Report

A recombinant receptor-binding domain in trimeric form generates protective immunity against SARS-CoV-2 infection in nonhuman primates

Limin Yang,[1,7,*] Deyu Tian,[1,7] Jian-bao Han,[4,7] Wenhui Fan,[1] Yuan Zhang,[1] Yunlong Li,[1] Wenqiang Sun,[1] Yanqiu Wei,[1] Xiaodong Tian,[2] Dan-dan Yu,[3,4] Xiao-li Feng,[4] Gong Cheng,[5,6] Yuhai Bi,[1,2] Yong-tang Zheng,[3,4,*] and Wenjun Liu[1,2,5,*]

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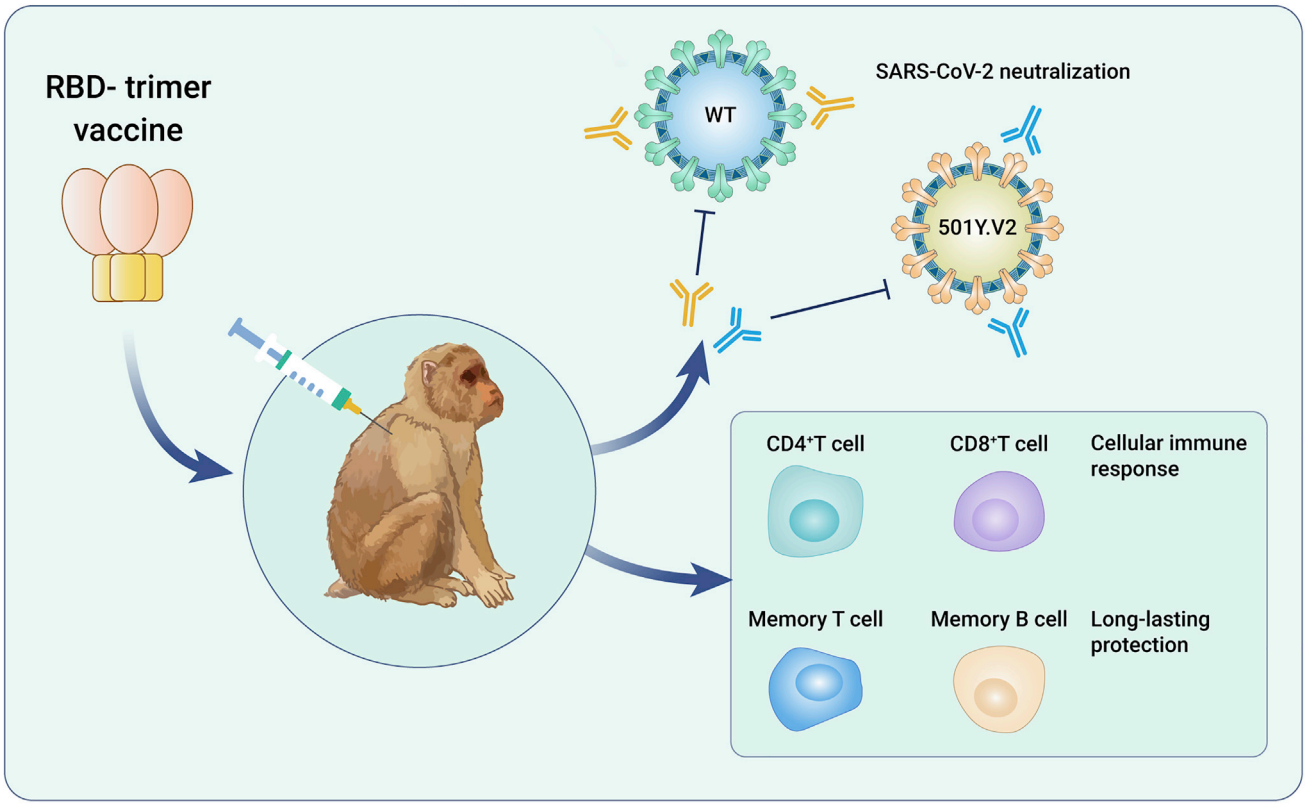
Graphical abstract



Public summary

- A SARS-CoV-2 trimeric vaccine candidate demonstrates safe, long-lasting, broad, and significant immunity protection in nonhuman primates
- The vaccine-induced antibodies can effectively neutralize the SARS-CoV-2 501Y.V2 variant
- A booster vaccination can quickly activate the memory immune response to avoid re-infection

Report

A recombinant receptor-binding domain in trimeric form generates protective immunity against SARS-CoV-2 infection in nonhuman primates

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A safe and effective vaccine is critical to combat the COVID-19 pandemic. Here, we developed a trimeric SARS-CoV-2 receptor-binding domain (RBD) subunit vaccine candidate that simulates the natural structure of the spike (S) trimer glycoprotein. Immunization with the RBD trimer-induced robust humoral and cellular immune responses, and a high level of neutralizing antibodies was maintained for at least 4.5 months. Moreover, the antibodies that were produced in response to the vaccine effectively cross-neutralized the SARS-CoV-2 501Y.V2 variant (B.1.351). Of note, when the vaccine-induced antibodies dropped to a sufficiently low level, only one boost quickly activated the anamnestic immune response, conferring full protection against a SARS-CoV-2 challenge in rhesus macaques without typical histopathological changes in the lung tissues. These results demonstrated that the SARS-CoV-2 RBD trimer vaccine candidate is highly immunogenic and safe, providing long-lasting, broad, and significant immunity protection in nonhuman primates, thereby offering an optimal vaccination strategy against COVID-19.

Keywords: COVID-19; SARS-CoV-2; RBD; subunit vaccine; NHPs; variants

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified as the causative agent for the novel coronavirus disease (COVID-19),[1] and the ongoing worldwide epidemic has resulted in the infection of more than 2.3% (over 176 million people) of the world's population, causing severe economic burden and hindering social development worldwide. There is an urgent need to develop safe and effective vaccines to end the current health crisis. Researchers worldwide are racing to develop COVID-19 vaccines; more than 200 vaccine candidates are in development, and 102 are in clinical trials.[2] Multiple vaccine development strategies have been pursued simultaneously, including viral vectors,[3–5] inactivated whole viruses,[6,7] DNA,[8,9] RNA,[10–12] subunits,[13,14] virus-like particles,[15] and live attenuated viruses (World Health Organization [WHO]). Vaccines developed by different approaches have distinct advantages and application limitations.

SARS-CoV-2 is a member of the *Betacoronavirus* genus of the *Coronaviridae* family of enveloped RNA viruses and is homologous to SARS and Middle East respiratory syndrome (MERS) coronaviruses. The viral surface spike (S) glycoprotein mediates receptor binding and cell entry and is the primary target for vaccine design.[16] To mimic the native S trimer structure, an artificially designed S trimer was constructed by fusing the C-terminal region of human type Iα collagen, and this construct was used for vaccine research.[17] Previous studies have found that both SARS-CoV and MERS-CoV display antibody-dependent enhancement (ADE), which may increase the risk of vaccinations enhancing viral infection.[18,19] The primary mechanism is that the non-neutralizing antibodies (non-NAbs) produced in response to the vaccine mediate virus infection via the fragment crystallizable (Fc) receptor.[19] A recent study has proved that antibodies against the S protein N-terminal domain enhanced the binding capacity of S protein to the cell surface receptor angiotensin-converting enzyme 2 (ACE2) and infectivity of SARS-CoV-2.[20] Hence, the potential ADE effect needs to be carefully considered in designing COVID-19 vaccines. To mitigate the ADE effect, minimizing non-neutralizing epitopes and keeping only the critical neutralizing epitope to elicit robust protective immunity is a solution. The receptor-binding domain (RBD) located at the C terminus of the S1 subunit has thus attracted attention. Several lines of evidence have indicated that RBD-specific antibodies could minimize the ADE effect,[21] and most discovered potent NAbs target the RBD region.[22,23] SARS-CoV-2 initiates viral replication by binding via the RBD to ACE2. Hence, the RBD can be used as a vaccine target to block viral attachment. In addition, the RBD possesses T cell epitopes, which can induce antiviral T cell responses.[12,13] However, the RBD has a low molecular weight, which leads to its weak immunogenicity.[14] Some researchers have tried to express the RBD by fusing it with the Fc domain or tandem repeat and preparing it as a dimer, thereby greatly enhancing its immunogenicity.[14,24] Some evidence has shown that the NAb level in SARS-CoV-2-infected humans significantly declines from the second month and can even be lost,[25] causing those individuals to be reinfected.[26] This short-lasting antibody protection poses a severe challenge for vaccine development. In addition, SARS-CoV-2 is characterized by a high mutation rate, and a growing body of evidence has shown that the 501Y.V2 variant (B.1.351), which emerged in South Africa, severely reduced the protective efficacy of mRNA vaccines.[27–29] Therefore, a vaccine strategy that can produce persistent and broad protection is particularly preferred.

In this study, we designed and developed an RBD trimer as a candidate SARS-CoV-2 subunit vaccine. A single immunization elicited the rapid production of protective NAbs in all rhesus macaques. Booster immunization

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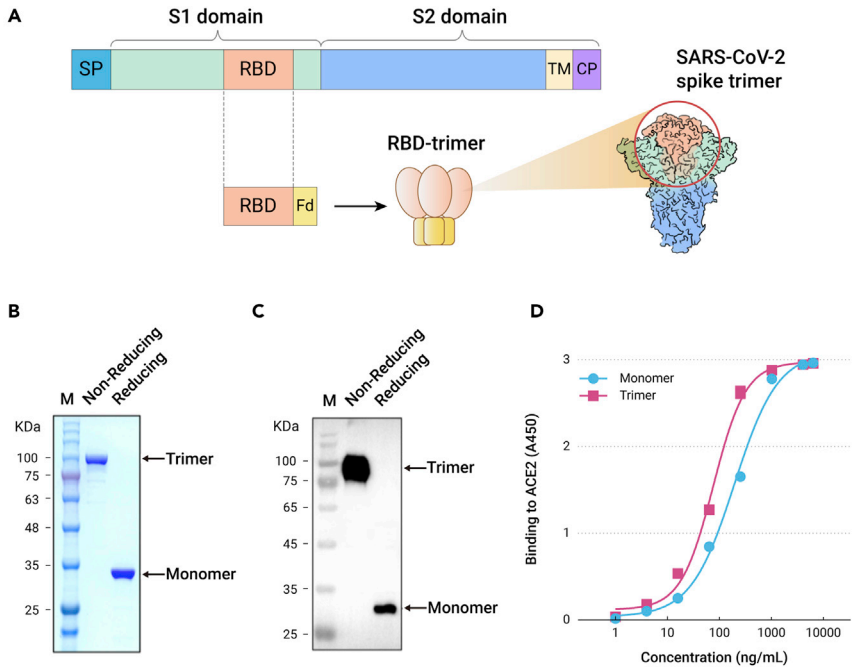

Figure 1. Characterization of the SARS-CoV-2 RBD trimer
(A) Schematic design of the SARS-CoV-2 RBD trimer.
(B and C) The purified recombinant protein was analyzed using SDS-PAGE (B) and western blotting with a polyclonal antibody against SARS-CoV-2-S1 (C). M, Marker. Ten micrograms of recombinant protein was loaded in each lane.
(D) Binding of RBD monomer or trimer to ACE2 as determined by ELISA. Nonlinear regression was performed using a log (agonist) versus normalized response curve and a variable slope model ($R^2 > 0.95$).

induced a robust immune response, and high NAb titers, $CD4^+$ as well as $CD8^+$ T cell immune responses developed. The immune protection period lasted for at least 4 months (neutralization titer >100). Moreover, booster immunization could immediately activate the memory immune response, allowing an immunized individual to regain immune protection within a week. In addition, the vaccine-induced antibodies presented cross-neutralizing activity against the 501Y.V2 variant with slightly decreased potency. Our vaccine candidate conferred significant protection against SARS-CoV-2 infection in vaccinated nonhuman primates (NHPs).

RESULTS

Construction of a SARS-CoV-2 RBD trimer vaccine

To simulate the native SARS-CoV-2 S protein trimeric form and improve conformational homogeneity, a natural trimerization domain of T4 bacteriophage fibritin (foldon) was fused to the C terminus of the SARS-CoV-2 RBD protein (Figure 1A). An interleukin-10 (IL-10) signal peptide was added to the N terminus of the RBD to improve peptide secretion. The recombinant protein, named RBD trimer, was ectopically expressed in human embryonic kidney (HEK293) cells. The RBD trimer was purified by Ni-NTA affinity chromatography and gel filtration. The purity and antigenicity of the recombinant protein were assessed by SDS-PAGE (Figure 1B) and western blot (Figure 1C) assays under reducing and nonreducing conditions. With the help of foldon, the recombinant RBD protein polymerized into a stable homogeneous trimer (~100 kDa), which dissociated into monomers (~33 kDa) under reducing conditions. According to the N-terminal sequence of the RBD trimer, the IL-10 signal peptide was efficiently cleaved off. The binding affinity of RBD trimer or monomer for recombinant ACE2 protein was determined using enzyme-linked immunosorbent assay (ELISA). The RBD trimer demonstrated higher binding affinity than the monomer (Figure 1D). We chose the AddaVax adjuvant, an MF59-like nanoemulsion, to prepare our vaccine formula to better stimulate the cellular immune response. The AddaVax-adjuvanted RBD trimer protein was used throughout the following immunization experiments in animals.

Immunization with the RBD trimer elicited robust humoral and cellular immune responses in rhesus macaques

To investigate the immunogenicity of the SARS-CoV-2 RBD trimer vaccine, five male rhesus macaques (3–6 years old) were vaccinated with either RBD trimer or phosphate-buffered saline (PBS) as placebo. The animals were intramuscularly immunized with 50 μg of RBD trimer with AddaVax adjuvant (n = 3, animal numbers: 140829, 140271, 163957) or PBS (n = 2, animal numbers: 17,361, 17321) at 0, 3, and 21 weeks. All macaques were phlebotomized as outlined in Figure 2A. Serum was isolated for ELISA and neutralizing activity tests, in which NAbs were measured by a standard plaque-reduction neutralization test, a lentiviral vector pseudoneutralization test (LVV-PsN), and a surrogate virus neutralization test (sVNT) based on antibody-mediated blockage of the binding of ACE2 to the RBD. The average neutralizing titers of the vaccinated animals reached approximately 100 (50% effective concentration, EC_{50})/200 (sVNT) and 1,000 (EC_{50})/4,000 (sVNT) after the first and second immunizations, respectively, and then gradually decreased to 130 (EC_{50})/400 (sVNT) at week 21. Considering a previous report that NAbs can achieve full immune protection as long as the live virus neutralizing titer reaches 50 (EC_{50}),[6] these data suggest that our RBD trimer vaccine can induce rapid and durable protective immunity for no less than 4.5 months in macaques. The second booster immunization produced extremely high neutralizing titers (2,600 EC_{50}, 8,000 sVNT) (Figures 2B, 2C, and S1A). Similar to the NAb results, RBD-specific ELISA IgG titers in the vaccination group gradually increased and peaked after the booster immunizations (Figure 2D). To evaluate whether the 501Y.V2 variant is resistant to neutralization by serum from animals vaccinated with RBD trimer, we tested the neutralizing activity of serum against the original SARS-CoV-2 strain (wild type [WT]) and 501Y.V2 variant on day 7 post-second boost using the LVV-PsN assay. The results showed that the neutralizing titers (half-maximal inhibitory concentration, NT_{50}) against the 501Y.V2 variant was approximately 1,030, which was approximately 30% lower than that against the WT strain (NT_{50} = 1,500) (Figures 2E and S1B), suggesting that our vaccine can elicit strong cross-NAbs against the 501Y.V2 variant. Neither RBD-specific binding antibodies nor NAbs were detected in the sham-vaccinated macaques.

For evaluation of SARS-CoV-2-specific T cell responses, peripheral blood mononuclear cells (PBMCs) were isolated after the second dose, and the $CD4^+$ and $CD8^+$ T cells producing interferon-γ (IFN-γ) were measured by enzyme-linked immunospot (ELISPOT) and intracellular cytokine staining assays. The level of IFN-γ secreted by PBMCs significantly increased in the SARS-CoV-2-S1 stimulation group compared with the sham group (140–440 spots/10^6 cells from 7 to 21 spots/10^6 cells, Figure 2F). Moreover, the proportion of IFN-γ-secreting $CD4^+$ T cells (IFN-γ^+/$CD4^+$) among all $CD4^+$ T cells increased from 0.12% to 0.32%–0.44% (Figures 2G and 2I), and the

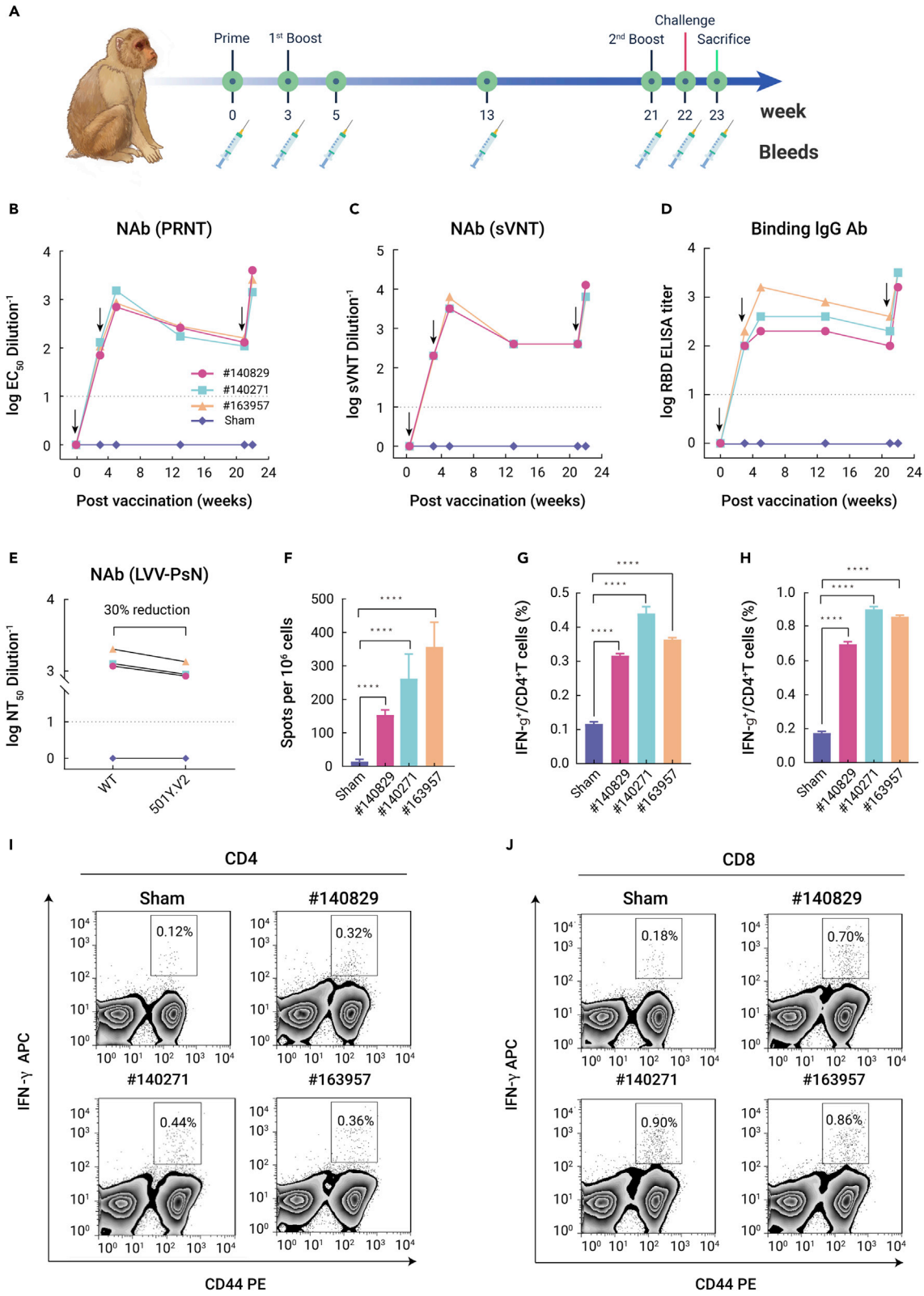

Figure 2. Immunogenicity of the RBD trimer in rhesus macaques (A) Flow chart of the experimental design. Solid lines indicate weeks of immunization (black), challenge (red), and sacrifice (green), and syringes indicate sampling weeks. (B–D) (B and C) NAb titers were measured in serum samples by plaque-reduction neutralization test (PRNT) assay (B) and a surrogate virus neutralization test (sVNT) (C), and (D) an RBD-specific ELISA was used to assess binding antibody titers. (E) Neutralization of the original Wuhan-Hu-1 (WT) and 501Y.V2 variant viruses by the second boost serum was measured with the use of a recombinant lentiviral-based pseudovirus neutralization assay (LVV-PsN). Measurements below the detection limit were assigned a value of 1. (F–J) (F) PBMCs isolated from the immunized and sham groups 2 weeks after the first booster immunization were collected for IFN-γ analysis by ELISPOT assay after stimulation with S1 protein. The proportion (G and I) of $CD4^+$ T cells producing IFN-γ among all $CD4^+$ T cells and the proportion (H and J) of $CD8^+$ T cells producing IFN-γ among all $CD8^+$ T cells were evaluated by intracellular cytokine staining. The dotted lines indicate the detection limit. Data are shown as the means ± SEM (standard errors of the mean). p values were determined by Student's t test (****$p < 0.0001$).

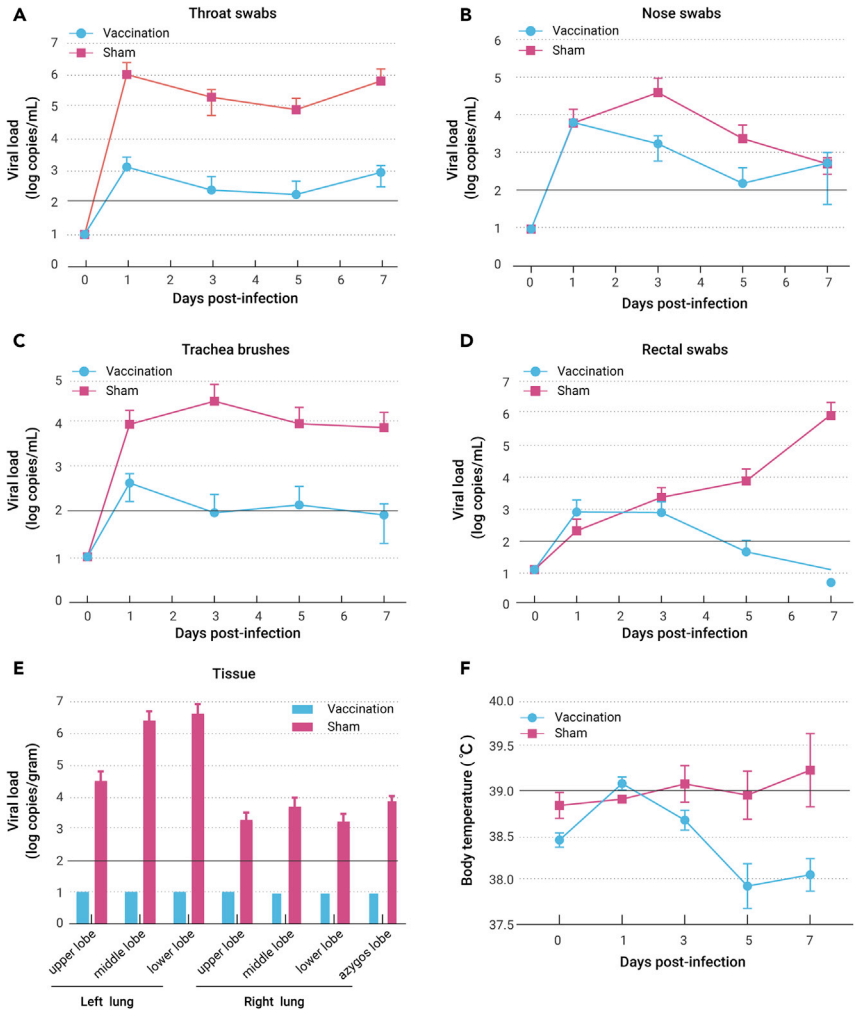

Figure 3. Protective efficacy of the RBD trimer vaccine in rhesus macaques Macaques were intranasally and tracheally challenged with SARS-CoV-2. (A–E) Viral loads in throat swabs (A), nasal swabs (B), tracheal brushes (C), rectal swabs (D), and lung tissues (E) were measured using qRT-PCR.
(F) The body temperature of animals in each group after infection. The dotted lines indicate the detection limit. Measurements below the limit of detection were assigned a value of 10. The solid lines represent the upper limit of the standard body temperature reference value. Data are shown as the means ± SEM.

proportion of IFN-γ-secreting $CD8^+$ T cells (IFN-γ^+/$CD8^+$) reached 0.70%–0.9% from 0.18% (Figures 2H and 2J), demonstrating that immunization with RBD trimer developed a virus-specific cellular immune response.

The RBD trimer vaccine provides significant protection against SARS-CoV-2 challenge in rhesus macaques

We next assessed the protective efficacy of the RBD trimer vaccine against SARS-CoV-2 infection in rhesus macaques; all macaques were intranasally and tracheally challenged with 1×10^7 $TCID_{50}$ of the SARS-CoV-2 20SF107 strain 9 days after the second booster vaccination (Figure 2A). Throat, nose, and rectal swabs, and tracheal brush specimens, were collected at different times during post-infection. All animals were euthanized 7 days post-infection (dpi) to assess the viral load and lesions in the respiratory tissues. High concentrations of viral RNA were detected in swab specimens from the throat, nose, and rectum swabs, and tracheal brushes, of the control animals throughout the experimental period (Figures 3A–3D). The viral loads in nasal swabs and tracheal brushes of control macaques gradually decreased after peak values at 2 dpi. However, the viral loads in anal swabs increased gradually from 2 dpi, suggesting that the virus was excreted through the digestive tract. In contrast, a small amount of viral RNA was detected in the swabs and tracheal brushes at 1 dpi and then quickly dropped to an extremely low level in the RBD trimer-immunized macaques. All seven different lung lobe tissues from the control macaques showed high levels of viral genomic RNA. In contrast, no viral RNA was detected in the immunized macaques, indicating that our RBD vaccine candidate could confer full protective immunity in lung tissues (Figure 3E). In addition, no clinical signs were found in the immunized macaques after challenge, while the control macaques exhibited symptoms of infection, including cough, malaise, and fervescence (Figure 3F).

Histopathological analysis of the fixed lung tissues was performed with hematoxylin-eosin staining. No visible pathological change or inflammatory cell infiltration was observed in the lung tissues from immunized macaques (Figures 4A and 4B). In contrast, severe histopathological changes were found in the control macaques, including apparent thickened alveolar walls, mononuclear inflammatory cell infiltration, focal exudation, hemorrhage, and architecture disappearance (Figures 4C and 4D). Immunofluorescence staining assays showed that multiple pneumocytes in the lung sections from the control macaques were positive for SARS-CoV-2 S protein staining, especially along the airway (Figures 4G and 4H). In contrast, no viral antigen was detected in the lung tissues from the immunized animals (Figures 4E and 4F).

To further evaluate memory humoral and T cell immune responses in vaccinated macaques after challenge, paired serum and PBMCs were collected from vaccinated animals before and after SARS-CoV-2 challenge, and changes in the NAb titer and the percentage of $CD4^+$ and $CD8^+$ T cells producing cytokines in response to SARS-CoV-2 were analyzed. The results showed that the NAb titers did not change 1 week after viral challenge, indicating that no anamnestic humoral immune response was induced (Figure 5A). Previous studies showed that, when the vaccine candidate fails to generate a sufficient protective immune response, the challenge will enhance NAb titers[12]; therefore, our vaccine protection is highly effective. Simultaneously, the proportions of $CD4^+$ and $CD8^+$ T cells secreting IFN-γ, IL-2, IL-4, and tumor necrosis factor alpha (TNF-α) increased significantly (approximately 1.5- or 2.5-fold increase in $CD4^+$ or $CD8^+$ T cells), suggesting that

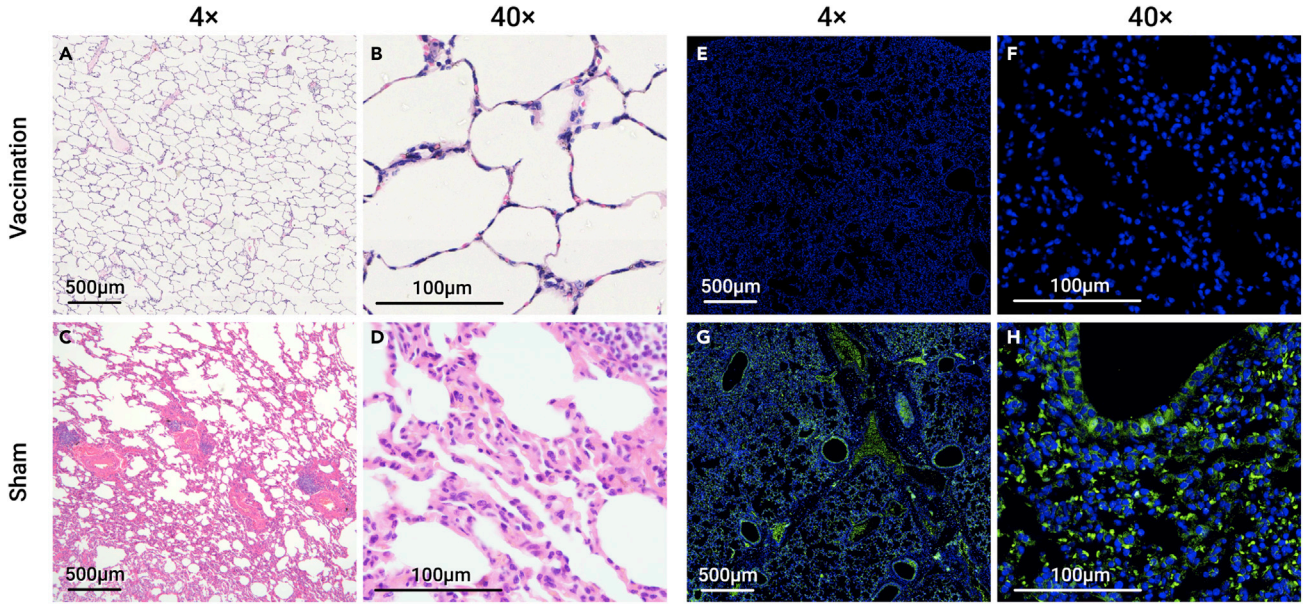

Figure 4. Hematoxylin-eosin (H&E) and immunofluorescence staining analysis of lungs from rhesus macaques Macaques immunized with RBD trimer or PBS were sacrificed at 7 dpi. (A–D) Lung tissue sections were stained with H&E. (E–H) Immunofluorescence staining detection of the SARS-CoV-2-S protein. Images of different tissue pathologies at low magnification (4×) and higher magnification (40×) are shown. Scale bars, 100 μm in (B, F, D, H) or 500 μm in (A, C, E, G).

the SARS-CoV-2-specific memory T cell immune response led to cytokine secretion after infection (Figures 5B–5E). Virus-induced type 1 cytokines (IFN-γ, IL-2, and TNF-α) can promote potent cytotoxic T lymphocyte responses, which facilitate clearance of infected cells and infectious virus.

Hematology and serum biochemistry of all macaques were assessed at different times after the infection. The number of platelets in the control macaques increased and reached a peak at 5 dpi, indicating hemorrhage in the tissues (Figure S2), which suggested blood cell exudation in the lung blood vessels. In addition, the number of lymphocytes in the control macaques slightly increased after infection, indicating virus infection. The concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the serum of the control macaques increased and exceeded the upper reference range at 1 and 2 dpi, suggesting that these macaques had liver injury after SARS-CoV-2 challenge (Figure S3). In contrast, the hematology and biochemical values of immunized macaques stayed within a normal range after viral challenge.

DISCUSSION

The severe COVID-19 pandemic and the precipitously increasing number of deaths worldwide necessitate the urgent development of SARS-CoV-2 vaccines. Global researchers are racing toward safe and effective vaccines against COVID-19 based on multiple platforms, and some vaccines have received emergency use authorization, including mRNA vaccines (Moderna, Pfizer/BioNTech), inactivated virus vaccines (Sinopharm, Sinovac), and adenovirus vector-based vaccines (AstraZeneca, CanSino, Johnson & Johnson). The mRNA vaccines demonstrate excellent protective efficacy (>90%), but they need to be stored in ultralow temperature conditions, specifically, −20°C (Moderna) or −70°C (Pfizer). The cold chain can account for 80% of the cost of vaccination, and this temperature requirement poses a challenge for deployment as a large amount of cold chain equipment is needed. In addition, studies have shown that newly emerged SARS-CoV-2 variants can escape the neutralizing responses of mRNA vaccines.[27–29] This increases the risk of infection after vaccination. Adenovirus vector vaccines require high doses to achieve the expected immune protection due to pre-existing immunity, which increases the risk of side effects. Moreover, they were found to be associated with rare cases of blood clots after vaccination.[30] Inactivated vaccines can induce antibody production but cannot effectively elicit a cellular immune response. Although antibodies have been shown to play a critical role in protection against coronavirus infections, the T cell response is still indispensable for virus clearance, decreasing severe illness, and prognostic recovery.[31] Subunit vaccines are considered the safest vaccine strategy. Hepatitis B and human papillomavirus subunit vaccines, which have been widely administered, showed good safety. A SARS-CoV-2 RBD-Fc fusion subunit vaccine can induce a strong humoral immune response in NHPs but fails to develop a T cell response. After challenge, high concentrations of viral RNA were detected in the lungs of vaccinated NHPs, suggesting that the vaccine did not provide adequate protection.[24] Another RBD tandem repeat dimer vaccine was also unable to elicit a T cell response in a mouse model.[14]

To improve the immunogenicity of the recombinant RBD protein and better mimic the native trimeric SARS-CoV-2 S structure, we fused a trimer motif to make the RBD form a stable trimer structure; the construct will not dissociate even if the temperature is above 60°C, and there was no change after 3 months of storage at 4°C. Western blot analysis showed that, compared with the monomer, the trimer binds to the SARS-CoV-2 S1 polyclonal antibody more strongly, suggesting that the conformational epitope is better retained. NHPs can obtain protective NAb levels (EC_{50} titer of 100) with a single dose of our candidate vaccine, indicating that it can be used for emergency vaccination of high-risk populations. There are few reports about the persistence of NAb responses induced by COVID-19 vaccine candidates. To evaluate the candidate's protection persistence, our immunity cycle spanned half a year. The results showed that, 18 weeks after the second vaccination, the antibody level gradually decreased to an EC_{50} NAbs titers of 130. Although the antibody level was much lower than that immediately after vaccination, it was still higher than the protective antibody level (≥50 EC_{50}),[6] suggesting that this vaccine can achieve no less than 4.5 months of long-lasting humoral protection. Moreover, the vaccine-induced cellular immune response also helps extend the immune protection period due to the virus-specific memory T cells that can be maintained for a long time.[32] Considering that SARS-CoV-2 may coexist with humans for a long time, a vaccine that can generate acute and durable immune protection is of great value.

Due to the high variability in SARS-CoV-2, multiple novel variants have recently been identified and are now spreading worldwide, such as D614G, 501Y.V1 (B.1.1.7), 501Y.V2 (B.1.351), 501Y.V3 (P1), and 452R.V3

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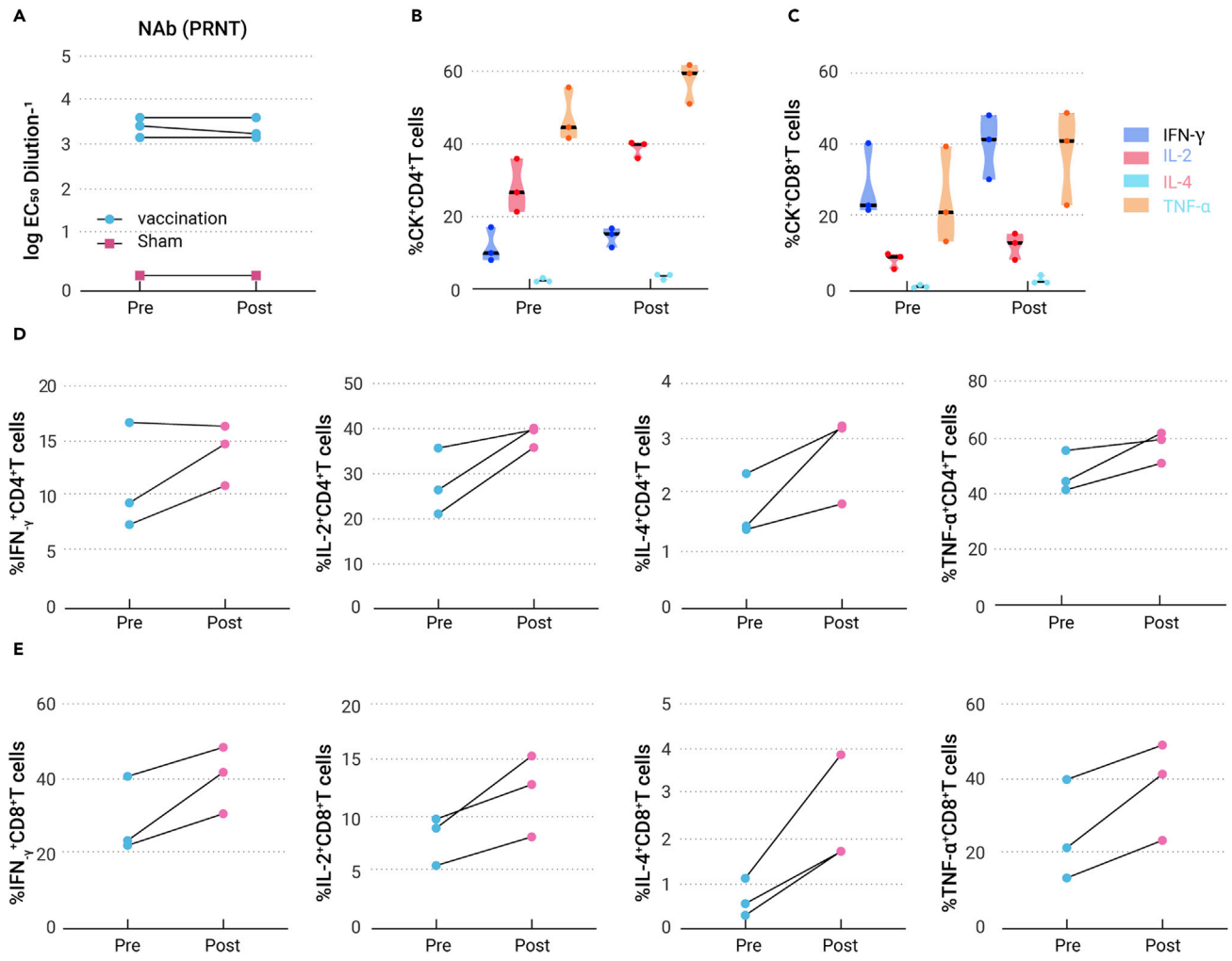

Figure 5. NAb and memory T cell responses induced by SARS-CoV-2 infection in vaccinated rhesus macaques Serum and PBMCs were collected and isolated from macaques before infection (Pre) and 7 dpi (Post). (A) Serum NAb titers were measured by PRNT assay. Cells were stained to assess intracellular production of IFN-γ, IL-2, IL-4, and TNF-α. (B and C) The grouped violin plot graphs show the mean changes in the total percentage of $CD4^+$ and $CD8^+$ T cells expressing the indicated cytokine. (D and E) Each line represents an individual animal.

(B.1.617), which appear to be more easily transmitted.[33,34] There is a growing concern that variants harboring RBD mutations could impair the protective efficacy of vaccines.[28,29,35] Therefore, the broadly protective effects of the vaccine are also a focus of our attention. Previous research reported that squalene-based adjuvants, such as MF59 or AF03, could increase the broadly protective potency of the influenza virus vaccine by stimulating a cross-neutralizing humoral response.[36,37] Compared with aluminum adjuvants, squalene-based adjuvants have been shown to induce a more balanced humoral and cellular immune responses.[38] Therefore, we chose the AddaVax adjuvant, a squalene-based oil-in-water emulsion similar to MF59, to formulate our candidate vaccine. Excitingly, the antibodies produced in response to our vaccine showed good cross-neutralizing activity against the N501Y.V2 variant with slightly decreased potency. In addition, high titers of NAbs better prevent the immune escape of evolving variants,[28] which should be another reason why our vaccine has good cross-neutralization activity. The enhanced breadth of protective response is valuable in the ongoing pandemic because it is difficult to predict the subsequent emergence of variants. The emerging variants, including N501Y.V2, N501Y.V3, P2, P3, and 452R.V3, showed resistance to vaccine-induced NAbs, notably N501Y.V2; thus we evaluated its cross-neutralization activity with RBD trimer-induced antibodies. In addition, although our RBD trimer vaccine formula induced a potent immune response in NHPs, it induced a weak immune response in the BALB/c mouse model. This result is contrary to a previous report of an inactivated vaccine.[6] We think that this adjuvant or our formula is not suitable for the mouse model, which leads to immune weakness. Considering the international 3R principle (replacement, reduction, and refinement), we aimed to minimize the number of NHPs, so we only used five experimental monkeys in this project. Due to the limitation of the number of experimental animals, most of our data cannot reach significant differences.

NAbs are considered to play a vital role in preventing SARS-CoV-2 infections. However, the T cell immune response is also a critical effector in virus clearance and clinical symptom alleviation.[31] Moreover, previous studies have shown that the duration of the memory T cell response induced by infection is much longer than that of antibody level alterations, so cellular immunity is crucial for the durability of vaccine protection.[32,39,40] In addition, as the level of NAbs gradually decreases after vaccination or infection, non-NAbs may promote infection via the ADE effect. Nevertheless, virus-specific T cell immunity can reduce this potential risk. An ideal vaccine should be able to induce humoral and cellular immune responses in a balanced manner. Compared with the RBD monomer, the RBD trimer generated a higher level of NAbs, resulting in more potent protection. It is surprising that, by formulating an AddaVax adjuvant, our vaccine candidate also demonstrated a strong $CD4^+$ and $CD8^+$ T cell immune response in NHPs; the T helper 1 (T_H1) cells and cytotoxic T cells promote the elimination of virus-infected cells, conferring robust protection. SARS-CoV-2 infects hosts mainly in the

respiratory tract and lung, and pneumonia is the typical symptom of COVID-19.[41,42] In this study, sham-immunized macaques showed severe pneumonia, whereas macaques immunized with our vaccine candidate exhibited significant protection. Furthermore, no viral RNA or S protein was detected in the lungs of the immunized macaques by qPCR or immunostaining assays, which proved that the vaccine candidate conferred full immunity protection. SARS-CoV-2 attacks host organs beyond the lung; liver injury has also been recorded.[43] In this work, after challenge, the ALT and AST values of the unimmunized animals increased, indicating liver lesions caused by SARS-CoV-2. There were no abnormalities in the vaccinated animals, which means that our vaccine candidate may protect the animals from liver injury. In short, our candidate vaccine can effectively protect the organs of NHPs from virus attacks.

Compared with young people, the frail middle-aged population has higher risks of severe COVID-19 and a higher mortality rate, and the virus replicates more efficiently in their lungs and other organs.[44,45] Therefore, the development of vaccines that offer protection to middle-aged and frail people is particularly valuable. Thus, our vaccination group included a frail macaque (no. 140829, 6 years old, 4.8 kg), which was only half the weight of macaques of the same age. This animal can better simulate the candidate vaccine's immune and protective efficacy in frail people. Compared with the other two macaques, the frail macaque had a slightly weaker immune response and lower antibody levels during the first two vaccinations. However, after the third immunization, its immune responses were no different from those of the other macaques. This frail monkey was also fully protected by RBD trimer vaccination, suggesting that it could provide adequate protection against COVID-19 to middle-aged and frail people.

Since the antibody level induced by SARS-CoV-2 infection or vaccination does not last very long, when the antibody level in an individual decreases to a level insufficient to achieve immune protection, there is a risk of re-infection. If there is another outbreak, how can individuals quickly obtain protective immunity? Our results proved that only one booster immunization could quickly result in immune protection for the vaccinated NHPs. Notably, after the COVID-19 pandemic is under control, if SARS-CoV-2 appears again, people who have been vaccinated can be immediately administered a booster immunization to avoid another large-scale outbreak.

In summary, our RBD-based subunit vaccine candidate induced robust humoral and cellular immune responses and protected NHPs against SARS-CoV-2 infection, and it also showed good safety. There were no abnormalities in body temperature, weight, clinical signs, pathology, hematology, or biochemical indicators during vaccination and viral challenge. These data proved the possibility that our RBD trimer is a promising candidate vaccine to prevent COVID-19.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.M.Y. and W.J.L.; methodology, L.M.Y., D.Y.T., J.-B.H., and W.H.F.; investigation, L.M.Y., Y.Z., W.Q.S., Y.L.L., X.D.T., Y.Q.W., D.-D.Y., and X.-L.F.; visualization, L.M.Y., Y.H.B., and G.C.; funding acquisition, L.M.Y., W.J.L., and Y.H.B.; project administration, L.M.Y. and W.J.L.; supervision, W.J.L., Y.-T.Z., and Y.H.B.; writing – original draft, L.M.Y. and D.Y.T.; writing – review & editing, W.J.L., Y.H.B., and G.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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