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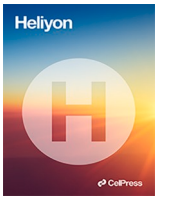

Research article

Development and evaluation of vaccination strategies for addressing the continuous evolution SARS-CoV-2 based on recombinant trimeric protein technology: Potential for cross-neutralizing activity and broad coronavirus response

Peng Du [a,*,1], Ning Li [b,1], Shengjun Tang [b,1], Zhongcheng Zhou [b,1], Zhihai Liu [b], Taorui Wang [a], Jiahui Li [b], Simiao Zeng [b], Juan Chen [c,**]

[a] *Faculty of Medicine, Macau University of Science and Technology, Macau, China*
[b] *Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, 510620, China*
[c] *Reproductive Medicine Center, Guangdong Second Provincial General Hospital, #466 Xin-Gang-Zhong-Lu, Haizhu District, Guangzhou, 510317, China*

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ABSTRACT

Given the significant decline in vaccine efficacy against Omicron, the development of novel vaccines with specific or broad-spectrum effectiveness is paramount. In this study, we formulated four monovalent vaccines based on recombinant spike trimer proteins, along with three bivalent vaccines, and five monovalent vaccines based on recombinant spike proteins. We evaluated the efficacy of different vaccination regimens in eliciting neutralizing antibodies in mice through pseudovirus neutralization assays. Following two doses of primary immunization with D614G, mice received subsequent immunizations with Omicron (BA.1, BA.2, BA.4/5) boosters individually, which led to the generation of broader and more potent cross-neutralizing activity compared to D614G boosters. Notably, the BA.4/5 booster exhibited superior efficacy. Following two doses of primary immunization with Omicron (BA.1, BA.2, BA.4/5), mice were subsequently immunized with one dose of D614G booster which resulted in broader neutralizing activity compared to one dose of Omicron (BA.1, BA.2, or BA.4/5). In unvaccinated mice, full-course immunization with different bivalent vaccines induced broad neutralizing activity against Omicron and pre-Omicron variants, with D614G&BA.4/5 demonstrating superior efficacy. However, compared to other variants, the neutralizing activity against XBB.1.5/1.9.1 is notably reduced. This observation emphasizes the necessity of timely updates to the vaccine antigen composition. Based on these findings and existing studies, we propose a vaccination strategy aimed at preserving the epitope repertoire to its maximum potential: (1) Individuals previously vaccinated or infected with pre-Omicron variants should inoculate a monovalent vaccine containing Omicron components; (2) Individuals who have only been vaccinated or infected with Omicron should be inoculated a monovalent vaccine containing pre-Omicron variants components; (3) Individuals without SARS-CoV-2 infection and vaccination should inoculate a bivalent vaccine comprising both pre-Omicron and Omicron components for primary immunization. Additionally, through

* Corresponding author.
** Corresponding author.
E-mail addresses: dupeng5@mail2.sysu.edu.cn (P. Du), chenjuan91@hotmail.com (J. Chen).
[1] Joint first authors.

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cross-inoculation of SARS-CoV-2 D614G spike trimer protein and SARS-CoV-1 spike protein in mice, we preliminarily demonstrated the possibility of cross-reaction between different coronavirus vaccines to produce resistance to the pan-coronavirus.

1. Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), initially identified in China in 2019, quickly evolved into a global pandemic, leading to the spread of COVID-19. In response to this worldwide crisis, a variety of vaccines have been developed and deployed, including inactivated virus vaccines, mRNA vaccines, recombinant protein vaccines, adenovirus vector vaccines, etc. These approved vaccines have demonstrated considerable efficacy against the ancestral strain of SARS-CoV-2, as evidenced by both in vitro neutralization and large-scale population-scale studies [1]. Given that the spike protein plays a major role for binding to host cells to initiate viral entry, development efforts primarily focus on utilizing the spike protein as an antigen for designing effective SARS-CoV-2 vaccines [2].

The replication characteristics of positive-strand RNA viruses result in continuous mutations of SARS-CoV-2, particularly in the S protein, posing challenges to the effectiveness of existing vaccines. In November 2021, WHO convened a special meeting and classified the novel SARS-CoV-2 variant B.1.1.529, firstly reported in South Africa, as a Variant of Concern (VOC) named Omicron. Since its emergence, Omicron has rapidly disseminated ， displacing other variants globally and precipitating a resurgence of infections [3,4]. Over time, numerous sub-lineages within Omicron have emerged. Original Omicron BA.1 was succeeded by BA.2 which further evolved into BA.2.12.1, BA.2.75, BA.2.75.2, BA.4 and BA.5. BA.4 and BA.5 share identical spike protein sequences (hereafter referred to as BA.4/5), which subsequently evolved into BA.4.6, BF.7, BQ.1 and BQ.1.1, eventually superseding BA.4/5 as the dominant epidemic strain in certain regions. Following this, the XBB variant, a recombinant of two sub-branches of BA.2 (BJ.1 and BM.1.1.1), replaced BQ as the dominant epidemic variant [5]. The spike protein serves as the primary locus of mutation in SARS-CoV-2. In comparison to previous VOCs (Alpha, 10 mutations; Beta, 10 mutations; Gamma, 12 mutations; Delta, 10 mutations), Omicron (BA.1, 39 mutations; BA.2, 31 mutations; BA.4/5, 34 mutations; BQ.1.1, 37 mutations, XBB.1.5/1.91, 42 mutations) exhibits a notably higher number of mutations within the spike protein (Fig. 1). Mutations in the spike protein typically contribute to enhanced immune evasion and/or transmission efficiency of the virus [6–12]. The unprecedented amino acid changes observed in Omicron variants result in evident immune escape from prior immunity, leading to recurrent COVID-19 outbreaks [13–19]. Recent studies have highlighted the emergence of additional Omicron variants, which exhibit an even greater ability to evade pre-existing immunity [13,20,21]. Various efforts were made to strengthen the power of vaccination. For example, administering monovalent vaccines containing Omicron components or bivalent vaccines comprising both original strain and Omicron components alongside existing immunity may represent effective strategies for eliciting robust cross-neutralizing antibody responses [21,22].

mRNA-based SARS-CoV-2 vaccines have captured significant attention from both the public and the scientific community. Their expedited development process has revolutionized vaccine production in addressing future pandemic threats. However, achieving global COVID-19 vaccine equity remains a challenge for most mRNA-based platforms and production methods. Recombinant protein vaccines play a crucial role in this endeavor. Although they have several disadvantages compared to mRNA vaccines, including longer production time for new variants and more complex manufacturing and quality control processes, their well-established and secure

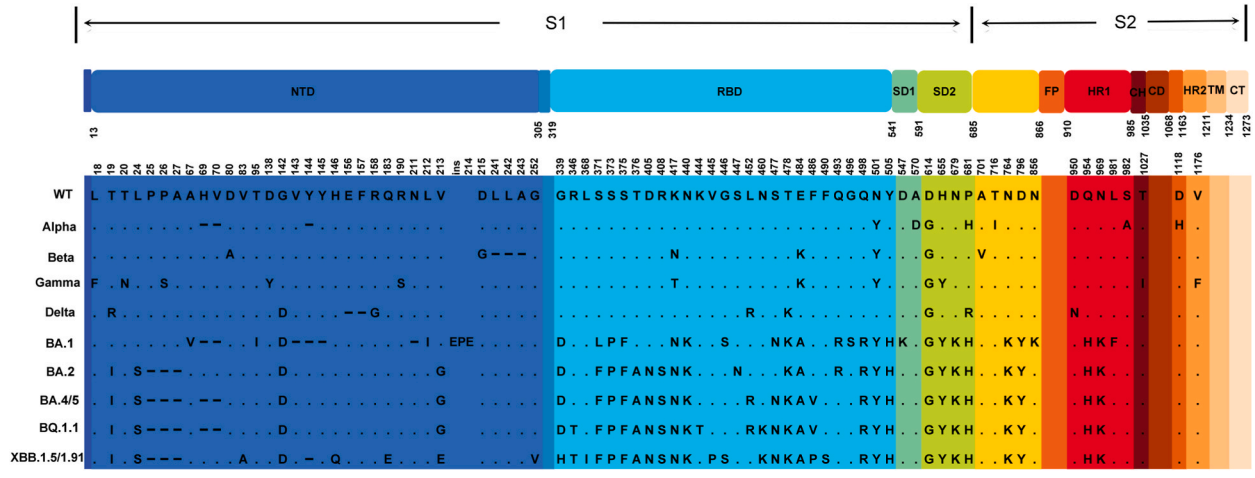

Fig. 1. Diagram of SARS-CoV-2 variants spike mutations. NTD, N-terminal domain; RBD, receptor-binding domain; SD1, subdomain 1; SD2, subdomain 2; FP, fusion peptide; HR1, heptad repeat 1; CH, central helix; CD, onnector domain; HR2, heptad repeat 2; TM, transmembrane region; CT, cytoplasmic tail.

production technology, coupled with global manufacturing systems, holds promising potential for bridging the COVID-19 vaccine accessibility gap, particularly in low- and middle-income countries across Africa, Latin America, and Southeast Asia [23–25].

The spike protein presents challenges in recombinant production due to its metastable nature. Stabilizing the prefusion state of viral fusion glycoproteins could enhance their recombinant expression by potentially preventing triggering or misfolding associated with adopting the more stable postfusion structure. Additionally, prefusion-stabilized viral glycoproteins have demonstrated enhanced efficacy as immunogens compared to their wild-type (WT) counterparts [26–28]. Previous studies have identified proline substitutions are effective in increasing the stability of the coronavirus spike protein [26]. The most stable form, named HexaPro (with mutations F817P, A892P, A899P, A942P, K986P, and V987P), was determined through comparisons of various proline substitutions [29]. Substituting RRAR with GSAS at the furin cleavage site has been shown to maintain the integrity of S1 and S2 subunits [30]. The BA.1 represents the earliest Omicron variant, while BA.2 and BA.4/5 serve as starting points for further evolution within most Omicron subvariants. Therefore, we first compared the immunogenicity of four spike proteins (D614G, BA.1, BA.2, BA.4/5) in monomeric form (expressed as D614G spike, BA.1 spike, BA.2 spike, BA.4/5 spike) and spike trimer protein (containing HexaPro and furin cleavage site mutations)(expressed as D614G, BA.1, BA.2, BA.4/5). Based on these initial comparisons, we developed four monovalent vaccines (D614G, BA.1, BA.2, BA.4/5) and three bivalent vaccines (D614G&BA.1, D614G&BA.2, D614G&BA.4/5) based on the recombinant trimer spike protein to investigate the neutralizing effects of different vaccination combinations against major SARS-CoV-2 variants in mice. Subsequently, we particularly explored the potential for inducing pan-coronavirus immunity by cross-immunization with SARS-CoV-2 D614G spike trimer protein and SARS-CoV-1 spike protein in mice. Our findings provide insights into serum cross-neutralization activity upon exposure to diverse variants of spike protein and contribute to designing effective vaccination strategies and developing pan-coronavirus immunization programs.

2. Methods

2.1. Mice immunization

The spike protein (40589-V08B4, 40589-V08B33; Sino Biological, and CG239, CG246, CG207-01; vazyme) or spike trimer protein (40589-V08H8, 40589-V08H26, 40589-V08H28, 40589-V08H33; Sino Biological) was individually dissolved in 50 μl PBS (protein content of different vaccines as indicated in corresponding figure captions). Subsequently, 50 μL aluminum hydroxide gel (Aluminum content: 10 mg/ml) adjuvant (Alhydrogel adjuvant 2 %, vac-alu-250; InvivoGen) was added and the mixture was vigorously shaken for 5 min to prepare monovalent vaccines. The D614G and other variant spike trimer proteins were dissolved in 50 μl PBS and mixed with an equal volume of aluminum hydroxide gel adjuvant. After vigorous shaking for 5 min, bivalent vaccines were prepared. Proteins 40589-V08B4 and 40589-V08B33 were produced using Baculovirus-Insect Cells, and the purity of the purified proteins was assessed using SDS-PAGE. Proteins CG239, CG246, and CG207-01 were produced using HEK293 Cells, and the purity of the purified proteins was assessed using SDS-PAGE. Proteins 40589-V08H8, 40589-V08H26, 40589-V08H28, and 40589-V08H33 were produced using HEK293 Cells, and the purity of the purified proteins was assessed using SEC-HPLC. All proteins were tested for endotoxin content using the LAL assay. The control group received a combination of 50 μl PBS and 50 μl aluminum hydroxide gel adjuvant. BALB/c female mice (6–8 weeks old), purchased from Gempharmatech-GD for immunization, were intramuscularly injected with the respective vaccines as planned (in the figure legends of Figs. 2, 3, 4, 5 and 6). Serum samples were collected by retroorbital hemorrhage at 3 or 4 weeks, 3 months, and 6 months after the last immunization, respectively. The mice were all fully anesthetized before sample collection. The sera were isolated by centrifugation at a speed of 4000 rpm and maintained at temperature of −80 °C.

2.2. Analysis of binding affinity of anti-SARS-CoV-2 or anti-SARS-CoV-1 antibodies derived from immunized mouse serum

Ninety-six-well enzyme-linked immunosorbent assay (ELISA) plates (9018; Corning) were coated with SARS-CoV-2 spike protein (40589-V08B4, 40589-V08B33; Sino Biological, and CG239, CG246; vazyme), SARS-CoV-1 spike protein (CG207-01; vazyme), and SARS-CoV-2 spike trimer protein (40589-V08H8, 40589-V08H28, 40589-V08H26, 40589-V08H33; Sino Biological). The plates were blocked with bovine serum albumin and washed with wash buffer. Serum samples were diluted at ratios of 1:50 or 1:150 and subsequently serially diluted with diluent buffer at a ratio of 1:3, resulting in a total of twelve gradients. These diluted samples were then added to their respective wells, incubated at 37 °C for 1 h, and subjected to three washes. Subsequently, the antibodies were detected using goat anti-mouse IgG H&L (alkaline phosphatase) (ab7069; Abcam), which was further diluted at a ratio of 1:1000 and incubated at room temperature for 30 min. Following three additional washes, an alkaline phosphatase substrate solution (pNPP)(P7998-100 ML; Sigma-Aldrich) was added to each well and incubated at room temperature for 15–20 min before being quenched with 3 M NaOH. Finally, the optical density was measured at a wavelength of 405 nm.

2.3. Preparation and quantification of pseudoviruses

The spike gene sequences of D614G, Alpha, Beta, Gamma, Delta, BA.1, BA.2, BA.4/5, BQ.1.1, XBB.1.5/1.9.1, SARS-CoV-1 and WIV1–CoV (WIV1, a beta coronavirus found in bats) were synthesized or generated through site-directed mutagenesis methods and subsequently cloned into the pcDNA3.1 (+) vector. The polymerase chain reaction (PCR) mix and recombinase enzyme used in this

study were High Fidelity DNA Polymerase Mix (P525; Vazym) and Exnase II (C214; Vazyme) respectively. The pcDNA3.1 (+)-spike, pLOVE-luciferase-EGFP and psPAX2 plasmids amplified by *Escherichia coli* were purified using the EasyPure HiPure Plasmid MaxiPrep Kit (EM121; TransGen Biotech). A total of 12 μg of pLOVE-luciferase-EGFP plasmid, 26 μg of psPAX2 plasmid and 2 μg of pcDNA3.1 (+)-spike plasmid were co-transfected into 100 mm cell culture dishes containing 8×10^6 293T cells using Lipofectamine 3000 (L30000015; Invitrogen). After transfection for a period of 6–8 h, the transfection cell culture medium was replaced with fresh medium (10 ml), followed by collection of supernatant containing pseudoviruses after 48 h post-transfection. The collected supernatant was subjected to centrifugation at 4 °C for 10 min at a speed of 200×*g*, followed by filtration through a membrane filter with a pore size of 0.45 μm. RNA extraction from pseudoviruses was performed using the TaKaRa minibest Viral RNA/DNA Extraction Kit Ver.5.0 (9766; TaKaRa). Reverse transcription was carried out using the HiScript III All-in-one RT SuperMix Perfect for qPCR kit (R333-01; Vazyme). The titer determination for pseudoviruses was conducted utilizing the TransLvTM Lentivirus qPCR Titration Kit (FV201; TransGen Biotech).

2.4. Cell culture

The HEK293T (CL-0005; Procell) and 293T-ACE2-TMPRSS22 (CL0015: VectorBuilder) cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) culture medium (C11995500BT; Gibco) with 10 % FBS (10099141C; Gibco) and 1 % penicillin-streptomycin (15140122; Gibco), at a temperature of 37 °C in a 5 % CO_2 incubator. The cells were subcultured every 3–4 days using 0.25 % trypsin for digestion (25200072; Gibco).

2.5. Pseudovirus neutralization assay

The methodology for pseudovirus neutralization assay has been previously validated in our and other research studies as an accurate means of assessing the levels of neutralizing antibodies in samples [11,19,31,32]. The specific methodology is outlined as follows. The 293T-ACE2-TMPRSS2 cells (1×10^4/well) were seeded in a 96-well white cell culture plate and incubated for 12 h befor the experiment. The mice sera were diluted with DMEM including 10 % FBS in round-bottom cell culture plates, followed by pre-incubation with SARS-CoV-2 pseudovirus (2×10^4 RLU) at 37 °C for 1 h. Each plate also included six virus control wells (without sera) and six control wells (without virus and sera). After incubation, the mixture was transferred to a white plate and further incubated at 37 °C for an additional 12 h before replacing the medium. After 48 h, luciferase substrate (11404ES80; Yeasen) was added to each well and the fluorescence signal was measured using LumiStation 1800 (Shanghai Flash Biotechnology Co., Ltd). The median effective dose (ED_{50}) of the sample was calculated using the Reed-Muench method [33].

2.6. Quantification and statistical analysis

The software GraphPad Prism 8.0.1 was used for data visualization and statistical analysis. The results were presented as means ± standard error of the mean (SEM). Mann-Whitney test was utilized with a significance level set at p 0.05 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$).

3. Results

3.1. Spike trimer protein as a more suitable antigen component for recombinant protein vaccines against coronaviruses

Previous studies have demonstrated that the HexaPro mutation and GSAS replacement of the furin cleavage site in the WT spike protein enhances its stability, maintaining it in the pre-fusion conformation. This structural modification promotes increased immunogenicity of the WT spike protein [29,34]. Therefore, we initially evaluated the immunogenicity of both spike protein and spike trimer protein (containing HexaPro and furin cleavage site mutations) from four variants of SARS-CoV-2 (D614G, BA.1, BA.2, BA.4/5). BALB/c mice (6–8 weeks old) were administered a vaccine containing 5 μg of corresponding proteins along with aluminum hydroxide adjuvant at weeks 0, 4, and 6. Serum samples were collected four weeks after the final injection for ELISA and Neutralization assays (Fig. 2A). Our findings revealed that, following multiple injections, they exhibited minimal neutralizing activity, although the four unprocessed spike proteins (excluding HexaPro and furin cleavage site mutations) elicited robust levels of IgG-binding antibodies against their corresponding spike proteins, comparable to those induced by spike trimer proteins (Fig. 2B and C). Conversely, the four spike trimer proteins not only induced high levels of IgG-binding antibodies but also demonstrated significant neutralizing antibody titers (Fig. 2C). To confirm the generalizability of these observations, we conducted a parallel experiment using unprocessed SARS-CoV-1 spike proteins. The results indicated that SARS-CoV-1 spike proteins similarly stimulated substantial levels of IgG-binding antibodies but displayed negligible neutralizing activity (Fig. 2C). Collectively, our data unequivocally demonstrated that unprocessed coronavirus spike proteins are unsuitable as antigenic components for recombinant protein vaccines targeting coronaviruses.

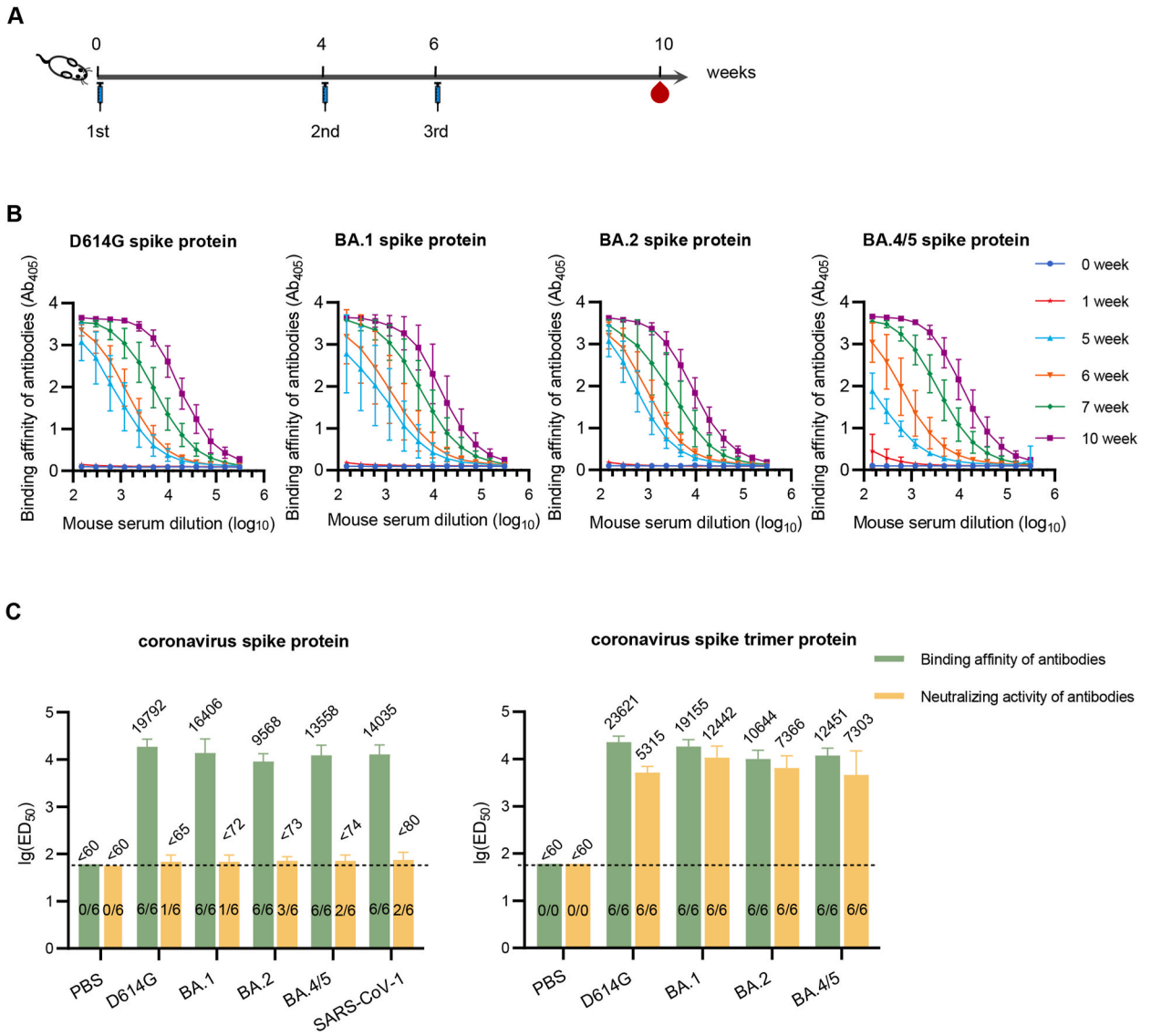

Fig. 2. Spike trimer protein as more suitable antigen component for recombinant protein vaccines against coronaviruses **A** Immunization process. Each group of mice inoculated one dose of the corresponding recombinant protein vaccine (in the form of D614G, BA.1, BA.2, BA.4/5, SARS-CoV-1 spike protein or spike trimer protein) at the initiation of the experiment at 0, 4, and 6 weeks respectively. Each vaccine dose contained 5 μg of the respective protein and serum samples were collected four weeks after completion of the third vaccination (n = 6). **B** ELISA results of mouse serum at various time points post-inoculation with D614G, BA.1, BA.2, BA.4/5 or SARS-CoV-1 spike protein vaccines demonstrated the induction of high levels of IgG-binding antibodies against the corresponding spike proteins. **C** The binding antibody titers (ELISA tests) and the neutralizing antibody titers (neutralization assay) were compared following immunization with the spike protein vaccine (left) and spike trimer protein vaccine (right). All tests are conducted using proteins or pseudovirus corresponding to the inoculated vaccine. The upper values denote the mean ED50 for each data group, and the dashed lines represent the limit of detection. Below the dashed line is the proportion of positive samples to total samples in each group (number of positive samples/total number of samples). Samples with an ED_{50} lower than a 50-fold dilution were denoted as 50 for statistical analysis. ED_{50}, median effective dose. Notably, the spike trimer protein includes HexaPro mutant and furin cleavage site mutants, distinguishing it from the spike protein which lacks these mutations. The control group (PBS) was administered injections containing a mixture of phosphate buffer and aluminum hydroxide adjuvant.

3.2. Broader immunity induced by three Omicron (BA.1, BA.2, BA.4/5) booster following two-dose D614G vaccines

During the progression of the SARS-CoV-2 epidemic, most individuals have undergone immunization with a vaccine derived from the Wuhan-Hu-1 or have been infected by pre-Omicron variants. To investigate the impact of different booster immunizations on pre-existing immunity, we prepared four boosters (D614G, BA.1, BA.2, BA.4/5) following two doses of D614G vaccination. Four weeks after completing the booster immunization, mouse serum was collected and neutralizing antibody activity in the serum was assessed (Fig. 3A). As shown in Fig. 3B, the findings revealed that while the D614G booster significantly increased neutralizing antibody titers

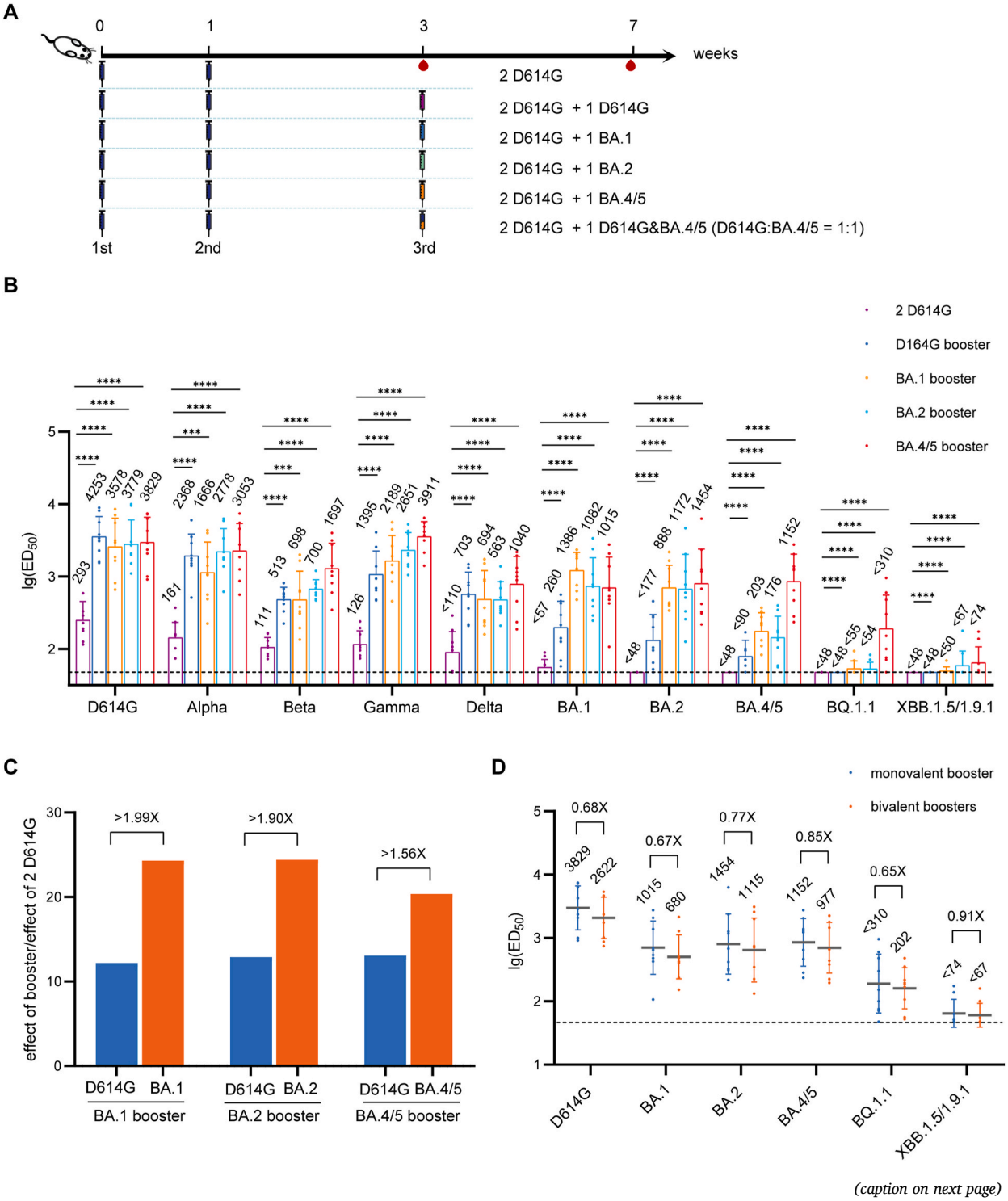

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Fig. 3. Broader immunity induced by three Omicron (BA.1, BA.2,BA.4/5) booster following two-dose D614G vaccines **A** Immunization process. The mice in each group were inoculated with one dose of the D614G monovalent vaccine at week 0 and week 1, respectively. Subsequently, they were inoculated with one dose of the D614G, BA.1, BA.2, BA.4/5 monovalent vaccines, or the bivalent D614G&BA.2 vaccine at week 3. Serum samples were collected during weeks 3 and 7 of the experiment (n = 9). Each dose of the monovalent vaccine contains 10 μg of the corresponding protein, while the bivalent D614G&BA.2 vaccine contains 5 μg each of D614G and BA.2 proteins. **B** Neutralizing activity of mouse serum from different monovalent vaccine enhancement strategies. The values presented represent the average ED_{50}. **C** Each booster demonstrated higher neutralizing antibody titers for neutralizing corresponding Omicron mutants than for D614G mutant. The value indicated the difference in the fold change of titer. **D** Comparison of neutralizing activity of sera from mice immunized with enhanced BA.2 monovalent vaccine and enhanced D614G&BA.2 bivalent vaccine. The values below the line represent the mean of ED_{50} for each data set, and the values above the line represent the fold change of titer against different inoculation Plans. The dashed lines represented the limit of detection. In the statistical analysis, an ED_{50} lower than a 50-fold dilution was denoted as 50 for these samples.

against pre-Omicron variants, its effect on Omicron was limited. Additionally, for pre-Omicron variants (excluding D614G), all three Omicron boosters (BA.1, BA.2, BA.4/5) exhibited similar effects to the D614G booster, but they also significantly increased the immune response against Omicron variants. Furthermore, each booster demonstrated higher neutralizing antibody titers for neutralizing corresponding Omicron variants than for D614G variant (Fig. 3C). Among the booster regimens, the BA.4/5 booster exhibited superior broad-spectrum efficacy. However, the neutralizing antibody titers in mice vaccinated with this booster remained lower against the BQ.1.1 and XBB.1.5/1.9.1 variants compared to those against earlier-emerged variants (Fig. 3B). Furthermore, we conducted a comparative analysis between the monovalent (BA.4/5 booster) and bivalent boosters (D614G&BA.4/5 booster), revealing that the bivalent vaccine booster displayed slightly less effective (but not statistically significant) against both the D614G and Omicron (BA.1, BA.2, BA.4/5, BQ.1.1, XBB.1.5/1.9.1) variants when compared to the monovalent vaccine (Fig. 3A and D). These findings suggested that for individuals previously vaccinated with or infected by pre-Omicron variants, a monovalent vaccine containing Omicron components might confer higher neutralizing antibody titers compared to other vaccines, including monovalent vaccines containing pre-Omicron components or bivalent vaccines containing both pre-Omicron and Omicron components.

3.3. Potential necessity of booster vaccination with early strain spike protein in individuals unexposed to pre-Omicron strains and vaccines

Given the complete replacement of early variants by the Omicron subvariants and its apparent ability to evade previous immunization, we investigated the immunization effect of three monovalent vaccines containing Omicron (BA.1, BA.2, BA.4/5), individually. Our neutralization experiments revealed that all three Omicron variants exhibited robust immunogenicity comparable to that of D614G, with BA.1 showing the highest level of immunogenicity (Fig. 4A). Furthermore, compared to three doses of D614G vaccination, the three Omicron monovalent vaccines elicited stronger neutralizing responses against the Omicron subvariants (Fig. 4A). Specifically, BA.1 immune serum effectively neutralized both BA.1 and BA.2, while BA.2 showed efficacy against these two variants as well as BA.4/5. Furthermore, BA.4/5 displayed efficacy not only against these three variants but also against BQ.1.1 and XBB.1.5/1.9.1 (Fig. 4A). However, despite the superior immunization effect of the three monovalent Omicron vaccines against the Omicron subvariants, limited effect was displayed against early strains (Fig. 4A). This implied that individuals lacking infection or vaccination against previous Omicron variants may not be adequately protected against reinfection with early variants, which could potentially be more pathogenic. Consequently, these populations may require a spike protein vaccine containing early strains. Subsequently, we investigated the effect of administering a booster dose of the D614G vaccine to mice previously vaccinated with two doses of the corresponding monovalent vaccines (BA.1, BA.2 or BA.4/5). The results indicated that while the D614G booster maintained similar effects as the corresponding Omicron (BA.1, BA.2, BA.4/5) booster for the Omicron subvariants, it significantly enhanced the immunization effect against early variants (Fig. 4B–D).

3.4. A bivalent vaccine regimen as the optimal option for populations unexposed to SARS-CoV-2 virus and vaccine

Reinfection with B.1.1.529 after prior Wuhan Hu-1 infection resulted in diminished T cell recognition and did not lead to an increase in cross-reactive neutralizing immunity [35]. The initial exposure to SARS-CoV-2 antigens can significantly influence future immune responses. Hence, to enhance the safeguarding of the epitope repertoire against different VOCs, employing a bivalent vaccine approach may be a more favorable strategy for primary immunization. We compared the immunogenicity of monovalent and bivalent vaccination strategies during primary immunization (Fig. 5A). In pseudovirus neutralization assay, mice vaccinated with monovalent D614G exhibited insufficient neutralization capacity against Omicron (BA.1, BA.2, BA.4/5, BQ.1.1, XBB.1.5/1.9.1) pseudoviruses. Similarly, sera from mice immunized with monovalent BA.1, BA.2, or BA.4/5 exhibited limited neutralizing efficacy against pre-Omicron variants (Fig. 4), whereas those immunized with bivalent vaccines (D614G&BA.1, D614G&BA.2, or D614G&BA.4/5) demonstrated broader immune coverage (Fig. 5B). Among them, D614G&BA.4/5 displayed the most extensive immune coverage. However, mice inoculated with bivalent D614G&BA.4/5 still exhibited lower titers of neutralizing antibodies against XBB.1.5/1.9.1 (6.2 times lower than BA.4/5). The results indicated that XBB.1.5/1.9.1 might possess a higher capability to evade immune responses

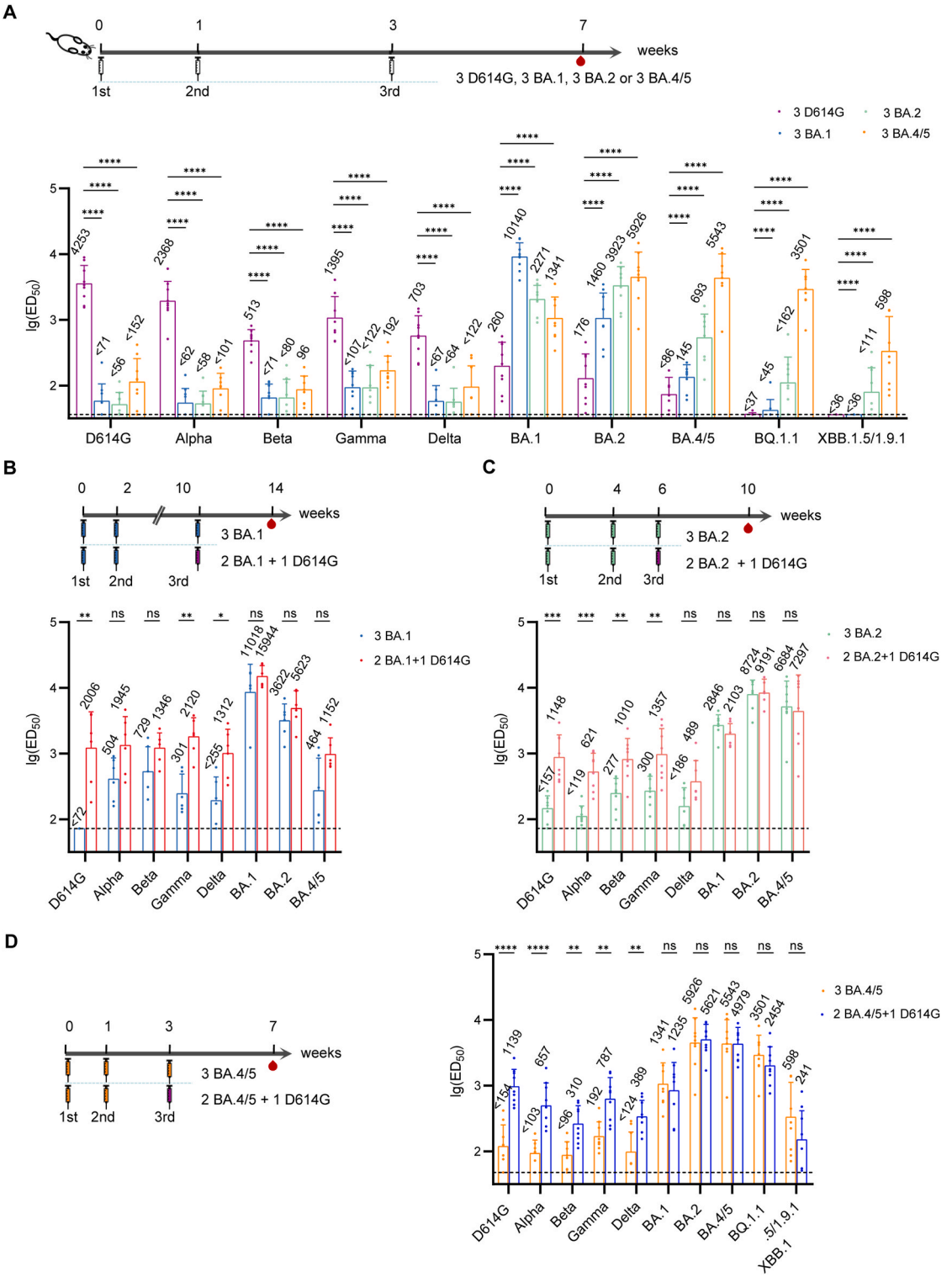

Fig. 4. Potential necessity of booster vaccination with early strain spike protein in individuals unexposed to pre-Omicron strains and vaccines **A** Neutralizing activity of sera from mice fully vaccinated with monovalent vaccine. The vaccination schedule was displayed in the upper panel. Each dose contained 10 μg of the corresponding protein (n = 9). **B** Neutralizing activity of sea from mice fully vaccinated with 3 doses of BA.1 vaccine and boosted with D614G vaccine after 2 doses of BA.1 vaccine. The vaccination schedule was displayed in the upper panel. Each dose contained 5 μg of the corresponding protein (n = 6). **C** Neutralizing activity of sera from mice fully vaccinated with 3 doses of BA.2 vaccine and boosted with D614G vaccine after 2 doses of BA.2 vaccine. The vaccination schedule was displayed in the upper panel. Each dose contained 5 μg of the corresponding

protein (n = 8). **D** Neutralizing activity of sera from mice fully vaccinated with 3 doses of BA.4/5 vaccine and boosted with D614G vaccine after 2 doses of BA.4/5 vaccine. The vaccination schedule was displayed in the left panel. Each dose contained 10 μg of the corresponding protein (n = 9). The values presented represent the average ED_{50}. The dotted line indicated the detection limit, which was expressed as the lowest dilution factor for these samples in the statistical analysis.

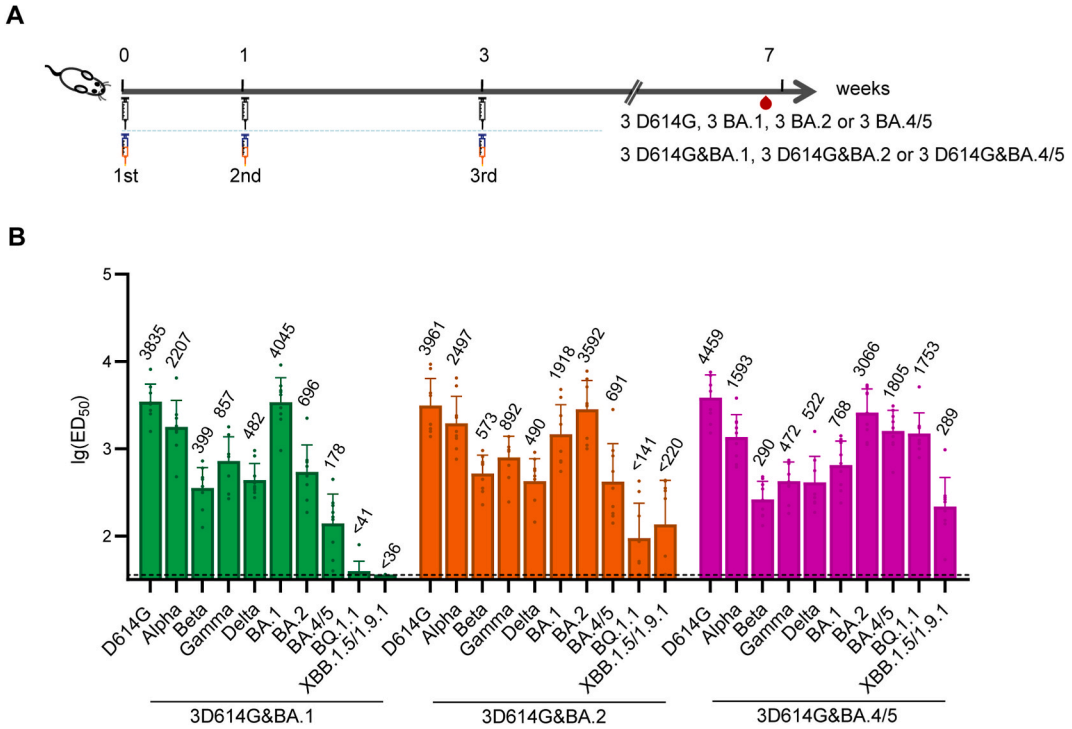

Fig. 5. Bivalent vaccine regimen as the optimal option for populations unexposed to SARS-CoV-2 virus and vaccine **A** Immunization process. **B** Sera neutralizing activity of mice fully immunized with one of three bivalent vaccines (D614G&BA.1, D614G&BA.2 or D614G&BA.4/5). The inoculation schedules were presented below the horizontal line, with each vaccine dose containing 5 μg of both D614G and its corresponding Omicron protein (n = 9). The value represented the mean of ED_{50} for each data group. The dashed lines represented the limit of detection. In the statistical analysis, an ED_{50} lower than a 36-fold dilution was denoted as 36 for these samples.

compared to other variants, which was consistent with observations in human studies [13,36,37]. Therefore, the utilization of bivalent recombinant protein vaccines incorporating both pre-Omicron and Omicron spike proteins may induce a wider range of serum neutralizing activity. However, attention should also be given to updating the antigen component following changes in epidemic variants.

3.5. Cross-vaccination for broad protection against SARS-CoV-2 variants and other coronaviruses: a potential approach

Since the emergence of Omicron, the rate of SARS-CoV-2 evolution has significantly accelerated. To address the challenge posed by its increasingly potent immune evasion, efforts have been made to update vaccines. Current vaccine development is based on epidemic variants, which inherently results in a time lag in updating vaccines compared to the virus's evolutionary pace. Therefore, it is imperative to investigate the vaccination strategy for pan-coronaviruses. Pre-existing cross-reactive immune responses have been observed in individuals prior to SARS-CoV-2 exposure, potentially resulting from previous human coronavirus (HCoVs) cross-infections [38–42]. This implied that the co-infection of an individual with different coronaviruses could elicit the production of pan-coronavirus antibodies, thereby offering insights into the development of a pan-coronavirus vaccination scheme. In this study, we employed SARS-CoV-2 D614G spike trimer protein and SARS-CoV-1 spike protein recombinant vaccines to devise vaccination strategies and assess their feasibility for pan-coronaviruses (Fig. 6A). Consistent with our predictions, mice inoculated with three doses of D614G spike trimer protein exhibited robust neutralizing activity against D614G (titer, 4571), limited neutralizing activity against BA.1 (titer, 224), and negligible neutralizing activity against SARS-CoV-1 and WIV1. On the other hand, mice inoculated with three doses of SARS-CoV-1 spike protein exhibited negligible neutralizing activity against D614G, BA.1, SARS-CoV-1, and WIV1. Interestingly, a significant enhancement in the neutralizing activity against all four pseudoviruses was observed when mice received three doses of SARS-CoV-1 spike protein vaccination following completion of the initial three doses of D614G spike trimer protein vaccination. Particularly remarkable was the substantial enhancement in neutralization potency against SARS-CoV-1 (>8.1-fold) and WIV1 (>3.6-fold), which previously exhibited negligible or weak neutralization capability, as well as BA.1 (>5.8-fold) that initially

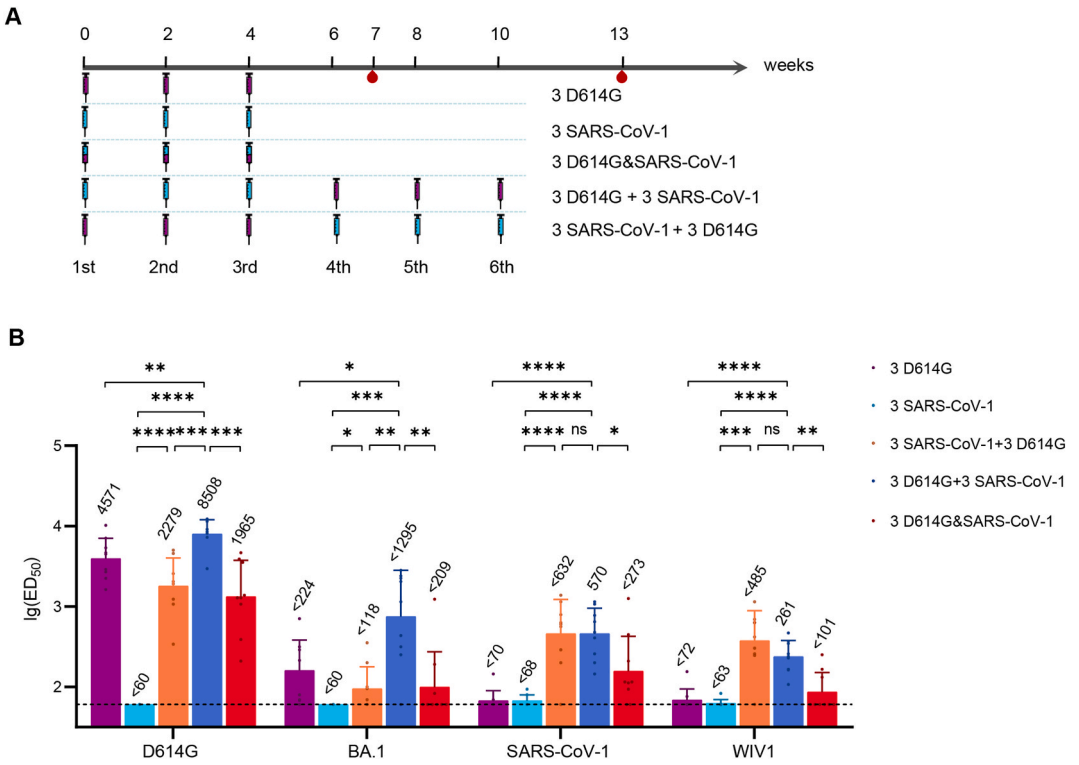

Fig. 6. Cross-reactivity between SARS-CoV-2 and other coronaviruses may offer broad protection against future SARS-CoV-2 variants and other coronaviruses**A.** Immunization process. The serum samples were collected at week 3 following the completion of the entire vaccination program in each group. Each dose of the monovalent vaccine contained 3 μg of protein, while the bivalent vaccine contained a total of 6 μg protein, with 3 μg for each individual protein (n = 9). **B** Sera neutralizing activity in mice subjected to various inoculation regimens. The value represented the mean of ED_{50} for each data group. The dashed lines represented the limit of detection. In the statistical analysis, an ED_{50} lower than a 60-fold dilution was denoted as 60 for these samples. ED_{50}, median effective dose. The antigen contained in the SARS-CoV-1 vaccine was spike protein, and the antigen contained in the D614G protein vaccine was spike trimer protein.

demonst0rated low efficacy in neutralization. This implied that sequential inoculation of SARS-CoV-2 and SARS-CoV-1 could confer broad protection against coronaviruses (Fig. 6B). Subsequently, we assessed the sera-neutralizing activity in mice immunized with three doses of SARS-CoV-1 spike protein followed by three doses of D614G spike trimer protein. We found that the neutralizing activity of SARS-CoV-1 and WIV1 was significantly enhanced (>9.3-fold and >7.8-fold, respectively) following vaccination with three doses of D614G spike trimer protein. However, the neutralizing activity of D614G alone exhibited a 2-fold decrease, while that of BA.1 showed a reduction by more than 1.9 times compared to mice vaccinated with three doses of D614G spike trimer protein alone (Fig. 6B). Furthermore, we assessed the immune effect of the bivalent vaccine inoculated with three doses of D614G&SARS-CoV-1 (D614G: SARS-CoV-1 = 1:1, with a total protein content twice that of the monovalent vaccine). Our findings revealed a moderate neutralizing effect against D614G (titer, 1965) and a low neutralizing effect against SARS-CoV-1 (titer, 168) (Fig. 6B). These results suggest that an initial inoculation with three doses of D614G spike trimer protein followed by three doses of SARS-CoV-1 spike protein may represent the most effective approach to enhance antibody cross-reactivity.

4. Discussion

Compared to mRNA vaccines, recombinant protein vaccines involve the direct vaccination of purified antigens in humans. Therefore, stricter requirements are imposed to the spatial conformation of antigens. Consequently, we initially evaluated the immunogenicity of spike protein and spike trimer protein derived from four SARS-CoV-2 variants (D614G, BA.1, BA.2, BA.4/5), as well as SARS-CoV-1 spike protein. Our findings revealed that while all four SARS-CoV-2 spike proteins and the SARS-CoV-1 spike protein elicited substantial levels of IgG-binding antibodies, they exhibited minimal neutralizing activity. In contrast, the four spike trimer proteins induced robust levels of neutralizing antibodies, possibly due to the presence of HexaPro and furin cleavage site mutants within coronavirus proteins [29]. This observation suggests that the initial step in developing recombinant protein vaccines for both the currently circulating SARS-CoV-2 virus and potential future coronaviruses should prioritize the design of spike proteins with a stable prefusion conformation.

We investigated the immune effects of different monovalent vaccine boosters (D614G, BA.1, BA.2, BA.4/5) following two doses of D614G immunization. Similar to mRNA vaccine boosters, the D614G booster exhibited significant efficacy only against pre-Omicron

variants, while the Omicron booster significantly enhanced neutralizing activity against all variants [43]. Compared to BA.1 or BA.2 booster regimens, the BA.4/5 booster regimens exhibited superior enhancement effects. However, mice receiving booster vaccinations still demonstrated lower titers of neutralizing antibodies against BQ.1.1 and XBB.1.5/1.9.1, indicating the significance of timely antigen updates in maintaining efficacy. Additionally, when comparing the two booster regimens of BA.4/5 and D614G&BA.4/5, we observed a slight drop of enhancement effect of bivalent vaccines on both D614G and Omicron compared to monovalent vaccines, consistent with other findings [43]. Studies have shown that enhancement with antigens different from WT variant mainly recalls WT vaccination-induced memory B cells and masks the generation of mutant-specific B cells from scratch, which hinders the generation of appropriate humoral immunity against newly emerging variants [44]. This phenomenon may be further exacerbated when bivalent vaccines containing components of the original strain are employed for booster immunization [45]. Based on our findings and existing research, we proposed that individuals previously vaccinated or infected with pre-Omicron variants should administer booster doses of monovalent vaccines incorporating Omicron components. Furthermore, the antigen components should be promptly updated following prevailing epidemic conditions.

Following full-course immunization with three doses of Omicron (BA.1, BA.2, BA.4/5) monovalent vaccines in mice, it was observed that although these vaccines demonstrated enhanced immune effect against Omicron sublineages, their efficacy against early variants was limited, consistent with findings reported for mRNA-based vaccines [46]. This suggested that individuals lacking prior exposure to pre-Omicron variants and vaccinations may not be adequately protected against reinfection by early variants, which could possess heightened pathogenicity [47–49]. Consequently, this subgroup of individuals might necessitate a booster with a spike protein vaccine incorporating early variants. Our study suggested that the D614G booster, in the context of Omicron immunization, may enhance neutralizing antibody titers against both the Omicron sublineages and early variants. Therefore, it is crucial to closely monitor the potential re-emergence of early variants and strengthen surveillance on viral evolution and epidemic dynamics. In the event of a potential re-emergence of previous Omicron variants or diminished resistance due to delayed access to Omicron adaptive vaccines, individuals who have solely received vaccination or been infected with Omicron and subsequent variants should be administered a booster dose of monovalent vaccines containing pre-Omicron components.

In light of the absence of effective measures to completely eradicate SARS-CoV-2, many governments, including those of China and the United States, have canceled the order to mitigate its spread, signaling the likelihood of its coexistence with humans for an extended period. Consequently, it becomes imperative not only to bolster the immunity of individuals with prior exposure but also to consider the immunological status of neonates born during the Omicron pandemic. The SARS-CoV-2 virus has exhibited enhanced evasion of prior immunity over time. The widespread vaccination with the vaccine based on the Wuhan-1 strain and the establishment of immune responses through previous infections have contributed to the evolutionary transition from Wuhan-1 to Omicron. However, neonates born during the Omicron pandemic today face a distinct immunological background. Given the potential for localized outbreaks within this population due to the propensity of educational and medical settings to facilitate newborn gatherings, it is crucial to address this issue proactively. Our study demonstrated that bivalent vaccination could confer broad neutralizing effects against both Omicron and pre-Omicron variants during primary immunization. Considering the heightened pathogenicity associated with pre-Omicron variants [47–49], it is imperative to contemplate administering bivalent vaccines containing components targeting both original and Omicron variants for neonates born after the emergence of Omicron pandemic, This approach ensures effective safeguarding against diverse epitope repertoires.

To monitor potential side effects, we assessed the weight of mice was assessed during multiple vaccination regimens. The results revealed no significant disparity in mouse weight between the control and experimental groups throughout the entire study (Fig. S1), indicating that our vaccine does not have adverse long-term effects on weight. Additionally, we examined the duration of neutralizing antibodies in mice, finding that these antibodies remained consistently high for six months following completion of the vaccination regimen (Fig. S2). By comparing multiple schemes, we observed that low-dose, long vaccination intervals yielded superior or equivalent neutralizing antibody titers compared to high-dose, short vaccination intervals (Fig. S3). Furthermore, we also found that ELISA is incapable of accurately reflecting the levels of neutralizing antibodies against different SARS-CoV-2 variants in serum, potentially due to the presence of numerous non-neutralizing binding antibodies in the body (Fig. S4).

The emergence of SARS-CoV-2 variants and the potential for future coronavirus outbreaks necessitate the development of a pan-coronavirus vaccine. Observations of cross-reactivity with other coronaviruses in individuals suggested the possibility of eliciting a pan-coronavirus response through vaccination, possibly due to receptor-binding degeneracy or epitope structural similarity [50]. In this study, we investigated the potential induction of a pan-coronavirus response in mice using the SARS-CoV-2 D614G spike trimer protein and SARS-CoV-1 spike protein. Our findings demonstrated that repeated vaccination with different coronaviruses effectively elicited a pan-coronavirus response in mice. Although the proportion of neutralizing antibodies targeting multiple coronaviruses might be limited, further characterization and analysis of these antibodies lay the foundation for developing novel pan-coronavirus-neutralizing antibodies or vaccines. Additionally, we observed that distinct vaccination regimens had a significant impact on the final immune outcomes despite employing the same antigen. Notably, pre-vaccination with SARS-CoV-1 spike protein resulted in a substantial reduction in the efficacy of subsequent D614G vaccination against SARS-CoV-2. This observation is consistent with previous findings, suggesting that pre-existing immune imprinting influences the efficacy of subsequent immunization [51–53]. Although SARS-CoV-1 spike failed to induce effective neutralizing antibodies, it could generate high-affinity binding antibodies that cross-react with D614G spike trimer protein influencing germinal center and memory B cell selection. This change is facilitated by two mechanisms: (1) reducing the activation threshold for B cells to allow abundant lower-affinity clones to participate in the immune response; and (2) directly masking their cognate epitopes [44].

We acknowledged several limitations of this study: (1) The experiment was exclusively conducted in female BALB/c mice, necessitating further investigations involving male mice and larger study cohorts to extrapolate these findings; (2) Due to the

constraints on serum volume, we were unable to include the latest variants (XBB.1.16, EG.5) in our experimental assessment of immune protection breadth; (3) Our analysis did not encompass non-neutralizing antibodies or cellular immunity, both of which could potentially impact protective immune responses; (4) The utilization of mice as an experimental model facilitated rapid detection and multiple comparisons, but validation across alternative animal models and populations is indispensable for confirming these observations.

In this study, we conducted an extensive investigation into the potential use of recombinant protein-based vaccines for addressing the Omicron pandemic. Based on our findings, we propose three specific vaccination regimens tailored to different scenarios. Our results demonstrated that both booster immunization with a monovalent vaccine and primary immunization with a bivalent vaccine elicited broad and persistent neutralizing responses, highlighting the promising prospects of recombinant protein vaccines. However, even the most effective immunization using BA.4/5 monovalent or bivalent vaccine significantly reduced the titer of neutralizing antibodies against XBB.1.5/1.9.1, underscoring the importance of timely antigen updates. Additionally, we provided preliminary evidence for the potential induction of pan-coronavirus immunity through cross-reactivity among different coronavirus vaccines. Our findings are expected to enhance comprehension of the impact of exposure to diverse spike protein mutations on serum cross-neutralizing activity and contribute to the formulation of vaccination strategies and development of pan-coronavirus immunization regimens.

Ethics statement

This study was approved by Guangzhou Medical University and the ethics committee: G2023-418.

Data availability

Data included in article/supp. material/referenced in articles.

CRediT authorship contribution statement

Peng Du: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ning Li:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology. **Shengjun Tang:** Methodology, Investigation, Formal analysis, Data curation. **Zhongcheng Zhou:** Validation, Supervision. **Zhihai Liu:** Methodology, Investigation. **Taorui Wang:** Project administration. **Jiahui Li:** Project administration, Methodology. **Simiao Zeng:** Project administration, Methodology, Conceptualization. **Juan Chen:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34492.

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