5 h. HRP-conjugated rabbit anti-pig IgG antibodies (Abcam, Cambridge, UK) or HRP-conjugated goat anti-pig IgA antibodies (Abcam, Cambridge, UK) were added at a 1:2000 dilution and incubated at 37 °C for 1 h after washing with PBST. Plates were incubated with 3, 3′, 5, 5′-tetramethylbenzidine for 10 min. Finally, the reaction was stopped with 2 M sulfuric acid and the absorbance was read at 450 nm with a micro plate reader.

2.5. Mucosal immune cell development by immunostaining

Ileum tissues were fixed with Bonn's liquid, embedded in paraffin and sectioned at a thickness of 5 μm. PPs are important inductive sites for the initiation of innate and adaptive immune responses, and enhance the host's immunological barrier (Kiyono and Fukuyama, 2004; Tsai et al., 2010). The size of the PPs is an important indicator of their development (Lasa-Saracibar et al., 2014; Sato et al., 2006). Tissue samples were stained with hematoxylin and eosin. PP area measurement was conducted manually using Image-Pro Plus software. Intraepithelial lymphocytes (IELs) were located at the first sites, encountering and defending against pathogens in the intestinal tract.

Immunohistochemical (IHC) assessment was performed using with the SABC kit (Boster Bioscience, Wuhan, China). Hydrogen peroxide was used to deactivate intrinsic peroxidase. Antigen retrieval was performed in an array of buffers. Sections were incubated with diluted goat anti-pig IgA (1:100, Abcam) or rabbit anti-pig CD3 (1:400, Abcam, Cambridge, UK) antibodies overnight at 4 °C. Subsequently, slides were incubated with an ABC-based system, with biotinylated goat anti-mouse IgG or anti-rabbit IgG as a secondary antibody with DAB as a chromogen. After staining with DAB, slides were imaged using a digital camera (Leica-DM4000B).

For immunofluorescence staining, sections from paraffin-embedded tissue were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. Briefly, the fixed filters were incubated with the primary antibodies overnight at 4 °C after being blocked with 5% BSA for 1 h. Then, incubation with secondary antibodies was conducted for 1 h at room temperature. $CD4^+$ cells were labelled with FITC rabbit anti-pig CD4a (Santa Cruz, California, USA). $CD8^+$ cells were labelled with mouse anti-pig CD8 alpha (Abcam, Cambridge, UK) followed by Alexa Fluor 594-conjugated goat anti-mouse IgG (Multiscience, Hangzhou, China), and sections were sealed with coverslips for examination. Following DAPI staining, sections were visualized with CLSM (LSM 710, Zeiss, Germany).

2.6. Antiviral assessment via plaque reduction neutralization tests

The occurrence of PEDV neutralizing antibodies in piglet serum and washing fluid was assayed using plaque reduction neutralization tests (PRNT). Neutralizing antibody assays were performed as described in previous research (Chua et al., 2013). Briefly, serum was diluted in RPMI-1640 containing 2% FBS (virus diluent) at a ratio of 1:10, and washing fluid was diluted at a ratio of 1:1. Mixtures of serum or intestinal wash liquid were incubated with 300 PFU of virus at 37 °C for 1 h. Treated viruses were then transferred onto Vero cell monolayers at 37 °C for 1 h. Cell monolayers were then washed with virus diluent, and overlaid with agar overlay medium at 37 °C in a 5% CO_2 incubator. Plaques were visualized by staining the monolayer with 0.5% crystal violet in 25% formaldehyde solution at 72 h or 96 h post-infection. Plaque images were acquired with ultraviolet radiation -light in Tanon 5200 Multi.

2.7. $CD4^+$ $CD8^+$ T cell flow cytometry

Isolated single-cells were obtained from piglet intestines and stained

with anti-CD3-APC (BD Biosciences, California, USA), anti-CD4-FITC (BD Biosciences, California, USA), anti-CD8-PE (BD Biosciences, California, USA) (1:100 dilution) for 30 min on ice, and then washed twice with PBS. Flow cytometry was performed using a FACSC6 (BD Biosciences) instrument, and analyzed using FlowJo software. A total of 10,000 lymphocytes were acquired per sample.

In addition, IMALs were cultured in 96-well culture plates at a density of 10^6 cells/100 μL, following re-stimulation with recall antigen PEDV WIV (5 μg/mL) at 37℃ for 5 days. The collected cells were stained with mouse anti-pig CD4-FITC and CD8-PE cell surface molecules for 30 min at 4 ˚C. Cells were then stained with rabbit anti-human CCR9 antibodies (Abcam, Cambridge, UK), followed by incubation with anti-IgG dylight 488 secondary antibodies (Multiscience, Hangzhou, China). The amino acid homology and nucleotide sequence homology of CCR9 were 91% and 89%, respectively. Also, we confirmed rabbit anti-human CCR9 mAb to be cross-reactive with porcine CCR9 by Western blotting (data not shown). Cell supernatants were harvested for cytokine assays *in vivo*.

2.8. Cytokine assays

Levels of IL-6, IL-1β, and IFN-γ were assessed by ELISA according to manufacturer's instructions (Abcam, Cambridge, UK). Briefly, each standard and sample was added to the appropriate wells and incubated overnight at 4 ˚C. Liquid was discarded and washed 5 times with 1X wash buffer. After the last wash, biotinylated detection antibodies were added to the wells and incubated for 1 h at room temperature with gentle shaking. After washing 5 times, HRP-conjugated Streptavidin was added to each well for 45 min at room temperature (RT) with gentle shaking. Finally, 100 μL of TMB One-Step Substrate reagent was added to each well and incubated for 30 min at RT in the dark. After adding Stop Solution to each well, data was immediately acquired on an automated ELISA plate reader at 450 nm.

2.9. Real-time quantitative PCR analysis

To identify the impact of B.s on IECs in pigs, we evaluated whether B.s affected TLR2, TLR4 and IL-6 expression in IPEC-J2. IPEC-J2 was incubated in the presence or absence of B.s and *Lactobacillus* (Lac) (10^8 CFU) for 24 h. The mRNA levels of TLR2, TLR4, and IL-6 were determined with real-time PCR. Total RNA was extracted from the ileum and colon mucosa using TRIzol reagent (Takara, Shiga, Japan) following the manufacturer's guidelines. Gene expression data was collected using the BIOER PCR System and analyzed using the $2^{-\Delta\Delta Ct}$ method (Yin et al., 2015). Relative TLR2, TLR4 and IL-6 mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. The primer sequences will be made available upon request.

2.10. Statistical analyses

Data are presented as the means + S.E. GraphPad Prism V6.0 (San Diego, CA, USA) was used for statistical analyses. One-way analysis of variance (ANOVA) with Dunnett's test was used to analyze the significant differences between means. P-Values less than 0.05 ($P < 0.05$) was considered significant; and p-values less than 0.01 ($P < 0.01$) was considered highly significant.

3. Results

3.1. B.s plus PEDV WIV increased PEDV specific IgA and IgG levels

The immunization procedure was performed as shown in Fig. 1B. We found that serum PEDV-specific IgG (Fig. 1C) antibody titers induced by B.s plus PEDV WIV increased compared to PEDV WIV alone from day 7 to 56, except on day 28. Significant increases were observed from day 7 to 14 and day 35 to 42 after boost immunization, when compared to inactivated PEDV in piglets. In addition, the levels of PEDV specific IgA both in saliva (Fig. 1D) and feces (Fig. 1E) increased on day 7 to 42, and significantly increased from day 14 to 28. In contrast, low levels of PEDV-specific IgA were detected in saliva and feces after 42 days and no significant vaccination response was observed. However, vaccine specific IgA levels in nasal washing fluid were under the detection limit (OD450 < 0.2), and did not increase over time (data not shown). IHC results also revealed IgA-secreting cells mainly gathered around crypts or in the villous lamina propria (Fig. 1F). In the ileum, the number of IgA-secreting cells of the B.s plus PEDV WIV and PEDV WIV treatment groups were significantly higher than controls ($P < 0.05$) (Fig. 1G). Together, these results indicated that B.s plus PEDV WIV effectively induced systemic and local immune responses after oral immunization.

3.2. B.s increased the production of PEDV neutralizing antibodies by oral immunization

To confirm the efficacy of the induced antibodies against the virus, the neutralizing activity of the mucosal fluid and serum against PEDV infection was valuated *via* PRNT assays. Serum of the B.s plus PEDV WIV group displayed a powerful ability to induce neutralizing antibodies, and the reduction in plaques was statistically significant (Fig. 2A) ($P < 0.05$). Similarly, intestinal liquid samples prepared from piglets in the B.s plus PEDV WIV group also displayed significantly reduced plaque numbers (Fig. 2B) compared to the group with oral PEDV WIV alone ($P < 0.01$). These results suggested that both mucosal and systemic immune responses induced by feeding piglets effectively inhibited viral infection.

3.3. B.s plus PEDV WIV enhanced intestinal local mucosal immunity

Peyer's patches play an essential role in antigen uptake through specialized epithelial M cells, initiating immune responses and inducing the production of IgA precursors (Radloff et al., 2017). IELs located between epithelial cells and formed the front line of immune defenses against invading pathogens (Cheroutre et al., 2011). We found the area of PPs (Fig. 3A and D) and percentage of IELs (Fig. 3B and E) both markedly increased in the B.s plus PEDV group ($P < 0.01$). In the cross-sectional view, $CD3^+$ T cells were observed gather mainly in the epithelial layer and villous lamina propria stained with a deep yellow-brown color (Fig. 3C). Assessment of $CD3^+$ T cells in the epithelial layer for each 2500 μm^2 area revealed significant differences (Fig. 3F) ($P < 0.01$). These results indicated that oral immunization with B.s could effectively increase the local concentration of lymphocytes in the piglet ileum.

3.4. B.s plus PEDV WIV increased the amount of $CD4^+CD8^+$ memory T cells in IMALs

In swine, $CD4^+CD8^+$ T cells have been identified as antigen-specific memory cells that can be induced by vaccination (Ober et al., 1998). Thus, it is crucial to determine the role and biologic significance of peripheral $CD4^+$ $CD8^+$ T cells in swine, as they could potentially contribute to the adaptive immune response against invading pathogens (Nascimbeni et al., 2004). Previous studies have revealed that only $CD4^+$ $CD8^+$ T lymphocytes show a significant proliferative response (Summerfield et al., 1996; Zuckermann and Husmann, 1996). PEDV-specific $CD4^+$ $CD8^+$ T lymphocytes have been shown to be memory T lymphocytes, and B.s has been observed to prime $CD4^+$ $CD8\alpha^+$ memory T lymphocytes during vaccination by flow cytometry (FACS) (Fig. 4A) and immunofluorescence assays (Fig. 4C, D and E). The frequency of $CD4^+$ $CD8\alpha^+$ T cells gated from $CD3^+$ T cells were higher in the PEDV WIV plus B.s group than PEDV WIV alone or PBS group (Fig. 4B) ($P <$ 0.01). These results demonstrated activated

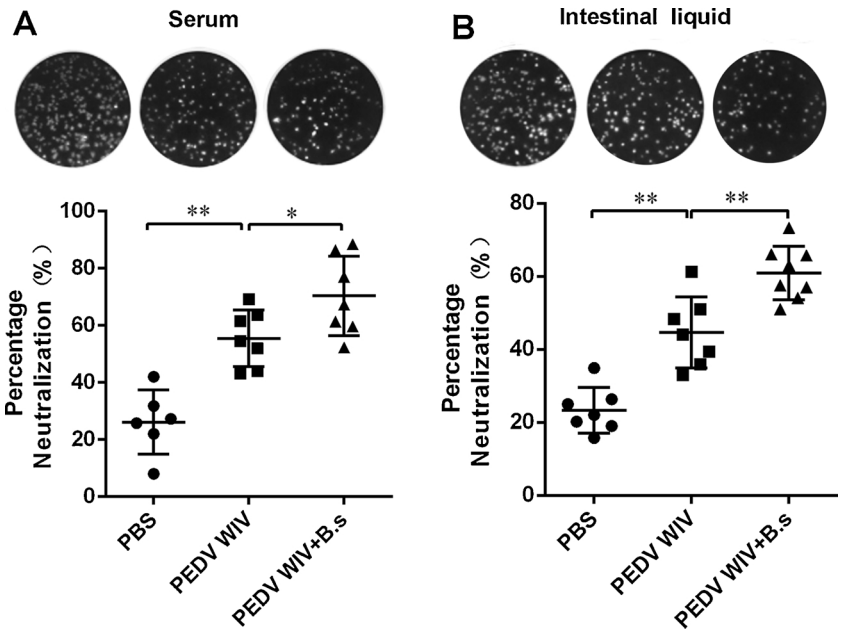

Fig. 2. Induction of PEDV neutralizing antibodies by PEDV WIV with B.s vaccine.

Occurrence of anti-PEDV neutralizing antibodies in the serum and intestinal wash fluid of piglets immunized with the vaccine was determined using plaque reduction neutralization tests (PRNTs). **(A)** Serum from immunized piglets was diluted 1:10 and tested against live PEDV Zhejiang08. **(B)** Intestinal washing fluid from piglets after oral immunization was tested against live PEDV Zhejiang08. Neutralization activity of serum or ileum wash buffer was expressed as the percentage of virus neutralization, based on plaque determinations. Data represent the means ± S.E. of four samples. $^{*}P < 0.05$; $^{**}P < 0.01$.

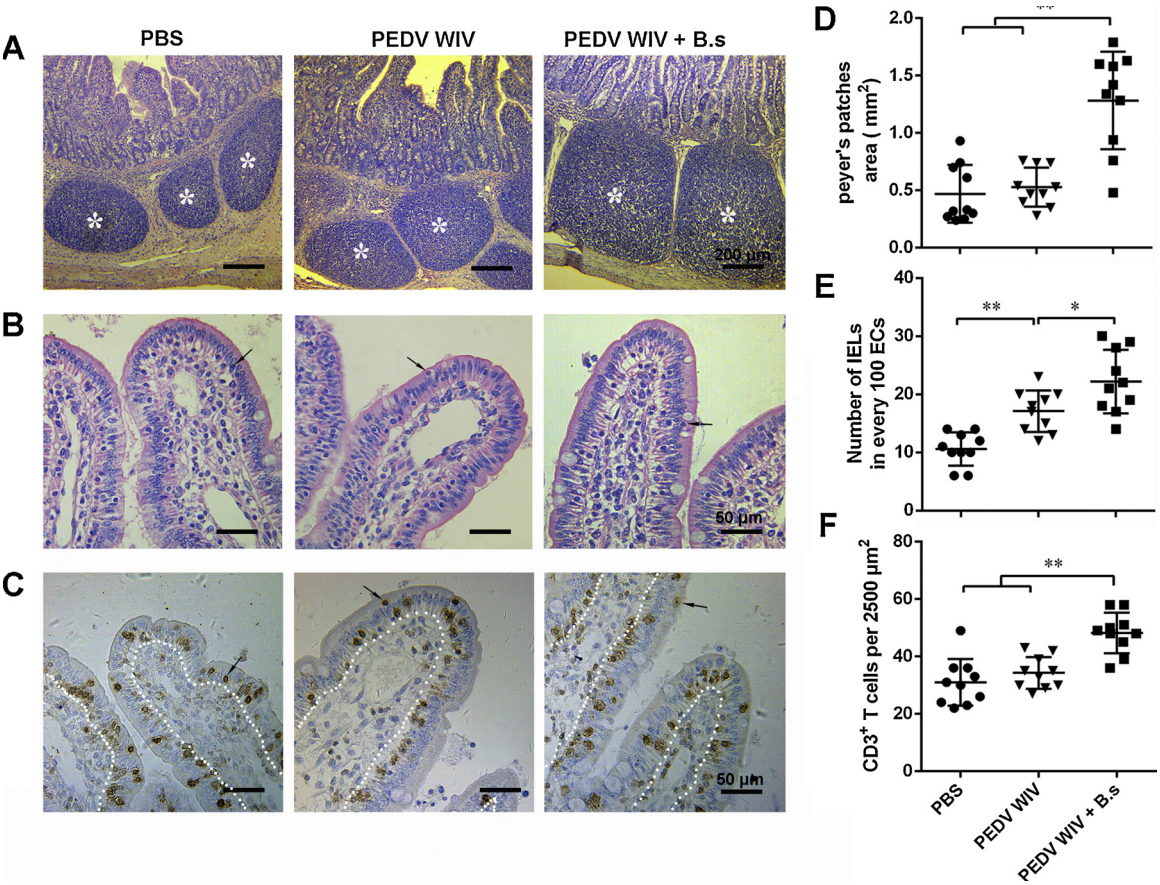

Fig. 3. B.s oral administration promotes intestinal development in piglets.

Five-day-old piglets were given PBS, PEDV WIV or PEDV WIV plus B.s. The small intestine and colon were removed and HE staining was performed. (A, D) Each area of the PPs was obtained from one experimental pig. (B, E) IELs per 100 epithelial cells (ECs) were counted in the ileum villi. (C, F) $CD3^{+}$ T lymphocytes in the ileum were stained using anti-CD3-HRP, then counterstained with hematoxylin (blue), and then analyzed. Data represent the means ± S.E. of four samples. $^{*}P < 0.05$; $^{**}P < 0.01$. Scale bars: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

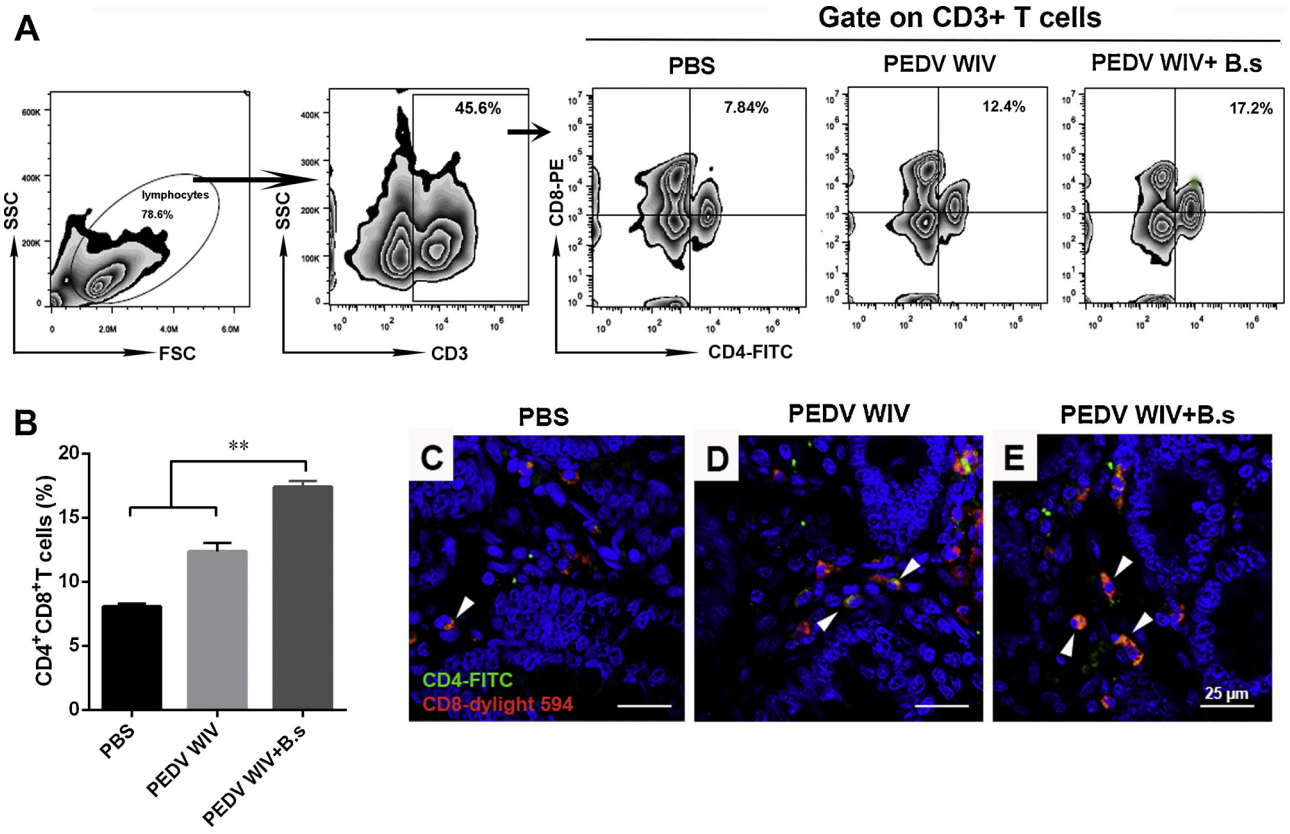

Fig. 4. The amount of CD4$^+$CD8$^+$ memory cells increases after oral vaccination in intestinal mucosal-associated lymphocytes.
(A, B) Frequencies of CD3$^+$ CD4$^+$ CD8$^+$ T cells in the small intestinal mucosal cells of the immunized piglets were analyzed by flow cytometry. **(C–E)** The expression of CD4-FITC (green) or CD8$^+$ (Alexa Fluor 594 red) of the indicated piglets was evaluated with confocal microscopy. Data represent the means ± S.E. of four samples. *$P < 0.05$; **$P < 0.01$. Scale bars: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

extrathymic CD4$^+$ CD8α$^+$ memory T lymphocytes could be induced by B.s.

3.5. B.s assisted PEDV WIV in the promotion of lymphocyte proliferation and CCR9 expression

Lymphocytes are believed to play a crucial role in the induction of mucosal immunity at effector sites, and IMALs can effectively prevent or directly limit infection (Iwata et al., 2004). After stimulation, virus-specific T cells are known to acquire tissue-homing capacity and migrate to the site of infection (Nascimbeni et al., 2004). IMALs were isolated from immunized piglets and re-stimulated with the same PEDV WIV *in vitro* to assess lymphocyte proliferation. The frequency of CD4$^+$ CD8α$^+$ double positive T cells in the PEDV WIV plus B.s group increased ($P < 0.01$) (Fig. 5A). Furthermore, FACS data obtained from the ileum showed that the expression of CCR9 on IMALs from PEDV WIV plus B.s group was remarkably enhanced compared to other groups (Fig. 5C, D) ($P < 0.05$). Previous report has showed CCR9 expression is found only in Peyer's patches and appears critical for gut homing as the unique features for memory cells (Stenstad et al., 2006). Additionally, upon comparison of the effects of cytokine for re-stimulation, no significant increase in IL-2 (Fig. 5E) or IFN-γ (Fig. 5F) was observed. Thus, we concluded that B.s plus PEDV WIV had PEDV-specific effects on T-cell proliferation.

3.6. B.s could enhance the expression of TLR 2, TLR 4 and the secretion of IL-6

In order to evaluate the functionality of the T cell responses after vaccination of B.s *in vivo*, cytokines assay was performed. The effects of oral vaccination on the level of IL-6 (Fig. 6A), TNF-α (Fig. 6B) and IL-1β (Fig. 6C) in the ileum mucosa on day 35 were shown in Fig. 6. Levels of IL-6 ($P < 0.05$) in the ileum of piglets on day 35 were higher than in control and recipient piglets. No significant difference was observed for the secretion of TNF-α and IL-1β. TLR2 and TLR4 serves as a costimulatory receptor for participating in the maintenance of T cell memory (Cui et al., 2014; Komai-Koma et al., 2004). In addition, we used IPEC-J2 co-incubated with B.s to verify the results *in vivo*. The relative mRNA expression of TLR2, TLR4 and IL-6 in IPEC-J2 were displayed in Fig. 7. Compared to medium alone, TLR2, TLR4 and IL-6 in the IPEC-J2 were significantly increased ($P < 0.01$) after incubation with B.s (10^8 CPU) for 24 h.

4. Discussion

Whole inactivated PEDV vaccine trials in pigs have been shown to be safe and immunogenic (Collin et al., 2015), but the inactivated virus does not appear to induce effective intestinal mucosal immunity (Xiao and Daniell, 2017). In previous studies, both attenuated and inactivated PEDV vaccines have been developed, but the transmission of the PEDV virus has not been controlled, and the associated high mortality and loss of productivity have persisted (Langel et al., 2016; Song et al., 2007). The ultimate goal of vaccination does not only rely on the magnitude of the humoral immune response, but also the ability to stimulate protective immunological memory (Benoun et al., 2016). Nevertheless, few studies have addressed whether the choice of adjuvant can influence mucosal memory cells development following vaccination.

In this study, we showed that B.s plus PEDV WIV increased the levels of IgA in saliva and IgG in serum, which were maintained at high levels after PEDV WIV re-challenge at 30 d (Fig. 1). The capability to

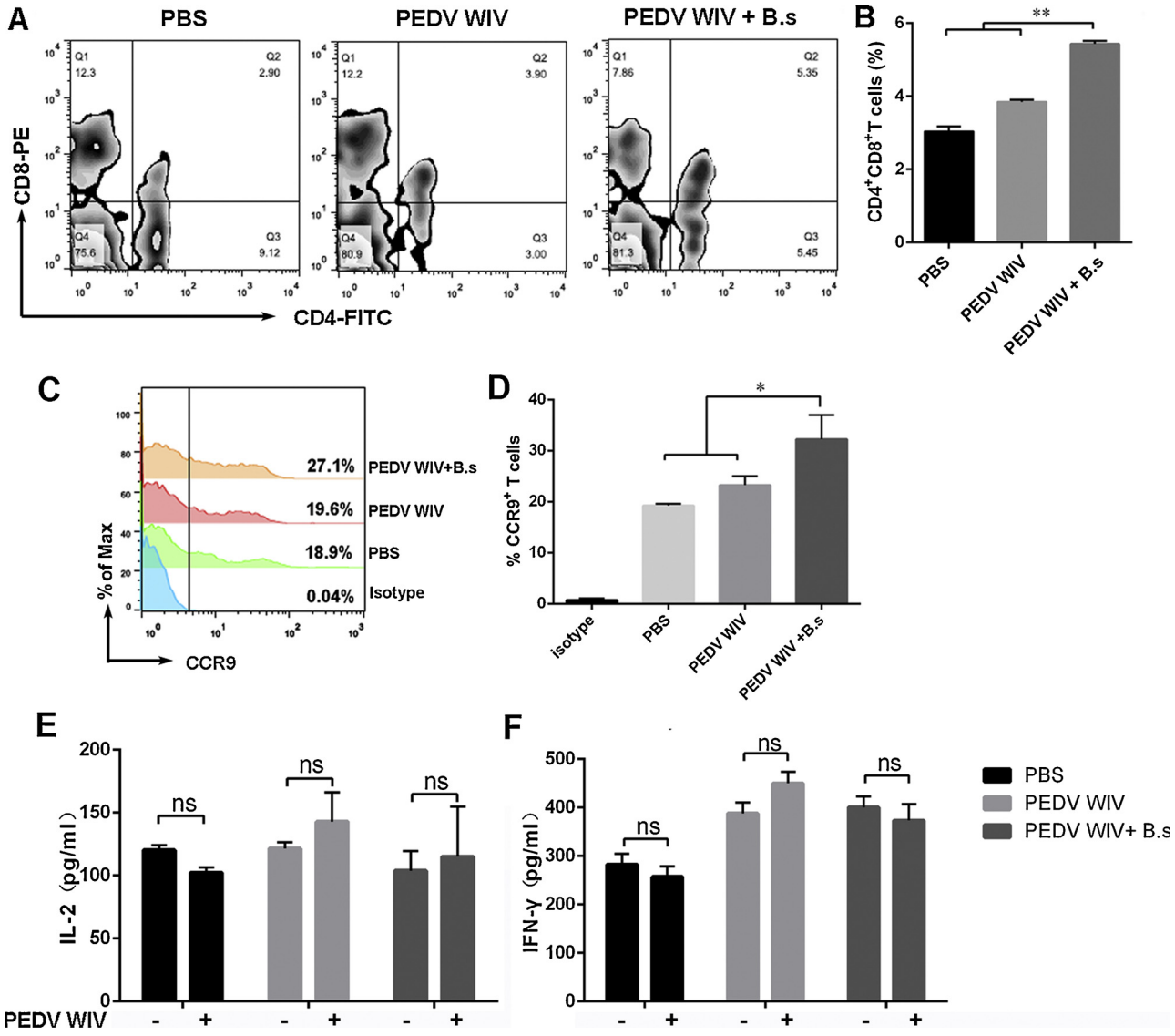

Fig. 5. B.s promotes lymphocyte proliferation and CCR9 expression.
Isolated lymphocyte cells were treated with or without PEDV (2 μg/ml) for 5 days. **(A, B)** The frequency of $CD4^+$ $CD8^+$ cells by recall antigen in the small intestinal mucosa of immunized piglets were analyzed with flow cytometry (FACS). **(C, D)** Gut-homing specificity was examined with FACS by measuring the expression of dylight 488 – CCR9 in cells. **(E, F)**Intestinal mucosal-associated lymphocytes from immunized piglets were stimulated with 5 μg/mL recall antigen for 5 days, and T-cell cytokines IL-2 and IFN-γ were measured by ELISA. Data represent the means ± S.E. of four samples. $^*P < 0.05$; $^{**}P < 0.01$.

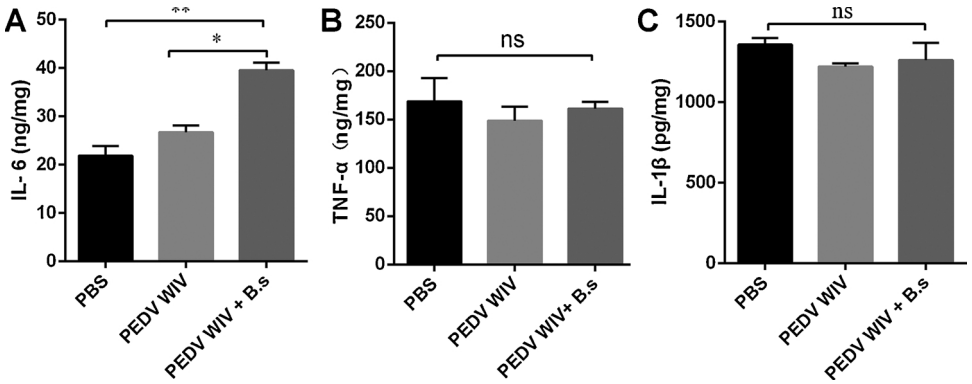

Fig. 6. Determination of cytokines produced by piglets in the intestine.
Mucosal fluid was gathered by vortexing intestinal tissues in tubes. Supernatants were collected and tested for IL-6 **(A)**, TNF-α **(B)** and IL-1β **(C)** by ELISA. Data represent the means ± S.E. of four samples. $^*P < 0.05$; $^{**}P < 0.01$.

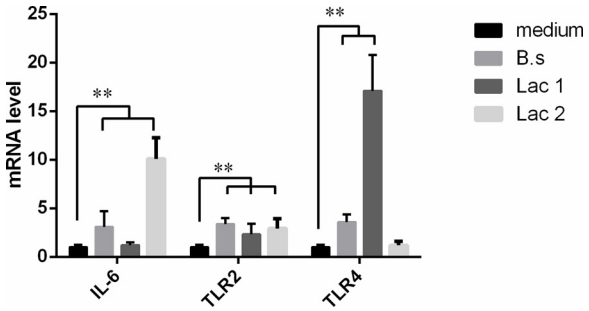

Fig. 7. Levels of TLR2, TLR4 and IL-6 in IPEC-J2 after treatment with B.s. IPEC-J2 was treated with B.s and Lac (10^8 CFU) for 24 h. mRNA levels of TLR2, TLR4 and IL-6 were assessed with real-time quantitative PCR (RT-qPCR). Data are represented as the means ± S.E. from three experiments. *$P < 0.05$; **$P < 0.01$.

initiate an effective recall response upon re-exposure to the same pathogen is an important aspect of immunological memory (Ratajczak et al., 2018). Similarly, our results suggested that oral immunization with PEDV plus B.s led to the accumulation of memory $CD4^+CD8\alpha^+$ T cells *in vivo* (Fig. 4). Nevertheless, $CD4^+CD8\alpha^+$ T cells were contained as specific subsets and their function could not be evaluated separately in this investigation. In addition, we were not able to detect significant increases in cytokine production such as IL-2 and IFN-γ after antigen recall. One possible explanation is that the secretion of cytokines peaks at different time points, or that their production occurs at a lower threshold than previously thought. In previous studies, the expression of the tissue-homing marker CCR9 was found in PPs and appeared critical for gut mucosal memory homing (Bemark et al., 2016). In this study, $CD4^+CD8^+$ memory cells were observed in swine consistent with the expression of CCR9. Recent evidence also has demonstrated that local antigen presentation and PEDV-induced tissue activation are both required for the recruitment of memory cells into tissues, and for the final imprinting of a resident memory CCR9 phenotype (Topham and Reilly, 2018). Another possibility is that dendritic cells (DCs) may also generate or combine with other cytokines to promote gut-homing molecule expression, including mucosal IL-6, which enhances gut-homing receptor expression in lymphocytes (Saurer et al., 2007).

Furthermore, the mucosal immune system properly balances pathogen surveillance as lower immunogenic potent adjuvants and delivery platforms are required for effective mucosal vaccination (Fujkuyama et al., 2012; Rhee et al., 2012). In this study, B.s could enhance the development of PPs (Fig. 2A). Previously, proliferation of cells in the follicles of PPs has been identified as a good indicator of antigen stimulation (David et al., 2003). Moreover, neutralizing antibodies can be used to assess the quality correlation of vaccine efficacy for many licensed vaccines (Zinkernagel, 2001). Assessment of PEDV-neutralizing activity showed that the B.s plus PEDV WIV group had the highest neutralization potential, highlighting it as a promising vaccine candidate against PEDV infection.

In ensemble, our results demonstrated that B.s combined with PEDV WIV upregulated the number of $CD4^+CD8\alpha^+$ memory cells and CCR9 homing molecules to guide lymphocyte migration in the intestinal tract. As a candidate immunopotentiator, B.s plus PEDV WIV could enhance both mucosal and systemic immunity, which renders it a great alternative for the oral vaccination of newborn piglets against PEDV.

Author Contributions

LH study conception and design, data analysis and interpretation, manuscript writing. JW and EZ performed most of the immunization and YW performed *in vitro* immunogenicity experiments. QY and YL conceived the study and revised the manuscript with LH. QY study conception and design, financial support, administrative support, data analysis and interpretation, manuscript writing, final approval of the manuscript.

Competing interests

The authors declare no competing financial interests.

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