REPORT

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Precise modification of the surface charge of antigen enhances vaccine immunogenicity

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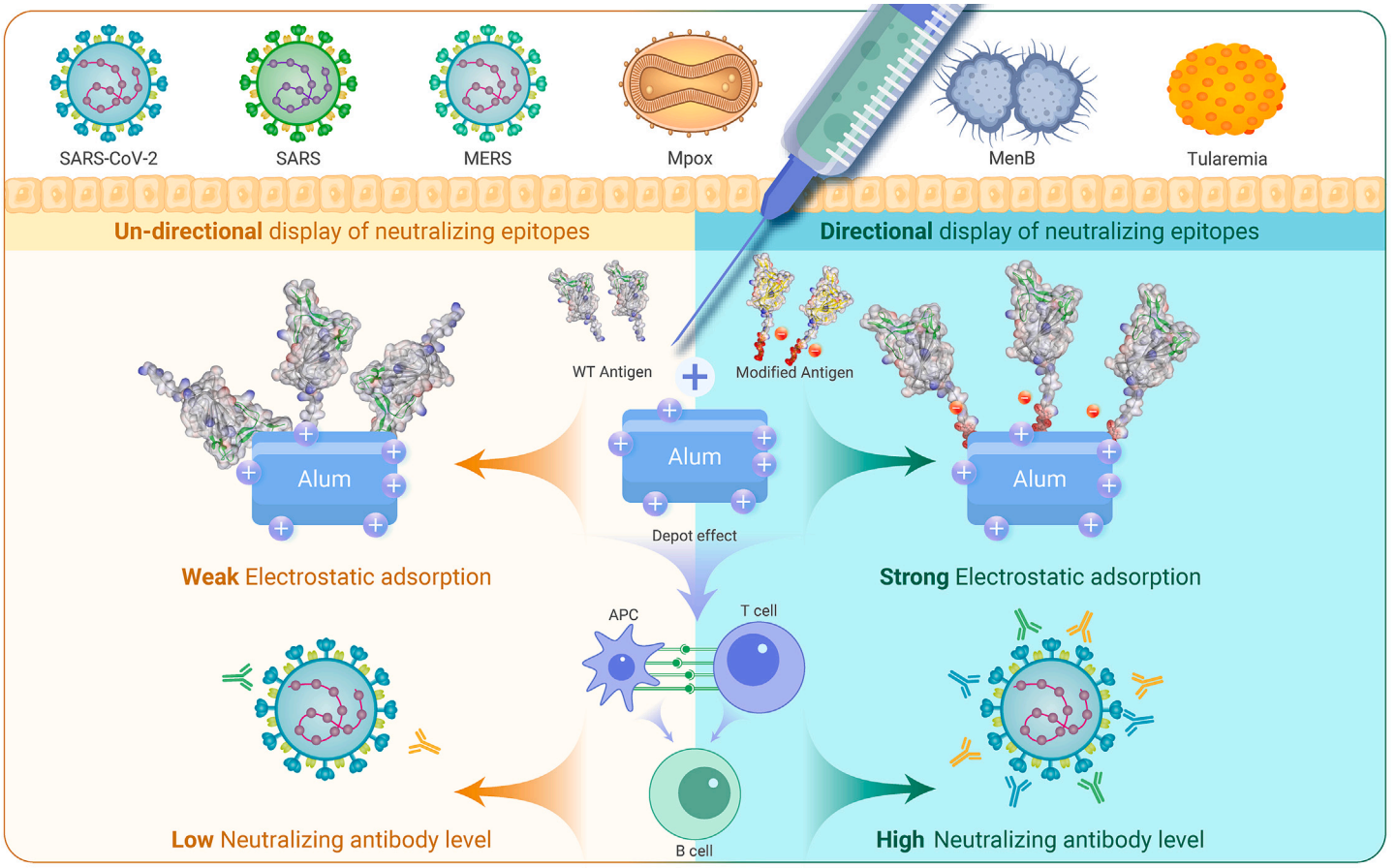
GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Charge modification of antigen significantly enhances vaccine immunogenicity.
- The adsorption of modified antigen to the alum adjuvant is dramatically improved.
- The neutralizing epitopes of modified antigen are directionally displayed.
- This novel approach can be applied to optimize traditional alum-adjuvanted vaccines.

REPORT

Precise modification of the surface charge of antigen enhances vaccine immunogenicity

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Aluminum (alum) adjuvant is the most extensively used protein subunit vaccine adjuvant, and its effectiveness and safety have been widely recognized. The surface charge of the antigen determines its electrostatic adsorption to alum adjuvant, which directly affects the immune efficacy of the protein vaccine. In our study, we precisely modified its surface charge by inserting charged amino acids into the flexible region of the SARS-CoV-2 receptor-binding domain (RBD), achieving electrostatic adsorption and a site-specific anchor between the immunogen and alum adjuvant. This innovative strategy extended the bioavailability of the RBD and directionally displayed the neutralizing epitopes, thereby significantly enhancing humoral and cellular immunity. Furthermore, the required dose of antigen and alum adjuvant was greatly reduced, which improved the safety and accessibility of the protein subunit vaccine. On this basis, the wide applicability of this novel strategy to a series of representative pathogen antigens such as SARS-RBD, MERS-RBD, Mpox-M1, MenB-fHbp, and Tularemia-Tul4 was further confirmed. Charge modification of antigens provides a straightforward approach for antigenicity optimization of alum-adjuvanted vaccines, which has great potential to be adopted as a global defense against infectious diseases.

INTRODUCTION

Vaccines are the surest means in the fight against infectious diseases.[1] The recently emergent coronavirus pandemic has spread at an unprecedented scale and speed, creating new challenges for vaccine development. Protein subunit vaccines may play an essential role in fighting against pandemics due to their safety and effectiveness; moreover, from a practical perspective, protein vaccines can be produced rapidly and do not require ultra-low temperature storage.[2] However, the efficacy of protein vaccines is highly related to the conjunction between antigen and adjuvant.[3,4] Currently, the majority of approved subunit vaccines use aluminum as the main adjuvant component due to its minimal safety reactogenicity and economical manufacturing cost.[4,5]

Adsorption of antigen to the alum adjuvant through hydrophobic, electrostatic, or ligand exchange may directly contribute to the immune-enhancing efficacy of the antigen-adjuvant complex.[5,6] Changing the particle size distribution of the alum adjuvant itself, the ratio of adjuvant-antigen, and the composition of the buffer could regulate the degree of adsorption, which in turn affects the efficacy of alum-adjuvanted vaccines.[7–9] Recently, it has been reported that chemical modification of proteins with phosphonate linkers or phosphorylated serine (pSer) significantly enhances the binding anchorage between antigen and alum adjuvant via a ligand exchange mechanism.[10–12]

Herein, we propose an innovative strategy that dramatically improves the adsorption of antigens to the alum adjuvant. By inserting negatively charged amino acid fragments into the flexible region of the SARS-CoV-2 receptor-binding domain (RBD), we could precisely modify its surface charge, extend the bioavailability of the antigen, and directionally display the neutralizing epitopes and thus significantly enhance the humoral and cellular immunity. This strategy could also be applied to a series of representative pathogenic antigens such as SARS-RBD, MERS-RBD, Mpox-M1, MenB-fHbp, and Tularemia-Tul4. The precise charge modification of immunogen is expected to provide a simple and direct way to design novel protein subunit vaccines by enhancing antigen-adjuvant adsorption, thus driving a robust immune efficacy.

RESULTS

Charge-modified SARS-CoV-2 RBD induced a strong and durable immune response

RBD, an attractive candidate for the SARS-CoV-2 subunit vaccine with moderate immunogenicity, was formulated with alum hydroxide (AH) adjuvant in several COVID-19 vaccines.[13–15] However, we have noticed that both RBD (pI = 8.96) and AH adjuvant have a positive surface charge under physiological conditions, which may directly result in the weak electrostatic adsorption and limited efficacy of these vaccines. Herein, by inserting different amounts of negatively charged amino acids, namely aspartic acid (Asp), at the flexible region (C terminus) of RBD, we designed three different RBD charge mutants with a pI ranging from 8.56 to 6.40 (Figure 1A). All mutated immunogens, especially the RBD-9Asp, show high purity and a notably high yield and thermostability (150 mg/L) (Figures 1B and S1).

To assess the immunogenicity of the RBD mutants, we immunized BALB/c mice with the antigens combined with AH adjuvants and boost on day 14 (Figure 1C). Compared with wild-type RBD, all the mutated RBD could elicit a robust humoral immune response, with a significant increase in RBD-binding IgG titer following RBD-9Asp vaccination (Figure 1D). The addition of charges to the RBD-9Asp did not bring further improvement in immunogenicity (Figure S2). Similarly, the pseudovirus-neutralizing antibody (NAb) titer stimulated by RBD-9Asp:alum was nearly two orders of magnitude higher than that of the wild-type, which is consistent with the data for specific antibodies (Figure 1E). To investigate the rapid and long-term immune response, we performed an immune evaluation to monitor changes in serum antibodies. The remarkable seroconverting could be detected in mice vaccinated with RBD-9Asp on day 7 after the first dose. A high antibody level was maintained at nearly 10^4 even on day 240 after vaccination, significantly higher than what was observed in the wild-type RBD group (Figure 1F).

Populations with low immune potency, such as the elderly, have a high risk of severe COVID-19 symptoms and have been the focus of vaccination.[16] To confirm the applicability of RBD-9Asp to the elderly, aged C57BL/6 mice were prepared for immunization. As expected, the level of RBD-9Asp-elicited antibodies was reduced in the aged mouse model compared with that in young mice. However, the RBD-9Asp still drives a robust humoral immune response, with a titer 27-fold greater than that in the wild-type RBD group (Figure 1G).

Optimizing the antigen and adjuvant dosage of a subunit vaccine is crucial for inducing a strong immune response.[17] To determine the efficient dosage of antigen and adjuvant, we immunized BALB/c mice with 5, 1, or 0.2 μg of RBD and 50, 20, or 2 μg of alum. The antibody titer in RBD-9Asp was heightened with an increasing antigen dosage (Figure 1H). There was no significant difference in RBD-binding IgG between the 0.2 μg RBD-9Asp and 5 μg wild-type RBD. Moreover, the increased RBD-binding IgG titer can still be kept at an extremely low alum dosage (2 μg).

In light of the well-established propensity of alum adjuvant to favor Th2-type immune responses, we investigated the potential impact of the modified antigens on this bias. The modified antigens enhanced both IgG1 and IgG2a, but predominantly IgG1, indicating that immune response remained Th2 biased (Figure S3).

To assess the cellular immune potency, the spleens of mice were removed after 56 days of immunization, processed for splenocyte collection, and stimulated with the RBD peptides pool. Compared with the wild-type RBD, the cytokine secretion level of splenocytes after RBD-9Asp immunization was significantly

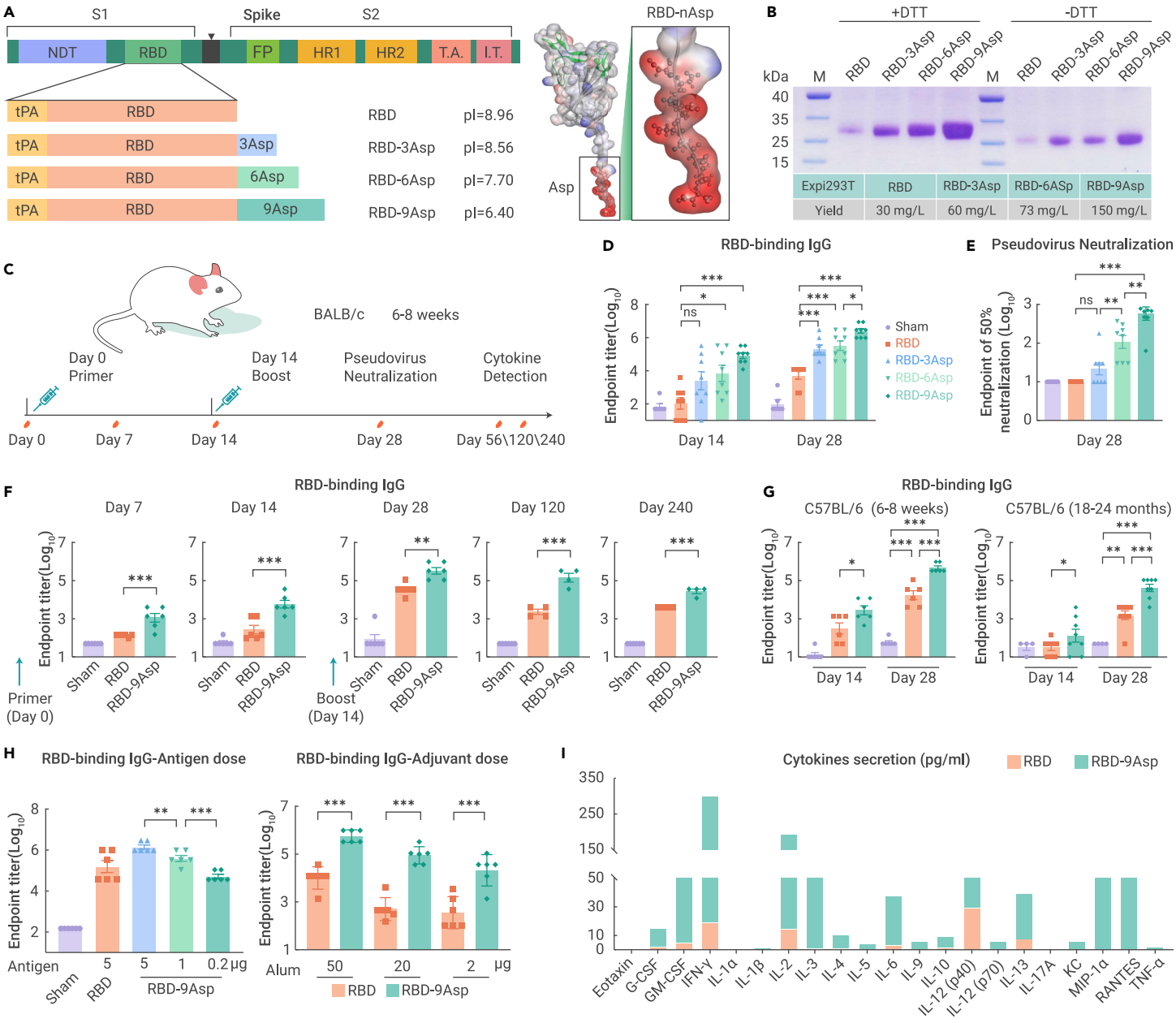

Figure 1. Immunogenicity of charge-modified RBD with alum adjuvant in mice (A) Schematic diagram of charge-modified RBD, which was achieved by inserting charged amino acids (Asp) into its C-flexible region, with blue (positive) and red (negative) representing the electrostatic potential regions. (B) The mutated protein was purified and verified by SDS-PAGE. (C) Schematic diagram of immunization and serum sample collection. (D and E) RBD-specific IgG titers and NAb titers were tested via an ELISA and a pseudovirus-based neutralization assay, respectively. (F) The rapid and long-term immune response. (G) The immune response in the aged mouse model. (H) Determination of the efficient dosage of antigen and adjuvant. (I) Cytokine secretion in mice splenocytes after stimulation with the RBD peptides pool antigen. The spleens of the mice were removed at 56 days after immunization and processed for splenocyte collection and stimulation. Data are expressed as the mean ± SEM. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to test differences between groups. p values <0.05 were considered statistically significant (*p < 0.05; **p < 0.01; ***p < 0.001).

upregulated (Figure 1I). In particular, the levels of cytokines such as IL-1β, INF-γ, IL-4, IL-6, and IL-10, associated with the Th1 and Th2 immune response, were elevated (Figure S4), which demonstrated that the RBD-9Asp could be a preeminent and durable immunogen in synergy with alum.

Charge-modified RBD bound to alum tightly by electrostatic adsorption

We hypothesized that charge-modified RBD and alum might form a complex structure by electrostatic adsorption, as shown in Figure 2A. The C-terminal electronegative RBD-9Asp could be "captured" by alum, with the directional display of receptor-binding sites, and the "up" conformation of RBD could be stabilized. To test this hypothesis, we first conducted antigen adsorption and release experiments *in vivo* and *in vitro* as described previously.[11,18] For the *in vitro* experiment, we mixed wild-type RBD or RBD-9Asp with alum in phosphate buffer (PB). The rate of antigen adsorption was calculated by detecting the proportion of protein in the supernatant after centrifugation. Then, 10% mouse serum was added to the buffer, and the release of antigen was detected after incubation. We showed that RBD was slowly adsorbed with alum in the PB solution and reached about 80% adsorption after 1 h (Figure 2B). RBD-9Asp, on the other hand, quickly achieved 100% adsorption within half an hour. In contrast, most of the RBD dissociated from the alum half an hour after adding serum, while RBD-9Asp maintained strong binding and only about 10% dissociated from alum within the same period.

For the *in vivo* experiment, we monitored the release of antigen-adjuvant mixtures in mice using an *in vivo* imaging system. We found that the sustained release of RBD-9Asp at the mouse injection site was more significant than that of RBD, as the fluorescence efficiency extinction time was more delayed (Figure 2C), which confirmed that the adsorption and release of protein antigens and adjuvants were optimized and improved after modification.

We then tested whether charge-modified RBD could engender stable binding to alum without disrupting key epitopes on the antigen. We used the immobilized

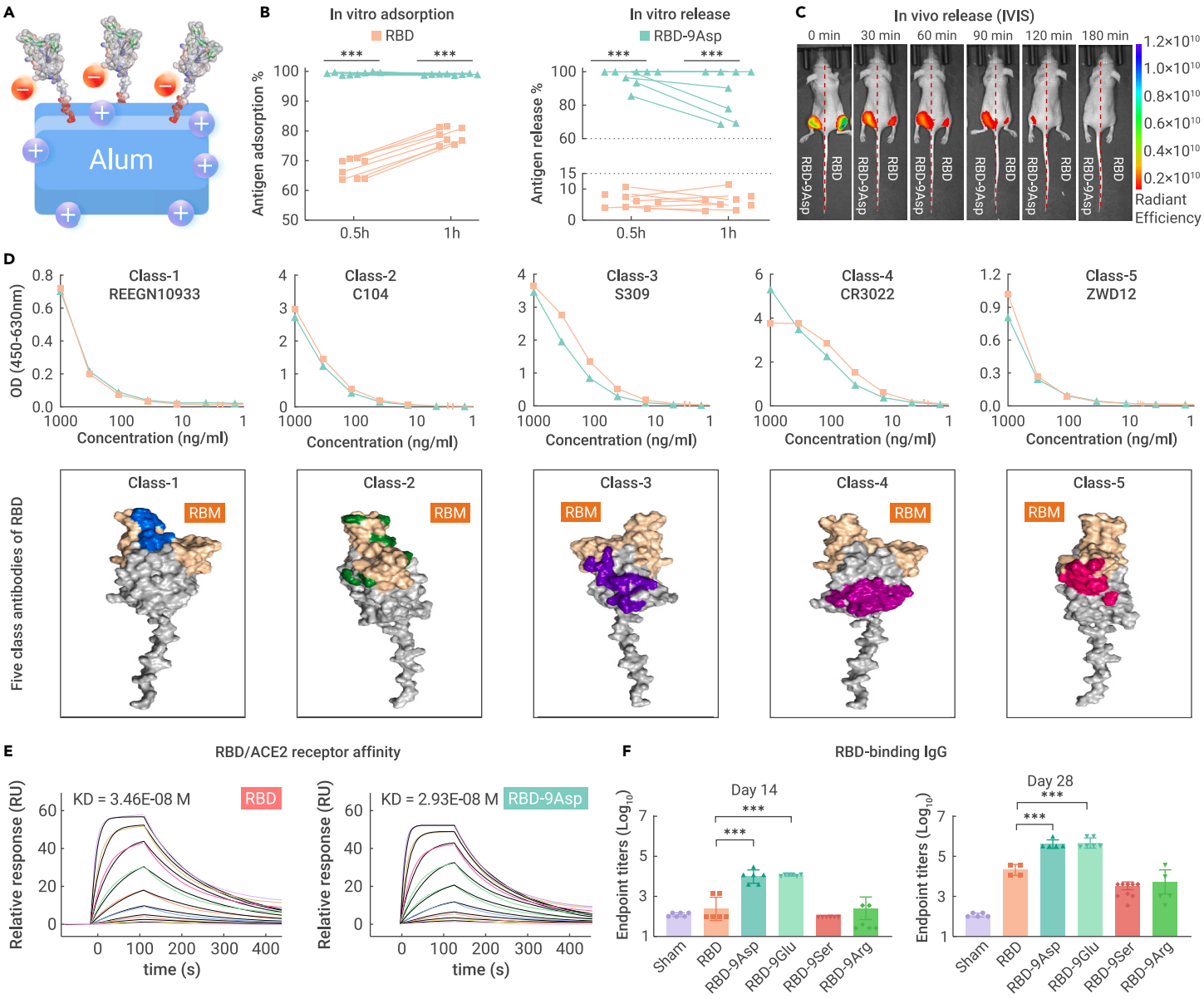

Figure 2. Charge modification enhances antigen-adjuvant adsorption (A) Charge modification for RBD promotes the strong electrostatic adsorption of the antigen on the surface of the alum adjuvant. (B–C) Adsorption and release detection of RBD/RBD-9Asp adjuvanted with AH *in vivo* and *in vitro*. (D) The binding profiles of monoclonal antibodies from five classes against RBD. (E) Affinity detection of RBD/RBD-9Asp and the receptor ACE2 by BIAcore. (F) ELISA-tested serum IgG response titers of day 14/28 to different mutated RBD. Data were expressed as the mean ± SEM. Unpaired t test (B) and one-way ANOVA followed by Tukey's post-hoc test (F) were used to test differences between groups. p values <0.05 were considered statistically significant (*p < 0.05; **p < 0.01; ***p < 0.001).

RBD/RBD-9Asp to capture monoclonal antibodies from five classes (including REEGN10933-Class1, C104-Class2, S309-Class3, CR3022-Class4, and ZWD12-Class5).[19–21] With an increased concentration of monoclonal antibodies, all showed similar binding to the RBD/RBD-9Asp, proving that the charge modification did not destroy the immunogenicity and structure from the unmodified RBD (Figure 2D). The BIAcore assay also revealed that the binding of RBD-9Asp to the hACE2 receptor was not disrupted but maintained a similar affinity to RBD (Figure 2E).

Finally, we considered inserting other exogenous amino acids at the flexible region (C terminus) of RBD, such as glutamic acid (Glu), arginine (Arg), and serine (Ser) (Figure S5A). After introducing another negatively charged amino acid (Glu) to the surface of the RBD (called RBD-9Glu), its immune potency was also improved when adjuvanted with AH (Figure 2F). In contrast, the positively charged amino acid Arg (called RBD-9Arg) and non-charged amino acid Ser (called RBD-9Ser) had no enhancing effect on the immune response. These observations further illustrate that the negatively charged modification may enhance the electrostatic adsorption of the antigen to the positively charged AH adjuvant.

Charge-modified RBD-Dimer ulteriorly induces broad-spectrum cross-neutralizing antibodies against subvariants

Since it has been reported that dimeric RBD antigens are more effective than monomers,[14,22,23] we examined the effect of charge modification on dimeric antigens. Based on the design of the RBD single-chain dimer, we then designed and expressed the RBD-9Asp-Dimer (Figure S6). RBD-9Asp-Dimer consist of two copies of RBD-9Asp tethered by their flexible N and C termini, without introducing an exogenous linker. Furthermore, according to the structure model predicted by AlphaFold2 and accuracy estimated by DeepUMQA3,[24,25] the RBD-9Asp-Dimer might display the neutralizing epitope of RBD properly while maintaining a strong negative charge on the opposite surface (Figures 3A and S7B).

To evaluate the immunogenicity of the RBD-9Asp-Dimer, we conducted a prime-boost immunization on BALB/c mice. Similarly, RBD-9Asp-Dimer and RBD-9Asp induced a significantly higher level of binding antibodies than the wild-type RBD (Figure 3B), consistent with the IFN-γ spot results (Figure 3D). In the live virus neutralization assay, the RBD-9Asp-Dimer could elicit additional NAbs based on RBD-9Asp (Figure 3C). Overall, we confirmed that the combination of charge modification and dimer protein

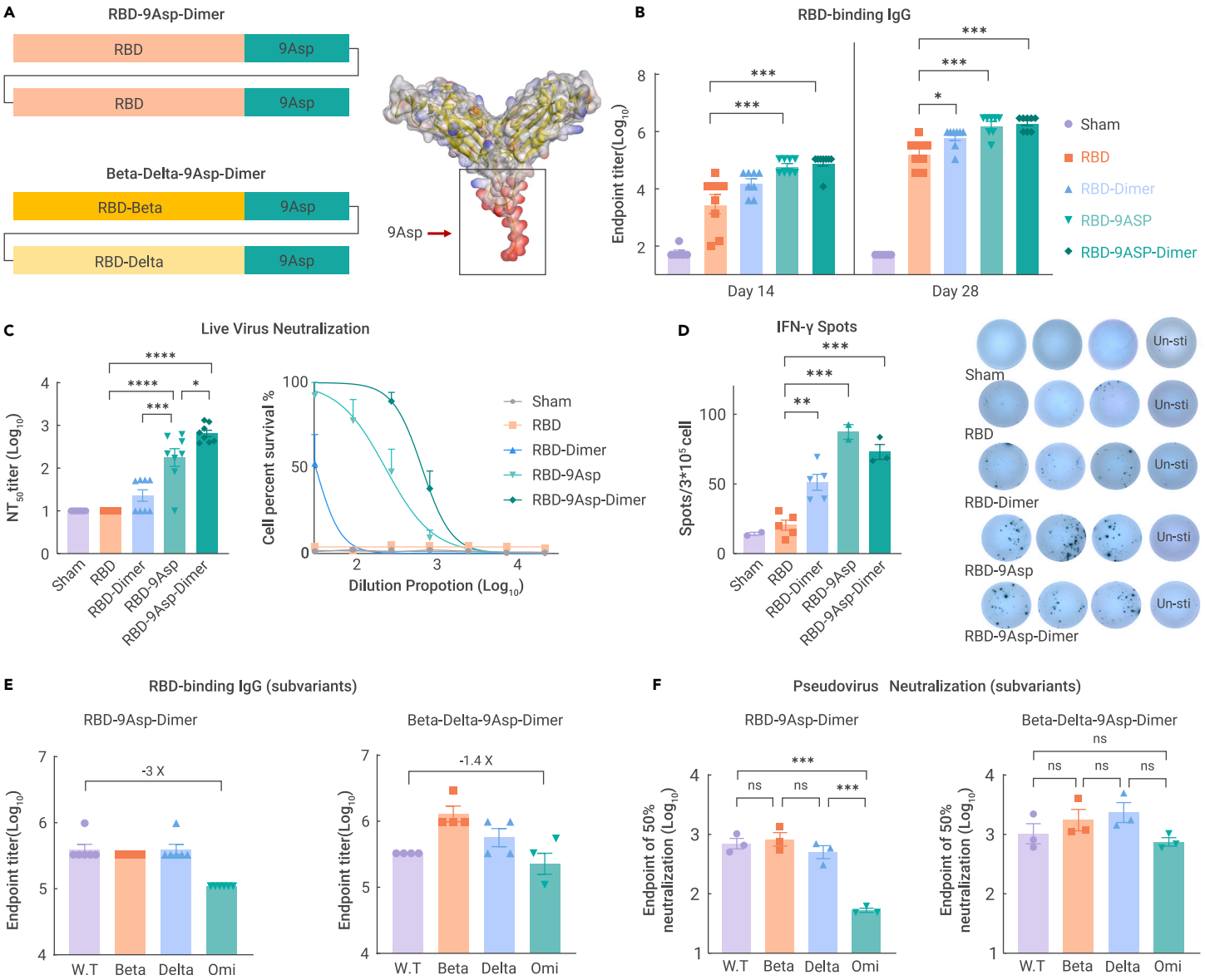

Figure 3. Immunogenicity of charge-modified RBD-Dimer with alum adjuvant in mice (A) Schematic diagram of charge-modified RBD-Dimer design and structure simulated by AlphaFold2. (B) ELISA-tested serum IgG response titers of day 14/28 to different mutated RBD. (C) The SARS-CoV-2 live virus NT50 was tested for serum collected on day 28; NAbs of serum against the live virus are shown as serial dilution curves. (D) IFN-γ secretion was detected by ELISpot cytokine. (E and F) The serum IgG response titer and the NAb titers at day 28 against a different variant of SARS-CoV-2 were measured. Data are expressed as the mean ± SEM. One-way ANOVA followed by Tukey's post-hoc test was used to test differences between groups. p values <0.05 were considered statistically significant (*p < 0.05; **p < 0.01; ***p < 0.001).

design could have an enhanced synergistic effect on the level of NAbs in RBD subunit vaccines.

Immune evasion still exists in the context of worldwide vaccination, especially because the mutation is concentrated on RBD, significantly reducing the existing COVID-19 vaccines' protective efficacy.[26,27] Here, to further improve the immunogenicity of RBD and expand the spread coverage of variants, we screened the variant of concern (VOC) subvariants and linked the RBD_{Beta}-9Asp and RBD_{Delta}-9Asp sequence as a dimer in end-to-end design, namely the Beta-Delta-9Asp-Dimer (Figure 3A).

We have noticed that after two shots of immunization, the IgG titers of the Beta-Delta-9Asp-Dimer only had a 1.4-fold decrease against Omicron BA.1, demonstrating the satisfactory coping ability of emergent mutant strains (Figures 3E and S8A). Furthermore, there was no significant difference between Beta, Delta, and Omicron BA.1 in the pseudovirus neutralization assay, further attesting that the design can stimulate broad-spectrum NAbs response (Figures 3F and S8B).

Charge modification strategy applies to a variety of pathogen antigens

To further confirm the applicability range of the charge modification strategy, we selected several antigens from various pathogens, including SARS-RBD, MERS-RBD, and Mpox-M1 from virus and MenB-fHbp and Tularemia-Tul4 from bacteria.[28–31] Their charge-modified antigens were prepared using a eukaryotic or prokaryotic expression system according to their properties, and immunogenicity was then assessed (Figures S4B and S4C). According to the structure model predicted by AlphaFold2, all charged fragments inserted are generally located at the flexible end of the protein structure. Consistent with prior results, RBD_{SARS}-9Asp, RBD_{MERS}-9Asp, $M1_{Mpox}$-9Asp, and Tularemia-Tul4-9Asp adjuvanted with AH maintained better immunogenicity after charge modification (Figures 4A–4D). This demonstrates that charge modification is a potential design strategy that can be generalized beyond the SARS-CoV-2 RBD antigen.

In contrast to AH, aluminum phosphate (AP) is another common alum adjuvant with a negative electrical charge and thus is well suited for the adsorption of positively charged antigens.[5] To verify the suitability of the charge modification strategy for both AH and AP adjuvants, we next selected an antigen with neutral charge properties. FHbp (pI = 7.25), a highly immunogenic protein that elicits a humoral response against *Neisseria meningitides*, is one of the main components of two current MenB vaccines (MenB-4C, the AH adjuvanted vaccine; MenB-fHbp, the AP adjuvanted vaccine).[31] By inserting negatively charged amino acids (Asp) or positively charged amino acids (Arg) at the flexible region

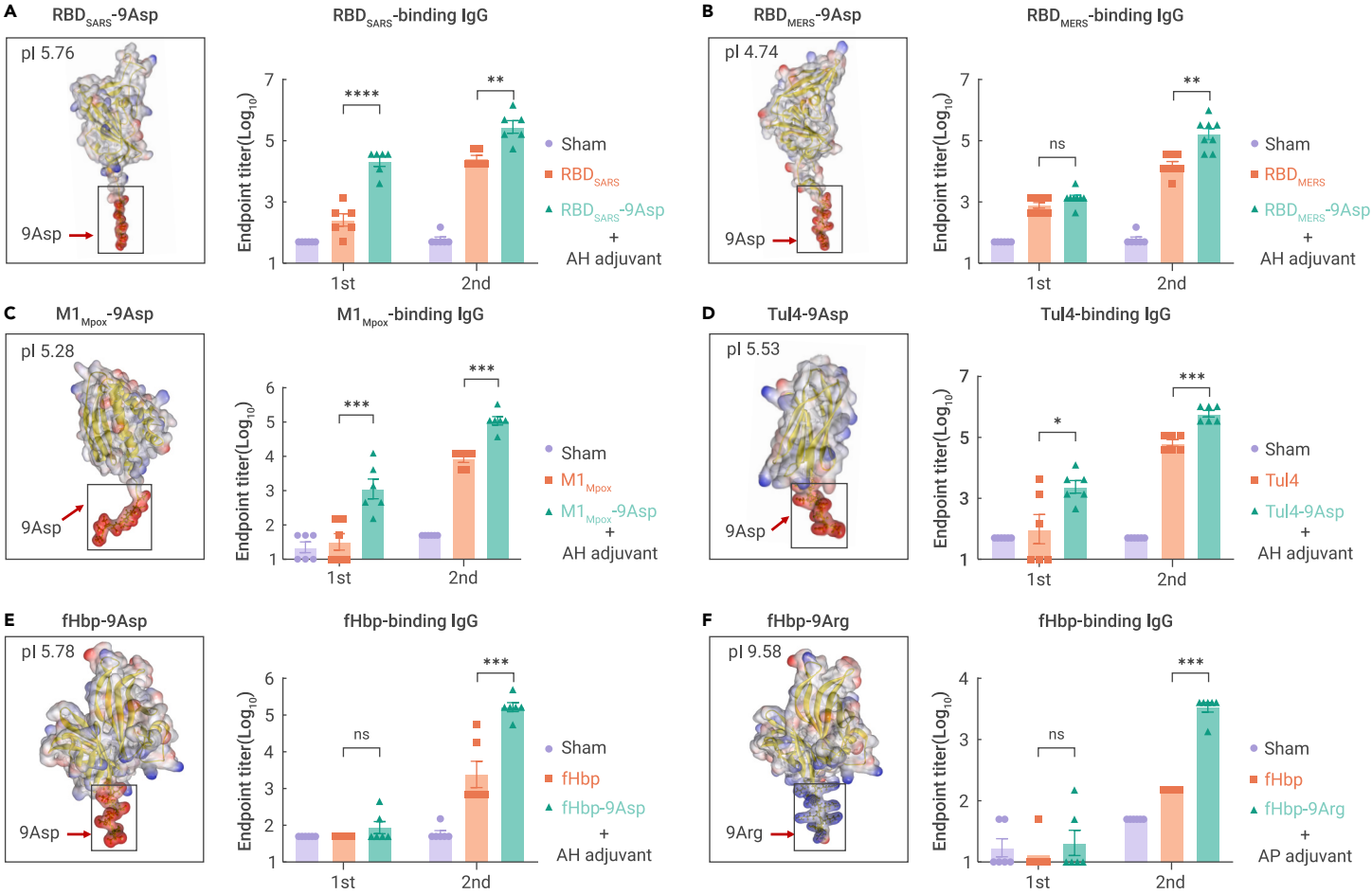

Figure 4. The wide applicable scope of charge modification strategy ELISA-tested serum IgG response titers of day 14/28 to (A) RBD$_{SARS}$-9Asp, (B) RBD$_{MERS}$-9Asp, (C) M1$_{Mpox}$-9Asp, (D) Tul4-9Asp, and (E) FHbp-9Asp, all adjuvanted with AH, and the IgG titer of (F) FHbp-9Arg adjuvanted with AP. The structure of antigen proteins was predicted using AlphaFold2 with default parameters. Data were expressed as the mean ± SEM. One-way ANOVA followed by Tukey's post-hoc test was used to test differences between groups. p values <0.05 were considered statistically significant (*p < 0.05; **p < 0.01; ***p < 0.001).

(C terminus) of fHbp, we designed two different charge mutants with a pI ranging from 5.78 (fHbp-9Asp) to 9.58 (fHbp-9Arg) (Figure S4C). FHbp-9Asp:AH and fHbp-9Arg:AP immunization groups could significantly increase the immune responses compared with WT-type fHbp, especially the positive modification fHbp-9Arg:AP, where the IgG titer was nearly 80-fold higher than that in WT-type fHbp:AP (Figures 4E and 4F).

DISCUSSION

Vaccination is a proven, safe, cost-effective way to protect against existing and emerging infectious diseases.[1,32] Compared with the traditional vaccines that consist of intact pathogens, protein subunit vaccines use protein fragments from the disease-causing pathogens; they are safer and easier to manufacture but often induce a low immunogenic response.[2,3] To overcome this problem, adjuvants such as alum have been incorporated into billions of vaccines administered to millions of people each year.

The actual mechanism by which alum induces high levels of antibodies remains elusive.[4,5] Earlier studies have shown that alum mediates its adjuvant effect primarily through a "depot effect" mechanism involving the slow release of antigens from the immune site. Thus, adsorption can play a role in the interaction between antigens and alum adjuvants, directly affecting the efficacy of protein subunit vaccines.[6,33,34] Recently, a pSer chemical coupling modification strategy has been proposed, which could enhance the binding anchorage between antigen and alum adjuvant utilizing the ligand exchange mechanism.[10–12]

In this work, we describe a novel strategy of precise modification of surface charge for antigen in subunit vaccine design. We could precisely modify its surface charge by inserting charged amino acids into the flexible region of the SARS-CoV-2 RBD. Our experimental results have shown that the RBD-modified antigen combined with alum adjuvant elicited rapid (7 days after single injection), efficient (>100-fold), and durable (up to 240 days) specific immune responses in a mouse model compared with the original RBD antigen. Notably, the immune efficacy of the vaccine was maintained at a low antigen level of 0.2 μg/mouse or at a low alum adjuvant level of 2 μg/mouse, which greatly increased the accessibility of the vaccine. In addition, since the adverse events associated with vaccines are generally dose dependent, lowering the dose substantially without losing immunogenicity should have obvious advantages.

Elderly populations with low immune potency are the focus of vaccination efforts. We evaluated the immunogenicity of charge-modified RBD with alum adjuvant in aged mice. The result shows that RBD-9Asp still drives a robust humoral immune response in the aged mouse model compared with WT-type RBD and has a high potential for vaccinating the elderly population. Though the synergistic immune efficacy of RBD-9Asp with alum has been significantly improved after surface charge modification, we consider further expanding its ability to deal with a pandemic. Based on the design of the RBD single-chain dimer, we next used the charge modification strategy, as well as updates of important VOC variants. The designed Beta-Delta-9Asp-Dimer antigen could stimulate a broad-spectrum NAbs response against various emerging SARS-CoV-2 variants, including the Omicron variant.

In investigating the potential mechanism by which the RBD-9Asp enhances immunogenicity, we conducted antigen release experiments *in vivo* and *in vitro*. It was confirmed that the adsorption and release of protein antigens and adjuvants were optimized and improved after charge modification. The strong adsorption and slow release might promote the activation and transport of antigen-presenting cells to lymphoid tissue, improving the bioavailability of vaccines and enhancing their immunogenicity. While the modification of the antigens enhanced the overall immunogenicity, it did not alter the Th1/Th2 bias of the immune response.

When creating vaccines against pathogens, there is often a desire to direct an immune response toward a particular conformational epitope on an antigen.[35] As we hypothesized, charge-modified RBD and alum might form a complex structure by electrostatic adsorption (Figure 2A). The C-terminal electronegative RBD-9Asp could be "captured" by alum, with the directional display of antigen binding sites, and the "up" conformation of RBD could be stabilized. The surface charge of the modified RBD has improved electrostatic adsorption with alum, which could benefit the epitope focusing and prolong the release of antigens.

This study still has some limitations. In the charge modification of various pathogen antigens, the fixed amount of charged amino acids (9Asp or 9Arg) was inserted at the C terminus of antigens. Inserting different amounts of charged amino acids and selecting different sites in the flexible region of the antigen for precise charge modification based on the antigen's structure are worthy of further exploration. In the current stage of our research, the rough shape of the RBD-9Asp-Dimer and Beta-Delta-9Asp-Dimer was observed under the microscope using negative staining. The 2D characteristics of protein particles were mainly "V" type, which was generally consistent with our expectations (Figures S9 and S10). The more refined experimentally determined structures would provide valuable insights into the impact of modifications on the antigen conformation, which could also help to address how antigen-targeted display enables epitope focusing and the following antigen processing and presentation.

PB was selected as a buffer solution to formulate the antigens with AH in this study. The influence of different buffers on the efficacy of charge-modified antigens warrants further investigation. Regarding safety and tolerability, we assessed the potential effects of the antigen modifications on the mice by monitoring detailed blood biochemistry, complete blood count, and body weight following immunization (Tables S1 and S2; Figure S11). We did not observe any pathological conditions or fatalities among the mice from the immune and control groups. Given that the inserted residues constitute a minor modification, we do not expect any substantial safety concerns to arise. Our current study primarily focused on the overall immune response and antibody production elicited by the modified antigens adsorbed to the aluminum adjuvant. As neutralization antibody tests and protective efficacy evaluation of antigens are crucial, we look forward to further refining these aspects in subsequent research.

To sum up, our results provide a proof of concept that the strategy of precise charge modification of antigen is expected to provide a simple and direct way to design novel protein subunit vaccines by enhancing antigen-adjuvant adsorption and epitope focusing, thereby driving a robust immune efficacy. The charge modification strategy is applicable and flexible for various pathogen antigens. Optimizing traditional alum-adjuvanted vaccines using this unique approach may create an innovative approach to cope with an emerging pandemic, particularly given the focus on the SARS-CoV-2 pandemic since 2020.

MATERIAL AND METHODS

Materials and methods related to this work are available in the supplemental information.

DATA AND CODE AVAILABILITY

All data are available in the main text or the supplemental information.

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DECLARATION OF INTERESTS

C.W., J.X., X. Zai, C.Z., Z.Z., F.Z., Y.Z., J.Z., Y. Yin, Y. Yang, R.L., and Y.L. are listed as inventors on a pending patent application for charge-modification vaccines.

SUPPLEMENTAL INFORMATION

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