

Immunological evaluation of an inactivated SARS-CoV-2 vaccine in rhesus macaques

Hongbo Chen,^{1,6} Zhongping Xie,^{1,6} Runxiang Long,^{1,6} Shengtao Fan,^{1,6} Heng Li,^{1,6} Zhanlong He,^{1,6} Kangwei Xu,^{5,6} Yun Liao,¹ Lichun Wang,¹ Ying Zhang,¹ Xueqi Li,¹ Xingqi Dong,² Tangwei Mou,¹ Xiaofang Zhou,³ Yaoyun Yang,¹ Lei Guo,¹ Jianbo Yang,¹ Huiwen Zheng,¹ Xingli Xu,¹ Jing Li,¹ Yan Liang,¹ Dandan Li,¹ Zhimei Zhao,¹ Chao Hong,¹ Heng Zhao,¹ Guorun Jiang,¹ Yanchun Che,¹ Fengmei Yang,¹ Yunguang Hu,¹ Xi Wang,¹ Jing Pu,¹ Kaili Ma,¹ Lin Wang,⁴ Chen Cheng,¹ Weiguo Duan,¹ Dong Shen,¹ Hongling Zhao,¹ Ruiju Jiang,¹ Xinqiang Deng,¹ Yan Li,¹ Hailian Zhu,¹ Jian Zhou,¹ Li Yu,¹ Mingjue Xu,¹ Huijuan Yang,¹ Li Yi,¹ Zhenxin Zhou,¹ Jiafang Yang,¹ Nan Duan,¹ Huan Yang,¹ Wangli Zhao,¹ Wei Yang,¹ Changgui Li,⁵ Longding Liu,¹ and Qihan Li¹

¹Institute of Medical Biology, Chinese Academy of Medicine Sciences & Peking Union Medical College, Yunnan Key Laboratory of Vaccine Research and Development on Severe Infectious Diseases, Kunming 650118, China; ²Yunnan Provincial Infectious Disease Hospital, Kunming 650301, China; ³Yunnan Center for Disease Control and Prevention, Kunming 650034, China; ⁴Kunming Third People's Hospital, Kunming 650041, China; ⁵National Institute of Food and Drug Control, Beijing 100050, China

Because of the relatively limited understanding of coronavirus disease 2019 (COVID-19) pathogenesis, immunological analysis for vaccine development is needed. Mice and macaques were immunized with an inactivated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccine prepared by two inactivators. Various immunological indexes were tested, and viral challenges were performed on day 7 or 150 after booster immunization in monkeys. This inactivated SARS-CoV-2 vaccine was produced by sequential inactivation with formaldehyde followed by propiolactone. The various antibody responses and specific T cell responses to different viral antigens elicited in immunized animals were maintained for longer than 150 days. This comprehensive immune response could effectively protect vaccinated macaques by inhibiting viral replication in macaques and substantially alleviating immunopathological damage, and no clinical manifestation of immunopathogenicity was observed in immunized individuals during viral challenge. This candidate inactivated vaccine was identified as being effective against SARS-CoV-2 challenge in rhesus macaques.

INTRODUCTION

Since the end of last year, a new species of coronavirus, a contagious agent capable of causing acute and severe respiratory infection and pneumonia through airborne transmission, has rapidly caused global public concern and even fear.^{1–3} This coronavirus was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the World Health Organization (WHO).⁴ Updated data indicate that more than 40 million infections have occurred worldwide,⁵ and the mortality rate is approximately 3%–10%, with most deaths occurring in the elderly population.^{6,7} In this emergency pandemic situation,^{8–10} vaccine development is urgently needed.^{11,12} However, as SARS-CoV-2 is a new viral agent with an unknown infection mechanism and an unclear interaction with the immune system,

the evaluation of vaccines requires a comprehensive analysis of the immunological index observed in immunized animals, which involves the characterization of the antigenic component of this virus and its immunogenicity, the protective immunity elicited by the viral antigen via certain immune processes, the period in which immunity persists, and whether the process of developing immunity against a viral antigen is associated with immunopathogenesis during viral infection.^{13,14} Based on these considerations, the following hypothetical model was used as the basis for the present study: SARS-CoV-2 infects cells via the binding of its spike (S) protein to angiotensin converting enzyme 2 (ACE2) molecules on the cell membrane, indicating that the S protein, particularly the receptor-binding domain (RBD) that directly interacts with ACE2, is a major viral antigen that elicits a neutralizing antibody response,^{15,16} while the nucleocapsid (N) protein, the other major structural component, functions as an antigenic stimulator of innate and adaptive immune responses.^{17,18} Our work here describes an inactivated SARS-CoV-2 vaccine that was developed based on the aforementioned model, and the characterized immune response revealed the various antibody responses and specific T cell responses to different viral antigens that were maintained

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⁶These authors contributed equally

Correspondence: Qihan Li, Institute of Medical Biology, Chinese Academy of Medicine Sciences & Peking Union Medical College, Yunnan Key Laboratory of Vaccine Research and Development on Severe Infectious Diseases, Kunming 650118, China.

E-mail: liqihan@imbcams.com.cn

Correspondence: Longding Liu, Institute of Medical Biology, Chinese Academy of Medicine Sciences & Peking Union Medical College, Yunnan Key Laboratory of Vaccine Research and Development on Severe Infectious Diseases, Kunming 650118, China.

E-mail: liuld@imbcams.com.cn

Correspondence: Changgui Li, National Institute of Food and Drug Control, Beijing 100050, China.

E-mail: changguili@aliyun.com



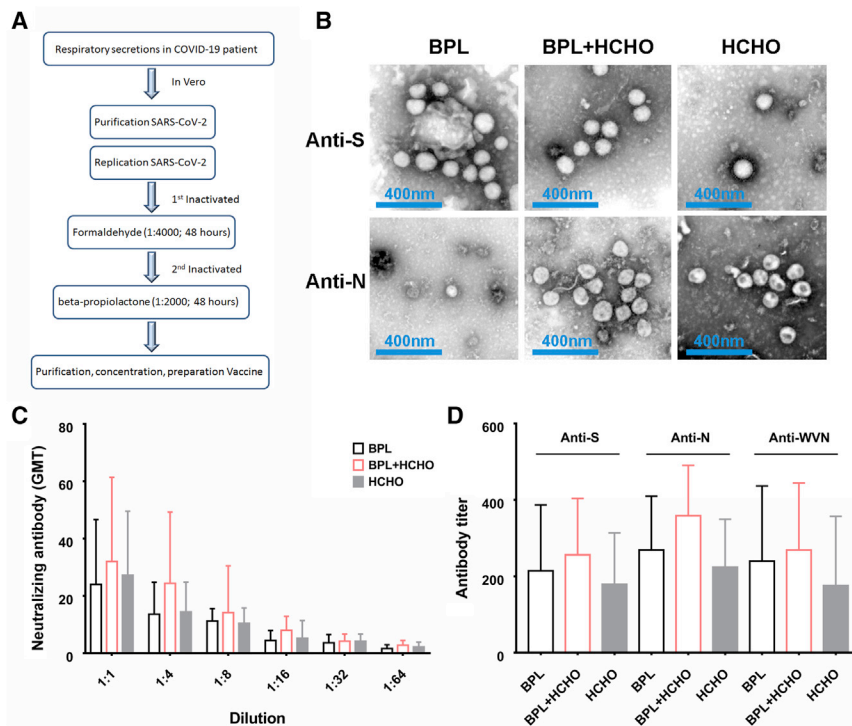


Figure 1. Design of the SARS-CoV-2 inactivated vaccine and analysis of its antigenicity and immunogenicity

(A) Technical strategy used to produce the SARS-CoV-2 inactivated vaccine. (B) The interaction of inactivated SARS-CoV-2 antigens exposed to BPL, BPL-HCHO, or HCHO treatment with anti-S and anti-N antibodies. The inactivated viral antigens exposed to treatment with the three inactivators were coincubated with the anti-S or anti-N antibody for 2 h at 37°C in PBS and observed with an electron microscope (25,000X). The result was based on observations of 50 fields with a 90% coincidence rate. (C) Neutralizing antibodies induced by a single intraperitoneal injection of the inactivated vaccine in gradient doses in BALB/c mice. $n = 5$ mice per group. Geometric means \pm SDs. (D) Specific antibodies against the S and N proteins and the virion induced by inactivated vaccine treated with different inactivators in BALB/c mice. The mice were immunized with the vaccine at a dose of 100 EU. $n = 5$ mice per group. Geometric means \pm SDs.

for longer than 150 days. Importantly, this immune response was capable of defending against viral infection in rhesus macaques immunized with the vaccine without any possible immunopathology-related damage, as indicated by histopathological observations and the detection of specific cytokines. Based on the data, the immunity elicited by the vaccine in macaques exhibited comprehensive immunological characteristics and protected animals under viral challenge, suggesting that it may be applicable in a clinical trial.

RESULTS

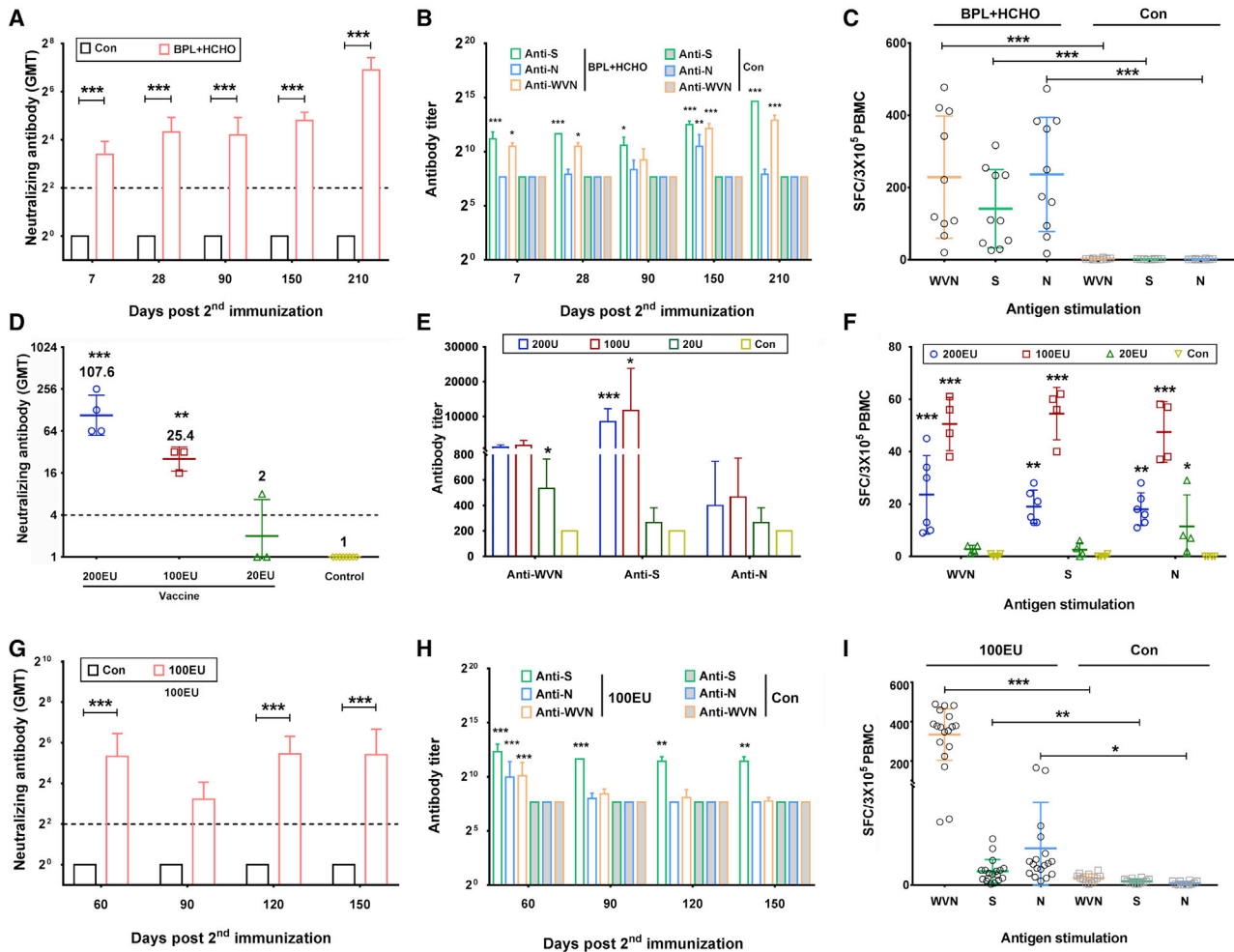
Antigenic design of an inactivated SARS-CoV-2 vaccine based on an analysis of viral antigenicity and immunogenicity

According to the currently available data, several structural proteins with different features that might be related to viral immunogenicity are incorporated in the SARS-CoV-2 virion.^{19–21} We first investigated the antigenicity of the viral structural proteins by performing a series of immunologic tests using convalescent serum from patients with COVID-19 (coronavirus disease 2019) to design the antigenic formula. Not only the S protein but also the N protein and other proteins were recognized by the serum, as the virus was neutralized by the antibodies contained in the sample; this recognition was more extensive than that observed by immune blotting with a monoclonal antibody (mAb) against the S or N protein (Figure S1). Furthermore, a technical evaluation of the vaccine showed that an appropriate inactivation process enables virions to present S and N antigens. This inactivation process included an initial inactivation step with formalin (1:4,000) for 48 h upon harvesting the virus, a purification step, and a second inactivation step with beta-propiolactone (BPL; 1:2,000) for 48 h (Figure 1A). In the subsequent electronic micro-

scopic observation, the virions inactivated by the treatment with both agents were simultaneously recognized by antibodies specific for N and S, but the control virions inactivated by treatment with only one of the two inactivators were recognized by only either the anti-S or the anti-N antibody (Figure 1B). These results were further evaluated in immunized BALB/c mice after a single intraperitoneal injection of the inactivated vaccine in gradient doses. The detection of neutralizing antibodies suggested that the vaccine inactivated by treatment with both inactivators elicited a higher antibody titer than the vaccines inactivated by treatment with only one of the two control inactivators at each dilution of antigen (Figure 1C). The enzyme-linked immunosorbent assay (ELISA) results indicated the induction of antibodies specific for the S protein, N protein, and virions (Figure 1D). Collectively, these data support the antigenicity and immunogenicity of our inactivated SARS-CoV-2 vaccine.

Dynamic immune response elicited by the antigens of the SARS-CoV-2 vaccine in mice and rhesus macaques

Based on the antibody response in mice that were immunized intraperitoneally with the SARS-CoV-2 inactivated vaccine once (Figures 1C and 1D), further immunization of mice with the vaccine in two intramuscular inoculations at an interval of 14 days was analyzed using antibody assays from days 7 to 150 after booster immunization. Based on the results, the elicited antibody response, including neutralizing antibodies and specific antibodies against viral proteins in these immunized mice, presented a dynamic variation and was consistently positive for up to 5 months, eliciting a T cell response against the viral S or N protein or the virion at day 150 (Figures 2A–2C). Increasing neutralizing antibody titers were observed on day 7 after booster inoculation, with geometric mean titers (GMTs) of 107.6, 25.4, and 2 in 10 macaques from group A immunized with the three doses (200, 100, and 20 ELISA units [EU], respectively) on days 0 and 14 (Figure 2D).



Moreover, titers of less than 1:4 were observed in 2 macaques from the low-dose group (i.e., 20 EU). This result suggests a dose dependence for immunization with this vaccine. However, ELISA with plates coated with the S or N protein or the purified virion indicated no obvious differences in the titers of anti-S, anti-N, and anti-virion antibodies (Figure 2E). Furthermore, an enzyme-linked immunosor-

bent spot (ELISPOT) analysis of interferon- γ (IFN- γ) specificity at day 7 post immunization showed a positive T cell response with no dose dependency after stimulation with the S or N antigen (Figure 2F). In a further study, 18 macaques from group C immunized with 100 EU/dose by the same procedure were monitored for 5 months and showed a dynamic trend of a neutralizing antibody response

Table 1. Clinical symptoms of immunized monkeys during viral challenge

Clinical symptoms	Challenge on 7 days after booster				Challenge on 150 days after booster	
	Control		Vaccine		Control	Vaccine
	(n = 10)	200 EU (n = 4)	100 EU (n = 3)	20 EU (n = 3)	(n = 12)	100 EU (n = 18)
Fever	100.0 (69.2, 100.0)	0.0 (0.0, 60.2)	0.0 (0.0, 70.8)	0.0 (0.0, 70.8)	66.7 (34.9, 90.1)	0.0 (0.0, 18.5)
Cough	0.0 (0.0, 30.8)	0.0 (0.0, 60.2)	0.0 (0.0, 70.8)	0.0 (0.0, 70.8)	0.0 (0.0, 26.5)	0.0 (0.0, 18.5)
Reduced food intake	90.0 (55.5, 99.7)	0.0 (0.0, 60.2)	0.0 (0.0, 70.8)	0.0 (0.0, 70.8)	83.3 (51.6, 97.9)	0.0 (0.0, 18.5)
Reduced activity frequency	90.0 (55.5, 99.7)	0.0 (0.0, 60.2)	0.0 (0.0, 70.8)	0.0 (0.0, 70.8)	83.3 (51.6, 97.9)	0.0 (0.0, 18.5)
Diarrhea	0.0 (0.0, 30.8)	0.0 (0.0, 60.2)	0.0 (0.0, 70.8)	0.0 (0.0, 70.8)	0.0 (0.0, 26.5)	0.0 (0.0, 18.5)
Vomiting	0.0 (0.0, 30.8)	0.0 (0.0, 60.2)	0.0 (0.0, 70.8)	0.0 (0.0, 70.8)	0.0 (0.0, 26.5)	0.0 (0.0, 18.5)

Data are shown as % (95% CI).

associated with specific antibodies against viral proteins, which was maintained for up to 150 days postinfection (dpi; [Figures 2G and 2H](#)), while ELISPOT detection supported the existence of immune memory ([Figure 2I](#)).

The immunoprotective effect induced by the vaccine antigen inhibits viral replication in macaques and substantially alleviates pathological damage to tissues

We observed various indicators related to the pathogenesis of SARS-CoV-2 infection in macaques after viral challenge to evaluate the effectiveness of this vaccine. The monkeys in groups A and B were challenged at 7 dpi and macaques in groups C and D were challenged at 150 dpi to confirm the effectiveness of the vaccine ([Figure S2](#)). In the challenge test for groups A and B, the 10 immunized macaques and 10 control macaques treated with the adjuvant controls were intranasally challenged with wild-type virus at a titer of 2×10^5 CCID₅₀ (50% cell culture infective dose) per animal. After viral challenge, the manifestations; the viral load in pharyngeal secretions, nasal secretions, and anal swab samples; and viremia were monitored daily ([Table 1](#); [Figures 3A and 3B](#)). Compared with the 10 macaques in the adjuvant control group, no immunized macaques exhibited an obvious fluctuation in body temperature ([Table 1](#)). The detection of viral loads indicated that viral shedding in the nasal cavity, i.e., the site of viral challenge, in macaques immunized with the inactivated vaccine was noticeably lower than that in the positive controls, indicating that the viral load decreased from 3×10^3 copies/100 μ L on day 1 to less than 50 copies/100 μ L on day 2 and was maintained at that level through 15 dpi, and the viral loads in pharyngeal and anal swabs were always less than 50 copies/100 μ L ([Figure 3A](#)). On the other hand, values of 10^3 – 10^4 copies/100 μ L and higher were detected in the pharyngeal, nasal, and anal swabs from macaques in the adjuvant control group for at least 8–9 days ([Figure 3A](#)). In the analysis of peripheral blood, no viral genome copies were detected in macaques from the inactivated vaccine group, but the copy number peaked on days 5–7 in macaques from the adjuvant control group ([Figure 3A](#)). Furthermore, we observed viral loads in tissues and pathological lesions in these tissues in immunized macaques, which were sacrificed under anesthesia on days 3, 5, 7, 9, or 15 after viral challenge. All tissue samples collected from these challenged animals,

including 5 adjuvant control-treated macaques, were examined to detect viral loads and for pathological observations. Based on the qRT-PCR results, the viral genome copy numbers in almost all samples of various tissues from the macaques in the vaccine immunization group were less than 50 copies/100 mg, and only the viral genome copy numbers in spleens were greater than 100 copies/100 mg ([Figure 3B](#)). Interestingly, the two macaques that were immunized with a low dosage of the vaccine, presented a neutralizing antibody titer lower than 1:4, and were positive for specific antibodies against the S and N proteins showed low viral loads that were similar to those of other immunized macaques with a positive neutralizing antibody response to viral challenge. For the macaques in groups C and D, viral challenge was performed at 150 dpi, followed by euthanasia on days 2, 4, 6, 8, 10, and 12 after viral challenge. Observation of all the challenged animals did not reveal any clinical manifestations in immunized macaques ([Table 1](#)). A similar decreasing trend in the viral loads detected in nasal and pharyngeal swabs from animals in group A was associated with lower viral loads detected in anal swabs and blood ([Figure 3C](#)). The examination of all tissue samples from sacrificed animals suggested obviously lower viral loads than those of the control macaques in group D ([Figure 3D](#)). The histopathological observation showed slight and nonspecific inflammatory reactions in the lung tissues from all immunized macaques, including a few local aggregations of inflammatory cells, inflammatory exudates in some alveoli and bronchioles, slight epithelial tissue hyperplasia in a few alveolar tissues, and slight congestive reactions, and these slight histological changes recovered quickly after days 5–7 ([Table 2](#); [Figure S3](#)). However, lung tissues from macaques in the adjuvant control group exhibited more severe inflammatory reactions, which were recognized as indicators of interstitial pneumonia that persisted for 10 additional days based on histopathological observations ([Table 2](#); [Figure S3](#)). These results suggest that a valid immunoprotective effect was induced and maintained in vaccinated macaques.

Detection of some immune cells and cytokines related to immunopathogenicity

Because COVID-19 is an acute and severe inflammatory disease of the respiratory system,^{1,22} safety concerns regarding SARS-CoV-2 vaccines generally focus on immunopathology-related events

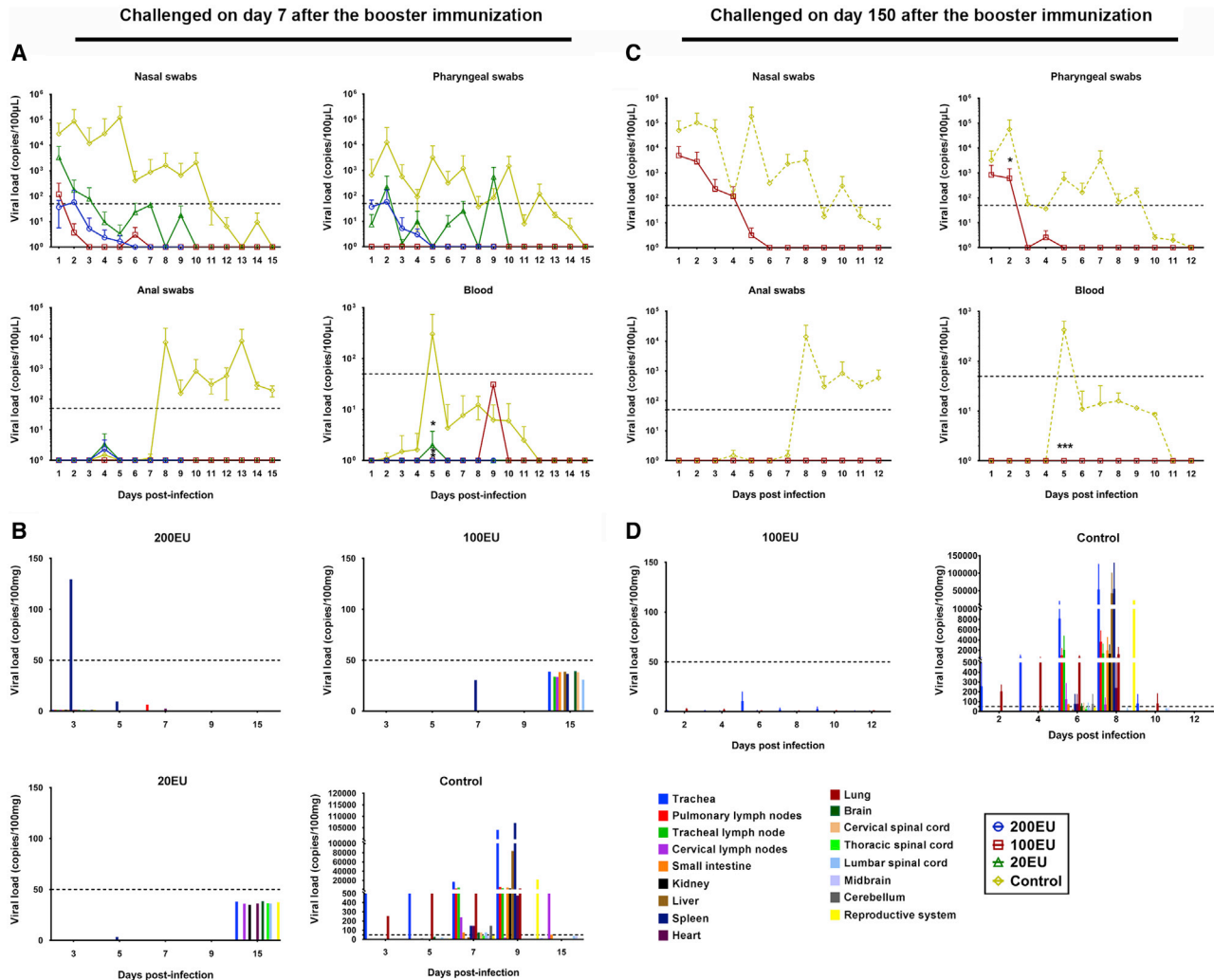


Figure 3. The integrated immune response elicited by the inactivated vaccine limits viral replication in the respiratory and/or alimentary tracts of rhesus macaques

(A and B) The viral loads in pharyngeal secretions, nasal secretions, anal swabs, and blood (A) and various organs (B) of monkeys that were immunized and challenged on day 7 after the booster immunization (groups A and B) were monitored. (C and D) The viral loads in pharyngeal secretions, nasal secretions, anal swabs, and blood (C) and various organs (D) of monkeys that were immunized and challenged on day 150 after the booster immunization (groups C and D) were monitored. A viral copy number of less than 50 copies/100 μ L (dotted lines) was considered negative. The results are presented as means \pm SDs. 200 EU (n = 4), 100 EU (n = 3), 20 EU (n = 3), and Con (n = 10) challenged on day 7 after the booster, and 100 EU (n = 18) and Con (n = 12) challenged on day 150 after the booster. Two-way ANOVA was used to determine significant differences between the vaccine group and the control group (Al(OH)₃ adjuvant). *p < 0.05; ***p < 0.001 versus the Control group.

occurring in immunized individuals in the setting of pandemics.¹³ Thus, we monitored the dynamic alterations in the peripheral blood mononuclear cell (PBMC) population in all macaques immunized with the inactivated vaccine during the first (groups A and B) and second (groups C and D) viral challenges. At 7 dpi, we observed increases in the proportions of T cells, natural killer (NK) cells, T cells with IFN- γ specificity, and regulatory T (Treg) cells; in particular, we observed increases in the percentages of IFN- γ -specific T cells and Treg cells on days 9 and 5 post viral challenge, respectively (Figure 4A), while no increases in the levels of cytokines, such as inter-

leukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α ; Figure 4B), which have been identified to be increased in peripheral blood from patients with COVID-19, were observed.^{23,24} After the second viral challenge at 150 dpi, similar percentages of immune cell populations and alterations in cytokine levels were observed (Figures 4C and 4D). The results observed in immunized animals differed significantly from the results observed in control animals (Figures 4A–4D). In addition, in the toxicity test, there were no significant pathological changes in the major tissues and organs of rats immunized with the inactivated vaccine (300 EU) compared to the control group (treated

Table 2. Pathological changes in major tissues and organs of rhesus macaques immunized with the inactivated vaccine and adjuvant control after challenge with the live virus.

Tissues	Control (n = 22)	Vaccine			Challenge on 150 days after booster
		Challenge on 7 days after booster			
		200 EU (n = 4)	100 EU (n = 3)	20 EU (n = 3)	100 EU (n = 18)
Lung	+	-	-	-	-
Trachea	+	-	-	-	-
Pulmonary lymph nodes	+	-	-	-	-
Tracheal lymph nodes	+	-	-	-	-
Cervical lymph nodes	+	-	-	-	-
Brain	-	-	-	-	-
Heart	±	-	-	-	-
Liver	±	-	-	-	-
Spleen	±	-	-	-	-
Intestinal	-	-	-	-	-

+ indicates that all experimental animals had pathological changes; - indicates that all experimental animals had no pathological changes; ± indicates that some experimental animals had pathological changes but some had no pathological changes.

with only the Al(OH)₃ adjuvant; Figure S4). Thus, immunopathology-related adverse events have not been considered to occur in immunized individuals—at least in nonhuman primates.

DISCUSSION

The SARS-CoV-2 pandemic has led to 40 million infection cases worldwide, and this serious situation is driving the rapid development of a suitable vaccine.^{3,25} An immunological question that must first be addressed to satisfy this urgent need for a vaccine is whether and how the antigen composition in a vaccine elicits effective immunity. However, no clear antigenic analysis of different viral structural proteins is available. We thus investigated the antigenicity of our inactivated vaccine with convalescent serum from patients with COVID-19 and observed various interactions among antibodies and viral structural proteins. The data from this study suggest that the S antigen elicits neutralizing antibodies and forms a barrier against viral infection, and the N antigen may also play an important role in stimulating immune memory.^{26,27} This result led to our antigenic design of an inactivated vaccine that exposes the immune system to both the S and N proteins upon the immunization of animals. This antigenic mode enables the elicitation of an integrated immune response composed of neutralizing antibodies, specific antibodies, and T cell responses against the S and N proteins and the virion in mice and macaques that were maintained for at least 5 months, and alterations in antibodies were observed in this period. In the viral challenge test with macaques at 7 dpi, a specific immunoprotective effect was observed in animals immunized with high and intermediate doses of the vaccine,

resulting in the production of neutralizing antibodies with GMTs of 107 and 25, respectively, and was associated with specific T cell responses to the S and N proteins and the virion as antigens. Interestingly, similar protective effects on macaques immunized with the low dose of the vaccine were observed; these macaques had a neutralizing antibody GMT of 2 and a T cell response similar to those observed in the high- and intermediate-dose groups. In the challenge test at 150 dpi, the immunoprotective effect on immunized animals was again confirmed. Based on these results, first, the immune response induced by our vaccine antigen provides systematic immune protection against viral infection by not only eliciting increases in neutralizing antibody titers but also inducing a specific T cell response associated with increased anti-N and anti-virion antibody levels. We postulated that integrated immunity involving antibodies against various viral proteins elicited by antigens of SARS-CoV-2 or its vaccine might be needed for the effective prevention of viral infection, at least in macaques. Second, the immunity elicited by our vaccine in macaques was maintained for at least 5 months, and even alteration of antibody levels was observed in this period. Collectively, all the data reported in this study logically lead to the conclusion that the S antigen and other viral antigens may play roles in the activation of innate immunity and the subsequent activation of the specific anti-viral immune response because viral proteins such as the N protein interact with pattern recognition receptors (PRRs) in cells, while the S protein substantially alters the production of neutralizing antibodies. If this hypothesis is confirmed, a valid systematic immunoprotective response to SARS-CoV-2 infection should include at least anti-S and anti-N antibodies, and innate immunity may have a substantial contribution to this response and should be considered in vaccine development.

MATERIALS AND METHODS

Virus and cells

The SARS-CoV-2 strain used in the present study was isolated from the respiratory secretions of an adult male patient at Yunnan Hospital of Infectious Diseases in Kunming in January 2020. The virus proliferated in Vero cells (ATCC, Manassas, USA) and was purified by plaque cloning. The cloned virus was identified via genomic sequencing and named KMS-1 (GenBank No: MT226610.1). Vero cells were cultured in DMEM (Corning, NY, USA) containing 5% fetal calf serum (FCS; HyClone, Logan, USA).

Viral infectivity titration

All experimental procedures were performed according to BSL3 requirements. Virus samples were serially diluted 10-fold with serum-free DMEM (Corning, NY, USA). Different dilutions of the virus were added to a 96-well plate. Each dilution (100 µL/well) was added simultaneously to 8 wells. Then, 100 µL of the Vero cell suspension (2.5×10^5 cells/mL) was added to each well. After the plate was incubated at 37°C in a 5% CO₂ atmosphere for 7 days, the cytopathic effect (CPE) was observed and assessed with an inverted microscope (Nikon, Tokyo, Japan). According to the classic method of viral titration, this method is appropriate for SARS-CoV-2 titration because the virus induces a typical CPE in Vero cells, depending upon the amount

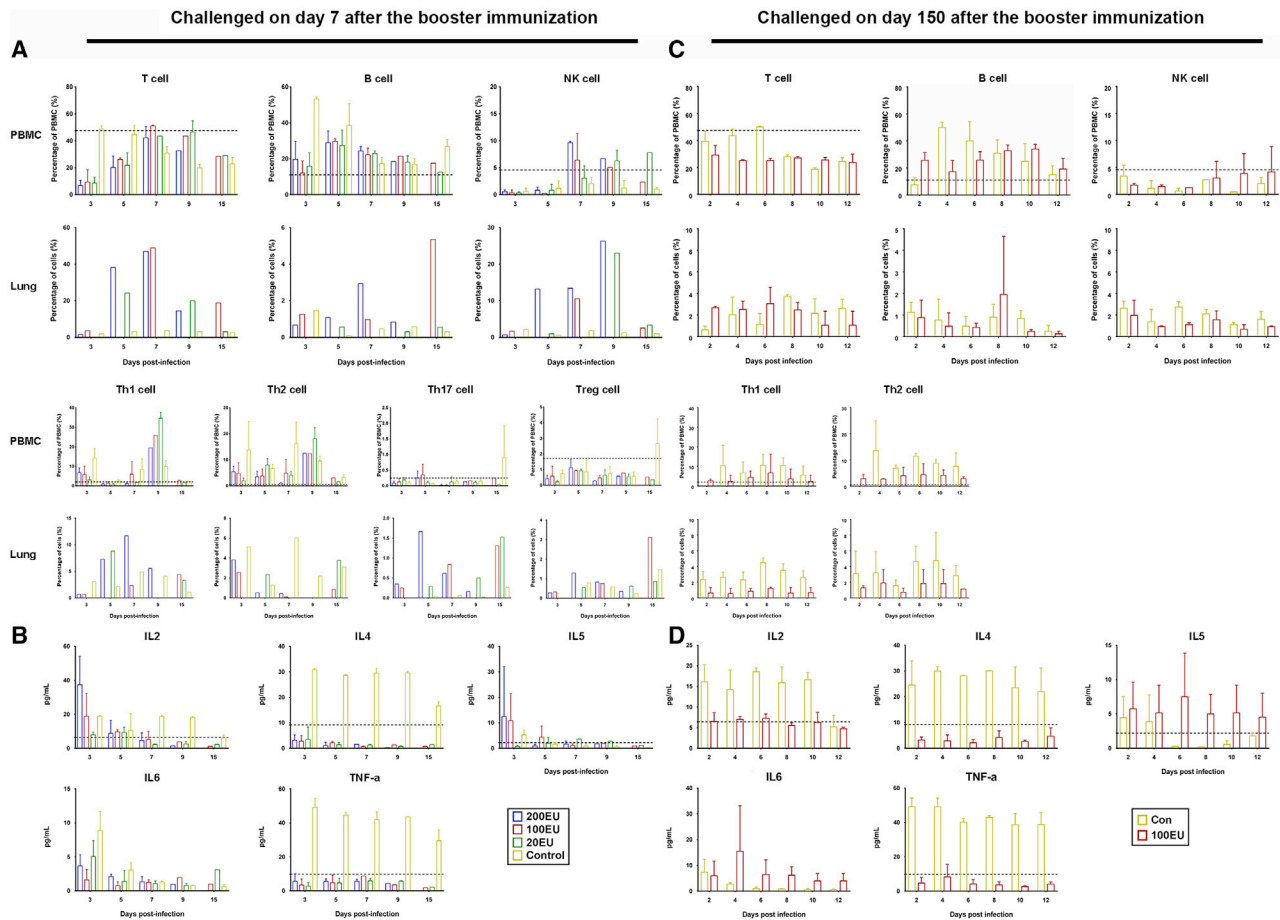


Figure 4. The dynamic levels of immune cells and cytokines are maintained in the normal range in macaques immunized with the inactivated vaccine during viral challenge

(A) Percentages of T, NK, B, Th1, Th2, Th17, and Treg cells among PBMCs or in lungs from rhesus macaques that were immunized and challenged on day 7 after the booster immunization (groups A and B). (B) Serum IL-2, IL-4, IL-5, IL-6, and TNF- α levels in rhesus macaques that were immunized and challenged on day 7 after the booster immunization (groups A and B). (C) Percentages of T, NK, B, Th1, Th2, Th17, and Treg cells among PBMCs or in lungs from rhesus macaques that were immunized and challenged on day 150 after the booster immunization (groups C and D). (D) Serum IL-2, IL-4, IL-5, IL-6, and TNF- α levels in rhesus macaques that were immunized and challenged on day 150 after the booster immunization (groups C and D). The normal range (referring to the normal macaques monitored during the same period) is indicated with dotted lines. 200 EU (n = 4), 100 EU (n = 3), 20 EU (n = 3), and Con (n = 10) challenged on day 7 after the booster, and 100 EU (n = 18) and Con (n = 12) challenged on day 150 after the booster. The results are presented as means \pm SDs.

of virus inoculated and the elapsed time (Figure S5A), as supported by the measurement of plaque-forming units (Figure S5B).

Inactivated vaccine

The inactivated SARS-CoV-2 vaccine was developed by the Institute of Medical Biology (IMB), Chinese Academy of Medical Sciences (CAMS). Briefly, the viral seed strain used to develop the vaccine was inoculated into a monolayer of Vero cells in an environment compliant with BSL3 requirements. The harvested virus was initially centrifuged at 5,000 rpm for 10 min to remove cell debris, and dual inactivation of the virus harvested from Vero cells was performed with formaldehyde (HCHO, 1:4,000; 48 h), followed by treatment with BPL propiolactone (1:2,000; 48 h) with an ultrafiltration-mediated concentration step and purification by column chromatography

between treatments. This process of inactivation simultaneously revealed the viral S protein and other viral proteins. The viral antigen content was measured using ELISA. A solution of the inactivated virus was then emulsified in 0.5 mg/mL Al(OH)₃ (determined based on the amount of aluminum ion) adjuvant to constitute the final vaccine product that was manufactured at a high dosage (200 EU/0.5 mL), medium dosage (100 EU/0.5 mL), and low dosage (20 EU/0.5 mL).

Ethical approval

Human studies

Convalescent serum samples were collected from patients diagnosed with pneumonia caused by the novel coronavirus at Yunnan Hospital of Infectious Diseases, Kunming Third People's Hospital, and the

CDC of Xianyang, Hubei Province. All patients provided informed consent. The protocols were reviewed and approved by the Experimental Management Association of the IMB, CAMS (approval number: DWSP 202003 004).

Animal studies

The animal experiment was designed and performed according to the principles in the “Guide for the Care and Use of Laboratory Animals” and “Guidance for Experimental Animal Welfare and Ethical Treatment.” The protocols were reviewed and approved by the Experimental Animal Management Association of the IMB, CAMS (approval number: DWSP 202003 005). All animals were fully under the care of veterinarians at the IMB, CAMS.

Animals

Mice

4-week-old female BALB/c mice (Beijing Vital River Laboratory Animal Technologies, Beijing, China) were housed in a laboratory (ABSL-3) within the specific pathogen-free facility at the IMB, CAMS. Mice were anesthetized by inhalation of 2% isoflurane for all procedures, and every effort was made to minimize suffering.

Monkeys

Rhesus macaques (age 1.5–2 years) were bred and fed a diet of pellets (IMB, CAMS, China) and fresh fruit in a laboratory (ABSL-3) at the IMB, CAMS.

Immunization

Mice

135 mice were included in this experiment. In the first stage, 95 mice were randomly divided into four groups, namely, the HCHO-inactivated ($n = 30$), BPL-inactivated ($n = 30$), BPL+HCHO-inactivated ($n = 30$), and control ($n = 5$) groups. For each group, the vaccine was diluted from an initial concentration of 100 EU (the viral antigen concentration determined by ELISA) per animal to 3.125 EU per animal by doubling the dilution and intraperitoneally inoculating once; each dilution was administered to 5 mice in the three experimental groups, and 5 mice in the control group were inoculated with $\text{Al}(\text{OH})_3/\text{PBS}$. Blood samples were collected for assays of neutralizing antibodies and specific antibodies against the viral protein on day 28 after injection. In the second stage, 40 mice were randomly divided into two groups and intramuscularly immunized with the vaccine at 100 EU per mouse or the adjuvant control according to the immunization procedure on days 0 and 14. Blood samples were collected for the detection of neutralizing antibodies and specific antibodies on days 7, 28, 90, 150, and 210 after the booster injection.

Monkeys

50 macaques were included in this experiment. In the first stage, 20 rhesus macaques were randomly divided into two groups: group A included 10 animals that were immunized intramuscularly with the vaccine at 200 EU ($n = 4$), 100 EU ($n = 3$), or 20 EU ($n = 3$) according to the immunization procedure on days 0 and 14; groups B contained 10 animals that were inoculated with the $\text{Al}(\text{OH})_3$ adjuvant alone as a

control. In the second stage, group C included 18 macaques that were each immunized intramuscularly with the vaccine containing 100 EU on days 0 and 14; group D included 12 macaques that were inoculated with $\text{Al}(\text{OH})_3$ as the adjuvant control. The macaques were tested for specific antibodies and the T cell response in blood samples collected at the following time points after the booster injection: day 7 (in groups A and B) and days 30, 60, 90, and 150 (in groups C and D).

Viral challenge

Macaques that had been immunized with the inactivated vaccine or treated with the adjuvant control were infected with SARS-CoV-2 (2×10^5 CCID₅₀/macaque) via nasal spray according to the requirements of BSL3. All animals were monitored daily for clinical signs. Pharyngeal secretions, nasal secretions, anal swabs, and blood samples were obtained daily after infection. Blood was collected under appropriate anesthesia to alleviate pain and minimize suffering.

For the animals examined in the first stage, one macaque from the 200 EU-dose vaccine group was euthanized at 3, 5, 7, and 9 dpi. In the 100 EU-dose vaccine group, one macaque was euthanized at 3, 7, and 15 dpi. In the 20 EU-dose vaccine group, one macaque was euthanized at 5, 9, and 15 dpi. In the adjuvant control group, one macaque was euthanized at each of the time points listed above (3, 5, 7, 9, and 15 dpi). The blood and organs of the euthanized macaques were obtained for various experiments. The remaining 5 macaques in the adjuvant control group were used to observe clinical manifestations and viral shedding. For the animals analyzed in the second stage, three macaques from group C and two macaques from group D were euthanized at 2, 4, 6, 8, 10, and 12 dpi for various analyses.

2D protein electrophoresis and western blot analysis

Purified virus samples were resuspended in 2D lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, and 2% IPG buffer). Total protein was quantified using a PlusOne 2D Quant Kit (GE Healthcare Europe GmbH, Freiburg, Germany) according to the manufacturer's instructions. Approximately 200 μg of protein was first separated based on the pI values on immobilized linear gradient strips (Immobiline DryStrip, Amersham Biosciences Europe GmbH, Freiburg, Germany) covering a pH range of 4–7, followed by separation on 12% SDS-PAGE gels. Proteins were transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% bovine serum albumin (BSA)-Tris-buffered saline/Tween 20 (Tris-HCl, 100 mM, pH 7.5; NaCl, 0.9%; and Tween-20, 0.2%) and reacted with convalescent serum, an anti-S protein antibody (Cat# 40591-T62; SinoBiological, Beijing, China), an anti-N protein antibody (Cat# 40143-R001; SinoBiological) and a horseradish peroxidase (HRP)-labeled secondary antibody (Cat# ab6721; Abcam, MA, USA) to visualize the proteins according to the standard protocol of the enhanced chemiluminescence (ECL) reagent (Cat#: P0018AM; Beyotime, Shanghai, China).

Neutralizing antibody test

Heat-inactivated serum from mice or macaques was serially diluted and coinoculated with live virus (100 lgCCID₅₀/well) for 2 h at

37°C. Then, 100 µL of the Vero cell suspension (10^5 cells/mL) was added to the mixture. The plates were then incubated at 37°C in a 5% CO₂ atmosphere for 7 days. The CPEs were observed and assessed with an inverted microscope (Nikon) to determine the neutralizing antibody titer of the serum. The GMTs of the neutralizing antibodies were measured.

ELISA

Separate wells of 96-well ELISA plates (Corning, NY, USA) were coated with the S protein (Cat# PNA002; expressed in HEK293 cells; Sanyou Biopharmaceuticals, Shanghai, China), the N protein (Cat# PNA006; expressed in HEK293 cells; Sanyou Biopharmaceuticals) and purified viral antigen at a concentration of 5 µg/well and incubated at 4°C overnight. Then, the plates were blocked with 5% BSA-phosphate-buffered saline (PBS), incubated with serially diluted serum samples, and visualized by a reaction with an HRP-conjugated antibody (Cat# ab6728, Cat# ab112767; Abcam, MA, USA) and TMB substrate (Cat# PR1200; Solarbio, Beijing, China) using previously described methods.²⁸ The absorbance of each well was measured at 450 nm in an ELISA plate reader (BioTek Synergy4; GeneCompany, Beijing, China), and the following equation was used: resulting OD (optical density) = (experimental well OD) - (mock well OD). The preimmunization sera of animals were used as the negative controls. A serum sample with an OD value at least 2.1-fold higher than the mean OD value of the negative control sera was defined as antibody positive, while a sample with an OD value less than 2.1-fold the mean OD value of the negative control sera was regarded as antibody negative. The endpoint titers were defined as the highest serum dilution showing a positive OD value compared with the negative control. The GMT was calculated as the geometric mean of the endpoint titers of positive sera in the same group.

ELISPOT

An ELISPOT assay was performed with the Mouse or Monkey IFN-γ ELISPOT Kit (Cat# 3321-4AST-2 and Cat# 3421M-4AST-2; Mabtech, Cincinnati, OH, USA) according to the manufacturer's protocol. Briefly, PBMCs were isolated from blood using a lymphocyte isolation technique (Ficoll-Paque PREMIUM; GE Healthcare, Piscataway, NJ, USA) and plated in duplicate wells. Three different stimulators, namely, purified SARS-CoV-2 antigen, recombinant S protein (Cat#PNA002; Sanyou Biopharmaceuticals), and recombinant N protein (Cat# PNA006; Sanyou Biopharmaceuticals), were added to separate wells. The positive control was phytohemagglutinin (PHA). The plate was incubated at 37°C for 24 h, after which the cells were removed and the spots developed. The colored spots were counted with an ELISPOT reader (CTL, Shaker Heights, OH, USA).

Electron microscopy

Purified inactivated SARS-CoV-2 preparations were coincubated with convalescent serum or with a mAb against the S protein (mAb-S; Cat#40592-R001; Solarbio, Beijing, China) or the N protein (mAb-N; Cat# 40143-R001; Solarbio, Beijing, China) at 37°C for 24 h,

stained with 1% phosphotungstic acid and observed using a transmission electron microscope (Hitachi, Kyoto, Japan).

Quantitation of the viral load with qRT-PCR

Total RNA was extracted from blood and tissue samples obtained from experimental macaques with TRIzol reagent (Cat# DP424; Tiangen, Beijing, China). qRT-PCR was performed using a One Step PrimeScript RT-PCR Kit (Cat# RR064B; Perfect Real Time; TaKaRa) according to the manufacturer's protocol. The primers used for qRT-PCR were selected to specifically amplify the E and ORF1ab sequences in the SARS-CoV-2 genome (Table 1). Viral copies were quantified from *in vitro* synthesized RNA, and the quantity was reported as the relative copy number calculated using the following equation: ($[\mu\text{g of RNA}/\mu\text{L}]/[\text{molecular weight}] \times \text{Avogadro's number} = \text{viral copy number}/\mu\text{L}$).²⁹

Histopathological examinations

The organs of experimental macaques were fixed with 10% formalin, embedded in paraffin, sliced into 4-µm sections, and stained with hematoxylin and eosin (H&E). Morphology was assessed with an inverted microscope (Nikon).

Immune cell populations

PBMCs were isolated from macaques using a lymphocyte isolation technique (Cat# 17-5442-02; Ficoll-Paque PREMIUM; GE Healthcare). Anti-CD3 (Cat# 552127; BD Biosciences, USA), anti-CD20 (Cat# 555623; BD Biosciences), and anti-CD16 (Cat# 302016; Biolegend, China) antibodies were added to the PBMCs. The mixtures were incubated at room temperature (RT) for 30 min in the dark. Reagents for red blood cell lysis (Cat# 349202; BDIS) and membrane permeabilization (Cat# 554714; BD Biosciences) were added sequentially. After two washes with PBS, the cells were resuspended in PBS and detected using a flow cytometer (BD, USA). T cells (CD3⁺), B cells (CD20⁺), and NK cells (CD16⁺) were evaluated. Furthermore, the T cells were typed into T helper (Th) 1, Th2, Treg, and Th17 cells. PBMCs were coincubated with an anti-CD4 antibody (BD) at RT for 30 min. Red blood cell lysis (BD) and membrane permeabilization (BD) reagents were then added. After incubation with red blood cell lysis and membrane permeabilization reagents and two washes with PBS, the cells were labeled with anti-FOXP3 (Cat#560047; BD Biosciences), anti-IL-4 (Cat#500824; BioLegend), anti-IFN-γ (Cat# 554700; BD), and anti-IL-17A (Cat# 50-7178-42; eBioscience, CA, USA) antibodies for 30 min at RT and were then washed once. The percentages of immune cells were determined using a flow cytometer (BD). Th1 cells (CD4⁺/IFN-γ⁺), Th2 cells (CD4⁺/IL-4⁺), Treg cells (CD4⁺/FOXP3⁺), and Th17 cells (CD4⁺/IL-17A⁺) were assessed.

Cytokine assay

The levels of IL-2, IL-4, IL-5, IL-6, TNF-α, and IFN-γ in the serum of experimental macaques were measured with a Non-Human Primate (NHP) Th1/Th2 Cytokine Kit (Cat# 557800; BD Biosciences) according to the manufacturer's protocol. Briefly, serum was added to a tube containing detection beads. Then, PE detection reagent was added,

and the mixtures were incubated at RT for 2 h in the dark. After washing, the beads were resuspended in wash buffer and analyzed in a flow cytometer (BD). The levels of these cytokines were calculated from a standard curve.

Statistical analysis

The data are presented as the means and standard deviations (SDs). GraphPad Prism software (San Diego, CA, USA) was used for statistical analyses. A *t* test was performed to compare the difference between the two groups with one factor. Two-way ANOVA was performed to compare the differences between the groups with multiple factors. The association of antibodies specific for the S protein, the N protein, and the whole virion (WVN) was determined using Spearman rank correlation analysis.

Data availability

All data generated or analyzed during this study are included in this published article (and its [supplemental information files](#)).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtm.2021.08.005>.

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AUTHOR CONTRIBUTIONS

Q.L., L.L., and C.L. conceived and designed the experiments. H.C., Z.X., R.L., S.F., H.L., Z.H., K.X., Y. Liao, L.W., X.L., T.M., Y.Y., L.G., Jianbo Yang, H.Z., X.X., J.L., Y. Liang, D.L., H.Z., G.J., F.Y., Y.H., X.W., C.C., X.Deng, Y. Li, H.Z., J.Z., H.Y., Jiafang Yang, and W.Z. performed the experiments. Q.L., L.L., Y.Z., and Z.M.Z. analyzed the data. X.Dong, X.Z., C.H., Y.C., J.P., K.M., L.W., W.D., D.S., H.Z., R.J., L.Y., M.X., L. Yi, Z.Z., N.D., H. Yang, and W.Y. contributed reagents/materials/analysis tools. Q.L. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests. The corresponding authors had full access to all the data generated in the present study and assume full responsibility for the final submission of this manuscript for publication.

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