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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) is the causative agent of porcine epidemic diarrhea, a highly contagious enteric disease of swine. The Spike (S) protein is one of the main structural proteins of PEDV capable of inducing neutralizing antibodies in vivo. Herein, we generated three distinct DNA constructs in the eukaryotic expression plasmid pVAX1; one encoding the S protein [pVAX1-(PEDV-S)], the second encoding the N-terminal fragment (S1) [pVAX1-(PEDV-S1)] containing potent antigenic sites, and the third expressing the porcine interleukin-18 (pIL-18) [pVAX1-(IL-18)]. Immunofluorescence assays in BHK-21 cells demonstrated successful protein expression from all 3 constructs. Kunming mice were injected separately with each of these constructs or with a pVAX1-(PEDV-S1)/pVAX1-(IL-18) combination, an attenuated PEDV vaccine, or vector only control. Animals were examined for T lymphocyte proliferation, anti-PEDV antibodies, IFN-γ and IL-4 protein levels, and cytotoxic T cell function in mouse peripheral blood and spleen. In all cases, results showed that pVAX1-(PEDV-S) and the combination of pVAX1-(PEDV-S1) with pVAX1-(IL-18) induced the strongest responses; however, pIL-18 had no adjuvant effects when given in combination with pVAX1-(PEDV-S1).

1. Introduction

Porcine epidemic diarrhea virus (PEDV) causes porcine epidemic diarrhea (PED) which causes very high mortality in newborn piglets. The disease was first reported in England in 1971 (Oldham, 1972). It has since been reported in many areas worldwide. The first link between the disease and the PEDV was identified in 1977 (Pensaert and Bouck, 1978). The virus is an enveloped coronavirus with a positive-stranded RNA genome that encodes four major structural proteins; spike (S), nucleocapsid (N), membrane (M) and envelope (E) proteins. Although conventional inactivated and attenuated vaccines are widely used in some areas in China, such vaccines have some drawbacks such as recovery of virulence, spread of viruses, high cost and poor protection efficacy. Therefore, development of novel and effective vaccines is necessary for control of PED.

Direct injection of plasmid DNA has been considered and evaluated as a candidate strategy for vaccination. A great number of studies have shown that eukaryotic expression plasmids can induce protective immune responses (Ding et al., 2005; Lillehoj et al., 2005; Li et al., 2011). It is well known that the S protein of coronavirus plays a crucial role in the induction of neutralizing antibodies and has been used in the preparation of effective vaccines (Gomez et al., 2000; Tuboly and Nagy, 2001). The N-terminal portion of the S protein of several coronaviruses has been shown to contain key antigenic sites responsible for eliciting humoral and cellular immune responses (Delmas et al., 1986; Gebauer et al., 1991). Interleukin-18 (IL-18) is a cytokine that was first cloned in 1995 from livers of propionibacterium acnes-treated mice (Okamura et al., 1995). Functionally, IL-18 induces IFN-γ production, stimulates proliferation of activated T cells, and enhances activities of NK cells among other things (Muneta et al., 2000).

In this study, eukaryotic expression plasmids encoding the full-length S protein, the N-terminal fragment of PEDV, or recombinant porcine IL-18 (pIL-18) were constructed. Using a mouse model, we compared host immune responses triggered by the full-length S protein and its N-terminal fragment (S1) in the presence or the absence of pIL-18 to determine if IL-18 offered any adjuvant effects to the stimulation provided by the full-length or truncated forms

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Table 1
Experimental parameters and cytokine levels in the blood of immunized mice ($\bar{X}_A - \bar{X}_B \pm s$). Mice were immunized with pVAX-(IL-18) (A), pVAX1-(PEDV-S) (B), pVAX1-(PEDV-S1) (C), pVAX1-(PEDV-S1) plus pVAX1-(IL-18) (D), attenuated PEDV vaccine (E), PBS (F) or pVAX1 (G). The number of mice, DNA dosage, and number of immunizations are indicated. The levels of IFN-γ in the serum of immunized mice were analyzed at 42 dpi using commercially available ELISA kits.

Group	Number of mice	Vaccine category	Immunizing dose	Number of treatments	IFN-γ (pg/ml)	IL-4 (pg/ml)
A	28	pVAX1-(IL-18)	100 μg	3	1257.52 ± 49.49	116.04 ± 5.656
B	28	pVAX1-(PEDV-S)	100 μg	3	1126.86 ± 64.34	202.17 ± 12.02
C	28	pVAX1-(PEDV-S1)	100 μg	3	1079.63 ± 38.89	54.45 ± 1.414
D	28	pVAX1-(PEDV-S1) + pVAX1-(IL-18)	100 μg 100 μg	3	1678.24 ± 142.1*	105.99 ± 4.949
E	28	Attenuated PEDV vaccine	100 μg	3	215.07 ± 13.43	106.39 ± 4.242
F	28	PBS	100 μg	3	133.34 ± 8.485	58.54 ± 6.363
G	28	pVAX1	100 μg	3	167.64 ± 38.89	62.64 ± 3.535

* $p < 0.01$ (highly significant), compared with PBS, pVAX1, pVAX1-(IL-18), pVAX-(PEDV-S1) or attenuated PEDV vaccine.

of the S protein. These data may facilitate the development of efficacious viral gene vaccines.

2. Materials and methods

2.1. Construction of plasmids containing genes encoding PEDV-S, PEDV-S1 or SwIL-18

The plasmid EasyT-S containing the full-length S gene of PEDV strain CV777 was constructed in our laboratory by common cloning techniques and used as a template for PCR amplification. A sense primer P1 (5′-GGGGGGATCCATGAGGTCTTTAATTTAC), and two antisense primers P2 (CCCCGAATTCTTAAATACTCATACTAAA) and P3 (5′-CCCCGAATTCTCATCTCTGCACGTGGAC) which contain BamHI (P1) and EcoRI (P2 and P3) restriction enzyme sites (underlined), were used for PCR. The amplification profile for the full length S gene (primers P1/P2) was as follows: 95 °C for 5 min followed by 30 cycles of 94 °C for 2.5 min, 56.8 °C for 2.5 min, and 72 °C for 5 min. A final extension of 72 °C for 10 min was performed at the end of the cycling period. The amplification profile for PEDV-S1 gene (primers P1/P3) was the same as for the PEDV-S gene except that the annealing temperature was raised to 54.8 °C. Plasmid pUC18-(IL-18) containing the full-length swine interleukin 18 gene was used as template for PCR. The sense and antisense primers P4 (5′-GGGGGGATCCATGTACTTTGGCAAGCTT) and P5 (5′-CCCCGAATTCCTAGTTCTTGTTTTGAACA) included BamHI and EcoRI restriction enzyme sites (underlined), respectively. The PCR profiles included 95 °C for 5 min followed by 30 cycles of 94 °C for 0.5 min, 56.5 °C for 0.5 min, and 72 °C for 1 min followed by a final extension of 72 °C for 10 min. All PCR products were purified, restriction enzyme digested and ligated into the similarly digested eukaryotic expression vector, pVAX1 (Invitrogen, USA). After transformation, clones were picked and validated by DNA sequencing. The plasmids were designated pVAX1-(PEDV-S), pVAX1-(PEDV-S1) and pVAX1-(IL-18), respectively.

2.2. Preparation of plasmid DNA and immunofluorescence of transformed BHK-21 cells

Plasmid DNAs were chromatographically purified from bacterial lysates (Qiagen, Germany), precipitated, and then washed with ethanol. All plasmids were dissolved to 1 μg/μl in 0.1 M PBS. BHK-21 cells from American Type Culture Collection (ATCC) were cultured to 90% confluency in 6-well plates at 37 °C (approx. 24 h) then transformed with 3 μg of pVAX1-(PEDV-S), pVAX1-(PEDV-S1), pVAX1-(IL-18), or pVAX1 using lipofectamine 2000. The next day, indirect immunofluorescence assays were performed with modifications as described (Liu et al., 2009; Sui et al., 2010; Li et al., 2011). Formalin fixed cells were incubated for 1 h with polyclonal antisera (1:200 dilution in 1% BSA) against PEDV (prepared in our laboratory) or pIL-18 (prepared in our laboratory). After washing with PBS, the cells were incubated in the dark with FITC-labeled goat anti-rabbit IgG (1:1000) for 1 h. The green fluorescence signals were analyzed by fluorescence microscopy (Leica, Germany).

2.3. Immunization of mice

All animal studies were pre-approved by the Animal Ethics Committee of Northeast Agricultural University, China (approval ID 1155-NCET-005). Prior to DNA vaccination, 6-week-old Kunming mice (Harbin Veterinary Research Institute) were partitioned into 7 groups (Table 1) and injected in the medial vastus muscle with lidocaine hydrochloride (27 ga needle; 50 μl of 0.8% (v/v) in PBS). After 15 min, the mice received by similar injection, 100 μg of pVAX1-(PEDV-S), pVAX1-(PEDV-S1), pVAX1-(IL18), pVAX1-(PEDV-S1) + pVAX1-(IL18) (100 μg each), attenuated vaccine PEDV, pVAX1, or PBS. The mice were boosted twice, each at 2-week intervals as shown in Table 1.

2.4. Antibody detection and T lymphocyte analysis

Peripheral blood was collected by orbital bleeds at 3 h and then at 7, 14, 21, 28, 35 and 42 days post-immunization (dpi); serum and T lymphocytes were subsequently prepared. For detecting PEDV-specific antibody, recombinant PEDV-S1 protein purified from pET-(PEDV-S1) transformed bacterial cells was diluted to 50 μg/ml in 0.05 M $NaHCO_3$. An ELISA was performed according to Liu et al. (2009) with modification according to Li et al. (2011). To assess antibody binding, ELISA wells were incubated with *o*-phenylenediamine dihydrochloride substrate for 15 min. The reactions were terminated with 50 μl 2 M H_2SO_4 and the wells read at 490 nm.

Peripheral blood lymphocytes were purified using lymphocyte separation solution (Invitrogen, USA) according to the manufacturer's instructions. Cells were suspended to 1×10^7 cells/ml in RPMI 1640 medium containing 10% serum and further prepared as described (Li et al., 2011). Prepared cells were then incubated at 4 °C with FITC-conjugated anti-CD4+ T cell antibody or PE-conjugated anti-CD8+ T cell antibody (1:1000 dilutions) (Zhongshan, China) for 30 min. After incubation, the cells were washed with cold PBS (3×), suspended in PBS and subjected to flow cytometry. Splenic lymphocytes were similarly prepared and analyzed.

2.5. Proliferation of T lymphocytes from immunized mice

Peripheral blood mononuclear cells and splenocytes from vaccinated and control mice were prepared for lymphocyte proliferation assays as described (Li et al., 2011). Prepared cells (50 μl of 2×10^6 cells/ml) were suspended in RPMI1640 containing 10% serum then transferred to 96-well, flat-bottom plates. To each well, 50 μl of medium containing either 20 μg/ml purified recombinant

PEDV-S1 protein or Concanavalin A (Con A; Sigma) was added; all treatments were performed in triplicate. Plates were incubated for 72 h, supplemented with 10 μl/well of 3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), then incubated for an additional 4 h. Reactions were terminated by adding an equal volume of DMSO and incubating the plates at room temperature for 10 min. Proliferation was determined from OD_{490} values.

2.6. Neutralization of PEDV with immune mouse sera

To determine if mice generated PEDV-neutralizing antibodies, sera (1:100 dilution, 50 μl) from DNA-vaccinated mice were mixed with an equal volume of PEDV (10^5 pfu/ml) at 37 °C. After 1 h incubation, the treated viruses were used to infect cultured African green monkey kidney (Vero) cells in 96-well plates. The plates were incubated at 37 °C in a 5% CO_2 atmosphere and examined daily for 3 days for PEDV-specific cytopathic effects (CPE).

2.7. Indirect detection of IFN-γ and IL-4

Serum IFN-γ levels were analyzed with an IFN-γ detection kit (Excell Bio, China) according to the manufacturer's instructions. A standard curve was generated using control IFN-γ diluted in PBS at different concentrations beginning with 10,000 pg/ml, followed by twofold serial dilutions between 2000 pg/ml and 61.25 pg/ml. Dilutions were subsequently coated onto ELISA plates overnight at 37 °C. Sera (1:100) from 42 dpi mice were also coated onto ELISA plates and used as primary antibodies in a parallel experiment to evaluate the virus-derived IFN-γ response. HRP-conjugated goat-anti mouse (1:2000) was used as secondary antibody in both the control experiment and in the analysis of the mouse sera. The OD_{490} values and therefore pg/ml of IFN-γ in immunized mice were determined relative to the IFN-γ standard curve.

Serum IL-4 levels were similarly analyzed using an IL-4 detection kit (Excell Bio, China). Control IL-4 was serially diluted twofold in PBS between 500 pg/ml and 7.8 pg/ml then coated onto ELISA plates at 37 °C overnight. The ELISA was performed as above and OD_{490} values (pg/ml) were determined relative to an IL-4 standard curve.

2.8. Cytotoxicity (CTL) assay

Cytotoxicity was analyzed using a lactate dehydrogenase (LDH) release assay kit according to the manufacturer's instructions (Jiancheng, Nanjing, China) using splenic lymphocytes and blood from 42 dpi mice. Lymphocytes, suspended in complete RPMI 1640 medium were used as effector cells. Simultaneously, target Vero cells were infected with PEDV at a titer of 100TCID50 for 36 h at 37 °C under 5% CO_2. The effector cells were mixed the sensitized target cells at 25:1, added to each well of a 96-well round-bottom microplate, then incubated for 6 h at 37 °C. After centrifugation at 1500 rpm for 10 min, 100 μl of supernatant was collected and transferred to a fresh 96-well flat-bottomed plate, followed by the addition of 100 μl/well of LDH assay reagent. The mixture was allowed to incubate for 15 min at 37 °C after which the OD_{490} was measured. Spontaneous release of LDH was determined using samples prepared from target cells cultured in medium alone; maximum LDH release was measured using samples prepared by lysis of target cells in medium containing 1% (v/v) Triton X-100. The CTL was calculated using the following equation: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. All experiments were performed in triplicate.

2.9. Statistical analysis

Statistical analysis of the data was performed using SPSS 11.5 software; $p < 0.05$ and $p < 0.01$ were defined as statistically significant and statistically very significant, respectively.

3. Results

3.1. In vitro expression of pVAX1-(PEDV-S), pVAX1-(PEDV-S1) and pVAX1-(IL-18)

Sequence analysis of the subcloned pVAX1-(PEDV-S), pVAX1-(PEDV-S1) and pVAX1-(IL-18) showed 100% identity with GenBank accession numbers AF353511.1 (S and S1) and U58142 (IL-18), respectively. We demonstrated by immunofluorescence assays that the pVAX1-(PEDV-S), pVAX1-(PEDV-S1) and pVAX1-(IL-18) plasmids were successfully transformed into the BHK-21 cells and expressed (Fig. 1).

3.2. T lymphocyte proliferation in blood and spleen

The specificities of blood and spleen T lymphocytes stimulated with purified recombinant PEDV-S and -S1 were analyzed with MTT assays. Analyses using PBMC showed that at 14–42 dpi, the proliferation in mice immunized with pVAX1-(PEDV-S1) and pVAX1-(IL-18) was highly significant ($p < 0.01$) relative to PBS and pVAX1-(IL-18) controls (Fig. 2A). Between 28 and 42 dpi, proliferation of PBMC from mice immunized with pVAX1-(PEDV-S1) plus pVAX1-(IL-18) was significantly higher ($p < 0.05$) than proliferation in mice immunized with pVAX1-(PEDV-S1) alone; no significant differences ($p > 0.05$) were observed between the groups receiving pVAX1-(PEDV-S) or pVAX1-(PEDV-S1).

As shown in Fig. 2B, by 14 dpi the proliferation levels of spleen T lymphocytes treated with pVAX1-(PEDV-S) were highly significant ($p < 0.01$) relative to PBS or pVAX1-(IL-18) controls. At 28 dpi, the proliferation levels using pVAX1-(PEDV-S1) in the presence of pVAX1-(IL-18) were also highly significant ($p < 0.01$). By 42 dpi, changes in proliferation levels relative to controls remained highly significant ($p < 0.01$) using pVAX1-(PEDV-S1) with pVAX1-(IL-18); however, no significant differences ($p > 0.05$) were observed between control mice and those immunized with pVAX1-(PEDV-S1) alone.

3.3. Changes in CD4+ and CD8+ T lymphocytes

Flow cytometry data showed that the levels of CD4+ and CD8+ T lymphocytes in peripheral blood gradually increased beginning at 14 dpi and extended through 28 dpi. However, by 42 dpi, T lymphocyte populations in question decreased to control levels (Fig. 3A). The levels of CD4+ cells in mice co-immunized with pVAX1-(IL-18) were significantly higher ($p < 0.01$) than those in mice administered pVAX1-(PEDV-S1) only at 28 dpi. Between 14 and 28 dpi, the number of CD4+ T cells from mice immunized with pVAX1-(PEDV-S1) and pVAX1-(IL-18) was significantly higher ($p < 0.05$) than mice immunized with vaccine only. In contrast, this difference was not significant ($p > 0.05$) at 42 dpi. At 28 dpi, the levels of CD8+ cells in mice co-immunized with pVAX1-(PEDV-S1) and pVAX1-(IL-18) were significantly higher ($p < 0.01$) than those in mice administered pVAX1-(PEDV-S1) only.

When spleen CD4+ lymphocyte numbers were examined by flow cytometry (Fig. 3B) the results again showed that the group receiving pVAX1-(PEDV-S1) and pVAX1-(IL-18) had a highly significant impact ($p < 0.01$) on proliferation at 28–42 dpi where proliferation peaked at 28 dpi and decreased thereafter. Analyses of spleen-derived CD8+ T cells in animals treated with

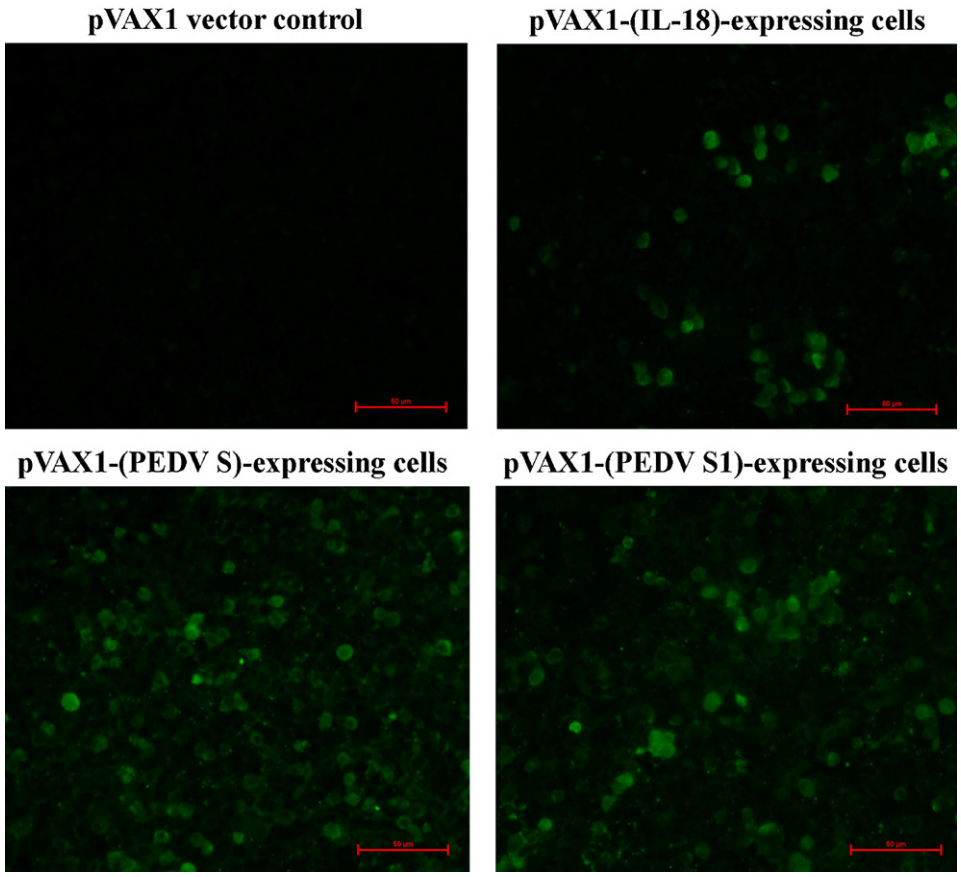

Fig. 1. Immunofluorescence analysis of cells transfected with recombinant plasmids. BHK-21 cells were transfected with pVAX, pVAX1-(IL-18), pVAX1-(PEDV-S) or pVAX1-(PEDV-S1). Transient expression of proteins within the transfected cells was detected with anti-PEDV antibody [pVAX, pVAX1-(PEDV-S) and pVAX1-(PEDV-S1)] or with anti-IL-18 antibody [pVAX1-(IL-18)]. The green signals reflect positive expression of proteins of interest. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pVAX1-(PEDV-S1) alone compared with those treated with pVAX1-(PEDV-S1)/pVAX1-(IL-18) showed significant changes ($p < 0.05$) at both 14 and 42 dpi.

3.4. Antibody detection in immunized mice

Serum antibody levels against PEDV were examined in mice treated with plasmid DNA using an indirect ELISA (Fig. 4). In general, between 7 and 42 dpi, the levels of PEDV antibodies in mice immunized with pVAX1-(PEDV-S1) and pVAX1-(IL-18) were significantly higher ($p < 0.01$) than those receiving PBS or empty vector. Also, at 21–42 dpi antibody levels induced by the combination of pVAX1-(PEDV-S1) and pVAX1-(IL-18) were significantly higher ($p < 0.01$) than those induced by pVAX1-(PEDV-S1) alone. Over the same period, the level of PEDV antibody in mice immunized with pVAX1-(PEDV-S) was highly significant ($p < 0.01$) relative to PBS and empty vector. Also, antibody levels induced by pVAX1-(PEDV-S) were highly significant ($p < 0.01$) relative to those induced by pVAX1-(PEDV-S1) alone beginning at 21 dpi; by 28–42 dpi, this significance decreased somewhat ($p < 0.05$).

3.5. Viral neutralization assay

Virus neutralizing assays showed that the serum from mice treated with pVAX1-(PEDV-S1) plus pVAX1-(IL-18) developed 1:40 and 1:20 neutralizing antibodies that significantly ($p < 0.01$) neutralized the virus relative to pVAX1-(PEDV-S1) only and control immunized mice (Fig. 5). Significant neutralization was observed at serum dilutions of both 1:20 and 1:40.

3.6. Changes in the levels of serum IFN-γ and IL-4 in immunized mice

Changes in serum IFN-γ and IL-4 levels in immunized mice were analyzed using ELISA. The results showed that the levels of IFN-γ in mice treated with pVAX1-(PEDV-S), pVAX1-(PEDV-S1), pVAX1-(IL-18) or the pVAX1-(PEDV-S1)/pVAX1-(IL-18) combination were higher than in mice treated with PBS or pVAX1. The levels of IFN-γ induced by pVAX1-(PEDV-S1) plus pVAX1-(IL-18) were significantly higher than other groups ($p < 0.01$) (Table 1); however, they did not match the additive effects of pVAX1-(IL-18) and pVAX1-(PEDV-S1) given separately. In like manner, approximately 2× increases were observed in the levels of IL-4 in all treatment groups except for pVAX1-(PEDV-S) which exhibited 4× increases over controls and pVAX1-(PEDV-S1) which did not induce any IL4 production.

3.7. Activity of CTL in blood and spleen

Cytotoxicity was analyzed using the LDH release assay. The results showed that CTL function in the peripheral blood of the mice treated with pVAX1-(PEDV-S1) in combination with pVAX1-(IL-18) was significantly higher than that observed with pVAX1-(PEDV-S1) alone ($p < 0.01$). However, as with other studies, these values were mirrored by the additive effects of pVAX1-(PEDV-S1) and pVAX1-(IL-18) given separately. In like manner, CTL function in spleen cells treated with pVAX1-(PEDV-S1) and pVAX1-(IL-18) was significantly greater ($p < 0.01$) than spleen cells from mice treated with pVAX1-(PEDV-S1) alone (Fig. 6).

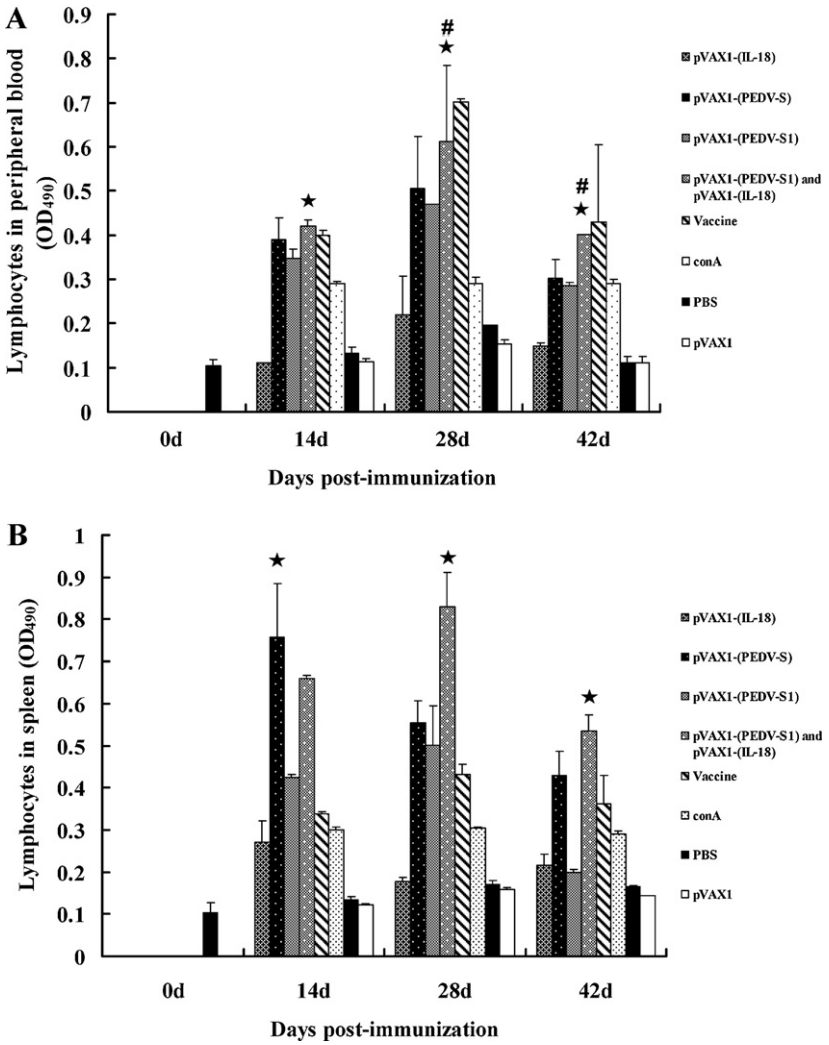

Fig. 2. Changes in T lymphocyte numbers in the peripheral blood and spleens of immunized mice. Mice were immunized with pVAX1-(IL-18), pVAX1-(PEDV-S), pVAX1-(PEDV-S1) and a combination of pVAX1-(PEDV-S1) and pVAX-(IL-18). In addition the attenuated PEDV vaccine, Con A, the empty vector pVAX1, and PBS were used as controls. The proliferation of T lymphocytes in mouse peripheral blood (A) and spleen (B) was analyzed by conventional MTT assays using recombinant PEDV-S1 protein and Con A as stimulating agents. The *y*-axis represents the lymphocyte proliferation index (OD_{490}). $^{*}p < 0.01$ (highly significant), compared with PBS group and pVAX1 group; $^{\#}p < 0.05$ (significant), compared with pVAX1-(PEDV-S1) immunized group.

4. Discussion

Interleukin-18 is a cytokine known to improve the proliferation of T cells, and enhance activities of NK cells and CTL (Micallef et al., 1996; Okamura et al., 1995; Tsutsui et al., 1997; Matsui et al., 1997). Furthermore, in porcine mucosal tissues IL-18 may advance immune responses to invading pathogens (Foss et al., 2001). As such, we hypothesized that IL-18 might act as a suitable adjuvant to help protect against a Th1 inducing pathogen, i.e. PEDV. For this reason, we evaluated synergistic effects between the PEDV-S1 and pIL-18 by means of DNA vaccination. Preliminary experiments demonstrated via immunofluorescence assays that the cloned and transiently transfected S and S1 genes along with the pIL-18 gene were indeed capable of expressing the protein constructs in BHK-21 mouse cells.

Cytotoxic T-cell activities and IgG levels can provide readouts for immune stimulation and protect hosts from virus infection by neutralizing viral antigens, respectively. In this study, spleen and blood-derived lymphocytes from immunized mice clearly showed that co-immunization with pVAX1-(PEDV-S1) and pVAX1-(IL-18) significantly induced T cell proliferation relative to controls (<0.01). Cytotoxic T-cell activity was also validated indirectly by monitoring cell lysis through increased LDH levels resulting from damaged cells (Yokozawa et al., 2000). Elevated LDH levels were observed in all but control treatments where the highest activities occurred in pVAX1-(PEDV-S1) and pVAX1-(IL-18) immunized mice. Changes in the serum IgG levels in particular between 7 and 35 dpi clearly showed that the plasmid constructs induced effective humoral responses capable of neutralizing the virus and that this neutralizing effect was strongest for the full-length S protein. However, unexpectedly, pVAX1-(IL-18) functioned only in tandem with pVAX1-(PEDV-S1) to affect both proliferative and virus neutralizing responses but exerted no adjuvant effects. This is likely the result of different and unrelated mechanisms that gave rise to these changes.

It is well accepted that following polarization, Th1 cells secret IFN-γ and IL-2 that mediate cellular immune responses, and Th2 cells secret IL-4 and IL-10 that help facilitate humoral immune responses where IFN-γ and IL-4 levels are inversely regulated. In the studies presented herein, the levels of IFN-γ were significantly elevated in all non-control treatment groups. Unexpectedly, the levels of IL-4 though low, were also elevated relative to controls

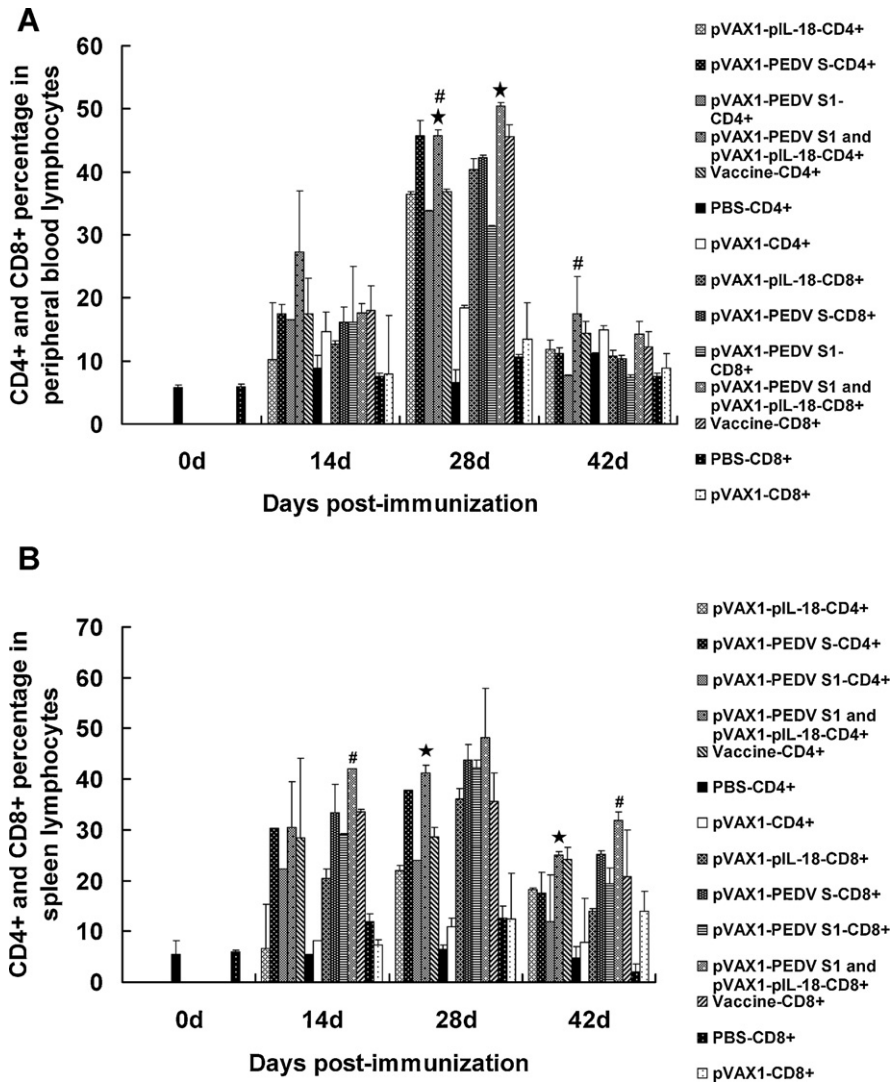

Fig. 3. Changes in CD4+ and CD8+ T lymphocytes in the peripheral blood and spleens of immunized mice. Lymphocytes were collected from mice treated with, pVAX1-(IL-18), pVAX1-(PEDV-S), pVAX1-(PEDV-S1) or a combination of pVAX1-(PEDV-S1) and pVAX-(IL-18). The attenuated PEDV vaccine, Con A, the empty vector pVAX1, and PBS were used as controls. Cells were subjected to flow cytometry to assess the numbers of CD4+ and CD8+ T lymphocytes in the blood (A) and spleens (B) of treated mice. In Fig 3A, $^{\star}p < 0.01$ (highly significant) compared with pVAX1-(PEDV-S1) treated animals; $^{\#}p < 0.05$ (significant), compared with attenuated PEDV vaccine. In (B), $^{\star}p < 0.01$ (highly significant) compared with the PBS and pVAX1 groups; $^{\#}p < 0.05$ (significant), relative to pVAX1-(PEDV-S1) group and pVAX1-(IL-18) group.

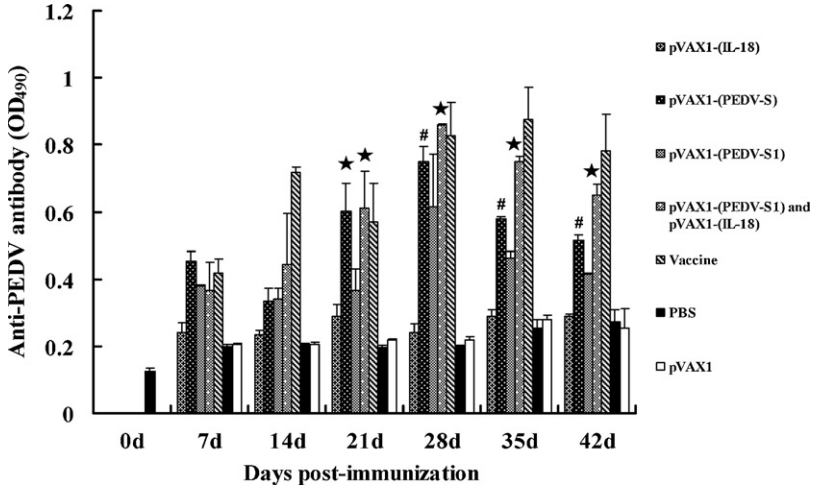

Fig. 4. Anti-PEDV antibody levels in mice treated with pVAX1-(PEDV-S1) and pVAX1-(IL-18). Anti-PEDV serum antibodies in pVAX1-(PEDV-S), pVAX1-(PEDV-S1) and/or pVAX1-(IL-18) immunized mice were detected by indirect ELISA at different time points following the injection of the plasmid DNAs. The OD_{490} was monitored as a function of time over a period of 42 days. $^{\star}p < 0.01$ (highly significant), compared with PBS, pVAX1 and pVAX1-(PEDV-S1) treatment groups; $^{\#}p < 0.05$ (significant), relative to PBS, pVAX1 and pVAX1-(PEDV-S1) groups.

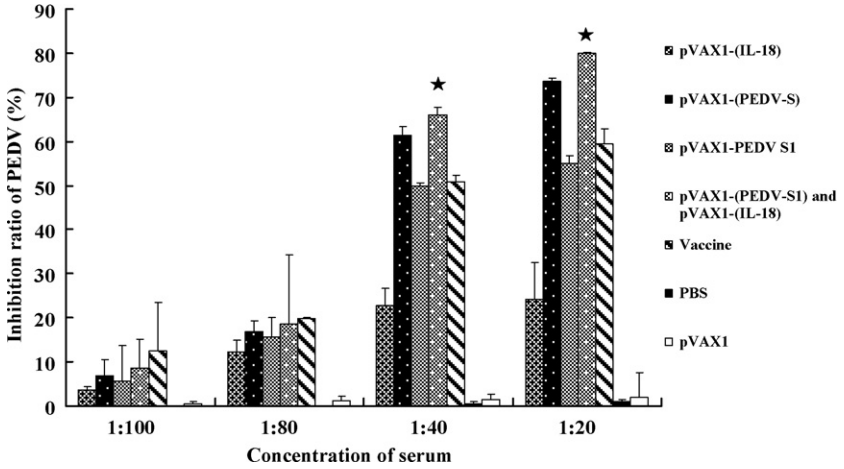

Fig. 5. Levels of neutralizing antibodies in mice treated with pVAX1-(PEDV-S1) and pVAX1-(IL-18). Anti-PEDV antibodies in immunized mice were detected by indirect ELISA at different serum dilutions following the injection of pVAX1-(IL-18), pVAX1-(PEDV-S), pVAX1-(PEDV-S1) and a combination of pVAX1-(PEDV-S1) and pVAX-(IL-18). The attenuated PEDV vaccine, Con A the empty vector pVAX1, and PBS were used as controls. The OD_{490} was monitored as a function of time over a period of 42 days. $^*p < 0.01$ (highly significant) relative to the pVAX1-(PEDV-S1) only group.

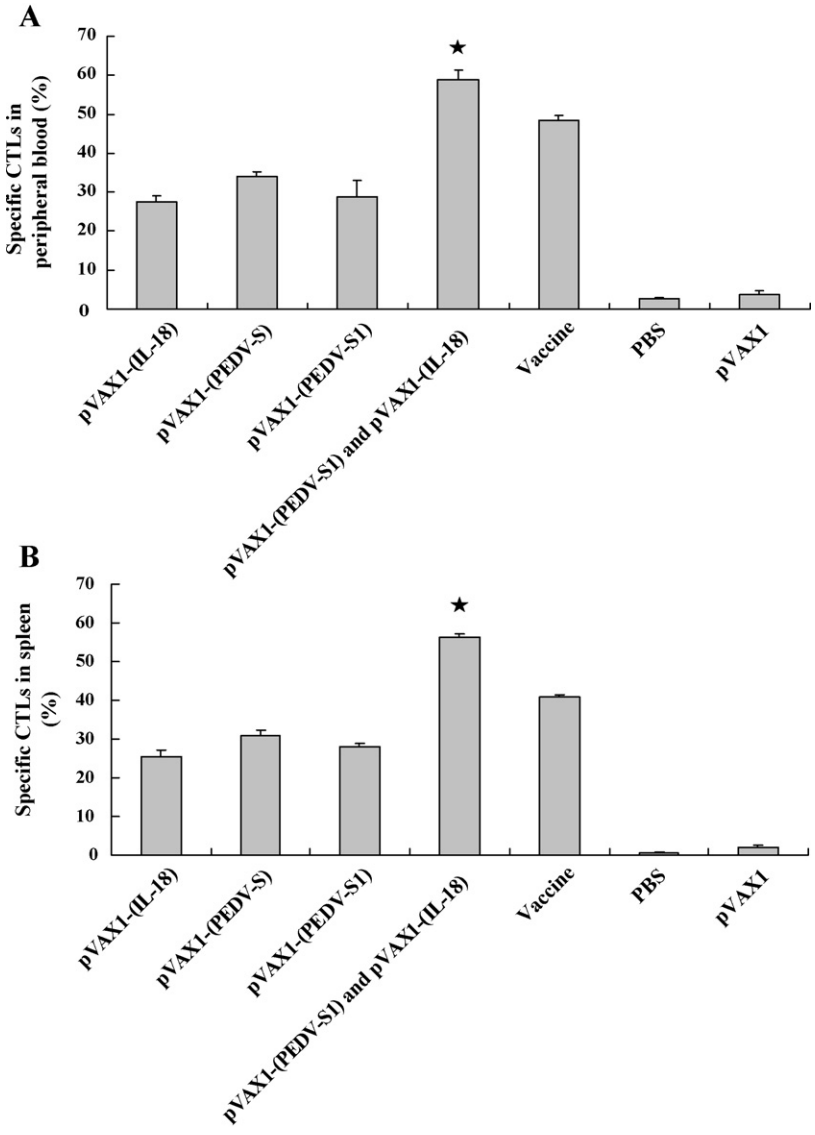

Fig. 6. Activity of CTLs in the peripheral blood and spleen. Cytotoxicity was analyzed using the LDH release assay. The release of LDH which is directly proportional to CTL activity was monitored as a function of time over period of 42 days. $^*p < 0.01$ (highly significant) relative to the pVAX1-(PEDV-S1) immunized group.

in all treatment groups except animals given pVAX1-(PEDV-S1) which generated no IL4 response. The dual elevation of IFN-γ and IL-4 has been observed elsewhere in GI nematode-infected cattle (Canals et al., 1997) and in cattle infected with the bovine viral diarrhea virus (Waldvogel et al., 2000) or vaccinated against the bovine respiratory syncytial virus (Miao et al., 2004). It is still unclear as to why such a deviation from the norm occurred here; however, some studies suggest that the level of infection and therefore the level of antigen presentation can affect Th1/Th2 profiles (Claerebout et al., 2005). These data in conjunction with virus neutralization studies suggest that IFN-γ does not play a major role in protection.

To the best of our knowledge, this is the first time that the immunological efficacies triggered by full-length S protein or the S1 portion of PEDV were compared using a mouse model. In contrast to our work on TEGV (Ren et al., 2006), results presented here showed that the full-length S gene unilaterally induced a better immune response than the truncated form of the protein; however, this may result from poor protein folding or less than optimal presentation of the shortened form to the host immune system. Future studies will examine this phenomenon more closely while investigating additional cytokines that enhance antibody production rather than Th1 type cytokines before attempting to transfer this technology to studies in swine.

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