SARS-CoV-2 protein subunit vaccination of mice and rhesus macaques elicits potent and durable neutralizing antibody responses

Graphical abstract

Highlights
- Neutralizing antibodies are detectable after one adjuvanted spike protein immunization
- Boosting yields higher neutralizing titers than observed in seropositive human donors
- Neutralizing antibody titers remain high for at least 4 months after the last boost
- Immunization stimulates spike-specific memory B cells

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In brief
Mandolesi et al. show that immunization with adjuvanted prefusion-stabilized SARS-CoV-2 spike glycoprotein yields potent antibody responses in mice and macaques. Neutralizing antibodies are detectable after one immunization. Boosting results in exceptional potency, with neutralizing titers exceeding (>10-fold) those observed in seropositive humans and remaining high during a 4-month follow-up.
SARS-CoV-2 protein subunit vaccination of mice and rhesus macaques elicits potent and durable neutralizing antibody responses

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SUMMARY

The outbreak and spread of SARS-CoV-2 (severe acute respiratory syndrome–coronavirus–2) is a current global health emergency, and effective prophylactic vaccines are needed urgently. The spike glycoprotein of SARS-CoV-2 mediates entry into host cells, and thus is the target of neutralizing antibodies. Here, we show that adjuvanted protein immunization with soluble SARS-CoV-2 spike trimers, stabilized in prefusion conformation, results in potent antibody responses in mice and rhesus macaques, with neutralizing antibody titers exceeding those typically measured in SARS-CoV-2 seropositive humans by more than one order of magnitude. Neutralizing antibody responses were observed after a single dose, with exceptionally high titers achieved after boosting. A follow-up to monitor the waning of the neutralizing antibody responses in rhesus macaques demonstrated durable responses that were maintained at high and stable levels at least 4 months after boosting. These data support the development of adjuvanted SARS-CoV-2 prefusion-stabilized spike protein subunit vaccines.

INTRODUCTION

As of February 2021, over 109 million cases of severe acute respiratory syndrome–coronavirus–2 (SARS-CoV-2) infection have been confirmed, with more than 2.4 million coronavirus disease 2019 (COVID-19)-related deaths recorded.1 Cases continue to increase globally despite unprecedented public health measures and lockdowns. Effective prophylactic vaccines are urgently required. Adjuvanted recombinant protein subunit vaccines have excellent safety profiles and represent a proven vaccine platform for eliciting protective immune responses to viral infections, including human papillomavirus (HPV), hepatitis B virus (HBV), and influenza A virus.

The spike glycoprotein of SARS-CoV-2 mediates receptor binding and entry into target cells and is the primary target for vaccine design. The receptor-binding domain (RBD) is a stable subunit within the spike glycoprotein (Figure 1A) responsible for angiotensin-converting enzyme 2 (ACE2) binding2-4 that can be expressed as an independent domain.6-8 While the RBD is a major target for neutralizing antibodies,9-11 antibodies specific for spike epitopes outside the RBD are also capable of neutralization.12,13

To evaluate the use and immunogenicity of recombinant protein subunit vaccines for SARS-CoV-2, we immunized C57BL/6J mice (N = 24) with either a trimeric form of the spike ectodomain or with RBD, produced in 293-F cells. The RBD domain was expressed as an Fc-fusion protein, which was cleaved and the RBD subsequently purified by size-exclusion chromatography. We previously showed,18 using cryoelectron microscopy (cryo-EM), that such spike preparations are well folded and maintained in the trimeric, prefusion conformation, consistent with the original report.15

Mice were immunized with varying doses of antigen (range: 5–50 μg) in 50 μL AddaVax (InvivoGen), a squalene-based oil-in-water emulsion analogous to MF59. MF59 is licensed, safe, and effective in humans,19 and increases the immunogenicity of an influenza vaccine in the elderly.20 Mice were boosted twice, at 3-week intervals, beginning 4 weeks after prime (Figure 1A). In both the low-dose (5 μg) and high-dose (25 μg) groups, a single immunization with prefusion-stabilized spike elicited a strong spike-specific immunoglobulin G (IgG) antibody response, detected by ELISA (Figures S1A and S1B), as early as 4 weeks after boosting.
antibody titers for the spike-immunized mice were significantly higher than those for RBD-immunized mice after both the first (p < 0.01) and second (p < 0.01) boosts. At matched doses, 5 μg spike elicited significantly higher neutralizing antibody titers than 5 μg RBD after the first (p < 0.01) and second (p < 0.01) boosts, and 25 μg spike elicited significantly higher neutralizing antibody titers than 25 μg RBD after the first (p < 0.01) but not after the second boost (p > 0.05). Pooling doses and neutralization titers increased over each immunization for spike (all p < 0.01) and between weeks 7 and 10 for RBD (p < 0.01), but not between weeks 4 and 7 (p > 0.05). Across all of the groups, spike-specific IgG titers correlated strongly with pseudovirus neutralization, although neutralization was detectable only above a threshold half-maximal effective concentration (EC_{50}) (Figure S1G).

Next, we immunized three rhesus macaques (Macaca mulatta) with adjuvanted trimeric prefusion-stabilized spike glycoprotein over ~4-week intervals (Figure 2A) and characterized the titers and kinetics of binding and neutralizing antibodies. Macaques were immunized intramuscularly with 100 μg spike protein in 75 μg Matrix-M (Novavax AB), a saponin-based adjuvant developed for clinical use. Neutralizing antibody responses were already detectable 2 weeks after a single dose, reaching ID_{50} titers in the range of 90–300 at 4 weeks. Two weeks after a first boost, the neutralizing antibody responses were extremely potent, with ID_{50} titers peaking at ~10,000 in all 3 macaques. An additional boost 3 weeks later did not raise the peak neutralization potency above that obtained with only 2 immunizations, suggesting diminishing returns of a third spike protein-based dose with this interval after the second injection (Figures 2C and 2D).

A SARS-CoV-2 strain harboring a D614G mutation in the spike protein is prevalent globally. We examined the neutralizing antibody responses elicited in immunized macaques against the D614G variant and observed neutralizing titers that were comparable those observed against the wild-type (vaccine) strain (Figure S1I).

While a standard neutralizing antibody assay has not been universally adopted, comparisons to a common reference point, such as seropositive human cohorts assayed in the same way, can calibrate titers. After 2 prefusion-stabilized spike protein immunizations, geometric mean ID_{50} neutralizing antibody titers in macaques were >1 order of magnitude higher than those measured in sera from SARS-CoV-2 seropositive humans analyzed within 1 month after a positive PCR test (Figure 2D). The ID_{50} neutralizing antibody titers were also substantially higher than those elicited by other immunization platforms that afforded macaques partial or complete protection from challenge in other studies.

The spike-directed IgG binding titers elicited in the macaques (Figure 2B) correlated strongly with the virus neutralizing activity (R^2 = 0.9478; Figure S1H). Recent data show that many neutralizing monoclonal antibodies isolated from SARS-CoV-2 convalescent individuals display low levels of somatic hypermutation (SHM), providing one possible explanation for the rapid development of neutralization in immunized animals. The clonality, antibody germline VDJ usage, and level of SHM that characterizes vaccine-induced SARS-CoV-2 spike-induced antibody
responses will be a matter of interest, both in the macaque model and in human vaccine trials.

Reported neutralizing antibody titers have varied substantially across different vaccine platforms. In animal models, inactivated virus, DNA-based vaccines, and adenovirus-vectorized vaccines elicited peak neutralizing antibody titers similar to or lower than those seen in convalescent sera. While high-dose mRNA-based vaccinations elicited potent neutralizing antibody responses in mice, neutralizing antibody titers elicited in Phase I/II human trials were markedly lower. Immunization of mice with mRNA-encoding membrane-anchored stabilized spike led to reduced or undetectable viral loads in the lungs and nasal turbinates in a dose-dependent manner, and susceptible transgenic mice were protected from lethal challenge. Other preclinical vaccine studies using nucleic acid platforms elicited immune responses that protected against disease but not against infection. For example, RBD- and spike-encoding DNA vaccines led to reduced viral loads in the nose and lungs of challenged macaques. Immunizations with recombinant spike protein subunits, including RBD and S1 subunits, elicited strong neutralizing antibody responses and could protect non-human primates from infection.

In our study, two inoculations of Matrix-M-adjuvanted SARS-CoV-2 spike trimers also generated robust antigen-specific memory B cell responses in all three macaques. Their presence was determined by in vitro stimulation of memory B cells into antibody-secreting plasma cells and enumeration by B cell ELISpot analysis, as previously described. The frequency of total IgG-secreting cells per million PBMCs. Error bars depict standard deviation about the mean.
with protection in a number of vaccine settings.49–51 For SARS-CoV-2, the passive transfer of neutralizing monoclonal antibodies underlie the reduced protection, with an overall efficacy of 95% following the administration of mRNA vaccines, which may directly relate this to sustained protection. Furthermore, even though antibodies alone can protect against challenge, but the precise relationship between antibody titer and protection is not yet established. Therefore, while we show that protein immunization results in neutralizing titers that remain well above 1 in 500 for months after immunization, we cannot directly relate this to sustained protection. Neutralization antibody titers were exceptionally high across different immunization routes and with different adjuvants, highlighting that the SARS-CoV-2 spike protein represents a robust immunogen.

### Limitations of study

One major limitation of the present study is the lack of SARS-CoV-2 challenge. We predominantly use serum neutralizing antibody responses to assess the robustness of vaccine-induced B cell responses, as it is known that antibodies alone can protect against challenge, but the precise relationship between antibody titer and protection is not yet established. Therefore, while we show that protein immunization results in neutralizing titers that remain well above 1 in 500 for months after immunization, we cannot directly relate this to sustained protection. Furthermore, we show that protein immunization results in neutralizing titers that remain well above 1 in 500 for months after immunization, we cannot directly relate this to sustained protection. Neutralization antibody titers were exceptionally high across different immunization routes and with different adjuvants, highlighting that the SARS-CoV-2 spike protein represents a robust immunogen.

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be reduced. Finally, our study was designed very early in the pandemic before anything was known about the immunogenicity of the spike protein. Recent studies⁴⁷,⁴⁸ suggest that our rhesus macaque immunization dose was probably higher than necessary to achieve robust neutralizing antibodies.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2021.100252.

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**AUTHOR CONTRIBUTIONS**

Conceptualization, D.J.S., M.M., G.B.K.H., and B.M.; formal analysis, D.J.S., M.M., G.B.K.H., and B.M.; investigation, D.J.S. performed the neutralization assays and the mouse ELISAs; J.M. performed the mouse immunizations and bleeds; M.M. and P.P. coordinated the NHP immunizations, processed samples, and performed ELISAs; P.P. performed the B cell ELISpot; and K. Le- nart, M.M., and M.A. performed the T cell intracellular staining; resources, L.H. and L.P.V. produced RBD and spike immunogens; C.K. and D.J.S. produced pseudovirus; and X.C.D. contributed data from seropositive humans; visualization, D.J.S., L.H., and B.M.; writing – original draft, D.J.S. and B.M.; writing – review & editing, D.J.S., K. Loré, J.M.C., G.M.M., B.M., and G.B.K.H.; funding acquisition, B.M., G.B.K.H., and G.M.; supervision, B.M., G.B.K.H., J.M.C., and G.M.M.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Goat anti-Mouse IgG-HRP | Southern Biotech | Cat#1013-05; RRID: AB_2794190 |
| Goat anti-Monkey IgG-HRP | Nordic MUbio | GAMonIgG(Fc)/PO |
| Goat anti-Human IgG, Fcγ | Jackson ImmunoResearch | Cat#109-005-008; RRID: AB_2337534 |
| Goat anti-Human IgG, Fcγ-Biotin | Jackson ImmunoResearch | Cat#109-065-008; RRID: AB_2337623 |
| Anti-CCR7 (clone G043H7) BV421 | BioLegend | Cat#353207; RRID: AB_10915137 |
| Anti-CD8a (clone RPA-T8) BV711 | BioLegend | Cat#301044; RRID: AB_2562906 |
| Anti-CD4 (clone S3.5) PE-Cy5.5 | Invitrogen | Cat#MHCD0418; RRID: AB_10376013 |
| Anti-CD45RA (clone 5H9) BV650 | BD Biosciences | Cat#740608; RRID: AB_2740308 |
| Anti-CD69 (clone TP1.55.3) ECD | Beckman Coulter | Cat#6607110; RRID: AB_1575978 |
| Anti-CD3 (clone SP34-2) APC-Cy7 | BD Biosciences | Cat#557757; RRID: AB_396863 |
| Anti-IFNγ (clone B27) AF700 | BioLegend | Cat#506515; RRID: AB_961353 |
| **Biological samples** |        |            |
| Serum from mice | This study | N/A |
| Plasma from NHPs | This study | N/A |
| PBMCs from NHPs | This study | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Recombinant SARS-CoV-2 Spike | This study | N/A |
| Recombinant SARS-CoV-2 RBD | This study | N/A |
| SARS-CoV-2 Overlapping peptides pool | JPT peptide technologies | Cat# PM-WCPV-S |
| Matrix-M | Novavax AB | N/A |
| AddaVax | InvivoGen | Cat# vac-advx-10 |
| Enterokinase, His, Bovine | GenScript | Cat# Z03004-500 |
| SIGMAFAST OPD | SigmaAldrich | Cat# P9187 |
| 3,3’,5,5’-tetramethylbenzidine (ELISA TMB Stabilized Chromogen) | Invitrogen | Cat# SB02 |
| Gibco FreeStyle MAX Reagent | Thermo Fisher Scientific | Cat# 16447100 |
| Lipofectamine 3000 | Invitrogen | Cat# L3000075 |
| Recombinant soluble CD40-L | PeproTech | Cat# 310-02 |
| Recombinant human IL-21 | PeproTech | Cat# 200-21 |
| Streptavidin-alkaline phosphatase | Mabtech AB | Cat# 3310-10-1000 |
| Staphylococcal enterotoxin B | SigmaAldrich | Cat# S4881 |
| BCIP/NBT-plus substrate | Mabtech AB | Cat# 3650-10 |
| Brefeldin A | Invitrogen | Cat# B7450 |
| **Critical commercial assays** |        |            |
| Cytofix/Cytoperm | BD Biosciences | Cat# 554714; RRID: AB_2869008 |
| LIVE/DEAD Fixable blue | Invitrogen | Cat# L23105 |
| Bright-Glo Luciferase Assay System | Promega | Cat# E2620 |
| **Experimental models: Cell lines** |        |            |
| Human: Gibco FreeStyle 293-F cells | ThermoFisher Scientific | Cat# R79009 |
| Human: HEK293T-ACE2 | Hanke et al.18 | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to the lead contact, Ben Murrell (benjamin.murrell@ki.se).

Materials availability
Plasmids generated in this study will be made available on request, but we may require a completed Materials Transfer Agreement.

Data and code availability
The published article includes all data generated or analyzed during this study, and summarized in the accompanying tables, figures and supplemental materials.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics statement
The animal work was conducted with the approval of the regional Ethical Committee on Animal Experiments (Stockholms Norra Djurförsöksetiska Nämnd). All animal procedures were performed according to approved guidelines.

Mice
Twenty-four 8-and-a-half-week-old C57BL/6J mice (Jackson Laboratory - 12 males and 12 females) were used in immunization experiments. Mice were housed at the Comparative Medicine animal facility (KM) at Karolinska Institutet in individually ventilated cages. Mice had access to food and water ad libitum and cage enrichment included shredded cardboard and paper rolls. Cage and water changes were performed on a weekly basis and general monitoring of all mice was performed on a daily basis by technical staff. Experiments were approved by the Swedish Board of Agriculture (ethical permit number N4/16). Immunogens were diluted in sterile PBS, emulsified in AddaVax (InvivoGen) and injected subcutaneously (s.c.) in the flanks of mice at weeks 0, 4 and 7. Each arm
PBS-T, developed for 15 minutes at room temperature using 200 secondary antibody (Southern Biotech) diluted 1:5,000 in PBS-T was added to each well. Plates were washed 6 times with PBS-T, and 100 μL of blocking solution containing 2%(w/v) non-fat milk powder in 1X PBS, and washed 6 times with PBS-T. Serum samples serially diluted in blocking solution were added and plates were incubated overnight at 4°C. Plates were washed 6 times with PBS-T, and 100 μL of a goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Southern Biotech) diluted 1:5,000 in PBS-T was added to each well. Plates were washed 6 times with PBS-T, developed for 15 minutes at room temperature using 200 μL per well of peroxidase substrate (o-phenylenediamine dihydrochloride, SIGMAFAST, SigmaAldrich), and read at 450 nm in an Asys Expert 96 plate reader (Biochrom). EC_{50} titers were calculated using a Bayesian logistic curve fitting approach, allowing plate-specific minimum and maximum sigmoid parameters to account for differences between plates, and sample specific slope and offset parameters. EC_{50} titers were calculated from the posterior median value midway between the plate minimum and maximum.

Rhesus macaques
One male and two female rhesus macaques (Macaca mulatta) of Chinese origin, 4-5 years old, were housed at the Astrid Fagraeus Laboratory at Karolinska Institutet. Housing and care procedures complied with the provisions and general guidelines of the Swedish Board of Agriculture. The facility has been assigned an Animal Welfare Assurance number by the Office of Laboratory Animal Welfare (OLAW) at the National Institutes of Health (NIH). The macaques were housed in groups in 14 m² enriched cages. They were habituated to the housing conditions for more than six weeks before the start of the experiment and subjected to positive reinforcement training in order to reduce the stress associated with experimental procedures. The macaques were weighed at each sampling. All animals were confirmed negative for simian immunodeficiency virus (SIV), simian T cell lymphotropic virus, simian retrovirus type D and simian herpes B virus. All immunizations and blood samplings were performed under sedation with 10-15 mg/kg ketamine (Ketaminol 100 mg/ml, Intervet, Sweden) administered intramuscularly (i.m.). For macaque immunizations, stabilized spike trimer (100 μg) was mixed in 75 μg of Matrix-M (Novavax AB). Macaques were immunized intramuscularly (i.m.) with half of the dose administered in each quadricep at weeks 0, 4 and 9. Blood samples were collected pre-immunization and at weeks 2, 4, 6, 9, 11, 19, 23 and 27.

Cell lines
HEK293T cells
HEK293T (human, female) cells were used to produce lentiviral pseudotyped viruses. HEK293T cells transduced to overexpress human ACE2 (HEK293T-ACE2) were used for pseudotyped virus neutralization assays. Both HEK293T cell lines were cultured in a humidified 37°C incubator (5% CO₂) in Dulbecco’s Modified Eagle Medium (GIBCO) supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin, and were passaged when nearing confluency using 1X Trypsin-EDTA.

FreeStyle 293-F cells
FreeStyle 293-F cells (Thermo Fisher Scientific), also derived from HEK293 cell line, were used for protein production, where cells were cultured in FreeStyle Expression Medium (Thermo Fisher Scientific) in a shaking incubator (135 RPM) at 37°C (8% CO₂). All cell lines tested negative for mycoplasma by PCR.

METHOD DETAILS

Protein production
The plasmid for expression of the SARS-CoV-2 prefusion-stabilized spike ectodomain was kindly provided by Jason McLellan. This plasmid was used to transiently transfect FreeStyle 293-F cells using the FreeStyle MAX reagent (Thermo Fisher Scientific). The spike ectodomain was purified from filtered supernatant on Strept-Tactin XT resin (IBA Lifesciences), followed by size-exclusion chromatography on a Superdex 200 in 5 mM Tris pH 8, 200 mM NaCl.

The RBD domain (RVQ-VNF) of SARS-CoV-2 was cloned upstream of an enterokinase cleavage site and a human Fc. This plasmid was used to transiently transfect FreeStyle 293-F cells using the FreeStyle MAX reagent. The RBD-Fc fusion was purified from filtered supernatant on Protein G Sepharose (Cytivia) respectively, and the RBD was purified by size-exclusion chromatography on a Superdex 200 (Cytivia) in 5 mM Tris pH 8, 200 mM NaCl. Proteins were re-buffered into PBS prior to immunization.

See Figure S3A–S3C for size exclusion chromatograms and SDS-PAGE analysis of purified proteins.

Mouse ELISAs
ELISA plates (Nunc MaxiSorp, Thermo Fisher Scientific) were coated overnight at 4°C with 100 μL of preflushed-stabilized spike protein at a concentration of 1 μg/ml in 1x PBS. After six times washing with washing buffer (0.05% Tween-20 in PBS; PBS-T), plates were blocked for 90 minutes at room temperature with 200 μL of blocking solution containing 2%(w/v) non-fat milk powder in 1X PBS and washed 6 times with PBS-T. Serum samples serially diluted in blocking solution were added and plates were incubated overnight at 4°C. Plates were washed 6 times with PBS-T, and 100 μL of a goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Southern Biotech) diluted 1:5,000 in PBS-T was added to each well. Plates were washed 6 times with PBS-T, developed for 15 minutes at room temperature using 200 μL per well of peroxidase substrate (o-phenylenediamine dihydrochloride, SIGMAFAST, SigmaAldrich), and read at 450 nm in an Asys Expert 96 plate reader (Biochrom). EC_{50} titers were calculated using a Bayesian logistic curve fitting approach, allowing plate-specific minimum and maximum sigmoid parameters to account for differences between plates, and sample specific slope and offset parameters. EC_{50} titers were calculated from the posterior median value midway between the plate minimum and maximum.
Macaque ELISAs
ELISA plates were coated with prefusion-stabilized spike protein as described above and blocked for 1 hour at room temperature with 200 μL blocking solution containing 5% (w/v) non-fat milk powder in 1× PBS. Plasma samples serially diluted in blocking solution were added and incubated for 2 hours at room temperature. Plates were washed 6 times with PBS-T and antibody-antigen interaction was detected using 100 μL HRP-conjugated anti-mouse IgG Fc (Nordic MUBio) diluted 1:20,000 in PBS-T. Plates were washed 6 times with PBS-T, developed using 50 μL of 3',5'-tetramethylbenzidine (TMB) substrate solution (Invitrogen) per well and stopped using 50 μL of 1M sulphuric acid per well. OD was read at 450 nm in an Asys Expert 96 plate reader (Biochrom). EC50 values were computed as for the mouse ELISAs.

Pseudotyped neutralization assays
Pseudotyped neutralization assays were adapted from protocols previously validated to characterize the neutralization of HIV57 but with the use of HEK293T-ACE2 cells, and as previously described.18 Pseudotyped lentiviruses displaying the SARS-CoV-2 pandemic founder variant or D614G mutant spike protein (harboring an 18 amino acid truncation of the cytoplasmic tail) and packaging a luciferase reporter gene were generated by the co-transfection of HEK293T cells using Lipofectamine 3000 (Invitrogen) per the manufacturer’s protocols. Media was changed 12–16 hours after transfection, and pseudotyped viruses were harvested at 48- and 72-hours post transfection, filtered through a 0.45 µm filter, and stored at −80 °C until use. Pseudotyped viruses sufficient to generate ~100,000 RLUs were incubated with serial dilutions of serum for 60 min at 37 °C in a 96-well plate, and then ~15,000 HEK293T-ACE2 cells were added to each well. Plates were incubated at 37 °C for 48 hours, and luminescence was then measured using Bright-Glo (Promega) per the manufacturer’s protocol, on a GM-2000 luminometer (Promega). ID50 titers were interpolated as the reciprocal serum dilution at which relative light units (RLUs) were reduced by 50% relative to control wells in the absence of serum using Prism 9 (GraphPad Software). Appropriate interpretation of these ID50 values requires linearity of the luciferase signal as the input virus decreases, which we show in Figure S3D. Statistical comparisons of pseudovirus neutralization ID50 titers between mouse groups were conducted with Mann-Whitney U tests, and between time points with Signed Rank tests, both implemented in the HypothesisTests.jl Julia package. Rhesus Macaque serology measurements were repeated in triplicate (with the geometric mean for the measure of central tendency, and geometric standard deviation for plotted error bars) but just once for the mouse samples, due to sample volume limitations.

B cell ELISpot assay
96-well multiscreen IP filter ELISpot plates (Millipore) were activated with 70% ethanol for 30 s and washed twice with 1× PBS. The plates were coated overnight at 4 °C with 10 μg/ml anti-human Fcγ (Jackson ImmunoResearch). The plates were washed 3 times with 1× PBS and blocked for 2 hours with complete media. Serially diluted PBMCs were plated and incubated overnight at 37 °C, 5% CO2. Cells were previously cultured for 72 h in complete media supplemented with 2.5 μg/ml CpG B oligodeoxynucleotides (Invivogen), 1 μg/ml sCD40-L (PeproTech) and 50 ng/ml IL-21 (PeproTech) in 48-well plates. The plates were washed 6 times with PBS-T and incubated for 90 minutes with the following biotinylated probes: 2.5 ng/ml goat anti-human Fcγ (Jackson ImmunoResearch), 1 μg/ml preadsorbed spike protein or 3 μg/ml ovalbumin to detect, respectively, total IgG and antigen-specific IgG. The plates were washed 6 times with PBS-T and incubated for 45 minutes with streptavidin-alkaline phosphatase (Mabtech AB) diluted 1:1,000. The plates were washed 6 times with PBS-T, developed with 50 μL nitro-blue tetrazolium 5-bromo-4-chloro-3-indolylphosphate substrate (Mabtech AB) for 5 minutes in the dark and stopped by washing with sterile H2O. The plates were dried and spots were counted using an Immunospot analyzer (Cellular Technology Ltd.).

Analysis of T cell responses
Cryopreserved rhesus PBMCs were thawed and rested for 3 hours at 37 °C in a 5% CO2 incubator. After rest, 2×106 PBMCs were added per well in 96-well U-bottom plate and cultured at 37 °C and 5% CO2 in the presence of SARS-CoV-2 S overlapping peptide pool (OLP, JPT Peptide Technologies) at 2 μg/mL or recombinant Spike trimer 57 at 10 μg/mL. For each animal, DMSO at an equal concentration to the peptide pool was used as a negative control, and Staphylococcal Enterotoxin B (Sigma-Aldrich) was used as a positive control. Ninety minutes after culture start, Brefeldin A (Sigma-Aldrich) was added to every well and the culture continued for 14-16 hours at 37 °C and 5% CO2. The PBMCs were stained using LIVE/DEAD Fixable Blue kit (Invitrogen), followed by staining with a surface marker antibody panel (Table S1). The cells were permeabilized using Cytofix/Cytoperm solution (BD Biosciences) and stained with a panel of antibodies against intracellular proteins (Table S1). Stained cells were fixed using 1% formaldehyde and acquired with LSRFortessa flow cytometer (BD Biosciences). Data were analyzed using FlowJo software v10.7.1.

QUANTIFICATION AND STATISTICAL ANALYSIS

FlowJo V10.7.1, GraphPad Prism V9.0.0 and Julia V1.5.3 were used to perform data and statistical analyses, unless otherwise stated. Statistical details of the experiments are provided in the respective figure legends or in the dedicated methods section.