ORIGINAL ARTICLE

Immunogenicity and protective efficacy of a DNA vaccine inducing optimal expression of the SARS-CoV-2 S gene in hACE2 mice

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Abstract

The wide spread of coronavirus disease 2019 (COVID-19) has significantly threatened public health. Human herd immunity induced by vaccination is essential to fight the epidemic. Therefore, highly immunogenic and safe vaccines are necessary to control SARS-CoV-2, whose S protein is the antigenic determinant responsible for eliciting antibodies that prevent viral entry and fusion. In this study, we developed a SARS-CoV-2 DNA vaccine expressing the S protein, named pVAX-S-OP, which was optimized according to the human-origin codon preference and using polyinosinic–polycytidylic acid as an adjuvant. pVAX-S-OP induced specific antibodies and neutralizing antibodies in BALB/c and hACE2 transgenic mice. Furthermore, we observed 1.43-fold higher antibody titers in mice receiving pVAX-S-OP plus adjuvant than in those receiving pVAX-S-OP alone. Interferon gamma production in the pVAX-S-OP-immunized group was 1.58 times ($CD3^{+}CD4^{+}IFN$-$gamma^{+}$) and 2.29 times ($CD3^{+}CD8^{+}IFN$-$gamma^{+}$) lower than that in the pVAX-S-OP plus adjuvant group but higher than that in the control group. The pVAX-S-OP vaccine was also observed to stimulate a Th1-type immune response. When, hACE2 transgenic mice were challenged with SARS-CoV-2, qPCR detection of N and E genes showed that the viral RNA loads in pVAX-S-OP-immunized mice lung tissues were 10^4 times and 10^6 times lower than those of the PBS control group, which shows that the vaccine could reduce the amount of live virus in the lungs of hACE2 mice. In addition, pathological sections showed less lung damage in the pVAX-S-OP-immunized group. Taken together, our results demonstrated that pVAX-S-OP has significant immunogenicity, which provides support for developing SARS-CoV-2 DNA candidate vaccines.

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Introduction

COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has generated an unprecedented public health crisis [10, 27], and its rapid global spread has resulted in a pandemic with more than 539 million laboratory-confirmed cases as of June 20, 2022.

SARS-CoV-2 belongs to the genus *Betacoronavirus* of the family *Coronaviridae* and is closely related to the severe acute respiratory syndrome coronavirus (SARS-CoV) and several bat coronaviruses [27]. SARS-CoV-2, SARS-CoV, and Middle East respiratory syndrome coronavirus (MERS-CoV) are the three most life-threatening viruses among the human coronaviruses, and SARS-CoV-2 has the highest spread potential [10]. It is an enveloped, single-stranded positive-stranded RNA virus with a diameter of about 60 nm to 140 nm, which is composed of the structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N) [18]. The S protein is a class I fusion protein that binds

to angiotensin converting enzyme 2 (ACE2) as a receptor and triggers fusion of the viral membrane with the cell membrane [6, 12].

Since we still do not fully understand SARS-CoV-2 pathogenicity, the consequences of repeated epidemics will cause unacceptably high mortality, a severe economic burden, and major changes in our lifestyle. The potential for viruses to achieve pandemic spread is diminished by establishing high levels of herd immunity in the population. Vaccination is the most appropriate approach to prevent repeated or sustained epidemics. The World Health Organization (WHO) (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/covid-19-vaccines) has approved some vaccines for emergency use, and some new SARS-CoV-2 vaccines have entered the clinical research stage [5, 7, 26]. At present, inactivated vaccines, recombinant protein vaccines, and adenovirus vector vaccines have been applied [13, 30], whereas the DNA vaccine ZyCoV-D was approved for emergency use in India. This was the first worldwide approval for a DNA vaccine to be marketed [17]. Furthermore, the DNA vaccine INO-4800 has entered clinical phase III. Protein antigens are expressed in host cells after DNA vaccination. The presentation process of DNA vaccines is similar to that of attenuated vaccines, which induce cellular and humoral immunity [28].

Different types of SARS-CoV-2 vaccines have their own advantages and disadvantages. DNA vaccines have the advantages of easy design and preparation, strong stability, and low cost, and their use has led to good research progress in tumor treatment, autoimmunity, and infectious disease prevention [9, 21]. Although DNA vaccines are in the research stage, they have not been reported to induce Th2-skewed immune responses, as is observed for protein vaccines [15]. A recent study showed that hACE2 transgenic mice are a suitable model for SARS-CoV-2 infection, with increasing viral load over time. In this study, we developed the novel DNA vaccine pVAX-S-OP and studied its immunogenicity in hACE2 transgenic mice by measuring specific neutralizing antibodies and determining their subclasses. We also demonstrated a pVAX-S-OP-induced T cell response by measuring IFN-γ production by mouse spleen lymphocytes.

Materials and methods

Virus, cells, and adjuvant

HEK293 (human embryonic kidney) and Vero E6 (African green monkey kidney cells) cells, maintained in our laboratory, were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin (10,000 U/mL)-streptomycin (10,000 µg/mL). Polyinosine-polycytidylic acid (1 mg/mL) was used as as a vaccine adjuvant.

SARS-CoV-2 strain BetaCoV/Beijing/IME-BJ01/2020 was originally isolated by the State Key Laboratory of Pathogens and Biosecurity [32]. All experiments involving infectious SARS-CoV-2 were performed in a level-3 facility.

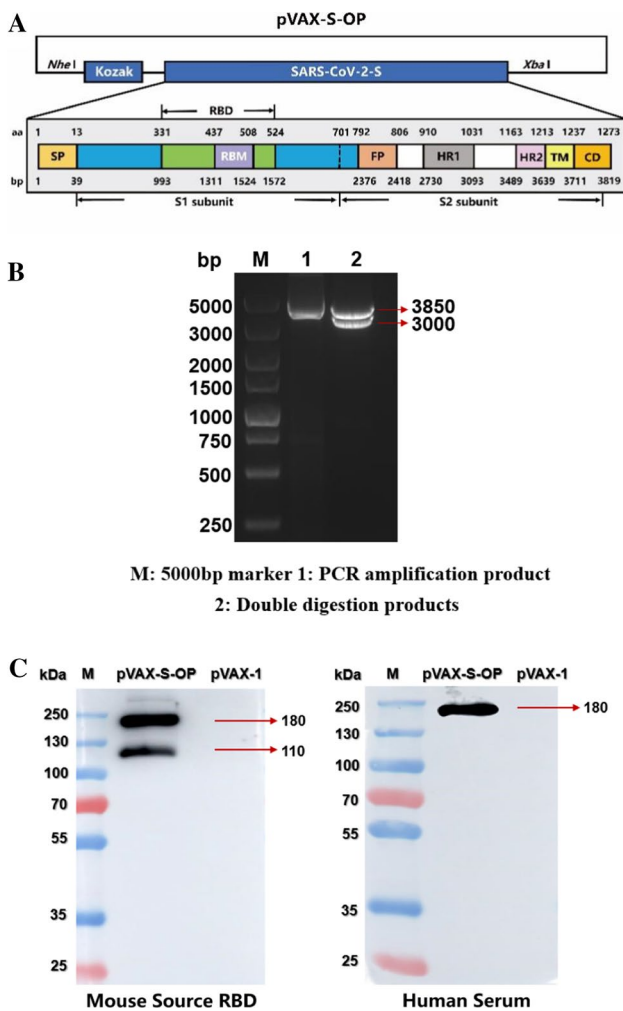### Structure optimization and construction of the candidate vaccine

We performed codon optimization of the S gene of SARS-CoV-2 Wuhan-Hu-1 (GenBank no. MN908947.3) according to the human-origin expression codon preference. The optimization included changing the GC content, codon use adjustment, codon use distribution, addition of restriction enzyme recognition sites, and avoidance of specific sequences (Supplementary Fig. S1), after optimization, about 1000 nucleotides were changed, but the amino acid sequence was not changed. To increase the efficiency of translation initiation, we added a Kozak sequence after the upstream restriction site, and NheI and XbaI restriction sites were added upstream and downstream of the optimized S (S-OP) sequence. S-OP was inserted into the pVAX-1 vector, using the seamless cloning method (Fig. 1A).

Expression of the spike protein after transfection of HEK293 cells with pVAX-S-OP was verified by Western blotting with human serum and mouse receptor-binding domain (RBD) antibodies prepared in our laboratory, using the pVAX-1 alone as a control.

Animal immunization experiments

Female 6-week-old BALB/c mice (Jintai Meidi Biotechnology Co., Ltd., Jilin Province) were randomly divided into five groups in each experiment. Mice were randomly distributed into experimental groups (n = 8). Female 6-week-old hACE2 mice were randomly distributed into experimental groups (n = 6). The grouping and immunization doses of BALB/c mice are shown in Table 1. Based on the results obtained with BALB/c mice, pVAX-S-OP combined with adjuvant was used to immunize hACE2 transgenic mice, using PBS as a negative control. The grouping and immunization doses of hACE2 mice are shown in Table 2.

All of the mice were immunized by intramuscular injection of both hindlimbs using a sterile insulin syringe (0.3 × 8mm, U-40/30G). Serum samples were collected each week after immunization (days 7, 14, 21, 28, 35, and 42), and a booster immunization was given 21 days after the initial immunization (Fig. 2A).

Fig. 1 Construction of the candidate vaccine and detection of spike protein expression. (A) Schematic diagram of the DNA candidate vaccine structure encoding the SARS-CoV-2 spike protein. The optimized S gene was inserted into the vector pVAX using NheI and XbaI restriction sites. (B) PCR amplification and double enzyme digestion. Q5 high-fidelity DNA amplification enzyme was used to amplify the target gene to obtain the expected fragment. (C) Verification of the expression of the SARS-CoV-2 S protein in HEK293 cells by Western blot analysis. Incubation with a mouse antibody against the RBD, two target bands were formed, the lower of which represents the cleaved S1 protein.

Table 1 Immunization groups and vaccine doses in BALB/c mice

Group	Name	Dose
A	pVAX-S-OP	200 μg/200 μL
B	pVAX-S-OP+ Ad	200 μg/100 μL +100 μL
C	pVAX-1	200 μg/200 μL
D	pVAX-1+ Ad	200 μg/100 μL +100 μL
E	PBS	200 μL

Table 2 Immunization groups and vaccine doses in hACE2 mice

Group	Name	Dose
F	pVAX-S-OP+ Ad	200 μg/100 μL +100 μL
G	PBS	200 μL

Immunogenicity analysis

Detection of specific antibodies

A COVID-19 mouse IgG antibody detection kit (Guangzhou Da rui Biotechnology Co., Ltd.) was used to test serum samples each week (days 7, 14, 21, 28, 35, and 42) after immunization. Serum samples were initially diluted 1:100, after which twofold serial dilutions were prepared, following the supplier´s instructions. The cutoff was the mean OD value of duplicate negative control wells + the mean OD value of duplicate positive control wells × 0.2. Samples with an OD value greater than or equal to the cutoff were judged to be positive.

Detection of IgG1 and IgG2a antibodies

Serum samples collected on day 42 were tested. A specific antibody detection kit was used to detect IgG1 and IgG2a antibodies, replacing the COVID-19 enzyme-labeled substance in the kit with IgG1 (goat pAb to Ms IgG1 (HRP), lot: GR3254189-11 Abcam) and IgG2a (goat pAb to Ms IgG2a (HRP), lot: GR3248508-9 Abcam) antibodies. IgG1 and IgG2a were diluted 1:40,000 before use according to the supplier´s instructions.

Detection of neutralizing antibodies

Neutralizing antibodies were detected by the microneutralization method. Serum samples were collected each week (days 7, 14, 21, 28, 35, and 42) after immunization for testing and inactivated at 56 °C for 30 min, and supernatants were collected after centrifugation at 6000 g for three minutes. Serum samples were initially diluted 1:20, after which twofold serial dilutions were prepared. The diluted serum was mixed with 100 $TCID_{50}$/mL virus (50 μL:50μL), added to a 96-well plate, and incubated at 37 °C for two hours. Next, 50 μL of a suspension containing 5000 Vero E6 cells was added to each well, the cells were cultured further at 37 °C, and the neutralizing antibody titer was determined by observing the cytopathic effect at each dilution, usually for 4-5 days.

Fig. 2 The immune response in pVAX-S-OP-vaccinated BALB/c mice. (A) Schematic diagram of immunization of BALB/c mice. The number of immunizations was 2 doses, and the immunization interval was 21 days. (B) Analysis of antibody production in specific BALB/c mice. The chart shows that the antibody levels in the immunized groups gradually increased and reached a maximum at 42 days. No antibody was detected in the negative controls. (C) Cellular immune responses in pVAX-S-OP-vaccinated BALB/c mice. Intracellular cytokine staining assays demonstrated IFN-γ^+, CD4$^+$, and CD8$^+$ T cell responses. Significant differences were found between the pVAX-S-OP plus adjuvant group and the PBS group. (***, $P < 0.001$; ****, $P < 0.0001$).
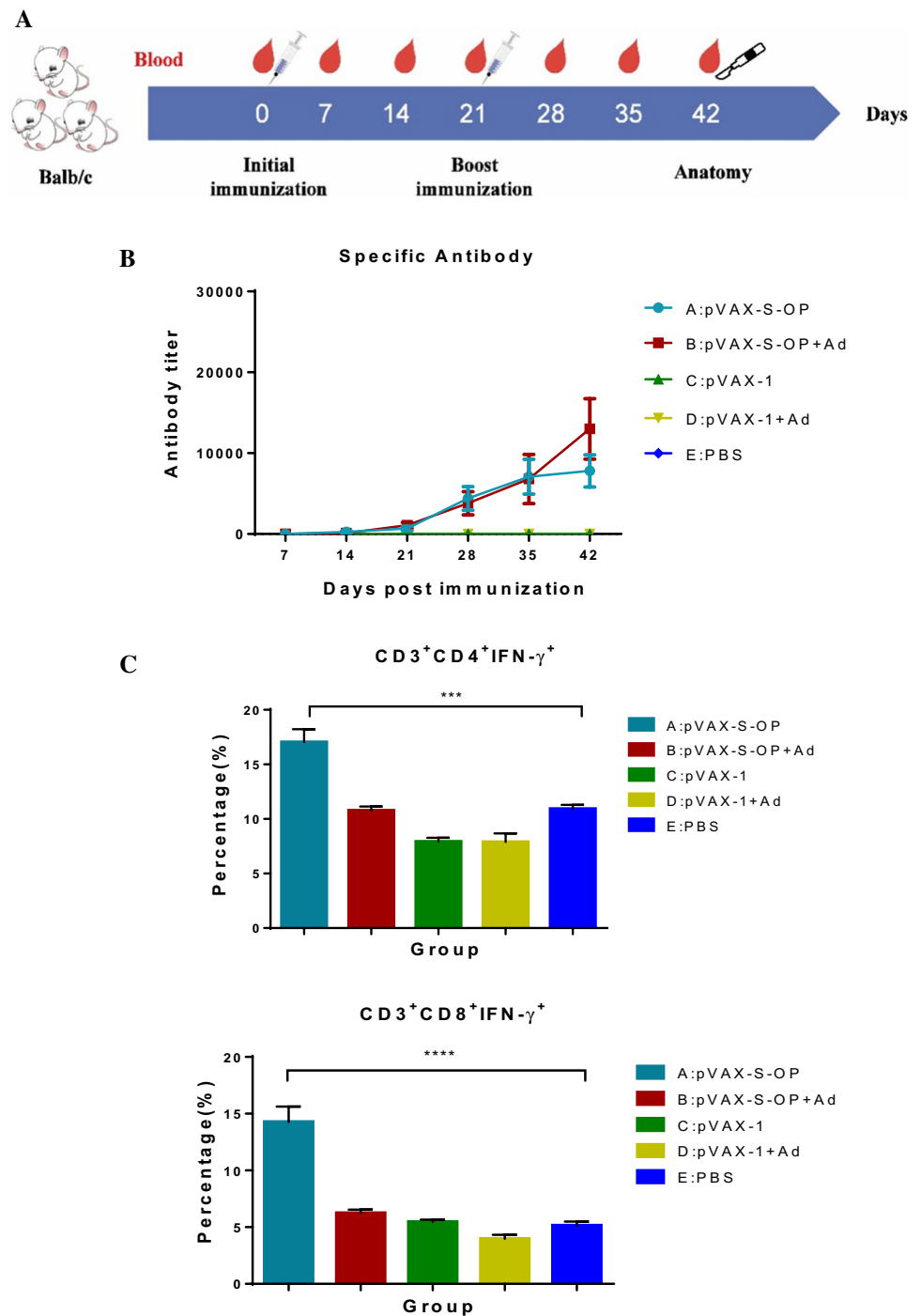
Analysis of intracellular interferon-gamma (IFN-γ)

On day 42, mice were euthanized by cervical dislocation, their spleens were removed, and their lymphocytes were isolated using a mouse lymphocyte separation kit (lot: LDS1090P Tianjin Haoyang Biological Manufacture Co., Ltd.) according to the supplier´s instructions. Next, we incubated 2×10^6 lymphocytes in 12-well plates, after which we added 50 μL of inactivated virus ($1 \times 10^{5.5}$ TCID$_{50}$ virus

titer) to each well and kept the plates at 37 °C for 48 h. After this, we added 1 μL of 1000× brefeldin A to each well and continued incubation for six hours. The cell suspension was then collected and incubated with 2.5 μL of CD3 (PE/Cyanine7 anti-mouse CD3ε antibody, 0.2 mg/mL, lot: 100320, Biolegend), 0.5 μL of CD4 (FITC anti-mouse CD4 antibody, 0.5 mg/mL, lot: 100510, Biolegend), and 1.25 μL of CD8 (APC anti-mouse CD8 antibody, 0.2 mg/mL, lot: 100712, Biolegend) antibodies. After incubation, the cells were fixed

and the membranes permeabilized, after which the cells were incubated with interferon gamma (IFN-γ) antibodies and evaluated by flow cytometry.

Virus challenge

All immunized hACE2 transgenic mice were anesthetized with tribromoethanol and challenged by tracheal injection of 30 μL virus with a titer of $1 \times 10^{5.5}$ TCID$_{50}$/mL. After five days of challenge, mice were euthanized using an overdose of anesthetic before being dissected, and their lung tissues were used for subsequent experiments (Fig. 3A). Lung tissues were fixed in 4% paraformaldehyde solution, and paraffin-embedded sections were prepared. Hematoxylin and eosin (H&E) staining was then used to identify pathological changes. Images were observed and captured by light microscopy. A 0.03-g sample of lung tissue was homogenized, and after centrifugation, the supernatant was used for RNA extraction. Viral RNA in tissue homogenates was extracted using a QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany). Virus copy numbers were determined by RT-qPCR, using a HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme Biotech, Nanjing, China). Primers were designed based on the N and sgE gene sequences of SARS-CoV-2, and the viral RNA load in the lung tissues was determined by the TaqMan fluorescent quantitative PCR method [23].

Results

Structure optimization and construction of candidate vaccine

A fragment with a size of 3850 bp was amplified by PCR, which is consistent with the size of the S-OP. We obtained two bands of 3850 bp (S-OP) and 3000 bp (pVAX-1) after double digestion of pVAX-S-OP with restriction enzymes (Fig. 1B). Expression of the spike protein was confirmed by Western blotting, and a band of the expected size (180 kDa) was obtained. As expected, we did not detect any proteins product in the cells transfected with the empty pVAX-1 vector (Fig. 1C).

Induction of specific antibodies in BALB/c mice

Specific antibodies were initially detected on day 14 after immunization, and their levels increased significantly after a booster immunization. On day 42, the antibody titers in group B (1:13,000) were 1.67 times higher than those in group A (1:7800). No specific antibodies were detected in the three negative control groups (Fig. 2B).

Cellular immunogenicity in BALB/c mice

The level of IFN-γ produced by CD3$^+$CD4$^+$T cells in group A was 1.55 times higher than that in group E ($P < 0.001$), whereas that produced by CD3$^+$CD8$^+$T cells in group A was 2.77 times higher than that of group E ($P < 0.0001$). Furthermore, IFN-γ production was lower in group B than in group A (Fig. 2C), which may be due to the effect of the adjuvant. These results indicate that the candidate vaccine pVAX-S-OP induces a cellular immune response.

Induction of specific and neutralizing antibodies in transgenic hACE2 mice

Immunization with pVAX-S-OP plus adjuvant resulted in the production of specific and neutralizing antibodies in transgenic hACE2 mice. Specific antibodies were initially detected on day 14 after immunization and gradually increased. On day 42, we observed the highest antibody titer of 1:102,400. The trend of neutralizing antibody production was similar to that of specific antibodies, and the highest neutralizing antibody titer was 1:134. No specific or neutralizing antibodies were observed in group E (Fig. 3B and C).

## Th1-type immune response in transgenic hACE2 mice

The ratio of log (IgG1)/log (IgG2a) in immunized mice was 0.785. Since this ratio is less than 1, it can be concluded that the response induced by the candidate vaccine pVAX-S-OP was biased towards a Th1 immune response (cellular immunity) [4, 16, 31] (Fig. 3D and E).

## Determination of the viral RNA load by TaqMan qPCR

In this study, two standard curves were established for the N gene and the sgE gene: $y = -3.29x + 45.949$ (N gene) and $y = -3.739x + 37.643$ (sgE gene). According to the qPCR test based on the N gene, the amount of viral RNA in the pVAX-S-OP plus adjuvant group was $10^{3.96}$ times lower than that in the PBS group, and it was observed that the vaccine effectively reduced the viral RNA load in the lung tissue ($P < 0.0001$) (Fig. 4A).

The test based on the sgE gene showed that the viral RNA level in the pVAX-S-OP plus adjuvant group was $10^{5.31}$ times lower than in the PBS group ($P < 0.001$) (Fig. 4A), indicating that the candidate vaccine pVAX-S-OP might have the effect of inhibiting virus replication *in vivo*.

Fig. 3 Immune response in pVAX-S-OP-vaccinated hACE2 mice. (A) Schematic diagram of immunization of hACE2 mice. The number of immunizations was 2 doses, and the immunization interval was 21 days, with virus challenge after 2 doses of immunization. (B, C) Analysis of specific antibody and neutralizing antibody production in hACE2 mice. The chart shows that specific and neutralizing antibody levels of the immunized group gradually increased, reaching a maximum at 42 days, and there was no change in the PBS group. (D, E) Subtype analysis showing that the candidate DNA vaccine induced a Th1-type immune response.
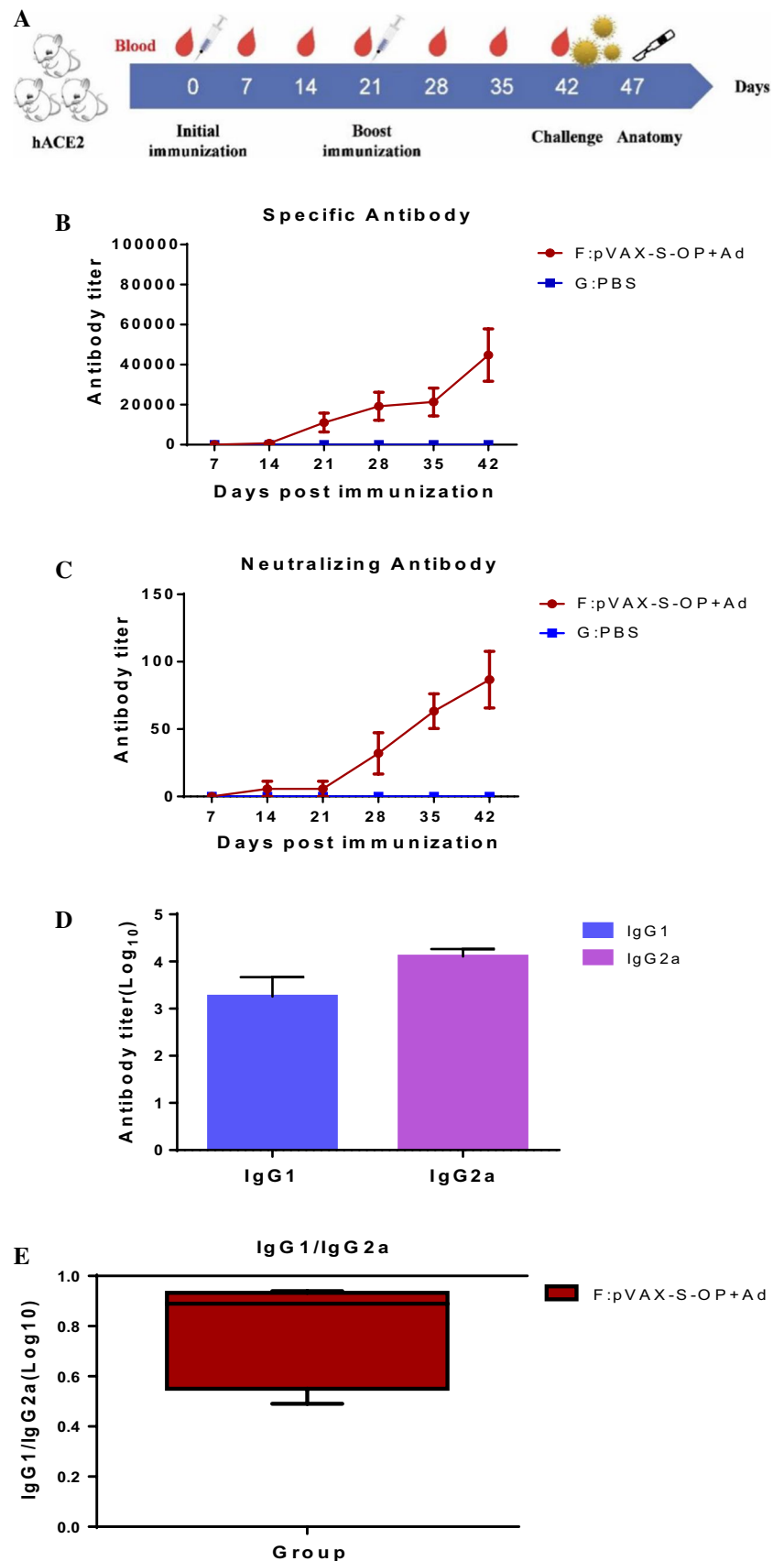
Fig. 4 Protection of pVAX-S-OP against SARS-CoV-2 challenge in hACE2 mice. (A) Viral RNA load analysis. The bar chart shows that there was a significant difference between the experimental group and the PBS group, with the viral load in the experimental group significantly reduced. (***, $P < 0.001$; ****, $P < 0.0001$). (B) Pathological section analysis. Significant inflammatory changes were seen in lung tissue of the PBS group, and the alveolar wall structure was clear in the pathological sections of the experimental group.
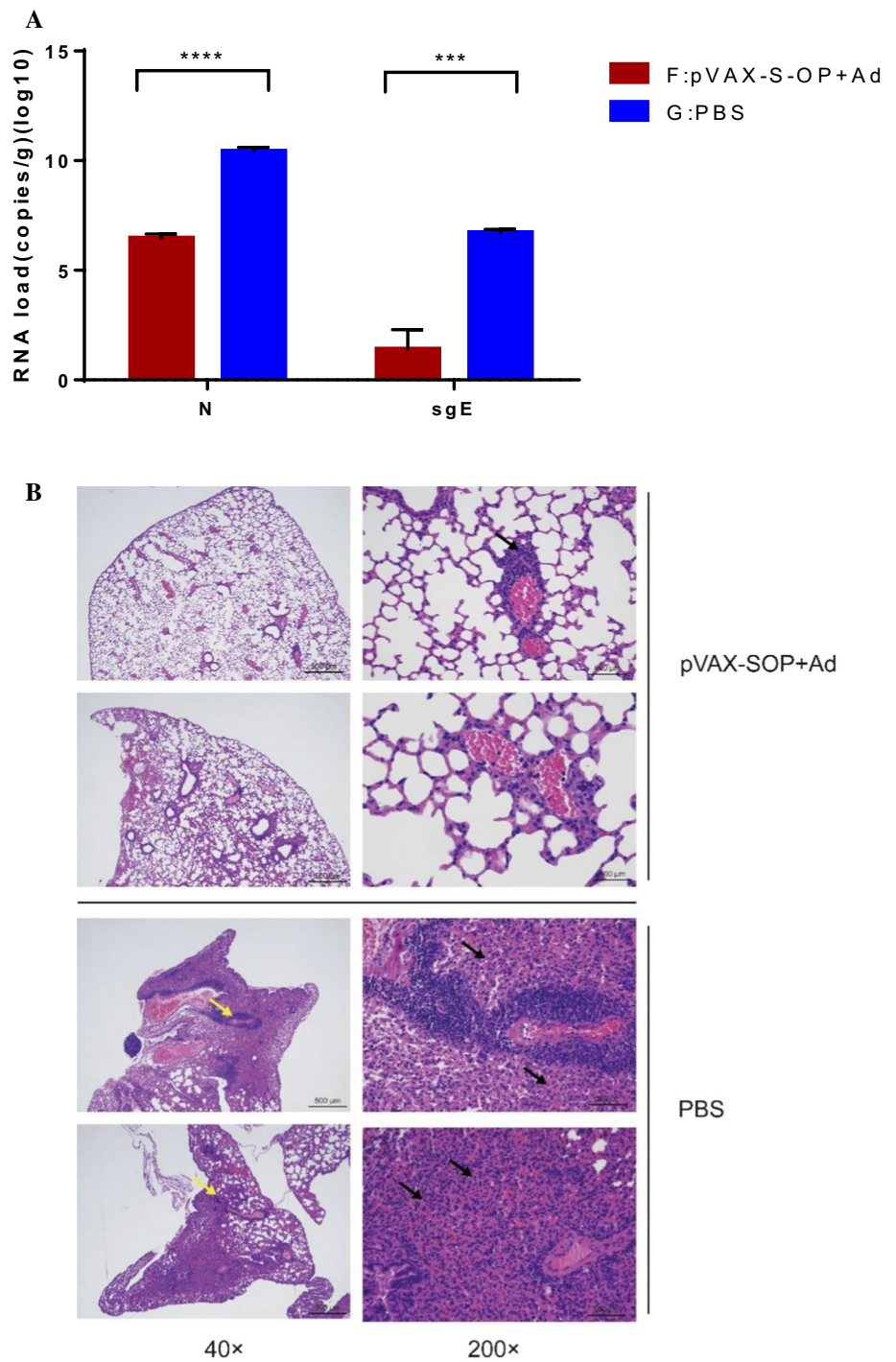
Histopathological analysis

Pathological sections from group F did not show abnormalities. The structure of the alveolar wall was clear, and no obvious inflammatory changes were observed. In group G, a large area of alveolar wall thickening, accompanied by infiltration with a large number of lymphocytes and neutrophils (black arrow) was observed. Inflammatory cell infiltration around blood vessels and pulmonary edema could also be observed (Fig. 4B).

Discussion

The World Health Organization declared the outbreak of COVID-2019 to be a Public Health Emergency of International Concern on January 30, 2020, and a pandemic on March 11, 2020 [10]. The pandemic of COVID-19 and the rapid increase in the death toll urgently demanded the development of effective SARS-CoV-2 vaccines. As of February 4, 2022, a total of 194 vaccines were in pre-clinical development, and 141 vaccines were in clinical development, including 16 DNA vaccines (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/covid-19-vaccines).

The use of codon-optimized genes increases the level of protein expression about 5 to 20 times, when compared to the corresponding wild-type genes [8], and this significantly stimulates the host's immune system, thereby enhancing DNA vaccine immunogenicity. The selection of the expression vector is also critical for a DNA vaccine. In our study, pVAX1 was used as the vaccine vector, which contains a strong cytomegalovirus promoter and a ??bovine growth hormone?? (BGH) polyA signal for expression of recombinant proteins at high levels in mammalian cells. Moreover, pVAX1 is the only vector plasmid recommended by the U.S. Food and Drug Administration (FDA) to be used in human trials [24].

As with all human coronaviruses, the S protein is the primary antigenic determinant responsible for eliciting antibodies that prevent viral entry and fusion [14]. Previous studies have shown that the complete S protein induces higher levels of neutralizing antibodies than the S1 or S2 subunit or the RBD [1, 20]. In our study, a SARS-CoV-2 DNA vaccine expressing the complete S protein was developed. Human serum and mouse RBD antibodies prepared in our laboratory were used to confirm the successful expression of pVAX-S-OP by Western blotting. Human immunity against coronaviruses is primarily mediated by the production of neutralizing antibodies, which may be sufficient to confer protection from re-infection [1]. We used two doses of the DNA vaccine for immunization, and its immunogenicity was evaluated. By measuring specific antibodies and neutralizing antibodies, two we found that pVAX-S-OP was immunogenic in two types of mice and that its immunogenicity was increased by the presence of adjuvant.

The cellular immune responses elicited by many vaccines have been demonstrated to be crucial for acute viral clearance, and protection from subsequent coronavirus infections is largely mediated by humoral immunity [19]. Therefore the ideal COVID-19 vaccine should be designed to avoid inducing non-neutralizing antibodies and biased Th2-type cellular immune responses. The Th1-type

immune response is important for antiviral immunity [11, 25], and DNA vaccines have been shown to favor Th1 over Th2 [29]. In the present study, we measured IgG1 and IgG2a and found the log (IgG1)/log (IgG2a) ratio to be less than 1, indicating that the pVAX-S-OP vaccine induces a Th1-biased response, in agreement with previous studies [4, 16, 31].

Almansour et al. used the S gene to make a DNA vaccine candidate named S.opt.FL, which, after three or four doses, induced the production of specific antibodies with a titer of 1:10-1:10,000 [1], whereas the candidate vaccine pGX27-S described by Seo et al. induced specific antibodies with a titer of 1:1000-1:10,000 after two doses [22]. The pVAX-S-OP candidate vaccine described here produced specific antibodies with a titer of 1:102,400 after two doses, which is significantly higher than that induced by the S.opt.FL and pGX27-S candidate vaccines.

The protective effect of our candidate vaccine against SARS-CoV-2 infection was evaluated based on histopathological examination and measurement of viral RNA levels in the lungs of mice. hACE2 mice immunized with pVAX-S-OP showed good protection, with reduced viral loads in the lung and reduced tissue inflammatory damage. The candidate vaccine pSARS2-S was shown previously to reduce the viral load in lung tissue by a factor of $10^{1.37}$ [3]. In the present study, the viral loads in the lungs of pVAX-S-OP-immunized hACE2 mice were $10^{3.96}$-fold lower than in the negative controls. Histopathological examination showed no obvious pathological changes in the lung tissue of pVAX-S-OP-immunized mice, whereas a large area of alveolar wall thickening, accompanied by a large amount of lymphocyte and neutrophil infiltration, was observed in the controls. This result was consistent with those of previous studies [2, 10] and confirmed that the pVAX-S-OP vaccine was protective against challenge with SARS-CoV-2.

Conclusion

Our findings highlight the importance of optimizing the spike protein sequence in SARS-CoV-2 DNA vaccines and provide evidence of protection against SARS-CoV-2 infection in hACE2 mice.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval Animal experiments in this study were performed in compliance with the Animal Ethics Procedures and Guidelines of the People's Republic of China. All of the animal protocols in this study were approved by the Animal Welfare and Ethics Committee of the Changchun Veterinary Research Institute, Chinese Academy of Agricultural Sciences (approval number: IACUC of AMMS-11-2021-033).

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