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PRRSV GP4 subunit vaccine combined with adenovirus heterologous prime-boost immunization strategy induced a significant immune response in mice

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Abstract

Background The porcine reproductive and respiratory syndrome virus (PRRSV) continues to cause widespread infections in the pig industry worldwide. Currently, multiple PRRSV vaccine candidates are in preclinical or clinical trials, and each has different advantages and limitations. Glycoprotein 4 (GP4) is rich in epitopes, which can induce the body to produce neutralizing antibodies, plays a vital role in causing the host immune response, and is a key target for PRRSV vaccine development. In this study, we developed a novel candidate vaccine immunization strategy combining a subunit vaccine with an adenovirus vector vaccine through prokaryotic and eukaryotic systems expressing GP4.

Results In this study, predictive analysis of PRRSV GP4 antigen structures in two expressed modes, and the results showed good antigenicity. The PRRSV GP4 subunit vaccine, as well as the adenovirus vector-based vaccine, were successfully constructed. In the immunization experiment of mouse models, a heterologous primary-boost immunization strategy was implemented: primary immunization with the GP4 subunit vaccine, and boost immunization was followed by an adenovirus vector vaccine. The safety assessment revealed that all candidate vaccine groups demonstrated good safety profiles. With an indirect enzyme-linked immunosorbent assay (ELISA) and neutralizing antibodies, mice in the combined immunization group developed higher levels of PRRSV-specific antibodies with significantly higher neutralizing antibody titers than mice alone. IgG subtype analysis indicated that the proteome favors the Th2-type immune response, while the adenoviral group favors the Th1-type immune response. The secretion levels of cytokines IL-4, IFN-γ, and TNF-α were significantly higher in the serum of the combined immunization group than in the immune group alone. Moreover, the cellular immune response test results showed that the combined immune group significantly enhanced the splenic lymphocyte proliferation

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capacity, IFN-γ secretion level, and cytokine transcript level. These findings suggest that the heterologous primary-boost immunization strategy of the PRRSV GP4 subunit vaccine developed here, in combination with the adenovirus vaccine, successfully induced strong humoral and cellular immune responses in mice.

Conclusions In this study, the PRRSV GP4 subunit and adenovirus vector vaccine were successfully constructed and induced high levels of PRRSV-specific neutralizing antibody and cellular immune responses in mouse models by a heterologous primary-boost immunization strategy. These results support the clinical development of the PRRSV vaccine and bring new hope for PRRSV prevention and control strategies in the swine industry.

Keywords PRRSV, Glycoprotein 4, Subunit vaccine, Adenovirus, Heterologous prime-boost immunization strategy, Vaccine

Background

PRRS is an infectious disease caused by the PRRSV, which causes serious harm to the pig industry [1]. The disease was first reported in the United States in 1987, followed by subsequent reports of related cases in Europe, Asia, and other regions. In 1991, the first European-type strain of PRRSV was isolated in the Netherlands, named Lelystad virus [2]; in 1992, American scientists isolated the North American strain named VR-2332 [3]. PRRSV infection can lead to severe reproductive disorders in pregnant sows, such as abortion, premature birth, and stillbirth, and cause respiratory symptoms in piglets and finishing pigs, leading to continuous high morbidity and mortality, which has a significant impact on the global pig industry [4]. In China, the PRRSV virus was first reported and isolated in 1995. Around 2006, an outbreak of "high fever" characterized by high fever, high morbidity, and high mortality was confirmed to be a highly pathogenic PRRSV variant [5, 6]. Since then, PRRSV has been one of the key diseases for prevention and control in China's pig industry, posing a continuous threat to the stable development of the pig industry.

Developing effective vaccines has become a critical strategy to address PRRSV's severe challenges to prevent and control the disease. Vaccines against PRRSV mainly include inactivated and live attenuated vaccines [7]. The inactivated vaccine was one of the first vaccines used for PRRSV control, and its main advantage was high safety. However, the inactivated vaccine is relatively weak and requires multiple vaccinations for an adequate immune response. In addition, inactivated vaccines mainly induce humoral immunity, with a weak cellular immune response and limited cross-protection ability against heterologous strains [8]. Live attenuated vaccines can stimulate the body to produce strong cellular and humoral immunity responses and protect against the homologous strain. However, live attenuated vaccines also have specific safety risks, such as the possibility of reversion to virulence. In addition, live attenuated vaccines may recombine with wild strains to produce new mutant strains, increasing the complexity of disease prevention and control [9].

The GP4 protein, as the key envelope protein of the PRRSV virus, plays a decisive role in the structure and function of the virus [4]. The protein is a transmembrane glycoprotein composed of multiple domains and contains numerous glycosylation sites, which are crucial for the protein's correct folding, stability, and immunogenicity [10]. The GP4 protein exists on the surface of the virus's outer membrane as a trimer; together with other viral structural proteins, it constitutes the virus's spike structure. These spike structures endow the virus with unique morphological characteristics and play a key role in the interaction between the virus and host cells [11]. The GP4 protein plays an indispensable role in the process of viral infection. It is located on the surface of the viral envelope and can specifically bind to host cell receptors, promoting the adsorption and penetration of the virus [12]. Furthermore, the GP4 protein is rich in antigenic epitopes, capable of inducing the production of neutralizing antibodies in the host, playing a significant role in inducing host immune responses [13, 14]. It is a key target for PRRSV vaccine development. Subunit vaccines have become a key direction in vaccine development due to their high safety and efficacy [15]. Adenovirus vectors, with their efficient gene delivery capabilities and strong immune activation properties, combined with the immunogenicity of the GP4 protein, are expected to elicit a more intense and durable immune response through a heterologous primary-boost immunization strategy.

The heterologous prime-boost immunization strategy employed in this study, by combining the advantages of the PRRSV GP4 subunit vaccine and recombinant adenoviral vector vaccine, successfully elicited a robust immune response against PRRSV GP4 in mice. This strategy increased the level of neutralizing antibodies and potentially induced a broad cellular immune response, providing strong support for effectively controlling PRRSV infection.

Results

Analysis of antigen structure

In our study, we focused on the bioinformatic structural analysis of the PRRSV GP4 gene. Since prokaryotic cells

do not possess organelles unique to eukaryotic cells, such as the endoplasmic reticulum, they are unable to properly process proteins that have signal peptides. This lack of processing can adversely affect the biological activity and function of the protein. Therefore, in the prokaryotic expression system, we have removed the N-terminal signal peptide fraction of the GP4 sequence. To further understand the vaccine candidates' structural properties, we used PSIPRED and SOPMA tools to perform a detailed prediction analysis of the secondary structures of the two different expression vaccine candidates, Pro-GP4 and Ad5-GP4. The study showed that candidate vaccine Pro-GP4 was mainly composed of α helix (38.46%), extended chain (15.38%), β turn (1.02%), and random turn (44.23%); candidate vaccine Ad5-GP4 mainly consists of α helix (39.89%), extension chain (16.29%), β turn (2.81%) and random turn (41.01%). These data are visually presented in Fig. 1A and B, respectively. Next, we modeled and generated the tertiary structure of the vaccine candidates using the Robetta server, the results of which are presented in Fig. 1C. To assess the confidence of these models, we performed Z-score scoring using the ProSA tool, which showed a Z-score of -3.71 and −4.54 for Pro-GP4 and Ad5-GP4, respectively, and these scores are shown in Fig. 1D. The Ramachandran diagram indicates that the Pro-GP4 most favored regions [A, B, L] is

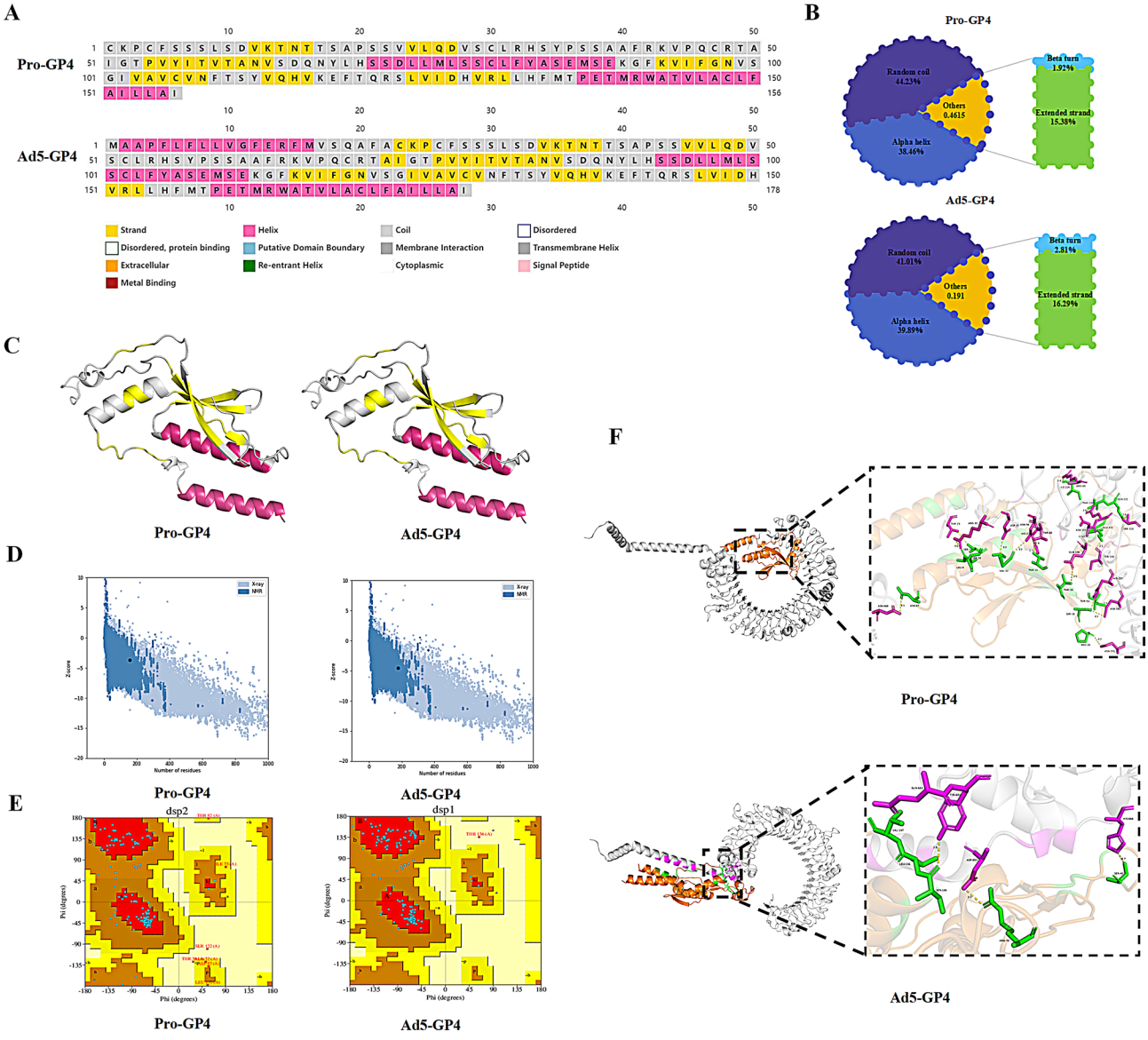

Fig. 1 Bioinformatics prediction analysis of GP4 proteins. (**A**, **B**) The secondary structure of candidate vaccines is predicted and analyzed using SOPMA (**A**) and PSIPRED (**B**). Different colors represent corresponding information about the secondary structure of a protein. (**C**) Modeling evaluation of the tertiary structure of candidate vaccines. (**D**)The Z score of the refined model ProSA SEB map is -3.71 and −4.54. (**E**) Ramachandran plot for the candidate vaccine construct. (**F**) Docking results of vaccines candidates with the TLR3 molecule

85.4%, additional allowed regions [a, b, l, p] is 13.9%, and disallowed regions is 0.7%. The Ad5-GP4 most favored regions [A, B, L] is 81.1%, additional allowed regions [a, b, l, p] is 14.6%, generously allowed regions [a, b, l, p] is 3.0%, disallowed regions is 1.2% (Fig. 1E). Furthermore, we observed a vaccine candidate with a central deep binding site in TLR3. We amplified specific amino acid residues in the local region, a phenomenon graphically depicted in Fig. 1F. These specific amino acid residues showed different distribution patterns and frequencies in the Pro-GP4 and Ad5-GP4 vaccines. Our structural analysis of the PRRSV GP4 gene has revealed insights into the secondary and tertiary structures of Pro-GP4 and Ad5-GP4 vaccines. This provides a basis for further study on their immunogenicity against PRRSV.

Construction, purification, and characterization of the GP4 subunit vaccine

The GP4 gene with the signal peptide sequence removed was successfully inserted into the expression plasmid pSumo-mut, and the detailed steps and results of this process are demonstrated in the schematic diagram of Fig. 2A. To ensure that the inserted sequence is entirely consistent with the template sequence, DNA sequencing analysis was performed, and the results confirmed the correctness of the sequence. Next, IPTG was used as an inducer to initiate the expression of the GP4 gene, and the expressed protein was purified using Ni-NTA affinity chromatography. SDS-PAGE analyzed the purified protein samples, and the results showed that the molecular weight of the protein was approximately 35 kDa, which is consistent with the expected size. To further verify the specificity of the protein, a Western Blot experiment was performed using an antibody against the His tag, and the results also showed a band at 35 kDa, which is consistent with the results of the SDS-PAGE analysis (see detailed results in Fig. 2B).

Construction and characterization of the GP4 adenovirus vector vaccine

We have successfully integrated the complete GP4 gene into the shuttle plasmid pAdTrack-CMV (Fig. 2A) and performed in vitro homologous recombination with the backbone plasmid (Supplementary Fig. 1C and D). Through identification by single enzyme digestion with PacI, the correctness of homologous recombination was confirmed, and the recombinant adenovirus plasmid was successfully constructed (Supplementary Fig. 1). Subsequently, the transfection was carried out in 293AD cells. Within 10 to 14 days, the first generation of recombinant adenovirus gradually began to induce cytopathic effects, characterized by visible changes such as cell shrinkage and aggregation. Under the fluorescence microscope, the cells exhibited a comet tail-like distribution of green fluorescence; the blank cell control group did not show any fluorescence signal, whereas, under bright-field microscopy, significant cytopathic effects were visible (Fig. 2C). This proves that the recombinant adenovirus has been successfully packaged in the cells. GAPDH was further selected as the internal reference, and PRRSV-positive serum was used as the primary antibody for Western Blot verification, showing the target band of about 19.7 kDa (Fig. 2D), indicating that the recombinant adenovirus was immunogenic.

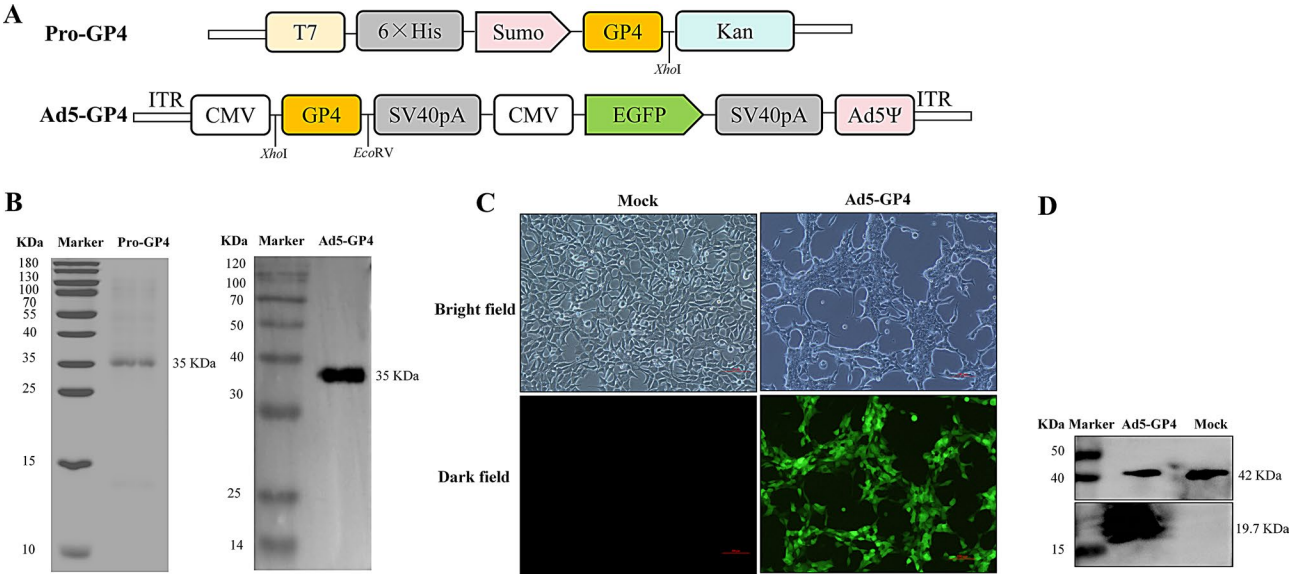

Fig. 2 Design and purification of recombinant proteins. (**A**) Schematic diagrams of Pro-GP4 and Ad5-GP4. (**B**) SDS-PAGE analysis of purified Pro-GP4 and Western blot analysis of purified Pro-GP4 using a 6×His-tag antibody. (**C**) Fluorescence identification of Ad5-GP4. (**D**) Western blot analysis of Ad5-GP4 using PRRSV positive serum, β-actin as an internal reference antibody

Safety evaluation of the vaccine candidates

After primary and booster immunization of the mice, none of the vaccine groups showed significant adverse effects, and no mortality occurred. However, during the continuous monitoring of mouse body weight, we noticed that Ad5-GP4 mice lost body weight significantly on the second day after the two immunizations and began to recover gradually on the third day (Fig. 3B). This phenomenon may be related to the properties of adenoviral vectors as live viruses, which, as heterologous substances, may induce a stress response. Although the Ad5-GP4 vaccine caused a temporary decrease in mouse body weight after primary and secondary immunization, it did not damage the long-term health of the mice, showing good performance of the Ad5-GP4 vaccine in terms of safety.

Anti-GP4 antibodies induced by vaccine candidates

To detect antibody responses to recombinant proteins in animal models, we collected mouse serum every two weeks to detect specific antibodies. The results indicate that the subunit vaccine groups Pro-GP4 and HPBS-GP4 rapidly generated GP4-specific antibodies, with antibody levels quickly reaching peak values, and no significant differences were observed between the two groups at different time points. In contrast, the Ad5-GP4 group, commercial vaccine group, and control group exhibited highly significant differences in antibody levels compared to the HPBS-GP4 group ($p < 0.001$). Additionally, the inactivated virus group showed no significant difference compared to the HPBS-GP4 group at days 28 and 42 (Fig. 4A). This phenomenon may be related to the fact that the antigen we used for the ELISA coating was the prokaryotic GP4 protein. In contrast, the Ad5-GP4 group used the complete GP4 total antigen, and the inactivated PRRSV group and the commercial vaccine group had more complex antigenic components.

Immune subtype induced by vaccine candidates

IgG1 and IgG2a were detected in the serum of postimmunization mice using the indirect ELISA technique to investigate the subtype characteristics of the immune response. In the IgG1 subtype analysis, the results showed that the Pro-GP4 group, the HPBS-GP4 group, and the inactivated virus group all exhibited relatively high IgG1 levels. Specifically, on day 28, the Pro-GP4

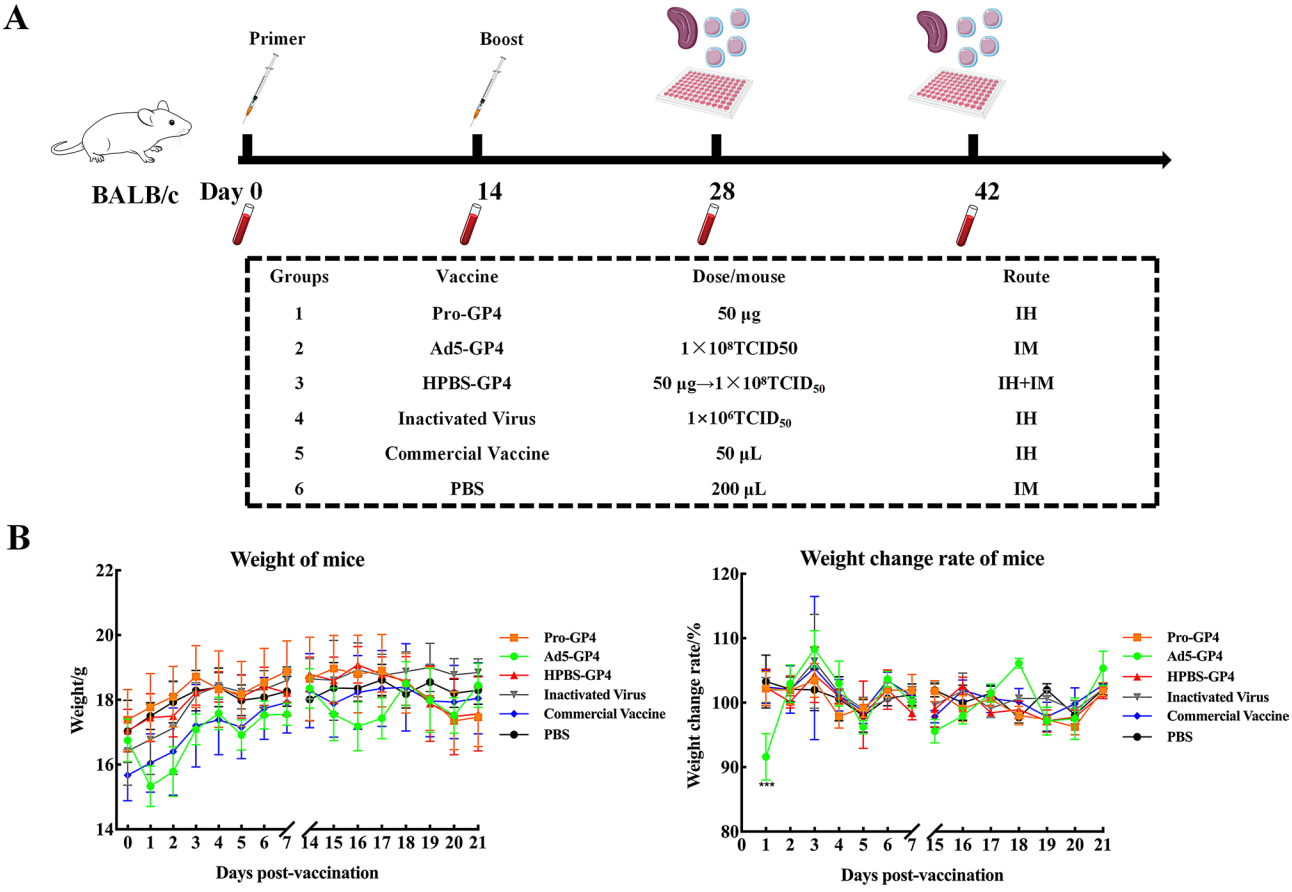

Fig. 3 Immune procedure and safety evaluation in mice. (**A**) Schematic of the vaccination schedule, dose, and route for the mouse experiment. SC represents subcutaneous inoculation, while IM stands for intramuscular inoculation. (**B**) Safety evaluation of body weight in mice

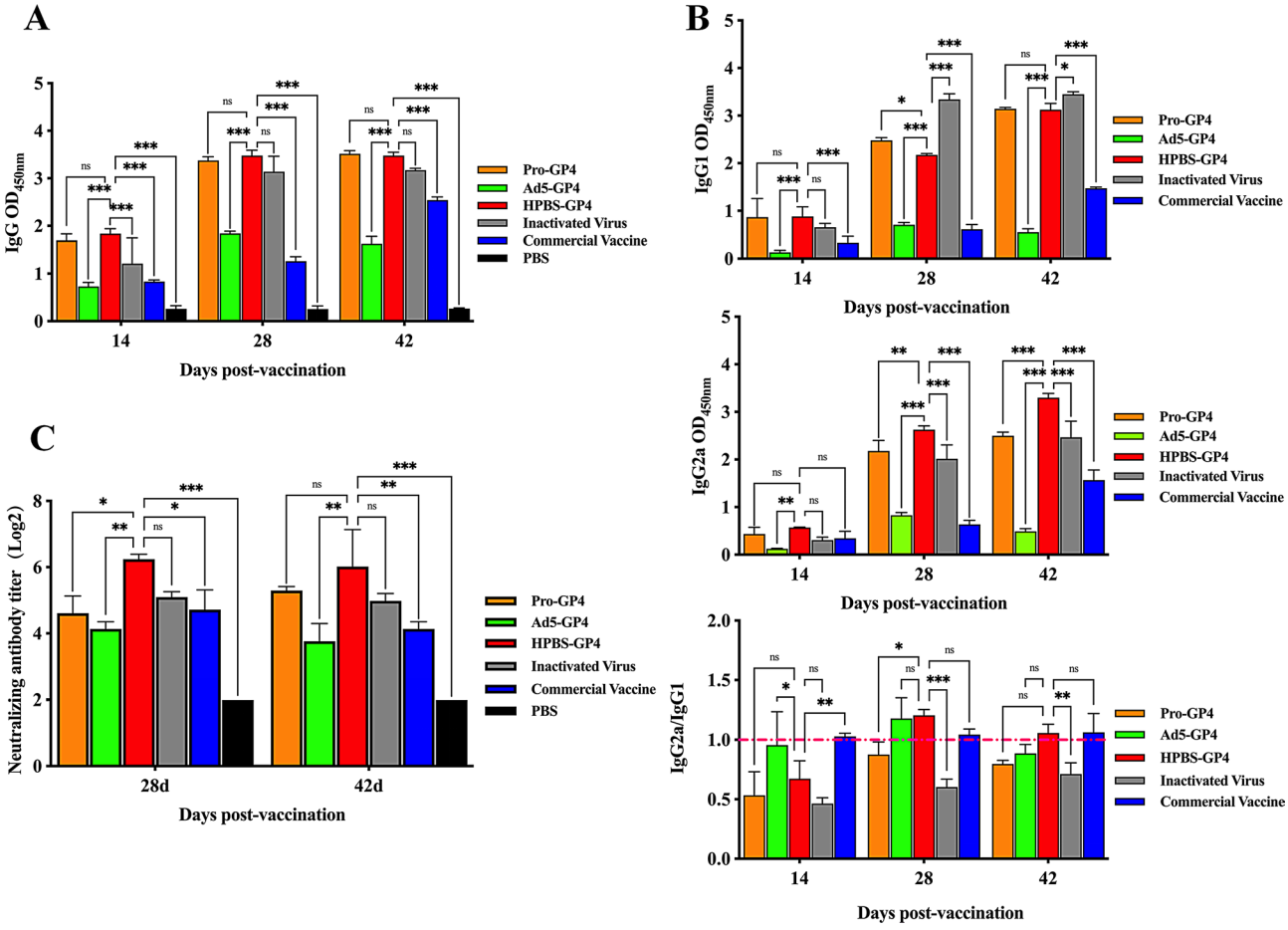

Fig. 4 Humoral immune responses of vaccinated mice. (**A**) The IgG levels of the GP4-specific antibody. (**B**) Specific antibody IgG subtype levels, including IgG1, IgG2a, and IgG2a/IgG1 ratios. (**C**) Levels of the neutralizing antibodies. The data are presented as mean ± SD in each group. ns indicates $p \geq 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$

group and the inactivated virus group displayed varying degrees of significantly higher IgG1 levels compared to the HPBS-GP4 group; whereas on day 42, the IgG1 level of the inactivated virus group remained significantly higher than that of the HPBS-GP4 group ($p < 0.05$). However, in the IgG2a subtype analysis, the HPBS-GP4 group demonstrated higher IgG2a levels, showing significant differences compared to the other four groups on both day 28 and day 42 ($p < 0.001$). Further analysis of the IgG2a/IgG1 ratio revealed that the IgG2a/IgG1 ratio in the Pro-GP4 group and inactivated virus group was less than 1, whereas the IgG2a/IgG1 ratio in the HPBS-GP4 group was greater than 1 (Fig. 4B). These findings suggest that vaccine formulations containing the A206 adjuvant may preferentially induce a Th2-type immune response, whereas adenovirus vector vaccines may tend to promote a Th1-type immune response.

Neutralizing antibody levels

Furthermore, we determined the levels of neutralizing antibodies at days 28 and 42 after immunization. We found that both time points showed the highest neutralizing antibody titers in the HPBS-GP4 group. On days 28 and 42, there was no difference between the HPBS-GP4 group and the PRRSV inactivated virus group, but varying degrees of differences were observed among the Pro-GP4 group, Ad5-GP4 group, commercial vaccine group, and PBS control group ($p < 0.05$) (Fig. 4C). These differences may reflect variations in the mechanistic and efficacy of neutralizing antibody induction among vaccines from different sources.

Cellular immunologic response

To assess the cellular immune response, we performed an in-depth analysis of lymphocyte proliferation and IFN-γ ELISpot in mice at days 28 and 42 after immunization to determine the vaccine-induced cellular immune activity. Under stimulation by GP4-specific proteins, the SI values of the HPBS-GP4 group was the highest among the five experimental vaccines, showing varying degrees of difference compared to the inactivated virus group, Pro-GP4 group, Ad5-GP4 group, and commercial vaccine

group ($p < 0.05$) (Fig. 5A). In the splenic lymphocytes of mice from different immunization groups, we observed that under GP4-specific protein stimulation, the HPBS-GP4 group exhibited more stronger GP4-specific IFN-γ responses, with a significantly different higher number of IFN-γ -secreting cells than the other five groups (Fig. 5B and C). These findings further support that heterologous immunization-booster immunization strategies can significantly enhance the body's cellular immune response.

Cytokine assays

To investigate cytokine changes in mice after vaccination, we determined the transcript levels of splenic lymphocyte-specific cytokines at day 42. The results showed that the IL-4 transcription level showed no significant difference in IL-4 between HPBS-GP4 and Ad5-GP4, exhibited varying degrees of significant differences compared to the other four groups; the IFN-γ and TNF-α levels were significantly higher than those of the other five groups (Fig. 6A-C). Meanwhile, we measured the cytokine secretion levels in serum on day 42 post-vaccination. The results showed that the IL-4, IFN-γ, and TNF-α secretion levels in the HPBS-GP4 vaccine group were significantly higher than those in the other five groups ($p < 0.001$) (Fig. 6D-F). These changes in cytokine transcript levels reflect the dynamic process of the immune response in mice after vaccination, but also further confirm the effectiveness of the HPBS-GP4 vaccine in inducing specific cellular immune responses.

Discussion

The PRRS disease has profoundly impacted the pig industry worldwide in the last 30 years [16]. This disease has caused a substantial economic loss to the pig industry, mainly due to its high morbidity and mortality and a significant decline in growth performance [17]. Despite the considerable research resources and efforts invested globally towards developing effective vaccines against this disease, controlling and eradicating the PRRS remains an ongoing challenge.

The PRRSV GP4 gene is crucial in inducing neutralizing antibodies and cellular immune responses. The structural analysis of the GP4 protein revealed several immunogenic epitopes recognized by the host immune system. These epitopes are critical for developing effective vaccines, as they can stimulate the production of antibodies that can neutralize the virus and prevent its entry into host cells. Furthermore, the GP4 gene also plays a significant role in modulating the cellular immune response, mainly through the induction of IFN-γ-producing T-cells. These T-cells are essential for clearing virus-infected cells and controlling viral replication. DNA shuffling of PRRSV GP4 can broaden the ability of chimeric viruses to induce cross-neutralizing antibodies against heterologous PRRSV strains [18]. After feeding pigs with transgenic Arabidopsis expressing the GP4 protein from PRRSV, humoral and cellular immune responses against PRRSV were generated in the pigs, which verifies that the GP4 protein can serve as a platform for an effective subunit vaccine [19]. The PRRSV GP4 antigen was expressed through a plant expression system for the preparation of subunit vaccines, which effectively induced the production of virus-neutralizing antibodies in mice [20]. A research team has meticulously designed a nanoparticle vaccine containing specific epitopes of the PRRSV GP4 key protein, which can effectively activate mouse T cells, induce particular antibody production in piglets, and significantly enhance PRRSV-specific IFN-γ levels, demonstrating significant protective effects against PRRSV challenge [21].

As a commonly used prokaryotic expression system, the *Escherichia coli (E. coli)* expression system plays a vital role in vaccine development [22]. The *E. coli* expression system, being highly efficient, fast, cheap, and easy to operate and control [23], enabled us to prepare GP4 protein in large quantities, providing sufficient material for subsequent vaccine purification, identification, and animal experiments. Although the *E. coli* expression system has shown many advantages in vaccine development, one of the challenges associated with the *E. coli* expression system is the potential misfolding or aggregation of the expressed proteins, which may affect their immunogenicity and functionality [24]. The adenovirus expression system also has its unique value and potential. Adenoviral vectors can efficiently transduce multiple cell types, including immune cells, inducing a strong immune response [25]. Furthermore, adenovirus vectors have a large loading capacity and can carry and express large foreign gene fragments [26]. In this study, we expected to develop a more efficient and safer PRRS vaccine by combining the efficient preparation capacity of the *E. coli* expression system and the powerful immune induction capacity of the adenovirus expression system.

Heterologous immunization strategies, namely, sequential vaccination with vaccines prepared by different expression systems to induce more extensive and durable immune responses, have become a hot topic in current vaccine research [27, 28]. In heterologous immunization strategies, two different types of vaccines activate the immune system through various pathways, which can identify and attack pathogens from multiple angles, thus reducing the possibility of immune escape from pathogens [29, 30]. Studies have shown that applying a heterologous prime-boost immunization strategy, which involves the co-administration of European-type PRRSV DNA and recombinant vaccinia virus expressing GP3 and GP5, can elicit an effective immune protective response [31].

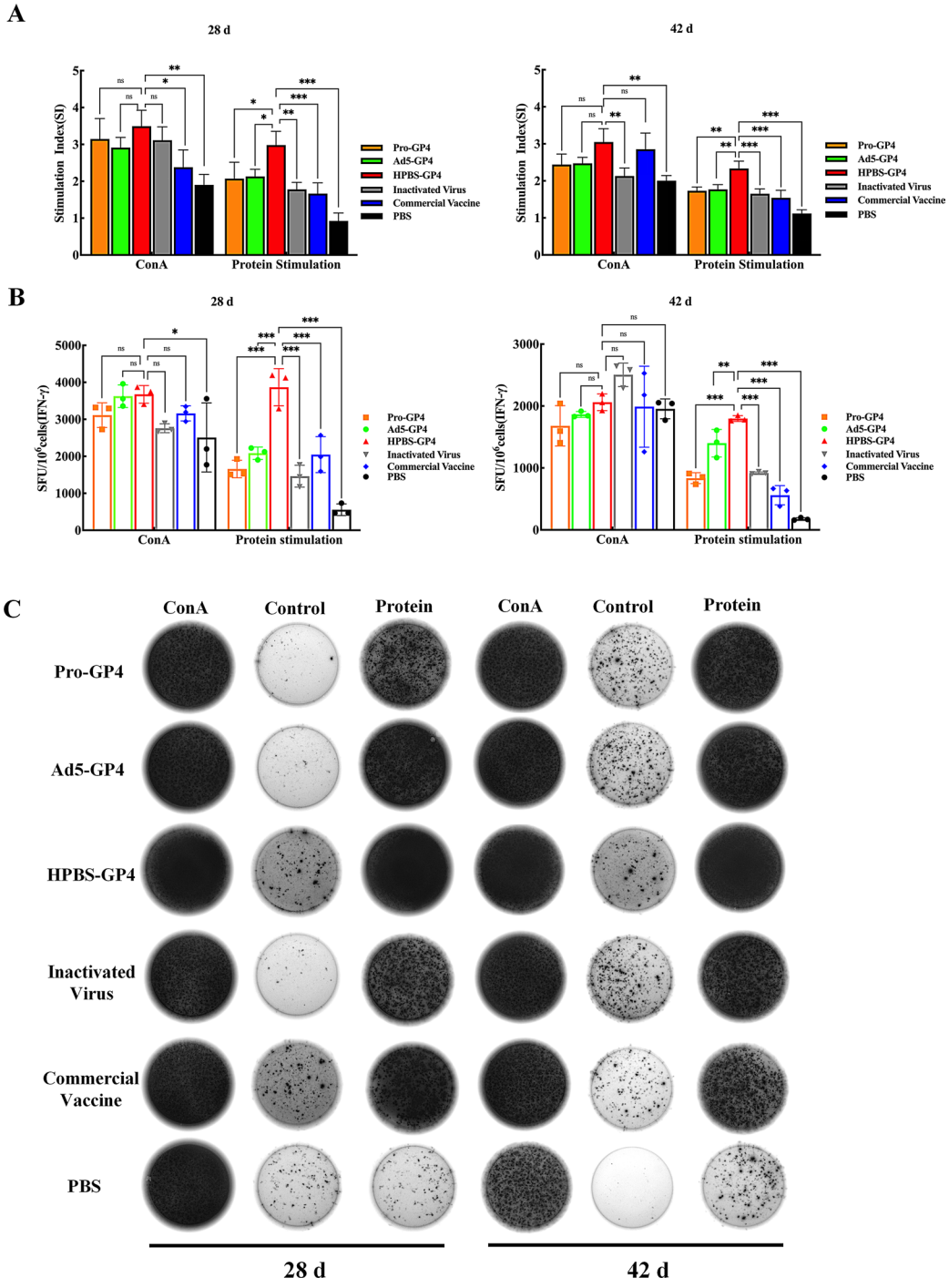

Fig. 5 Cellular immune responses of vaccinated mice. (**A**) Lymphocyte stimulation indices. (**B**) The levels of IFN-γ secretion by splenic lymphocytes were determined using ELISpot. (**C**) Spotted pore of splenic lymphocytes secreting IFN-γ. The data are presented as mean ± SD in each group. ns indicates $p \geq 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$

The subunit vaccine expressed by *E. coli* mainly activates B cells to produce humoral immunity and specific antibodies; the adenovirus vector vaccine can infect various cells and express antigens, activate CD8 + T cells, and induce cellular immunity. Combining the two can realize the dual activation of humoral and cellular immunity, forming more comprehensive immune protection. This study used the GP4 subunit vaccine prepared by the *E. coli* expression system for primary immunization in the combined immune group. This vaccine is safe and generally applicable for primary vaccination in most populations. Subsequently, we expect that GP4 adenovirus

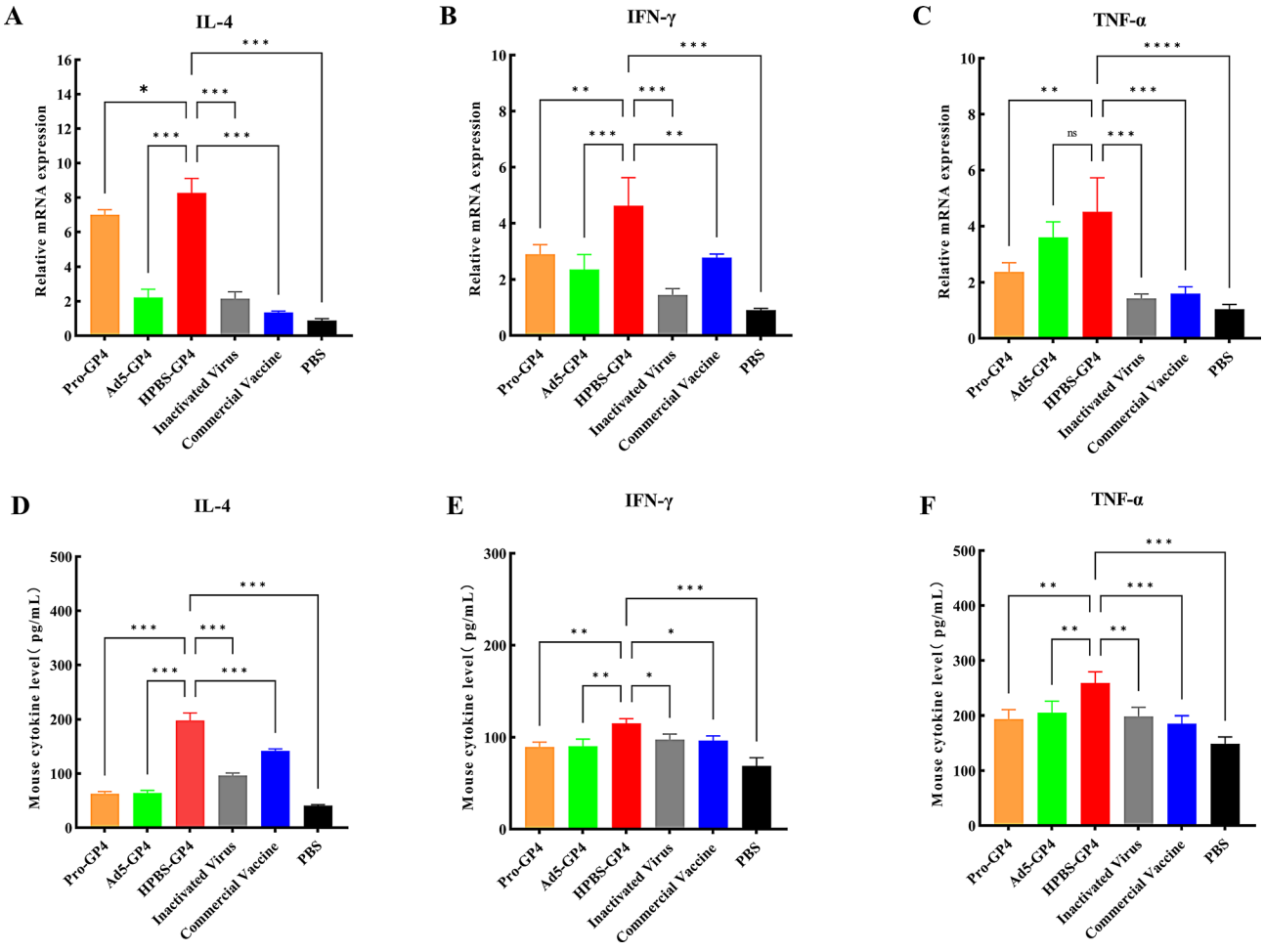

Fig. 6 Cytokine levels. The mRNA expression levels of the mouse splenic lymphocyte-specific cytokines IL-4 (**A**), IFN-γ (**B**), and TNF-α (**C**). Serum content of cytokine IL-4 (**D**), IFN-γ (**E**), and TNF-α (**F**). The data are presented as mean ± SD in each group. ns indicates $p \geq 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$

vector vaccines prepared with an adenovirus expression system for booster immunization will induce a higher level of specific antibodies and a stronger cellular immune response. This strategy significantly enhances the vaccine's immunogenicity and helps reduce the risk of immune escape that a single system might cause. The adenovirus expression system has shown significant advantages in eliciting strong immune responses; however, the use of adenovirus as a primary immunization method still has potential limitations. As a live viral vector, adenovirus has immunogenicity, which may lead to an immune response against adenovirus upon initial exposure, thereby affecting the efficacy of subsequent booster immunizations. Although this study did not observe any significant adverse reactions, as a live virus, adenovirus theoretically still has potential pathogenicity and transmission risks. Additionally, adenoviral vectors' preparation and purification process is relatively complex and costly, which may limit their application in regions with limited resources. Therefore, when using adenovirus for primary immunization, it is necessary to comprehensively evaluate its advantages and disadvantages, weighing the pros and cons. In future research, we can further explore how to optimize the design of adenoviral vectors and reduce their immunogenicity and safety risks while improving their preparation efficiency and cost-effectiveness. Moreover, other immunization strategies and technical approaches, such as the use of adjuvants and adjustments to the immunization schedule, can be considered to enhance the immunogenicity and safety of vaccines further. Based on the *E. coli* and adenovirus expression systems, exploring heterologous immunization strategies is profoundly significant for developing the PRRS vaccine.

It is noteworthy that Th1-type immune responses are typically associated with cell-mediated immune reactions, which are particularly important for clearing viral infections, whereas Th2-type immune responses are primarily related to humoral immunity, which aids in the production of antibodies [32]. In this study, the

combination of the subunit vaccine Pro-GP4 with the A206 adjuvant significantly enhanced IgG1 levels, indicating that this combination may be more suitable for situations requiring a strong humoral immune response, such as the prevention or treatment strategies for certain antibody-mediated diseases. Furthermore, the Th1-type immune responses induced by the HPBS-GP4 group may make them candidates for vaccines against viral infectious diseases that require cellular immune responses.

Cytokines such as IFN-γ, IL-4, etc., can enhance adaptive immune responses and significantly regulate innate and adaptive immunity [33]. In this study, we measured the expression levels of key cytokines such as IFN-γ, IL-4, and TNF-α, as well as the cytokine content in serum, aiming to evaluate the impact of different vaccine formulations on regulating immune responses. IFN-γ, as the core cytokine of the Th1-type immune response, showed significantly high expression in the HPBS-GP4 group, consistent with the strong Th1-type immune response they induced. IL-4, as the representative cytokine of Th2-type immune response, increased dramatically in the Pro-GP4 combined with the A206 adjuvant groups and the HPBS-GP4 group, further confirming the promoting effect of this combination on humoral immunity (Fig. 6). Furthermore, TNF-α, as a cytokine with multiple functions, also has a relatively high expression and content in the HPBS-GP4 group, which may be related to the inflammatory response induced by the vaccine in this group. It is noteworthy that by detecting the ability of individual cells to secrete IFN-γ through ELISpot, we found that the lymphocytes in the HPBS-GP4 group have more robust IFN-γ secretion activity, indicating that this vaccine formulation can more effectively activate Th1-type immune responses. This discovery is consistent with the results we previously obtained by measuring the expression levels of cytokines, further emphasizing the advantages of HPBS-GP4 in inducing a strong and durable Th1-type immune response. Although the Ad5-GP4 group had lower levels of specific IgG antibodies in the initial phase, the high expression of IFN-γ secreted by individual cells suggests to us that this group of vaccines may exert antiviral effects through non-antibody-mediated pathways, such as directly activating immune cells. In summary, our study reveals the impact of different vaccine formulations on cytokine levels. It provides essential clues for understanding the types of immune responses induced by vaccines and potential antiviral mechanisms.

The sequential immunization strategy of PRRSV GP4 subunit and adenovirus vector vaccine has shown enhanced humoral and cellular immune responses in a mouse model (Figs. 4 and 5). Pro-GP4, being a product of prokaryotic expression systems, consequently lacks glycosylation modifications. In contrast, when Ad5-GP4 is expressed in mammalian cells, glycosylation not only enhances its structural rigidity and stability but also significantly influences the exposure and conformation of its antigenic epitopes. The differences in glycosylation between the two expression systems directly regulate the intracellular processing and expression pathways of antigens, and may also generate unique complementary effects in heterologous immunization. Compared to the sole application of the GP4 subunit vaccine or adenovirus vector vaccine, the heterologous immunization strategies demonstrated significant superiority in terms of serum antibody levels and cellular immune responses. This phenomenon may be attributed to the subunit vaccines activating the body's immune system and forming immune memory cells. Adenovirus vector vaccines can reactivate these memory cells during the booster immune phase, promoting their rapid proliferation and differentiation, thereby triggering a more robust immune response. Although this study has achieved significant results, it inevitably has some limitations. Firstly, this experiment was only preliminarily explored on a mouse model. Given the substantial differences in physiological and immunological characteristics between mice and pigs, the actual effects and safety of this sequential immunization strategy in pigs still need further validation. Secondly, there is significant room for optimization in the immune protocol, including the optimal interval between primary and booster immunization, precise dosing of vaccines, etc. Future research should comprehensively evaluate the immune effect, safety, and actual protective efficacy against PRRSV infection of this sequential immunization strategy in pigs, and improve the preparation process of adenoviral vectors and subunit vaccines to enhance the stability of the vaccine, strengthen immunogenicity, and increase targeting.

This study verified the immune responses elicited by the sequential immunization strategy of the PRRSV GP4 subunit vaccine followed by the adenovirus vector vaccine in mice. The results indicated that this strategy could induce a high level of serum antibody production and a potent cellular immune response. Notably, the sequential approach of administering the GP4 subunit vaccine first, followed by a booster with the adenovirus vector vaccine, demonstrated the optimal immune effect. The findings of this study provide innovative strategies and insights for developing new PRRSV vaccines and suggest their potential application value in the prevention and control of PRRS. However, the actual effect of this immunization strategy in pigs still needs further research validation.

Conclusions

Our study provides comprehensive data on the antigenic structure and immune responses induced by a novel PRRSV vaccine candidate. The bioinformatic predictions and experimental validations demonstrate the potential

of this vaccine in eliciting robust neutralizing antibody responses and cellular immune reactions. The significant increase in IFN-γ secreting cells and lymphocyte proliferation observed in vaccinated mice suggests a strong Th1-type immune response, crucial for clearing viral infections. These findings advance our understanding of PRRSV immunology and pave the way for developing more effective vaccines against this economically significant swine disease.

Methods

Cells, viruses, and animals

Human embryonic kidney 293 cell line (293AD) and monkey embryonic kidney epithelial cells (Marc145) were maintained in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and penicillin-streptomycin (100 μg/mL) (Biosharp, Hefei, China). The PRRSV XJSW-2021 strain (GenBank Accession number: OR247780.1) was isolated and preserved in our laboratory. Four-to-six-week-old BALB/C female mice were purchased from the Laboratory Animal Center, Henan Province, China.

Bioinformatic prediction

Prediction of the secondary structure of candidate vaccines was made using the servers SOPMA (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) and PSIPRED 4.0 (http://bioinf.cs.ucl.ac.uk/psipred/). The preliminary prediction of the candidate vaccine's tertiary structure was completed using Robetta (https://robetta.bakerlab.org/submit.php). The candidate vaccine's tertiary structure model was tested globally through the server ProSA web (https://prosa.services.came.sbg.ac.at/prosa.php). The server PROCHECK (http://hdock.phys.hust.edu.cn/) was used to generate Ramachandran graphs. Predicting the interactions between TLR-3 (UniProt: V9P0U7) as a receptor and the candidate vaccine proteins as a ligand was made using the server HDOCK (http://hdock.phys.hust.edu.cn/). The computer software PyMOL was used to determine the interface between the vaccine ligand and TLR-3 receptor and whether there are hydrogen bonds and hydrophobic interactions between amino acid residues.

Prokaryotic expression and protein purification

The nucleotide sequence of the vaccine constructs was codon-optimized to enhance their fitness in the *E. coli* expression system. The optimized gene sequence was synthesized, integrated into the expression vector pSumo-Mut, and transferred into *E. coli* ArcticExpress (DE3) host cells. Protein expression was induced at 0.2 mM IPTG at 16℃, and overnight incubation was performed. The expressed products were purified and concentrated using a high-affinity Ni-NTA resin (GenScript, Nanjing, China). The concentration of purified recombinant protein was determined using the BCA Protein Assay kit (Thermo Fisher, MA, USA). In addition, the quality of proteins was assessed by SDS-PAGE and protein blotting. For the Western blot analysis, the mouse anti-His tag monoclonal primary antibody (Proteintech, Wuhan, China) and the goat anti-mouse IgG-HRP (H + L) antibody (Proteintech, Wuhan, China) were used.

Expression and characterization of the recombinant adenovirus

The nucleotide sequence of the vaccine construct was codon-optimized to enhance its fitness in eukaryotic expression systems. The optimized gene sequence was synthesized and integrated into the shuttle vector pAdTrack-CMV, followed by homologous recombination into *E. coli* BJ5183 competent cells (containing skeletal plasmid pAdeasy-1) for in vitro recombination. The recombinant correct plasmids were subsequently transfected into 293AD cells, screened by PCR and fluorescence identification, and expanded in culture to determine $TCID_{50}$. In addition, the quality of recombinant proteins was assessed by western blotting. Western blot analysis used PRRSV-positive serum and goat anti-swine IgG-HRP (H + L) antibody (Proteintech, Wuhan, China).

Immunization protocol for mouse animals

Female BALB/c mice that were four-to-six weeks old were randomly divided into 6 groups ($n = 10$). Among them, subunit vaccines and inactivated viruses were mixed with laboratory-prepared A206 adjuvant at a 1:1 ratio for preparation. Figure 3A shows the vaccination regimen. Among them, the HPBS-GP4 group adopted a heterologous prime-boost immunization strategy, with the primary immunization being subcutaneous inoculation of the subunit vaccine Pro-GP4, while the booster immunization involved intramuscular inoculation of the adenovirus vaccine Ad5-GP4. One group of mice served as the control and received vaccination with PBS an intramuscular inoculation route. For 7 days after primary and booster immunization, mice were observed for dietary behavior and mental status and were weighed regularly to assess the safety of vaccine candidates. Mouse sera were collected from the tail vein every 14 days until day 42 to detect the levels of specific and neutralizing antibodies. The mouse was euthanized on days 28 and 42 following vaccination, and splenocytes were collected and detected using an ELISpot assay.

ELISA

The PRRSV GP4-specific IgG antibody was detected in mouse serum after immunization using an indirect enzyme-linked immunosorbent assay (ELISA). PRRSV GP4 protein was pre-coated with ELISA plates at 1.0 μg/

mL and incubated overnight. They were blocked in PBST containing 5% skim milk powder for 2 h, 37℃. Serum samples were diluted, added to the plates, and incubated at 37℃ for 2 h. After washing, 1:10,000 diluted HRP-labeled anti-mouse IgG antibody (Proteintech, Wuhan, China) was added and incubated at 37℃ for 1 h. Tetramethylbenzidine (TMB) substrate was added for 15 min (Solarbio, Beijing, China), and the reaction was terminated with a termination solution. The absorbance values were measured at 450 nm using a microplate reader (Tecan, San Jose, CA).

Neutralization assay

In brief, a 2-fold serial dilution of mouse serum was incubated with PRRSV for 1 h at 37℃, after which this virus-serum mixture was moved to each well pre-inoculated with 10,000 Marc-145 cells. In contrast, uninoculated cells were set as a negative control. After two h incubation at 37℃, the upper culture media was removed, DMEM maintenance medium containing 2% FBS was added to the wells, continued at 37℃ for 3 to 7 days, and cytopathologies were continuously observed and recorded. The serum levels of possible neutralizing antibodies were analyzed using the Reed-Muench calculation.

Isolation of the lymphocytes

After euthanizing BALB/c mice via cervical dislocation, they were immersed in 75% alcohol for disinfection, and then the spleen was extracted to dissect the fascia under sterile conditions. The spleen was weighed and chopped, ground with a 70 μm cell screen, the cell suspension was collected in 15 mL centrifuge tubes, and the supernatant was centrifuged and resuspended for later use. The separation solution was added to the centrifuge tube, and the spleen single-cell suspension was carefully added and separated by centrifugation. The milky lymphocyte layer was moved to a new centrifuge tube, the washing solution was added, and the supernatant was discarded after centrifugation. Cells were resuspended, and the supernatant was discarded after centrifugation. Cells were resuspended in 1640 broth containing 10% FBS and diluted for cell counting. The counting results adjusted the cell concentrations to 10^6 cells/mL.

ELISpot

Isolated mouse splenic lymphocytes were prepared into a single-cell suspension. Subsequently, the ELISpot Plus plate (Mabtech, STH, Sweden) was detected using the mouse IFN-γ. The RPMI-1640 medium containing 10% FBS was incubated for 30 min at room temperature to prevent non-specific adsorption.1 × 10^6 splenic lymphocytes were added to each well and stimulated with GP4 protein for 36 to 48 h. The ConA protein stimulation was used as a positive control, and the PBS stimulation was used as a negative control. The spots were scanned and quantified by an enzyme-linked immunopunctometer. Finally, to calculate the spot-forming units (SFU) per million cells, the number of places in the negative control group was deducted from the number of spots in the experimental group to obtain the final experimental data.

Table 1 Primers used in this study

Primer name	Primer sequences (5′-3′)	Product size (bp)
Pro-GP4	F: GCAAACCGTGTTTTAGCAGC	477
	R: TGGCAAACAGACATGCCAGA	
Ad5-GP4	F: CCTCTTGGTTGGTTTTG	549
	R: CCAACAGTATGGCAAAA	
IL-4	F: GCCATATCGACGGATGCGACAA	128
	R: GGTGTTCTTCGTTGCTGTGAGGA	
IFN-γ	F: GCCACGGCACAGTCATTGA	201
	R: TGCTGATGGCCTGATTGTCTT	
TNF-α	F: AGCCCACGTCGTAGCAAACCAC	446
	R: ACACCCATTCCCTTCACAGAGC	
GAPDH	F: CACGGCAAATTCAACGGCACAGTC	200
	R: ACCCGTTTGGCTCCACCCTTCA	

Lymphocyte proliferation assay

The concentration of spleen cells was adjusted to 10^6 cells/mL; splenic lymphocytes from the same mouse were selected from three wells, one well without cells, and only medium as a blank group. Three groups were set for 1 μg ConA, 1 μg of GP4 protein, and RPMI-1640 culture, and cultured for 24 h. Add 10 μL of CCK-8 solution to each well; the culture was continued for 1–4 h, and the absorbance was measured at 450 nm using a microplate reader. The SI was calculated as follows: SI = (Stimulation-Control)/(No-stimulation-Control).

Transcriptional levels of cytokines in lymphocytes

Mouse spleen cells were adjusted to 10^7 cells/mL, spread into 12-well plates with 1 μg GP4 protein stimulation and RPMI-1640 medium control, and cultured for 24 h. Collect cells, extract cellular RNA according to the Total RNA Extraction Kit (TransGen, Beijing, China) instructions, reverse transcribe into cDNA, perform quantitative analysis using mouse IL-4, IFN-γ, and TNF-α primers, calculate results using the $2^{-\Delta\Delta Ct}$ method, and use GAPDH as an internal reference. Primer information is shown in Table 1.

Quantification of cytokines in the serum

Sera were collected 28 days and 42 days post-immunization to detect IL-4, TNF-α (Lunchangshuo, Xiamen, China), and IFN-γ (Elabscience, Wuhan, China). Each step of the cytokine detection procedure followed the instructions of each kit.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 10.2 (GraphPad Software, CA, USA) software. The data are presented as mean ± standard deviation (SD) in each group. If the data follow a normal distribution, homogeneity of variance is tested, and one-way analysis of variance (ANOVA) is used for comparisons among multiple groups, with post hoc pairwise comparisons conducted using Dunnett's multiple comparison test (for groups compared against a single control). If homogeneity of variance was not met, Dunnett's T3 test (a modified version for unequal variances) was used for pairwise comparisons instead. The nonparametric Kruskal-Wallis test was applied for non-normally distributed data, with subsequent pairwise comparisons performed using Dunn's multiple comparison test. Differences were deemed significant when $p < 0.05$.

Abbreviations

PRRS	Porcine reproductive and respiratory syndrome
PRRSV	Porcine reproductive and respiratory syndrome virus
GP4	Glycoprotein 4
HPBS	Heterologous prime-boosting strategy
Ad5	Adenovirus 5
E. coli	*Escherichia coli*
His	Histidine
IPTG	Isopropyl-β-d-thiogalactoside
DNA	DeoxyriboNucleic Acid
Ni	NTA-Nickel Nitrilotriacetic Acid
SDS	PAGE-Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
kDa	kiloDalton
PBS	Phosphate-buffered saline
HRP	Horseradish peroxidase
ELISA	Enzyme-Linked Immunosorbent Assa
ELISpot	Enzyme-linked Immunospot Assay
SFU	Spot-forming units
SI	Stimulation index
ConA	Concanavalin A
IL-4	Interleukin-4
IFN-γ	Interferon-γ
TNF-α	Tumor necrosis factor-α
ELISA	Enzyme-linked immunosorbent assay

Supplementary Information

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Supplementary Material 1: Supplementary Fig. 1 Construction and identification of GP4 vaccine. (**A**) Electrophoretic identification of the Pro-GP4 gene. (**B**) Prokaryotic expression plasmid pSumo-GP4 double enzyme digestion identification. (**C**) Electrophoretic identification of Ad5-GP4 gene. (**D**) Adenoviral vector shuttle plasmid pAdTrack-GP4 double digestion identification. (**E**) Adenovirus vector homologous recombination, *pacI* single enzyme digestion identification. (**F**) Adenovirus vaccine Ad5-GP4 intracellular genetic stability passage identification.

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Author contributions

HHL and WXZ analyzed the data and wrote the manuscript. HHL designed the experiments. WXW, YJQ, MGX, ZWL, XXG, and ADW played a role in performing the study. ZCM and CFC conceived and supervised the program. WZ contributed materials. All authors read and approved the final manuscript.

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Data availability

All data are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

All animal experiments in this study were conducted under the guidelines of the Shihezi University Institutional Animal Care and Use Committee and approved by the Animal Ethics Committee of Shihezi University (No. 2023 – 246).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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