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Vaccine 40 (2022) 4231–4241

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Immunogenicity and protective efficacy of SARS-CoV-2 recombinant S-protein vaccine S-268019-b in cynomolgus monkeys

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ARTICLE INFO

Article history:
Received 10 January 2022
Received in revised form 4 May 2022
Accepted 30 May 2022
Available online 6 June 2022

Keywords:
Coronavirus disease 2019
Cynomolgus monkeys
Recombinant protein vaccine
S-268019-b
Severe acute respiratory syndrome coronavirus 2

ABSTRACT

The vaccine S-268019-b is a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike (S)-protein vaccine consisting of full-length recombinant SARS-CoV-2 S-protein (S-910823) as antigen, mixed with the squalene-based adjuvant A-910823. The current study evaluated the immunogenicity of S-268019-b using various doses of S-910823 and its vaccine efficacy against SARS-CoV-2 challenge in cynomolgus monkeys. The different doses of S-910823 combined with A-910823 were intramuscularly administered twice at a 3-week interval. Two weeks after the second dosing, dose-dependent humoral immune responses were observed with neutralizing antibody titers being comparable to that of human convalescent plasma. Pseudoviruses harboring S proteins from Beta and Gamma SARS-CoV-2 variants displayed approximately 3- to 4-fold reduced sensitivity to neutralizing antibodies induced after two vaccine doses compared with that against ancestral viruses, whereas neutralizing antibody titers were reduced >14-fold against the Omicron variant. Cellular immunity was also induced with a relative Th1 polarized response. No adverse clinical signs or weight loss associated with the vaccine were observed, suggesting safety of the vaccine in cynomolgus monkeys. Immunization with 10 μg of S-910823 with A-910823 demonstrated protective efficacy against SARS-CoV-2 challenge according to genomic and subgenomic viral RNA transcript levels in nasopharyngeal, throat, and rectal swab specimens. Pathological analysis revealed no detectable vaccine-dependent enhancement of disease in the lungs of challenged vaccinated monkeys. The current findings provide fundamental information regarding vaccine doses for human trials and support the development of S-268019-b as a safe and effective vaccine for controlling the current pandemic, as well as general protection against SARS-CoV-2 moving forward.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiological agent of coronavirus disease 2019 (COVID-19), was first identified in Wuhan, China in December 2019. The World Health Organization (WHO) subsequently declared the outbreak a Public Health Emergency of International Concern on January 30, 2020 and a pandemic on March 11, 2020. COVID-19 remains a global health crisis, causing a critical need for effective intervention and prevention of SARS-CoV-2 infections [1]. This demonstrates the critical need for effective interventions and prevention strategies in order to control and resolve this global pandemic.

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https://doi.org/10.1016/j.vaccine.2022.05.081
0264-410X/

The development of efficacious vaccines against SARS-CoV-2 is the best approach for controlling its spread and curbing the pandemic [2]. As reviewed by Harrison et al. [3], SARS-CoV-2 is a member of the Coronaviridae family with its virion containing four structural proteins, including the spike (S) protein. The S protein forms a trimer anchored to the viral membrane and directly contacts the cellular receptor angiotensin-converting enzyme 2 (ACE2) on the host cell through its receptor-binding domain (RBD) [2,4]. As neutralizing epitopes have been identified not only on RBD, but also on the N-terminal domain (NTD), full-length S protein is consider a preferred target antigen of the immune system [5,6]. The characteristics and biological function of the S protein makes it an obvious vaccine target. Not surprisingly, S protein-based vaccines are under active development and are some of the most widely approved vaccines for COVID-19. As of April 1, 2022, WHO reports there are 153 COVID-19 vaccines in clinical development and 196 vaccines in pre-clinical development, with 51 protein subunit vacccine candidates being in clinical phase [7].

We are actively developing S-268019-b, an adjuvanted vaccine for COVID-19 consisting of antigen S-910823 mixed with the squalene-based, oil-in-water emulsion adjuvant A-910823. S-910823 is a recombinant full-length SARS-CoV-2 S protein based on amino acid sequenes of the Wuhan-Hu-1 isolate (Genbank MN908947) and has mutations in the furin cleavage site to inhibit S-protein cleavage into S1 and S2 subunits, as well as two substituted proline residues to improve prefusion conformaton stability of the S-protein trimers [8–10]. Although most relevant cell lines for insect cell-based recombinant protein expression are known to be persistently infected with various adventitious viruses, the S-910823 protein is generated using a baculovirus expression system (BEVS) with rhabdovirus-free insect cells. The use of BEVS technology is a feasible platform to manufacture recombinant antigens and is currently employed for production of several commercial vaccines against influenza and human papilloma viruses [11,12]. Previous studies suggest the balance between Th1 and Th2 immune responses may correlate with potential risks of vaccine-associated disease enhancement (VDE) [13–16]. Thus, the oil-in-water emulsion-based adjuvant A-910823 was screened through exploratory studies and selected in part based on profiles of immunogenicity and Th1/Th2 balance. The current study evaluated the immunogenicity, protective efficacy, and S-910823 dose dependency of the novel S-268019-b vaccine in cynomolgus monkeys. The findings will provide critical fundamental information on vaccine dosing for human trials and insight toward the goal of developing a safe and efficient vaccine against SARS-CoV-2.

2. Methods

2.1. Cell lines, SARS-CoV-2, and pseudoviruses

Transmembrane serine protease 2 (TMPRSS-2)-expressing VeroE6 (VeroE6/TMPRSS2) cells [17] were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) and maintained in culture medium of Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% heat inactivated fetal bovine serum (FBS) and 1 mg/mL gentamicin (Genticin). SARS-CoV-2 JPN/TY/WK-521 (WK-521, accession no. EPI_ISL_408667), which was isolated from the throat swab of a traveler who had returned from Wuhan on January 31, 2020, was provided by the National Institute of Infectious Diseases (NIID), Japan [17]. SARS-CoV-2 JPN/TY/38-873 (Omicron, accession no. EPI_ISL_7418017) was also provided by NIID, Japan. The WK-521 and Omicron strains were propagated on VeroE6/TMPRSS2 cells and virus stocks prepared, which were titered by tissue culture infectious dose 50 ($TCID_{50}$) assays using VeroE6/TMPRSS2 cells. Lenti-X 293 T cells were purchased from Takara Bio (Shiga, Japan) and used to generate the lentivirus-based D614G, Alpha, Beta, Gamma, Delta, and Omicron pseudoviruses [18]. Details on the pseudoviruses are provided in the Supplementary Material.

2.2. Vaccine (S-268019-b) preparation

The S-268019-b vaccine (Shionogi & Co., Ltd., Osaka, Japan) consists of the recombinant protein S-910823 (Shionogi & Co., Ltd.) and the squalene-based, oil-in-water emulsion adjuvant A-910823 (Shionogi & Co., Ltd.). The recombinant S-910823 protein was expressed using BEVS as previously described [11,12]. Briefly, baculovirus-infected cells were suspended in a buffer containing a nonionic detergent in order to extract the S-910823 protein. The cell extract was centrifugated and the supernatant filtered using a depth filter. The S-910823 protein was purified by affinity chromatography and hydrophobic interaction chromatography. Host cell DNA and baculoviruses were removed by Q membrane chromatography. Tangential flow filtration was used for protein concentration and buffer exchange. The S-910823 protein was prepared at 0, 4, 10, 20, 40, and 100 μg/mL in vehicle consisting of 0.02% polysorbate 20 (PS20) buffer (15 mM phosphate buffer, 150 mM sodium chloride, 0.02% w/v PS20, pH 7.5).

2.3. Vaccination of cynomolgus monkeys

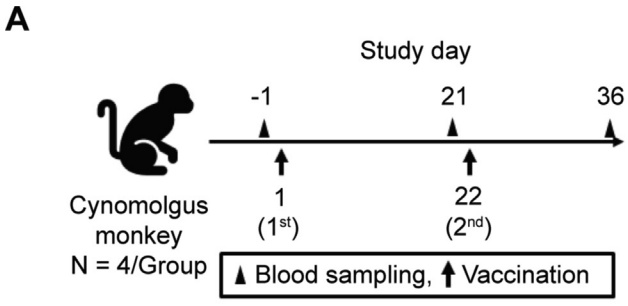
Twenty-eight female Cambodian cynomolgus monkeys (*Macaca fascicularis*), 3 to 7 years of age and weighing 2.1 to 8.0 kg at the begining of a 3-d acclimation period, were obtained from the stock colony of Shin Nippon Biomedical Laboratories, Ltd. Drug Safety Research Laboratories (Kagoshima, Japan). The overall study design is shown in Fig. 1A, with full details provided in the Supplementary Material. Briefly, the monkeys were randomly assigned to seven experimental groups (n = 4/group). Vaccines were administered intramuscularly in a volume of 500 μL. The day prior to the first dosing was day −1 with the first day of dosing being day 1. The second vaccine dose was administered 3 weeks later (day 22). According to the experimental group, the animals received either vehicle (PS20 buffer) only, S-910823 (1, 2.5, 5, 10, or 25 μg) in vehicle plus A-910823 (S+A-910823), or 10 μg S-910823 in vehicle without A-910823 (S-910823-only) for each vaccination. The animals were observed for 5 weeks (through day 36). The dosing schedule and route were in accordance with the intended clinical application. Blood samples were drawn from the femoral veins at the volumes and time points specified for the various analyses. Sample preparation is described in the Supplementary Material.

2.4. Anti-S and anti-RBD IgG analysis

Antibody titers were measured using enzyme-linked immunosorbent assays (ELISA) as described in the Supplementary Material. Sera collected on days −1, 21, and 36 were evaluated for anti-S-protein and anti-RBD IgG. All samples were analyzed in duplicate. Absorbance values of A_{405} minus A_{490} were calculated and the mean and coefficient of variation (CV) of absorbance of the duplicate samples were determined. The mean value was regarded as the analysis value of each sample. The highest dilution factor with an analysis value ≥the cut point was considered the antibody titer.

2.5. Neutralizing antibody titer testing

Neutralizing antibody levels in the monkey sera collected at days −1, 21, and 36 were analyzed against live SARS-CoV-2 WK-



	Study day				
	-1	1	21	22	36
Vaccination		✓		✓	
Anti-S protein antibody (ELISA)	✓		✓		✓
Anti-RBD antibody (ELISA)	✓		✓		✓
Neutralizing antibody against live virus	✓		✓		✓
Neutralizing antibody against pseudovirus					✓
Analysis of PBMC cytokines	✓		✓		✓

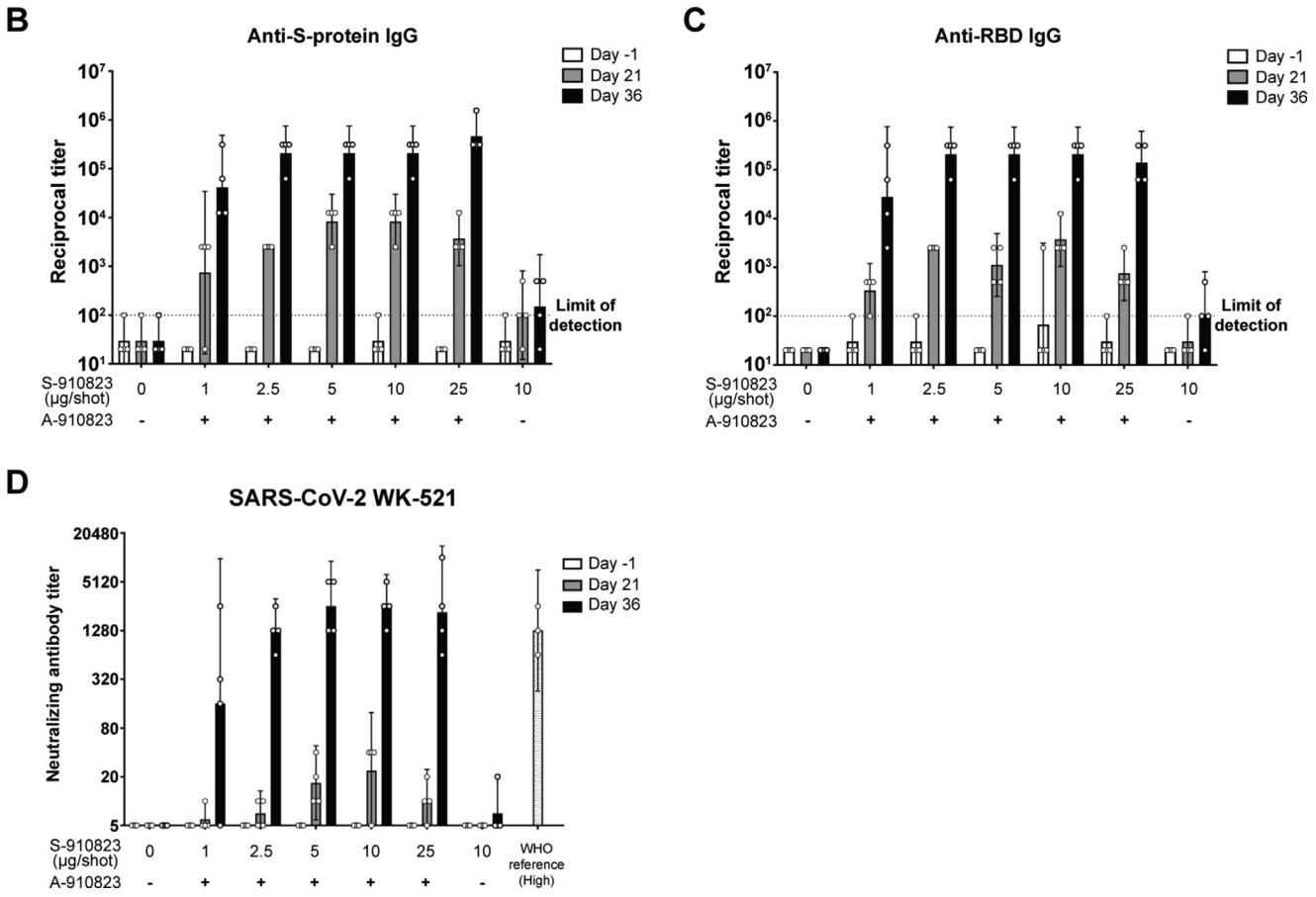


Fig. 1. Immunogenicity of vaccine S-268019-b. (A) Schematic overview of the vaccination schedule and experimental groups. Cynomolgus monkeys (n = 4/group) were vaccinated on day 1 (D1) and again 3 weeks later on day 22 (D22) with vaccine S-268019-b containing various doses of SARS-CoV-2 recombinant S-protein S-910823 as antigen and adjuvant A-910823. Vaccinations were performed intramuscularly in a total volume of 500 μL. The animals were monitored for weight and clinical signs, and blood was collected as indicated for analysis of antibody content, hematology, and peripheral blood cell types. **(B and C)** Anti-SARS-CoV-2 antibody levels in cynomolgus monkeys immunized with vaccine S-268019-b. The animals were vaccinated on day 1 and again 3 weeks later on day 22 with vaccine S-268019-b containing various doses of SARS-CoV-2 recombinant S-protein S-910823 as antigen and adjuvant A-910823. Sera were collected on day −1 (white bar), day 21 (gray bar), and day 36 (black bar) and analyzed by ELISA for **(B)** anti-SARS-CoV-2 S-protein antibody and **(C)** the receptor binding domain (RBD). Open circles represent titers of individual animals. Each bar represents geometric mean titer (GMT) with error bars indicating 95% confident interval. **(D and E)** Neutralizing antibody levels in cynomolgus monkeys immunized with vaccine S-268019-b. **(D)** The animals were vaccinated on day 1 and again 3 weeks later on day 22 with vaccine S-268019-b containing various doses of SARS-CoV-2 recombinant S-protein S-910823 as antigen and adjuvant A-910823. Sera were collected on day −1 (white bar), day 21 (gray bar), and day 36 (black bar) and analyzed for the ability to neutralize SARS-CoV-2 WK-521. World Health Organization (WHO) reference plasma 20/150 of pooled human convalescent plasma (1,473 international unit/mL) was used as a positive control.

521 and the Omicron strain using VeroE6/TMPRSS2 cells as described in the Supplementary Material. Briefly, 50 μL of each serum sample was mixed with an equal volume of WK-521 or Omicron virus suspension, and each 100 μL mixture was then added to individual culture-plate wells. The plates were incubated for approximately 1 h at room temperature for neutralization. After incubation, 3×10^4 VeroE6/TMPRSS2 cells in 100 μL of assay medium were added to each well (100 and 10,000 $TCID_{50}$/well for WK-521 and Omicron, respectively). The plates were then incubated at 37 °C with 5% CO_2 for 3 days. The assay was performed in duplicate for each sample. After the 3 day incubation, living cells were examined using CellTiter-Glo 2.0 (G9243; Promega, Madison, WI, USA) according to the manufacturer's instructions. WHO International Reference Panel of human pooled convalescent plasma, High [20/150: 1,473 international unit (IU)/mL)] and Negative [20/142], were used as positive and negative controls, respectively [19]. The WHO International Reference Panel High [20/150] is prepared from UK patients that were mainly infected with SARS-CoV-

2 Wuhan strain in June 2020 [20]. The International Standard was determined by neutralizing assays using live viruses and pseudoviruses of the Wuhan-Hu-1 isolate (Genbank accession number MN908947). The neutralizing antibody titers in the serum samples were calculated as the reciprocal of the highest dilution at which viable cell percentage was ≥50%. Geometric mean titer (GMT) of the serum samples was also calculated for each group. Details regarding the preparation, titration, and use of the lentivirus-based SARS-CoV-2 pseudovirus variants are provided in the Supplementary Material.

2.6. Preparation of peripheral blood mononuclear cells (PBMCs)

Blood was collected (3 mL) via the femoral vein from all animals on days −1, 21, and 36 using a syringe containing heparin sodium. The PBMCs were isolated using Ficoll-Paque Plus (GE Healthcare Life Sciences, USA) and a LeucoSep tube (Grainer Bio-One, Frickenhausen, Germany). The number of cells were counted using trypan blue staining and their density adjusted to 2.0×10^6 cells/mL with Cellbanker 1 (Nippon Zenyaku Kogyo Co., Fukushima, Japan). The cells were prepared as 1 mL aliquots and stored at −70 °C until use.

2.7. Analysis of cytokine production from PBMCs

Frozen PBMCs samples from individual cynomolgus monkeys were thawed and prepared as 1×10^6 living cells/mL in RPMI 1640 medium supplemented with L-glutamine, fetal calf serum, penicillin–streptomycin, HEPES, sodium pyruvate, MEM Non-Essential Amino Acids Solution, GlutaMAX™ Supplement, and 2-mercaptoethanol. The PBMCs were cultured with or without SARS - CoV 2 S-protein peptides and cytokine production from the cells were detected using a Monkey IFN-γ/IL-4/IL-5 FluoroSpotFLEX Kit (Mabtech AB, Nacka Strand, Sweden). The S-protein peptides used were PepTivator® SARS-CoV-2 Prot_S, PepTivator® SARS-CoV-2 Prot_S1, and PepTivator® SARS-CoV-2 Prot_S+ (Miltenyi Biotec, Bergisch Gladbach, Germany) at 0.15 μg/mL. These peptide cocktails almost completely span the S-protein. The assays were performed in triplicate for each sample. The plates were scanned and imaged using an ImmunoSpot Analyzer with ImmunoSpot 7.0 software (Cellular Technology Ltd., Cleveland, OH, USA). Image analysis was performed using ImmunoSpot 7.0 software (Cellular Technology Ltd.). The number of spots observed were averaged for wells of individual animals under the same conditions (e.g., sampling date, cytokine, with/without antigen peptide). The number of antigen-specific spots for each cytokine at each sampling day was also determined for individual animals. The results are presented as spot-forming cells (SFC)/1×10^6 PBMCs.

2.8. SARS-CoV-2 challenge experiments

2.8.1. Infection of vaccinated cynomolgus monkeys

To monitor body temperature throughout the virus challenge study, the monkeys were anesthetized 3 or 4 weeks before virus infection with a mixture of 50 mg/mL ketamine and 20 mg/mL xylazine (2:1; 0.2 mL/kg) and intraperitoneally implanted with small temperature probes that measure and record temperature (DST micro-T: 8.3 × 25.4 mm; Star-oddi, Gardabaer, Iceland). The probes were retrieved at necropsy. Eight or 13 weeks after the second immunization, the monkeys were infected under anesthesia with $10^{6.5}$ $TCID_{50}$ SARS-CoV-2 in 1.8 mL of culture medium through a combination of intranasal and intratracheal inoculations (1.5 mL intranasally and 0.3 mL intratracheally).

2.8.2. Sample collection

Approximately 7 days before the challenge infection, the monkeys were anesthetized with ketamine and were transferred from the quarantine room to the biosafety level 3 (BSL3) animal facility. On days 0, 1, 4, and 7 post experimental infection, clinical samples, including nasopharyngeal swabs, throat swabs, rectal swabs, and blood, and the weight of the monkeys were collected under anesthesia. Sera were prepared and stored at −80 °C until use. Ethylenediaminetetraacetic acid (EDTA) and heparinized blood samples were used immediately for hematologic tests. The monkeys were sacrificed by exsanguination under excess ketamine anesthesia at 7 days post infection and then necropsied. Clinical and tissue samples were collected for viral titration, viral RNA analysis, and histopathological analysis. The tissue samples included the lungs, trachea, hilar lymph nodes, tonsils, cervical lymph nodes, mesenteric lymph nodes, small intestine, and large intestine.

2.8.3. Clinical scores of infected monkeys

The monkeys were observed once daily for clinical signs and scored for dietary intake of food pellets (0–5) and fruits (0–5), drinking water intake (0–5), behavior in front of regular observers, such as standing up, showing interest in activity outside their cage and receivng attention, intimidation, up and down movement (0–5), and stool consistency, including color, stiffness, form, volume, and frequency (0–5).

2.8.4. Measurement of SARS-CoV-2 genomic and subgenomic RNA levels

Total RNA was extracted from swab samples and tissue homogenates using TRIzol LS or TRIzol reagents (Thermo Fisher Scientific) and a Maxwell RSC 48 automated nucleic acid purification system (Promega, Madison, WI). The RNA was then used to quantify the SARS-CoV-2 genome. The copy number of viral genomic RNA and subgenomic RNA transcripts in the samples were estimated by real-time reverse transcription polymerase chain reaction (RT-PCR) using a QuantiTect Probe RT-PCR Kit (Qiagen, Venlo, Netherlands) and LightCycler 480 system (Roche, Basal, Switzerland). Specific details regarding the RT-PCR primers and probes are provided in the Supplementary Material.

2.8.5. Histopathology

Tissue samples collected during necropsy were immersed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Histopathological scoring was performed with respect to viral infection, bronchiolitis, acute interstitial pneumonia, lymphoid lesions, eosinophilic pneumonia, and thrombus. The scoring system was as follows: Bronchiolitis (0, normal; 1, a few sites of epithelial degeneration and cell debris in a few bronchi; 2, many sites of degenerated epithelia and necrotic debris without inflammation; 3, inflamed epithelium with slight peribronchial inflammatory cell infiltration; 4, hyperplastic epithelium with luminal cells and massive peribronchial inflammatory cell infiltration); Acute interstitial pneumonia (0, normal; 1, accumulations of macrophages with some alveolar wall thickening; 2, localized regions of interstitial mononuclear or neutrophil infiltrates with airspace macrophages; 3, extensive interstitial cell infiltrates with airspace macrophages; 4, massive diffuse alveolar damage); Lymphoid lesions (0, normal; 1, lymphoid aggregates with germinal centers in bronchial associated lymphoid tissue (BALT); 2, mononuclear cell aggregates around a few blood vessels; 3, mononuclear aggregates around several blood vessels; 4, extensive mononulcear aggregates around blood vessels and alveoli); Eosinophilic pneumonia (0, normal; 1, eosinophilic infiltrates around bronchi; 2, eosinophilic infiltrates around bronchi and blood vessels; 3, eosinophilic infiltrates around bronchi, blood vessels, and alveoli; 4, massive eosinophilic pneumonia); Thrombus (0, normal; 1, a few fibrin deposits detected in alveoli; 2, many fibrin deposits detected in alveoli; 3, many fibrin deposits detected in

alveoli and small vessels; 4, thrombus observed in medium or large sized vessels).

2.9. Statistical analysis

Tukey's multiple comparison test was performed for analysis of the clinical scores of infected monkeys and the SARS-CoV-2 genome and subgenomic RNA transcript analyses.

3. Results

3.1. Immunogenicity of S-268019-b in cynomolgus monkeys

To assess the immunogenicity of S-268019-b, cynomolgus monkeys were administered S-268019-b twice, at a 3-week interval (Fig. 1A), and anti-spike IgG serum titers before and after vaccinations were measured (Fig. 1B). The titers two weeks after the second vaccination increased dose-proportionally in animals receiving S+A-910823, with 2.5 μg S-910823 inducing near peak titers. Meanwhile, 10 μg S-910823 without A-910823 induced IgG titers approximately 1,000-fold less than those of 10 μg S+A-910823, indicating that A-910823 enhanced the immunogenicity of 10 μg S-910823. Similarly, anti-RBD IgG titers also peaked following administration of 2.5 μg S+A-910823 (Fig. 1C). To evaluate the functional quality of the antigen-specific antibody induced by S-268019-b, we next evaluated the levels of neutralizing antibodies (Fig. 1D). Consistent with the anti-S-protein and anti-RBD IgG levels, a dose-dependent increase in neutralizing antibody against ancestoral SARS-CoV-2 WK-521 was observed. The GMT for 5 μg and 10 μg S+A-910823 demonstrated the highest neutralizing antibody titers of approximately 2,560. This was comparable with that of the titer for the WHO "High" reference sample of pooled convalescent plasma prepared from UK patients infected with wild-type SARS-CoV-2 [19]. Although the ages of the monkeys ranged from three to seven years, no correlation was seen between immune response and age.

Obvious changes were not observed throughout the study regarding clinical signs, body weight, immunological cell populations, or hematological values (Supplementary Table S1, Supplementary Fig. S1–S4). Transient elevation of C-reactive protein (CRP) was noted on days 2 and 23 post-dosing in the groups adminstered S+A-910823; however, this was not considered to be dose-dependent and all had recovered by day 29 (Supplementary Fig. S2). Although eosinophil counts increased on day 23 after the second dosing in the group administered 1 μg S+A-910823, this change reverted to basal levels by day 36 (Supplementary Fig. S3A). This transient increase in eosinophils was possibly due to temporal production of cytokines and the migration of eosinophils at the injection site. Overall, these results suggested that S+A-910823 vaccination displayed safe profiles for non-human primates.

To evaluate neutralizing antibody titers elicited by S-268019-b against variant SARS-CoV-2 viruses, we employed recombinant lentiviruses pseudotyped with coronavirus S protein derived from D614G, Alpha, Beta, Gamma, Delta, and Omicron strains (Fig. 2A). Analysis of neutralizing antibody levels of day 36 sera from monkeys (n = 8) twice vaccinated with 10 μg S+A-910823 at a 3-week dosing interval revealed that 50% pseudovirus neutralization titers ($pVNT_{50}$) against the parent D614G virus, and Alpha and Delta variants were comparable, while those against Beta, Gamma, and Omicron variants were decreased 3-fold to 14-fold (Fig. 2A). We also evaluated neutralizing antibody titers against live WK-521 and Omicron viruses (Fig. 2B). Consistent with the $pVNT_{50}$ results, neutralizing antibody titers against the Omicron variant was decreased approximately 25-fold compared with that against WK-521.

3.2. Analysis of PBMC cytokines

To investigate induction of a cellular immune response and the potential effect of S-268019-b vaccination on Th1/Th2 polarity, PBMCs derived from monkeys vaccinated with S+A-910823, S-910823-only, or vehicle were pre-cultured with SARS–CoV–2 S-protein peptides and the production of Th1 (IFN-γ) and Th2 (IL-4, IL-5) cytokines by the PBMCs were then assessed using FluoroSpot assays. Representative images of the IFN-γ/IL-4/IL-5 FluoroSpot assay are shown in Fig. 3A, and quantitative analysis results are shown in Fig. 3B–3D. None of the cytokines were detectable in cultures of PBMCs from animals vaccinated with vehicle or S-910823-only. However, antigen-specific responses for each of the cytokines demonstrated higher trends on day 36 compared to those on day 21 in all S+A-910823-vaccinated groups. Th1 cytokine (IFN-γ)-producing cells were observed in all S+A-910823-vaccinated groups with the cytokine level being dose-dependent on S-910823 (Fig. 3B). Individuals with PBMCs that produced Th2 cytokines (IL-4, IL-5) were also observed in all the S+A-910823-vaccinated groups. However, SFC values for the Th2 cytokines in the S+A-910823-vaccinated animals tended to be lower than those for the Th1 cytokine (Fig. 3C and 3D). In the animals vaccinated with 5 μg or 10 μg S+A-910823, the SFC values for IFN-γ were 4.4-fold to 4.8-fold higher than those for IL-4 and 1.9-fold to 2.2-fold higher than those for IL-5 (Fig. 3E). These results suggested that S-268019-b enhanced both Th1 and Th2 responses in the primates and did not induce a Th2-dominant phenotype.

3.3. SARS-CoV-2 challenge of vaccinated cynomolgus monkeys

To assess the protective efficacy of S-268019-b from SARS-CoV-2 infection and the potential of VDE, we performed a challenge study using monkeys previoulsly immunized intramuscularly with two doses of vehicle, two doses of 10 μg S+A-910823, or two doses of 10 μg S-910823-only at a 21-day interval (Fig. 4A). The ratios of clinical score, body weight, and respiratory rate of monkeys after challenge infection to those before infection were evaluated (Fig. 4B). The clinical score ratio dropped during the initial 2 days after infection with SARS-CoV-2 in the vehicle group and S-910823-only group, and then began to recover. The monkeys immunized with S+A-910823 showed a slight decrease in the clinical score ratio, but the extent was smaller compared with that for the vehicle and S-910823-only vaccinated monkeys. An obvious difference was seen between the clinical score ratios of the S+A-910823 group and the S-910823-only, but not S+A-910823 group and the vehicle, at 1 day post-infection ($p < 0.001$). The body weight and respiratory rate ratios did not show any remarkable changes among the three groups after challenge infection (Fig. 4B).

Blood biochemistry values of the challenge infected monkeys are shown in Supplementary Fig. S5. Overall, the monkeys immunized with S+A-910823 did not show any meaningful changes blood biochemistry, although a temporal elevation of CRP value was observed on day 1, which was lower than that of the vehicle group. After infection with SARS-CoV-2, all the monkeys showed temporary higher body temperatures during the night following inoculation. Then, two monkeys from the vehicle group and three monkeys from the S-910823-only group had 0.5℃ higher temperatures within 2 days post infection, while none of the S+A-910823 immunized monkeys exhibited an elevated temperature beyond the first night (Supplementary Fig. S6). One of the monkeys from the vehicle group showed hypothermia. After SARS-CoV-2 inoculation, lymphocyte counts transiently decreased and then recovered in all three experimental groups, but there were no obvious differences among the groups (Supplementary Fig. S7).

We next evaluated the neutralizing antibody titer in serum samples from the monkeys administrated S+A-910823 and subse-

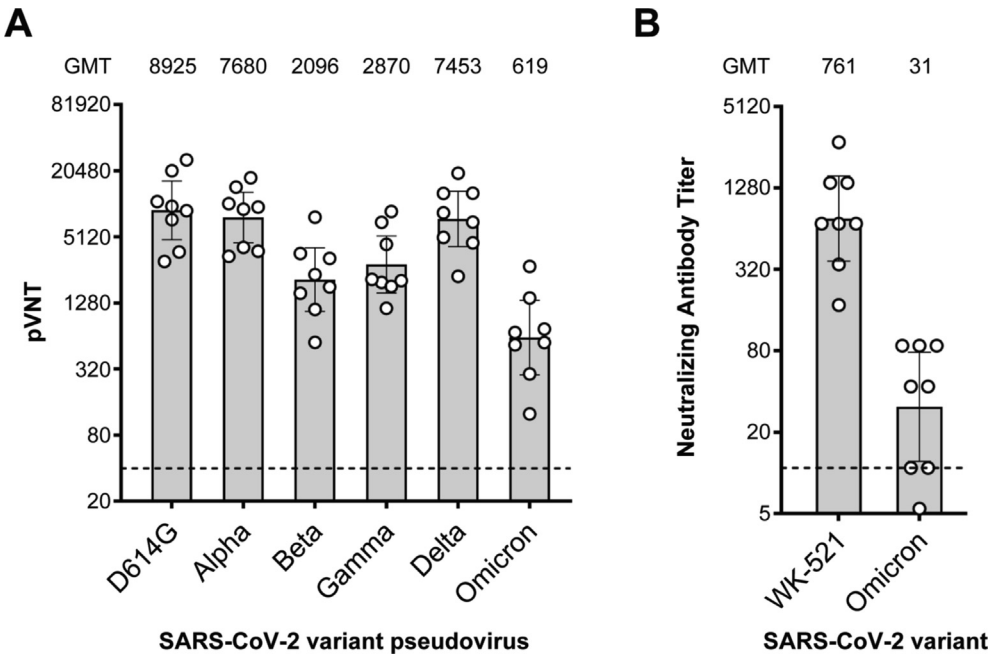


Fig. 2. Neutralizing antibody levels in cynomolgus monkeys immunized with vaccine S-268019-b against SARS-CoV-2 variants. Day 36 sera collected from cynomolgus monkeys vaccinated on day 1 and day 22 with S-268019-b containing 10 μg of antigen S-268019 and adjuvant A-910823 were analyzed. The 50% pseudovirus-neutralization titers (pVNT50) against SARS-CoV-2 variant pseudotyped lentiviruses (**A**) and neutralizing antibody titers against live SARS-CoV-2 (**B**) are shown. Open circles represent titers of individual animals. Each bar represents the geometric mean titer (GMT) with error bars indicating 95% confident interval.

quently challenged with SARS-CoV-2 after 8 to 13 weeks post second vaccination (Fig. 4C). In the challenged vehicle group, neutralizing antibody titers in the serum samples were below the lower limit of detection in all samples. Substantial levels of neutralizing antibody titer were detected before infection, and the levels were unchanged on days 1, 4, and 7 post infection in the S+A-910823-administered group.

The copy numbers of virus genomic and subgenomic RNAs in swab samples are shown in Fig. 4D. The average amount of genomic RNA in nasopharyngeal swab samples from monkeys of the vehicle and S-910823-only groups were approximately 1×10^7 copy/mL on day 1 and 1×10^5 copy/mL on day 7, which was higher than that for the throat swab samples and rectal swab samples. Meanwhile, the average copy number of genomic RNA in nasopharyngeal swab samples from monkeys of the of S+A-910823 group was approximately 1×10^6 copy/mL on day 1 and 4×10^3 copy/mL on day 7, which were both lower than those of the vehicle and S-910823-only groups. In comparison, the average copy number of subgenomic RNA transcripts in the nasopharyngeal swab samples of the S+A-910823 group was approximately 1×10^4 copy/mL on day 1, which was again lower than that of the vehicle group (approximately 1×10^6 copy/mL). The copy number of virus genomic and subgenomic RNAs in tissue homogenates on day 7 are shown in (Supplementary Fig. S8). The copy numbers for the S+A-910823 group were lower than those for the vehicle group in each sample. In addition, the copy number of viral genomic RNA and subgenomic RNA of the S-910823-only group were lower than those of the vehicle group.

Inflammatory cytokines and chemokines levels in the serum of the three groups of SARS-CoV-2 challenged animals are shown in (Supplementary Fig. S9). While the concentrations of IL-6, IL-1rα, and MCP-1 were increase on day 1 in the challenged vehicle group, they were not increased in the challenged S+A-910823 group.

Histopathological examination revealed mild interstitial pneumonia in the lungs of all the monkeys of the vehicle group following challenge infection (Fig. 5A, upper panels). All of the monkeys showed viral antigen positive cells in the lesions; however, only slight and focal intrestitial pneumoneia was observed in the lungs of the S+A-910823 group with no viral antigen positive cells (Fig. 5A, middle panels). For some animals, mild or slight mononuclear infiltration was present around blood vessels in the challenged S+A-910823 group. One monkey in the S-910823-only group exhibited severe pulmonary edema associated virus infection (Fig. 5A, lower panels). Other monkeys exhibited a few viral antigen-positive cells associated interstitial pneumonea with eosinophil and/or neutrophil infiltraion. The average of scores of thrombus and eosinophilic pneumonia of the challenged S+A-910823 group were lower than that of the challenged vehicle and S-910823 groups (Fig. 5B).

4. Discussion

In this study, we demonstrated that a new COVID-19 vaccine, S-268019-b, induced anti-S and anti-RBD IgG expression, and the production of neutralizing antibodies against SARS-CoV-2 and its variants in monkeys. S-268019-b also induced PBMCs to produce cytokines upon re-stimulation with S-protein peptides. Immunization with S-268019-b was also shown to protect cynomolgus monkeys against SARS-CoV-2 challenge, without any safety concerns based on hematological analysis, cytokine and chemokine profiles, and histopathological analysis. These results support the efficacy of S-268019-b as a novel vaccine against SARS-CoV-2 for eventual use in clinical settings.

In evaluating vaccines, appropriate animal models are essential. As thoroughly reviewed by Muñoz-Fontela and colleagues [21], non-human primate models have been evaluated as models of SARS-CoV-2 infection and pathogenesis and shown to demonstrate beneficial traits. For instance, they support SARS-CoV-2 replication in the upper respiratory tract, lower respiratory tract, and other organs; develop pneumonia with bilateral involvement, ground glass opacities, focal edema, and inflammation; immunologically seroconvert, develop neutralizing antibodies, induce T-cell immu-

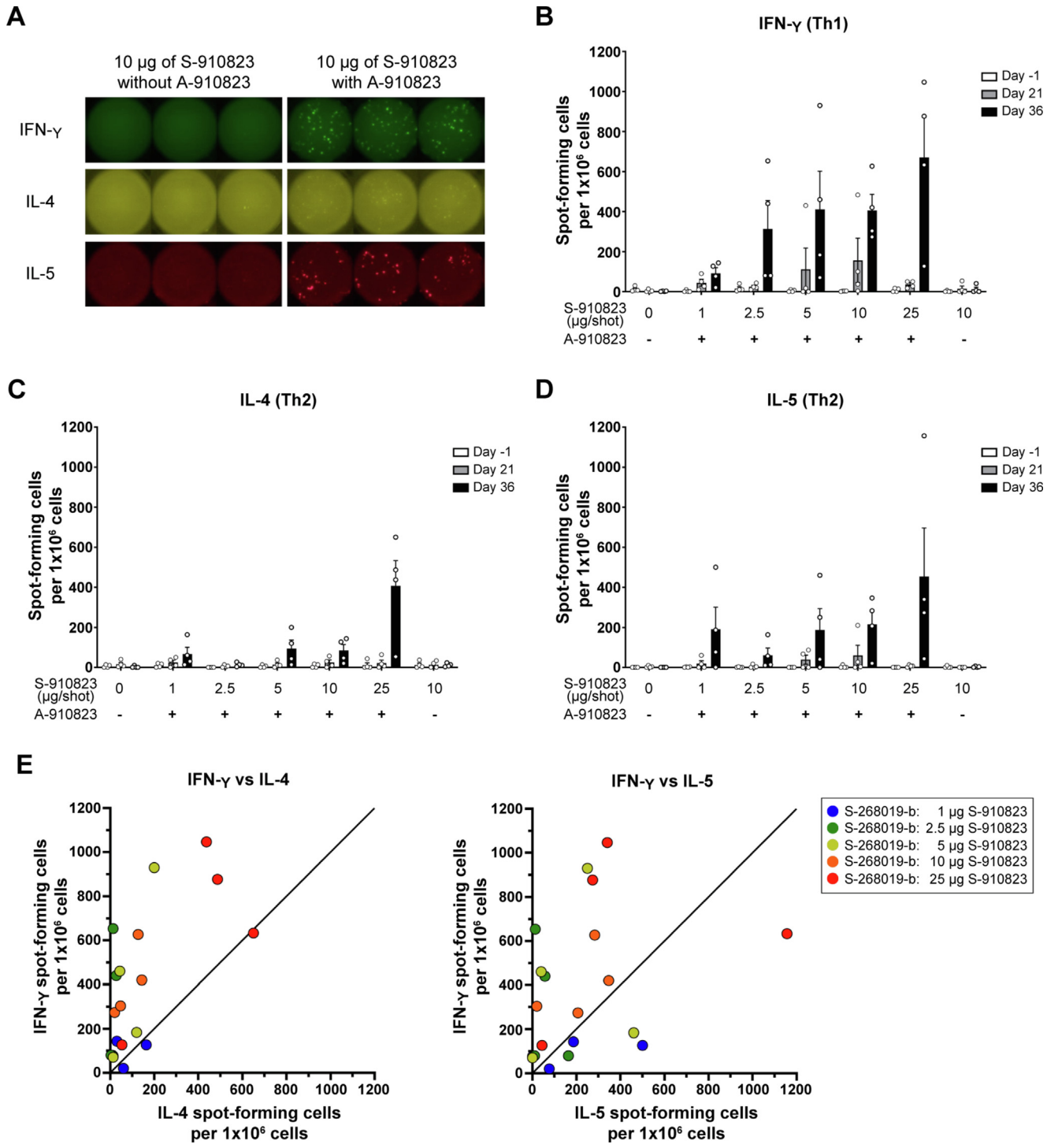
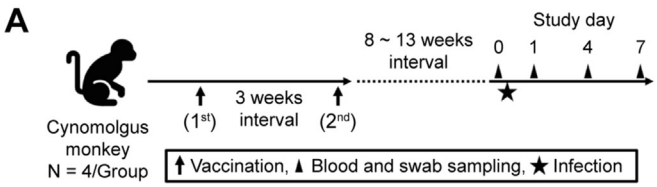


Fig. 3. Cytokine production by peripheral blood monocytic cells (PBMCs) of cynomolgus monkeys immunized with vaccine S-268019-b. The animals were vaccinated on day 1 (D1) and again 3 weeks later on day 22 (D22) with vaccine S-268019-b containing various doses of SARS-CoV-2 recombinant S-protein S-910823 as antigen and adjuvant A-910823. PBMCs were collected at the indicated time points and analyzed using FluoroSpot assays for Th1 cytokine IFN-γ, Th2 cytokine IL-4, and Th2 cytokine IL-5. (**A**) Representative images of IFN-γ/IL-4/IL-5 FluoroSpot assay results for groups vaccinated with S-268019-b containing 10 μg S-910823 with or without A-910823. Individual images of assay results were captured for IFN-γ (top image), IL-4 (central image), and IL-5 (lower image). Results are shown as spot-forming cells (SFC) per 1×10^6 cells for IFN-γ **(B)**, IL-4 **(C)**, and IL-5 **(D)** for the various vaccine antigen doses at day −1 (white bar), day 21 (gray bar), and day 36 (black bar). Open circles represent titers of individual animals. Each bar represents mean values with error bars indicating standard error of the mean (SEM). (**E**) Comparison of Th1 cytokine IFN-γ vs Th2 cytokine IL-4, and Th1 cytokine IFN-γ vs Th2 cytokine IL-5. Each colored circle represents an individual animal vaccinated with S-268019-b containing the indicated dose of antigen S-910823.

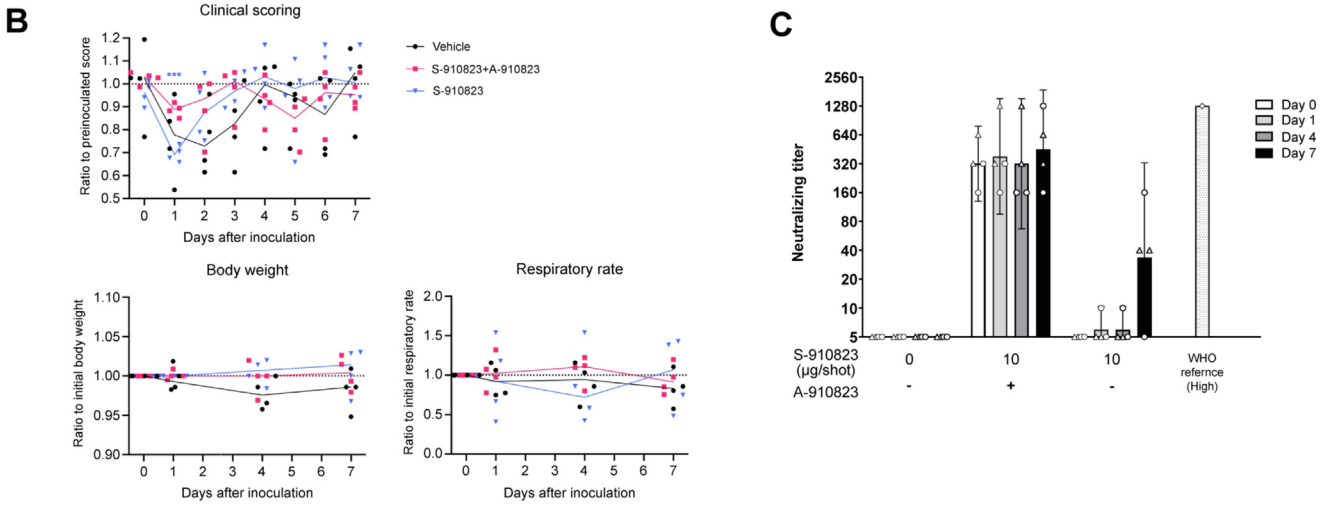
nity and pro-inflammatory cytokines, and exhibit more severe disease in older individuals [22–26]. This supports our choice to use cynomolgus monkeys as an *in vivo* model to evaluate the COVID-19 vaccine S-268019-b, which is under clinical development (Phase 1/2 Study: jRCT2031210269; Phase 2/3 Studies: jRCT2031210383 and jRCT2031210470).

To evaluate immunogenicity and antigen-dose dependency, the high-end amount of antigen was set at 25 μg/dose, which is a suf-



	Study day							
	0	1	2	3	4	5	6	7
Clinical scoring	✓	✓	✓	✓	✓	✓	✓	✓
Body weight, respiratory rate	✓	✓			✓			✓
Neutralizing antibody against live virus	✓	✓			✓			✓
Viral genomic RNA, subgenomic RNA	✓*	✓			✓			✓
Histopathological evaluation								✓

* Throat and rectal swab samples



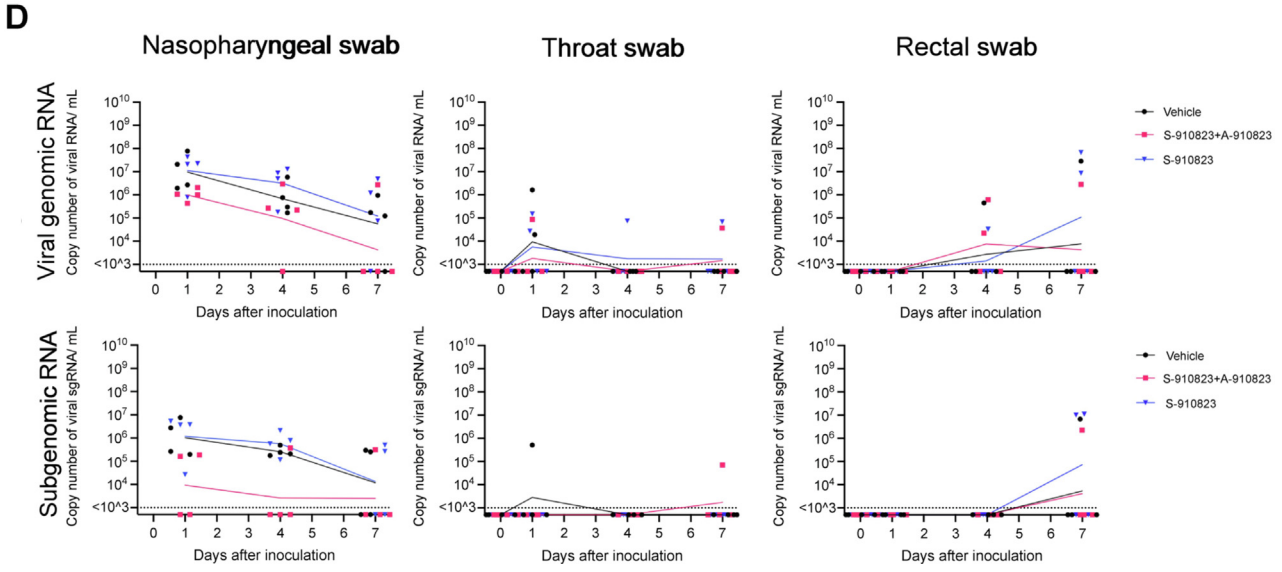


Fig. 4. Protective efficacy of S-268019-b immunization against SARS-CoV-2 infection in cynomolgus monkeys. (**A**) Schematic representation of the vaccination schedule and SARS-CoV-2 challenge experiments. Cynomolgus monkeys (n = 4/group) were vaccinated twice at 3 weeks interval as shown in Fig. 1A., followed by SARS-CoV-2 challenge 8–13 weeks later after the second vaccination. Blood and swab samles were collected on days 0, 1, 4, and 7 post infection and subjected to the indicated analyses. (**B**) Ratios of clinical scoring, body weight, and respiratory rate of immunized monkeys after challenge infection with SARS-CoV-2 relative to those prior to infection. Each symbol indicates the ratio of an individual monkey. Lines represent the mean clinical score ratio. ***p < 0.001 by Tukey's multiple comparison test (S-910823+A-910823 vs S-910823 at 1 day post infection). (**C**) Neutralizing titers of sera collected from immunized monkeys. The serum samples were collected on day 0 just prior to infection, and on days 1, 4, and 7 post infection. Each point represent an individual neutralizing titers against SARS-CoV-2 and the bars represent the geometric mean titers. The error bars indicate a 95% confidence interval. The far-right open bar represents the neutralizing antibody titer of the WHO International Reference Panel High sample (20/150). The limit of detection was set as 10 for monkey sera. (**D**) Copy numbers of virus genomic and subgenomic RNA in swab samples collected from vaccinated monkeys challenged with a SARS-CoV-2 infection. Each symbol indicates the copy number of viral genomic RNA (upper panels) and subgenomic RNA (lower panels) of individual monkeys. Lines represent the mean copy numbers. No significant differences were found by the Tukey's multiple comparison test.

ficient level at which antigen-specific immunogenicity was noted in another in-house study with cynomolgus monkey and mouse models. The lower amounts were set at 10, 5, 2.5, and 1 μg/dose for both primary and second shots. Our current results demonstrated this was an appropriate range of dosing as the animals exhibited immunological responses with most of the doses when A-910823 was included as an adjuvant in the formulation. Neutralizing antibody titer reached a peak with 5 μg S+A-910823. However, induction of Th2 cytokines was greater in the group treated with 25 μg S+A-910823 compared with that in the other groups. In previous reports, inactivated SARS-CoV and S-protein vaccines induced not only neutralizing antibodies, but also eosinophilic

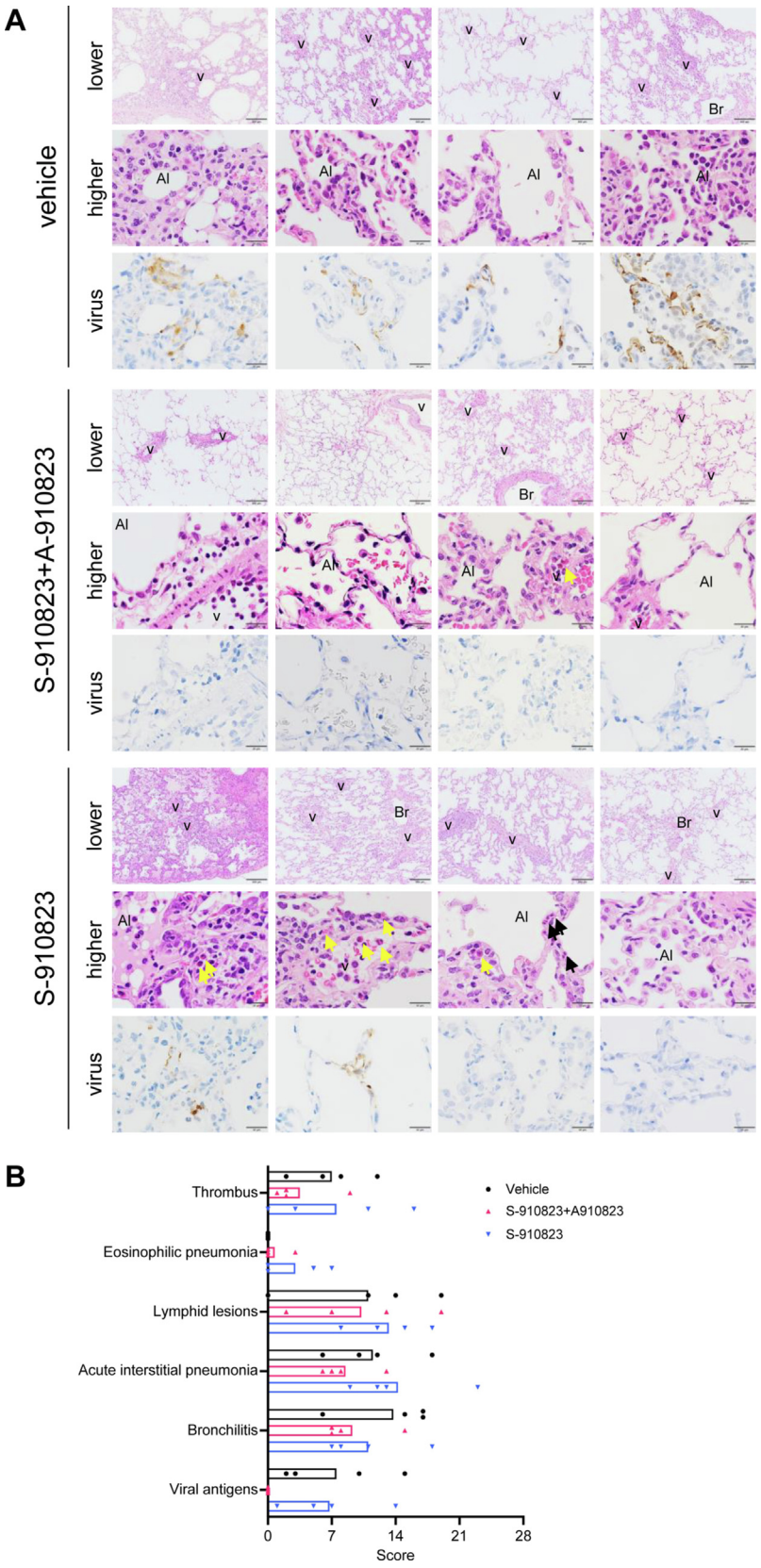


Fig. 5. Histopathological evaluation of cynomolgus monkey lung tissue after infection with SARS-CoV-2. (**A**) Representative histopathological findings of the lungs of cynomolgus monkeys 7 days post-infection. Upper and middle rows are low- and high-magnification, respectively, of hematoxirin and eosin stained paraffin-embedded lung tissue sections. Bottom rows of each panel show high-magnification of representative immunohistochemistry results using an anti-SARS-CoV-2 nucleocapsid protein-specific antibody. Yellow arrows indicate eosinophils; black arrows indicate neutrophils. Al, alveoli, Br, bronchi, v, vessels. The bars represent 200 μm at low magnification and 20 μm at high magnification. (**B**) Histopathological scores of pulmonary tissues from immunized monkeys after SARS-CoV-2 challenge infection. Each point represents the histopathological score of an individual monkey. The bars represent mean scores of each experimental group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

immunopathology in the lungs of mice upon challenge infection, which was related to insufficient induction of antibodies against SARS-CoV and a skewed immune reponse toward a Th2 response [13,15,27,28]. Given that the relatively greater induction of Th2 cytokines by 25 μg S-910823 may elevate risks for VDE, we concluded that the appropriate dose of S-910823 in the cynomolgus monkey model was 5 or 10 μg/dose.

S-268019-b induced potent neutralizing antibody titers against SARS-CoV-2, which were comparable to titers of the "High" (20/150) WHO International Reference Panel. According to WHO, the titer of the 20/150 reference panel is higher than that of the WHO International Standard (20/136) [19]. The 20/136 standard has recently been used to estimate correlates of protection against COVID-19 in clinical trials, and the neutralizing antibody titer of the 20/136 standard has been shown to be higher than that induced by viral vector-based or mRNA-based vaccines [29–31]. Given the neutralizing antibody titer induced by S-268019-b was comparable to that of 20/150, S-268019-b is expected to have the potential to provide substantial protection against COVID-19. In addition, we showed that immunization with S-268019-b containing 10 μg of S-910823 provided protective efficacy against challange SARS-CoV-2 infection. As the neutralizing antibody titer declined to approximately 320 in immunized monkeys at 8 to 13 weeks post second vaccination, this basal level of neutralizing antibody titer may be adequate for preventing SARS-CoV-2 infection as the challenged monkeys were protected. This suggests the protective efficacy elicited by S-268019-b was retained for several months. However, further studies correlating neutralizing antibody titer and protecton against SARS-CoV-2 infection are still required.

Although S-268019-b elicited potent neutralizing antibody against an original SARS-CoV-2 virus, reaching levels comparable to those of the WHO International Reference Panel High 20/150 (1473 international unit/mL), Beta and Gamma variants were approximately 3-fold more resistant compared to the original viruses, and the Omicron variant was approximately 14-fold more resistant. The reduced neutralizing antibody titers were comparable to those of currently marketed vaccines, such as BNT162b2 and mRNA-1273 [32,33]. Given the antigen of S-268019-b is based on full-length S protein and anti-RBD antibodies are well-correlated with neutralizing antibody titer, RBD mutations may have been responsible for the reduced neutralizing antibody titers. Therefore, continuous monitoring of mutations that affect neutralizing antibody titer is important.

Previous studies suggest the balance between Th1 and Th2 immune responses may correlate with potential VDE risk [13,15,27,28]; therefore, we aimed to develop a vaccine that induces a Th1 > Th2 balanced immune response. We confirmed antigen-specific IFN-γ production from PBMCs of S-268019-b immunized monkeys, whereas Th2-related IL-4 and IL-5 production were lower than that of Th1-related IFN-γ. These results suggest that S-268019-b induced a Th1/Th2 balanced immune response, and did not predominantly enhance a Th2 type immune response. Accordingly, we concluded the risk of S-268019-b vaccination inducing VDE attributed to allergic inflammation-related cytokine production would be limitted. As VDE is a potential complication hindering the successful development of vaccines against viruses, the COVID-19 pandemic has fueled concerns that VDE may hinder the safety of SARS-CoV-2 vaccines [13,15,27,28]. However, previous studies using non-human primate models to evaluate adjuvanted SARS-CoV-2 vaccines with S-protein as antigen have reported no indication of VDE upon virus challenge [23,24]. Regardless, careful monitoring will still be needed to evaluate the potential risk of VDE in clinical trials of S-268019-b in humans.

The current study had limitations. First, potential species-related differences exist between SARS-CoV-2 infections in humans and non-human primates, such as cynomolgus monkeys [26]. Second, the number of cynomolgus monkeys in each group was small, limiting some quantitative analyses. Third, since a model to estimate the potential risk of VDE has not been established in cynomolgus monkeys, it is possible that the current results underestimated the actual potential risks [16].

In conclusion, this study is the first evaluation of immunogenicity of the COVID-19 vaccine S-268019-b in non-human primates. The S-268019-b vaccine, which is composed of SARS-CoV-2 S-protein and A-910823 adjuvant, induced neutralizing antibody and cellular immunity in cynomolgus monkeys after the second vaccine administration. The findings also confirmed the safety of the vaccine in cynomolgus monkeys. Overall, the results support the clinical evaluation of S-268019-b in effort to provide a safe and efficient tool in combatting the worldwide health threat of COVID-19.

Ethical Approval/Informed Consent

All procedures using SARS-CoV-2 were conducted in a BSL3 laboratory. Experiments using recombinant DNA and pathogens were approved by the Committee for Experiments using Recombinant DNA and Pathogens at Shionogi & Co., Ltd. and NIID, Japan. For the immunogenicity studies, the animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC 055-870) of Shin Nippon Biomedical Laboratories in Japan, which is accredited by AAALAC International. All animal protocols were in accordance with the animal welfare bylaws of Shin Nippon Biomedical Laboratories. The SARS-CoV-2 challenge studies were carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. All animal experiments were conducted in strict compliance with animal husbandry and welfare regulations and were approved by the Committee on Experimental Animals at NIID, Japan (approval no. 521006). All experimental animals in the BSL3 animal facilities were handled according to the guidelines of the biorisk management of NIID, Japan (approval no. 21-43).

Declaration of Competing Interest

MH, TH, HM, KD, NS, KY, TSo, and SO are employees of Shionogi & Co., Ltd. The other authors declare that they have no competing interests.

Acknowledgements

We acknowledge Masataka Fumoto, Eiji Nikaido, Jun Hirose (Shionogi & Co., Ltd.), and all members of UMN Pharma Inc. for generating the antigens and vaccine formulation. We thank Yusuke Kawazu and the members of Shionogi TechnoAdvance Research Co., Ltd. for excellent technical assistance. We are grateful to Akira Ainai, Midori Ozaki, Takiko Yoshida, and Dai Izawa for technical assistane, and Yasushi Ami, Yuriko Suzaki, Kenzo Yonemitsu, Toshihiko Harada and the members of the Management Department of Biosafety and Laboratory Animals for support with the BSL3 facillity at NIID, Japan. We thank Satoshi Kojima, Yoshikazu Ishimoto, and Haruna Hayashi (Shionogi & Co., Ltd.) for support in development of the manuscript. Editorial support in the form of medical writing, assembling tables, and creating high-resolution images based on the authors' detailed directions, collating author comments, copyediting, fact checking, and referencing was provided by Editage, Cactus Communications.

Funding

This study was supported by internal funding from Shionogi & Co., Ltd. and the Japan Agency for Medical Research and Develop-

ment (AMED) under Grant Number JP21nf0101626 to Shionogi & Co., Ltd. and JP19fk0108112 to HH. Medical writing assistance was funded by Shionogi & Co., Ltd.

Author contributions

MH: Neutralizing antibody titer testing with live viruses, project design, data analysis and interpretation, visualization, original draft. NN: Study design for challenge infection, methodology, investigation, visualization, original draft. TH: Project design, data analysis and interpretation. HM: Neutralizing antibody titer testing with live viruses. KD: FluoroSpot assays. NS: Neutralizing antibody titer testing with pseudovirses. KY: Neutralizing antibody titer testing with live viruses. NI-Y: Investigation for challenge infection. NS-S: Investigation for challenge infection. YSa: Investigation for challenge infection. MS: Investigation for challenge infection. NK: Investigation for challenge infection. TA: Investigation for challenge infection. YSu: Investigation for challenge infection. SW: Investigation for challenge infection. HA: Investigation for challenge infection. TSo: Consultation. TSu: Study design, Investigation for challenge infection. SO: Conceptualization and supervision. HH: Conceptualization and supervision.

Unblinded statements

All animal experiments were approved by the SNBL Institutional Animal Care and Use Committee (Approval No. IACUC055-870) and performed in accordance with the animal welfare bylaws of SNBL. The animal care and manipulations are detailed in the Supplementary Material. The SARS-CoV-2 challenge studies were carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. All animal experiments were conducted in strict compliance with animal husbandry and welfare regulations and were approved by the Committee on Experimental Animals at NIID, Japan (approval no. 521006).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.05.081.

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