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Article

Adjuvant-Protein Conjugate Vaccine with Built-In TLR7 Agonist on S1 Induces Potent Immunity against SARS-CoV-2 and Variants of Concern

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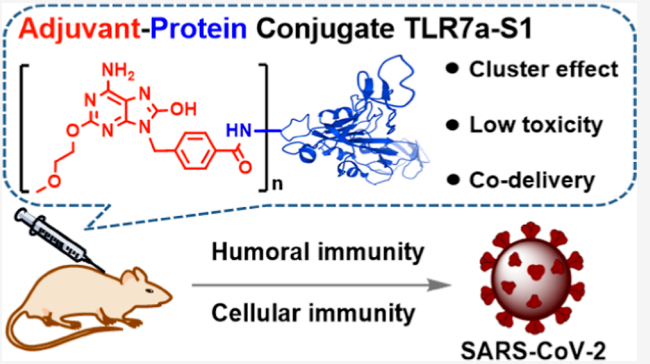
ABSTRACT: With the global pandemic of the new coronavirus disease (COVID-19), a safe, effective, and affordable mass-produced vaccine remains the current focus of research. Herein, we designed an adjuvant-protein conjugate vaccine candidate, in which the TLR7 agonist (TLR7a) was conjugated to S1 subunit of SARS-CoV-2 spike protein, and systematically compared the effect of different numbers of built-in TLR7a on the immune activity for the first time. As the number of built-in TLR7a increased, a bell-shaped reaction was observed in three TLR7a-S1 conjugates, with TLR7a(10)-S1 (with around 10 built-in adjuvant molecules on one S1 protein) eliciting a more potent immune response than TLR7a(2)-S1 and TLR7a(18)-S1. This adjuvant-protein conjugate strategy allows the built-in adjuvant to provide cluster effects and prevents systemic toxicity and facilitates the co-delivery of adjuvant and antigen. Vaccination of mice with TLR7a(10)-S1 triggered a potent humoral and cellular immunity and a balanced Th1/Th2 immune response. Meanwhile, the vaccine induces effective neutralizing antibodies against SARS-CoV-2 and all variants of concern (B.1.1.7/alpha, B.1.351/beta, P.1/gamma, B.1.617.2/delta, and B.1.1.529/omicron). It is expected that the adjuvant-protein conjugate strategy has great potential to construct a potent recombinant protein vaccine candidate against various types of diseases.



KEYWORDS: *TLR7 agonist, adjuvant-protein conjugate, recombinant protein vaccine, COVID-19, SARS-CoV-2*

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a huge number of infections and deaths worldwide. With the outbreak of variants, especially the B.1.617.2/delta and B.1.1.529/omicron, global public health is facing a new serious situation.[1,2] Although some COVID-19 drugs have been approved for emergency use to control the epidemic,[3−5] effective vaccines are still regarded as the important method of preventing and treating the COVID-19.

At present, several types of vaccine, mainly including inactivated virus, mRNA, viral vectors, and recombinant proteins, have been approved for marketing, in addition to more than 300 vaccine candidates in pre-clinical or clinical studies.[6] Among them, the subunit vaccine has attracted much attention due to the safety and stability. Similar to other coronaviruses, including SARS-CoV and Middle East Respiratory Syndrome Coronavirus (MERS-CoV), the transmembrane spike (S) protein of SARS-CoV-2 plays an important role in viral infection of host cells.[7] As an essential component of S protein, the S1 subunit bearing receptor binding domain is responsible for recognizing the host cell surface angiotensin-converting enzyme 2 (ACE2) receptor and contains most of the neutralizing epitopes, while S2 is responsible for cell fusion.[7,8] Therefore, the S1 subunit protein is expected to be an ideal target for the design of anti-SARS-CoV-2 vaccines.[9−12]

However, the S1 protein has been limited in the development of SARS-CoV-2 subunit vaccines due to its weak immunogenicity. To overcome this limitation, it is necessary to combine the antigen with adjuvants to induce a high level of immune response. As the "danger signal" of the immune system, adjuvants play a critical role in regulating the strength and type of immune response.[13] The generally utilized adjuvants in SARS-CoV-2 vaccines include aluminum hydroxide,[14−16] stimulator of interferon genes (STING) agonist,[17,18] invariant natural killer T (iNKT) cell agonist,[19] and toll like receptor (TLR) agonist: Pam_3CSK_4,[20] MPLA,[9,21−23] imidazo-

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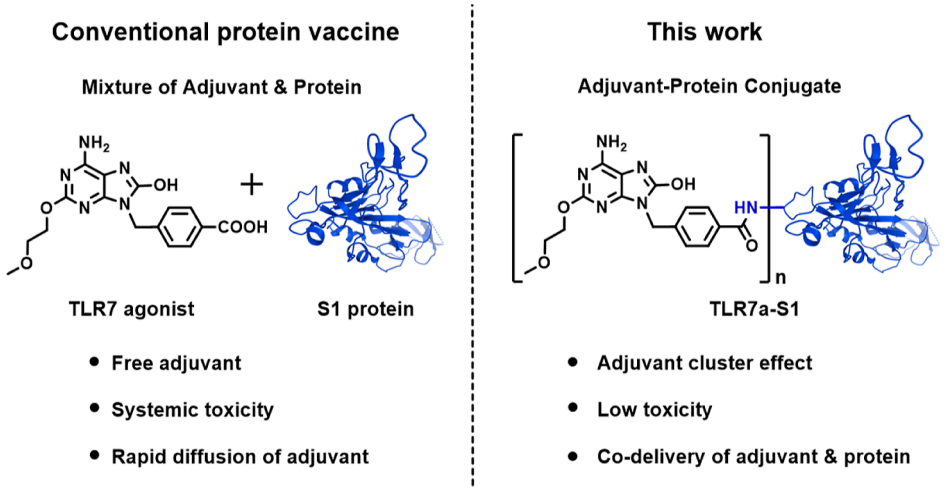
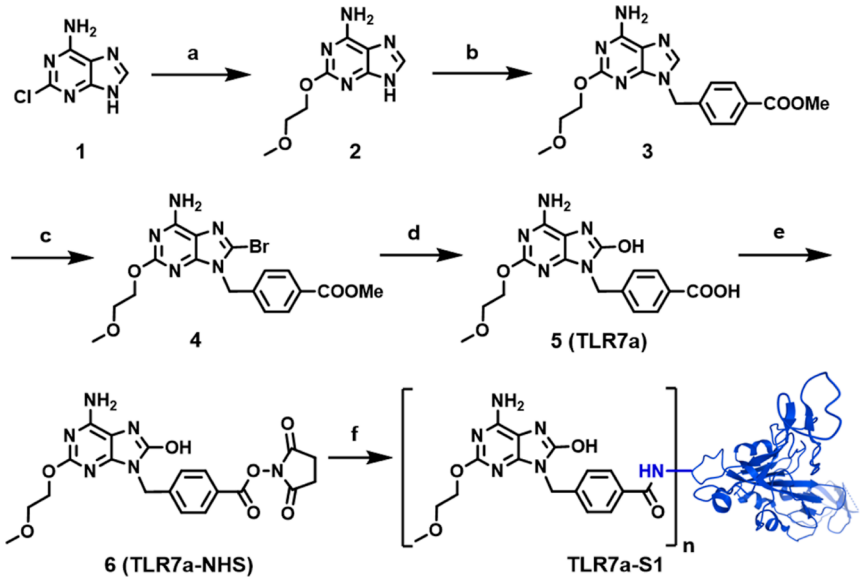


Figure 1. TLR7 agonist and the SARS-CoV-2 S1 protein (PDB code: 7A92) are formulated as a stable, biological active adjuvant-protein conjugate.

Scheme 1. Synthesis of TLR7 Agonist (TLR7a) and Adjuvant-Protein Conjugate TLR7a-S1[a]



[a](a) 2-methoxyethan-1-ol, Na, 140 °C; (b) methyl 4-(bromomethyl)benzoate, K_2CO_3, DMF, 60 °C; (c) Br_2, $CHCl_3$; (d) 6 M NaOH, MeOH, 100 °C; (e) 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), 1-hydroxypyrrolidine-2,5-dione (NHS), dry DMF; (f) S1 protein, PBS.

quinoline,[24] CpG,[9,25−27] and so forth. And most molecular adjuvants, especially TLR agonists, are physically mixed with antigens. However, free small molecular TLR agonists are usually difficult to be utilized in vivo, mainly due to the challenges with the systematic toxicity of cytokine syndrome caused by small molecule's rapid diffusion.[28] Previous studies have shown that the strategy of covalently linking the adjuvant to antigen by chemical approaches is effective in avoiding systemic toxicity and also more efficient in terms of co-delivery of antigen and adjuvant to antigen-presenting cells (APCs).[29−33]

TLRs recognize a range of different pathogen-associated molecular patterns and arouse the immune systems. Among them, TLR7 is located within endosomal compartments of the cell and is able to recognize and bind the single-stranded RNA from bacteria and viruses. Stimulation of TLR7 results in the activation of interferon regulatory factors and the production of type 1 interferons (IFNs) and pro-inflammatory cytokines.[34,35] Some adenine analogues, such as SM360320 and UC-1V150, were reported to be TLR-selective and activate immune cells via the TLR7 signaling pathway.[28,36,37] Similarly, as an adenine analogue, TLR7a was TLR-selective, triggering only TLR7 but no significant activity on TLR8.[38] These TLR7 ligands as built-in adjuvant components of vaccines are able to alter their own pharmacokinetic profile, avoid the systemic toxicity caused by rapid systemic transmission, and trigger efficient immune responses in mouse models.[30,38−41]

Based on the above considerations, we developed a SARS-CoV-2 subunit vaccine candidate using the strategy of adjuvant-protein conjugate, in which the TLR7 agonists were covalently conjugated to the recombinant S1 protein (Figure 1). The built-in TLR7 agonists were able to provide the cluster effect with a lower dose and avoid systematic toxicity caused by small molecule's diffusion to a certain extent. In addition, the adjuvant-protein conjugate can be co-delivered to and processed by the same antigen-presenting cell, thus eliciting

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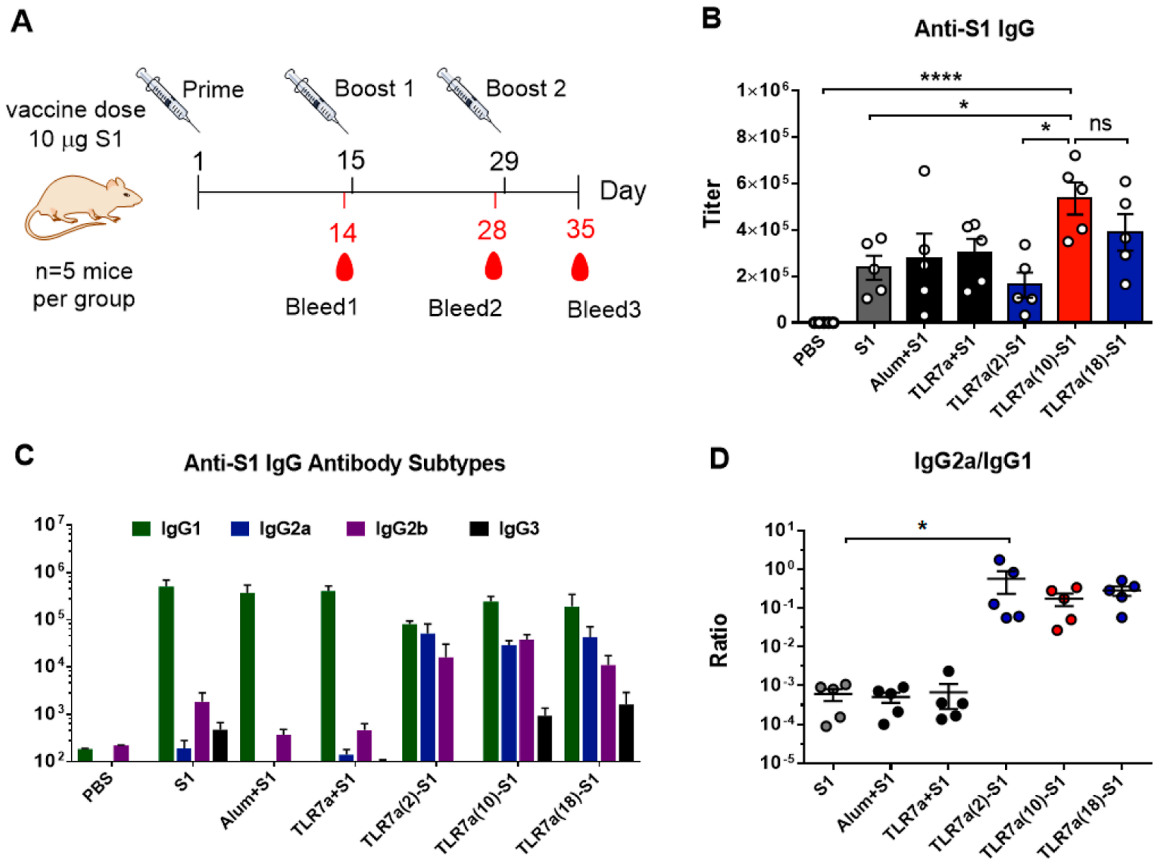

Figure 2. TLR7a(10)-S1 vaccine induces potent S1-specific antibody response in mice. (A) The vaccination schedule consists of a prime dose and two booster doses; (B) total anti-S1 IgG antibody on day 35; (C) IgG1, IgG2a, IgG2b, and IgG3 antibody subtypes on day 35; (D) ratio of IgG2a/IgG1. Results are shown as the mean ± SEM of five BALB/c mice in each group. Differences between groups were indicated by one-way ANOVA. The asterisk mark indicates a statistically significant difference (ns: no significant difference; *$P < 0.05$; ****$P < 0.0001$).

a potent immune response. In order to obtain the optimal immune response, herein vaccines consisting of different amounts of built-in adjuvants were prepared and immunologically evaluated as well.

RESULTS AND DISCUSSION

Preparation of the Adjuvant-Protein Conjugate. Based on the previous preparation strategy,[30,40] the synthesis of TLR7 agonist (compound **5**) and adjuvant-protein conjugate TLR7a-S1 is outlined in Scheme 1. Considering the safety and efficacy of the adjuvant, an optimized dose of the adjuvant (10 nmol per mouse) was chosen for the admix group (TLR7a + S1). In the preparation of TLR7a-S1, the carboxyl group of the TLR7a was reacted with 1-hydroxypyrrolidine-2,5-dione (NHS) to form TLR7a-NHS (compound **6**), which was used to covalently couple to S1 protein (Schemes 1 and S1). After reacting TLR7a-NHS with S1 protein in molar ratios of 10:1, 20:1, and 30:1, respectively (Schemes 1 and S2), the number of adjuvants coupled to S1 protein was determined by MALDI-TOF-MS to be approximately 2, 10, and 18 (Figure S1). In addition, the resulting adjuvant-protein conjugates were further analyzed by high-performance liquid chromatography (HPLC), and the retention times were 16.987, 17.150, and 17.282 min, respectively (Figure S2).

Evaluation of Anti-S1 Antibodies. The mice were immunized by subcutaneous injection every 2 weeks, and antibody titers in serum were evaluated (Figure 2A, Table S1). As shown in Figure 2B, compared with the S1 group, mice immunized with TLR7a + S1 group (10 nmol TLR7 agonists mixed with 10 μg S1 protein) elicited slightly higher IgG titers. TLR7a(10)-S1 group triggered a high antibody titer level of anti-S1 IgG (endpoint titer up to 535326), approximately 3.2-fold higher than the S1 group. Compared to the TLR7a + S1 group, mice immunized with TLR7a(10)-S1 elicited the nearly equivalent IgM titers (Figure S3) but higher IgG titers, which suggested that TLR7a as a built-in adjuvant can induce a switch in antibody class from IgM to IgG. Notably, the S1-specific antibody response was consistently most potent over time in mice inoculated with TLR7a(10)-S1 (Figure S4). In addition, comparing different amounts of the built-in adjuvant, the subunit vaccine TLR7a(10)-S1 elicited the highest titer of IgG antibody. We guess that the weaker immune response induced by the TLR7a(2)-S1 group was due to the lower adjuvant dose. In the TLR7a(18)-S1 group, a large number of adjuvant may cause excessive modification of the immunogenic protein, and some adjuvants may be linked to the important epitope sites for antibody recognition, resulting in weak recognition of the S1 protein by the immune system. These results showed that TLR7a(10)-S1 could effectively enhance the humoral immunity, which may be due to the combined effect of "adjuvant cluster effect" and "co-delivery of antigen and adjuvant".

Evaluation of Antibody Subtypes. In general, the ability to elicit a broad range of IgG subtypes is critical for antiviral vaccines. We tested the four IgG antibody subtypes in serum (Figures 2C and S5). Similar to the subtype distribution of

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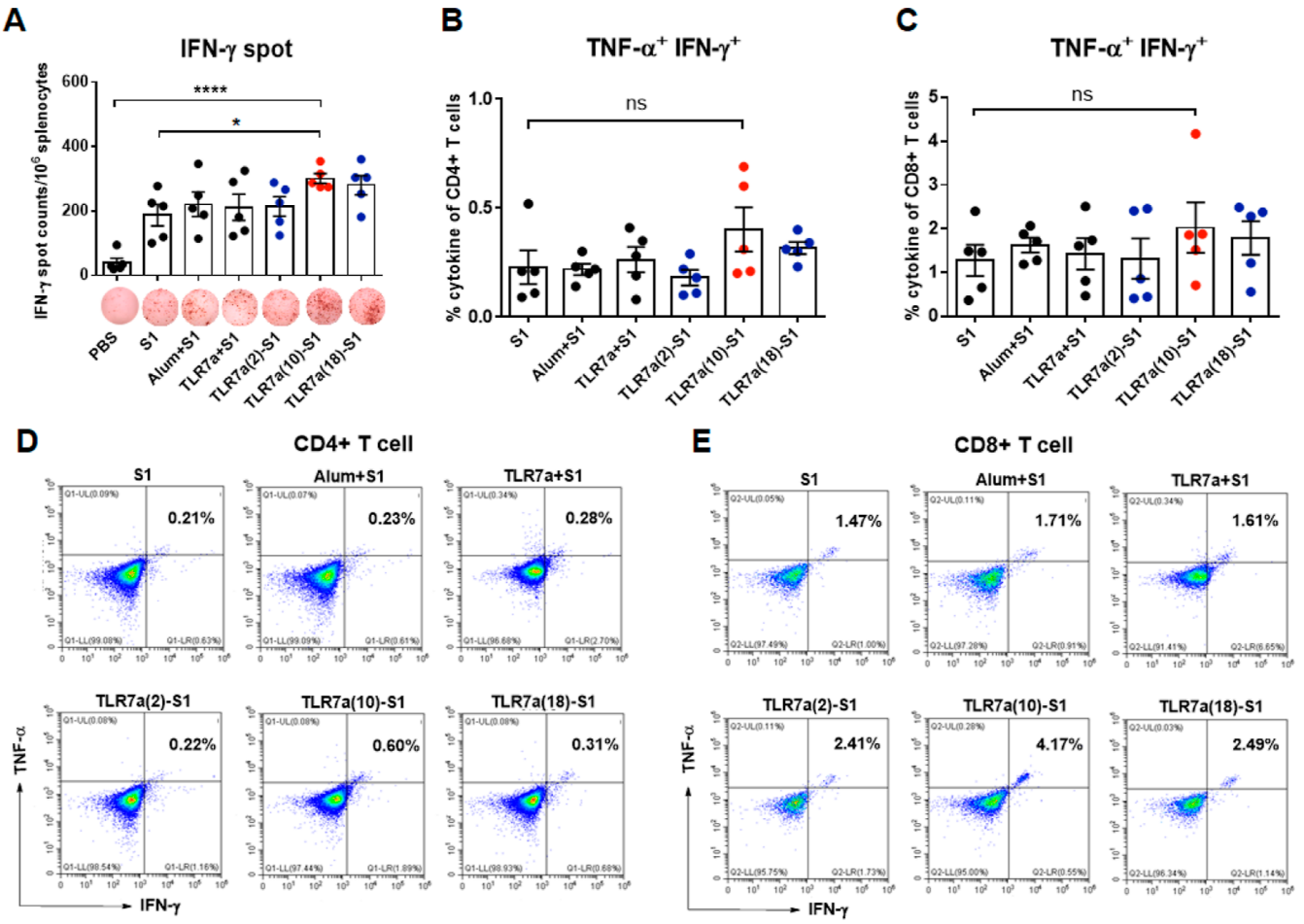

Figure 3. TLR7a(10)-S1 improves the T cell immune responses in mice. (A) IFN-γ ELISpot assay of spleen sample. (B,C) Proportion of CD4+ and CD8+ T cells secreting IFN-γ and TNF-α cytokines. (D,E) Representative flow cytometry dot plot of CD4+ and CD8+ T cells. Results are shown as the mean ± SEM of five mice in each group. Differences between groups were indicated by one-way ANOVA. The asterisk mark indicates a statistically significant difference (ns: no significant difference; *$P < 0.05$; ****$P < 0.0001$).

Alum + S1, the TLR7a + S1 group mainly elicited high levels of IgG1 titers with litter IgG2a, IgG2b, and IgG3, which represent a Th2-skewed immune response. On the contrary, covalently conjugated small-molecule TLR7a to S1 protein induced significantly high IgG2a and IgG2b titers. Notably, even the TLR7a(2)-S1 group, the lowest dose of TLR7a adjuvant covalently conjugated to S1 protein, elicited significantly higher titers of IgG2a and IgG2b antibody subtypes than the group mixed with optimal dose (10 nmol) of TLR7a. As shown in Figure 2D, we also calculated the IgG2a/IgG1 ratio, which is an indicator of the Th1/Th2 responses.[42] The IgG2a/IgG1 ratio was significantly increased in built-in adjuvant groups compared with the Alum + S1 group, and Alum adjuvant was identified as an adjuvant that could elicit only a Th2-type immune response.[43] All results suggested that the strategy of built-in adjuvants with several TLR7a molecules could induce a better balance between the Th1 and Th2 immune responses.

Cytokine-Producing T Cells. Cellular immunity plays a crucial role in the prevention and treatment of viral infections.[44,45] To explore the T cell immune response, we collected the spleen samples from mice on day 35. The capabilities of different vaccines were demonstrated through IFN-γ enzyme-linked immunospot (ELISpot) assay. As shown in Figure 3A, mice immunized with S1 protein mixed with TLR7a or Alum adjuvant induced a slightly higher number of spots than the no-adjuvant S1 group. Furthermore, the splenocytes from mice immunized with TLR7a(10)-S1, stimulated with an overlapping peptide library, increased the release of IFN-γ by nearly 1.7-fold compared to the S1 control group.

Next, we tested the percentage of CD4+ and CD8+ T cells secreting IFN-γ and TNF-α cytokines (Figure 3B–E). Under the same gating strategy, a high proportion of CD4+ and CD8+ T cells secreting TNF-α and IFN-γ double-positive cytokines was observed in TLR7a(10)-S1 group (0.40 and 2.03%). Comparing vaccines containing different numbers of built-in adjuvants, the subunit vaccine TLR7a(10)-S1 showed slightly higher CD4+ and CD8+ percentages than TLR7a(2)-S1 (0.18, 1.43%) and TLR7a(18)-S1 (0.32, 1.79%). This result suggested that TLR7a(10)-S1 vaccine showed a tendency to induce cellular immunity more effectively. This may be mainly due to the chemical conjugation of the TLR7 agonist and S1 protein to ensure their co-delivery to APCs and subsequently to induce robust T cell immunity.[46]

Neutralization Activity of Wild-Type Pseudovirus. Neutralizing antibody titer is a key indicator for evaluating the effect of antivirus vaccines. An accurate and efficient method of testing the neutralizing antibody is pseudovirus neutralization assay. As expected in Figure 4A, serum from the

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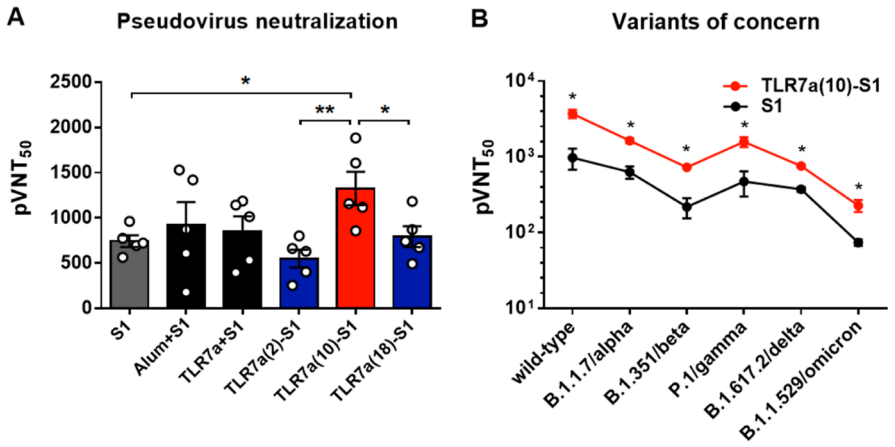

Figure 4. TLR7a(10)-S1 induces robust neutralizing antibody response in mice. (A) Pseudovirus neutralization ID_{50} ($pVNT_{50}$) titers measured by the pseudovirus expressing SARS-CoV-2 spike protein. (B) $pVNT_{50}$ values of TLR7a(10)-S1 and S1 groups against SARS-CoV-2 variants of concern. Data were analyzed for comparison against the S1 group. Results are shown as the mean ± SEM of five BALB/c mice in each group. Differences between groups were indicated by one-way ANOVA for (A) and unpaired Student's *t* tests for (B). The asterisk mark indicates a statistically significant difference (ns: no significant difference; $*P < 0.05$; $**P < 0.01$).

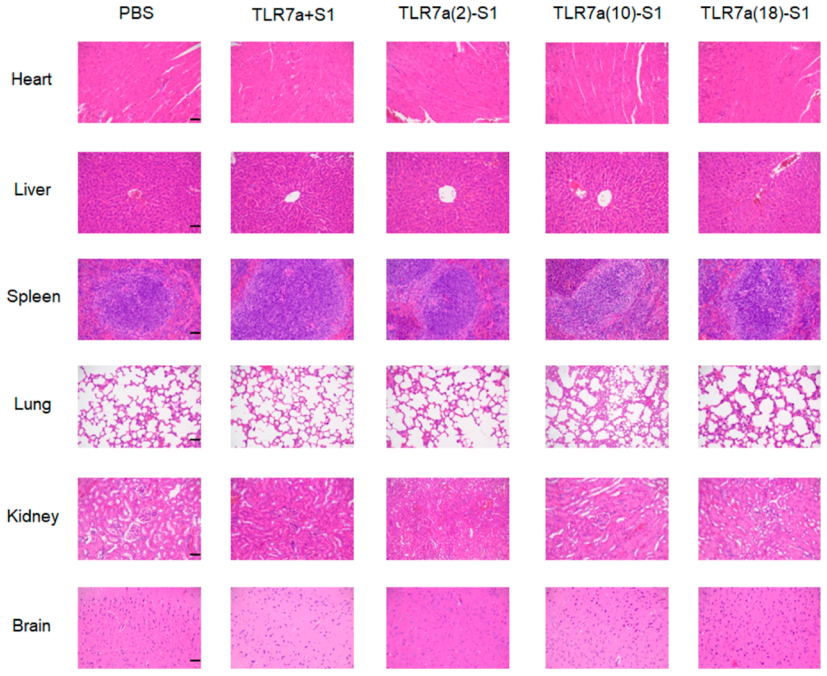

Figure 5. Adjuvant-protein conjugate TLR7a(10)-S1 is safe in mice. Histological sections (H&E staining) of the major organs of immunized mice, including heart, liver, spleen, lung, kidney, and brain. Scale bar = 100 μm.

mice immunized with TLR7a(10)-S1 demonstrated a robust ability of neutralization. The pseudovirus neutralization ID_{50} ($pVNT_{50}$) titers of S1, Alum + S1, TLR7a + S1, and TLR7a(10)-S1 were 743, 921, 854, and 1324, respectively. In addition, the $pVNT_{50}$ titer of TLR7a(10)-S1 group was significantly better than that of TLR7a(2)-S1 and TLR7a(18)-S1. Antibodies elicited by TLR7a(10)-S1 effectively neutralized the virus and prevented infection of the host cells, demonstrating its ability to provide protective immunity against SARS-CoV-2.

Neutralization Activity of Variants of Concern. Due to the pandemic of variants caused by the high mutagenicity of the SARS-CoV-2 virus, the ability to neutralize variants has become an important indicator for evaluating vaccines, especially for variants of concern, including B.1.1.7/alpha, B.1.351/beta, P.1/gamma, B.1.617.2/delta, and B.1.1.529/omicron.[1,47] For the pseudovirus neutralization assay, the $pVNT_{50}$ values of PBS control mouse sera against SARS-CoV-2 and variants of concern were less than 50 (data not shown). Serum from mice immunized with TLR7a(10)-S1 was neutralized against the B.1.1.7/alpha ($pVNT_{50}$ = 1640), B.1.351/beta ($pVNT_{50}$ = 728), P.1/gamma ($pVNT_{50}$ = 1580), B.1.617.2/delta ($pVNT_{50}$ = 755), and B.1.1.529/omicron ($pVNT_{50}$ = 227) (Figure 4B). The $pVNT_{50}$ values of TLR7a(10)-S1 group against all variants of concern were higher than those of S1 group. For the new omicron variant, TLR7a(10)-S1 maintained its neutralizing activity, although the neutralizing titer decreased 16.3-fold compared to wild-type pseudovirus, probably due to immune escape caused by many mutant sites in the S1 subunit protein.[48]

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Safety Evaluation of Vaccine Candidates. We estimated the safety of the vaccines in mice. The mice showed no obvious discomfort, such as scratching the inoculation site. Also, the inoculated mice showed no significant hair or behavioral changes compared to healthy mice during daily feeding. As indicated in Figure 5, histological examination of spleens from mice immunized with TLR7a(10)-S1 showed no structural disruption of the white pulp and no increased cellularity in the red pulp. Moreover, no significant differences were observed in the histological appearance of the liver, lung, heart, and kidney samples collected from each group compared to the PBS group. These results indicated that the adjuvant-protein conjugate TLR7a(10)-S1 has good biological safety as a COVID-19 vaccine candidate.

CONCLUSIONS

In conclusion, we successfully prepared the adjuvant-protein conjugate TLR7a-S1 as vaccine candidate, in which the molecular TLR7 agonist was covalently conjugated to S1 protein. In this vaccine construct, the built-in adjuvant molecule TLR7a provided adjuvant cluster effect and exhibited low toxicity, and the conjugation also provided co-delivery of adjuvant and antigen. As the number of built-in TLR7a increased, a bell-shaped reaction was observed for the first time in three TLR7a-S1 conjugates, with TLR7a(10)-S1 eliciting a more potent immune response than TLR7a(2)-S1 and TLR7a(18)-S1. By optimizing the number of built-in adjuvants, we found that the vaccine candidate TLR7a(10)-S1 triggered a more robust humoral immunity and a more balanced Th1/Th2 immune response in mice. In addition, TLR7a(10)-S1 exhibited good cross-neutralization ability against all variants of concern. However, the viral challenge and the longevity of the immune response are required in further studies. This adjuvant-protein conjugate vaccine strategy provides a good option for the antiviral vaccine development.

METHODS

Immunization of Mice. Animal experiments were approved by the Ethics Review Committee for Life Science Study of Central China Normal University and were performed in accordance with all national or local guidelines and regulations. Female BALB/c mice (6−8 weeks) were purchased and fed daily by Experimental Animal Centre of Huazhong Agriculture University (Wuhan, China). All mice were divided into six groups of five mice each. Each mouse was vaccinated three times with 2 week interruptions by subcutaneous injection, and blood was collected on days 14, 28, and 35. Serum was collected by centrifugation (8000 rpm, 4 °C) for 15 min and stored at −30 °C.

Enzyme-Linked Immunosorbent Assay of Antibody Titers. Recombinant S1 protein was coated on 96-well plates (Corning 3590) with 1 μg/mL of carbonate buffer (pH 9.5) at 4 °C overnight. The next day, the plates were washed three times with PBST [0.05% (v/v) Tween 20 in PBS] and blocked with 3% (w/v) BSA in PBS at 37 °C. After washing three times with PBST, mouse serum was diluted with PBS solution containing 0.1% BSA, added to the plates, and incubated for 1 h at 37 °C. Then, the plates were washed three times, incubated with diluted anti-mouse IgG/IgM/IgG1/IgG2a/IgG2b/IgG3 HRP-labeled secondary antibody for 1 h at 37 °C, washed five times with PBST, and treated with TMB solution for 5−10 min at room temperature. Finally, the reaction was stopped with 2 M H_2SO_4 and the absorbance (450 nm) was measured with a microplate reader.

ELISpot Assay. Spleens of immunized mice were removed, grinded, and filtered through a cell strainer. Next, cells were collected by centrifugation and lysed with a lysis solution to remove the red blood cells. Splenocytes suspensions were added to ELISpot plates pre-coated with IFN-γ capture antibody at 10^6 cells/100 μL per well. Then, an overlapping peptide library of spike protein was used to stimulate the cells for 24 h. After incubation, the cells were lysed and biotinylated detection antibody and streptavidin-HRP were added. Plates were treated with AEC solution at 37 °C for 30 min. Last, the reactions were terminated by washing the plates with deionized water and spots were counted using the ELISpot reader after natural drying.

Intracellular Cytokine Staining and Flow Cytometry. Mouse splenocytes were added to 24-well plates at 10^6 cells per well. The cells were stimulated with the overlapping peptide library for about 3 h. Next, monensin and brefeldin A were added to block protein transport and the plates were incubated for 12 h at 37 °C in a 5% CO_2 incubator. Cells were collected by centrifugation and stained with anti-CD3, anti-CD4, and anti-CD8 markers for 30 min at 0 °C. After washing, the cells were fixed and permeabilized and stained with anti-TNF-α and anti-IFN-γ markers for 30 min at 4 °C. Cells were analyzed with a CytoFLEX S flow cytometer (Beckman Coulter).

Pseudovirus Neutralization Assay. The heat-inactivated mice serum, twofold diluted with Opti-MEM, was added to plates (Corning 3610). The SARS-CoV-2 spike protein pseudovirus (Yeasen Biotech, Cat: 11906ES50) was diluted with Opti-MEM and incubated with serum samples for 1 h at room temperature. The medium was mixed with an equal volume of pseudovirus as the negative control. HEK293T cells overexpressing ACE2 were counted, and the cell concentration was adjusted to 3×10^5 cells/mL with a complete medium. At the end of co-incubation with serum samples and pseudovirus, the prepared ACE2-HEK293T cells were removed and 50 μL of cell suspension was added to each well. After incubation for 48 h, the cells were lysed with a lysis solution at room temperature for 15 min, and luciferase activity was measured by the Luciferase Reporter Gene Assay Kit (Yeasen Biotech, Cat: 11401ES60). Pseudovirus neutralization ID50 titers ($pVNT_{50}$) were calculated with 50% relative light units (RLU) compared with the virus control. Similarly, B.1.1.7/alpha (GM-0220PV33), B.1.351/beta (GM-0220PV32), P.1/gamma (GM-0220PV47), B.1.617.2/delta (GM-0220PV45), B.1.1.529/omicron (GM-0220PV84), and wild-type pseudovirus (GM-0220PV07) cross-neutralization assay were carried out. The heat-inactivated mice serum was serially diluted and co-incubated with pseudovirus. Next, the mixture was incubated with HEK293T-ACE2 cells (GM-C09233) for 48 h. The luminescence was measured using a Bio-Lite Luciferase Assay System (GM-040501B), and RLUs were read using a Spark multimode microplate reader (Tecan).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.2c00259.

MALDI-TOF-MS spectrum; HPLC spectrum; anti-S1 IgM antibody; anti-S1 IgG antibody over time; IgG

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subtypes after three immunizations; composition of each vaccine candidate; and experimental details, materials and methods, and NMR spectra for TLR7a-NHS (PDF)

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Author Contributions

†R.-Y.Z. and S.-H.Z. contributed equally to this work.

Author Contributions

R.Y.Z., S.H.Z., and J.G. designed experiments, analyzed the data, and prepared the manuscript. J.W., Y.W., and X.G.Y. developed the experimental methods and discussed the results. R.Y.Z., C.B.H., and R.R.F. performed experiments. G.F.Y. and J.G. supervised the research and the entire project. All authors reviewed and approved the version for publication.

Notes

The authors declare no competing financial interest.

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