Vaccine: X 16 (2024) 100444

Contents lists available at ScienceDirect

Vaccine: X

journal homepage: www.elsevier.com/locate/jvacx

Development of an RBD-Fc fusion vaccine for COVID-19

Yisheng Sun [a,b,1], Qiaomin Li [a,1], Yuanyuan Luo [a,1], Hanping Zhu [b], Fang Xu [b], Hangjing Lu [b], Pingping Yao [b], Zhen Wang [b,*], Wenbin Zhao [a,*], Zhan Zhou [a,c,*]

[a] Innovation Institute for Artificial Intelligence in Medicine and Zhejiang Provincial Key Laboratory of Anti-Cancer Drug Research, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China

[b] Key Lab of Vaccine, Prevention and Control of Infectious Disease of Zhejiang Province, Zhejiang Provincial Center for Disease Control and Prevention, Hangzhou 310015, China

[c] The Fourth Affiliated Hospital, Zhejiang University School of Medicine, Yiwu, 322000, China

ARTICLE INFO

Keywords:
SARS-CoV-2
Heterologous
RBD
Delta variant
Fc fusion

ABSTRACT

Although the global pandemic of SARS-CoV-2 has passed, there are still regional outbreaks that continue to jeopardize human health. Hence, there is still a great deal of interest in developing an efficient vaccine that can quickly and effectively prevent reemerging outbreaks of SARS-CoV-2. Delta variant was once a dominant strain in the world in 2021, and we first constructed a recombinant RBD_{delta}-Fc fusion vaccine by coupling the RBD of Delta variant with the human Fc fragment. This Fc fusion strategy increases the immunogenicity of the recombinant RBD vaccine, with a long-lasting high level of IgG antibodies and neutralizing antibodies induced by RBD_{delta}-Fc vaccine. This RBD_{delta}-Fc vaccine, as well as the RBD-Fc vaccine prepared in our previously study, could trigger a durable immune effect by the heterologous boosting immunity, and the RBD-Fc induced a quicker humoral immune response than the homologous immunization with inactivated vaccines. In conclusion, the Fc fusion strategy has a significant role in enhancing the immunogenicity of recombinant protein vaccines, thus promising the development of a safe and efficient vaccine for the heterologous boosting against SARS-CoV-2.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused more than 770 million infections and 6.9 million deaths by 14th September 2023 [1]. Though the COVID-19 caused by SARS-CoV-2 was no longer declared as a Public Health Emergency of International Concern (PHEIC) by WHO on May 2023. It still remains a global health emergency. Numerous people are still infected by SARS-CoV-2 every day, and infection with SARS-CoV-2 can lead to pathological sequelae in multiple organs [1,2]. The SARS-CoV-2 virus mutated fast and lots of variants emerged such as Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2) and Omicron (B.1.1.529) variants since the outbreak of COVID-19 at late 2019 [3]. The Delta variant was once a dominant variant worldwide in 2021, and the infection of Delta variant could lead to more severe disease than that of Omicron [4]. Though the dominant variant was replaced by the Omicron due to its increased transmissibility nowadays [5], the Delta variant is still an important variant need to be noticed.

Vaccines play a key role in the prevention and control of COVID-19. A variety of vaccines against SARS-CoV-2, including inactivated vaccines, mRNA vaccines, and recombinant subunit vaccines, have been approved for market, of which inactivated vaccines are one of the most widely used vaccines, especially in China. According to statistics, China had received more than 300 million doses of inactivated COVID-19 vaccine by the end of 2022 [6]. However, the efficacy and duration of vaccine-induced immunity have been a concern. In randomized clinical trials, after two doses of inactivated COVID-19 vaccination, IgG titers peaked after one month, declined significantly after three months, and then declined sharply after six months [7].

Heterologous boosting with inactivated vaccine and other vaccines could induce high level of neutralizing antibodies with broad neutralizing capacity and long retention time [7]. Since immunization with two doses of inactivated vaccines was widely used in China, adenovirus vector vaccines, mRNA vaccines, and recombinant subunit vaccines can all be used as heterogenic boosters, with RBD being the primary target of vaccine-induced and naturally-acquired humoral immunity to SARS-

* Corresponding authors at: Innovation Institute for Artificial Intelligence in Medicine and Zhejiang Provincial Key Laboratory of Anti-Cancer Drug Research, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China (Z. Zhou).

E-mail addresses: wangzhen@cdc.zj.cn (Z. Wang), pharmacy_zwb@zju.edu.cn (W. Zhao), zhanzhou@zju.edu.cn (Z. Zhou).

[1] These authors made equal contributions to this work.

https://doi.org/10.1016/j.jvacx.2024.100444
Received 18 October 2023; Received in revised form 16 January 2024; Accepted 18 January 2024
Available online 21 January 2024

CoV-2, which makes RBD safer as a vaccine than either full-length Spike proteins or inactivated viruses [8–10]. However, recombinant RBD vaccines are the least active of these heterologous booster vaccines. Zhang et al. purposed that in the single-dose immunization experiment, the neutralizing antibody titer of the RBD vaccine heterologous boosted group was significantly higher than that of the inactivated vaccine homologous immunization group, but the RBD vaccine did not enhance T-cell immunity over inactivated vaccines [11]. In a sequential immunization experiment, the titer of neutralizing antibody in the RBD vaccine heterologous booster group was similar to that in inactivated vaccine homologous immunization group and much lower than that in adenovirus vector vaccine heterologous boosted group. Therefore, there is a need to improve the activity of RBD vaccines as heterologous boosters to provide a viable strategy for safer and more efficient prevention of SARS-CoV-2 and even other viruses. Unlike ZF2001 (a subunit vaccine product) combining two RBD protein as a dimeric RBD protein [12], the recombinant RBD-Fc vaccine was constructed by fusing RBD with the IgG Fc fragment. In our previous study, we had developed a recombinant RBD-Fc vaccine candidate based on the prototype SARS-CoV-2 RBD sequence, which showed good immunogenicity [13]. In this study, based on the RBD sequence of Delta variant, we first constructed a recombinant RBD_{delta}-Fc vaccine. The immunogenicity of this recombinant RBD_{delta}-Fc vaccine was measured in a two-dose immunization and a three-dose heterologous immunization. The dynamic response of the IgG and neutralizing antibody were monitored for 28 weeks. The IFN-γ-producing T cell response was also detected. Through all these experiments, we demonstrated the recombinant RBD-Fc vaccine could induce effective humoral and cellular immune response, which might be a candidate for the heterologous vaccination.

Materials and methods

Ethics statements

The experiments related to live SARS-CoV-2 viruses were approved by the Ethics Committee of the Zhejiang Provincial Center for Disease Control and Prevention (ZJCDC), and performed in the biosafety level 3 (BSL-3) lab of ZJCDC (2020–025, approval date 24 August 2020). The Animal experiments were approved by Animal Ethical and Welfare Committee of Zhejiang Chinese Medical University (IACUC-20211115–24, approval date 15 November 2021).

Virus and cell lines

The live SARS-CoV-2 virus strain (prototype) was isolated from the sputum of a male Covid-19 patient as mentioned previously [13]. Vero E6 cells and human embryonic kidney cells (HEK293T) were obtained from the National Collection of Authenticated Cell Cultures. Dulbecco's Modified Eagle's Medium (Gibco, MA, USA) was used to culture HEK293T, and Minimal Essential Medium (Gibco, MA, USA) was used to culture Vero E6 cells with supplementation of 10 % heat-inactivated fetal bovine serum (FBS, Gibco, MA, USA), 1 % penicillin–streptomycin (Solarbio, Beijing, China) at 37 °C, 5 % CO_2. The 50 % tissue culture infective dose ($TCID_{50}$) of the virus in Vero cells was calculated by the Reed and Muench method [13].

Gene cloning, expression and purification of recombinant proteins

Firstly, we introduced mutations (L452R, T478K) in the prototype RBD gene (NCBI Reference Sequence: YP_009724390.1) by PCR to obtain the RBD_{delta} gene. Next, we assembled the RBD_{delta} gene with the human Fc gene by overlapping PCR and ligated it into the pLVX vector. The RBD_{delta}-pLVX recombinant vector was verified by sequencing. Finally, the RBD_{delta}-pLVX recombinant vector was transferred into HEK-293 T cells by PEI (Polysciences, Niles, Illinois, USA) to express RBD_{delta}-Fc, which was then purified by protein A affinity column (GE Healthcare, Shanghai, China). The purified RBD_{delta}-Fc was verified by SDA-PAGE and fractionated for freezing at −80 ℃. The RBD-Fc (prototype) was prepared as mentioned previously [13].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

In short, 10 μg of purified RBD_{delta}-Fc containing 4 μL loading buffer and 1 % β-mercaptoethanol (β-ME) was heated in a 95 °C metal bath for about 10–15 min, which was then analyzed by SDS-PAGE. A constant voltage of 90 V for the concentrated gel was used, and an increased voltage to 120 V was used for the separation gel. The protein was stained with Coomassie Brilliant Blue and decolored with a bleach solution until the strip was clearly visible.

Flow cytometry analysis for Vero E6 cell binding ability

About 1×10^6 angiotensin converting enzyme-2 (ACE2)-positive 293 T cells was collected and then incubated them with purified RBD_{delta}-Fc (10 μg/mL) at 4 °C for 30 min. After phosphate buffered saline (PBS) washing for three times, the cells were incubated with 200 μL of FITC-labeled anti-human Fc secondary antibody (1:200 diluted in PBS) at 4 °C for 30 min, and then mean FITC fluorescence intensity on cell surface was detected using flow cytometer after washing 3 times with PBS.

Mouse experiments

Firstly, eight-week-old, female BALB/c mice were randomly assigned into 5 groups, and each group had 5 mice. High (8 μg) and low (2 μg) doses of RBD_{delta} (Sino Biological Company, China), and RBD_{delta}-Fc proteins, which were consistent with our previous work [13], were mixed with 0.5 mg/ml aluminum hydroxide adjuvant each were used to immunize mice intramuscularly. The immunizations were conducted on days 0 and 7. The sera and spleens were collected as the schedule below (Fig. 2A). PBS with the aluminum hydroxide at a concentration of 0.5 mg/ml was used as the control.

In the sequential immunization experiment, six groups of eight-week-old, female BALB/c mice were used, and each group contained 5 mice. Mice were immunized with two doses of inactivated SARS-CoV-2 vaccine on day 0 and 7. Then, on day 28, mice were boosted with inactivated PBS, inactivated SARS-CoV-2 vaccine, RBD (8 μg), RBD-Fc (8 μg) or RBD_{delta}-Fc (8 μg), individually. The sera and spleens were collected as the schedule below (Fig. 4A). The inactivated SARS-CoV-2 vaccine was prepared as mentioned previously [14].

Enzyme-linked immunosorbent assay (ELISA)

In short, the ELISA plates were coated with 50 ng/ml RBD (Sino Biological Company, China) per well at 4 °C overnight. Plates were blocked with 10 % FBS in PBS for 1 h at 37 °C, and 2-fold serially diluted sera were added to the ELISA plates for incubation for another hour. After washing 5 times, plates were incubated with rabbit anti-mouse IgG-HRP antibody at a dilution of 1:20000 (Abcam, Cambridge, UK) at 37 ℃ for 1 h. The color development reaction was carried out by adding TMB substrates (Solarbio, Beijing, China), and were stopped with 2 M H_2SO_4. Absorbance at 450 nm was read using a microplate reader, and an optical density at 450 nm (OD_{450}) value greater than 2.1-fold of the background value was regarded as positive.

Neutralization assay

The sera from immunized mice were incubated at 56 °C for 0.5 h for inactivation. Serially 2-fold diluted sera were mixed with the same volume of wild type SARS-CoV-2 virus suspension containing 100

$TCID_{50}$ at 37 °C for 1 h. Then, the virus-serum mixtures were transferred to 96-well plates seeded with confluence Vero E6 cells and incubated at 37 °C for another 3 days. After that, the cytopathic effect (CPE) of each well were observed, and the neutralization titers were calculated as the reciprocal of the highest serum dilution that can protect 50 % of cells from infection.

Enzyme-linked immunospot assay (ELISPOT)

The ELISPOT assays were performed using the mouse IL-4 ELISPOT kit and IFN-γ ELISPOT kit (BD Biosciences, USA). Twenty-eight weeks after the vaccination, mice from the two-dose immunization and the sequential immunization groups were all euthanized. Spleens were collected and the splenocytes were isolated by the cell strainers. Splenocytes (2×10^5 cells/well) were cultured in antibody pre-coated ELISPOT plates for the IL-4 or IFN-γ detection according to the kit's instructions. SARS-CoV-2 RBD (2 μg/well) was used as the stimulant in the assay. Spot-forming cells (SFCs) were imaged by a ChemiDoc XRS + imaging system (Bio-Rad, CA, USA), and enumerated by the Quantity One software.

Detection of cytokines

Splenocytes were obtained as mentioned in the ELISPOT section and seeded in a 96-well plate at a density of 2×10^5 cells per well. After stimulation with or without SARS-CoV-2 RBDdelta (2 μg/well) at 37 °C in 5 % CO_2 for 16 h, the supernatants of each well were collected and the concentrations of secreted IL-2, IL-6 and IL-10 were detected by ELISA (BD Biosciences, NJ, USA) kits.

Statistical analysis

All data were analyzed with GraphPad Prism 8.0. The Student's *t*-test was performed, and $p < 0.05$ between two groups considered as statistically significant.

Results

Construction and purification of SARS-CoV-2 RBD_{delta}-Fc recombinant proteins

We have prepared Fc-fused RBD prototype protein (RBD-Fc) in the previous study [13], and here we prepared Fc-fused RBD Delta variant protein (RBD_{delta}-Fc) using the same methods. Briefly, we coupled the RBD (spanning from 330 to 583 of the spike protein of the SARS-CoV-2) of the Delta variant with the human Fc fragment and used G4S as a linker sequence to connect the two fragments (Fig. 1A). The RBD_{delta}-Fc recombinant protein was purified by Protein A purification column (Fig. 1B), and then analyzed by SDS-PAGE (Fig. 1C). The theoretical molecular mass of reduced RBD_{delta}-Fc is about 52 kDa, but we found a protein band near 25 kDa, which was verified by mass spectrometry to be degradation products of RBD_{delta}-Fc. (Supplementary Table. S1). To further confirm whether RBD_{delta}-Fc formed the correct conformation, we evaluated its affinity for ACE2 using flow cytometry in Vero E6 cells (Fig. 1D). Compared with the control group, the fluorescence of the incubated RBD_{delta}-Fc recombinant protein group was shifted significantly, indicating that the purified protein had obvious affinity with the ACE2 receptor.

The immunogenicity of recombinant RBD_{delta}-Fc vaccine

To evaluate the immunogenicity of recombinant RBD_{delta}-Fc vaccine, BALB/c mice received two injections 7 days apart of RBD and RBD-Fc at different dose levels. Mice's sera were collected as the schedule (Fig. 2A), and the spleens were collected at week 28 after euthanizing. As Fig. 2B showed, one week after booster immunization (week 2), the seroconversion rate of IgG was 100 % in all groups. Over the next 26 weeks, the IgG antibody titers of the groups immunized with the RBD_{delta} vaccine remained relatively stable. However, in the RBD_{delta}-Fc groups, the IgG antibody levels at 6 weeks post vaccination were significantly higher than those at 2 weeks post vaccination. The highest

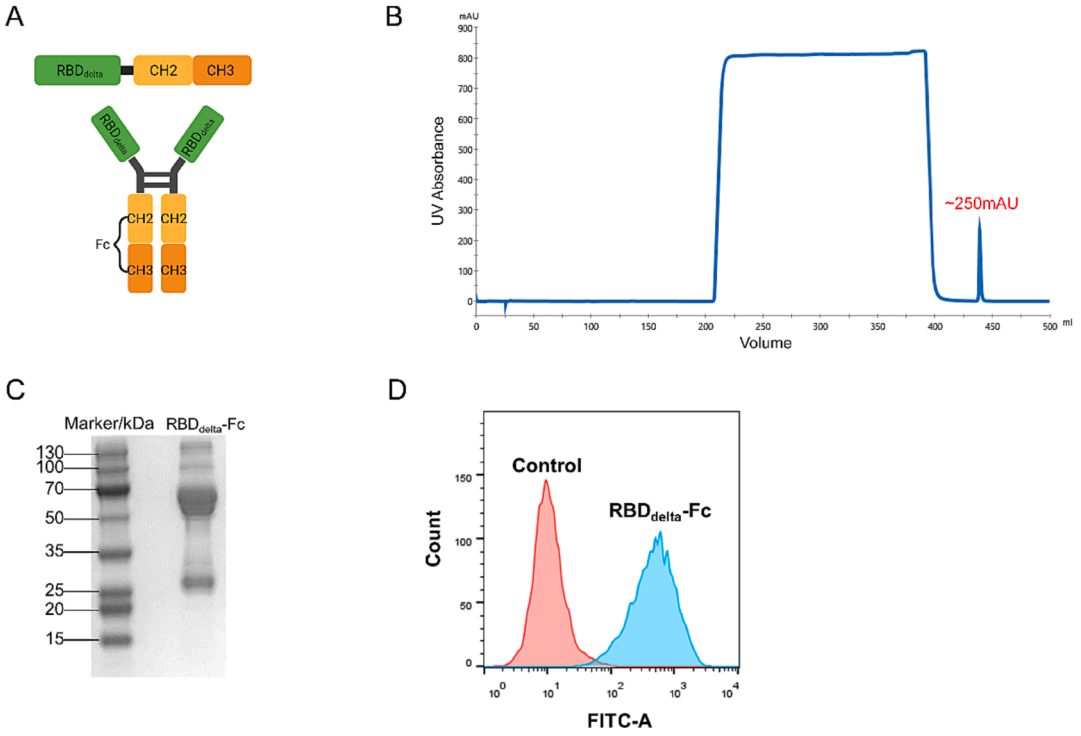

Fig. 1. Construction and purification of the RBD_{delta}-Fc recombinant protein. (A) Structure diagram of the RBD_{delta}-Fc recombinant protein. (B) Result of Protein A purification. The eluting peak was about 250 mAU. (C) SDS-PAGE analysis of purified RBD_{delta}-Fc under denaturing condition. The molecular weight of RBD_{delta}-Fc was about 52 kDa. (D) Flow cytometric evaluation of the affinity of RBD_{delta}-Fc for ACE2. Control: claudin-18.2 with Fc fragment.

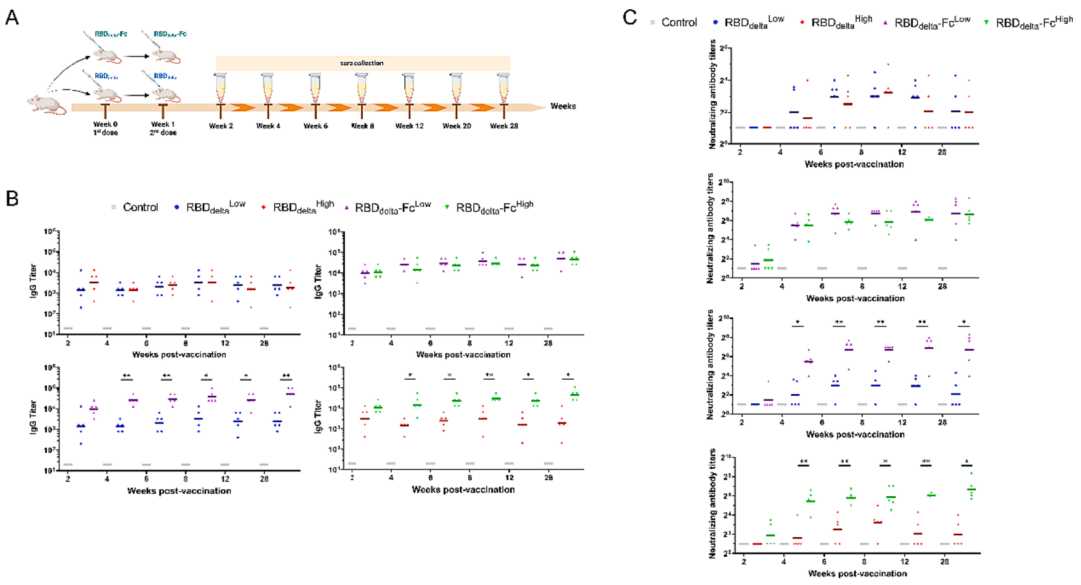

Fig. 2. The immunogenicity of RBD_{delta} and RBD_{delta}-Fc vaccines. (A) Timeline of the immune process and sera collection. The mice were divided into 5 groups, and each group was injected respectively with low-dose of RBD_{delta}, high-dose of RBD_{delta}, low-dose of RBD_{delta}-Fc, or high-dose of RBD_{delta}-Fc proteins, at week 0 and week 1. PBS with the aluminum hydroxide at a concentration of 0.5 mg/ml was used as the control. Mice sera was collected for the evaluation of (B) IgG titers and (C) Neutralizing antibody titers at week 2, 4, 6, 8, 12 and 28. Spleens were collected after euthanizing. * $p \leq 0.05$, ** $p \leq 0.01$.

geometric mean titers (GMTs) of IgG antibodies in the RBD_{delta}^{Low}, RBD_{delta}^{High}, RBD_{delta}-Fc^{Low} and RBD_{delta}-Fc^{High} groups were 4222, 3200, 51,200 and 77604, respectively. There was no significant difference in IgG titers between the high and low dose groups. But the IgG antibody levels in the RBD_{delta}-Fc groups were significantly higher than those in the RBD_{delta} groups, suggesting that RBD_{delta}-Fc has a superior ability to induce anti-SARS-CoV-2 humoral immunity compared with RBD_{delta}.

Neutralizing antibodies were detectable in the RBD-Fc groups at 1 week after booster immunization, whereas they were not detectable in the RBD groups until 3 weeks post booster immunization (Fig. 2C). Both in the RBD_{delta}^{Low} and RBD_{delta}^{High} groups, the neutralizing antibody titers (NATs) peaked at week 8, and the seroconversion rates were 80 %. At week 28, no NATs could be measured in 60 % samples of the RBD_{delta} groups at week 28. Differently, the GMTs of RBD_{delta}-Fc^{Low} and RBD_{delta}-Fc^{High} groups exhibited a significant increase at 4 weeks post vaccination, with a 100 % seroconversion rates. More importantly, the NATs of the two RBD_{delta}-Fc groups continued to increase slightly in the following 20 weeks, and remained at a high level at week 28. The highest GMTs of neutralizing antibody in the RBD_{delta}^{Low}, RBD_{delta}^{High}, RBD_{delta}-Fc^{Low} and RBD_{delta}-Fc^{High} groups were 9, 11, 139 and 101, respectively. The safety of our recombinant RBD_{delta}-Fc vaccine was demonstrated by the fact that we observed the lungs and livers of the mice by sectioning after two dose experiments and found no significant substantial damage (Supplementary Fig. S1, S2). These results suggested that the subunit vaccine based on RBD_{delta}-Fc fusion proteins had superior immunogenicity in mice than that of RBD_{delta} proteins.

The cellular immune response of the recombinant RBD_{delta}-Fc vaccine

To further evaluate the cellular immune response of recombinant RBD-Fc vaccine, the spleens of all immunized mice were collected at the end of the immunization process (week 28) and separated into individual splenocyte suspensions, which were stimulated for 16 h with or without SARS-CoV-2 RBD. The secretion amounts of IFN-γ and IL-4, which were detected by ELISPOT, had no significant difference between the RBD and RBD-Fc groups (Fig. 3B). The secretion levels of IFN-γ and IL-4 in the RBD_{delta} and RBD_{delta}-Fc groups were all significantly higher than those in the control group. As shown in Fig. 3C-3E, the concentrations of IL-2, IL-6, and IL-10 in the culture medium of splenocytes were also measured by ELISA. The concentrations of IL-6 in the RBD_{delta} groups were lower than those in the RBD_{delta}-Fc groups. The

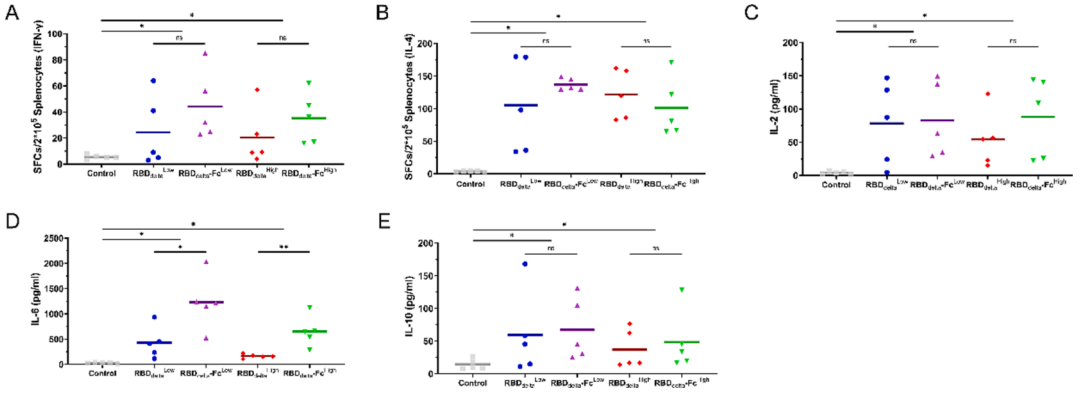

Fig. 3. Cellular immune response of the recombinant RBD_{delta} and RBD_{delta}-Fc vaccines. The concentrations of IFN-γ (A) and IL-4 (B) secreted by splenocytes was measured by ELISPOT. The release of IL-2 (C), IL-6 (D) and IL-10 (E) in the culture medium of splenocytes was determined by ELISA. * $p \leq 0.05$, ** $p \leq 0.01$, and ns ($p > 0.05$) indicated that the difference is not significant.

values of IL-6 in the RBD_{delta}^{Low}, RBD_{delta}^{High}, RBD_{delta}-Fc^{Low}, and RBD_{delta}-Fc^{High} groups were 430, 163, 1,231, and 653 pg/mL, respectively (Fig. 3D). As for the concentrations of IL-2 and IL-10, no significant difference was found between RBD_{delta} and RBD_{delta}-Fc groups. Besides, the concentrations of IL-2, IL-6, and IL-10 in RBD_{delta} and RBD_{delta}-Fc groups were all higher than those in the control groups. The results indicated that both the RBD_{delta} and RBD_{delta}-Fc vaccines could trigger a good cellular immunity in mice, and the RBD_{delta} fusion with Fc is also enhanced its ability to induce IL-6 secretion.

Heterologous boosting with RBD-Fc vaccine can effectively improve the immune response

To verify the effectiveness of heterologous immunization, BALB/c mice were divided into 6 groups (5 mice each) and vaccinated with PBS, two doses of inactivated vaccine, three doses of inactivated vaccine, two doses of inactivated vaccine plus RBD booster, two doses of inactivated vaccine plus RBD-Fc booster, or two doses of inactivated vaccine plus RBD_{delta}-Fc booster, respectively. The sequential immune process was shown in the schedule (Fig. 4A). The sera were collected as the schedule and the spleens were collected at week 28 after euthanizing. As Fig. 4B showed, 1 week after two-dose immunization (week 2), the

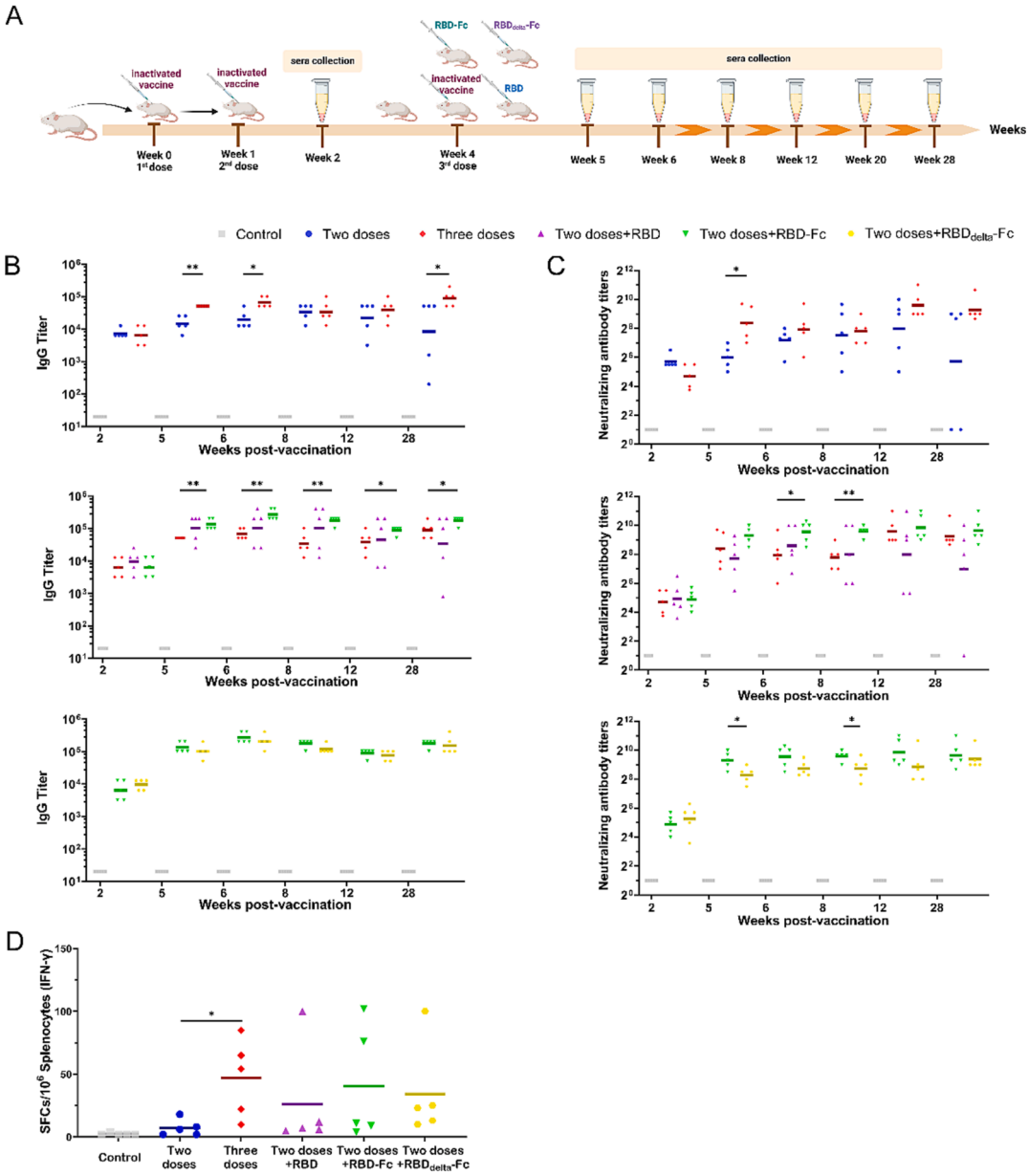

Fig. 4. Heterologous Boosting with RBD-Fc vaccine effectively improve the immune response. (A) Timeline of the immune process and sera collection. Mice were divided into 6 groups and immunized with two doses of inactivated SARS-CoV-2 vaccine at week 0 and week 1. Then, at week 4, mice were boosted with inactivated SARS-CoV-2 vaccine, RBD (8 μg), RBD-Fc (8 μg), or RBD_{delta}-Fc, respectively. PBS with the aluminum hydroxide at a concentration of 0.5 mg/ml was used as the control. The sera were collected for the evaluation of (B) IgG titers and (C) Neutralizing antibody titers at week 2, 5, 6, 8, 12 and 28. Spleens were collected after euthanizing. * $p \leq 0.05$, ** $p \leq 0.01$. (D) Cellular immune response in the homologous and heterologous immunization. Mice were immunized with PBS, two doses of inactivated vaccine, three doses of inactivated vaccine, two doses of inactivated vaccine + RBD (8 μg), two doses of inactivated vaccine + RBD-Fc (8 μg), or two doses of inactivated vaccine + RBD_{delta}-Fc (8 μg), respectively. PBS with the aluminum hydroxide at a concentration of 0.5 mg/ml was used as the control. The concentrations of IFN-γ secreted by splenocytes were measured by ELISPOT. * $p \leq 0.05$, ns ($p > 0.05$) indicated that the difference is not significant.

seroconversion rate of IgG was 100 % in all groups. One week after a third vaccination (week 5), the IgG titers were increased by an order of magnitude in all boosted groups, and remained at a relatively high level for the next 23 weeks. However, in the two doses group, the IgG titers peaked at week 8 and decreased gradually. The highest GMTs of IgG antibodies in the two doses, three doses, two doses + RBD, two doses + RBD-Fc, and two doses + RBD_{delta}-Fc groups were 33779, 89144, 102400, 270235, and 204800, respectively. The IgG titers in the two doses + RBD-Fc (prototype) group were significantly higher than those in the three doses group. However, no statistically significant difference in IgG titers was found between two doses + RBD-Fc and two doses + RBD_{delta}-Fc groups.

We further measured the neutralizing antibody titers of these six groups (Fig. 4C). One week after a two-dose immunization, the neutralizing antibody titers could be detected in all groups. One week after the second booster dose, the neutralizing antibody titers increased substantially. The neutralizing antibody titers in the two doses group followed a similar trend to its IgG antibody titers, which peaked at week 12 and then decreased. At week 28, 2 samples in the two doses group had no detectable NATs, while all the 5 samples in the three doses group maintained high neutralizing antibody levels. As for the sequential immunization groups, the neutralizing antibody level in the two doses + RBD-Fc group was higher than that in the three doses group at 6 and 8 weeks post vaccination. The neutralizing antibody level in the two doses + RBD-Fc group was also higher than that in the two doses + RBD_{delta}-Fc group at 5 and 8 weeks post vaccination. However, at 28 weeks post vaccination, there is no significant difference in the neutralizing antibody level between the two doses + RBD-Fc group and the two doses + RBD_{delta}-Fc group. The highest GMTs of neutralizing antibody in the two doses, three doses, two doses + RBD, two doses + RBD-Fc, and two doses + RBD_{delta}-Fc groups were 255, 776, 388, 926, and 672, respectively. These results showed that a third vaccination can effectively enhance the immune response, and the heterologous sequential immunization with RBD-Fc instead of inactivated vaccine as the booster vaccine could trigger a quicker immune response. As with the two-dose immunization experiments, we observed lung and liver slices from mice after consecutive experiments, and no substantial damage was detected again, demonstrating the safety of our recombinant subunit RBD_{delta}-Fc vaccine (Supplementary Fig. S1, S2).

The cellular immunity detection was performed as the above part mentioned (Results 'The cellular immune response of the recombinant RBDdelta-Fc vaccine'). Splenocytes were collected and stimulated with or without SARS-CoV-2 RBD for 16 h. The secretion levels of IFN-γ in the three doses group was significantly higher than that in the two doses group (Fig. 4D). And the secretion levels of IFN-γ in the RBD-Fc groups, two doses + RBD, two doses + RBD-Fc, and two doses + RBD_{delta}-Fc groups were not significantly different compared with that in the three doses group. The results showed that a third immunization with either the RBD-Fc vaccine or the inactivated vaccine could trigger a good cellular immune response.

Discussion

In this study, we first constructed a recombinant RBD-Fc subunit vaccine based on the RBD sequence of Delta variant. This recombinant RBD_{delta}-Fc vaccine could induce higher IgG and neutralizing antibody titers than the RBD_{delta} vaccine. The RBD_{delta}-Fc vaccine, as well as the RBD-Fc vaccine, was applied to the heterologous booster immunization. Both these two recombinant RBD-Fc vaccines could trigger good humoral and cellular immune response, and the neutralizing antibodies titers could be maintained at a high level for at least 28 weeks. Besides, heterologous boosting with the RBD-Fc vaccine after two doses of inactivated vaccines could induce a faster production of neutralizing antibodies than homologous booster immunization.

We observed that IgG titers and neutralizing antibody titers were about 10 times higher in the RBD_{delta}-Fc immunized group than in the RBD_{delta} immunized group. In addition, the results of cytokine expression analysis showed that the Fc fusion enhanced the ability of the RBD vaccine to stimulate cellular immunity. Fc is effective in prolonging the half-life of proteins, facilitating protein purification, and increasing the immunogenicity of target proteins, which may account for its improved immuno-efficacy of RBD [15]. More importantly, Fc might also promote vaccine uptake by antigen-presenting cells (APCs) [16]. It is well known that the processing of the vaccines after uptake by APCs is a prerequisite for their activity, and the expression of multiple Fc receptors on the surface of specialized APCs such as macrophages and dendritic cells may enhance the uptake of the RBD_{delta}-Fc vaccine [17], thereby boosting the RBD-stimulated immune capacity. So, compared with the RBD protein, the RBD-Fc fusion protein might have advantages to be used as a vaccine.

Heterologous booster strategy has been available for more than a decade and have been shown to be effective in previous vaccine development against emerging viruses such as HIV-1 [18] and influenza [19]. In the context of widespread vaccination with inactivated vaccines in China, enhancing immunity through heterologous boosting is a strategy to look forward to [20,21]. Zuo et al. purposed that heterologous vaccination strategy is beneficial for eliciting a broader immune response [22]. Ai et al. showed by single-cell analysis that the heterogeneous booster evoked functional B-cell responses, induced reactivation and activation of naïve B-cells, as well as activation and differentiation of plasma cells (IgA and IgG), which may account for the significant increase in humoral response after booster immunization [23]. In this study, after receiving two-dose inactivated vaccines, heterologous boosting with RBD-Fc vaccine could induce a higher level of IgG antibody and neutralizing antibody than homologous boosting at 6 and 8 weeks post vaccination, indicating a quicker humoral immune response than the homologous boosting. Besides, the GMT of IgG antibody at 28 weeks post-vaccination in the two doses plus RBD-Fc group was also higher than that in the three doses group. Interestingly, heterologous immunization with RBD-Fc vaccine trigger a higher level of neutralizing antibody than that with the RBD_{delta}-Fc vaccine at 5 and 8 weeks post vaccination, while the level of IgG antibody in both groups were not significantly different. Since there were two key mutations (L452R and T478K) in RBD sequence of the delta variant, the immunogenicity of the RBD_{delta}-Fc vaccine might be not the same as the RBD-Fc vaccine.

We also examined the secretion levels of IL-2, IL-4, IL-6, IL-10, and IFN-γ in mouse spleen cells in the two-dose immunological experiment, and IFN-γ in mouse spleen cells in the sequential immunization experiment, which mainly responded to the stimulatory effect of the vaccines on cellular immunity [24–26]. The results show that RBD-Fc vaccines can induce a cellular immune response, which is an indispensable component of the immune system to fight the SARS-CoV-2. It is worth noting that cellular immunity receives a strict limitation of MHC I [27,28], and human MHC I is quite different from mouse MHC I, whether RBD-Fc vaccine can elicit effective cellular immunity in humans remains to be investigated.

SARS-CoV-2 is an extremely fast-mutating virus [29] and a new offshoot of the Omicron variant EG. 5 has emerged currently, which has become the most prevalent variant in the U.S. now [30]. However, we should not only focus on the dominant variant recently, but also pay attention to the previously known variants especially the Delta variant. A recombinant strain named "Deltamicron", with an Omicron 21 K/BA.1 variant in a Delta 21 J/AY.4 lineage backbone, was declared to be found in south France [31]. It raised the concern about "supervirus". In this study, we first constructed a RBD_{delta}-Fc vaccine based on the Delta variant RBD sequence. It could induce a cellular immune response, and a high level of IgG antibodies and neutralizing antibodies, which could last for at least 28 weeks. The RBD_{delta}-Fc, as well as the RBD-Fc prepared in our previous study, could trigger a long-lasting immune effect by the heterologous boosting immunity, and the RBD-Fc induced a quicker humoral immune response than the homologous immunization.

This recombinant RBD fusion protein vaccine exhibited good immunogenicity in mice and might be a useful strategy for vaccine design in the future.

CRediT authorship contribution statement

Yisheng Sun: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Funding acquisition. **Qiaomin Li:** Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Yuanyuan Luo:** Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Hanping Zhu:** Investigation, Resources. **Fang Xu:** Investigation. **Hangjing Lu:** Investigation, Resources. **Pingping Yao:** Resources. **Zhen Wang:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Wenbin Zhao:** Conceptualization, Methodology, Writing – review & editing. **Zhan Zhou:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

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.

Data availability

Data will be made available on request.

Acknowledgments

We thank Yingying Huang in the Core Facilities, Zhejiang University School of Medicine, for the technical support provided. This work was supported by the Huadong Medicine Joint Funds of the Zhejiang Provincial Natural Science Foundation of China (LHDMZ22H300002), and the Basic Public Welfare Research Project of Zhejiang Province (LGF22C080002, LDT23H19013H19).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jvacx.2024.100444.

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