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Research Article

The DNA Vaccine Combining the Adjuvant Porcine IL-12 with the Spike Gene of Transmissible Gastroenteritis Virus Enhances the Immune Response in Swine

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Abstract

Transmissible gastroenteritis (TGE), cause by transmissible gastroenteritis virus (TGEV), is an acute digestive and highly infectious disease in piglets. In this study, porcine interleukin-12 immunological adjuvant combined with a DNA vaccine bearing the TGEV-S1 gene was used to immunize piglets, and assessed the immune response of piglets. The results showed that $CD4^+$ and $CD8^+CD28^+$ T lymphocytes were significantly increased in immunized piglets compared to control groups. In addition, the observed increase of IFN-γ suggested that T helper cells tended to convert to Th1 cells during immune response. HE stain and indirect immunofluorescence assays indicated obvious differences between the immunized piglets and the controls, suggesting that immunized piglets suffered less pathological changes relative to piglets in the control groups. Moreover, the levels of anti-TGEV and neutralizing antibodies in serum also indicated an effective immune response in the immunized piglets by which the pathogen was rapidly controlled. Altogether, our results suggest that the combination of pVAX1-(pIL-12) and pVAX1-(TGEV-S1) in a vaccine is capable of producing a significantly enhanced immune response in piglets.

Keywords: *Transmissible gastroenteritis virus, Spike protein, DNA vaccine, porcine interleukin-12 gene, Immunologic adjuvant*

Adjuvant Domuz IL-12 ile Kombine Transmissible Gastroenteritis Virüs Spike Geni Taşıyan DNA Aşı Domuzlarda Bağışıklık Yanıtı İyileştirir

Öz

Transmissible gastroenteritis virüs (TGEV)'un neden olduğu Transmissible gastroenteritis (TGE) akut sindirim sistemi hastalığı olup domuzlarda oldukça enfeksiyöz bir hastalıktır. Bu çalışmada, TGEV-S1 geni taşıyan DNA aşısı ile kombine olarak domuz interlökin-12 immunolojik adjuvant domuzları immunize etmek amacıyla kullanıldı ve domuzlarda meydana gelen immun yanıt değerlendirildi. Sonuçlar, kontrol grubu ile karşılaştırıldığında immunize edilen domuzlarda CD4+ ve CD8+CD28+ T lenfositlerin anlamlı derecede arttığını gösterdi. Ayrıca, artan IFN-γ immun yanıt süresince yardımcı T lenfositlerin Th1 hücrelerine dönüştüğüne işaret etmekteydi. HE boyaması ve indirek immunofloresans tekniği immunize edilenler ile kontrol arasında bariz farkların olduğunu gösterdi. Bu durum immunize edilen domuzların kontrol grubundakine oranla daha az patolojik değişikliklere maruz kaldığına işaret etmekteydi. Serumdaki anti-TEGV seviyesi ve nötralize edici antikorların miktarı immunize edilen domuzlarda daha etkili immun yanıtın oluştuğunu ve patojenin hızlı bir şekilde kontrol altına alındığını gösterdi. Elde edilen sonuçlar, pVAX1-(pIL-12) ve pVAX1-(TGEV-S1)'nin aşıda kullanılmasının domuzlarda immun yanıtı anlamlı oranda iyileştireceğini göstermiştir.

Anahtar sözcükler: *Transmissible gastroenteritis virüs, Spike protein, DNA aşı, domuz interlökin-12 geni, İmmunolojik adjuvant*

INTRODUCTION

Transmissible gastroenteritis (TGE), cause by transmissible gastroenteritis virus (TGEV), is an acute digestive and highly infectious disease in pigs, with vomiting, severe diarrhea, and dehydration being classical symptoms. Previous study showed that pigs of all breeds and ages are susceptible, but TGEV mainly infects suckling piglets

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and being frequently accompanied by other pathogens. Consequently, the mortality rate of suckling piglets might reach 100%. The mortality rate in older pigs will be lower, the absence of effective preventive methods greatly affected the pig industry, and causing significant economic loss [1-3].

TGEV is an enveloped coronavirus of the subfamily *Coronavirinae* of the family *Coronaviridae*. The subfamily *Coronavirinae* consists of four genera: the alpha-, beta, gamma-, and delta-coronavirus. The transmissible gastroenteritis virus (TGEV) belongs to the alphacoronavirus genus, which is characterized by a single-stranded positive-sense RNA genome [4,5]. The alphacoronavirus genome encodes for four structural proteins: the spike (S), nucleocapsid (N), membrane (M), and envelope (E) proteins and for three non-structural proteins (by the open reading frames ORF1, ORF3 and ORF7). The virion surface is composed of a number of spike (S) proteins which contain four antigenic sites. In this study, the TGEV-S1 gene was used in our DNA vaccine against TGEV. This gene encodes for a spike (S) protein, which contains antigen sites known to induce the production of neutralizing antibodies [6,7].

Currently, several countries have carried out monovalent and combined vaccines against TGE, but the inactivated vaccine does not induce an effective immune response and the attenuated vaccine has relatively high risks associated with virulence factors. Thus, a novel and effective vaccine to prevent TGEV needs to be developed [5]. The use of DNA vaccines may be an effective approach to protect swine from TGEV, and number of studies have suggested that the eukaryotic expression of plasmids could induce the immune response [8,9]. Moreover, many reports have also suggested that the activity of cytokine adjuvant can further enhance this effect. Cytokine interleukin (IL)-12 is known to regulate and promote cellular immunity and therefore, has attracted the attention of researchers as a potential immune-adjuvant [10,11]. In this study, the potential of the DNA vaccine bearing the TGEV S1 gene and the adjuvant pIL12 to prevent TGEV infection have been assessed in piglets, that may provides a theoretical basis for further research on DNA vaccines combined with immune-adjuvant.

MATERIAL and METHODS

Plasmid Construction

The full-length S gene of TGEV strain PUR46 was used as PCR template. PCR was carried out using a forward primer (a) (5′-GGGGAAGCTTGCCACCATGAAAAAACTATTTGTG-3′), and a reverse primer (b) (5′-CCCCGAATTCTTAGTTAGTTT GTCTAATA-3′) which contained a *Hin*dIII (a) and *Eco*RI (b) restriction enzyme site (underlined), respectively. The PCR parameters for TGEV-S1 gene amplification (primers P1/P2) were as follows: 95°C for 5 min, 30 cycles of 94°C for 1 min, 45.7°C for 1 min, and 72°C for 2 min followed by a final extension of 72°C for 10 min. Products were purified and subjected to restriction enzyme digestion, then ligated into the appropriate pVAX1 (Invitrogen, USA) eukaryotic expression vector. Similarly plasmid pVAX1-(pIL-12) ligated into the pVAX1 (Invitrogen, USA) eukaryotic expression vector by polymerase chain reaction (PCR), splicing by overlap extension (SOE)-PCR and restriction enzyme digestion, that had been constructed in our previous study [12].

Immunofluorescence Assays

The pVAX1, pVAX1-(pIL-12), or pVAX1-(TGEV-S1) were diluted at 1 μg/μL by 0.1M PBS (phosphate buffer saline), and the plasmids (1 μg) were transfected into BHK-21 cells by Lipofectamine2000 (Invitrogen) respectively, then cultured about 24h (5% CO2, 37°C), according to the method as described previously [12], the cells were fixed with 4% paraformaldehyde (w/v) and then incubated with anti-pIL-12 (p40) or anti-TGEV-S1 antibody (1:200). Finally the cells were incubated with fluorescein isothiocyanate-labelled (FITC) goat-anti rabbit immunoglobulin (1:200) in darkness, then, the green fluorescences were detected by fluorescence microscope (Leica, Germany).

Immunization

The seven-day-old piglets divided into five groups (n=4, *Table 1)*. All piglets were injected with 500 μL 0.2% lidocaine hydrochloride in the cervical muscle. After 15 min, the piglets were injected with 500 μL PBS (no treatment control group), or 500 μg plasmid (pVAX1, empty vector control group), (pVAX1-(pIL-12), pIL-12 treatment group), (pVAX1-(TGEV-S1), TGEV-S1 treatment group) in 500 μL

Table 1. *Experimental groups and immune interval*

Group	Number of Piglets	Vaccine Category	Immunizing Dose	Interval of Treatments
A	4	PBS	500 μL	7-days-old, 21-days-old, 35-days-old
B	4	pVAX1	500 μg	7-days-old, 21-days-old, 35-days-old
C	4	pVAX1-(pIL-12)	500 μg	7-days-old, 21-days-old, 35-days-old
D	4	pVAX1-(TGEV-S1)	500 μg	7-days-old, 21-days-old, 35-days-old
E	4	pVAX1-(pIL-12) + pVAX1-(TGEV-S1)	500 μg 500 μg	7-days-old, 21-days-old, 35-days-old

The five groups include PBS (A), pVAX1 (B), pVAX-pIL-12 (C), pVAX1-(TGEV-S1) (D) and pVAX1-(pIL-12) + pVAX1-(TGEV-S1) (E). number of piglets, vaccine category, and Immunizing dose are indicated

PBS, or 500 µg plasmid pVAX1-(pIL-12) + 500 µg plasmid pVAX1-(TGEV-S1) (combined pIL-12-TGEV-S1 treatment group) in 500 µl PBS respectively. The piglets were injected three times at 2-week intervals (7-days, 21-days, 35-days of age). At 42-days of age, the piglets were exposed to a virus challenge after which blood samples were collected for analysis [13,14], and collected the samples of blood and histology at 47-days. The animal study was performed in compliance with institutional guidelines and animal welfare. All animal experiments were approved by the Animal Ethics Committee of Northeast Agricultural University of China (Protocol number: SRM 16).

Analysis of T lymphocytes

After the lymphocytes were separated from peripheral blood, a lymphocyte proliferation assay was undertaken, as previously described [15,16]. In summary, the lymphocytes were diluted in RPMI1640 (Gibco, USA) and added to 96-well plates (10^6 cells in 50 µL/well). Then, recombinant TGEV-S1 protein (20 µg/mL) and concanavalin A (Sigma, Germany) were added as stimulating agents. After 48 h, WST-1 (6.5 mg/mL, 10 µL/well, Boster, USA) was added to each well. At this stage, samples were left to incubate for 4 h at 37°C in 5% CO_2, after which the viability of lymphocytes was assessed at OD 450 nm.

The peripheral blood lymphocytes (PBL) were re-suspended to 1×10^7 cells/mL and incubated separately with an anti-CD4+ T cell antibody (FITC-labelled) (BD, USA) and an anti-CD8+ T cell antibody (PE-labelled) (BD, USA), and simultaneously with both anti-CD8+ T cell antibody (PE-labelled) and an anti-CD28+ T cell antibody (APC-labelled). The incubation period lasted for 30 min (5% CO_2, 37°C). The specific lymphocytes subgroups were detected with flow cytometric analysis (BD, USA).

Detection of TGEV Antibody

The TGEV-specific antibody (IgG, IgA) from serum of vaccinated piglets were detected by enzyme-linked immunosorbent assay (ELISA), after conjugation with recombinant TGEV S1 protein (50 µg/ml diluted with 0.05 M $NaHCO_3$) in the ELISA plate overnight at 4°C [17], blocking for 1 h at 37°C and incubated with sera samples from piglets for 1 h at 37°C, then added Horseradish peroxidase -labelled goat anti-pig IgG (Proteintech, China) and the Horseradish peroxidase -labelled goat anti-pig IgA (Proteintech, China) respectively for 1 h at 37°C [18-20]. Plates were measured at OD 490 nm.

The efficacy of the TGEV-neutralizing antibodies in the serum of vaccinated piglets was determined by a viral neutralization test [21]. In summary, piglets' sera samples (double dilution from 1:20 to 1:160) were mixed with TGEV viral diluent (1 mL) of ($10^{-4.67}$/0.1 mL TCID50) at 37°C. Samples were left to incubate for 1 h, then added to a monolayer of swine testis (ST) cells in 24-well plates (5% CO2, 37°C) to assess the cytopathic effects during a period of 48 to 60 h.

Detection of Cytokines

Levels of IL-4 and interferon-γ in serum were detected with test kits, in accordance with the manufacturer's instructions (YuanYe, China) [22,23]. Standard curve using a dilution series of IL-4 (256 pg/mL, 128 pg/mL, 64 pg/mL, 32 pg/mL, 16 pg/mL, 0 pg/mL) and IFN-γ (400 pg/mL, 200 pg/mL, 100 pg/mL, 50 pg/mL, 25 pg/mL, 0 pg/mL) were prepared. These solutions were then incubated in ELISA plates for 1 h at 37°C alongside sera samples from the piglets. Horseradish peroxidase-labelled goat anti-pig immunoglobulin was added to each well and measured at OD 450 nm. The serum concentrations of IL-4 and IFN-γ were estimated using the standard curves.

Histological Analysis

Frozen sections of tissues were analyzed using indirect immunofluorescence and hematoxylin and eosin staining (HE) assays. In summary, samples were embedded in optimal cutting temperature (OCT) compound and sectioned (7 µm thick). The indirect immunofluorescence assay was based on the use of the polyclonal antibody of TGEV and fluorescein isothiocyanate-labelled (FITC) goat-anti rabbit immunoglobulin, and nucleuses were dyed by propidine iodide (PI). The HE stain was applied according to the standard method [24]. Tissue sections were observed under a microscope (Leica DM2000, Germany).

Statistical Analysis

Data analysis was performed by SPSS V13.0 software; "p" was defined by one-factor analysis of variance statistically.

RESULTS

The recombine pVAX1-(pIL-12) and pVAX1-(TGEV-S1) plasmids could expressed in mammalian cells, through observed the green fluorescences in *Fig. 1*A,B,C. The proliferation and differentiation of T lymphocytes were an important stage in immune response, so the T lymphocyte proliferation assay could reflect an immune state of organism. Results showed that *(Fig. 2A)*, T lymphocytes were stimulated with specific antigen TGEV-S1 protein and non-specific antigen ConA (not shown in the figure as a comparison), the TGEV-S1 and the pIL-12 + TGEV-S1 treatment groups had significantly increased, which might be due to a beneficial reaction and a favorable state of T lymphocytes.

The $CD4^+$T and $CD8^+$T lymphocytes were very valuable in immune system, and have different marker on the surface of T cells. The changes in $CD4^+$T and $CD8^+$T lymphocytes in peripheral blood from piglets in all experimental groups were evaluated by flow cytometry *(Fig. 3)*. The number of $CD4^+$T lymphocytes in the pIL-12 + TGEV-S1 group

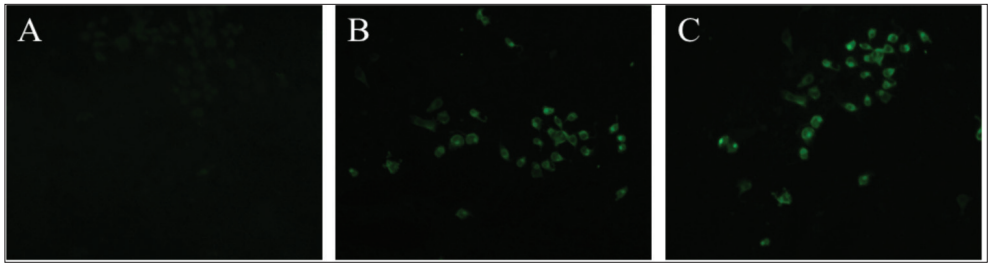

Fig 1. Immunofluorescence analysis of BHK-21 cells transfected with recombinant plasmids. BHK-21 cells were transfected with pVAX1-(pIL-12) and pVAX1-(TGEV-S1) respectively. (A) pVAX1 vector control, (B) pVAX1-(pIL-12) plasmids, (C) pVAX1-(TGEV-S1) plasmids. Transient expression of proteins were detected with anti-pIL-12 or anti-TGEV-S1 antibody. The green fluorescences indicated positive protein expression

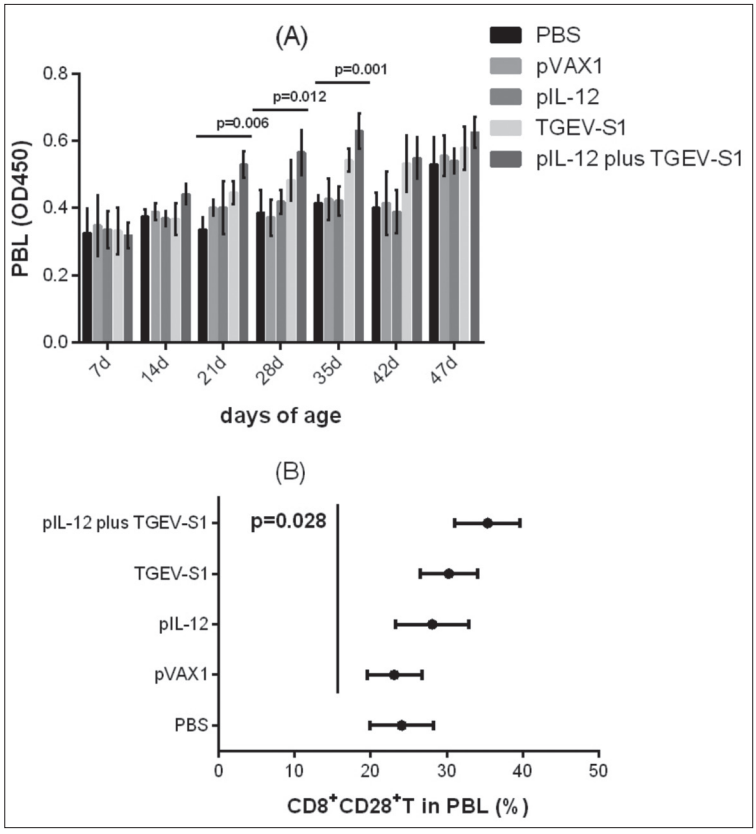

Fig 2. The proliferation of T lymphocytes and the quantity of $CD8^+CD28^+$T in peripheral blood. T lymphocytes proliferate in peripheral blood (PBL) of piglets (A) were analyzed by WST-1 assay. The y-coordinate represents the lymphocyte proliferate index (OD 450nm) in peripheral blood. The quantities of CD8+CD28+T in peripheral blood were determined by flow cytometry (B)

was considerably increased at 28 and 42 days of age. The number of $CD8^+$T lymphocytes, at 35 days of age, in the pIL-12 + TGEV-S1 group was also significantly increased, and at day 47, after virus challenge the number of $CD8^+$T lymphocytes in all groups were increased obviously, it would be an effective immune response by cytotoxic lymphocyte when the organism was infected by the virus.

Changes in $CD8^+CD28^+$T lymphocytes in peripheral blood from experimental animals at 35 days of age were also evaluated by flow cytometry *(Fig. 2B)*. The number of $CD8^+CD28^+$T lymphocytes in the pIL-12, TGEV-S1 and pIL-12 + TGEV-S1 groups showed a tendency to increase and the pIL-12 + TGEV-S1 group had a number of $CD8^+CD28^+$T lymphocytes significantly higher than those of the control groups.

The levels of TGEV-specific antibodies in the serum of immunized piglets were examined using an indirect ELISA assay *(Fig. 4A,B)*. In general, starting from 21 days of age, the TGEV-S1 and pIL-12 + TGEV-S1 groups showed a significant increase in IgG antibodies: Additionally, the IgG antibodies increased sharply in the same groups at day 47, after the exposure to the virus challenge test. However, the pIL-12 + TGEV-S1 and the TGEV-S1 groups did not differ significantly, between them regarding the IgG response in piglets. The IgA antibodies from the serum of immunized piglets at 35 days of age were significantly induced in the TGEV-S1 and the pIL-12 + TGEV-S1 groups *(Fig. 4B)* compared to other groups, although no difference was detected between the two treatment groups.

The levels of neutralizing antibody in serum of immunized piglets at 35 days of age were detected using virus neutralizing assays. The TGEV-S1 and pIL-12 + TGEV-S1 groups showed a more effective neutralizing antibody production *(Fig. 4C)*. Moreover, there was a significant difference between the TGEV-S1 and the pIL-12 + TGEV-S1 groups. The neutralizing antibody titers were 1:40.5 and 1:114.5 in the TGEV-S1 and pIL-12 + TGEV-S1 groups, respectively.

The levels of IFN-γ and IL-4 in different samples of serum, as analyzed by the ELISA method, are shown in *Fig. 5*. Starting at 21 days of age, the IFN-γ serum levels in the pIL-12, TGEV-S1 and pIL-12 + TGEV-S1 groups were significantly increased, The pIL-12 + TGEV-S1 group showed a noticeable increase in the IFN-γ serum level at day 35 and 42. The concentration of IL-4 did not vary significantly among groups.

The results of the HE stain and indirect immunofluorescence assays on frozen sections suggested significant differences

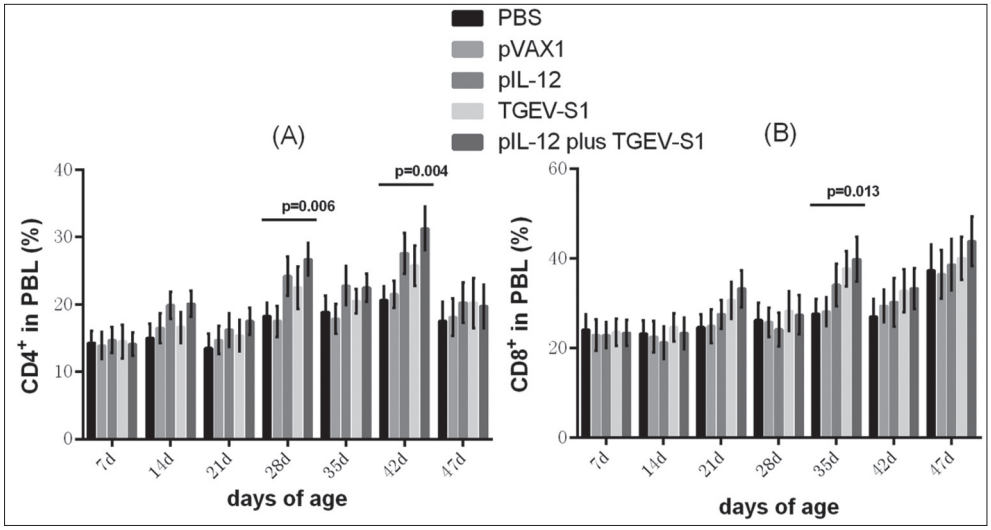

Fig 3. Changes of CD4⁺T and CD8⁺T in peripheral blood. Lymphocytes from the peripheral blood of piglets immunized with pVAX1-(TGEV-S1) and pVAX1-(pIL-12) were collected and subjected to flow cytometry to evaluate the proportions of CD4⁺T and CD8⁺T cells in peripheral blood lymphocytes (PBL)

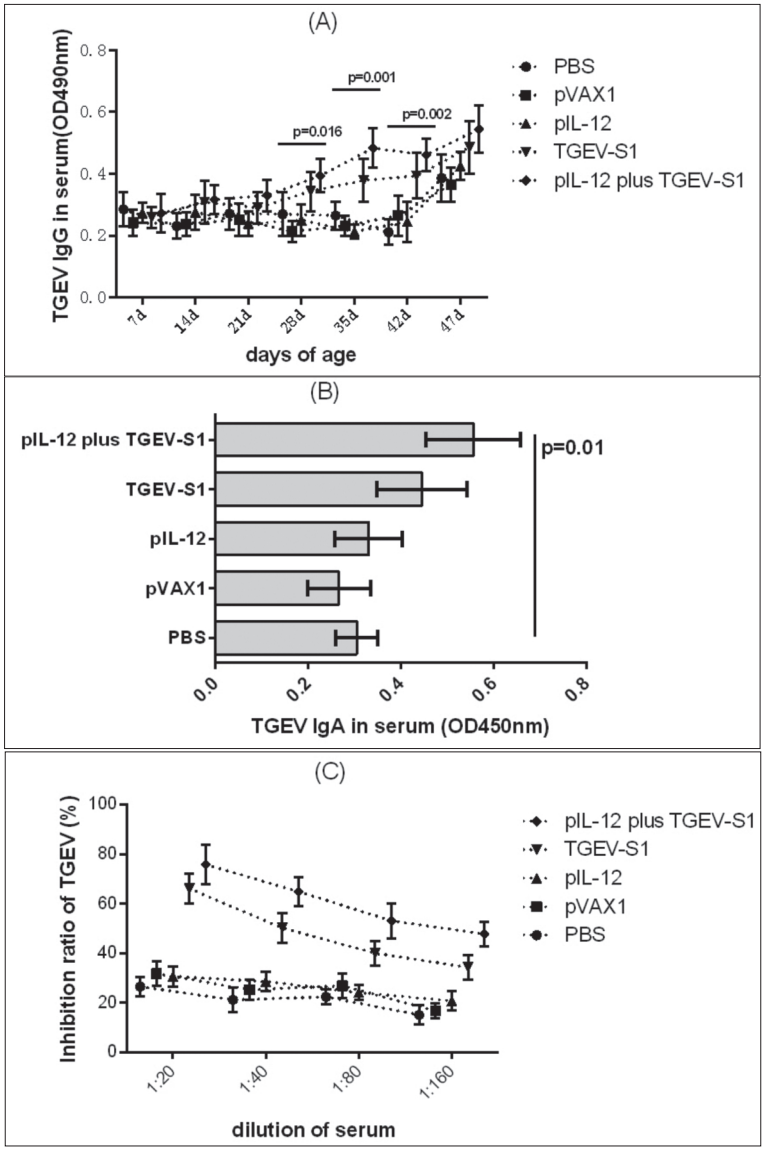

Fig 4. The specific antibody concentration of humoral immunity. The levels of Anti-TGEV IgG (A) and IgA (B) antibody in serum samples were detected by indirect ELISA. The levels of neutralizing antibodies in serum (C) were detected by virus neutralizing assays

(Fig. 6) regarding pathological change and viral replication rate after viral infection. The small intestine epithelium denaturation and endochylema vacuolization, some nucleus concentrate and smash or lysis, some inflammatory cells infiltration are conspicuous in HE stain of the control groups, and these pathological changes show an obvious weakening trend from A to E in *Fig. 6*. Moreover, these groups also have a different fluorescence signal obviously in the results of the indirect immunofluorescence, the red fluorescence represents nucleuses, and the green fluorescence indicated the content of viruses in the intestine, which also had an obvious decreasing trend from F to J in *Fig. 6*.

DISCUSSION

The S protein of TGEV has four antigenic epitopes near the N terminal portion (S1) [25]. The S1 gene has been reported to induce more impactful immune responses than the full-length S gene of TGEV in piglets [6]. Furthermore, the cytokine IL-12 is known to promote the differentiation of naive CD4⁺T cells into Th1, enhancing the activity of T cells and natural killer cells [26]. The cytokine IL-12 might also increase the production and activation of Th1-associated immunoglobulin (such as IgG2a) directly or through the effects of type-1 cytokines (such as IFN-γ) [27], and thus it has been widely used in nucleic acid vaccine research as an immunologic adjuvant [23,28,29]. In our study, the DNA vaccine for TGEV combining the TGEV S1 gene with pIL-12 as an immunologic adjuvant assessed its effect on the immune response in piglets.

T lymphocytes are main effector cells of the cellular and humoral immune response, which

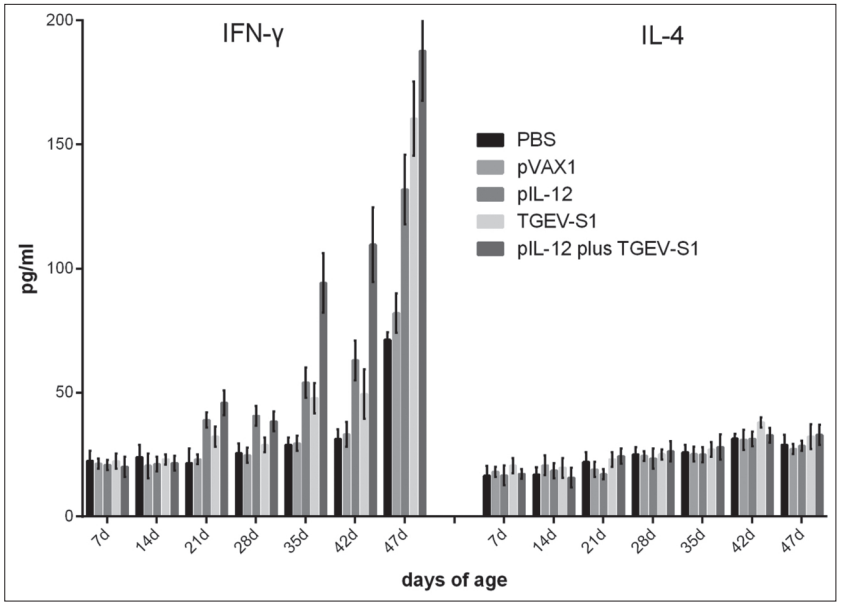

Fig 5. Changes of IFN-γ and IL-4 in serum. The levels of IFN-γ and IL-4 in serum samples were detected by ELISA

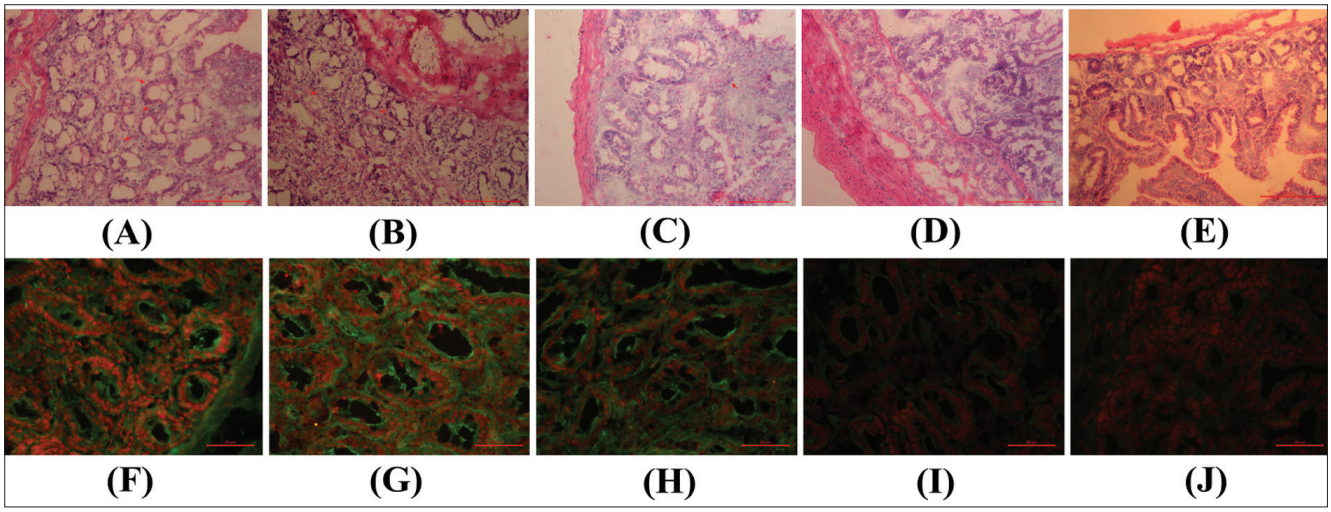

Fig 6. The result of froze sections in HE stain and indirect immunofluorescence. The froze sections of small intestines were treated with HE stain in PBS **(A)**, pVAX1 **(B)**, pIL-12 **(C)**, TGEV-S1 **(D)** and pIL-12 + TGEV-S1 **(E)** groups, and observed using 10×stage eyepiece and 20×stage objective. The froze sections of small intestines were detected by indirect immunofluorescence in PBS **(F)**, pVAX1 **(G)**, pIL-12 **(H)**, TGEV-S1 **(I)** and pIL-12 + TGEV-S1 **(J)** groups, and observed using 10×stage eyepiece and 40×stage objective

play an important role in the immunological regulation, including in the activation of effector T cells, helper T cells (Th) and cytotoxic T cells (Tc). The level of proliferation of T lymphocytes reflects the immunity level of an organism, after specific antigen stimulation. In this study, there was a conspicuous increase in the T lymphocytes in the TGEV-S1 and pIL-12 + TGEV-S1 groups.

$CD4^+$T lymphocytes are critical to the immune system. $CD4^+$T lymphocytes play a positive role in defense against the pathogen. The $CD8^+$T lymphocytes are another important subgroup. These are the pathogen "killer" of the immune system, which clean up the infected cells directly and mainly express in Tc cells. The T lymphocytes surface markers $CD8^+$ and $CD28^+$ represent the Tc cells [30,31]. All these T lymphocytes subgroups showed a degree of change in our study, among them, the increase in pIL-12 + TGEV-S1 group was particularly obvious. The $CD4^+$T and $CD8^+$T lymphocytes appeared a relative change when immune response attained a diverse stage. Furthermore, the $CD4^+$T lymphocytes could differentiate into Th1 and Th2, and secrete different cytokines. The IFN-γ and IL-4 proteins are markers for the Th1 and Th2 cells, respectively. In our study, the level of IFN-γ in the immunized piglets increased significantly, especially in pIL-12 + TGEV-S1 group, which did not occur with the level of IL-4. Consequently, Th1 cells were predominant in immune response [32,33].

The production of specific antibodies are important for humoral immune response. The specific antibodies might combine with the pathogen directly leading to pathogen devitalization or activate other defense pathways. In this

study, the levels of IgG and IgA antibodies indicate a more effective immune response in the TGEV-S1 and the pIL-12 + TGEV-S1 groups. The level of neutralizing antibodies also increased significantly in these two groups, especially the pIL-12 + TGEV-S1 group, to prevent pathogen spread.

In our report, the pIL-12 + TGEV-S1 group had the best cellular and humoral immune responses, except regarding the serum level of IL-4. The IFN-γ result indicates that the pIL-12 adjuvant mainly enhances the helper T cells differentiation into Th1. Th1 cells might induce the macrophage production and help the cytotoxic T lymphocytes ($CD8^+$ $CD28^+$) to eliminate intracellular pathogens. Moreover, the pIL-12 adjuvant showed increased levels of IgG3 and IgG2a antibody production in the serum [34], which may explain the high contents of specific antibody and neutralizing antibody in the serum of the pIL-12 + TGEV-S1 group. After the virus challenge, which initiated immune response, the pIL-12 + TGEV-S1 showed the most effective immune response to TGEV infection. It follows that pIL-12 was a advantageous immune adjuvant which could enhanced immune stimulation of antigen gene, induced the IFN-γ production, enhance the generation of cytotoxic T lymphocytes, promoted the cellular and humoral immune responses of piglets, and it could effectively improve the ability of TGEV S1 DNA vaccine to resist virus invasion, and contribute further application of DNA vaccine and pIL-12 immune adjuvant.

In summary, our study suggests that this newly developed DNA vaccine combining the adjuvant porcine IL-12 and the TGEV-S1 gene could enhance the immune response and prevent pathogen spreading through cellular and humoral immunity, that would be developed an effective means to prevent TGEV in swine.

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REFERENCES

1. Mou C, Zhu L, Xing X, Lin J, Yang Q: Immune responses induced by recombinant *Bacillus subtilis* expressing the spike protein of transmissible gastroenteritis virus in pigs. *Antiviral Res,* 131, 74-84, 2016. DOI: 10.1016/j.antiviral.2016.02.003

2. Gerdts V, Zakhartchouk A: Vaccines for porcine epidemic diarrhea virus and other swine coronaviruses. *Vet Microbiol,* 206, 45-51, 2017. DOI: 10.1016/j.vetmic.2016.11.029

3. Li P, Ren X: Reverse transcription loop-mediated isothermal amplification for rapid detection of transmissible gastroenteritis virus. *Currt Microbiol,* 62 (3): 1074-1080, 2010. DOI: 10.1007/s00284-010-9825-9

4. Lin CM, Gao X, Oka T, Vlasova AN, Esseili MA, Wang Q, Saif LJ: Antigenic relationships among porcine epidemic diarrhea virus and transmissible gastroenteritis virus strains. *J Virol,* 89 (6): 3332-3342, 2015. DOI: 10.1128/JVI.03196-14

5. Gelhaus S, Thaa B, Eschke K, Veit M, Schwegmann-Wessels C: Palmitoylation of the *Alphacoronavirus* TGEV spike protein S is essential for incorporation into virus-like particles but dispensable for S-M interaction. *Virology,* 464-465, 397-405, 2014. DOI: 10.1016/j.virol.2014.07.035

6. Ren XF: Monoclonal Antibody Against TGEV S 7F9. *Hybridoma,* 29 (4): 379-379, 2010. DOI: 10.1089/hyb.2010.0028.MAb

7. Meng FD, Zhao ZP, Li GX, Suo S, Shi N, Yin JC, Zarlenga D, Ren XF: Bacterial expression of antigenic sites A and D in the spike protein of transmissible gastroenteritis virus and evaluation of their inhibitory effects on viral infection. *Virus Genes,* 43 (3): 335-341, 2011. DOI: 10.1007/s11262-011-0637-1

8. Sheng Z, Gao N, Cui X, Fan D, Chen H, Wu N, Wei J, An J: Electroporation enhances protective immune response of a DNA vaccine against Japanese encephalitis in mice and pigs. *Vaccine,* 34 (47): 5751-5757, 2016. DOI: 10.1016/j.vaccine.2016.10.001

9. Wang C, Sun X, Suo S, Ren Y, Li X, Herrler G, Thiel V, Ren X: Phages bearing affinity peptides to severe acute respiratory syndromes-associated coronavirus differentiate this virus from other viruses. *J Clin Virol,* 57 (4): 305-310, 2013. DOI: 10.1016/j.jcv.2013.04.002

10. De Koker S, Van Hoecke L, De Beuckelaer A, Roose K, Deswarte K, Willart MA, Bogaert P, Naessens T, De Geest BG, Saelens X, Lambrecth BN, Grooten J: Inflammatory monocytes regulate Th1 oriented immunity to CpG adjuvanted protein vaccines through production of IL-12. *Sci Rep,* 7: 5986, 2017. DOI: 10.1038/s41598-017-06236-6

11. Jayanthi S, Koppolu BP, Nguyen KG, Smith SG, Felber BK, Kumar TKS, Zaharoff DA: Modulation of Interleukin-12 activity in the presence of heparin. *Sci Rep,* 7: 5360, 2017. DOI: 10.1038/s41598-017-05382-1

12. Li XL, Ren XF: Antibody against biologically active p40 subunit of porcine interleukin-12 expressed in *Escherichia coli. Hybridoma,* 29 (6): 489-494, 2010. DOI: 10.1089/hyb.2010.0074

13. Yu W, Grubor-Bauk B, Gargett T, Garrod T, Gowans EJ: A novel challenge model to evaluate the efficacy of hepatitis C virus vaccines in mice. *Vaccine,* 32 (27): 3409-3416, 2014. DOI: 10.1016/j.vaccine.2014.04.014

14. Wesley RD, Lager KM: Increased litter survival rates, reduced clinical illness and better lactogenic immunity against TGEV in gilts that were primed as neonates with porcine respiratory coronavirus (PRCV). *Vet Microbiol,* 95 (3): 175-186, 2003. DOI: 10.1016/S0378-1135(03)00150-0

15. Li GX, Shi N, Suo SQGW, Cui J, Zarlenga DT, Ren XF: Vaccination of mice with ORF5 plasmid DNA of PRRSV; enhanced effects by co-immunizing with porcine IL-15. *Immunol Invest,* 41 (3): 231-248, 2012. DOI: 10.3109/08820139.2011.614306

16. Suo SQGW, Ren YD, Li GX, Zarlenga D, Bu RE, Su DD, Li XL, Li PC, Meng FD, Wang C, Ren XF: Immune responses induced by DNA vaccines bearing Spike gene of PEDV combined with porcine IL-18. *Virus Res,* 167 (2): 259-266, 2012. DOI: 10.1016/j.virusres.2012.05.007

17. Meng F, Zhao Z, Li G, Suo S, Shi N, Yin J, Zarlenga D, Ren X: Bacterial expression of antigenic sites A and D in the spike protein of transmissible gastroenteritis virus and evaluation of their inhibitory effects on viral infection. *Virus Genes,* 43 (3): 335-341, 2011. DOI: 10.1007/s11262-011-0637-1

18. Shabani SH, Zakeri S, Salmanian AH, Amani J, Mehrizi AA, Snounou G, Nosten F, Andolina C, Mourtazavi Y, Djadid ND: Biological, immunological and functional properties of two novel multi-variant chimeric recombinant proteins of CSP antigens for vaccine development against *Plasmodium vivax* infection. *Mol Immunol,* 90, 158-171, 2017. DOI: 10.1016/j.molimm.2017.06.033

19. Denies S, Leyman B, Huysmans H, Combes F, Mc Cafferty S, Cicchelero L, Steppe M, De Temmerman J, Sanders NN: Evaluation of a xenogeneic vascular endothelial growth factor-2 vaccine in two preclinical metastatic tumor models in mice. *Cancer Immunol Immunother,* 66 (12): 1545-1555, 2017. DOI: 10.1007/s00262-017-2046-3

20. Dizaji NB, Basseri HR, Naddaf SR, Heidari M: Molecular characterization of calreticulin from Anopheles stephensi midgut cells and functional assay of the recombinant calreticulin with Plasmodium berghei ookinetes. *Gene,* 550 (2): 245-252, 2014. DOI: 10.1016/j.gene.2014.08.036

21. Pogranichnyy R, Yoon K, Harms P, Swenson S, Zimmerman J,

Sorden S: Characterization of immune response of young pigs to porcine circovirus type 2 infection. *Viral Immunol,* 13 (2): 143-153, 2000. DOI: 10.1089/vim.2000.13.143

22. Yazdanian M, Memarnejadian A, Mahdavi M, Motevalli F, Sadat SM, Vahabpour R, Khanahmad H, Soleimanjahi H, Budkowska A, Roohvand F: Evaluation of cellular responses for a chimeric HBsAg-HCV core DNA vaccine in BALB/c mice. *Adv Biomed Res,* 4:13, 2015. DOI: 10.4103/2277-9175.148296

23. Azizi H, Kazemi B, Bandehpour M, Mohebali M, Khamesipour A, Aryaeipour M, Yaghoobi H, Rokni MB: Modulation of the immune response to DNA Vaccine encoding gene of 8-kda subunit of *Echinococcus granulosus* antigen B using murine interleukin-12 plasmid in BALB/c mice. *Iran J Parasitol,* 11 (4): 480-489, 2016.

24. Sylla S, Cong YL, Sun YX, Yang GL, Ding XM, Yang ZQ, Zhou YL, Yang M, Wang CF, Ding Z: Protective immunity conferred by porcine circovirus 2 ORF2-based DNA vaccine in mice. *Microbiol Immunol,* 58 (7): 398-408, 2014. DOI: 10.1111/1348-0421.12158

25. Suo S, Wang X, Zarlenga D, Bu RE, Ren YD, Ren XF: Phage display for identifying peptides that bind the spike protein of transmissible gastroenteritis virus and possess diagnostic potential. *Virus Genes,* 51 (1): 51-56, 2015. DOI: 10.1007/s11262-015-1208-7

26. Noble A, Mehta H, Lovell A, Papaioannou E, Fairbanks L: IL-12 and IL-4 activate a CD39-dependent intrinsic peripheral tolerance mechanism in CD8+T cells. *Eur J Immunol,* 46 (6): 1438-1448, 2016. DOI: 10.1002/eji.201545939

27. Hamza T, Barnett JB, Li B: Interleukin 12 a key immunoregulatory cytokine in infection applications. *Int J Mol Sci,* 11 (3): 789-806, 2010. DOI: 10.3390/ijms11030789

28. Bagley KC, Schwartz JA, Andersen H, Eldridge JH, Xu R, Ota-Setlik A, Geltz JJ, Halford WP, Fouts TR: An Interleukin 12 adjuvanted herpes simplex virus 2 DNA vaccine is more protective than a glycoprotein d subunit vaccine in a high-dose murine challenge model. *Viral Immunol,* 30 (3): 178-195, 2017. DOI: 10.1089/vim.2016.0136

29. Maspi N, Ghaffarifar F, Sharifi Z, Dalimi A: Codelivery of DNA vaccination encoding LelF gene and IL-12 increases protection againstLeishmania majorinfection in BALB/c mice. *Parasite Immunology,* 38 (4): 228-235, 2016. DOI: 10.1111/pim.12310

30. He J, Duan S, Yu X, Qian Z, Zhou S, Zhang Z, Huang X, Huang Y, Su J, Lai C, Meng J, Zhou N, Lu X, Zhao Y: Folate-modified chitosan nanoparticles containing the IP-10 Gene enhance melanoma-specific cytotoxic $CD8^{+}CD28^{+}$ T lymphocyte responses. *Theranostics,* 6 (5): 752-761, 2016. DOI: 10.7150/thno.14527

31. Xenopoulos A, Pattnaik P: Production and purification of plasmid DNA vaccines: Is there scope for further innovation? *Expert Rev Vaccines,* 13 (12): 1537-1551, 2014. DOI: 10.1586/14760584.2014.968556

32. Maspi N, Ghaffarifar F, Sharifi Z, Dalimi A, Dayer MS: Immunogenicity and efficacy of a bivalent DNA vaccine containing LelF and TSA genes against murine cutaneous leishmaniasis. *Apmis,* 125 (3): 249-258, 2017. DOI: 10.1111/apm.12651

33. Escalona E, Saez D, Onate A: Immunogenicity of a multi-epitope DNA Vaccine encoding epitopes from cu-zn superoxide dismutase and open reading frames of *Brucella abortus* in Mice. *Front Immunol,* 8:125, 2017. DOI: 10.3389/fimmu.2017.00125

34. Arulanandam BP, O'Toole M, Metzger DW: Intranasal interleukin-12 is a powerful adjuvant for protective mucosal immunity. *J Infect Dis,* 180 (4): 940-949, 1999. DOI: 10.1086/314996