Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
30 (5): 641-651, 2024
DOI: 10.9775/kvfd.2024.32122

Research Article

Recombinant Porcine Interferon Alpha Enhances the Humoral and Cellular Immune Responses to Porcine Transmissible Gastroenteritis Virus Inactivated Vaccine in Piglets

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How to cite this article?
Yu HY, Gao DM, Du J, Su Y, Zhao J: Recombinant porcine interferon alpha enhances the humoral and cellular immune responses to porcine transmissible gastroenteritis virus inactivated vaccine in piglets. *Kafkas Univ Vet Fak Derg,* 30 (5): 641-651, 2024.
DOI: 10.9775/kvfd.2024.32122

Article ID: KVFD-2024-32122
Received: 07.04.2024
Accepted: 29.08.2024
Published Online: 10.09.2024

Abstract

In this study, the effect of recombinant porcine interferon alpha (rPoIFNα) on porcine transmissible gastroenteritis virus inactivated vaccine (TGEV IV) in terms of immunological augmentation was examined. Seven experimental piglet groups, including PBS group, rPoIFNα group, inactivated vaccine (IV) alone group, $4.0x10^4$ U rPoIFNα+IV group, $2.0x10^5$ U rPoIFNα+IV group,$1.0x10^6$ U rPoIFNα+IV group, $5.0x10^6$ U rPoIFNα+IV group, were divided. The piglets in each group received a secondary vaccination at 28 days following the initial immunization. By using the ELISA assay, neutralization assay, MTT assay, and flow cytometry, we measured anti-TGEV-specific antibody expressions, neutralization antibodies, as well as lymphocyte proliferation index (Stimulation index, SI), specific IL-4 and IFN-γ production, and T cell subpopulations (CD3+, CD4+, and CD8+). Piglets injected with IV supplemented with rPoIFNα at $1.0x10^6$ U or $5.0x10^6$ U developed significantly higher anti-TGEV-specific and neutralizing antibodies compared to those treated with IV alone. IV therapy with rPoIFNα at $1.0x10^6$ U or $5.0x10^6$ U can boost cellular immunity against TGEV by increasing SI, IL-4, IFN-γ, and the ratio of CD3+, CD4+, and CD8+ cell subgroups. The IV+$5.0x10^6$ U rPoIFNα group showed a considerably larger immune increase than the IV+$2.0x10^5$ U rPoIFNα group, suggesting that it works in a dose-dependent manner. Therefore, rPoIFNα at $1.0x10^6$ U or $5.0x10^6$ U enhances the immune response against TGEV IV and may function as an immune stimulant.

Keywords: Cellular immune response, Humoral immune response, Inactivated Vaccine (IV), Porcine transmissible gastroenteritis virus (TGEV), Recombinant porcine interferon alpha (rPoIFNα)

Introduction

Porcine transmissible gastroenteritis (TGE), a highly contagious disease in pigs, is caused by the pathogen of the porcine transmissible gastroenteritis virus (TGEV) [1,2], with the exception of a non-pathogenic variant "porcine respiratory coronavirus (PRCV)" whose spike gene is deleted in TGEV and associated with mild or subclinical respiratory tract infections [3]. TGEV is classified as a member of the coronavirus family within the nidovirus order [4]. If piglets encounter this virus, it will cause severe diarrhea and a high mortality rate. The pig industry is at risk from this important virus [5].

Immunization has a high-quality capacity to prevent the TGE outbreak and TGEV infection. The development of the TGEV vaccine is crucial for the therapy and prevention of TGEV infection [6].

Currently, the efficiency of the TGEV-inactivated vaccination (IV) is insufficient [7]. Due to their high level

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of safety, inactivated vaccines have been used extensively. However, they still have some drawbacks, such as short immunization duration and poor immunogenicity, the need for multiple booster doses, insufficient immunity in older animals, and strict production process safety regulations that drive up costs and cause unfavorable side effects [7-9]. Typically, adjuvants were needed in order for inactivated vaccines to be applied and have adequate immunogenicity [10]. Therefore, Boosting the TGEV vaccination's immune response is crucial for stopping and managing the spread of the TGE disease [11,12].

Various kinds of adjuvants have been employed to boost immunity and increase the efficacy of the TGEV IV vaccine, such as porcine interleukin-12 plasmid (IL-12 plasmid) and silicon nanoparticles (nano silicon particles) [13,14].

Studies on the adjuvant impact in the vaccination experiment have demonstrated that type alpha interferon, when administered parenterally with an inactivated vaccine formulation, has excellent adjuvant enhancement function [15].

According to studies, immune adjuvants like Freund's complete adjuvant and CpG use the interferon α of type I interferon as their main effector molecules. It significantly enhances the protective immunological response to influenza vaccines and animal foot-and-mouth disease (FMD) subunit vaccines, and leads those vaccinated individuals to develop large amounts of IgG2a antibodies [16,17].

According to research, when co-administered with an antigen, IFN-α/β can enhance the effects of a variety of immunological reagents, such as soluble proteins [18], inactivated vaccines [17], or recombinant DNA containing transgene materials [19].

Porcine interferon alpha (PoIFNα) has been demonstrated to possess cytokine (CK) properties and exhibit several fundamental biological roles. PoIFNα has long been the subject of research centered on its potent antiviral role, however, limited knowledge is known about its immunological function [20,21].

Previous studies have demonstrated that PoIFNα can generate an effective stimulating impact on porcine immune cells [22]. The recombinant porcine interferon alpha (rPoIFNα) prepared in *E. coli* has been demonstrated by way of us to inhibit the infection of TGEV *in vivo* [21] and *in vitro* [20].

We combined the soluble rPoIFNα made in our lab with the inactivated vaccine (IV) of TGEV in this study to further assess the viability of using recombinant porcine interferon in piglets [23]. We then co-injected them into pigs to explore the feasibility of developing a new mixed TGEV vaccine containing cytokine adjuvants.

Therefore, our research represents an *in vivo* examination of rPoIFNα's capacity to encourage the production of an immunological response to the TGEV-inactivated vaccine (IV) in pigs.

Through animal testing, This investigation aims to comprehend rPoIFNα's potential as an adjuvant alternative for TGEV IV. It also aims to offer a new option available for preventing piglets from contracting TGEV and reducing the incidence of TGE.

Material and Methods

Ethical Statement

The animal experiment project of this study was approved by Anhui Medical University's Bioethics Committee (Hefei, Anhui, People's Republic of China) (Approved Serial number. LLSC20180307, Approved date:01.03.2018). The authors also affirmed that they had followed with all applicable international requirements and standards regarding the use and care of laboratory animals for research purposes.

Reagents and Drugs

Experiments were conducted using Concanavalin A (ConA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) obtained from Sigma Co. Ltd. Fetal Bovine Serum (FBS) was procured from Gibco Corp, while the TGEV antibody detection package (ELISA kit) was obtained from Ingenasa Tech. Catalog serial number 11.TGE.K.3/2, manufactured by Ingenasa Tech in Madrid, Spain.

The TGEV IV oil emulsion, which served as the vaccine control group, was manufactured and procured from TECBOND Biological Products Co., Ltd., located in Chengdu, Sichuan, P.R. China.

The recombinant porcine interferon α Protein (rPoIFNα) was synthesized by Wuhu Interferon Bio-products Industry Research Institute Co., Ltd (Wuhu, Anhui, P.R. China) using the established research and development methodologies employed by our research group [23], it is frozen in an ultra-low temperature refrigerator at -80°C.

Immunization of Experimental Animals in Groups

In this study, 28 white SPF Landrace pigs that were 30 days old were used; they were grown and kept in the barrier facility of the Animal Experiment Center of Anhui Medical University. The pigs had been obtained from a commercial herd that no longer included TGEV. During the tests, they consumed food and liquids *ad-libitum* throughout the experiments.

ELISA tests and PCR tests have been used to screen and verify that all pigs did not comprise TGEV-specific antibodies and that all viral genomes of porcine reproductive and respiratory syndrome virus (PRRSV), porcine parvovirus (PPV), pseudorabies virus (PRV), porcine circovirus type 2 (PCV-2), classical swine fever virus (CSFV) and TGEV were negative. This was done to ensure that the experimental piglets (participants) did not carry TGEV and different pig-related pathogens [24-26].

The inactivated vaccine of TGEV was prepared with the following procedures:

(1) A TGEV was inoculated in ST cells at a cell density of 80% at a dosage of 5 MOI. Subsequently, an adequate quantity of MEM medium supplemented with 5% FBS was added, followed by incubation at a temperature of 37°C in a 5% CO_2 incubator for a duration of 24-72 h. The culture supernatant was collected when the cell lesion rate was not less than 80%, and then the culture supernatant underwent a series of three freeze-thaw cycles, followed by centrifugation at 3.000 rpm for a duration of 10 min. When the $TCID_{50}$ value was not less than $10^{-6.0}$, and then formaldehyde and Binary ethylenimine diethylenimine (BEI) were added to inactivate the virus. The final concentration of BEI was 5 mmol/L and the final mass fraction of formaldehyde was 0.2%.

After sufficient mixing, the mixture was placed at the temperature of 30°C, shaking for 72 h for virus inactivation; finally, the final concentration of 5 mmol/L of sterile sodium thiosulphate solution was added to terminate the inactivation for 1 h, and then mixed with sterile Tween-80 at a mass ratio of 24:1 mixed to make the aqueous phase;

(2) White oil, Spencer-80, and aluminum stearate were mixed in mass fraction 48:2:1 and autoclave sterilize was performed to make the oil phase;

(3) The aqueous phase and the oil phase were homogenized and emulsified at 10.000 rpm in a mass ratio of 1:1 for 3 min each time, and repeated 3 times, and then content determination test, sterility test, mycoplasma test, and exogenous virus test were carried out. When the test results were in accordance with the Veterinary Pharmacopoeia of the People's Republic of China, the described inactivated porcine transmissible gastroenteritis vaccine IV was obtained, and the commonly used dosage of the vaccine was 1 mL/piglet.

The piglets were allocated into seven groups, with each group consisting of four piglets, using a random assignment method. Each piglet belonged to 30-day-old SPF Landrace white piglet. Except for the first blank PBS control group and the second rPoIFNα control group, each of the third to seventh groups was injected with a mixture of 1 mL of TGEV IV and 1 mL of different doses of rPoIFNα. After the mixture (the adjuvant and IV) is evenly mixed, we inject it into the pig. The first blank control group received 2 mL PBS as an immunization, the second rPoIFNα control group received 1 mL PBS+1 mL $2.0x10^5$ U rPoIFNα, the third vaccine control group received the 1 mL PBS+1 mL TGEV IV, and the fourth group received a combination of 1 mL $4.0x10^4$ U rPoIFNα+1 mL TGEV IV. 1 mL TGEV IV+1 mL $2.0x10^5$ U rPoIFNα were administered together to Group 5. 1 mL TGEV IV+1 mL $1.0x10^6$ U rPoIFNα were administered together to Group 6. 1 mL TGEV IV+1 mL 5.0×10^6 U rPoIFNα were administered together to Group 7.

A total of 2 mL of each preparation was injected. The mass of piglets was measured and their body temperatures were documented at 8 a.m. on the test day. Then, in accordance with the experimental grouping strategy, intramuscular injections were delivered to each pig into its neck. All of the experimental pigs received a boost vaccination on the 28th day following their first immunization. Secondary immunization of each group of piglets was performed with a vaccine solution of whose composition and quality were the same as the first immunization of each group (PBS/rPoIFNα+IV). The study design and experimental grouping schemes are shown in *Fig.1*.

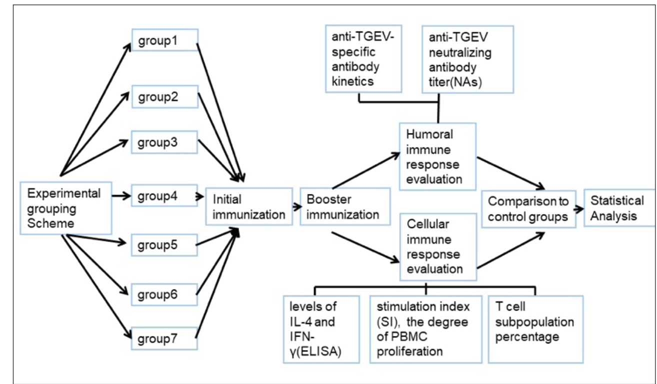

Fig 1. Study design and experimental grouping scheme

Clinical Symptom Tracking

(1) Clinical symptom recording: Following vaccination, the experimental pigs' mental state, hunger, respiratory health, skin color, and other clinical indicators were observed each day.

(2) Measurement of body temperature: After the experiment began, the pig's body temperature was recorded daily at 8 a.m. and 8 p.m., and the recording was kept up until the study's ending.

(3) Measurement of body weight: After the trial began, the piglets' body weights were assessed every six days at 8:00 a.m. (Daily Gain/Original Weight, RDWG, relative daily weight gain) was used to compute relative weight changes.

Pig Blood Sampling

Test tubes containing aseptic vacuum lithium heparin (150 USP units) were utilized to collect blood samples at specific time intervals: 1, 7, 14, 21, 28, 35, 42, and 56 days post-inoculation (dpi). Blood samples that were not subjected to anticoagulation were collected for the purpose of serum separation. The obtained sera were then kept at -20°C in a low-temperature refrigerator.

Separation of Peripheral Blood Mononuclear Cells (PBMC)

PBMCs were extracted from pig heparinized blood using a density gradient centrifugation technique, as previously reported [27,28].

Test for PBMC Proliferation Specific to TGEV

Cultured PBMC cells were stimulated using purified TGEV antigens. As a negative control, PBMC that had not been activated by the TGEV antigen was employed. Positive controls included PBMC activated with the mitogen ConA (10 g/mL).

The proliferation response of PBMC cells was assessed using the common MTT methodology. The stimulation index (SI) was employed to quantify the extent of peripheral blood mononuclear cell (PBMC) proliferation. It is calculated by dividing the average value of the stimulated data by the average value of the unstimulated control [28].

Measuring the Antibody Titers Against TGEV

An enzyme-linked immunosorbent assay (ELISA) kit (Catalog Serial No: 11.TGE.K.3/2, Ingenasa Tech, Madrid, Spain) was employed following the guidelines provided by the manufacturer to detect antibodies specific to TGEV in pig serum samples. The titer was reported using the S/P ratio [21,25,29]. The serum sample was deemed to be positive when the result was 0.4 or above, and it was deemed to be negative when the value was less than 0.4.

The Quantification of Serum TGEV Neutralizing Antibodies (Nas)

The serum neutralization (SN) assay was conducted in accordance with the previously reported experimental methodology [30]. Serial 1:2 dilutions of all pig serum samples were performed. The 200 $TCID_{50}$ TGEV virus suspension was then combined with the serial serum dilutions, incubated at 37°C for 1 h, and then transferred to a PK-15 cell monolayer in a 96-microwell cell culture plate. The CPE formed in each microwell was then examined following a 72-h incubation period. The TGEV-neutralizing antibody titer in each serum sample, reported as ND_{50}, was determined using the Reed-Muench formula [31] (The maximum dilution required to achieve a 50 percent neutralization of viral activity.).

Experiment to Determine Cytokines

Based on the expression patterns of the cytokines they release, Th cell subpopulations may be identified. Interleukin-2 (IL-2), Interferon-gamma (IFN-γ), and lymphotoxin are the primary products of Th1 cells, whereas IL-13, IL-10, IL-6, IL-5, and IL-4 are the main output products of Th2 cells. Following stimulation, the levels of peripheral blood lymphocyte proliferation and the release of interferon-alpha (IFN-γ) and interleukin-4 (IL-4) can serve as indicators of the progression of acquired immunity. Porcine IL-4/IFN-γ was measured using ELISA kits purchased from Adlitteram Diagnostic Laboratories Corporation. The sensitivity of the test was determined to be 1 pg/mL using Adlitteram Diagnostic Laboratories (ADL, San Diego, CA, USA) after following the instructions provided by the manufacturer. The measurement of optical density was conducted at a wavelength of 450 nm using an automated Bio-Rad Model 450 microplate ELISA reader manufactured by Bio-Rad in Hercules, CA, USA [32-35].

Peripheral Blood Lymphocyte Flow Cytometric Analysis

Resuspended PBMCs were supplemented with antibodies against the porcine CD molecule (anti-CD3ε-FITC, anti-CD4α-PE, and anti-CD8-SPRD) at a density of 1×10^7 cells/mL using different fluorescent dyes. The source of the antibodies is Southern Biotech, located in Birmingham, AL, USA. Using the flow cytometry technique, the frequency of CD_8^+, CD_4^+, and CD_3^+ immune cells was quantitatively examined (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical Analysis

To conduct the statistical study, the researchers employed SPSS 17.0 Software (SPSS Inc., Chicago, IL, USA). The mean and standard deviation (SD) results of the experiment were provided. The t-test was employed to conduct a comparison between two groups, whereas the one-way analysis of variance (one-way ANOVA) was utilized to compare variables across many groups. If the P-value is less than 0.01, the observed differences are considered to be statistically significant.

Results

Each Experimental Pig was Not Infected with Swine-Related Viruses

Both the ELISA and the PCR screen test results confirmed that none of the pigs tested exhibited antibodies specific to the porcine transmissible gastroenteritis virus (TGEV). Additionally, the results indicate the absence of porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PRV), porcine parvovirus (PPV),

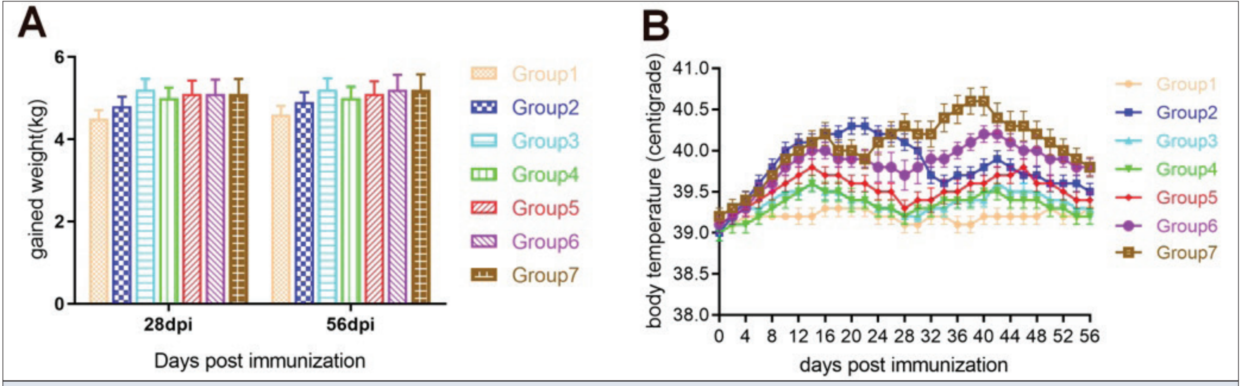

Fig 2. Piglets need to be monitored for clinical signs at various time points following initial and booster vaccinations. These monitors include, but are not limited to, recording the change in the average weight growth of the piglets (A) and the average rectal temperature change of the piglets (B). Body weight growth was recorded in kilograms, while fever was defined as when a piglet's rectal temperature reached or exceeded 41°C. The data are reported as mean±standard deviation (SD), with each group containing four piglets, respectively

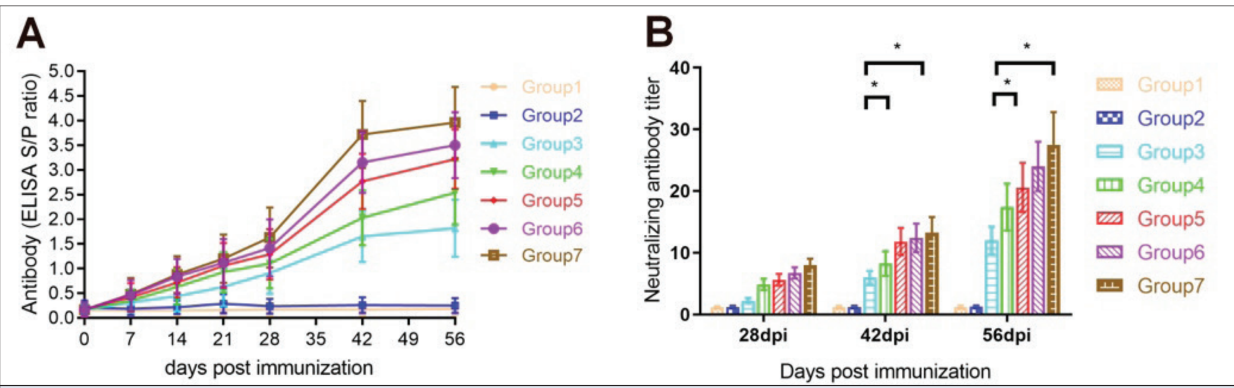

Fig 3. Levels of anti-TGEV specific antibodies (A) and titers of anti-TGEV neutralizing antibodies (B) in vaccinated experimental piglets at different times. According to the grouping scheme, seven groups of experimental piglets received intramuscular injections of different biochemical reagents, such as PBS alone, or IV alone, or rPoIFNα alone, or a combination of $4.0x10^4$U rPoIFNα+IV, or a combination of $2.0x10^5$U rPoIFNα+IV, or a combination of $1.0x10^6$ U rPoIFNα+IV, or a combination of $5.0x10^6$U rPoIFNα+IV. The levels of anti-TGEV specific antibodies and the titers of neutralizing antibodies in the blood samples from each animal group were assessed using the ELISA assay and the serum neutralization (SN) assay. The S/P ratios were employed to quantify the levels of anti-TGEV-specific antibodies, whereas the ND_{50} (The maximum dilution required to achieve a 50 percent neutralization of viral activity) form was utilized to measure the levels of anti-TGEV neutralizing antibodies. The outcomes were reported as the mean±SD (standard deviation). The asterisk (*) indicates a significant difference (P<0.01) between the two groups

porcine circovirus type 2 (PCV-2), classical swine fever virus (CSFV), or TGEV viral genomes. This measure was implemented to ensure the absence of TGEV and other pig-associated viruses in the piglets comprising the trial participants [24-26].

Clinical Signs in Each Pig Group Underwent Experimentally

Following the first inoculation and booster immunization, the pigs were weighed at various intervals.

Although there were little observed differences in the body weight growth (weight gain) of the piglets among the groups, the overall size of the piglets increased *(Fig. 2-A)*. Furthermore, none of the piglets in any group had a fever, and their rectal temperatures were all lower than 41°C *(Fig. 2-B)*. The aforementioned findings indicate that the vaccination administration did not provide any significant impact on the clinical performance of the experimental piglets in either of the groups.

Specific Anti-TGEV Antibody Titers

Fig. 3-A illustrates the kinetics of the fluctuating levels of TGEV-specific antibody responses in seven experimental groups, as assessed through the use of ELISA assay.

Pig groups inoculated with IV+$1.0x10^6$ U and IV+$5.0x10^6$ U rPoIFNα at 28 dpi exhibited significant antibody production against TGEV. Moreover, the production of these antibodies experienced a substantial increase subsequent to the booster immunization.

The IgG levels observed in the IV+$1.0x10^6$ U rPoIFNα group and the IV+$5.0x10^6$ U rPoIFNα group were found to be considerably elevated compared to the IV alone group at 42 dpi and 56 dpi (P<0.01).

At various points after vaccination, the IV+$1.0x10^6$ U rPoIFNα group and the IV+$5.0x10^6$ U rPoIFNα group's piglets showed greater antibody titers than the IV immunization alone group's. It is deduced that rPoIFNα

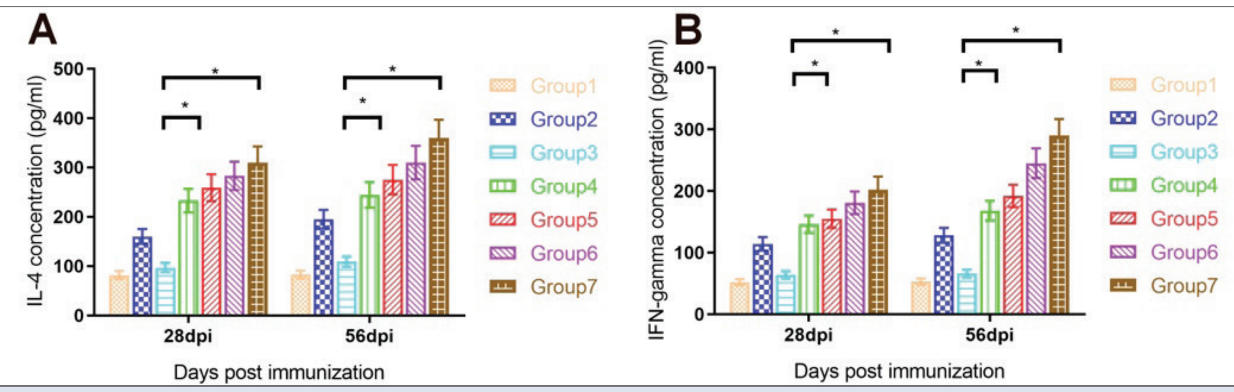

Fig 4. The concentrations of IL-4 (A) and IFN-γ (B) in the PBMC supernatant of experimental piglets at 28 and 56 dpi were measured using ELISA assays. The columns in the table present the average and variability (standard deviation) of the cytokine levels. These levels were measured in triplicate for a group of four subjects. The asterisk (*) indicates a significant difference (P<0.01) between the two groups

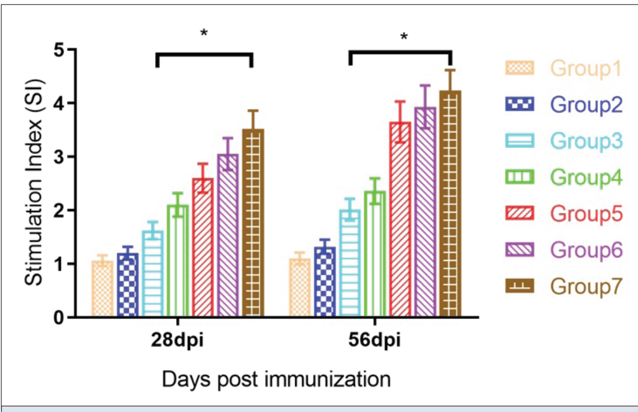

Fig 5. The values of the stimulation index (SI) represent the amounts of PBMC proliferation in each of the experimental groups. The group mean±SD is provided, and the SI value, which was determined in triplicate, is included in each column. ConA serves as a positive control, and the asterisk (*) indicates a statistically significant difference (P<0.01) between the two groups

probably works in a dose-dependent manner to increase antibody response.

Neutralizing Antibodies are Determined Using the SN Test

Overall, the neutralizing antibody titers from the IV+1.0x10^6 U rPoIFNα group and IV+5.0x10^6 U rPoIFNα group were considerably greater than those from the IV alone group at 28 dpi and 56 dpi *(Fig. 3-B)*.

Pigs inoculated with 2.0x10^5 U of rPoIFNα or PBS alone failed to produce any neutralizing antibodies against TGEV. Compared to the IV alone group, the IV+2.0x10^5 U rPoIFNα group was able to develop moderate higher amounts of neutralizing antibodies. Furthermore, the group treated with IV+1.0x10^6 U rPoIFNα and the group treated with IV+5.0x10^6 U rPoIFNα exhibited higher levels of neutralizing antibodies compared to the group treated with IV alone. This is indicative of an enhanced humoral immune response to TGEV.

Specific Cytokines Secreted in Porcine PBMC Supernatant After TGEV Antigen Stimulation

The production levels of IFN-γ and IL-4 in the supernatant of swine PBMC were assessed using commercially available ELISA kits. This evaluation was conducted after stimulating the PBMCs with TGEV antigen.

The findings of the study indicate that the co-administration of rPoIFNα and IV vaccine can effectively elevate the production levels of IL-4 and IFN-γ. These cytokines play a crucial role in stimulating Th1 and Th2 immune responses and augmenting cell-mediated immunity (CMI) in piglets at various time intervals following immunization *(Fig. 4-A,B)*. This is done when the piglets' peripheral blood lymphocytes are stimulated with the TGEV antigen.

RPoIFNα Induces Proliferation of the TGEV-Specific PBMCs (SI)

The rPoIFNα-mediated lymphocyte proliferation levels

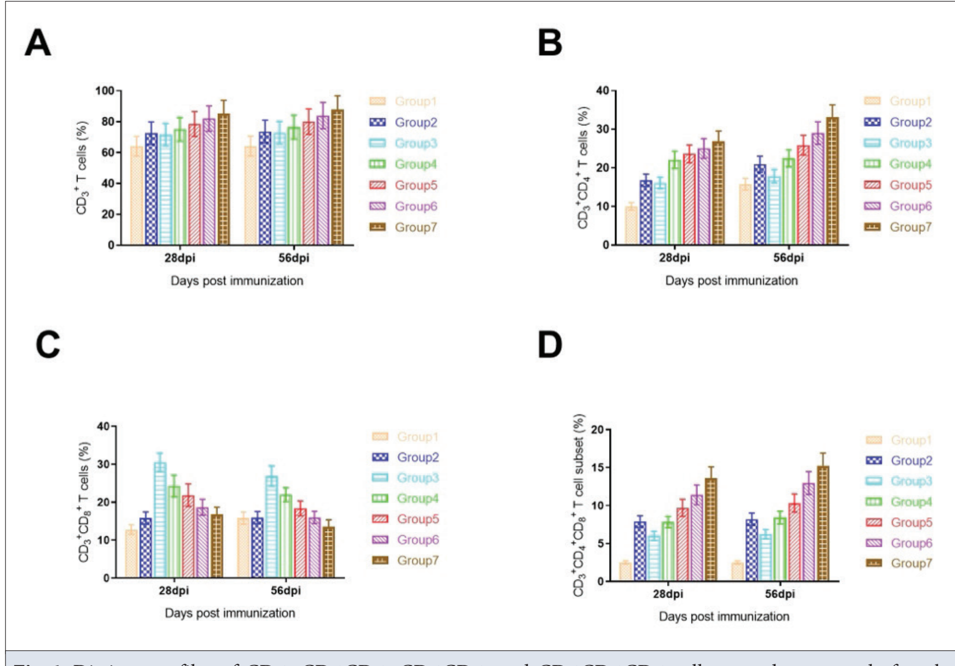

Fig 6. Distinct profiles of CD_3^+, $CD_3^+CD_4^+$, $CD_3^+CD_8^+$, and $CD_3^+CD_4^+CD_8^+$ cells were documented after the initial vaccination and subsequent booster immunization procedures. Panel A exhibits the proportion of CD_3^+ cells; Panel B exhibits the proportion of $CD_3^+CD_4^+$ cells; Panel C exhibits the proportion of $CD_3^+CD_8^+$ cells; and Panel D exhibits the proportion of $CD_3^+CD_4^+CD_8^+$ cells

(expressed in stimulation index, SI) were assessed using the MTT colorimetric test *(Fig. 5)*. The piglet cells from the IV+1.0x10^6 U rPoIFNα group and the IV+5.0x10^6 U rPoIFNα group produced higher stimulation index (SI) at 28dpi, respectively, in comparison to the group receiving PBS alone and the group receiving IV alone. The statistical analysis revealed significant differences among the groups (P<0.01). Specifically, the SI of the IV+5.0x10^6 U rPoIFNα group exhibited the greatest level at 56 dpi (P<0.01) in comparison to both the PBS-alone group and the IV-alone group. Based on the established association between PBMC proliferative response and cell-mediated immune response (CMI), the findings of this study indicate that the inclusion of rPoIFNα enhances the CMI of the IV administration in piglets.

Flow Cytometry Analysis of T Cell Subpopulations in Piglets' Blood Specimens.

The evolving profiles of the CD_3^+, $CD_3^+CD_4^+$, $CD_3^+CD_8^+$, and $CD_3^+CD_4^+CD_8^+$ T cell subpopulations are shown in *Fig. 6*. Early in the post-first vaccination interval, $CD_3^+CD_8^+$ and $CD_3^+CD_4^+CD_8^+$ T cell counts increased somewhat in the majority of samples. At 56 dpi, Piglets' blood showed a notable increase in their values following booster vaccination. Among groups, differences were statistically significant (P<0.01). At 28 dpi, these growth values' arguments commenced.

Discussion

Numerous investigations in recent years have focused on examining the impact of IFNα as adjuvants in prophylactic vaccinations. In 2006, de Avila Botton et al.[16] co-injected porcine IFN-α with the adenovirus containing the A24 capsid of the FMDV and the 3C enzymatic protease encoding region (Ad5-A24) into the pigs, the result could offer full protection, but the control group with only adenovirus could only postpone the development of viremia.

Similar to this, Cheng et al.[36] cloned the swine interferon alpha gene into the pcDNA3.1 vector, created a recombinant vector, and administered it to pigs together with the protein vaccine for porcine FMD. This led to the induction of many neutralizing antibodies that are specific for FMD as well as a robust immunological response driven by T cells. However, the adjuvant-free control group can only stimulate a minimal cellular and humoral immune response [36]. It was also shown that interferon, when used as an adjuvant for protein vaccines, was able to stimulate a significant amount of inflammatory cytokines *in vitro* and activate a Th1-type immune response.

The pVAX1-gag-gp120 (DNA vaccine plasmid against HIV) and the pVAX1-IFN (eukaryotic expression plasmid) were both built by Jiang et al.[37] based on the eukaryotic expression vector pVAX1. The BALB/C mice were given an intramuscular injection with the mixed resulting vaccine. In the mouse spleen T cells, according to the findings in comparison to the control group without adjuvant, there were substantial increases in the percentages of the lymphocyte $CD_3^+CD_8^+$ and $CD_3^+CD_4^+$

subpopulations as well as the cytotoxic activity of spleen cytotoxic T cells [37].

Proietti et al.[17] revealed that endogenous type I interferon in mice was required for the Th1 type immune response elicited by conventional adjuvants. Interferon also showed strong adjuvant efficacy when paired with human influenza vaccination owing to the development of IgG2a and IgA.

In support of this claim, Bracci et al.[38] showed that IFN-α may have a positive adjuvant effect when the influenza vaccination is administered intramuscularly or mucosally.

IFN-α may also greatly boost the cellular immune response to the PRRSV vaccination, according to Charerntantanakul [22], who was searching for an appropriate immunological adjuvant for the pig PRRSV vaccine. Primary lymphocyte proliferation, cytotoxic T cell activity, peripheral blood lymphocyte IFN-γ production, and T cell reactivity to the PRRSV antigen are all markedly enhanced. Intriguing studies on the adjuvant impact of IFN-α on preventative vaccinations have also been published by de Avila Botton et al.[16], Le Bon et al.[18], and O'Brien et al.[39].

The development of neutralizing antibodies (NA) is a crucial component of humoral immunity, as it aids in the elimination of TGEV and the prevention of viral infections [6]. Within the context of the host immune response against TGEV infection, cell-mediated immunity (CMI) is also an essential component [21]. However, protective immunity against infection with heterologous TGEV strains may require a dual enhancement of CMI and antibody production [7]. These strongly indicate that the production of TGEV NA antibodies and virus-specific cellular immune responses collaborate to facilitate the elimination of the virus. This suggests that novel formulations of non-infectious vaccines and delivery systems are required to stimulate both humoral and cellular responses in order to effectively control TGE [7,11-14,21,35].

Our results indicate that the combination of rPoIFNα with the TGEV IV vaccine has the ability to enhance the immunological response. Our study specifically showed that when rPoIFNα was given along with an inactivated TGEV vaccination, it resulted in the activation of both Th1 and Th2 immune responses in pigs. rPoIFNα exhibits a highly favorable safety profile. The clinical signs of the pigs, such as weight increase and body temperature, were not impacted after immunization *(Fig. 2)*. After immunization, we also determined changes in parameters such as anti-TGEV-specific antibodies, NAs, cytokines IFN-γ and IL-4, lymphocyte proliferative activity (SI), and CD_3^+, CD_4^+, and CD_8^+ cell subpopulations.

The results showed the following findings:

(1) Regarding the TGEV-specific antibody titers, the rPoIFNα+IV group exhibited an earlier generation of TGEV-specific antibodies compared to the IV alone group. The group receiving both rPoIFNα and IV showed elevated levels of TGEV-specific antibodies compared to the group receiving IV alone. This indicates that rPoIFNα increases the antibody response specific to TGEV that is stimulated by the IV vaccine *(Fig 3-A)*.

(2) Regarding the levels of neutralizing antibodies, pigs vaccinated with rPoIFNα+IV showed a significant difference compared to those vaccinated with IV alone. This suggests that rPoIFNα boosts the production of neutralizing antibodies, which in turn may play a role in enhancing humoral immunity and providing protection against TGEV *(Fig 3-B)*.

(3) Th2 cytokines, such as IL-4, play a crucial role in boosting the humoral immune system, while Th1 cytokines, such as IFN-γ, mainly affect cell-mediated immune responses [40]. Regarding cytokines, the inclusion of rPoIFNα notably augmented the synthesis of IFN-γ and IL-4, indicating that rPoIFNα enhances both Th1 and Th2 reactions. This suggests that rPoIFNα enhances the efficacy of the TGEV vaccine and acts as an adjuvant for both the cellular and humoral immune responses to TGEV IV vaccination *(Fig 4)*.

(4) Regarding lymphocyte proliferative activities, our data demonstrated that the TGEV-specific lymphocyte proliferative response was considerably greater in the group that received the IV+rPoIFNα combination immunization compared to the group that received IV alone immunization ($P<0.05$) *(Fig. 5)*. Given that lymphocyte proliferative responses are typically linked to cell-mediated immunity (CMI), this implies that the administration of both IFN-α and IV can enhance the CMI of the TGEV IV vaccine.

(5) Regarding T cell subpopulations, we conducted a more detailed analysis of alterations in the subpopulations of CD_3^+, CD_4^+, and CD_8^+ cells using flow cytometry *(Fig. 6)*. During the early stage following the initial immunization, there was a small rise in the populations of CD_3^+, CD_4^+, and CD_8^+ cells in most groups. The quantities of CD_3^+ and CD_4^+ cells in pig blood exhibited a progressive increase in all groups at 28 dpi, followed by a considerable increase at 56 dpi subsequent to booster immunization.

We also found that the administration of rPoIFNα at dosages of $1.0x10^6$ U or $5.0x10^6$ U effectively boosted the immunological response of piglets when combined with the TGEV IV vaccination. More precisely, the inclusion of rPoIFNα in the TGEV IV vaccine resulted in improved

immune responses, both in terms of antibodies and cellular immune responses, when compared to the group that received only the TGEV IV vaccine. The results of our study indicated that the immune-boosting impact of rPoIFNα was more pronounced when administered at a dosage of $5.0x10^6$ U compared to a dosage of $1.0x10^6$ U. This suggests that the immunological response of piglets is enhanced by rPoIFNα in a dose-dependent manner. The long-lasting impact was most pronounced at a concentration of $5.0x10^6$ U. The results of our study align with the findings published by Tovey et al.[15] and Damjanovska et al.[41]. They noticed a considerable increase in IgG and IgA levels in bodily fluids when IFN-α was provided with inactivated influenza vaccine, and this increase was dependent on the dosage.

Our study showed that rPoIFNα exhibited good safety. After co-administration to piglets, the inactivated vaccine (IV) mixture including rPoIFNα may considerably increase the generation of antibodies, Th1 and Th2 cytokines, and PBMC proliferation when compared to TGEV IV alone. The piglets' immune response to TGEV was greatly enhanced by the combined administration. Furthermore, when the dose of rPoIFNα was increased, the immunological boost induced by the IV+$5.0x10^6$ U rPoIFNα group was significantly bigger than that of the IV+$2.0x10^5$ U rPoIFNα group.

Thus, the addition of rPoIFNα at doses of $1.0x10^6$ U or $5.0x10^6$ U to the IV vaccine resulted in higher levels of antibody titers, lymphocyte stimulation index (SI), IFN-γ and IL-4 secretion, and lymphocyte subpopulations compared to immunization with the TGEV IV vaccine alone. To our knowledge, this work represents the first investigation conducted in a living organism to assess the ability of rPoIFNα to enhance the immune response to the TGEV IV vaccine in piglets. The aforementioned results indicate that rPoIFNα has the potential to serve as an adjuvant, augmenting the immunological response to vaccines in farm animals.

Limitations of This Study: This study aimed to assess the suitability of rPoIFNα as an adjuvant for immune activation in vaccinations for farm animals. Nevertheless, additional field challenge tests are required to assess and thoroughly analyze the protective efficacy of rPoIFNa+IV on piglets.

In summary, rPoIFNα showed significant adjuvant effects when combined with TGEV- inactivated vaccines at doses of $1.0x10^6$ U or $5.0x10^6$ U. The results of our study show that rPoIFNα is an ideal adjuvant for immunostimulation of TGEV. The significance of our findings lies in the revelation of rPoIFNα's potential as an optimal immunostimulatory adjuvant for TGEV-inactivated vaccines.

Declarations

Availability of Data and Materials: The authors affirm that they can provide the data that supports the findings of this study if a legitimate request is made.

Acknowledgments: The authors are very thankful to the entire research staff members in Wuhu Interferon Bio-products Industry Research Institute Co., Ltd, (Wuhu, Anhui, P.R. China) for their outstanding work in technical assistance.

Funding Support: This research was funded by the University Synergy Innovation Program of Anhui Province of "Anhui Provincial Department of Education" (Grant No.GXXT-2022-057), the Wuhu City Science and Technology Plan Project of "Wuhu Municipal Government" (Grant No.2022ly12 and Grant No. 2023yf003) and Anhui Provincial Key Research and Development Program of "Anhui Provincial Department of Science and Technology" (Grant No 2023S07020021).

Competing Interests: The authors declared that there is no conflict of interest.

Ethical Approval: The animal experiment project of this study was approved by Anhui Medical University's Bioethics Committee (Hefei, Anhui, People's Republic of China). (Approved Serial number. LLSC20180307, Approved date:01.03.2018).

Declaration of Generative Artificial Intelligence (AI): The article and/or tables and figures were not written/created by AI and AI-assisted technologies.

Author Contributions: HYY: Conducted data inquiry, performed data analysis, and drafted the manuscript. DMG: Data acquisition, investigation, and methodology. JD: Conducted data analysis and performed experiments and methods. YS: focused on designing the study, conducting thorough data analysis, and providing critical feedback for revising the manuscript. JZ: Engage in the examination, creation, oversight, and revision of the manuscript. All authors revised and approved the final version of the manuscript.

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