Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Corbett KS, Flynn B, Foulds KE, et al. Evaluation of the mRNA-1273 vaccine against SARS-CoV-2 in nonhuman primates. N Engl J Med. DOI: 10.1056/NEJMoa2024671

Supplementary Appendix to a Manuscript Entitled

mRNA-1273 Vaccine against SARS-CoV-2 Infection in Nonhuman Primates

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ADDITIONAL METHODOLOGICAL DETAILS

SARS-CoV-2 virus

Rhesus macaques were challenged with SARS-CoV-2 (USA-WA1/2020 strain) from BEI Resources (NR-52281).

mRNA-1273 mRNA and LNP Production Process

A sequence-optimized mRNA encoding prefusion-stabilized SARS-CoV-2 S-2P protein was synthesized *in vitro* using an optimized T7 RNA polymerase-mediated transcription reaction with complete replacement of uridine by N1m-pseudouridine[1]. *In vitro* transcription source material included a DNA template containing the immunogen open-reading frame flanked by 5' UTR and 3' UTR sequences and was terminated by an encoded polyA tail. After transcription, the Cap 1 structure was added to the 5' end using Vaccinia capping enzyme (New England Biolabs) and Vaccinia 2'O-methyltransferase (New England Biolabs). The mRNA was purified by oligo-dT affinity purification, buffer exchanged by tangential flow filtration into sodium acetate, pH 5.0, and sterile filtered. The manufacturing process intermediate mRNA was kept frozen at –20 °C until further use.

The mRNA was encapsulated in a lipid nanoparticle through a modified ethanol-drop nanoprecipitation process described previously[2]. Briefly, ionizable, structural, helper, and PEG lipids were mixed with mRNA in acetate buffer, pH 5.0, at a ratio of 2.5:1 (lipids:mRNA). The mixture was neutralized with Tris-Cl, pH 7.5, sucrose was added as a cryoprotectant, and the final solution was sterile filtered. Vials were filled with formulated LNP and stored frozen at –70 °C until further use. The final drug product underwent analytical characterization, which included the determination of particle size and polydispersity, encapsulation, mRNA purity, double stranded RNA content, osmolality, pH, endotoxin, and bioburden, and the material was deemed acceptable for *in vivo* study.

Quantification of SARS-CoV-2 RNA and sgRNA

SARS-CoV-2 in lungs was quantified by plaque assay to determine infectious virus. PCR was used to determine viral RNA load in BAL and ND; both methods are previously-published[3]. To quantify nascent viral replication, sgRNA was detected in BAL and NS using previously-published methods[4].

Histopathology

NHP SARS-CoV-2 infected lung samples were processed per a standard protocol. Briefly, the tissues were fixed in 10% neutral buffered formalin, processed with Leica ASP6025 tissue processor (Leica Microsystems), embedded in paraffin, and sectioned at 5 µm for histological analysis. Tissue sections were stained with hematoxylin and eosin (H&E) for routine histopathology and then evaluated by a board-certified veterinary pathologist using an Olympus BX51 light microscope. Photomicrographs were taken using an Olympus DP73 camera.

Immunohistochemistry (IHC)

Immunohistochemical (IHC) staining was performed using formalin-fixed paraffin-embedded tissues sections (5 µm) obtained from lung tissues. IHC staining used a rabbit polyclonal SARS-CoV-2 (GeneTex, GTX135357) with dilution of 1:2000. Staining was carried out on the Bond RX (Leica Biosystems) platform according to manufacturer-supplied protocols. Briefly, 5µm-thick sections were deparaffinized and rehydrated. Heat-induced epitope retrieval (HIER) was performed using Epitope Retrieval Solution 1, pH 6.0, heated to 100°C for 20 minutes. Specimens were then incubated with hydrogen peroxide to quench endogenous peroxidase activity prior to applying the primary antibody. Detection with DAB chromogen was completed using the Bond Polymer Refine Detection kit (Leica Biosystems, DS9800). Slides were finally cleared through gradient alcohol and

xylene washes prior to mounting and placing cover slips. Sections were examined by a boarded-certified veterinary pathologist using an Olympus BX51 light microscope and photomicrographs were taken using an Olympus DP73 camera.

In-Situ Hybridization (ISH)

Chromogenic In-Situ Hybridization (cISH) was preformed using RNAScope technology. The target probe detecting positive-sense SARS-CoV-2 (RNAscope® 2.5 LS Probe- V-nCoV2019-S, 848568,) was designed by Advanced Cell Diagnostics (ACD). Epitope retrieval was performed on tissues with an application of Bond Epitope Retrieval Solution 2 (Leica Microsystems) for 15 min at 95 °C followed by a 15 min digestion with Protease III (ACD) at 40 °C. Tissues were hybridized with the probes for 2 hr and visualized with the RNAscope 2.5 LS Reagent Kit-RED (ACD) according to manufacturer's protocol.

Measurement of IgG in Sera

Total IgG in sera was determined by ELISA, similar to previously-published methods[7]. Briefly, Nunc-Immuno Microwell 96-well MaxiSorp-treated plates (Sigma-Aldrich) (ThermoFisher) were coated with SARS-CoV-2 S-2P (100 ng/well) or N protein (400 ng/well) in 1X PBS at 4°C for 16 hr. After standard washes and blocks, plates were incubated with duplicate serial dilutions (1:100, 4-fold, 8X) of heat-inactivated (HI) sera for 1 hr at room temperature (RT). Following washes, secondary anti-monkey IgG (H+L) HRP Secondary Antibody (ThermoFisher) in blocking buffer at a 1:10000 dilution (ThermoFisher) was added. 3,5,3′5′-tetramethylbenzidine (TMB) (KPL) substrate was used to detect Ab responses. The optical density (OD) of each well was read at 450 nm and 650 nm by SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices). Area under the curve (AUC) was calculated using Prism v8 (GraphPad), setting the baseline with our defined endpoint (average of negative control wells + 10STDEV) and taking the total peak area.

ACE2 Binding Inhibition Assay

MSD 384-well, 4-Spot Custom Serology SECTOR® plates precoated with RBD were generously supplied by the manufacturer. On the day of the assay, the plate was blocked for 30 min with MSD Blocker A (5% BSA) at RT. The blocking solution was washed off (3 cycles of PBS-T), and test samples were applied in duplicates to the wells at 1:40 dilution and allowed to incubate with shaking (1300 rpm) for 1 hr at RT. MSD Assay Diluent 100 in quadruplicate was used as negative control. MSD SULFO-TAG™ labeled ACE2 (supplied as 200X) was applied to the wells and allowed to associate with test samples and RBD within the assay wells for 1 hr at RT with shaking (1300 rpm). Plates were then washed (3 cycles of PBS-T) to remove unbound detection antibody. MSD GOLD™ read buffer substrate was applied to the wells, and the plate was entered into the MSD MESO Sector S 600 detection system. A current is applied to the plate and areas of well surface where RBD has complexed with SULFO-TAG™-ACE2 will emit light in the presence of the electrochemiluminescence (ECL) substrate. The MESO Sector S 600 detection system quantitates the amount of light emitted and reports the ECL unit response as a result for each test sample and control of the plate. The amount of signal emitted in wells containing no sample (assay diluent only) is evaluated as the maximal binding response. Reduction of ECL response from this maximal readout is directly proportional to the extent of competitive binding activity in the test sample.

Pseudovirus Neutralization Assay

To produce SARS-CoV-2 pseudoviruses, a codon-optimized CMV/R-SARS-CoV-2 S (Wuhan-1, Genbank #: MN908947.3) plasmid was constructed. Pseudoviruses were produced by co-transfection of plasmids encoding a luciferase reporter, lentivirus backbone, human transmembrane protease serine 2 (TMPRSS2), and S genes into HEK293T/17 cells (ATCC), and neutralization was assessed as previously described[8]. Briefly, triplicate HI sera was mixed with pseudoviruses, incubated, and then added to ACE2-expressing 293T

cells. Seventy-two hr later, cells were lysed, and luciferase activity (relative light units, RLU) was measured. Percent neutralization was normalized considering uninfected cells as 100% neutralization and cells infected with only pseudovirus as 0% neutralization. ID_{50} titers were determined using a log (inhibitor) vs. normalized response (variable slope) nonlinear function in Prism v8 (GraphPad).

Nanoluc Virus Reporter Assay

A full-length SARS-CoV-2 virus based on the Seattle Washington isolate was designed to express luciferase and was recovered via reverse genetics and described previously [9-11]. The virus was titered in Vero E6 USAMRID cells to obtain a relative light units (RLU) signal of at least 10X the cell only control background. Vero E6 USAMRID cells were plated at 20,000 cells per well the day prior in clear bottom black walled 96-well plates. Neutralizing antibody serum samples were tested at a starting dilution of 1:50 and were serially diluted 2-fold up to ten dilution spots. Antibody-virus complexes were incubated at 37°C with 5% CO_2 for 1 hr. Following incubation, media was removed, and virus-antibody dilution complexes were added to the cells in duplicate. Virus-only controls and cell-only controls were included in each neutralization assay plate. Following infection, plates were incubated at 37°C with 5% CO_2 for 24 hours. After the 24 hr incubation, cells were lysed, and luciferase activity was measured via Nano-Glo Luciferase Assay System (Promega), according to the manufacturer specifications. SARS-CoV-2 neutralization titers were defined as the sample dilution at which a 50% reduction in RLU was observed relative to the average of the virus control wells.

Intracellular Cytokine Staining (ICS)

Cryopreserved PBMC were thawed and rested overnight at 37°C in 5% CO_2. Cells were then stimulated with SARS-CoV-2 S protein (S1 and S2, homologous the vaccine insert) and Nucleoprotein (N) peptide pools (JPT Peptide Technologies, Inc.) at a final concentration of 2 µg/mL in the presence of monensin and costimulatory antibodies anti-CD28 and -49d (clones CD28.2 and 9F10, BD Biosciences) for 6 hr. Negative controls received an equal concentration of DMSO (instead of peptides) and co-stimulatory antibodies. ICS was performed as described[12] except the following monoclonal antibodies were added: PD-1 BUV737 (clone EH12.1, BD Biosciences) in place of PD-1 BV785, TNF-FITC (clone Mab11, BD Biosciences) in place of IL-5 BB515, and CD40L BV785 (clone 24-31, BioLegend). Aqua LIVE/DEAD kit (Invitrogen) was used to exclude dead cells. All antibodies were previously titrated to determine the optimal concentration. Samples were acquired on an BD FACSymphony flow cytometer and analyzed using FlowJo version 9.9.6 (Treestar, Inc.).

Statistical Analysis

Eight animals were chosen to have 90% power to detect a difference in viral loads in the BAL of about 1.3 $\log_{10}$ between the 100 µg and control, using a standard deviation of 0.75 $\log_{10}$ (estimated from published data[14]), and assuming 100 µg leads to at least as big an effect as 10 µg. A hierarchical testing procedure was prespecified in the protocol, where the 100 µg group was tested against PBS first, and then if and only if that was significant ($p<0.05$), the 10 µg group was tested against both PBS and 100 µg. The power calculation was based on this structure of hierarchical testing. Other exploratory comparisons use a Kruskal-Wallis followed by Holm's adjustment within families of pairwise group comparisons (including convalescent sera).

Dotted lines on graphs indicate assay limits of detection. ICS response rates were estimated using the MIMOSA[13] package, which uses a Bayesian hierarchical beta-binomial model to calculate the probability that each individual is responding to a specific stimulation; values that were judged to be a probable response, subject to a 0.05 false discovery rate constraint, are considered responses for these analyses. The model used a Beta (1,1) prior on the proportion of responders, and exponential (.0001) priors on the hyperparameters.

Graphs were generated using PRISM v8 (GraphPad Software) and analyses in R version 4.0.0. The data from this study were not used to decide whether or not to move forward with the vaccine. This was designed to be

an exploratory study of multiple different assays for further follow-up and consideration in potential efficacy trials.

SUPPLEMENTAL METHODS

Cell Lines

293T/17 (ATCC) and ACE2-expressing 293T cells (provided by Michael Farzan, Scripps Research Institute) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, and 1% penicillin/streptomycin at 37°C and 5% CO_2. Vero E6 cells used in NanoLuc virus reporter assay were cultured in DMEM supplemented with 10% Fetal Clone II and amphotericin B [0.25 µg/ml] at 37°C and 5% CO_2. Expi293 cells were maintained in manufacturer's suggested media.

SARS-CoV-2 S-2P, RBD, and S1_NTD Protein Expression and Purification

Vectors encoding SARS-CoV S-2P[5] were generated as previously described with the following small amendments. Proteins were expressed by transfecting plasmids into Expi293 cells using Expifectamine transfection reagent (ThermoFisher) and growing them in suspension at 37°C for 4-5 days. Cell culture supernatants were collected, buffer exchanged into 1X PBS, and protein was purified using Strep-Tactin resin (IBA). Size exclusion chromatography using Superose 6 Increase column (GE Healthcare) yielded final purified protein. For biotinylated SARS-CoV-2 S-2P, RBD, and S1_NTD protein expression, purification, and conjugation was performed as previously described[6].

SARS-CoV-2 N Protein Expression and Purification

A human codon-optimized secreted SARS-CoV-2 N gene (Genebank#: MN908947) was synthesized and cloned into the VRC8400 plasmid backbone with a N-terminal mIL2 leader and a C-terminal HRV3C cleavage site followed by an 6xHis-tag. After 4-day Expi293 cell transfection, cells were harvested, and clarified cell culture supernatant was filtered. The N protein is first purified with the Hispur Ni-NTA Resin (ThermoFisher) and subsequently with 500mM imidazole (Sigma). Eluted protein was purified by gel filtration on a Superdex 200 HiLoad 16/60 column (GE Healthcare) in 1X PBS.

Measurement of Antibody Isotype Responses in BAL

Total IgG and IgA antigen specific antibodies were determined by ELISA using MULTI-ARRAY 384-well streptavidin-coated plates (Meso Scale Discovery, MSD). Plates were blocked with 5% BSA in 1X PBS for 30 min at RT. After blocking, plates were washed with wash buffer (0.05% Tween-20 in 1X PBS). Plates were coated with 1 µg/mL SARS-CoV-2 S- 2P-Foldon-AVI-biotin protein, 0.18 µg/mL SARS-CoV-2 RBD-AVI-biotin protein, or 0.26 µg/mL SARS-CoV-2 NTD-AVI-biotin protein[6] in 1% BSA in 1X PBS for 1 hr at RT. After incubation, plates were washed. Serial dilutions of 10X concentrated BAL in 1% BSA, 0.05% Tween-20 in 1X PBS were added to wells and incubated for 1 hr at RT. Plates were washed 5X with wash buffer and incubated for 1 hr with 1 µg/mL of anti-human IgG sulfo-tag or 2 µg/mL of anti-human/NHP IgA sulfo-tag (MSD) in 1% BSA, 0.05% Tween 20 in 1X PBS. Plates were then washed 5X with wash buffer, and 1X Read T buffer was added to each well. Signals were read by MSD plate reader (Sector Imager 600).

Measurement of Cytokines and Chemokines in BAL

BALs were analyzed with Bead-Based Multiplex Assays using Luminex. Briefly, BALs were concentrated 10X and added neat in duplicate to MILLIPLEX MAP Nonhuman Primate Cytokine Magnetic Bead Panel (Millipore Sigma), and the standard assay was preformed following manufacturer's instructions. Fluorescence data were collected on MAGPIX with Bio-Plex Manager™ MP software (BioRad).

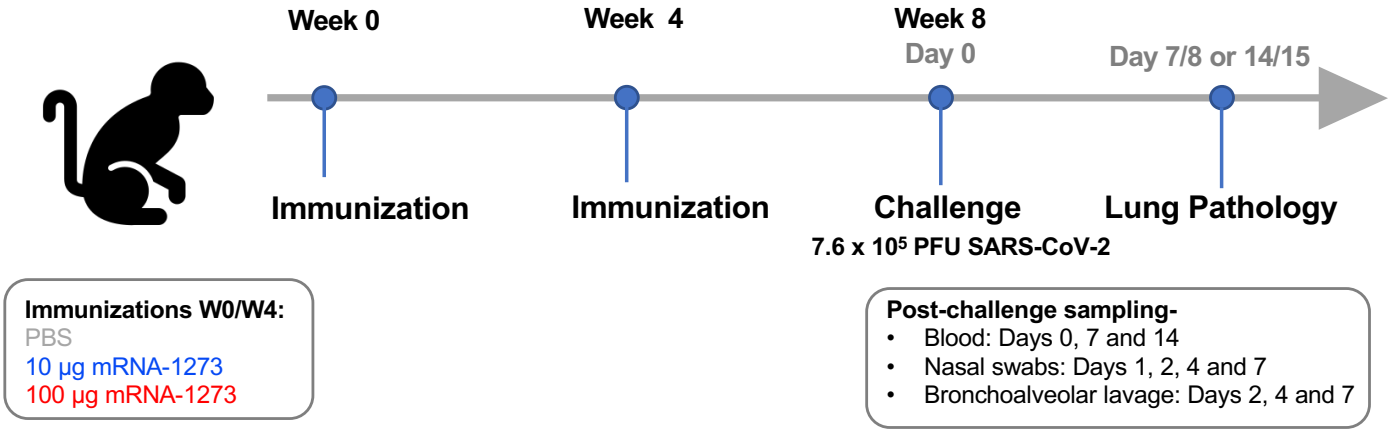

Figure S1. Study design: evaluation of immunogenicity and protective efficacy of mRNA-1273 in rhesus macaques. Rhesus macaques (N=8/group) were immunized at 0 and 4 weeks with PBS (gray) or mRNA-1273 (10 µg, blue or 100 µg, red) and challenged 4 weeks post-boost with a total of 7.6 x 10^5 PFU of SARS-CoV-2. Viral inoculum was administered as 5.7 x 10^5 PFU in 3 mL intratracheally (IT) and 1.9 x 10^5 PFU in 1 mL intranasally (IN) (0.5 mL into each nostril). Sera were collected pre-immunization, bi-weekly post-prime and post-boost, and at days 7 and 14 post-challenge. Nasal swabs (NS) and bronchoalveolar lavages (BAL) were collected post-challenge on days 1, 2, 4, and 7 and 2, 4 and 7, respectively. Lung pathology assessment was assessed on days 7 and 8 post-challenge.

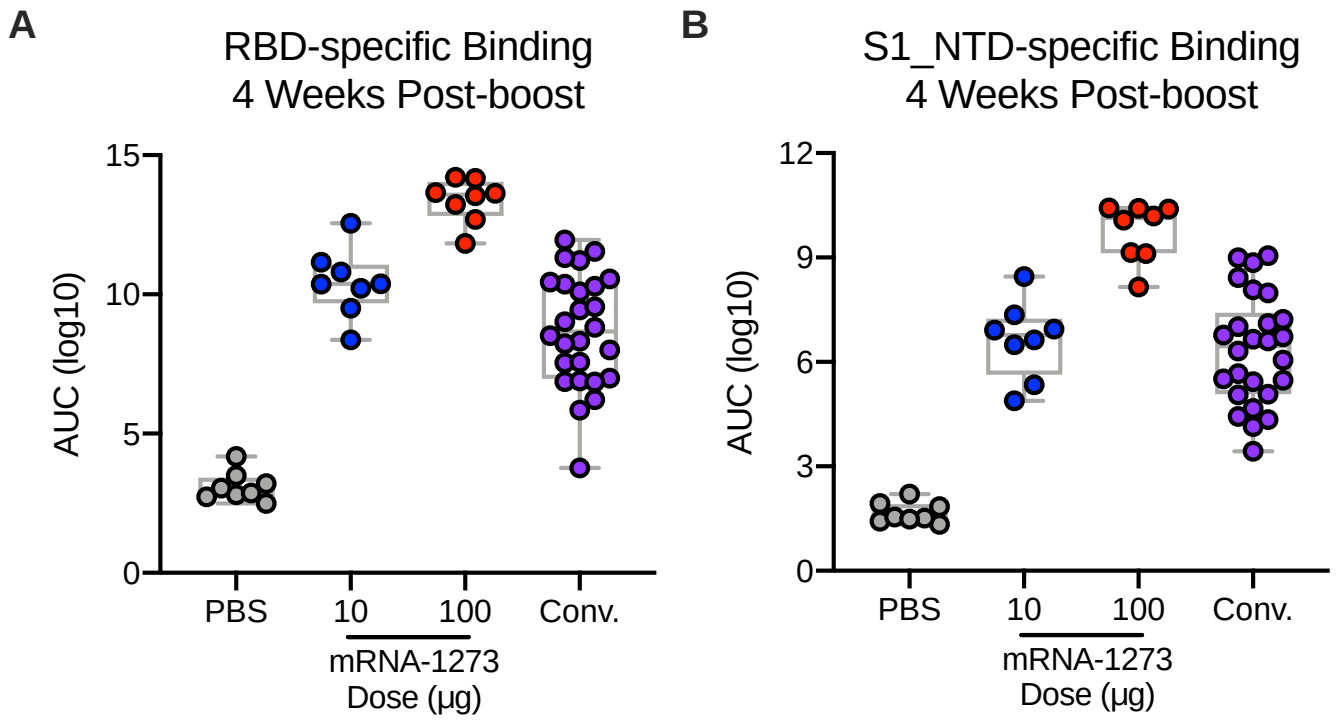

Figure S2. SARS-CoV-2 S antibody domain specificity following mRNA-1273 immunization in NHP. Rhesus Macaques were immunized according to Figure S1 with PBS (gray) or mRNA-1273 (10 µg, blue or 100 µg, red). Sera samples collected 4 weeks post-boost, immediately prior to challenge, and assessed for SARS-CoV-2 IgG specific to the RBD (A) or S1 N-terminal domain (NTD) (B) by MULTI-ARRAY ELISA. A panel of human SARS-CoV-2 convalescent sera (purple) was also assessed.

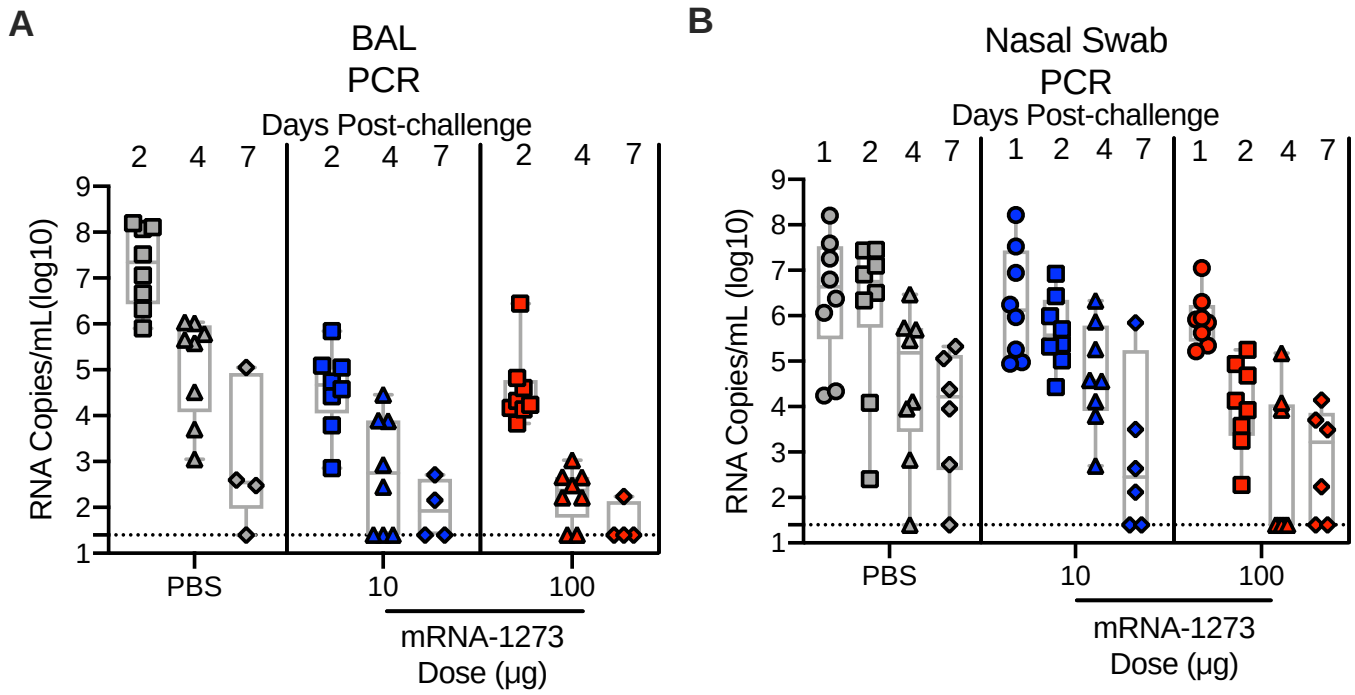

Figure S3. Efficacy of mRNA-1273 against upper and lower respiratory viral load. Groups (N=8) of Rhesus macaques were immunized with PBS (gray) or mRNA-1273 (10 µg, blue or 100 µg, red) and challenged, as described in Figure S1. BAL (A) and nasal swabs (B) were collected on days 1, 2, 4, and 7 post-challenge, where applicable, and viral load was assessed by PCR. Symbols represent individual NHP and overlap for equal values where constrained.

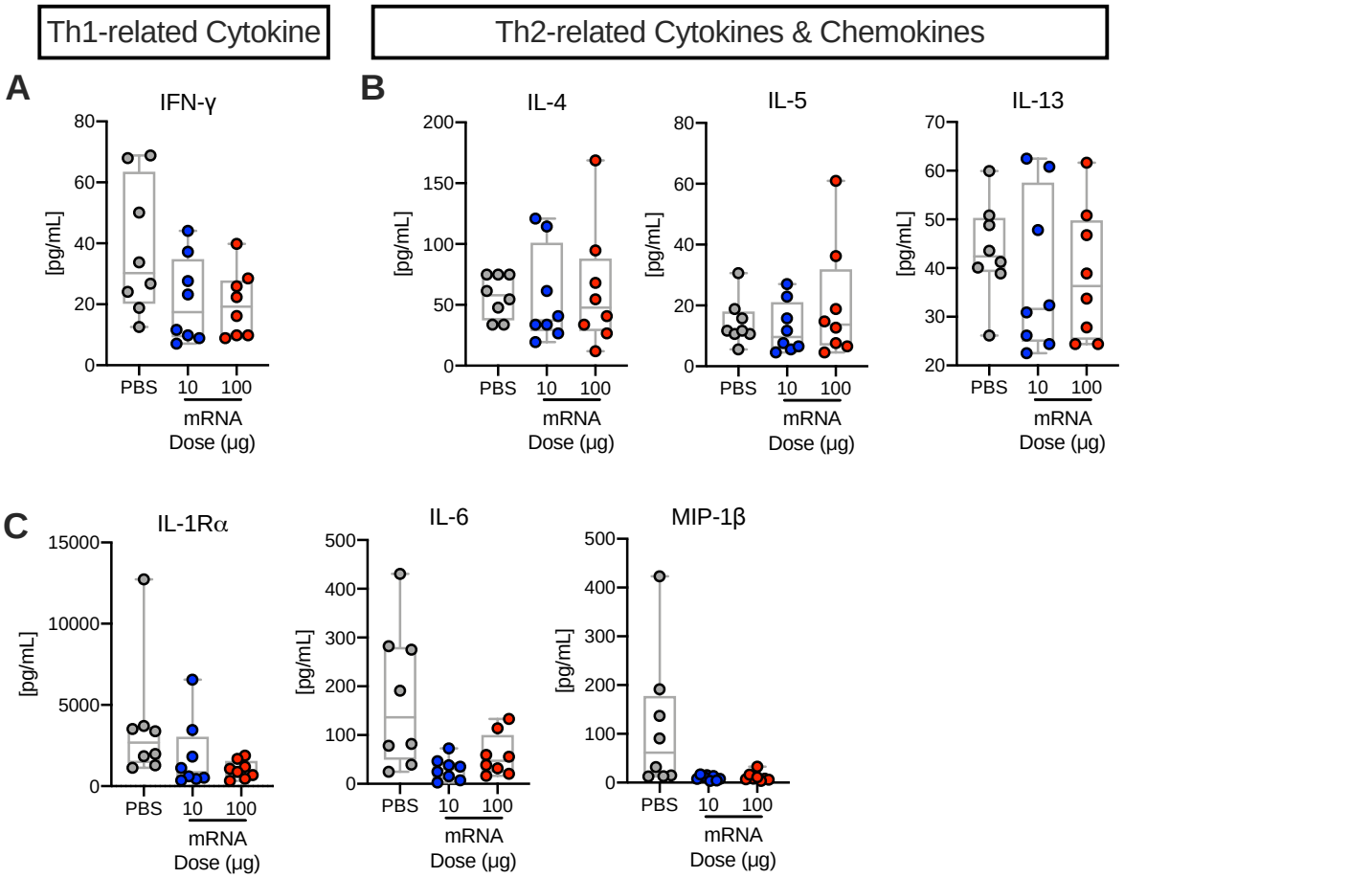

Figure S4. Post-challenge BAL cytokine and chemokine responses in mRNA-1273-immunized rhesus macaques. Rhesus macaques were immunized with PBS (gray) or mRNA-1273 (10 μg, blue or 100 μg, red) and challenged, as described in Figure S1. BAL collected on days 2 and 4 post-challenge were concentrated 10X and assessed for 23 chemokines and cytokines by MILLIPLEX® MAP. Graphs depict day 2 cytokines relevant to VAERD, i.e. Th1-related (A) or Th-2 related (B). Additionally, selected inflammatory cytokines and chemokines are shown in (C). Data for all cytokines and chemokines are available in **Table S2**.

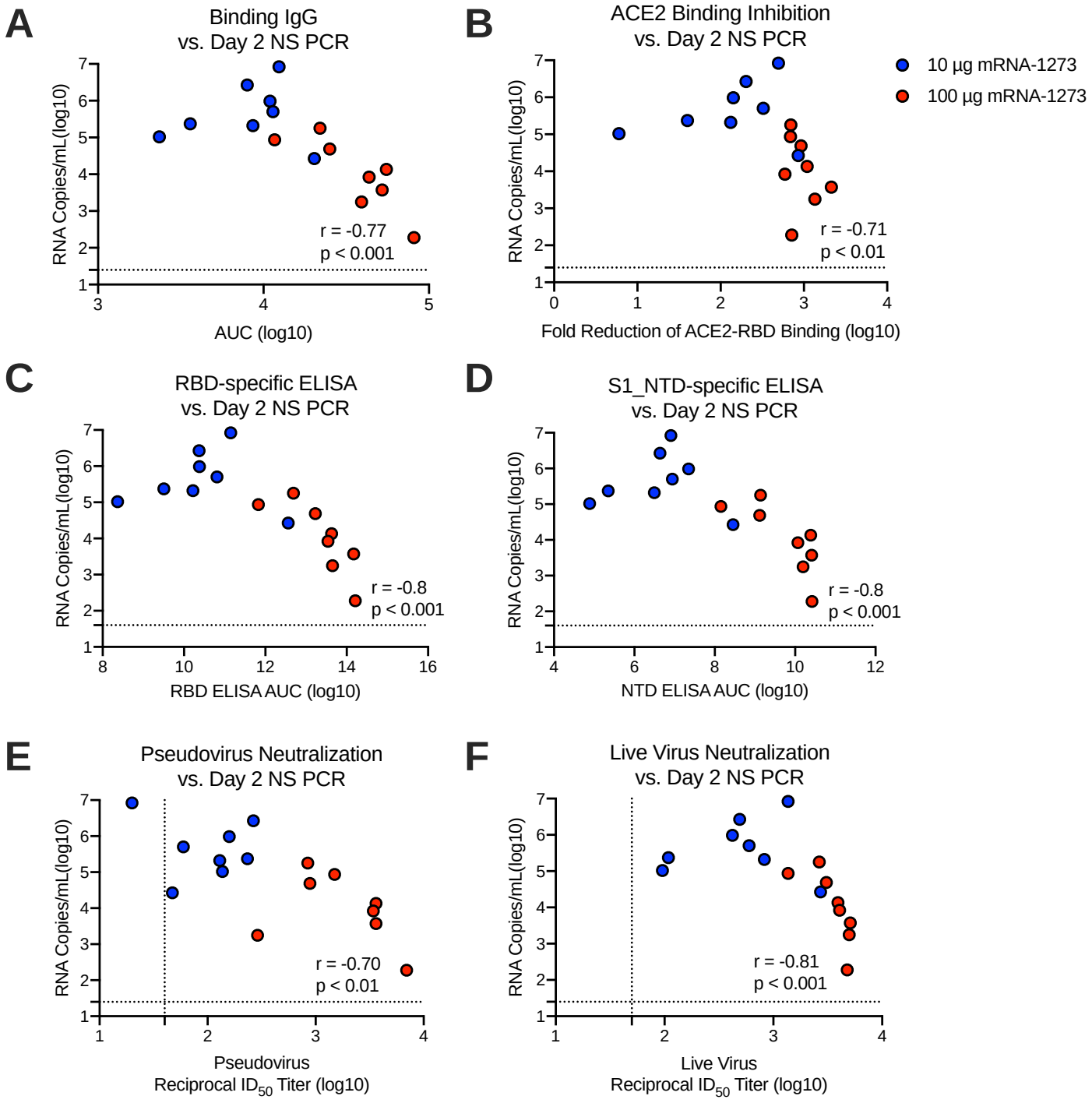

Figure S5. Pre-challenge binding and functional antibody correlations to post-challenge viral assessments. Rhesus macaques were immunized with PBS (gray) or mRNA-1273 (10 µg, blue or 100 µg, red) and challenged, as described in Figure S1. Sera were collected 4 weeks post-boost, immediately prior to challenge, and the following antibody measurements were correlated with day 2 post-challenge NS PCR. (A) S-specific IgG, (B) ACE-2 binding inhibition, (C) RBD-specific IgG, (D) S1_NTD-specific IgG, (E) pseudovirus neutralization, (F) live virus neutralization.

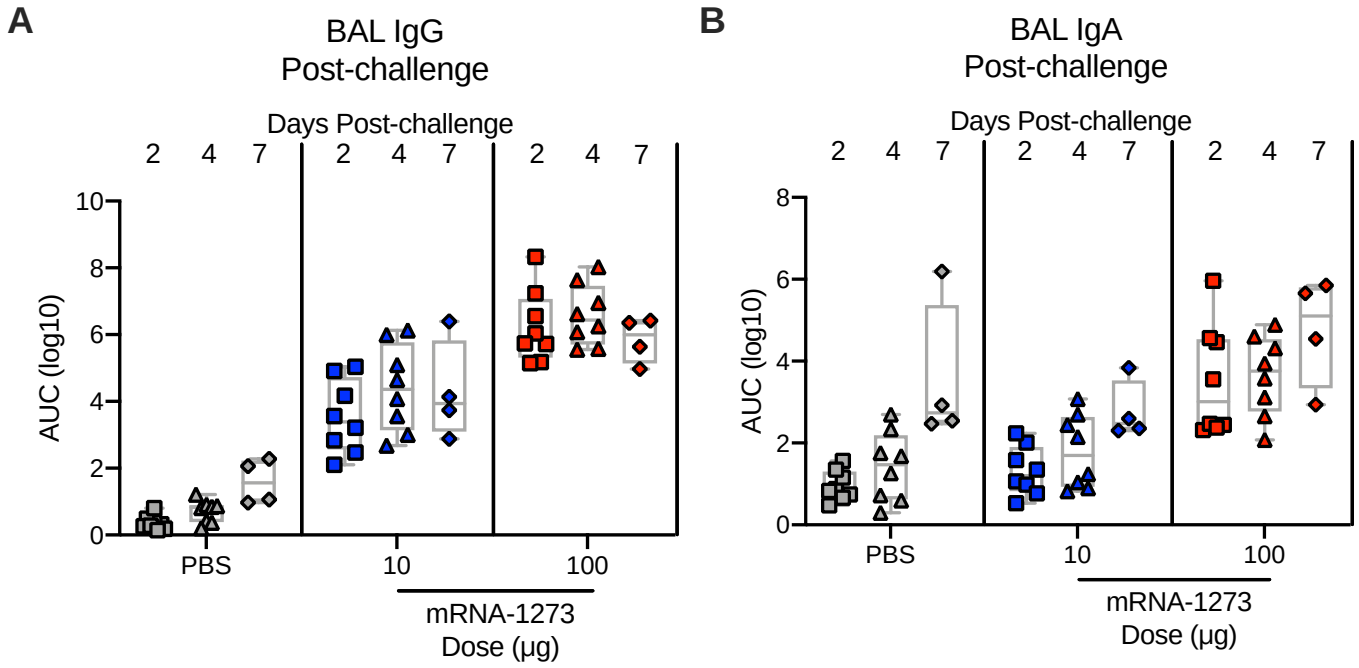

Figure S6. Post-challenge BAL antibody responses in mRNA-1273-immunized rhesus macaques. Rhesus macaques were immunized with PBS (gray) or mRNA-1273 (10 µg, blue or 100 µg, red) and challenged, as described in Figure S1. Post-challenge BAL samples were assessed for SARS-CoV-2 S-specific IgG (A) or IgA (B) by MULTI-ARRAY ELISA.

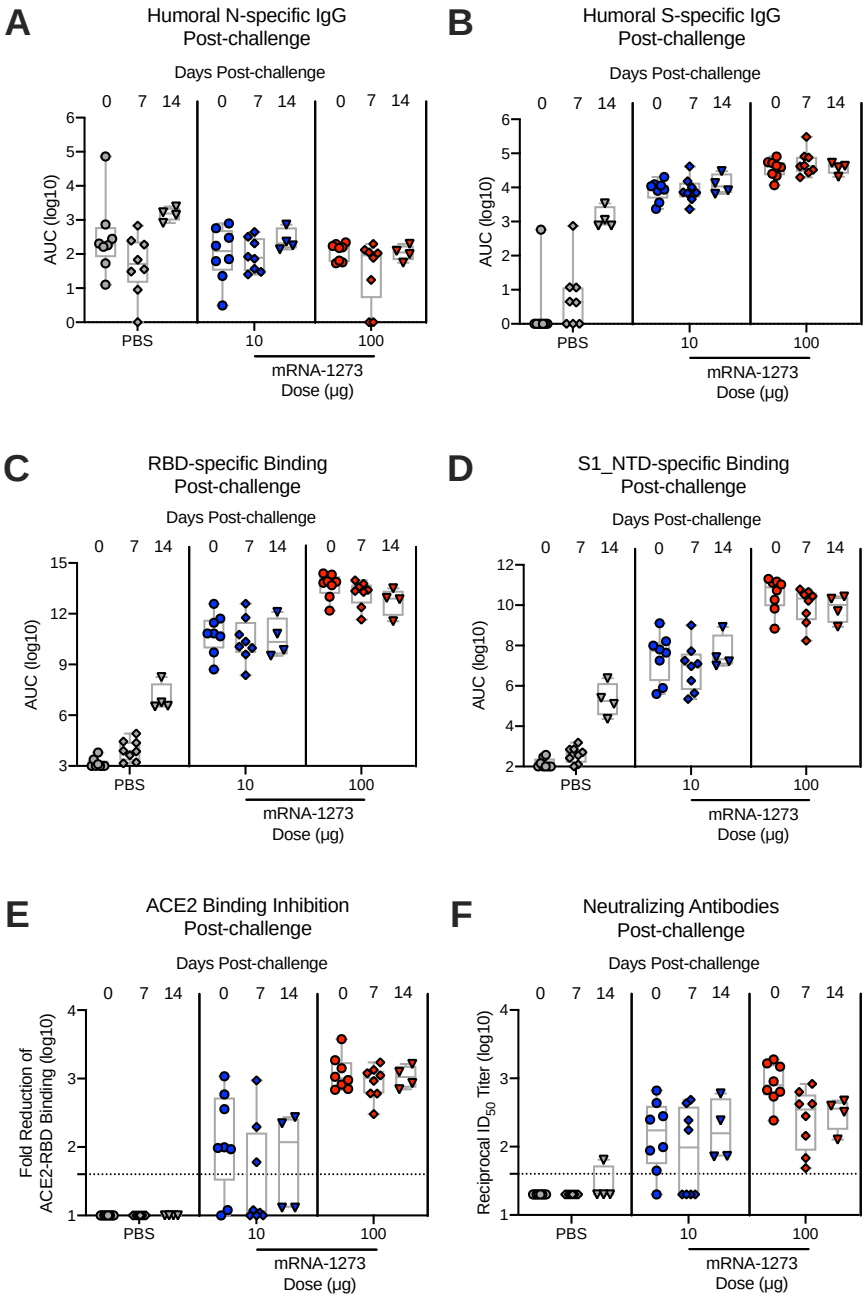

Figure S7. Post-challenge humoral antibody responses in mRNA-1273-immunized rhesus macaques. Rhesus macaques were immunized with PBS (gray) or mRNA-1273 (10 µg, blue or 100 µg, red) and challenged, as described in Figure S1. Sera were collected post-challenge on days 0 (4 weeks post-boost, immediately prior to challenge), 7, and 14 and assessed for SARS-CoV-2 N- (A), S- (B), RBD- (C), and S1_NTD-specific IgG (D) by ELISA and ACE2 binding inhibition (E) pseudovirus neutralization (F).

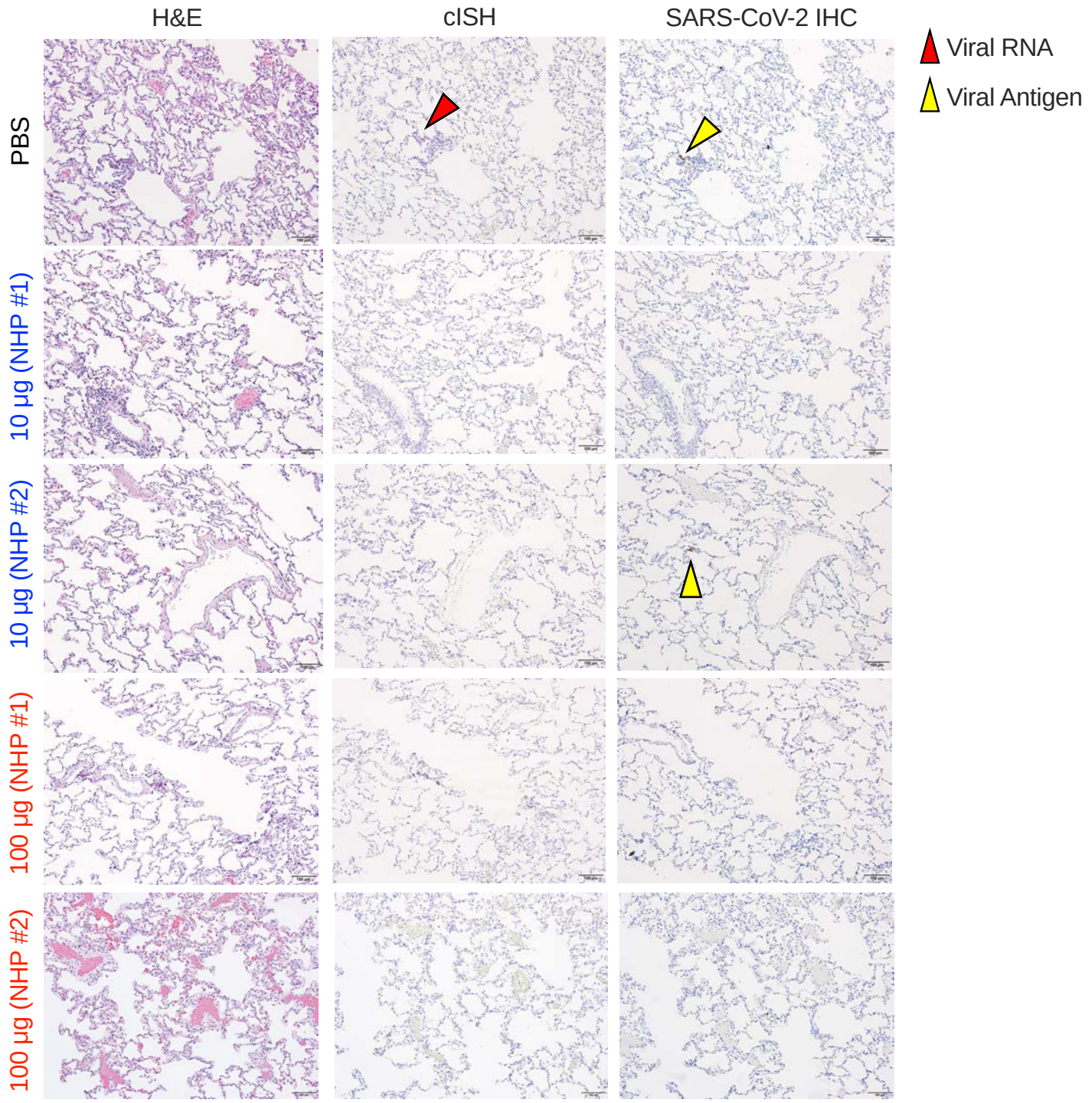

Figure S8. Lung histopathology and viral detection 8 days post-challenge in mRNA-1273-immunized rhesus macaques. Rhesus macaques were immunized and challenged as described in Figure S1. Eight days post-challenge lungs were harvested from 2 NHP per group and assessed for histopathology and evidence of viral infection; representative images taken at 10X magnification are shown for localization of virus by chromogenic In Situ Hybridization (cISH) and SARS-CoV-2 immunohistochemistry (IHC) in serial tissue sections. Lung sections from both NHP immunized with 10 and 100 µg mRNA-1273 are shown. Scale bars represents 100 µm (10X).

Table S1. Assessment of lung inflammation, viral RNA, and viral antigen following SARS-CoV-2 challenge of mRNA-1273-immunized rhesus macaques.

Day Post-challenge	mRNA Dose (μg)	Inflammation	ISH (RNA; R[1]/L[2])	IHC2 (SARS CoV-2 Ag; R/L)
7	PBS	moderate	+/- / +/-[3]	+ / +
7	PBS	moderate-severe	+ / +	+ / +
7	10	minimal-mild	-/-	-/-
7	10	mild-moderate	-/-	-/-
7	100	minimal-absent	-/-	-/-
7	100	minimal-absent	-/-	-/-
8	PBS	moderate-severe	+/- / -	+ / -
8	PBS	moderate	+/- / +	+ / +
8	10	minimal-mild	-/-	-/-
8	10	minimal-mild	-/-	[4]+/- / -
8	100	minimal-mild	-/-	-/-
8	100	mild-moderate	-/-	-/-
14	100	minimal-absent	-/-	-/-
14	100	minimal-absent	-/-	-/-
14	10	minimal-mild	-/-	-/-
14	10	minimal-absent	-/-	-/-
14	PBS	minimal-mild	-/-	-/-
14	PBS	mild-moderate	-/-	-/-
15	100	minimal-absent	-/-	-/-
15	100	minimal-absent	-/-	-/-
15	10	minimal-mild	-/-	-/-
15	10	minimal-mild	-/-	-/-
15	PBS	moderate	-/-	-/-
15	PBS	mild-moderate	-/-	-/-

Lung tissue was evaluated for the presence of inflammation, SARS-CoV-2 viral RNA, and viral antigen.
[1] lung left side
[2] lung right side
[3] – = not detected; +/- = occasional positive foci; + = multiple positive foci
[4] viral antigen detected in a single pneumocyte

Table S2. Nonhuman primate experimental data

Please see excel file *Manuscript data worksheets-Table S2.*

Table S3. Nonhuman primate model development data

Please see excel file *Manuscript data worksheets- Table S3*.

Table S4. Nonhuman primate clinical observations

Please see excel file *Manuscript data worksheets-Table S4.*

Table S5. Nonhuman primate weight and temperature data

Please see excel file *Manuscript data worksheets-Table S5.*

Table S6. Nonhuman primate complete blood cell count (CBC) data

Please see excel file *Manuscript data worksheets-Table S6.*

Table S7. Human convalescent demographics and data

Please see excel file *Manuscript data worksheets-Table S7.*

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