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THE CO-ADMINISTRATION OF POIL-2 WITH TGEV INACTIVATED VACCINE ENHANCES IMMUNE RESPONSE OF PIGLETS TO TGEV

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ABSTRACT

Porcine interleukin-2 (poIL-2) has not yet been demonstrated to be immune-enhancing against porcine transmissible gastroenteritis virus (TGEV) inactivated vaccine (IV), despite IL-2 having been proven to have immunological adjuvant effects for a variety of vaccinations. In this work, the impact of poIL-2 on TGEV IV in terms of immunological enhancement was investigated. Twenty four (24) SPF piglets were utilized and divided into six groups: PBS group, poIL-2 group, IV group, 10μg poIL-2+IV group, 50μg poIL-2 +IV group, and 250μg poIL-2+IV group. They received a second vaccine at 28 days point following the initial immunization. Serum and blood samples were obtained at various periods throughout the experiment. By using ELISA assay, neutralization assay, MTT assay, and flow cytometry assay, the TGEV-specific antibody expressions, neutralizing antibodies generations, interleukin-4 (IL-4), and interferon-gamma (IFN-γ) productions, peripheral blood mononuclear cells (PBMCs) proliferation response and lymphocyte phenotype subpopulations (CD_3^+, CD_4^+, and CD_8^+ immune cells) reflections were determined. The results showed that piglets inoculated with IV supplemented with poIL-2 significantly not only increased more piglet cellular immunity against TGEV by raising the degrees of IL-4, IFN-γ, Stimulation Index (SI), and the ratio of CD_4^+/CD_8^+ cell subgroups, but also promoted more humoral immunity against TGEV by increasing levels of anti-TGEV specific antibodies and neutralizing antibodies (NAs) than those piglets inoculated with the TGEV IV alone. Additionally, the results suggested that porcine interleukin-2 (poIL-2) may improve pigs' immune responses in a dosage-dependent way.Our study revealed that poIL-2 had an immune-enhancing effect on the immunization of TGEV IV, and it possessed the potential to be applied as an immune-stimulating agent.

Keywords: Inactivated vaccine (IV); adjuvant; porcine transmissible gastroenteritis virus (TGEV); porcine interleukin-2 (poIL-2); immune response.

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INTRODUCTION

Porcine transmissible gastroenteritis (TGE), a highly contagious illness in piglets, is brought on by the pathogen of porcine transmissible gastroenteritis virus (TGEV). (Ma *et al*., 2018; Xue *et al*., 2018) TGEV is one of the members of the nidovirus order that belongs to the coronavirus family. (Walker *et al*., 2021; Li *et al.,* 2023) Piglets will experience severe diarrhea and a high death rate if they become infected with this virus. It is a crucial virus that poses a threat to the pig husbandry industry. (Pan *et al*., 2021; Yan *et al.,*2023)

Immunization is very effective in preventing a TGE epidemic and TGEV infection. (Liu and Li, 2022) The development of the TGEV vaccine has the necessary

value for treating and preventing TGEV outbreaks or epidemics. (Li *et al*., 2019; Lin *et al*., 2016) Presently, a few sorts of TGEV vaccines have been developed and are available in the market, the majority of which are directed to the pregnant sows during incubation of their gestation to give lactogenic immunity to infant piglets. Nonetheless, low degrees of protection against TGEV were accounted for, and accordingly, we ought to in any case give extraordinary consideration to this virus infection. (Valko *et al.,* 2019)

Immunization is crucial for stopping and limiting the spread of the TGE disease. To prevent and control the spreading of the TGE illness, it is essential to strengthen the immunological activity of the TGEV vaccination. (Xue *et al*., 2019; Zhao *et al*., 2020) For the reason that inactivated vaccines are very safe, they have been used extensively in the field of animal-diseases prevention, however, they still have some disadvantages including poor immunogenicity and short duration of immunization, multiple doses of booster being required; unsatisfactory immunity in older animals; and high safety requirements for the production process, resulting in high costs and adverse side effects. (Zheng *et al.,* 2021**;** Guo *et al.,* 2020). At present, the efficiency of the TGEV inactivated vaccine (IV) is not very satisfactory (Chen *et al*., 2016; Zheng *et al*., 2021). Usually, the application of inactivated vaccines requires the addition of adjuvants in order to achieve their good immunogenicity. (Del Giudice, *et al.,* 2018) To increase the efficacy of the TGEV IV vaccination, several adjuvants, including silicon nanoparticles (nano silicon particles) and porcine interleukin-12 plasmid (IL-12 plasmid), have been utilized to boost the immune response (Jin *et al*., 2018; Li *et al*., 2019; Du *et al.,* 2023). However, we still hope to identify immunological adjuvants that are more effective, persistent, compatible, and diverse.

Cytokines are the most important signaling molecules that specifically and efficiently regulate immune cells and coordinate the immune response in animals, which can efficiently regulate immune response, inflammatory response, tissue repair, transplantation rejection, and hematopoietic function, etc., and play a very important role in controlling the differentiation, development, maturation, and activation of various types of immunologically active cells. (Saxton *et al.,* 2023) In recent years, with the advancement of biological technology and immunological technology, cytokines researches have made remarkable achievements. Many studies have demonstrated that cytokines can enhance the immune response of the animal body and reduce the occurrence of diseases, and have shown broad application prospects in the immune-prevention and immunotherapy of animal diseases.

IL-2 was the first cytokine with broad biological activity to be discovered. (Morgan *et al*., 1976) IL-2 used to be previously given the name of T-cell growth factor (TCGF). (Gaffen and Liu, 2004) It belongs to a lymphokine that helper T lymphocytes (Th) make in response to mitogens or certain antigens that stimulate them. Its primary biological functions include promoting cytotoxic T cell (CTL) and NK cell activity, long-term preservation of T lymphocyte clonal development in vitro, and induction and enhancement of gamma interferon generation. In addition to acting directly on these immunological cells, IL-2 can also promote or boost the secretion of a variety of lymphokines, including interleukin-4 (IL-4), colony-stimulating factor (CSF), and interferon (IFN). As a result, IL-2 is consequently imperative for the managing of the immune system. (Arenas-Ramirez *et al*., 2015; Liao *et al*., 2013) However, on the other hand, the immune-boosting impact of recombinant porcine interleukin-2 (poIL-2) on the inactivated vaccine of porcine transmissible gastroenteritis virus (TGEV IV) in piglets has not been described.

Therefore, we blended the soluble recombinant poIL-2 with the TGEV IV in this learn about to further examine the viability of the usage of recombinant poIL-2 in piglets. Because vaccine-induced immunity is separated into humoral and cellular immunity, this study aims to determine whether poIL-2 combined with TGEV IV treatment improves virus-specific humoral immune responses or virus-specific cellular immune responses in piglets. Our work is the first in vivo demonstration of poIL-2's ability to promote the generation of immune response to the TGEV IV in piglets. We hope that this may understand the potential of poIL-2 as an adjuvant candidate for the TGEV IV.

MATERIALS AND METHODS

Animal rights statement: The local Bioethics Committee of Anhui Medical University (Hefei city, Anhui province, P.R.China) had authorized the animal experiment project of this study. (Approbation No: LLSC20180307, Certification date: 2018.03.01).

Reagents and Drugs: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Concanavalin A (ConA) have been bought from Sigma Corporation. Porcine Transmissible Gastroenteritis Virus inactivated vaccine (TGEV IV) (in the form of oil emulsion) was bought from TECBOND Biological Products Co. Ltd (Chengdu, Sichuan, P.R.China). Porcine transmissible gastroenteritis virus (TGEV) antibody ELISA kit was bought from Ingenasa Tech. (Catalog serial No: 11.TGE.K.3/2, Ingenasa Tech, Madrid, Spain, Europe)

Before use, recombinant porcine interleukin-2, which was acquired from Beyotime Biotech (Catalog number P6461, Shanghai, P.R.China), was frozen at -80 ℃ in an ultra-low temperature freezer.

Immunization of experimental animals in groups: In this study, 24 30-day-old SPF Landrace large white pigs were utilized. Both ELISA test and PCR test were used to screen and confirm that no pigs contained antibodies specific to the porcine transmissible gastroenteritis virus (TGEV) and that no pseudorabies virus (PRV), porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2(PCV-2), classical swine fever virus (CSFV), or TGEV viral genomes were present. This was done to make sure the piglets (participants) in the trial were free of the TGEV and other pig-related viruses. (Jiang *et al*., 2010) The piglets were randomly divided into six companies, every consisting of 4 piglets. PBS was given to the first group as a control administration, 50µg of poIL-2 solution to the second, TGEV IV to the third at the commonly used dose, and a combination of 10µg poIL-2 and TGEV IV to the fourth, both TGEV IV and 50µg poIL-2 were given to fifth, both TGEV IV and 250 µg poIL-2 were given to the sixth group. Piglets were observed for 28 days after the initial vaccination, and on the 28th day the experimental piglets were vaccinated again, each experimental piglet received a booster vaccination and was observed for another 28 days, for a total time period of 56 days. Along with vaccination injection, piglets were weighed continuously, and their body temperatures were written down at 8:00 a.m. and 8:00 p.m. of each day.

Clinical symptom tracking of experimental animals: The piglets were weighed at various times following the initial vaccination and booster shot. The body weight increase of the piglets in each group differed only slightly. Besides, none of the piglets in either group had fevers (data not shown). These two above findings indicated that immunization had no influence on the clinical efficacy of the experimental piglets in either group.

Blood sampling from piglets: Using aseptic vacuum lithium heparin (150 USP units) test tubes, blood samples were obtained at 1, 7, 14, 21, 28, 35, 42, and 56 days after vaccination. Non-anticoagulated blood samples were taken for serum separation. Those serum samples from these non-anticoagulant specimens were collected and preserved at below -20℃.

Measurement of the specific antibody titers against TGEV: Following the manufacturer's instructions, an INGEZIM TGEV 2.0 ELISA detection assay kit (Catalog Serial No: 11.TGE.K.3/2, Ingenasa Tech, Madrid, Spain, Europe), which conjugated a monoclonal antibody (MAb) specific to site Ac of TGEV S protein (specific epitope of TGEV), building an enzymatic immunoassay (Capture Blocking ELISA assay), was adopted to detect TGEV-specific antibodies in samples of piglets' sera.

Briefly, before being used, the kits and samples were left at room temperature. Plate is coated with recombinant S protein of TGEV which is captured by a specific monoclonal antibody. 50µl of every serum sample and serum diluent must be added in duplicates to the proper wells. If the serum sample contains specific antibodies to the Transmissible Gastroenteritis Virus (TGEV), they will bind to all of the epitopes present. After that, plates were incubated for one hour at 37°C. Three rounds of washing solution were used for washing the plates. After an incubation period, a 100µl specific TGEV Mab-HRPO liquid (Conjugate B) was added to each well. Next, after sealing the plates, they were incubated at 37°C for 30 minutes. After six rounds of plate washing, 100 microliters of substrate (3,3', 5,5"-tetramethylbenzidine, TMB), were added to each well and allowed to react at room temperature for ten minutes. Once 100 µl of stop solution was added, the reactions were halted, and the plates were measured at 450 nm using a spectrophotometer. The Conjugate B will only bind to free epitopes. By adding a substrate that triggers a colorimetric reaction with horseradish peroxidase (HRPO), it is possible to determine if the labeled Mab is present or absent. The S/P ratio was used to report the titer. 0.4 was the positive/ negative cut-off value.

Determination of TGEV neutralizing antibodies (NAs): The serum neutralization (SN) quantitative measurement test was carried through using the experimental technique described in the prior literature. (Jiang *et al*., 2008) Briefly, test sera samples were applied in a twofold dilution series and blended equivalent volume of TGEV SC-T clinical isolate, which was gifted by Professor Zhi-Wen Xu from Sichuan Agricultural University (Yaan, Sichuan Province, P.R.China), containing 200 $TCID_{50}$ Virus (Gao *et al*., 2021). The prepared mixtures were transferred to PK-15 monolayers in quadruplicate on 96-well tissue culture plates after an hour of incubation at 37 °C. Following incubation, the plates were checked every day for up to five days to see if any cytopathic effects (CPE) had been shown. Neutralizing titers in the serum samples were calculated using the conventional Reed-Muench formula and were expressed as the ND_{50} values, which stand for the reciprocal of the highest dilution that prevented 50% of the cytopathic effects from occurring. (Reed and Muench, 1938) Porcine antiserum against TGEV served as a positive control in the meantime.

Test for peripheral blood mononuclear cells (PBMC) proliferation specific to TGEV: As previously reported, PBMCs were extracted from pig heparinized blood by using the density gradient centrifugation technique. (Linghua *et al*., 2007) Purified TGEV viral antigen was used to activate PBMC cells that had been cultured. As a negative control, PBMC cells only were suspended in

PBS, and not activated by the TGEV viral antigen or activated by ConA. ConA, a mitogen, was added to PBMC cells for stimulation at a dose of 10 μg/ml and served as a positive control.

Using the conventional MTT technique, the proliferation response of PBMC cells was evaluated. Briefly, Aliquots of PBMCs (10^5/well) were planted in triplicate wells of 96-well U-shaped-bottom plates. The cells were then individually stimulated for 48 hours using either PBS as a negative control, mitogen ConA (Sigma, St. Louis, MO) as a positive control, or the pure TGEV viral antigen as the experimental sample detection. 150μL of media was taken out of each well after 72 hours of culture in 5% CO_2 at 37°C. The wells were subsequently incubated for an additional 4 hours with 15 μL of newly made filtered MTT dye (5 mg/mL in PBS). Following incubation, 175 μL of 0.04N hydrochloric acid in isopropanol (Sigma) was added, and color development was incubated for 30 minutes at room temperature. Absorbance values were detected at 490 nm using a Bio-Rad Model 450 microplate ELISA reader (Bio-Rad Co., Ltd., Hercules, CA, USA). The stimulation index (SI), which is calculated by dividing the average of the experimental data by the average of the non-stimulated controls, was used to express the extent of lymphocyte proliferation.(Magden *et al*., 2020) It was a ratio of the average OD_{490nm} value in the stimulated wells to the average OD_{490nm} value in the non-stimulated wells. Reported results are the geometric mean values of the stimulation index (SI) plus standard deviations for triplicate detections, which served as an indicator for the proliferation degree of PBMC cells. (Mosmann, 1983)

Experiment for determining cytokines: The cell subpopulations can be distinguished based on the cytokines they release and the patterns they express. By generating substances that stimulate almost every other immune system cell, helper T cells are essential for proper immunological responses. These cells include macrophages and other effector cells that assault invasive pathogens (disease-causing agents); CTL that destroy cells transporting infectious agents; and B cells that create the antibodies required to combat infection. Instead of being a single kind of cell, helper T cells may be broadly classified into two subpopulations: Th1 and Th2 cells. These subpopulations differ greatly in terms of chemistry and function. The chemical messengers known as cytokines that these populations release allow for their differentiation. Th1 cells are mainly responsible for producing the cytokines interleukin-2 (IL-2), tumor necrosis factor-beta, and interferon-gamma (IFN-γ), which in turn activates cell-mediated responses (i.e., CTL and macrophages). The interleukins IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 are mostly produced by Th2 cells, and they aid in encouraging B cells to produce antibodies.

The pattern of change in the cell-mediated immune state can be seen in the levels of peripheral blood lymphocyte proliferation (SI) as well as the secretions of IFN-γ and IL-4 after stimulation.

In line with the manufacturer's recommendations, commercial pig IFN-γ/IL-4 ELISA detection kits were used to identify and evaluate the pig IFN-γ/IL-4 cytokine concentration in piglets' sera at various time points. (Du *et al*., 2012; Li *et al*., 2009)

Analysis of peripheral blood lymphocytes using flow cytometry: Anti-porcine CD molecule antibodies (anti-CD8-SPRD, anti-CD4α-PE, and anti-CD3ε-FITC) paired with various fluorescent dyes were added to resuspended PBMCs at a cellular density of 1×10^7 cells/ml solution (Southern Biotech, Birmingham, AL, USA).

The frequency of CD_8^+, CD_4^+, and CD_3^+ immune cells was quantified using the flow cytometry method (BD Biosciences, Franklin Lakes, NJ, USA). In summary, secondary fluorochrome-conjugated porcine CD-specific antibodies (anti-CD3ε-FITC, anti-CD4α-PE, and anti-CD8-SPRD) were used to surface-mark PBMCs. 1% paraformaldehyde was used to fix the surface immunostained cells, and then they were permeabilized at 4°C for 18 hours using the cell-permeabilization buffer (85.9% deionized water, 11% Ca^{2+} free or Mg^{2+} free PBS, 3% formaldehyde solution, and 0.1% saponin). Cells were rinsed with fluorescence-activated cell sorting (FACS) buffer containing 0.1% saponin. FACS Aria II (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometry was used to evaluate immunostained cells. The gating approach involves first gating the cells for the CD3 marker, and then gating the CD_4^+ and CD_8^+ markers for the cells that are CD_3^+ positive. Subsequently, the frequency of CD_3^+, $CD_3^+CD_4^+$, $CD_3^+CD_8^+$, and $CD_3^+CD_4^+CD_8^+$ cells within each group was determined.

Statistical analysis: SPSS 17.0 Statistic Software (SPSS Inc., Chicago, IL, USA) was employed for the statistical analysis and research in our study. The results of the experiments and measurements were calculated as mean±SD (standard deviation). Two groups were compared using the t-test, and multiple groups (more than two) were examined using the one-way analysis of variance (one-way ANOVA) test. When $P\leq0.05$, differences were deemed statistically significant.

RESULTS

Clinical signs in each experimental pig group: Piglets in each group grew larger, although the differences in their body weight gain (weight increase) were very small (Fig. 1A)(p=0.256, p>0.05). Additionally, the rectal temperatures of the piglets in all groups were below 41 degrees Celsius, and none of them had fevers (Fig 1B) (p=0.358, p>0.05). The aforementioned two findings

show that the immunization of poIL-2 displayed good safety.

Specific anti-TGEV antibody titers determination: Piglets from the groups of IV+50μg poIL-2 (p=0.021, $p\leq0.05$) and IV+250μg poIL-2 (p=0.018, $p\leq0.05$) demonstrated higher antibody titers at different stages following vaccination than those piglets from the IV alone inoculation group. (Fig 2A)

Piglet groups inoculated with IV+50μg poIL-2 and IV+250μg poIL-2 at 28 days post-inoculation (dpi) exhibited significant antibodies against TGEV, and these antibodies significantly increased following the booster vaccination. (Fig 2A)

It is inferred that the way in which poIL-2 enhances antibody response is likely in the manner of dose-dependent.

Neutralizing antibodies determination using the SN test: At 42 dpi and 56 dpi, the IV+50 g poIL-2 group (p=0.014, $p\leq0.05$) and the IV+250 g poIL-2 group (p=0.012, $p\leq0.05$) were able to produce higher levels of neutralizing antibodies than the IV alone group (Fig 2B), This means that high dose implements of poIL-2 increased the humoral immune response of TGEV. In contrast, the IV alone group produced little augment in the generation of neutralizing antibodies.

The particular cytokines IFN-γ and IL-4 secreted in the supernatant of porcine PBMCs upon stimulation: After stimulating pig PBMC with TGEV antigen, the concentrations of released IFN-γ and IL-4 in the supernatant of porcine PBMC cells were assessed using available commercial ELISA kits in order to monitor the generation of specific cytokines.

The results demonstrated that when the peripheral blood lymphocytes of the piglets were stimulated by the TGEV antigen, co-administration of poIL-2 and IV vaccine can significantly increase the secretion of both IL-4 (p=0.015, $p\leq0.05$) and IFN-γ (p=0.012, $p\leq0.05$), those were to both enhance Th1 and Th2 cytokine responses, therefore enhanced cell-mediated immunity (CMI) in piglets at various time points after immunization (Fig3A and Fig3B).

The relationship between poIL-2 induction and the proliferation of TGEV-specific PBMCs (SI): By performing the MTT colorimetric test, the poIL-2-mediated lymphocyte proliferation levels were evaluated (Fig 4).In comparison to the PBS-alone group and the IV-alone group, the piglet cells from the IV+50μg poIL-2 group (p=0.028, $p\leq0.05$) and IV+250μg poIL-2 group (p=0.019, $p\leq0.05$) produced greater SI at 14dpi and 28dpi, respectively.

When compared with the IV alone group, the IV+250μg poIL-2 group was able to produce the largest concentration of neutralizing antibodies. Besides, the SI of the IV+250μg poIL-2 group attained the greatest level at the time points of 42 dpi and 56 dpi (p=0.009, $p\leq$ 0.05).

This data demonstrates that the inclusion of poIL-2 enhances the CMI of inactivated vaccine (IV) in piglets, as PBMC proliferative response is often correlated to CMI.

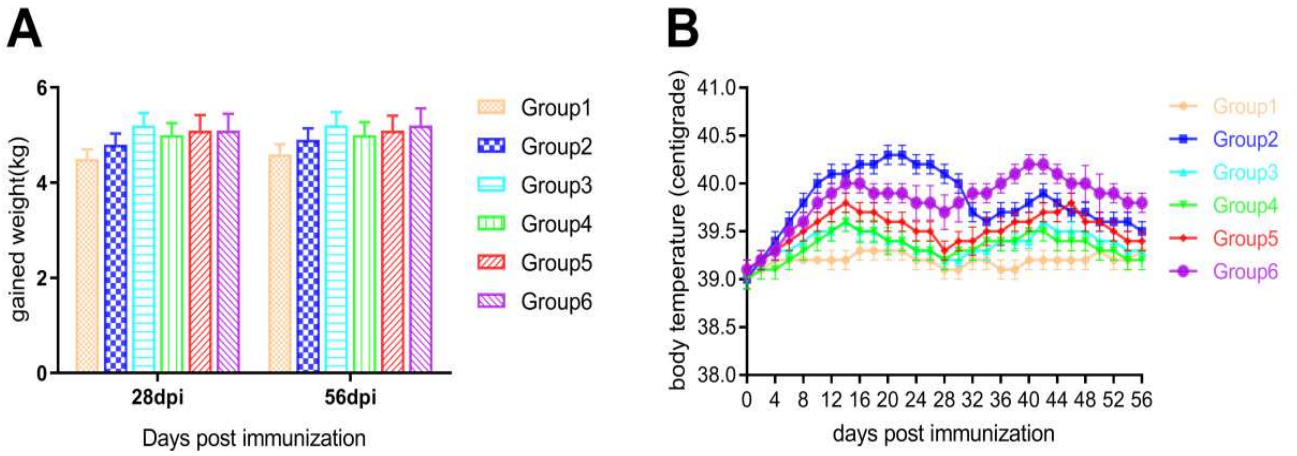

Fig.1A. The changing profile in the average weight growth of the pigs (the clinical symptoms monitoring of pigs) at various time periods following the first immunization and booster immunization. The increase in body weight was indicated in kilograms. The value was provided as the mean±SD, and each group contained four pigs.

Fig.1B. The changing profile in the average rectal temperature of the pigs (the clinical symptoms monitoring of pigs) at various time periods following the first immunization and booster immunization. The increase in body temperature was indicated in Degrees Celsius, and for experimental piglets, a rectal temperature of 41°C or above is considered to be fever. The value was provided as the mean±SD, and each group contained four pigs.

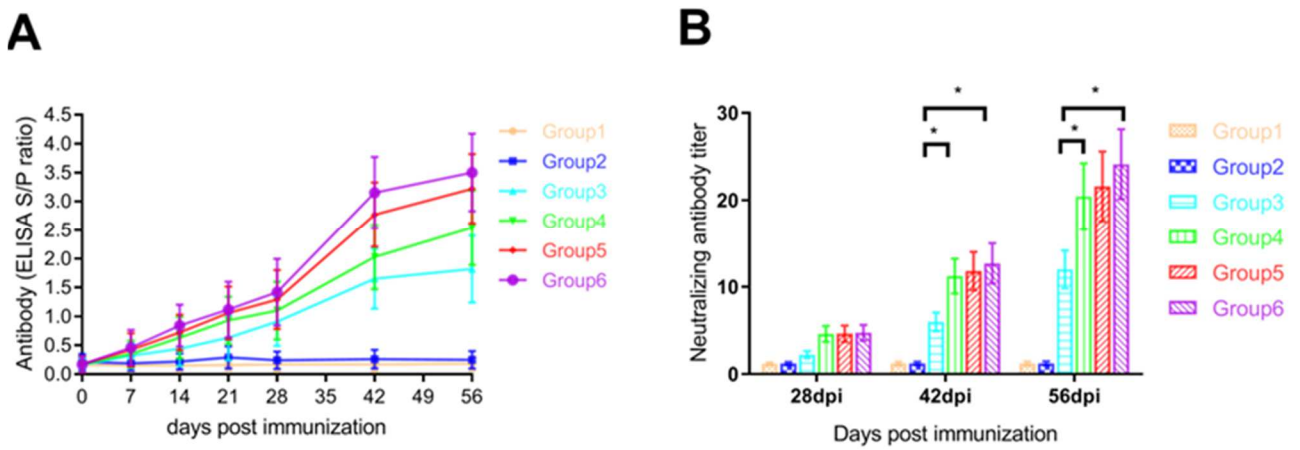

Fig.2A. The anti-TGEV-specific antibody kinetics at various time periods in vaccinated experimental piglets.

Analysis of the lymphocyte subpopulations in piglets' blood by flow cytometry: Fig 5 displays the changing profiles of different cell subpopulations (CD_3^+ immune cells, $CD_3^+CD_4^+$ immune cells, $CD_3^+CD_8^+$ immune cells, and $CD_3^+CD_4^+CD_8^+$ immune cells.). Most samples for T cell counts showed a slight rise in the immune cell groups containing $CD_3^+CD_8^+$ and $CD_3^+CD_4^+CD_8^+$ in the early post-first vaccination period. There was a considerable increase in its value in the blood of piglets at 56 dpi after booster vaccination (p=0.032, p≤0.05). This increase started at 28 dpi.

Six groups of experimental pigs were inoculated intramuscularly (i.m.) with various biochemical reagents, such as PBS alone or IV alone, poIL-2 alone, or poIL-2+IV, in accordance with the grouping system.

The anti-TGEV-specific antibody levels in each animal group's serum samples were assessed through an ELISA test. The S/P ratio was used to gauge the quantity of anti-TGEV-specific antibodies, and the findings were presented as mean± SD.

Fig.2B. The anti-TGEV neutralizing antibody titer at various time periods in vaccinated experimental piglets.

Six groups of experimental pigs were inoculated intramuscularly (i.m.) with various biochemical reagents, such as PBS alone or IV alone, poIL-2 alone, or poIL-2+IV, in accordance with the grouping system.

The neutralizing antibody titer levels in each animal group's serum samples were assessed through SN assay.

The reciprocal value of the final piglets' serum dilution that neutralized 100 $TCID_{50}$ (dosage) TGEV to 50% cytopathic effect (CPE) of the microwells was used to express the neutralizing antibodies' titer, which was displayed as mean±SD.

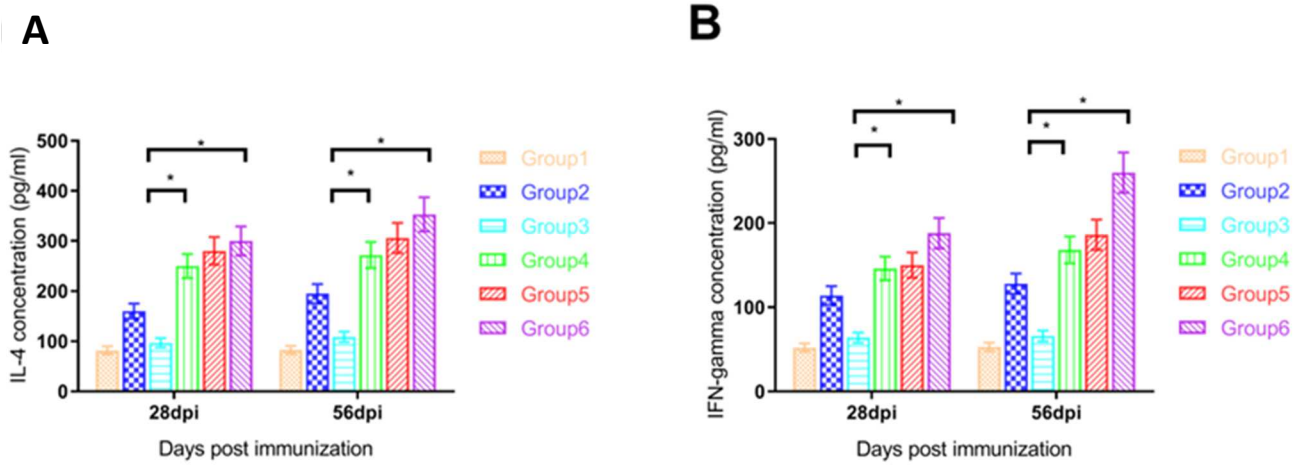

Fig.3. ELISA tests were applied to measure the concentrations (pg/ml) of specific cytokine IL-4 (A) and IFN-γ (B) in collected PBMC supernatant of experimental piglets at 28 dpi and 56 dpi.

Each column displays the mean±SD value of the cytokine levels obtained from each piglet group (n=4). The measurement was conducted in triplicate.

*signifies a significant distinction (p≤ 0.05) between the two groups.

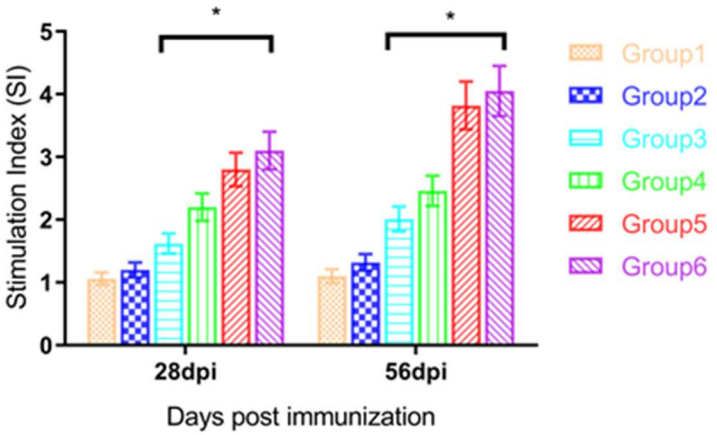

Fig.4. The stimulation index (SI) values, which represent the degree of PBMC proliferation in the various experimental groups, are shown in the colorful histogram.

The data is given as the group mean±SD, and each column contains the SI value that was calculated in triplicate.

ConA is a positive control, and *signifies a significant distinction ($p \leq 0.05$) between the two groups.

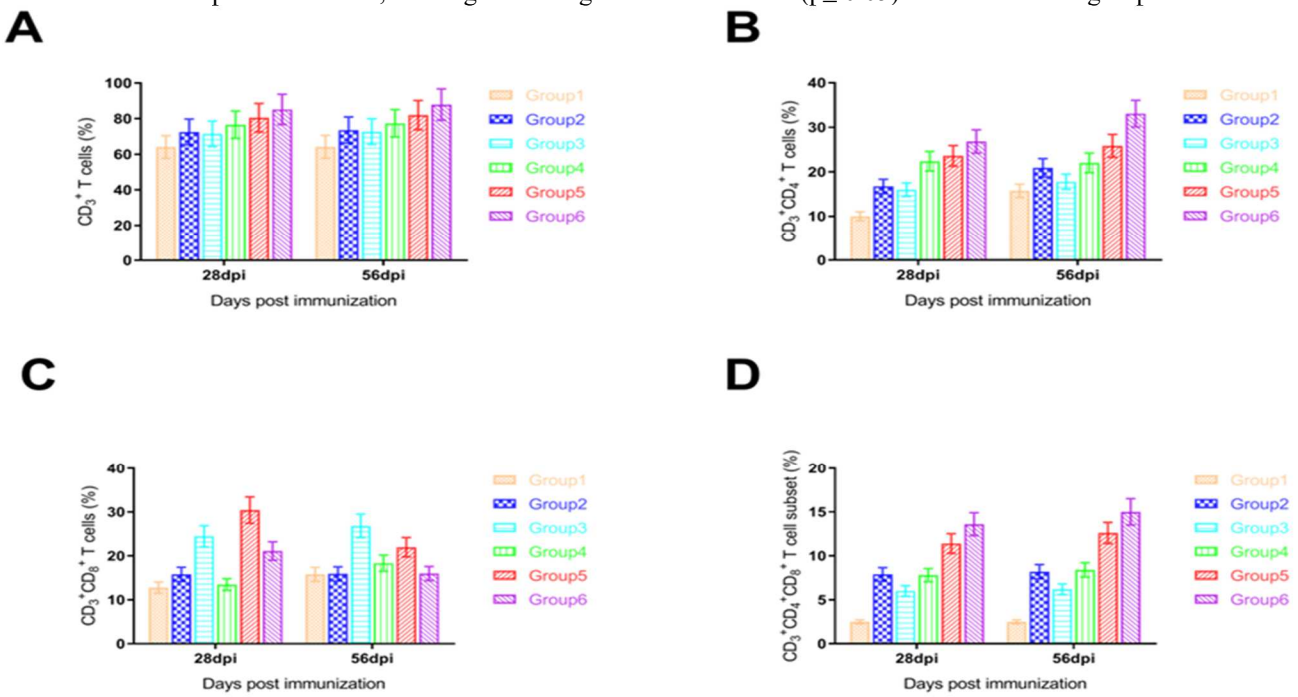

Fig.5. During the first vaccination and booster immunization, different proportion profiles and frequencies of CD_3^+, $CD_3^+CD_4^+$, CD_3+CD_8+, and $CD_3^+CD_4^+CD_8^+$ lymphocytes among the total immune cell populations were measured.

Panel A depicts the percentage of CD_3^+ immune cells; Panel B depicts the percentage of $CD_3^+CD_4^+$ immune cells; Panel C depicts the percentage of $CD_3^+CD_8^+$ immune cells; and Panel D depicts the percentage of $CD_3^+CD_4^+CD_8^+$ immune cells. The formula for values is mean ±SD.

DISCUSSION

In recent years, the effects of IL-2 as adjuvants in preventative vaccines have been the topic of many investigations.

Scientific researchers have found that interferon (IFN), lymphokines (IL-2, IL-3, IL-4, IL-5, etc.), monokine (IL-1, IL-6, IL-8, IL-10, IL-12, etc.) and other cytokines such as granulocyte macrophage-colony stimulating factor (GM-CSF)and tumor necrosis factor

(TNF), had immune adjuvants effect. Among them, IFN and IL-2 are the two most noticeable cytokines.(Arico and Belardelli, 2012; Decker and Safdar, 2011; Kamphorst *et al*., 2015; Rahman and Eo, 2012; Takaki *et al*., 2016)

Previously, Our study explored the interaction of porcine IFNα (interferon-alpha) with TGEV in pigs. (Gao *et al*., 2021; Zhao *et al*., 2017) and therefore, this study hoped to confirm that poIL-2 could produce a potent stimulatory effect on porcine immune cells against TGEV infection.

As an immune adjuvant, IL-2 protein has been confirmed by many studies to have an immune-enhancing effect. Lofthouse *et al.* have shown that the administration of recombinant IL-2 protein can significantly enhance both humoral and cellular immune responses of the body. (Lofthouse *et al*., 1996) Nunberg *et al*. discovered that when IL-2 and inactivated rabies vaccine were combined and administrated, the vaccine's protective effect on experimental mice was increased by 25 times in the face of challenge. (Nunberg *et al*., 1989) Thiagarajan *et al*. found that when poultry was vaccinated with the Newcastle disease vaccine, the number of IL-2 receptors in lymphocytes did not change significantly, but when the Newcastle disease vaccine was co-vaccinated with IL-2 protein, lymphocytes could rapidly differentiate and proliferate. (Thiagarajan *et al*., 1999) Reddy *et al*. reported that when low-dose recombinant bovine IL-2 was used together with the live herpes virus type I vaccine, the serum neutralizing antibody titer was increased by six times compared with the vaccine group alone, and the viral shedding after challenging was reduced by four times. (Reddy *et al*., 1989) Wong *et al*. discovered that IL-2 could decorate the body's immune response to FMDV infection. (Wong *et al*., 2002) Lin *et al*. found that the combination of porcine IL-2 recombinant protein and pseudorabies virus vaccine can significantly enhance antibody production and CTL cell activity. (Lin *et al*., 2005)

Using genetic engineering technology, the IL-2 gene and protective antigen gene can be transferred into two eukaryotic expression plasmids, or fused together in sequence and transferred into one plasmid/viral vector to construct a new type of genetically engineered vaccine to achieve immunization purposes.

Barouch *et al*. recombined the IL-2 gene into a plasmid to form a long-term low-level expression in the animal body, thereby acting as an adjuvant. (Barouch *et al*., 2004) Xin *et al*. injected a mouse nasal cavity with a mixture of an HIV DNA vaccine and an IL-2 recombinant plasmid; the results showed that the combination significantly enhanced the immune response, and the greater the dose of IL-2 recombinant plasmid, the greater apparent the effect. (Xin *et al*., 1998) Kim *et al*. co-injected DNA vaccines expressing gag/pol proteins of HIV and SIV into BAlB/c mice with pcDNA3 expression vector containing IL-2, respectively. The results show that IL-2 can induce higher levels of specific antibodies, shift the mice's immune response from Th1 type to Th2 type, and significantly enhance the proliferative response of Th cells, but no significant antigen-specific CTL response was observed. (Kim *et al*., 1999) Nobiron *et al*. co-immunized BAlB/c mice with the surface glycoprotein gene of the bovine diarrhea virus and IL-2 gene, the neutralizing antibody IgG2A was significantly enhanced, and the antigen-specific proliferation of spleen cells was also enhanced. (Nobiron *et al*., 2001) Chow *et al.* constructed a plasmid that conveyed the expression of IL-2 and the HBV protein simultaneously. After inoculating mice, it was observed that the anti-HBV antibody production and T cell proliferation response induced were drastically improved compared with the control group, indicating that the expressed IL-2 can promote the immune response to HBV nucleic acid vaccine. (Chow *et al*., 1997) Hazama *et al*. fused the IL-2 gene with the bovine herpes simplex virus gD gene, expressed the fusion protein in eukaryotic cells, and challenged the mice after immunization. As a result, the fusion protein could fully protect mice from virus attack, and the in vivo half-life of IL-2 was thus extended by about four times. In contrast, recombinant glycoproteins adjuvanted with aluminum hydroxide and without IL-2 produced only partial protection. (Hazama *et al*., 1993) When Rompato *et al*. co-immunized pigs with IL-2 plasmid and PRRSV vaccine, they found that it can significantly enhance the body's cellular and humoral immunity to PRRSV infection. (Rompato *et al*., 2006)

The application of the vaccinia virus as a viral expression vector was hampered by the following two major disadvantages: 1. Severe local reaction or side effects after vaccination; 2. The body produces fewer antibodies against the vaccinia virus. However, IL-2 can provide a strong guarantee for the safety of the vaccinia virus, Such as Ramshaw *et al*. and Flexner *et al*. have successively reported that a recombinant virus expressing IL-2 can be obtained by recombining mouse or human IL-2 with a poxvirus containing avian influenza hemagglutinin (HA) gene. Comparing this recombinant virus with other poxviruses without IL-2 gene, the results demonstrated that the insertion of IL-2 gene did not influence the HA expression level and its immunogenicity after inoculating mice with these two viruses, and could prevent the lethality of mice, indicating that the insertion of IL-2 cytokines can greatly improve the safety of live viral vectors. (Flexner *et al*., 1987; Ramshaw *et al*., 1987) Allen *et al*. co-expressed IL-2 and HSV viral proteins using a recombinant vaccinia virus vector. The experiments showed that the antibody level and lymphocyte proliferation ability of immunized mice were significantly improved. (Allen *et al*., 1990)

IL-2, as a crucial T-cell growth factor, is likely to be necessary for the transformation of naive cells into

Th1 or Th2 cells. It was formerly said that IL-2 tends to boost Th1-like responses but not Th2 responses when used as an adjuvant regularly. (Nunberg *et al*., 1989; Weinberg and Merigan, 1988) However, our findings indicate that poIL-2 boosts both Th1 and Th2 responses. Because Th cell subpopulations may be differentiated by the pattern of cytokines they produce. (Mosmann *et al*., 1986; Mosmann and Coffman, 1989) Th2 cytokines, such as IL-4, are crucial for enhancing humoral immune systems, while Th1 cytokines, such as IFN-γ, primarily influence cell-mediated immune responses. (Romagnani, 1991) In our study, The findings of our investigation indicate that poIL-2 has adjuvant effects on humoral and cellular immune responses to TGEV IV vaccination and that it also increases Th1 and Th2 responses. The substantial increases in IL-4 and IFN-γ measurements corroborate this.

Regarding the adjuvant effect in the vaccination experiment, investigations have shown that porcine interleukin-2 (poIL-2) has high-quality adjuvant enhancement action when combined with an inactivated vaccine formulation given through the parenteral route. The main mechanisms by which cytokine adjuvants work for an animal may be as follows: 1. After being injected into the animal body, IL-2 reacts with the antigenic substances and slows down the rate at which the antigen is hydrolyzed and metabolized, thus enhancing the antigen's sustained retention time in the body. 2. IL-2 can concentrate and adsorb antigens that were injected, making the antigen's action range larger, which in turn makes it easier for macrophages to phagocytose it. 3. When IL-2 is injected into the animal body, it increases the contact of lymphocytes, and this increases the activity of lymphocyte helper T cells. 4. When IL-2 is injected into the animal body, it enhances the dividing ability of the sensitized lymphocytes themselves; in addition, it causes the plasma cells in the immune system to secrete antibodies, i.e., the use of IL-2 convert substances that are not immunogenic into immunogens. 5. Cytokines can show cells their biological functions through paracrine or autocrine forms; at the same time, cytokines can renew the functions of the body's own target cells and stimulate the target cell membranes so that they can play their roles effectively. Therefore, the use of IL-2 can change the type of antibody production and also enhance the effect of antibodies. (Chen *et al.,* 2016; Gerdts and Zakhartchouk, 2017)

The effect of the poIL-2+IVs group in inducing cellular immunity is significantly stronger than that of all other groups, and in studies on humans and a variety of mammals, it has been shown that IL-2 promotes the proliferation and differentiation of T-lymphocytes and activated B-cells and induces the proliferation of natural killer (NK) cells, and lymphokine-activated killer (LAK) cells (Hennessy *et al.,* 1990) Besides, the application of IL-2 promotes the secretion of other cytokines. As a new type of immune adjuvant, IL-2 cytokine as an immune adjuvant has more obvious advantages than traditional vaccine adjuvants: it not only avoids the shortcomings of conventional adjuvants, but also reduces the side effects of some vaccines. It can also significantly increase the immunity effectiveness of viral, bacterial, and parasitic vaccines, improve antibody secretion, prolong antibody duration, stimulate Thl cell activity, enhance CTL activity, and enhance both humoral and cellular immunity. IL-2 can regulate the immune function of the body, change the intensity or type of immune response, and thus enhance the immunity effect of vaccines. It is a new type of adjuvant that people are generally optimistic about and has broad application prospects. (Lin *et al*., 2005; Wong *et al*., 2002; Wyckoff *et al*., 2005)

Furthermore, the present study found that IL-2 has the potential for immune enhancement when combined with the TGEV IV vaccine. Specifically, the body weight gain (Fig. 1A) and body temperature (Fig.1B) of piglets after immunization were not affected, with a good safety profile. In addition, we also measured the change curve of TGEV-specific antibodies and NAs after immunization. The finding revealed that for TGEV-specific antibody titers, the IL-2+IV group was produced earlier than the IV alone group, and the IL-2+IV group was also produced at a higher level than the IV alone group. There was a similar kinetic trend of change for TGEV NAs titers. Thus, IL-2 increases IV-elicited TGEV-specific antibody responses and neutralizing antibody levels (Fig. 2A and Fig.2B), and these enhancements may additionally make contributions to humoral immunity's protective effects.

Moreover, the poIL-2 we used was divided into three dose amount levels, namely IV + 10 μg poIL-2, IV + 50 μg poIL-2, and IV + 250 μg poIL-2, and the finding revealed that at the dose level of 250 μg poIL-2, the immune-enhancing effect of poIL-2 was greater and the boosting effect was more durable, suggesting that IL-2 would possibly improve pig immune response in a dose-dependent way. These findings are consistent with that of earlier researcher's report (Zhang *et al*., 2011)

According to our findings, poIL-2 displayed good safety. The inactivated viral vaccine (IV) mixture, including poIL-2, may considerably stimulate the proliferation of piglets PBMC and raise the level of Th1 cytokines and antibody production following co-administration to piglets when compared to TGEV IV alone. The combination considerably improves the pigs' immunological response to TGEV. Additionally, the immunological boost brought on by the IV+250μg poIL-2 group was noticeably greater than that of the IV+50μg poIL-2 group when the dose of poIL-2 was raised. According to this, poIL-2 may improve the piglets' immune response in a dose-dependent way.

The research mentioned above suggested that poIL-2, in the form of a protein or plasmid, might be used

as an adjuvant to boost vaccination immunological responses in pigs.

Conclusion: In conclusion, poIL-2 has an adjuvant effect when combined with IV at a dosage of 50μg poIL-2 or 250μg poIL-2. Our findings are significant because they show the potential of poIL-2 as a suitable immune-stimulatory adjuvant for the TGEV IV. However, to assess the protective impact of poIL-2+IV on piglets, more field trials were necessary.

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