A SARS-CoV-2 ferritin nanoparticle vaccine elicits protective immune responses in nonhuman primates

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The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants stresses the continued need for next-generation vaccines that confer broad protection against coronavirus disease 2019. We developed and evaluated an adjuvanted SARS-CoV-2 spike ferritin nanoparticle (SpFN) vaccine in nonhuman primates. High-dose (50-μg) SpFN vaccine, given twice 28 days apart, induced a T helper cell 1 (Th1)-biased CD4 T cell response and elicited neutralizing antibodies against SARS-CoV-2 wild type and variants of concern, as well as against SARS-CoV-1. These potent humoral and cell-mediated immune responses translated into rapid elimination of replicating virus in the upper and lower airways and lung parenchyma of nonhuman primates after high-dose SARS-CoV-2 respiratory challenge. The immune response elicited by SpFN vaccination and resulting efficacy in nonhuman primates support the utility of SpFN as a vaccine candidate for SARS-causing betacoronaviruses.

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has reached a milestone with the emergency use authorization, approval, and increasing availability of efficacious vaccines (1). Successes in rapid coronavirus vaccine development, however, have been tempered by the rise of viral variants of concern (VOCs) (2). The accelerating frequency with which variants are emerging raises the specter that host-selective pressures may drive the evolution of mutants to escape vaccine-elicited immunity (3). This concern, coupled with stringent cold-chain requirements for product stability and high unit costs for some vaccine platforms (4), justifies the continued development of cost-effective, thermostable vaccines that match currently authorized and approved vaccines in safety and efficacy and are also effective against a wide range of circulating variants and evolving strains, as well as species that may arise from zoonotic reservoirs in the future.

Self-assembling protein nanoparticle vaccines offer the advantage of multivalent antigen presentation, a property previously shown to augment immunogenicity over monovalent immunogens (5–7). Ferritin is a naturally occurring, ubiquitous, iron-carrying protein that self-oligomerizes into a 24-unit spherical particle (8). The threefold axis symmetry of the resulting polymer makes it conducive to conjugation and antigen display of trimeric glycoproteins, such as SARS-CoV-2 spike protein. Ferritin has been evaluated as a vaccine platform for several pathogens (9–11), most notably influenza, for which it has demonstrated immune potency and breadth (12, 13). As such, ferritin vaccines have advanced to phase 1 clinical trials as a strategy to target multiple influenza strains (14, 15).

The prefusion-stabilized form of the spike protein is the basis for most major SARS-CoV-2 vaccine candidates (16, 17). Although a correlative of protection from COVID-19 has not been conclusively defined, there is mounting evidence that neutralizing, and some fraction of non-neutralizing, antibodies against spike protein are necessary, if not sufficient, to confer protective immunity (18, 19). The most potent neutralizing antibodies are directed against the spike protein receptor binding domain (RBD), which mediates...
attachment to the primary host cell receptor, angiotensin-converting enzyme 2 (ACE2). Our prior assessment of a SARS-CoV-2 spike protein ferritin nanoparticle (SpFN) vaccine candidate, coformulated with a liposomal adjuvant, has demonstrated potent immunogenicity and protection against SARS-CoV-2 infection in mouse models (20). These data have provided a basis for evaluating SpFN immunogenicity and efficacy against viral replication and pathology in the airways and lungs of nonhuman primates (NHPs), a standard model for preclinical evaluation of SARS-CoV-2 vaccines (21).

RESULTS
A SARS-CoV-2 nanoparticle vaccine was designed and administered to NHPs

The SpFN vaccine was designed as a ferritin-fusion recombinant protein for expression as a nanoparticle and has been previously described in detail (20). Briefly, the spike protein sequence was derived from the Wuhan-Hu-1 genome sequence (GenBank accession number MN908947.3). The spike protein ectodomain was modified to introduce two proline residues (K986P and V987P) and the removal of the furin cleavage site (RRAS to GSAS), as previously described (16). To stabilize spike protein trimer formation on the ferritin molecule, the heptad repeat between stem hinge 1 and 2 regions (residues 1140 to 1161) was mutated to increase the coiled coil interactions. An adjuvant, Army Liposome Formulation containing QS-21 (ALFQ), was mixed with the SpFN vaccine at room temperature and administered to animals within 4 hours of mixing. Briefly, ALFQ is a liposome formulation comprising saturated phospholipids, dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG); cholesterol (Chol); and two adjuvants, synthetic monophosphoryl lipid A (3D-PHAD) (200 µg per dose) and QS-21 (100 µg per dose) in a molar ratio of 9:1:2:0.114:0.044 (22). QS-21 is a triterpenoid glycoside saponin derived from the bark of the Quillaja saponaria (soap bark) tree, found in Chile. QS-21 binds irreversibly to Chol in the liposomes and abrogates the toxicity seen with free QS-21 (23).

In this study, 32 male and female specific pathogen–free, research-naive Chinese-origin rhesus macaques (age 3 to 7 years) were distributed on the basis of age, weight, and sex into four cohorts of eight animals (table S1). Animals were vaccinated intramuscularly either with 50 or 5 µg of SpFN formulated with ALFQ or 1 ml of phosphate-buffered saline (PBS) in the anterior proximal quadriceps muscle. Animals were vaccinated on alternating sides with each dose in the series. Immunizations were administered twice, 4 weeks apart, or once, 4 weeks before challenge (fig. S1). Animals were challenged with 1 × 10⁶ 50% tissue culture infective dose (TCID₅₀) of SARS-CoV-2 (SARS-related coronavirus 2, isolate USA-WAI/2020) administered simultaneously by the intratracheal (1.0 ml) and intranasal (0.5 ml per nostril) routes.

SpFN vaccination elicits spike protein–specific T cell responses in NHPs

We measured longitudinal antibody responses in animals after each vaccination and after viral challenge. Total binding to SARS-CoV-2 prefusion-stabilized spike protein (S-2P) (16) increased from baseline to an area under the curve (AUC) of 679,213 and 1,646,288 at 4 weeks after two vaccinations with 5 and 50 µg of SpFN, respectively (Fig. 1A). Vaccination with a single 50 µg dose resulted in a 4-week AUC of 621,605. Binding responses were unchanged in vaccinated groups after viral challenge; in contrast, unvaccinated controls had a 200-fold rise (Fig. 1A). Neutralization was tested using spike protein pseudotyped HIV. Two doses of 5 or 50 µg of SpFN elicited reciprocal 50% inhibitory dilution (ID₅₀) pseudovirus neutralizing antibody geometric mean titers (GMTs) of 22,405 and 52,773, respectively, 2 weeks after the second vaccination, and plateaued at 12,171 and 22,527, 2 weeks later (Fig. 1B). Administration of a single dose of 50 µg of SpFN elicited a peak GMT of 4063. Authentic virus neutralization activity mirrored group differences seen in the pseudovirus assay but at slightly lower values (Fig. 1C).

We performed functional assessments of binding antibody responses by measuring the ability of serum samples to inhibit binding of RBD to the ACE2 receptor. Binding inhibition in the 5 µg– and 50 µg–vaccinated animals exceeded that among unvaccinated controls by a factor of 224 and 998, respectively (Fig. 1D). ACE2 competition in the single 50 µg-dose group was 291 times higher than controls. We compared neutralizing antibody responses elicited by the vaccine against a panel of convalescent plasma samples with the same pseudovirus neutralization assay. We found that two doses of either SpFN dose elicited neutralizing activity that was an order of magnitude higher than that of the convalescent serum samples (P < 0.01; Fig. 1E).

We used orthogonal approaches to assess binding antibody specificities to the spike S1 subunit domains. RBD and S-2P binding recapitulated the results of the ACE2 binding inhibition assay (fig. S2). Serum binding to the N-terminal domain, which may be a marker of additional protection through both neutralizing activity and non-neutralizing effector functions (24), was 500-fold higher compared to baseline across vaccine groups (fig. S3). We assessed the strength of RBD binding by biolayer interferometry, finding an increasing antibody on-rate association response throughout follow-up (fig. S4). Given the potential importance of auxiliary antibody functions for protection (25, 26), we assessed a suite of Fc-mediated antibody effector functions, including opsonization, antibody-dependent complement deposition (ADCD), antibody-dependent cellular phagocytosis (ADCP), antibody-dependent neutrophil phagocytosis (ADNP), and trogocytosis (a measure of antigen transfer) (27). All activity peaked at week 6 and was highest in the two-dose 50-µg SpFN group (fig. S5).

SpFN vaccination elicits spike protein–specific T cell responses in NHPs

CD4 T helper cell (T₄H) responses are important for respiratory virus vaccine development, given the theoretical concern and precedent for vaccine-associated enhanced respiratory disease and its association with a T₄H2-biased response (28). We focused our assessment of cell-mediated immunity on canonical cytokines expressed by T₄H1 [interferon-γ (IFN-γ), tumor necrosis factor–α (TNF-α), and interleukin-2 (IL-2)] and T₄H2 (IL-4 and IL-13) CD4 T cells. Robust T₄H1 responses were observed 4 weeks after the second vaccination in all vaccinated groups, except one animal in the 50-µg single-dose group (Fig. 2A). T₄H1 responses were poly-functional and variable but consistently high at week 8, ranging from 0.2 to 17%. T₄H2 and CD8 T cell responses were minimal or undetectable (Fig. 2B and fig. S6).

We interrogated key indicators of helper responses that support humoral immunity, such as IL-21, a cytokine secreted by follicular helper CD4 T cells that regulate the evolution of memory B cells (29). Five of eight animals dosed twice with 50 µg of SpFN had
detectable IL-21 responses, as did seven of eight animals given 5 µg of the vaccine (Fig. 2C). We also examined the expression of CD40 ligand (CD40L), a broad T cell activation marker expressed on the surface of CD4 T cells that promotes B cell maturation through antibody isotype switching (29). All but one animal receiving two doses of SpFN had detectable CD40L responses (Fig. 2D), indicating an engaged memory response.

SpFN vaccination reduces viral load after respiratory SARS-CoV-2 challenge

Rhesus macaques generally exhibit mild disease that does not fully recapitulate the severe pneumonia observed in many people with COVID-19 (21). Protective efficacy, therefore, was assessed virologically and pathologically. The primary virologic endpoint was based on the measurement of subgenomic mRNA (sgmRNA) for the envelope (E) gene region of the virus, an indicator of viral replication, in the upper [nasopharyngeal (NP) swabs and saliva] and lower airways [bronchoalveolar lavage (BAL) fluid]. sgmRNA concentrations (log_{10} copies per milliliter) of vaccinated animals were compared to controls. On the second day after simultaneous respiratory mucosal challenge by the intratracheal and intranasal routes, sgmRNA concentrations in the BAL fluid of control animals peaked at a mean of 10^6 copies/ml (Fig. 3A). In contrast, none of the eight animals that received two doses of 50 µg of SpFN had detectable...
that received a single dose of SpFN.

Fluid of all animals of the 5 μg vaccine group and all but one animal had sgmRNA at day 2. By day 4, sgmRNA was undetectable in the BAL fluid of all animals of the 5 μg vaccine group and all but one animal had sgmRNA at day 2. By day 4, sgmRNA was undetectable in the BAL fluid of all animals of the 5 μg vaccine group and all but one animal

Whereas sgmRNA concentrations reached a mean of 10^7 copies/ml in the NP swabs of control animals at day 2 after challenge, sgmRNA was undetectable in five of eight animals that received two doses of 50 μg of SpFN (Fig. 3B). All animals in this group had undetectable virus from day 4 onward, whereas virus persisted in the control animals through day 10. Five of eight controls had high concentrations of sgmRNA in saliva on day 2 after challenge, whereas virus was undetectable in all animals of the two-dose 50 μg

**SpFN vaccination provides protection from lung pathology after SARS-CoV-2 challenge**

One section of lung tissue from each lung lobe (six sections per animal) was fixed and stained at 1 to 2 weeks after virus challenge and evaluated under light microscopy and by immunohistochemistry (IHC). Unvaccinated control animals developed histopathologic evidence of multifocal, moderate interstitial pneumonia at 7 days after challenge (Fig. 4A). The pneumonia was characterized by type II pneumocyte hyperplasia, alveolar septal thickening, edema and necrotic debris, pulmonary macrophage infiltration, and vasculitis of smaller-caliber blood vessels. None of the vaccinated animals, however, had evidence of interstitial pneumonia (Fig. 4, B to D). IHC revealed viral antigen in alveolar pneumocytes and pulmonary macrophages in at least one lung section of every control animal (Fig. 4E). In contrast, no viral antigen was detected in any of the lung sections taken from vaccinated animals (Fig. 4, F to H).

**SpFN elicits broadly neutralizing antibodies that bind to VOCs**

We assessed the serum antibody responses elicited by the SpFN vaccine against four circulating SARS-CoV-2 VOCs: B.1.1.7 (alpha variant), B.1.351 (beta variant), P.1 (gamma variant), and B.1.617.2 (delta variant). Serum binding assessment by biolayer interferometry to the first three variant forms of SARS-CoV-2 RBD showed no change in binding to B.1.1.7 (N501Y mutation) or B.1.351 (K417N, E484K, and N501Y mutations) in the two-dose 50 μg SpFN group (fig. S8). Serum binding assessment by biolayer interferometry to these same variant forms of SARS-CoV-2 RBD showed no change in binding to B.1.1.7 (N501Y mutation) but a 25% reduction in binding to B.1.351 (K417N, E484K, and N501Y mutations) in the one-dose 50 μg SpFN group (fig. S8). We next assessed the serum neutralizing activity elicited by the SpFN vaccine against all four VOCs, including B.1.617.2 (delta variant). Serum samples from all vaccinated NHPs elicited potent neutralizing activity against all variants in two orthogonal virus neutralization assays. Neutralization capacity of the authentic B.1.1.7 virus variant was high across all vaccine groups (Fig. 5, A to C) and was significantly higher than neutralization of wild-type WA-1 in the two-dose
Neutralizing activity against the authentic B.1.351, P.1, and B.1.617.2 virus variants, however, was diminished slightly (Fig. 5, A to C), except in the two-dose 50 μg group (Fig. 5A). Neutralizing activity against the B.1.1.7 in an orthogonal pseudovirus assay revealed equivalent ID\textsubscript{50} GMTs to the WA-1 wild-type pseudovirus (Fig. 5, D to F). Reductions in neutralizing activity against the B.1.351 pseudovirus, however, were slightly more pronounced in the pseudovirus assay as compared to the authentic virus assay. For example, the reciprocal ID\textsubscript{50} GMT dropped fivefold in the two-dose 50 μg group but remained high at a value of 10,209 (Fig. 5D). The absolute neutralizing antibody titers were generally elevated after two doses of vaccine, irrespective of the virus variant against which they were measured (Fig. 5G).

We expanded the assessment of the immunogenicity breadth to the interrogation of neutralizing and non-neutralizing antibody and cellular immune responses against SARS-CoV-1. Binding of vaccinee serum samples to SARS-CoV-1 RBD, as measured by biolayer interferometry, was absent in controls but was relatively potent in vaccinated animals, binding at half the strength of that observed to SARS-CoV-2 RBD (Fig. 6A and figs. S3 and S4). Antibody-dependent cellular phagocytotic activity also increased in all vaccine groups, reaching a score that was 100-fold higher than baseline or compared to unvaccinated controls when measured 2 weeks after the last 50 μg SpFN vaccination (Fig. 6B). Two vaccinations with high-dose SpFN also yielded plaque reduction neutralizing activity against authentic SARS-CoV-1 with a reciprocal ID\textsubscript{50} GMT of 390 that was significantly increased above background (P = 0.01; Fig. 6C). An orthogonal pseudovirus neutralization assay exhibited some background activity in PBS controls. We minimized this background by analyzing neutralization activity at ID\textsubscript{90} and found that two doses of 50 μg of SpFN induced a SARS-CoV-1 neutralizing antibody titer that was sixfold higher (GMT, 667) than controls (Fig. 6D). CD4 T cell responses to SARS-CoV-1 spike, although lower in absolute percentage as compared to those against SARS-CoV-2, were still robust and Th1 biased (Fig. 6, E and F). The CD8 T cell response, in contrast, was minimal (fig. S9). Overall, the immune

Fig. 3. Viral replication was reduced in the lower and upper airways after SpFN vaccination and subsequent SARS-CoV-2 respiratory challenge. sgmRNA copies per milliliter were measured in the (A) BAL, (B) NP swabs, and (C) saliva of vaccinated and control animals for 2 weeks after intranasal and intratracheal SARS-CoV-2 (USA-WA1/2020) challenge. Specimens were collected on 1, 2, 4, 7, 10, and 14 days after challenge. Dotted lines demarcate assay lower limits of linear performance range (to 450 copies/mL). In the box plots, horizontal lines indicate the mean, and the top and bottom reflect the minimum and maximum.
response elicited by SpFN against SARS-CoV-1, although lower in magnitude, recapitulated the quality of response against SARS-CoV-2.

**DISCUSSION**

The recent success in the rapid development of safe and efficacious SARS-CoV-2 vaccines has been tempered by the emergence of virus variants to which vaccine-induced immunity has shown diminished potency or efficacy (30–33). Thus, there remains a need for next-generation vaccines that target the broadening antigenic diversity of SARS-CoV-2 and related coronaviruses. The major vaccines that have progressed to human efficacy trials and have been granted either approval or emergency use authorization all present the SARS-CoV-2 spike protein that is based on the genetic sequence of the Wuhan-Hu-1 isolate. All of these vaccines have demonstrated protective efficacy in NHPs against respiratory mucosal challenge with the closely matched USA-WA1/2020 (25, 34–38). These earlier animal studies, however, did not evaluate the neutralization capacity of serum against other coronavirus species. In the current study, we demonstrate that an adjuvanted recombinant nanoparticle vaccine, SpFN, elicited high titters of antibodies that neutralized SARS-CoV-2 and rapidly protected against SARS-CoV-2 infection. We also found that, compared to wild-type virus, SpFN elicited serum virus neutralizing activity that was either higher or equivalent against four major VOCs (B.1.1.7, B.1.351, P.1, and B.1.617.2) in an authentic virus neutralization assay, and equivalent or mildly diminished against two VOCs in a pseudovirus neutralization assay (B.1.1.7 and B.1.351). Last, SpFN induced robust neutralizing activity against SARS-CoV-1, a separate species that has 26% and 36% sequence divergence in the spike protein and S1 subunit, respectively (39), which is important for protection in animal models (40, 41).

SARS-CoV-2 vaccine efficacy studies in NHPs generally compare elicited antibody responses to those from patients who have recovered from COVID-19. We found neutralizing activity in the two-dose 50 μg SpFN group to be 10-fold higher than that in recovering patients. The lack of standardization across convalescent serum panels and the absence of head-to-head comparisons of candidate vaccines make it difficult to compare immunogenicity profiles. Direct comparisons come with a qualification that immunogenicity output assays have been in the process of harmonization. Bearing this caveat in mind, we found that SARS-CoV-2 antibody responses in animals vaccinated with high-dose SpFN were robust when set in the context of the entire vaccine landscape, to include genetic vaccines (34, 38), recombinant virus vector vaccines (25, 37), and adjuvanted protein subunit vaccines (35). Although direct quantitative comparisons of NHP vaccine studies can be difficult to interpret, we have made attempts to mitigate differences in assay outputs by analyzing specimens with orthogonal assays harmonized to consensus platforms. Specifically, the pseudovirus neutralization assay we developed for our immunogenicity assessments demonstrated equivalence to platforms used for the assessment of other major vaccines through participation in the SARS-CoV-2 Neutralizing Assay Concordance Survey 1 coordinated by the External Quality Assurance Program Oversight Laboratory/Virology Quality Assurance Program at the Duke Human Vaccine Institute (42).

Potent neutralizing antibody responses may offer advantages for both vaccine efficacy and durability. Thus far, neutralizing activity has been predictive of efficacy in human trials, because vaccines that generate lower antibody titers have diminished efficacy (43). An open question remains, however, regarding the length of immunity conferred by SARS-CoV-2 vaccines. Among viral infections for which neutralizing antibodies are the primary correlate of protection, peak titers have been shown to be predictive of durability and may serve as one of several indicators of the length of vaccine-elicited protective immunity (44–46). As such, SpFN may offer some measure of a durable immune response, although this will require empirical confirmation.

Overall, SpFN vaccination induced serum cross-neutralizing antibody responses. In addition, we found that serum binding to mutated SARS-CoV-2 RBD was either unaffected or mildly reduced. Other reports of nanoparticle vaccine approaches presenting RBD have also shown a breadth of neutralizing antibody responses against multiple sarbecoviruses (47–49). Similar to other studies, SpFN vaccination induces comprehensive binding and neutralizing antibody responses and a balanced cellular immune response against SARS-CoV-1. Although background neutralizing activity was high in one assay, neutralizing potency against SARS-CoV-1 was confirmed in an orthogonal virus neutralization assay. Still, we plan to test purified immunoglobulin G (IgG) from vaccine serum in both assays to confirm the background activity at baseline and in controls.

We hypothesize that the breadth of immune response elicited by the SpFN vaccine may be the result of several factors. First, the quantity of the polyclonal antibody response may surpass a threshold that overcomes resistance to neutralization of antigenically distinct virus variants. Second, repetitive, ordered display of antigen on a
The rapid elimination of replicating virus in the upper airways also may have implications for preventing viral transmission. Together, these findings support the further development of SpFN, which has add-on adjuvant performance in humans, we are now conducting a follow-on adjuvant comparison study in NHPs to evaluate the impact of ALFQ on immunogenicity potency and breadth. In addition, because NHPs do not exhibit the same degree of fidelity as Syrian golden hamsters in terms of developing severe COVID-19 disease (21), we have also found SpFN to protect against VOCs in challenge experiments in the latter model (52).

The interpretation of the results from this study is limited by several factors. First, we were able to evaluate the efficacy of our vaccine candidate only against challenge with the Wuhan-Hu-1 strain of SARS-CoV-2. This is because VOCs were only beginning to emerge and circulate when this study was conceived and initiated. Our assessment of the breadth of immune response was, instead, evaluated by serum neutralizing activity against a panel of SARS-CoV-2 VOCs and SARS-CoV-1. Second, we cannot define, from the design and outputs of this study, the relative contributions of the immunogen and adjuvant of our vaccine formulation to the potency and breadth of immune response observed. Last, we have yet to understand the role and repertoire of the humoral and cellular immune responses elicited by this SpFN. We are carrying out follow-on studies to examine the mechanisms of immunity conferred by our vaccine candidate.

The collective immune response elicited by SpFN translated into a robust and rapid reduction in replicating virus in the upper and lower airways of animals and resultant prevention of pulmonary pathology. It is notable that SpFN protected against a potent viral challenge, because replicating virus concentrations detected in the upper and lower airways of unvaccinated controls reached a mean of $10^4$ to $10^5$ copies/ml.

**Fig. 5. Neutralizing antibody responses are elicited against SARS-CoV-2 variants B.1.1.7, B.1.351, P.1, and B.1.617.2 by SpFN vaccination in rhesus macaques.** (A to C) Authentic virus and (D to F) pseudovirus neutralizing antibody responses were measured 2 weeks after the last SpFN vaccination with either two doses of 50 µg (A and D), 5 µg (B and E), or one dose of 50 µg (C and F). (G) Reciprocal ID<sub>50</sub> GMT against each VOC and fold change from wild-type neutralization (WA-1 or Wuhan-1) were assessed. Statistically significant fold change differences are indicated as follows: *P < 0.05; **P < 0.01; ***P < 0.001. Statistical comparisons were done by Kruskal-Wallis test followed by post hoc Dunn's test. In the box plots, horizontal lines indicate the geometric mean, and the top and bottom reflect the minimum and maximum. Dotted lines indicate the lower limit of detection. Only statistically significant differences at α = 0.05 are presented in the graphs. ND, not determined.

The self-assembling nanoparticle has been shown to drive an expanded germinal center reaction with resultant increases in B cell receptor mutation, affinity maturation, and plasma cell differentiation (5–7). Last, the adjuvant, ALFQ, may drive some of the breadth through CD4 T cell activation (50, 51), especially given the high Th1 response elicited by the coformulation. ALFQ, as compared to aluminum hydroxide (Alhydrogel), has previously demonstrated superior immunogenicity when administered with SpFN to C57BL/6 and BALB/c mice (20). Because NHPs are a more predictive model with respect to adjuvant performance in humans, we are now conducting a follow-on adjuvant comparison study in NHPs to evaluate the impact of ALFQ on immunogenicity potency and breadth. In addition, because NHPs do not exhibit the same degree of fidelity as Syrian golden hamsters in terms of developing severe COVID-19 disease (21), we have also found SpFN to protect against VOCs in challenge experiments in the latter model (52).

Despite this higher challenge, SpFN still protected lower airway viral burden and disease as early as within 1 day of virus inoculation. The rapid elimination of replicating virus in the upper airways also lends evidence for potential near-sterilizing immunity, which again may have implications for preventing viral transmission. Together, these findings support the further development of SpFN, which has now moved to human evaluation in a phase 1 clinical trial (53).
### Humoral and cellular immune responses to SARS-CoV-1 are elicited by SpFN vaccination of rhesus macaques

**Fig. 6.** Humoral and cellular immune responses to SARS-CoV-1 are elicited by SpFN vaccination of rhesus macaques. (A) Serum binding responses to SARS-CoV-1 RBD were measured by biolayer interferometry. (B) ADCP induction by serum samples was measured. (C and D) Serum samples were tested for neutralization (ID$_{50}$) of authentic SARS-CoV-1 (Urbani) (C) and for pseudo–SARS-CoV-1 (Urbani) neutralization (ID$_{50}$) (D). (E and F) SARS-CoV-1 (Urbani) spike protein–specific CD4$^+$ T$_{H1}$ (E) and T$_{H2}$ (F) responses were measured using peripheral blood mononuclear cells isolated 2 weeks after the last vaccination (week 6). The fraction of animals exhibiting a T$_{H1}$ or T$_{H2}$ response is shown below each plot. Statistical significance was assessed with a Kruskal-Wallis test followed by post hoc Dunn’s test. In the box plots, horizontal lines indicate the mean, and the top and bottom reflect the minimum and maximum. Only statistically significant differences at $\alpha = 0.05$ are presented in the graphs.

### Materials and Methods

#### Study design

Thirty-two male and female specific pathogen–free, research-naïve Chinese-origin rhesus macaques (age 3 to 7 years) were distributed—on the basis of age, weight, and sex—into four cohorts of eight animals (table S1). Sample sizes were set on the basis of animal availability and prior experience with other NHP vaccination/challenge studies. Animals were assigned to groups without a specific randomization scheme; however, no outliers were skewed to any one group (table S1). Animals were vaccinated intramuscularly with either 50 or 5 µg of SpFN, formulated with ALFQo, or 1 ml of PBS in the anterior proximal quadriceps muscle, on alternating sides with each dose in the series. Immunizations were administered twice, 4 weeks apart, or once, 4 weeks before challenge. Animals were challenged with virus stock obtained through Biodefense and Emerging Infections Research Resources Repository Resources (BEI Resources), National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH): SARS-related coronavirus 2, isolate USA-WA1/2020, NR-53780 (Lot#70038893). Virus was stored at $-80^\circ$C before use, thawed by hand, and placed immediately on wet ice. Stock was diluted to $5 \times 10^5$ TCID$_{50}$/ml in PBS and vortexed gently for 5 s before inoculation by combined intratracheal (1 ml) and intranasal routes (0.5 ml per nostril).

All procedures were carried out in accordance with institutional, local, state, and national guidelines and laws governing animal research included in the Animal Welfare Act. Animal protocols and procedures were reviewed and approved by the Animal Care and Use Committee of both the U.S. Army Medical Research and Development Command (USAMRDC; protocol 11355007.03) Animal Care and Use Review Office and the Institutional Animal Care and Use Committee of Bioqual Inc. (protocol number 20-092), where NHPs were housed for the duration of the study. USAMRDC and Bioqual Inc. are both accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and are in compliance with the Animal Welfare Act and Public Health Service Policy on Humane Care and Use of Laboratory Animals.
Vaccine and adjuvant design and production

The SpFN vaccine immunogen was produced by linking Helicobacter pylori ferritin to the C-terminal region of the prefusion-stabilized ectodomain (residues 12 to 1158) of the SARS-CoV-2 spike protein in a genetic fusion. The immunogen was expressed, purified, and characterized as previously described (20). Briefly, SpFN was derived from the Wuhan-Hu-1 strain genome sequence (GenBank MN9089473), synthesized by Genscript Inc., and subcloned into a modified cytomegalovirus promoter (CMV) expression vector. Exp293F cells (Thermo Fisher Scientific) were transiently transfected with SpFN plasmid DNA using ExpiFectamine 293 transfection reagent (Thermo Fisher Scientific). Cells were grown in polycarbonate baffled shaker flasks at 34°C and 8% CO₂ at 120 rpm. Cells were harvested 5 days after transfection by centrifugation at 3500g for 30 min. Culture supernatants were filtered through a 0.22-μm filter and stored at 4°C before purification using GNA lectin affinity chromatography. Briefly, 25 ml of GNA-lectin resin (Vector Labs) was used to purify SpFN from 1 liter of expression supernatant. GNA resin was equilibrated with 10 column volumes (CVs) of PBS (pH 7.4) followed by supernatant loading twice at 4°C. Unbound protein was removed by washing with 20 CVs of PBS buffer. Bound protein was eluted with 250 mM methyl α-D-mannopyranoside in PBS buffer (pH 7.4). SpFN was further purified by size exclusion chromatography using a 16/60 Superdex-200 purification column. Purification purity for all the proteins was assessed by SDS–polyacrylamide gel electrophoresis. Endotoxin concentrations for SpFN were assessed (Endosafe nexgen-PTS, Charles River Laboratories), and 5% (v/v) glycerol was added before filter sterilization with a 0.22-μm filter, flash-frozen in liquid nitrogen, and stored at −80°C. SpFN antigenicity was assessed by Octet biolayer interferometry with a set of antibodies that included CR3022 (20) and SR1-SR5 (potent neutralizing antibodies targeting the ACE2 binding site of the RBD; provided by S. Rajan, P.M. McTamney, and M.T. Esser of Astra Zeneca). Size and consistency of the SpFN immunogen were determined by negative-stain electron microscopy. These measurements ensured lot-to-lot consistency of the SpFN immunogen.

The adjuvant, ALFQ, was prepared as previously described (54). Briefly, ALFQ is a unilamellar liposome that contains saturated phospholipids, Chol, monophosphoryl lipid A, and the saponin QS-21. It is composed of DMPC, DMPG, Chol, 3D-PHAD (Avanti Polar Lipids), and QS-21 (Desert King). DMPC and Chol were dissolved in chloroform, and DMPG and 3D-PHAD were dissolved in chloroform:methanol at a ratio of 9:1. The lipids were mixed in a molar ratio of 9:12:2.0:0.114 (DMPC:DMPG:Chol:3D-PHAD), and the solvent was removed by rotary evaporation. 3D-PHAD and QS-21 doses were 200 and 100 μg, respectively. The lipids were suspended in Sorenson’s PBS (pH 6.2), microfluidized to form small unilamellar vesicles (SUVs), and filtered. QS-21 was solubilized in Sorenson’s PBS (pH 6.2), filtered, and added to the SUV to form ALFQ. The final lipid ratio was 9:12:2.0:0.114:0.044 (DMPC:DMPG:Chol:3D-PHAD:QS-21).

The immunogen was diluted in Dulbecco’s PBS (Lot#723188, Quality Biological) to 0.1 or 0.01 mg/ml and mixed 1:1 with 2× ALFQ liposomes on a tilted slow-speed roller at room temperature for 10 min, followed by incubation at 4°C for 50 min. All reagents were equilibrated to room temperature before use, and immunizations were performed within 4 hours of vaccine formulation. Each vaccine comprised a 1.0-ml solution of SpFN formulated with ALFQ.

Convolascent plasma samples

A panel of 41 human convalescent-phase plasma samples was obtained from BEI Resources Repository (n = 30), StemExpress (n = 7), and a Walter Reed Army Institute of Research institutional review board–approved leukapheresis protocol (no. 1386H) (n = 4), for which written informed consent was provided by participants. Samples were collected from males (n = 20) and females (n = 21) ranging in age from 31 to 71 years. Individuals donated plasma specimens about 4 to 8 weeks after laboratory-confirmed SARS-CoV-2 infection and had histories of asymptomatic-to-mild-to-moderate clinical presentation.

Binding antibody measurements

SARS-CoV-2–specific binding IgG antibody responses were measured using MULTI-SPOT 96-well plates from Meso Scale Discovery (MSD). Multiplex wells were coated with spike (S), RBD, and nucleocapsid (N) antigens at a concentration of 200 to 400 ng/ml and bovine serum antigen, which served as a negative control. Fourplex MULTI-SPOT plates were blocked with MSD Blocker A buffer for 1 hour at room temperature, shaking at 700 rpm. Plates were washed with buffer before the addition of reference standard and calibrator controls. Serum samples were diluted at 1:1000 to 1:100 000 in diluent 100 buffer (provided with the manufacturer’s kit) and then added to each of four wells. Plates were incubated with shaking at 700 rpm for 2 hours at room temperature and then washed. MSD SULFO-TAG anti-IgG antibody was added to each well. Plates were incubated for 1 hour at room temperature with shaking at 700 rpm and washed, and then MSD GOLD Read buffer B was added to each well. Plates were read by the Meso Sector SQ 120 reader. IgG concentration was calculated using DISCOVERY WORKBENCH MSD software and reported as arbitrary units (AU) per milliliter.

The ability of SARS-CoV-2 spike–specific binding antibodies to inhibit spike protein or RBD binding to the ACE2 receptor was also measured using MULTI-SPOT 96-well plates (MSD). Antigen-coated plates were blocked and washed as described above. Assay calibrator and samples were diluted at 1:25 to 1:1000 in MSD Diluent buffer and then added to the wells. Plates were incubated for 1 hour at room temperature, shaking at 700 rpm. ACE2 protein conjugated with MSD SULFO-TAG was added, and plates were incubated for 1 hour at room temperature, shaking at 700 rpm. Plates were washed and read as described above. Concentration (AU per milliliter) of inhibitory antibodies was calculated with DISCOVERY WORKBENCH MSD software.

Binding antibody measurements by Octet biolayer interferometry were made on biosensors of the Octet FortéBio Red96 instrument (Sartorius) that were hydrated in PBS before use. All assay steps were performed at 30°C with agitation set at 1000 rpm. Baseline equilibration of the anti–His-tag biosensors (HIS1K biosensors with a conjugated Penta–His antibody, Sartorius) was carried out with PBS for 15 s before SARS-CoV-2 RBD (30 μg/ml diluted in PBS) loading for 120 s. After dipping in assay buffer (15 s in PBS), biosensors were dipped in the serum samples (100-fold dilution) for 180 s. Binding response (nanometers) at 180 s was recorded for each sample.

SARS-CoV-1 and SARS-CoV-2 pseudovirus neutralization

The spike protein expression plasmid sequence for SARS-CoV-2 was codon-optimized and modified to remove an 18–amino acid
endoplasmic reticulum retention signal in the cytoplasmic tail to improve spike protein incorporation into pseudovirions (PSVs) and thereby enhance infectivity. SARS-CoV-2 PSVs were produced by cotransfection of human embryonic kidney (HEK) 293T/17 cells with a SARS-CoV-2 spike protein–expressing plasmid (pcDNA3.4), derived from the Wuhan-Hu-1 genome sequence (GenBank accession number MN908947.3) and HIV-1 (pNL4-3.Luc.R.E., NIH HIV Reagent Program, catalog no. 3418). Infectivity and neutralization titers were determined using ACE2-expressing HEK293 target cells (Integral Molecular) in a semiautomated assay format using robotic liquid handling (Biomek NXp, Beckman Coulter). Virions pseudotyped with the vesicular stomatitis virus G protein were used as a nonspecific control. Serum samples were diluted 1:10 in growth medium [10% fetal bovine serum (FBS), 2.5% Hepes, 0.5% gentamicin, and 0.1% puromycin in Dulbecco’s modified Eagle’s medium]; then, 25 μl per well was added, in triplicate, to a white 96-well plate. An equal volume of diluted SARS-CoV-2 PSV was added to each well, and plates were incubated for 1 hour at 37°C. Target cells were added to each well (40,000 cells per well), and plates were incubated for an additional 48 hours. Real-time light units were measured with the EnVision Multimode plate reader (PerkinElmer) using the Bright-Glo Luciferase Assay System (Promega). Neutralization dose–response curves were fitted by nonlinear regression using the LabKey Server, as previously described (35). Final titers are reported as the reciprocal of the dilution of serum necessary to achieve 50% (ID50) and 90% neutralization (ID90). Assay equivalency was established by participation in the SARS-CoV-2 Neutralizing Assay Concordance Survey run by the Virology Quality Assurance Program and External Quality Assurance Program Oversight Laboratory at the Duke Human Vaccine Institute, sponsored through programs supported by the National Institute of Allergy and Infectious Diseases, Division of AIDS.

**Authentic SARS-CoV-2 wild-type neutralization assay**

Authentic virus neutralization was measured using SARS-CoV-2 (2019-nCoV/USA_WA1/2020) that was obtained from the Centers for Disease Control and Prevention and passaged once in Vero CCL81 cells [American Type Culture Collection (ATCC)]. Rhesus serum samples were serially diluted and incubated with 100 focus-forming units of SARS-CoV-2 for 1 hour at 37°C. Serum–virus mixtures were added to Vero E6 cells in 96-well plates and incubated for 1 hour at 37°C. Cells were overlaid with 1% (w/v) methylcellulose in Minimum Essential Medium (Sigma–Aldrich). After 30 hours, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature then washed and stained overnight at 4°C with the antibody CR3022 (1 μg/ml) in PBS supplemented with 0.1% saponin and 0.1% bovine serum albumin. Cells were subsequently stained with horseradish peroxidase–conjugated goat anti-human IgG for 2 hours at room temperature. SARS-CoV-2–infected cell foci were visualized with TrueBlue peroxidase substrate (KPL) and quantified using an ImmunoSpot microanalyzer (Cellular Technologies). Neutralization curves were generated with Prism software (GraphPad Prism 8.0).

**Authentic SARS-CoV-2 variant and SARS-CoV-1 neutralization assay**
The SARS-CoV-2 viruses USA-WA1/2020 (WA1), USA_CA_CDC_5574/2020 (B1.1.7), hCoV-19/South Africa/KRISP-EC-K005321/2020 (B.1.351), hCoV-19/Japan/TY7-503/2021, and hCoV-19/USA/PHC658/2021 (B.1.617.2) were obtained from BEI Resources (National Institute of Allergy and Infectious Diseases, NIH) and propagated for one passage using Vero clone E6 cells. Virus infectious titer was determined by an endpoint dilution and cytopathic effect (CPE) assay on Vero-E6 cells as described previously (56, 57). An endpoint dilution microplate neutralization assay was performed to measure the neutralization activity of NHP serum samples. In brief, serum samples were heat-inactivated and subjected to successive threefold dilutions starting from 1:50. Triplicates of each dilution were incubated with SARS-CoV-2 at a multiplicity of infection of 0.1 in Eagle’s minimum essential medium with 7.5% inactivated fetal calf serum for 1 hour at 37°C. After incubation, the virus-antibody mixture was transferred onto a monolayer of Vero-E6 cells grown overnight. The cells were incubated with the mixture for about 70 hours. CPE of viral infection was visually scored for each well in a blinded fashion by two independent observers. The results were then reported as the percentage of neutralization at a given sample dilution. A SARS-CoV-1 authentic plaque reduction virus neutralization assay was performed similarly to previously described (58), with the following modifications. The starting dilution of serum was 1:5, and about 100 plaque-forming units of virus were used for virus and serum incubation. The overlay used after virus adsorption was Dulbecco’s modified Eagle’s medium (Gibco) containing 2% FBS and 20% methylcellulose. Plates were then incubated for 5 days, and after crystal violet staining, the washing step used water. Plaques were graded as follows: about 25 plaques/25% monolayer damage (MD; ±); about 50 plaques/ 50% MD (+); about 75 plaques/75% MD (++); and about 100 plaques/100% MD (+++). All negative control wells were solid monolayers.

**Antibody-dependent neutrophil phagocytosis**

Biotinylated SARS-CoV-2 prefusion-stabilized S trimer was incubated with yellow-green streptavidin–fluorescent beads (Molecular Probes) for 2 hours at 37°C. Ten microliters of a 100-fold dilution of beads and protein was incubated for 2 hours at 37°C with 100 μl of 8100-fold diluted plasma samples before addition of effector cells (50,000 cells per well). Fresh human peripheral blood mononuclear cells were used as effector cells after red blood cell lysis with ACK lysing buffer (Thermo Fisher Scientific). After 1 hour of incubation at 37°C, the cells were washed, surface-stained, and fixed with 4% formaldehyde solution (Tousimis), and fluorescence was evaluated on an LSRII flow cytometer (BD Biosciences). Antibodies used for flow cytometry included anti-human CD3 Alexa Fluor 700 (clone UCHT1) and anti-human CD14 aliphycocyanin cyanine dye 7 (APC-Cy7) (clone M6P9) from BD Biosciences, as well as anti-human CD66b Pacific Blue (clone G10F5) from BioLegend. A phagocytic score was calculated by multiplying the percentage of bead-positive neutrophils (side scatter high, CD3− CD14− CD66+ ) by the geometric mean of the fluorescence intensity of bead-positive cells and dividing by 10,000.

**Antibody-dependent cellular phagocytosis**

ADCP was measured as previously described (59). Briefly, biotinylated SARS-CoV-1 or SARS-CoV-2 prefusion-stabilized spike protein trimer was incubated with red streptavidin–fluorescent beads (Molecular Probes) for 2 hours at 37°C. Ten microliters of a 100-fold dilution of beads protein was incubated for 2 hours at 37°C with 100 μl of 8100-fold (SARS-CoV-2) or 900-fold (SARS-CoV-1) diluted plasma samples before addition of THP-1 cells (20,000 cells per well; MilliporeSigma). After a 19-hour incubation at 37°C, the cells were...
fixed with 2% formaldehyde solution (Tousimis), and fluorescence was evaluated on an LSRRII flow cytometer (BD Biosciences). The phagocytic score was calculated by multiplying the percentage of bead-positive cells by the geometric mean of the fluorescence intensity of bead-positive cells and dividing by 10,000.

**Antibody-dependent complement deposition**

SARS-CoV-2 spike protein–expressing expi293F cells were generated by transfection with linearized plasmid (pcDNA3.1) encoding codon-optimized full-length SARS-CoV-2 spike protein matching the amino acid sequence of the IL-15–CD8-IL1/2020 isolate (GenBank ACC# MN988713). Stable transfectants were single-cell–sorted and selected to obtain a high spike surface–expressing clone (293F-spike-S2A). ADCD was adapted from methods previously described (60). Briefly, SARS-CoV-2 spike protein–expressing expi293F cells were incubated with 10-fold diluted, heat-inactivated (56°C for 30 min) plasma samples for 30 min at 37°C. Cells were washed twice and resuspended in R10 media. During this time, lyophilized guinea pig complement (CL4051, Cedarlane) was reconstituted in 1 ml of cold water and centrifuged for 5 min at 4°C to remove aggregates. Cells were washed with PBS and resuspended in 200 μl of guinea pig complement, which was prepared at a 1:5 dilution in Gelatin Veronal buffer with Ca²⁺ and Mg²⁺ (IBB-300x, Boston BioProducts). After incubation at 37°C for 20 min, cells were washed in PBS and 15 mM EDTA (Thermo Fisher Scientific), fixed with 2% formaldehyde solution (Tousimis), and fluorescence was evaluated on an LSRII flow cytometer (BD Biosciences).

**Opsonization**

SARS-CoV-2 spike protein–expressing expi293F cells were generated as described above for the ADCY assay. SARS-CoV-2 spike protein–expressing cells were incubated with 200-fold diluted plasma samples for 30 min at 37°C. Cells were washed twice and stained with anti-human IgG phycoerythrin (PE), anti-human IgM Alexa Fluor 647, and anti-human IgA FITC (SouthernBiotech). Cells were then fixed with 4% formaldehyde solution, and fluorescence was evaluated on an LSRII flow cytometer (BD Biosciences).

**Trogocytosis**

Trogocytosis was measured using a previously described assay (27). Briefly, SARS-CoV-2 spike–expressing expi293F cells were stained with PKH26 (Sigma-Aldrich). Cells were then washed with and resuspended in R10 media. Cells were then incubated with 200-fold diluted plasma samples for 30 min at 37°C. Effector peripheral blood mononuclear cells were next added to the R10 media at an effector to target (E:T) cell ratio of 50:1 and then incubated for 5 hours at 37°C. After the incubation, cells were washed, stained with live/dead aqua fixable cell stain (Life Technologies) and CD14 APC-Cy7 (clone M5E9) for 15 min at room temperature, washed again, and fixed with 4% formaldehyde (Tousimis) for 15 min at room temperature. Fluorescence was evaluated on an LSRRII flow cytometer (BD Biosciences). Trogocytosis was evaluated by measuring the PKH26 mean fluorescence intensity of the live CD14⁺ cells.

**Intracellular cytokine staining**

Cryopreserved peripheral blood mononuclear cells were thawed and rested for 6 hours in R10 with Benzonase Nuclease (50 U/ml; Sigma-Aldrich). They were then stimulated for 12 hours with two pools of peptides spanning the spike protein of either SARS-CoV-2 or SARS-CoV-1 (1 μg/ml; JPT, PM-WCPV-S and PM-CVHSA-S, respectively) in the presence of brefeldin A (0.65 μl/ml; GolgiPlug, BD Biosciences Cytofix/Cytoperm Kit, catalog no. 555028), costimulatory antibodies anti-CD28 (1 μg/ml; BD Biosciences, catalog no. 555725) and anti-CD49d (1 μg/ml; BD Biosciences, catalog no. 555501), and CD107a (HA43, BD Biosciences, catalog no. 561348, lot 9143920 and 253441). After stimulation, cells were stained serially with LIVE/DEAD Fixable Blue Dead cell stain in PBS for 20 min at room temperature (Thermo Fisher Scientific, #L23105) and a cocktail of fluorescently labeled antibodies (BD Biosciences unless otherwise indicated) to cell surface markers CD4-PE-Cy5.5 (1:80; S3.5, Thermo Fisher Scientific, #MHCD0418, lot 2118390), CD8 BV570 (1:160; RPA-T8, BioLegend, #301038, lot B281322), CD45RA BVU995 (1:160; 5H9, #552888, lot 154382 and 259854), CD28 BV737 (1:20; CD28.2, #612815, lot 0113886), CCR7 BV650 (1:20; GO43H7, #533234, lot B297645), and HLA-DR BV480 (1.640; G46-6, #566113, lot 0055314) in 4% FBS in PBS for 20 min at room temperature. Intracellular cytokine staining was performed after fixation and permeabilization per the manufacturer’s instructions (BD Cytofix/ Cytperm, BD Biosciences) with CD3 APC-Cy7 (1:1282; SP4-3, #557757, lot 6140803), CD154 PE-Cy7 (1:40; 24-31, BioLegend, #31084, lot B264810 and B313191), IFN-γ Alexa Fluor 700 (700; 1:1282; B27, #506516, lot B187646 and B290145), TNF-α FITC (1:160; MAb11, #554512, lot 15360), IL-2 BV750 (1:160; MQ1-17H12, BioLegend, #566361, lot 0042313), IL-4 BB700 (1:320; MP4-25D2, lot 0133487 and 0308726), macrophage inflammatory protein–1β PE (1:160; D21-1351, #550078, lot 9296809), CD69 electron coupled dye (PE-Texas Red conjugate) (1:80; TP1.53.5, Beckman Coulter Life Sciences, #6607110, lot 7620070 and 7620076), IL-2 Alexa Fluor 647 (1:20; 3A3-N2.1, #560493, lot 9199272), IL-13 BV421 (1:20; JES10-5A2, #563580, lot 9322765, 210147, and 169570), and IL-17A BV605 (1:20; BL168, BioLegend, #512326, B289357). Sample staining was measured on a FACSymphony A5 SORP (Becton Dickinson), and data were analyzed using FlowJo v.9.9 software (Tree Star Inc.). The gating strategy is shown in fig. S10. CD4 and CD8 T cell subsets were pregated on memory markers before assessing cytokine expression as follows: single positive or double negative for CD45RA and CD28. Boolean combinations of cells expressing one or more cytokines were used to assess the total spike protein–specific response of memory CD4 or CD8 T cells. Responses from the two-peak pools spanning SARS-CoV-2 spike protein or SARS-CoV-1 were summed. Display of multicomponent distributions was performed with SPICE v6.0 (NIH).

**Total and sgRNA quantification**

Real-time quantitative polymerase chain reaction (RT-qPCR) was carried out for sgRNA and viral load RNA quantification from NP swab, BAL fluid, and saliva samples. Primers targeted the envelope (E) gene of SARS-CoV-2 (table S2). RNA was extracted from 200 μl of NP swab media or BAL specimens using the EZ1 DSP Virus kit (Qiagen) on the EZ1 Advanced XL instrument (Qiagen). Briefly, samples were lysed in 200 μl of ATL buffer (Qiagen) and transferred to the Qiagen EZ1 for extraction. Bacteriophage MS2 (ATCC) was added to the RNA carrier and used as an extraction control to monitor the efficiency of RNA extraction and amplification (61). Purified RNA was eluted in 90 μl of elution buffer (AVE). The RT-qPCR amplification reactions were performed in separate
well of a 96-well Fast plate for the three targets: sgRNA, RNA viral load, and MS2 RNA using 10 µl of extracted material, 0.72 µM primer, and 0.2 µM probe and 1x TaqPath 1-Step RT-qPCR (A15299, Life Technologies, Thermo Fisher Scientific). Amplification cycling conditions were as follows: 2 min at 25°C, 15 min at 50°C, 2 min at 95°C, and 45 cycles of 3 s at 94°C and 30 s at 55°C with fluorescence read at 55°C. An mRNA transcript for the SARS-CoV-2 E gene was used as a calibration standard. RNA copy values were extrapolated from the standard curve and multiplied by 45 to obtain RNA copies per milliliter. A negative control (PBS) and two positive controls (heat-inactivated SARS-CoV-2, ATCC, and VR-1986HK at 10^6 and 10^3 copies/ml) were extracted and used to assess the performance of both assays.

**Histopathology**
Formalin-fixed sections of lung tissue were evaluated by light microscopy and IHC. Lungs were perfused with 10% neutral-buffered formalin. Sections were processed routinely into paraffin wax, sectioned at 5 µm, and resulting slides were stained with hematoxylin and eosin. IHC was performed using the Dako Envision system (Dako Agilent Pathology Solutions). Briefly, after deparaffinization, and incubation at room temperature for 45 min. They were rinsed, with a mouse monoclonal anti–SARS-CoV nucleocapsid protein (Dako Agilent Pathology Solutions). Briefly, after deparaffinization, and incubation at room temperature for 45 min. They were rinsed, with a mouse monoclonal anti–SARS-CoV nucleocapsid protein (Dako Agilent Pathology Solutions). Briefly, after deparaffinization, and incubation at room temperature for 45 min. They were rinsed, with a mouse monoclonal anti–SARS-CoV nucleocapsid protein (Dako Agilent Pathology Solutions). Briefly, after deparaffinization, and incubation at room temperature for 45 min. They were rinsed, with a mouse monoclonal anti–SARS-CoV nucleocapsid protein (Dako Agilent Pathology Solutions).

**Statistical analysis**
Raw, individual-level data can be found in data file S1. Primary immunofluorescence outputs of binding and neutralizing antibody titers as well as T cell responses were compared across vaccination groups using Kruskal-Wallis test. Nonparametric pairwise comparisons between groups were made using the post hoc Dunn’s test. The same hierarchical analysis was applied to comparisons of sgRNA concentrations in the NP swabs and BAL fluids of vaccinated versus control groups. Statistical significance was preset at an α level of 0.05. The correlation between dependent T cell responses was assessed by nonparametric Spearman correlation (r).

**SUPPLEMENTARY MATERIALS**
www.science.org/doi/10.1126/scitranslmed.abi5735
Figs. S1 to S10
Tables S1 and S2
Data file S1

View/request a protocol for this paper from Bio-protocol.

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