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Subunit Nanovaccine with Potent Cellular and Mucosal Immunity for COVID-19

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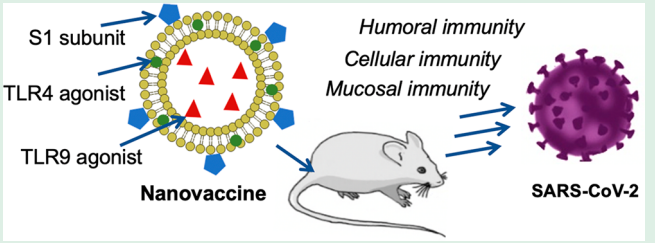
ABSTRACT: To combat the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, we formulated the S1 subunit of the virus with two adjuvants, amphiphilic adjuvant monophosphoryl lipid A for Toll-like receptor 4 and CpG oligodeoxynucleotide for Toll-like receptor 9, into cationic liposomes to produce a potent, safer, and translatable nanovaccine. The nanovaccine can efficiently elicit a humoral immune response and strong IgA antibodies in mice. The sera from the vaccinated mice significantly inhibit SARS-CoV-2 from infecting Vero cells. Moreover, relative to the free S1 with a traditional Alum adjuvant, the nanovaccine can elicit strong T-cell immunity by activating both $CD4^+$ and $CD8^+$ cells.



KEYWORDS: *COVID-19, cellular immunity, humoral immunity, liposome, nanoadjuvant, nanovaccine*

INTRODUCTION

The new emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a worldwide pandemic. As of August 16, 2020, the number of coronavirus-induced disease (COVID-19) cases is over 21 million, and COVID-19 has caused over 768 000 deaths. Cases are still increasing rapidly, and it is obvious that the outbreak of SARS-CoV-2 cannot be stopped unless effective vaccines can be developed. Great efforts have been made to develop vaccines against SARS-CoV-2 worldwide. The first research article on a vaccine for COVID-19, which is based on the deactivated virus, has been published recently.[1]

Here we develop an adjuvanted nanovaccine with the recombinant S1 subunit from SARS-CoV-2. It shows remarkable immunogenicity in mice, and the serum of the vaccinated mice efficiently blocks virus infection of the cells. The S1 subunit is one component of the SARS-CoV-2 transmembrane spike (S) glycoprotein, a homotrimer protruding from the viral surface.[2] Its function is to bind the host receptor, angiotensin-converting enzyme 2 (ACE2), and then trigger the S2 subunit of the S protein to fuse the viral membrane and the host cell membrane.[3,4]

Among different types of vaccines, the subunit vaccine is very attractive because it is safer and easier to produce. However, the subunit vaccines show a weak immune response *in vivo* due to their poor pharmacokinetics, easy degradation, and lack of other components of the whole virus, and they act as a pathogen-associated molecular pattern (PAMP) to initiate the immune response.[5] To overcome the drawbacks of the subunit vaccine, researchers have developed several strategies to enhance subunit antigen immunogenicity, including applying nanotechnology to codeliver antigens and molecular adjuvants.[6] Different types of carriers, such as synthetic polymers, chitosan, and liposomes, have been applied in the formulation of nanovaccine loading with subunit antigens and adjuvants for preclinical studies.[7] Here we applied cationic liposomes as a delivering vehicle,[8] in which CpG-oligodeoxynucleotides (CpG ODNs (CpG)) for Toll-like receptor 9 (TLR9) were loaded in its internal water depot, monophosphoryl lipid A (MPLA) for Toll-like receptor 4 (TLR4) was coassembled with lipid molecules, and the anionic S1 at neutral pH was adsorbed onto its cationic surface (Figure 1). Thus a potent subunit vaccine for anti-SARS-CoV-2 was easily produced. This vaccine can efficiently activate the humoral immune response in mice, and the sera from the vaccinated mice can significantly inhibit the virus infection of Vero cells. More importantly, strong T-cell immunity is also elicited, and both $CD4^+$ and $CD8^+$ cells are more strongly activated than the control. In addition, a higher amount of S1-specific IgA antibody can be detected in the nanovaccine-immunized group. All of the characteristics of the present nanovaccine

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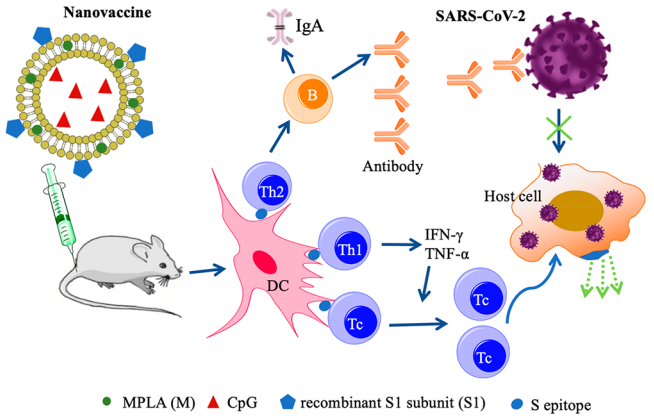

Figure 1. Schematic of liposomal subunit nanovaccine for anti-SARS-CoV-2.

show a unique advantage in protecting the host against SARS-CoV-2. Because the delivery materials and two adjuvants have been recognized by the U.S. Food and Drug Administration for clinical application, the vaccine shows a high technological translation for application.

PREPARATION AND CHARACTERIZATION OF THE NANOVACCINE

MPLA/CpG-loaded liposome particle, p(M+C), was produced via a thin-film hydration approach by using cationic 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), helper lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and cholesterol as carrier materials. Thus the obtained p(M+C) is a nanoparticulate adjuvant. The charge interaction has been commonly applied to load the proteins and nucleic acids onto the surface of the particles,[9−11] and thus we applied this facile method to generate vaccines. The p(M+C)−S1 vaccine was formed through mixing p(M+C) with a solution of S1. As shown in Figure 2A,B, the results of dynamic light scattering showed that the average particle diameter was ∼135 nm for p(M+C) and then changed to 158 nm for p(M+C)−S1. Compared with the surface charges of p(M+C), +48.1 mV, the ζ-potential of p(M+C)−S1 was decreased to +30.4 mV (Figure 2C) due to the charge neutralization. The entrapment efficiency of CpG and MPLA within p(M+C)−S1 was measured to be 95.8 ± 0.9 and 99.3 ± 0.2%, respectively, and the binding efficiency of S1 to the surface of the liposome particle was 12.6 ± 0.3%. These results suggested that adjuvant molecules CpG and MPLA could be efficiently loaded into liposome particles, and S1 was absorbed onto the surface of p(M+C) and a nanoparticulate vaccine was formulated. Such obtained p(M+C) and p(M+C)−S1 exhibited high colloidal stability after 11 weeks of storage at 4 °C (Figure 2D,E).

NANOVACCINE ELICITATION OF ROBUST S1-SPECIFIC ANTIBODY PRODUCTION

Humoral immunity is important to protect people from virus infection,[12] and the S1-specific antibody was evaluated in this study. BALB/c mice were immunized three times on day 0, day 14, and day 28 with different formulations, Alum + free S1 (A+S1), free MPLA + free CpG + free S1 (f(M+C+S1)), and p(M+C)−S1, with untreated mice as the control (Figure 3A). As shown in Figure 3B,C, compared with the A+S1 and f(M+C+S1), p(M+C)−S1 triggered a high antibody titer level of total IgG, IgG1, and IgG2a. In particular, the IgG2a level of the p(M+C)−S1- and f(M+C+S1)-treated groups was much higher than that of the A+S1 group at days 28, 42, and 65. At day 14, after the first vaccination, the IgG2a/IgG1 values of p(M+C)−S1 and f(M+C+S1), which reflect the polarizing ability of the T helper cells to the Th1 response, were 0.88 and 0.79, respectively, larger than that of A+S1 (0.50). Then, the value of IgG2a/IgG1 in all of the groups decreased from day 14 to day 28, whereas, the A+S1 group decreased more significantly. After the third immunization, the IgG2a/IgG1 values of the p(M+C)−S1 and f(M+C+S1) groups increased again and were retained up to day 65. On the contrary, the ratio of the A+S1 group further decreased to 0.06 at day 42 and was retained

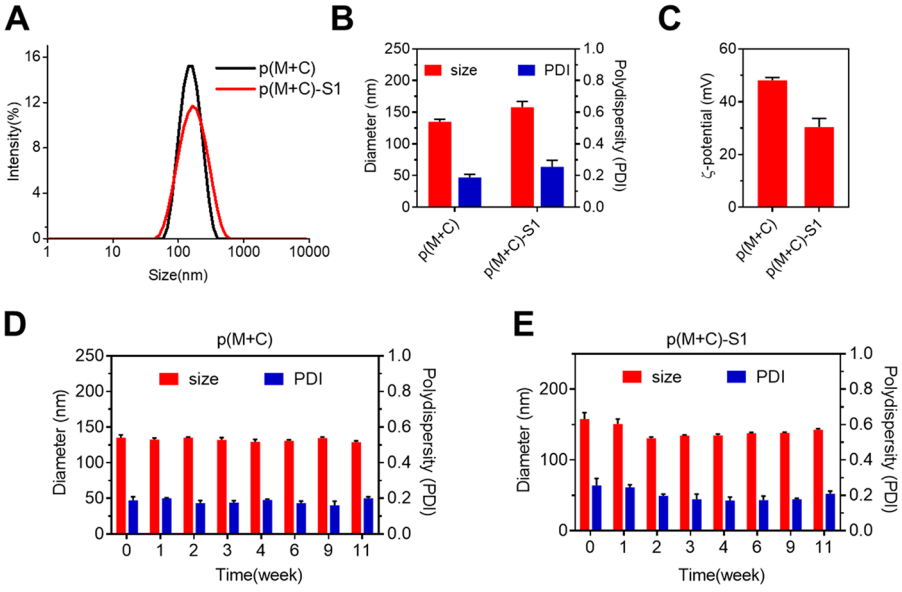

Figure 2. Characterization of the size and stability of the nanovaccine. (A) Dynamic light scattering profiles of p(M+C) and p(M+C)−S1. (B) *Z*-average diameter and PDI and (C) ζ-potential of p(M+C) and p(M+C)−S1. Storage stability of (D) p(M+C) and (E) p(M+C)−S1 under 4 °C conditions.

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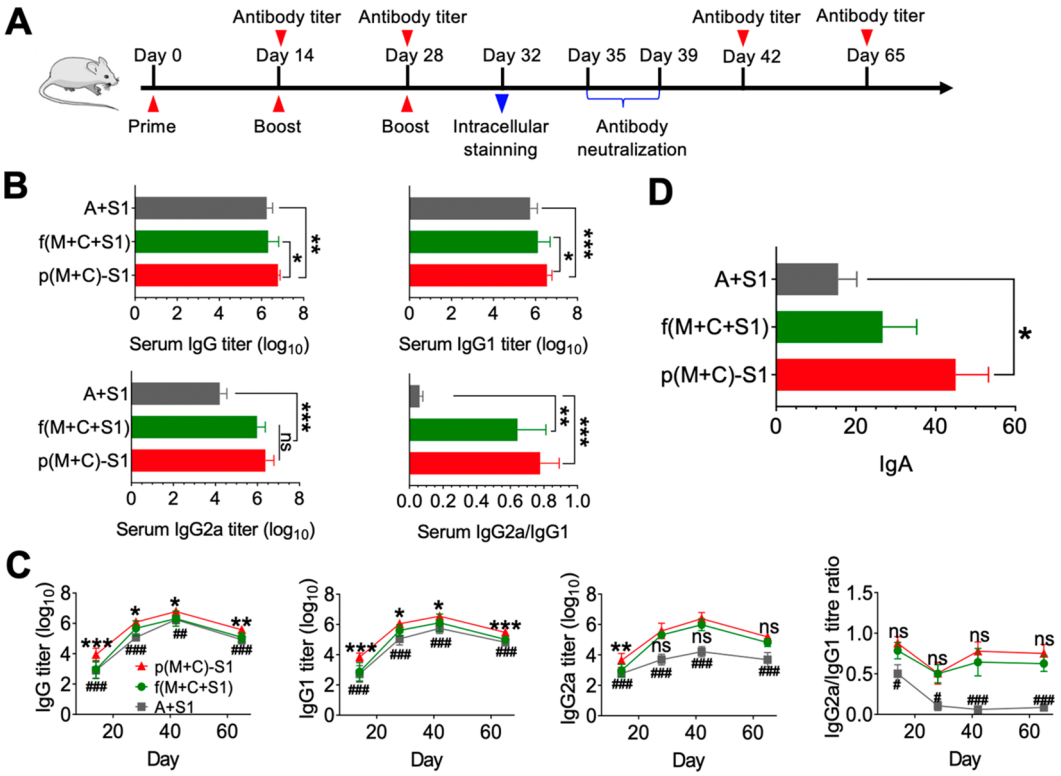

Figure 3. Nanovaccines elicit strong humoral immunity. (A) Schematic of the immunization strategy. (B) Quantitative comparison of the S1-specific production of IgG, IgG1, IgG2a and IgG2a/IgG1 on day 42. (C) Change of antibody titer over time (* represents the comparison of p(M+C)−S1 with f(M+C+S1); # represents the comparison of p(M+C)−S1 with A+S1), n = 10. (D) Quantitative comparison of the S1-specific production of IgA at day 65. Data are presented as the mean ± SEM (*P or #P < 0.05, **P or ##P < 0.01, ***P or ###P < 0.001).

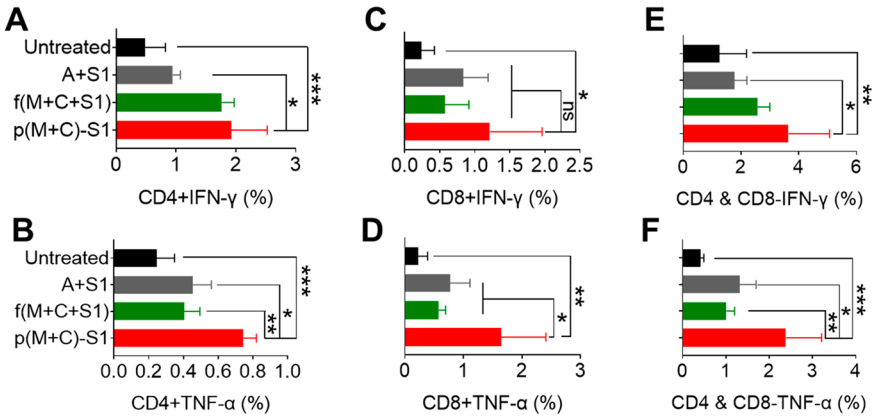

Figure 4. Nanovaccines elicit a potent T-cell response. (A,C) IFN-γ and (B,D) TNF-α expressed in $CD4^+$ and $CD8^+$ T cells in blood on day 32 after restimulation with different formulations of S1 (n = 10) by flow cytometry analysis. Total (E) IFN-γ and (F) TNF-α expressed in $CD4^+$ and $CD8^+$ T cells in blood on day 32 after restimulation with different formulations of S1 (n = 10) by flow cytometry analysis. Data are presented as the mean ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001).

until day 65. Altogether, the MPLA- and CpG-adjuvanted vaccine could polarize the T helper cells to Th1 dominant immunity, and the particulate vaccine of liposome could be more efficient than the free one, evoking more efficient cell immunity. Conversely, the traditional Alum adjuvant stimulated a Th2-dominant response. Additionally, the humoral immunity elicited by the particulate vaccine was higher than that of the other two groups. From nasopharynx mucus eluted from the mice, we found that p(M+C)−S1-immunized mice produced a higher amount of S1-specific IgA than the other groups (Figure 3D). Therefore, the p(M+C)−S1 nanovaccine could elicit stronger humoral, cellular, and mucus immunities than those of the Alum adjuvant group, which shows its high potency to be further developed.

As previously reported, the antibodies in the sera of the recovered patients of SARS disappeared after 1 year,[13,14] whereas the T cells were persistent in the patients for up to 6 to 11 years,[15,16] which indicated that cell immunity should be considered in the SARS-CoV-2 vaccine. Here, to investigate whether the nanovaccine can activate T cells, the characteristic cytokines secreted by T cells were evaluated. As shown in Figure 4A−D, the mice injected with p(M+C)−S1 induced a higher level of IFN-γ or TNF-α produced by $CD4^+$ T cells (1.92, 0.74%) and $CD8\alpha^+$ (1.21, 1.64%) than the mice treated

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with A+S1 producing $CD4^+$ T cells (0.94, 0.45%) and $CD8\alpha^+$ T cells (0.83, 0.77%), respectively. It is noteworthy that the $CD8^+$ T cell in f(M+C+S1) was not significantly activated compared with the other two groups, which may be due to the fact that the antigen in the free form could not be efficiently cross-delivered by MHC I.[17] The total IFN-γ and TNF-α cytokines secreted in $CD4^+$ and $CD8^+$ T cells of the p(M+C)−S1 group were 2.1 and 1.8 times those in the A+S1 group (Figure 4E,F). Thus it is further confirmed that p(M+C)−S1 could evoke cell immunity as well as a high humoral immunity, implying that the p(M+C)−S1 is a promising vaccine candidate for protecting the people from infection.

COVID-19 NEUTRALIZATION *IN VITRO*

The specificity of the antibody induced by vaccines is of importance. To test the neutralization ability of the serum antibody from the different groups, a microneutralization assay was performed using the SARS-CoV-2 strain CZ01 isolated from patients with Vero cells.[1] The mean neutralizing antibody titer against the CZ01 strain of the p(M+C)−S1 nanovaccine was >1024, which is significantly higher than that of the control. Also, it is higher than that of the A+S1 group (384) and the f(M+C+S1) group (768). The effective titer of antibody *in vitro* indicated that p(M+C)−S1 has potential for an *in vivo* neutralization challenge and preclinical trial.

DISCUSSION

As of April 2020, over 100 various vaccines are undergoing research and development worldwide. They include RNA vaccines, DNA vaccines, adenovirus-based vaccines, inactivated virus vaccines, recombinant/subunit protein vaccines, virus-like particle and peptide vaccines, and so on.[18] Among them, mRNA, adenovirus-based, and inactivated virus vaccines have been quickly moved into clinical development. Also, more than 20 protein vaccines are under investigation in different stages. However, it is still hard to find research articles on subunit vaccines, let alone how the subunit antigens are delivered.

Compared with other types of vaccines, the subunit protein vaccine is safer and cheaper to produce. Previous research on vaccines using the spike protein of severe acute respiratory syndrome coronavirus (SARS-CoV) as the subunit showed that the antibodies could effectively prevent the coronavirus from binding to the cell and undergoing membrane fusion, neutralizing the virus during infection.[19] The causative agent of the ongoing SARS-CoV-2 pandemic, along with SARS-CoV[20] and Middle East respiratory syndrome (MERS-CoV),[21] belongs to the *Betacoronavirus* genus and *Coronaviridae* family. The three zoonotic coronaviruses harboring a linear, single-stranded positive RNA genome cause the most deadly pneumonia in humans. Although the S-protein gene sequence of SARS-CoV-2 is <75% similar to the S-protein sequence of SARS-CoV, the cell membrane binding receptor is still ACE2.[3,22,23] Therefore, the S protein of SARS-CoV-2 still plays a major role in binding host cells and promoting membrane fusion; namely, the S protein can be used as a major target for subunit vaccine preparation.[24,25] However, in general, the immunogenicity of free subunit antigens is weak when used alone, and it is hard to stimulate a robust immune response. In addition to the antigen, the body identifies pathogenic microorganisms by PAMPs, such as Toll-like receptors (TLRs) for activating the innate immunity. As a result, proliferation and maturation of T cells effectively cause cellular immunity. Therefore, TLR agonists in the vaccine play a key role in the clearance of intracellular pathogens.[26] However, low-molecular-weight molecular adjuvants can enter blood vessels and cause a systemic inflammatory response. To overcome the problems of protein antigens and adjuvants, particulate antigens and agonist adjuvants as well as a delivery system are needed for a potent vaccine.

Here we applied cationic liposomes as carriers to anchor the S1 subunit of the virus onto their surface by the charge interaction, to entrap CpG into their internal aqueous phase, and to insert MPLA along their lipid bilayer. Thus we formulated the S1 protein and TLR agonists into a particulate vaccine with a diameter of ca. 150 nm. The results showed that the cell response as well as a robust humoral response are activated, and the serum antibodies of mice could efficiently neutralize the virus. The robust immune response should be attributed to the use of MPLA as a TLR4 agonist to induce the Th1 and Th2 response and CpG as a TLR9 agonist to strengthen the Th1 response. Moreover, a combination of MPLA and CpG in the p(M+C)−S1 nanovaccine is beneficial as it elicits both humoral and cell-mediated immune responses. In contrast, the subunit with Alum elicits only humoral immunity. It has been reported that cellular immunity played a critical role in decreasing the viral load of patients with SARS-CoV infection in 2003.[25] More importantly, it was found that virus-specific memory T cells persisted up to 6 to 11 years in recovered patients.[15,16] On the contrary, the virus-specific memory B cells were lacking in the patients, and the antibodies disappeared after 1 year.[27] This indicates that the cell immunity could perhaps be much more important in the SARS-CoV-2 development. Additionally, we showed that the nanovaccine could elicit a stronger mucus S1-specific IgA antibody than the traditional Alum group. It is reported that SARS-CoV-2 attaches to goblet and ciliated cells in the nose as an initial infection site to invade the host, and thus the mucus immunity may be meaningful to protect people from viral infection.[28]

Furthermore, it is known that liposome products for drug delivery, like AmBisome and Doxil, are on the market, and many more are in clinical trials. Thus the present lipid-based nanovaccine with the narrow size distribution and high biocompatibility is safe and easy for scale-up manufacturing for further application development.

In summary, we constructed a subunit antigen lipid nanovaccine for COVID-19. With the aid of MPLA and CpG in adjuvants, the nanovaccine efficiently induces robust humoral and $CD8^+$ and $CD4^+$ T-cell responses and also may provide potential mucosal protection. Furthermore, the vaccine shows an efficient *in vitro* neutralization effect. Because the lipid-based formulation technique has already been applied, this nanovaccine may have great potential for clinical applications. Moreover, MPLA/CpG-loaded liposomes alone can be used as nanoparticulate adjuvants of a coronavirus vaccine with different antigens to enhance an innate immunity and thus a cellular immune response.

MATERIALS AND METHODS

Materials. DOTAP and DOPE were purchased from A.V.T. (Shanghai) Pharmaceutical (Shanghai, China). Cholesterol was obtained from Shanghai Macklin Biochemical (Shanghai, China). Monophosphoryl lipid A (MPLA) was from Avanti Polar Lipids (Alabaster, AL). Inject Alum Adjuvant was purchased from Thermo Scientific (Rockford, IL). CpG ODN1826 was from InvivoGen (San

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Diego, CA). Recombinant SARS-CoV-2 spike protein (S1) was purchased from Sino Biological (Beijing, China). Antimouse PerCP-eFluor 710-CD3, antimouse FITC-CD4, antimouse APC-CD8α, antimouse PE-IFN-γ, antimouse PE/Cy7-TNF-α, and the Zombie Violet Fixable Viability Kit were purchased from Biolegend (San Diego, CA). HRP-conjugated antimouse IgG, IgA, IgG2a, and IgG1 were purchased from Abcam (Cambridge, England).

Preparation of Adjuvant-Loaded Liposome and Vaccine. Adjuvant-loaded liposome was prepared through a lipid film hydration approach with slight modifications.[29] In brief, DOTAP, DOPE, cholesterol, and MPLA (50 μg) with the weight ratio of 150:50:20:1 were dissolved in 5 mL of ethanol; then, a thin lipid film was obtained after the removal of the organic solvent by a vacuum rotary evaporator at 45 °C. Subsequently, this lipid film was rapidly hydrated with 5 mL of CpG aqueous solution (72.5 μg), followed by five repeated extrusions through a polycarbonate filter (0.1 μm, Whatman) for the preparation of the MPLA/CpG-loaded liposome (p(M+C)). Furthermore, p(M+C) was formulated with the S1 solution and then incubated at 4 °C overnight to obtain the vaccine, p(M+C)−S1. The particle diameter, polydispersity index (PDI), and ζ-potential were characterized by a Zetasizer Nano ZS Instrument (Malvern) at room temperature. The obtained p(M+C)−S1 was centrifuged in a 30 kDa ultrafiltration tube at 4 °C and 15 000g for 10 min, and the lower layer of the solution was taken out to detect the amount of free CpG and MPLA through a NanoDrop instrument (Thermo Scientific) and the Endotoxin Detection Limulus kit (Bioendo, China), respectively. The amount of S1 was determined using the Micro BCA protein assay kit (Thermo Scientific). The entrapment efficiency (EE) of S1, CpG, and MPLA was calculated using the following equation: EE% = (1 − amount of free drug/total amount of drug) × 100%.

Animal Immunization. Pathogen-free female BALB/c mice (5−8 weeks) were divided into four groups, and one group of untreated mice was used as a negative control. Mice of the other three groups were injected subcutaneously at the base of tail with A+S1, f(M+C+S1), and p(M+C)−S1, which contained S1 (20 μg per mouse), CpG (2.9 μg per mouse), and MPLA (2.0 μg per mouse). 14 and 28 days after the first immunization, each group of mice was boosted twice with the same dose of the corresponding formulations.

S1-Specific Antibody Titer Determination. Blood was collected from the orbital vein of the mouse and centrifuged at 5000 rpm for 10 min to collect the serum. 4.3 μg/mL of S1 antigen was coated on the ELISA plates overnight at 4 °C. Then, the plates were washed three times with PBST (0.05% Tween-PBS), and 3% BSA in PBS was added to block the plates at 37 °C for 2 h. After washing with PBST, diluted serum with corresponding multiples was added to the ELISA plates and incubated at 37 °C for 2 h. Subsequently, the plates were washed three times with PBST and incubated with 100 μL of diluted HRP-conjugated antibodies at 37 °C for 1 h; then, the plates were washed with PBST several times, 3,3′,5,5′-tetramethylbenzidine substrate was added, and the plates were incubated in the dark for 5−10 min. Finally, 50 μL of stock solution was added, and the ELISA plates were measured with a Multimode Microplate Reader. It was deemed positive when the OD value of the experimental group was greater than twice the negative OD value, and the final antibody titers were expressed by the serum dilution factor.

For IgA detection, the nasal lavage fluids and lung lavage fluids were collected on day 65 after the priming and centrifuged at 12 000 rpm for 10 min at 4 °C. As described, the supernatant was analyzed for IgA by ELISA. A nonimmunized group was used as a negative control.

Intracellular Cytokine Staining. The function of T cells in peripheral blood mononuclear cells was evaluated by detecting the secretion of the intracellular cytokines IFN-γ and TNF-α. 32 days after the first immunization, 150 μL of whole blood from mice of A+S1, f(M+C+S1), and p(M+C)−S1 was collected, and the leukocytes were collected by lysing the erythrocytes with ammonium-chloride-potassium (ACK) lysis buffer. The intracellular staining was conducted as previously described.[30]

Neutralizing Assay. The sera from different groups of mice were collected, and they were inactivated at 56 °C for 0.5 h. After two-fold dilution with the complete medium, they were incubated with 100 $TCID_{50}$ of SARS-CoV-2 virus in an incubator for 2 h at a ratio of 1:1. Then, (1 to 2) × 10^4 Vero cells were added to the serum−virus mixture and further incubated for 5 days. The cytopathic effect (CPE) was confirmed with a microscope, and the neutralizing titer was evaluated by the dilution number of 50% protective condition.

Statistical Analysis. All statistical analyses were performed using SPSS software 19.0 with one-way ANOVA, and $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicated the significant statistical differences.

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Author Contributions

$^{\perp}$L.L., Z.L., and H.C. contributed equally to this work. L.L. designed and conducted partial experiments and wrote the paper. Z.L., H.C., and H.L. conducted partial experiments and data analysis. Q.G. supplied the virus neutralization. G.G. supplied the discussion on immunology. F.C. supplied the animal support. Y.C. supervised and wrote the paper.

Notes

The authors declare no competing financial interest.

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