Immunization with synthetic SARS-CoV-2 S glycoprotein virus-like particles protects macaques from infection

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In brief
Sulbaran et al. find that formaldehyde cross-linked S lipid nanoparticles induce potent neutralizing antibody titers upon cynomolgus macaque vaccination. Notably, vaccinated animals develop sterilizing immunity as highlighted upon virus challenge. Thus, the study provides a path to induce sterilizing immunity correlating with mucosal immune responses, which are desired to prevent virus spreading.

Highlights
- S glycoprotein formaldehyde cross-linking stabilizes S in the prefusion conformation
- Vaccination of cynomolgus macaques with S lipid particles induces neutralization
- Vaccination protects macaques against a SARS-CoV-2 challenge
- Sterilizing protection correlates with nasopharyngeal anti-S IgG and IgA titers
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SUMMARY

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has caused an ongoing global health crisis. Here, we present as a vaccine candidate synthetic SARS-CoV-2 spike (S) glycoprotein-coated lipid vesicles that resemble virus-like particles. Soluble S glycoprotein trimer stabilization by formaldehyde cross-linking introduces two major inter-protomer cross-links that keep all receptor-binding domains in the “down” conformation. Immunization of cynomolgus macaques with S coated onto lipid vesicles (S-LVs) induces high antibody titers with potent neutralizing activity against the vaccine strain, Alpha, Beta, and Gamma variants as well as T helper (Th)1 CD4+ -biased T cell responses. Although anti-receptor-binding domain (RBD)-specific antibody responses are initially predominant, the third immunization boosts significant non-RBD antibody titers. Challenging vaccinated animals with SARS-CoV-2 shows a complete protection through sterilizing immunity, which correlates with the presence of nasopharyngeal anti-S immunoglobulin G (IgG) and IgA titers. Thus, the S-LV approach is an efficient and safe vaccine candidate based on a proven classical approach for further development and clinical testing.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a betacoronavirus closely related to SARS-CoV-1, is the etiological agent of coronavirus disease (COVID-19), which quickly developed into a worldwide pandemic1,2 causing more than five million deaths as of November 2021 (https://covid19.who.int/) and highlighting the urgent need for effective infection control and prevention.

An important correlate of protection of antiviral vaccines is the generation of neutralizing antibodies.3–5 The main SARS-CoV-2 target for inducing neutralizing antibodies is the spike (S) glycoprotein composed of the S1 subunit that harbors the receptor-binding domain (RBD) and the S2 membrane fusion subunit that anchors the S trimer in the virus membrane.6 RBD binding to the cellular receptor ACE2 (ACE2) leads to virus attachment, and subsequent S2-mediated fusion with endosomal membranes establishes infection.7,8 S is synthesized as a trimeric precursor polyprotein that is proteolytically cleaved by furin and furin-like proteases in the Golgi generating the non-covalently linked S1-S2 heterotrimer.9 The structure of S reveals a compact heterotrimer composed of the S1 N-terminal domain (NTD), the receptor-binding domain (RBD) , two subdomains, S2, the transmembrane region, and a cytoplasmic domain. The conformation of RBD is in a dynamic equilibrium between either all RBDs in a closed, receptor-inaccessible conformation or one or two RBDs in the “up” conformation.9,11–14 Only the S RBD in the up position allows receptor binding,15,16 which triggers the
SARS-CoV-2 58, 59 (PiCoVacc/CoronaVac, Sinovac). Numerous challenges in small animals and non-human primates 28,36–39 or are neca; Ad shown to provide in vivo protection against SARS-CoV-2 challenge in small animals and non-human primates 28,36–39 or are in clinical development and use.10

The magnitude of antibody responses to S during natural infection varies greatly and correlates with disease severity and duration.41,42 Basal responses are generally maintained for months43–45 or decline within weeks after infection,11 most notably in asymptomatic individuals.46 Thus, any vaccine-based approach aims to induce long-lasting immunity.

A number of animal models have been developed to study SARS-CoV-2 infection including the macaque model, which demonstrated an induction of innate, cellular, and humoral responses upon infection,17–51 conferring partial protection against reinfection.52,53 Consequently, many early vaccine candidates provided protection in the macaque model including the currently licensed vaccines based on S-specific mRNA delivery54,55 (BNT162b2, Pfizer/BioNTech; mRNA-1273, Moderna), adeno-virus vectors56,57 (ChAdOx1 nCoV-19, Oxford/AstraZeneca; Ad26.COV2.S, Johnson & Johnson), and inactivated SARS-CoV-258,59 (PICoVacc/CoronaVac, Sinovac). Numerous other approaches have been evaluated as well.60

Employing the classical subunit approach, S subunit vaccine candidates have generated different levels of neutralizing antibody responses in pre-clinical testing.61–65 Using self-assembly strategies of S or RBDs further increased immune responses66,67 and protected against infection.68–70

Antigens can be also presented via liposomes, which provide a highly controllable degree of multivalency and stability and a prolonged circulating half-life in vivo.71,72 Notably, liposomes coated with viral glycoproteins such as the HIV-1 envelope (Env) induced more efficient immune responses than did immunization with single glycoprotein trimers.73–75 This is in line with more efficient B cell activation and with the generation of germinal centers (GCs) by multivalent presentation of Env trimers versus soluble trimers.76

Here, we developed synthetic virus-like particles employing liposomes that are decorated with S glycoprotein trimers that have been treated by formaldehyde cross linking, which in turn stabilized S in the native conformation over a long period. Serum antibody recognition of cross-linked versus non-cross-linked S did not show significant binding differences. A small group of cynomolgus macaques was immunized with S-LVs, which produced high S-specific antibody titers and Th1 CD4+ T cell responses. Potent neutralization of wild-type (WT) SARS-CoV-2 and of Alpha pseudovirus variants was observed after two immunizations, while Beta and Gamma pseudovirus variants were neutralized at reduced potency. Challenge of the animals with SARS-CoV-2 demonstrated that S-LV immunization protected the animals from infection revealing no detection of genomic RNA (gRNA) upon infection in nasal and tracheal swabs nor in bronchoalveolar lavages (BALs), thus suggesting sterilizing immunity. This indicates that S-LVs are potential candidates for further clinical development of a safe protein-based SARS-CoV-2 vaccine.

RESULTS
S-LV formation and characterization
The S glycoprotein construct 2P8 was expressed in mammalian cells and purified by Ni2+-affinity and size-exclusion chromatography (SEC) (Figure S1A), with yields up to 10 mg/L using Expi293F cells. This produced native trimers as determined by negative staining electron microscopy and 2D class averaging of the single particles (Figure S1B). Since S revealed low thermostability (melting temperature [Tm] = 42 °C) as reported previously,18 it was chemically cross-linked with 4% formaldehyde (FA) producing a higher molecular weight species as determined by SDS-PAGE (Figure S1C). FA cross-linking preserved the native structure (Figure S1D) over longer time periods (Figures S1E) by increasing the thermostability to a Tm of 65 °C. The cryo-electron microscopy structure of FA-cross-linked S (FA-S) at 3.4 Å resolution (Figure S2; Table S1) revealed two major sites of cross linking (Figure 1A). RBD residues R408 and K378 cross-linked neighboring RBDs producing S trimers in the closed “RBD-down” conformation (Figures 1A and 1B). The second site introduced inter-S2 subunit bonds by cross-linking R1019 of the central S2 helix and/or S2 K776 with S2 HR1 K947 (Figure 1C). FA-S was incubated with liposomes (phosphatidylcholine 60%, cholesterol 36%, DGS-NTA 4%), and efficiently captured via its C-terminal His-tag. Free, unbound Fa-S was removed from the S proteoliposomes by sucrose gradient centrifugation (Figure S1F), and decoration of the liposomes with FA-S (S-LV) was confirmed by negative staining electron microscopy (Figure 1D).

S-LV immunization induced potent neutralizing antibody responses in cynomolgus macaques
S-LVs were produced for a small vaccination study of cynomolgus macaques to evaluate the immunogenicity and elicitation of neutralizing antibodies. Four cynomolgus macaques were immunized with 50 μg S-LVs adjuvanted with monophospholipid A (MPLA) liposomes by the intra-muscular route at weeks 0, 4, 8, and 12 and for the first, second, and third immunizations, respectively (Figure 2A). Sera of the immunized macaques were analyzed for binding to S, FA-S, and the RBD in 2 week intervals. The results revealed similar S-specific antibody (Ab) titers for all animals. S effective dose (ED50) titers increased from ~75 on week 4 to ~10,000 on week 6 and to ~20,000 on week 12, after the first, second, and third immunizations, respectively (Figure 2B). Slight reductions in titers were detected against FA-S (Figure 2C). Titers against RBD alone reached effective doses (ID50s) of ~100 on week 4 and ~4,500 on week 6, as well as slight increases on week 12 for some animals (Figure 2D). This suggests that the first and second immunizations induced significant RBD titers, while the third immunization boosted non-RBD antibodies since the week 12 S-specific titers were ~4 times higher than the RBD-specific titers in contrast to previous time points at which this ratio was lower (Figure 2C). A fourth immunization did not further boost antibody generation, and titers at week 22 were lower or comparable to week 12 titers (Figure...
We conclude that S-LV immunization induced primarily RBD-specific antibodies after the first and second immunizations, while the third immunization increased the generation of non-RBD antibodies significantly.

Serum neutralization titers using a WT pseudovirus were elicited in all four animals. At week 2 after the first immunization, ID50 titers between 100 and 1,000 were observed, which dropped close to baseline at week 4 but was significantly increased at week 6, 2 weeks after the second immunization demonstrating ID50s between 5,000 and 20,000. The ID50s then decreased at week 8 and increased from 20,000 to 40,000 at week 10, 3 weeks after the third immunization. At week 19, neutralization potency decreased but was still high, indicating that three immunizations induced robust neutralization titers. The fourth immunization boosted neutralization titers to the same level as the third immunization (Figure 3A).

Since antibody titers indicated the induction of high levels of RBD-specific antibodies, in order to understand the part of anti-RBD antibodies in serum neutralization, we depleted the serum at week 10 by anti-RBD affinity chromatography, resulting in no detectable RBD antibodies by ELISA. RBD-specific Ab-depleted serum showed 10% to 30% neutralization compared with the complete serum, indicating some level of non-RBD-specific neutralization. While RBD-specific Ab neutralization largely dominated in two animals, the fraction of non-RBD-specific Ab neutralization activity (Figure 3B) appeared greater in the other two, suggesting a participation of these Abs in the high neutralization titers (Figure 3A).

In order to determine the extent of S-LV-vaccination-induced protection, vaccinated and non-vaccinated animals (n = 4) were infected with the primary SARS-CoV-2 isolate (BetaCoV/France/IDF/0372/2020) with a total dose of 1 × 10^5 plaque-forming units (PFU). Infection was induced by combining intra-nasal (0.25 mL into each nostril) and intra-tracheal (4.5 mL) routes at week 24, 5 weeks after the last immunization. Viral loads in the control animal group peaked in the trachea at 3 days post-exposure (dpe) with a median value of 6.0 log_{10} copies/mL and in the nasopharynx between 4 and 6 dpe with a median copy number of 6.6 log_{10} copies/mL (Figure 4A). Viral loads decreased subsequently, and no virus was detected on 10 dpe in the trachea, while some animals showed viral detection up to 14 dpe in the nasopharyngeal swabs (Figure 4A). In the BALs, three control (Ctrl) animals out of four showed detectable viral loads at 3 dpe, and two of them remained detectable at 7 dpe with mean value of 5.4 and 3.6 log_{10} copies/mL, respectively. Rectal fluids tested positive in one animal, which also had the highest tracheal and nasopharyngeal viral loads (Figures S3A and S3B). Viral subgenomic RNA (sgRNA), which is believed to estimate the number of infected and productively infected cells collected with the swabs or during the lavage, showed peak copy numbers between 3/4 and 6 dpe in the tracheal and nasopharyngeal fluids, respectively (Figure 4B). In the BALs, the two animals presenting high genomic viral loads also showed detectable sgRNA at 3 and 7 dpe, with medians of 5.1 and 3.1 log_{10} copies/mL, respectively (Figure 4B).
In contrast to control animals, neither gRNA nor sgRNA was detected at any point in the vaccinated group (Figures 4A and 4B). The mean gRNA peaks in the trachea and nasopharynx (6.0 and 6.6 log_{10} copies/mL, respectively) of the control group were higher (p = 0.0286) than those of the vaccinated group. The area under the curve was also higher in the trachea of the control group (6.2 log_{10}, p = 0.286). In the BAL, the difference was not statistically significant due to the low number of animals.

The complete absence of viral RNA in the vaccinated group, both in the upper and lower respiratory tract, strongly suggested that sterilizing immunity was induced by vaccination. ID50 antibody titers against S, FA-S, and the RBD decreased slightly from the day of infection (week 24) to 4 weeks pe (Figures 5A, 5B, and 5C), although a small increase in Ab titers is observed at 1 week pe (week 25). Ab titers also correlated with a slight decrease in neutralization from week 24 to 4 weeks pe, although one animal showed a small increase in neutralization on week 25, 1 week pe (Figure 5D). This demonstrated that challenge of vaccinated animals further correlated with the presence of significant S- and RBD-specific IgG and IgA in nasopharyngeal fluids (Figures 2E and S4). This indicated that S-LV vaccination induced mucosal immunity that very likely contributed to the sterilizing effect of vaccination.

Similar to previous observations, during the first 14 dpe, all control animals showed mild pulmonary lesions characterized by non-extended ground-glass opacities (GGOs) detected by chest CT (Figure S5A). Vaccinated animals showed no significant impact of challenge on CT scores. The only animal showing a lesion score >10 was in the control group. Whereas all control animals experienced monocytoses between 2 and 8 dpe, probably corresponding to a response to infection, monocyte counts remained stable after challenge for the vaccinated monkeys (Figure S5B), in agreement with the absence of detectable anamnestic response in the latter animals.

The levels of CD4- and CD8-specific T cells were measured in both groups of animals. Before exposure, Th1-type CD4+ T cell responses were observed in all vaccinated macaques following ex vivo stimulation of peripheral blood mononuclear cells (PBMCs) with S-peptide pools (Figures 6, S6, and S7). None had detectable anti-S CD8+ T cells (Figure S8). No significant difference was observed at 14 dpe, also in agreement with the absence of an anamnestic response in vaccinated animals. In contrast, the anti-S Th1 CD4+ response increased post-exposure for most of the control animals (Figures 6 and S6).
gamma at week 8. Neutralization potency was increased after the third immunization (week 12), with median ID50s of ~5,000 (WT), ~8,000 (Alpha), ~800 (Beta), and 1,000 (Gamma). Neutralization titers did not increase after the fourth immunization at week 24 and started to decrease at week 28 (Figure 7). We conclude that three immunizations provided robust protection against the variants, although neutralization titers may have already been within the protective range after two immunizations for the three variants tested.

**DISCUSSION**

Many vaccines are under development in pre-clinical and clinical testing, and eight have been approved by regulatory agencies around the world. Here, we developed a two-component system employing the SARS-CoV-2 S glycoprotein coupled to liposomes. Since the stability of the WT SARS-CoV-2 S glycoprotein is low due to its tendency to spontaneously switch into its post-fusion conformation, SARS-CoV-2 S was stabilized by two proline mutations that enhanced stability. However, this S 2P version still showed limited stability over time, as reported, which may be due to cold sensitivity. We overcame the problem of stability by using FA cross-linking that increased the thermostability to 65°C, preserving the native S conformation over extended storage time periods. Furthermore, we detected two cross-linking sites by cryoelectron microscopy (cryo-EM), which showed that cross-linking locked the native S trimer in the closed, RBD-down conformation via covalent inter-protoner interactions, which prevent conformational changes leading to the post-fusion conformation. Notably, FA cross-linking is widely used in vaccine formulations. Stability has since been improved by engineering six proline mutations (S 6P), which increased the thermostability to 50°C, and by disulfide-bond engineering. Moreover, ligand binding renders S more stable.

Many previous studies have shown that immunogen multime-
and an mRNA vaccine (Pfizer/BionTech BNT162b2) showed 10–20 times lower titers compared with the S-LVs in macaques studies. Further, the Moderna mRNA-1273 (Moderna), S trimers (Clover Biopharmaceutical), and NVX-CoV2373 (Novavax) induced similar or higher titers in macaque studies. Median ID50 titers increased by a factor of 4 after the third immunization but did not amplify after the fourth immunization. The T cell response in the vaccinated group was biased toward Th1 CD4+ T cells, consistent with licensed or other experimental vaccines. Serum neutralization was already significant after the first immunization but increased by a factor of ~20 after the second immunization and by a factor of 3 after the third immunization, indicating that two immunizations with S-LVs may suffice to confer protection. Neutralizing antibody (nAb) titers decline within 10 weeks after the third immunization to the levels of week 8.
prior to the third immunization) and increase slightly after the fourth immunization to the median ID50 level attained after the third immunization.

Vaccination prevented lymphopenia and lung damage in animals infected with SARS-CoV-2 at a dose comparable or lower than in previous studies. Protection was sterilizing since no replication could be detected in the upper and lower respiratory tracts, suggesting that vaccination with S-LVs will prevent virus shedding and transmission. Sterilizing immunity likely correlates with mucosal antibody responses that protect the upper respiratory tract from infection. Since we detected significant IgG and IgA in nasopharyngeal fluids at the time of viral challenge, we propose that S-LV vaccination induces sterilizing protection by eliciting mucosal immune responses.

SARS-CoV-2 infection generates nAbs, with up to 90% targeting the RBD, which is immunodominant. Similarly, mRNA vaccination elicits predominantly RBD-specific nAbs. RBD antibodies can be grouped into three classes and seem to be easily induced by immunization, as many of them are generated by a few cycles of affinity maturation, indicating that extensive GC reactions are not required. Consistent with these findings, we show that RBD-specific antibodies are predominant after the first and second immunizations as indicated by similar S- and RBD-specific titers. However, after the third immunization, median S-specific ED50s are 3 times higher than RBD-specific ED50s 4 weeks after the third immunization. This trend is continued after the fourth immunization, which revealed a 3.5 times higher median ID50 for S than for RBD 5 weeks post-immunization. This thus suggests that more than two immunizations expand the reactive B cell repertoire that targets non-RBD S epitopes.

Current variants carry the B.1D614G mutation and have been reported to be more infectious. Although the D614G mutation alone was reported to increase neutralization susceptibility, further mutations present in Beta (B1.351, SA) and Gamma (P1, BR) reduce neutralization potencies of natural and vaccine-induced sera, while Alpha (B.1.1.7, UK) neutralization seems to be less affected. The reduction in neutralization potency of polyclonal plasma Abs is mainly affected by mutations within the three main epitopes in the RBD. In particular, the E484K mutation present in Beta and Gamma was reported to reduce neutralization by a factor of 10. Here, we show that S-LV vaccination produces robust neutralization of Alpha, Beta, and Gamma, although the median ID50s of Beta and Gamma neutralization are reduced 20- and 5-fold, respectively, after the second immunization compared with WT and Alpha. The third immunization boosted neutralization of Beta and Gamma, albeit with 6- and 3-fold reductions in potency,
respectively, compared with WT, which is slightly more potent than the median ID50 of vaccinated and hospitalized patient cohorts using the same assay setup.115

In summary, S-LV vaccination represents an efficient strategy that protects macaques from high-dose challenge. Although the animals have been challenged only after the fourth immunization, which did not boost Ab or neutralization titers significantly, our neutralization data suggest that the animals might have been protected after two immunizations. Furthermore, our data suggest that the third immunization increases S-protein Ab titers more significantly than RBD-specific Ab titers. This points to an increase of non-RBD antibodies, which may be beneficial for the neutralization of different variants. Although other regions within S, most notably NTD, are targets for mutation within new variants, S2 or other epitopes may be less prone to mutations due to conformational constraints. Therefore, future vaccination strategies should consider boosting non-RBD antibodies to compensate for the loss of neutralization activity targeting RBD in variants. Notably, SARS-CoV-2 memory B cells are present over a long time period in convalescent116,117 and mRNA-vaccinated individuals,118 which is in line with potential boosting strategies.

Limitations of study
We provide data that the immunization of macaques with synthetic S-glycoprotein-coated liposomes induced robust antibody and neutralization titers after two immunizations. SARS-CoV-2 challenge after four immunizations revealed complete protection by sterilizing immunity. First, a limitation of the study is that the challenge was performed only after the fourth immunization. Secondly, although the SARS-CoV-2 challenge infection was performed 5 weeks after the last immunization, we cannot predict the efficacy of protection months post-immunization. The third limitation of the study is the small number of animals used, which prevents robust statistical analyses of the results. Fourth, although we observe robust pseudovirus neutralization of Alpha, Beta, and Gamma variants, we cannot predict the efficacy of protection upon infection, which will require further studies.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100528.

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AUTHOR CONTRIBUTIONS

Conceptualization, W.W., G.S., R.L.G., and P.M.; funding acquisition, W.W., R.L.G., and R.W.S.; writing—original draft, W.W., R.L.G.; writing—review & editing, all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Horsedish peroxidase conjugated goat anti-monkey H+L | Invitrogen | Cat# PA1-84631 |
| Anti-IL2 PerCP5.5 (MO1-17H12) | BD Bioscience | Cat# 560708 |
| Anti-IL17a Alexa700 (N49-653) | BD Bioscience | Cat# 560613 |
| Anti-IFN-γ V450 (B27) | BD Bioscience | Cat# 560371 |
| Anti-TNF-α BV605 (Mab11) | Biolegend | Cat# 502936 |
| Anti-IL-13 BV711 (JES10-5A2) | BD Bioscience | Cat# 564288 |
| Anti-CD137 APC (4B4) | BD Bioscience | Cat# 550890; RRID:AB_2848292 |
| Anti-CD154 FITC (TRAP1) | BD Bioscience | Cat# 555699; RRID:AB_396049 |
| CD3 APC-Cy7 (SP34-2) | BD Bioscience | Cat# 557757; RRID:AB_396863 |
| CD4 BV510 (L200) | BD Bioscience | Cat# 563094 |
| CD8 PE-Vio770 (BW135/80) | Miltenyi Biotec | Cat# 130-113-721 |
| FastImmune™ CD28/CD49d | BD Bioscience | Cat# 130-113-159 |
| Live/Dead Fixable Blue Dead Cell | Thermofisher | Cat# L34962 |
| Anti-Monkey IgG (γ-chain specific)-Biotin antibody produced in goat | Sigma Aldrich | Cat# SAB3700768 |
| Anti-Monkey IgA (α-chain specific)-Biotin antibody produced in goat | Sigma Aldrich | Cat# SAB3700761 |
| **Bacterial and virus strains** |        |            |
| SARS-CoV-2 (hCoV-19/France/IDF0372/2020 strain) | Lescur et al. EPI_ISL_410720 (GISAID ID) |
| TOP10 Chemically Competent E. coli | Thermofisher | C404010 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Formaldehyde solution 36.5% | Sigma-Aldrich | Cat# F8775-25ML |
| Dithiothreitol | Sigma-Aldrich | Cat# 43819 |
| Streptavidin-PE | ThermoFisher Scientific | Cat# 12-4317-87 |
| EDC | ThermoFisher Scientific | Cat# A35391 |
| Sulfo-NHS | ThermoFisher Scientific | Cat# A39269 |
| Amphicline | Euromedex | Cat# EU0400-D |
| Polyethyleneimine (PEI-25K) | Polysciences | Cat# 23966-1 |
| L-α-phosphatidylcholine (PC) | Avanti Polar Lipids | 840051 |
| DGS-NTA(Ni) | Avanti Polar Lipids | 790404 |
| Cholesterol | Avanti Polar Lipids | 700000 |
| Bradford Protein Assay Reactive | BioRad | Cat# 500006 |
| Excel purification resin | Cytiwa | Cat# 17-3712-01 |
| PBS 10X | EuroMedex | Cat# ET330-A |
| Penicillin | Sigma-Aldrich | Cat# P3032-10MI |
| Streptomycin | VWR | Cat# 382-EU-100G |
| MPLA liposomes | Polymun Scientific | https://www.polymun.com/liposomes/reference-projects/ |
| Bovine Serum Albumin | Sigma-Aldrich | Cat# A2153-1000 |
| GlutaMax | Gibco | Cat# 35050061 |
| PepMix™ SARS-CoV-2 (Spike Glycoprotein) | JPT peptide Technologies | Cat# PM-WCPV-S |
| Poly-L-Lysine Hydrobromide | Sigma-Aldrich | Cat# P1399 |

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### Reagent or Resource Source Identifier

#### Critical Commercial Assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| SSIV Reverse Transcriptase | Thermo Fisher scientific | Cat# 18090050 |
| Ligation Sequencing Kit | Nanopore | Cat# M0494 |
| Q5 Hot Start DNA Polymerase | NEB | Cat# A14535 |
| Exp293™ Expression System Kit | Thermo Fisher scientific | Cat# A14535 |
| Nano-Glo Luciferase Assay System | Promega | Cat# N1130 |
| Nucleospin 96 Virus Core | Macherey-Nagel | Cat# 740452.4 |

#### Experimental Models: Cell Lines

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| FreeStyle™ 293-F Cells | Thermo Fisher scientific | Cat# R79007 |
| Expi293™ Cells | Thermo Fisher scientific | Cat# A14527 |
| VeroE6 | ATCC | ATCC®CRL 1586TM |
| HEK293T/ACE2 cells | Schmidt et al. | N/A |

#### Deposited Data

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| PDB | 7Q1Z | https://www.rcsb.org/ |
| EMBD | EMD-13776 | https://www.ebi.ac.uk/emdb/ |

#### Experimental Models: Organisms/Strains

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Cynomolgus Macaques | Cynologics | N/A |

#### Oligonucleotides

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Primers covid19 V3 | ARTIC network | http://artic.network/resources/ncov-amplicon-v3.pdf |
| RdRp-IP4 primer, forward – GGTAACCTGGTATGATTTCG | https://www.who.int/docs/default-source/coronaviruse/real-time-rtpcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn=3662fc6_b_2 | N/A |
| RdRp-IP4 primer, reverse – CGGTCAAGGTATATAGG | https://www.who.int/docs/default-source/coronaviruse/real-time-rtpcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn=3662fc6_b_2 | N/A |
| RdRp-IP4 primer probe P – TCATAACAACCCACGCGACAGG | https://www.who.int/docs/default-source/coronaviruse/real-time-rtpcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn=3662fc6_b_2 | N/A |
| sgLead SARS-CoV2-forward – CGATCTCTTGTAGATCTGTTCTC | Corman | N/A |
| E-Sarbeco-reverse primer – ATATTGCGAGCATGACGACACA | Corman | N/A |
| E-Sarbeco probe HEX-ACACTAGCCATCCTACTGCGCTTCG-BHQ1 | Corman | N/A |

#### Recombinant DNA

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Plasmid: Protein S | Wrapp et al. | N/A |
| Plasmid: RBD | NR-52309, BEI resources, NIAID, NIH | N/A |
| pHV-1NL43:ENV-NanoLuc plasmid | Schmidt et al. | N/A |
| SARS-CoV-2-ΔΔ19 plasmid | Schmidt et al. | N/A |
| gBlock B.1.1.7, B.1.351, and P.1 | Integrated DNA Technologies | N/A |
| Variants: B.1.1.7, B.1.351, and P.1 cloned in pCR3 plasmid | Caniels et al. | N/A |

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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Software and algorithms** | | |
| GraphPad Prism v7 | GraphPad | N/A |
| GraphPad Prism v8 | GraphPad | N/A |
| ProtParam | | http://web.expasy.org/ |
| Prometheus MT | Nanotemper | https://nanotempertech.com/prometheus/ |
| Flowjo v10 | Flowjo | N/A |
| INTELLISPACE PORTAL 8 software | Philips Healthcare | https://www.philips.fr/healthcare/product/HC881062/inellispace-portal-80-all-your-advanced-analysis-needs-one-comprehensive-solution |
| SerialEM | Mastronarde et al. | N/A |
| motioncor2 | Zheng et al. | N/A |
| RELION 3.1.2 | Zivanov et al. | N/A |
| GCTF | Zhang et al. | N/A |
| Biocres | Cardone et al. | N/A |
| DeepEMhancer | Sanchez-Garcia et al. | N/A |
| CHIMERA | Pettersen et al. | N/A |
| COOT | Emsley et al. | N/A |
| MOLPROBITY | Williams et al. | N/A |
| PHENIX | Adams et al. | N/A |
| CHIMERAX | Goddard et al. | N/A |
| **Others** | | |
| Superose 6 increase | GE Healthcare | 28-9909-44 |
| Superdex 75 | GE Healthcare | N/A |
| Whatman® Nuclepore™ Track-Etched Membranes | Merck | WHA111105 |
| FreeStyle Expression Medium | Thermo Scientific | Cat# 12338018 |
| Opti-MEM™ I Reduced Serum Medium | Thermo Scientific | Cat#31985062 |
| RPMI Medium 1640 | GIBCO | Cat# 21875-034 |
| DMEM | Sigma-Aldrich | Cat# D6429-500ML |
| Grilles Cu 400 mesh | Oxford Instruments | G2400C |
| Nucleobond PC 2000 EF | Macherey Nagel | Cat# 740549 |
| Nucleobond Xtra Midi EF | Macherey Nagel | Cat# 740420 |
| Nucleobond Xtra Mini Kit | Macherey-Nagel | N/A |
| ELISA microplates | Thermo Scientific | 10547781 |
| Protein A Sepharose ® | Abcam | Cat# ab193256 |
| Protein G Sepharose™ | Abcam | Cat# ab17061801 |
| Instant Blue Coomassie Protein Stain | Abcam | Cat# ab119211 |
| Amicon® Ultra-15 | Sigma-Aldrich | UFC903096 |
| Amicon® Ultra-30 | Sigma-Aldrich | UFC901096 |
| Capillars | | N/A |
| AKTA system | GE Healthcare | https://www.cytivalifesciences.com/en/us/shop/chromatography/chromatography-systems/akta-pure-p-05844 |
| TMB | Interchim | UP 664781 |
| Glomax | Turner BioSystems | Model# 9101-002 |
| Microplate 96 well half area white | Greiner bio-one | Cat# 675074 |

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Winfried Weissenhorn (winfried.weissenhorn@ibs.fr).

Materials availability
All reagents will be made available from the Lead Contact on request and after completion of a Materials Transfer Agreement.

Data and code availability
Data: The raw data supporting the findings of the study are available from the lead contact upon request. Structural data have been deposited at https://www.rcsb.org/ and https://www.ebi.ac.uk/emdb/. They are publicly available as of the date of publication. Accession codes are listed in the key resources table.

Code: This paper does not report original code

General statement: Any additional information required to reanalyze the data reported in this paper is available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
HEK293T (ATCC CRL-11268) and HEK293F (Thermo Fisher Scientific) and are human embryonic kidney cell lines. HEK293F cells are adapted to grow in suspension. HEK293F cells were cultured at 37°C with 8% CO2 and shaking at 125 rpm in 293FreeStyle expression medium (Life Technologies). HEK293T cells were cultured at 37°C with 5% CO2 in flasks with DMEM supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL) and penicillin (100 U/mL). HEK293T/ACE2 cells are a human embryonic kidney cell line expressing human angiotensin-converting enzyme 2. HEK293T/ACE2 cells were cultured at 37°C with 5% CO2 in flasks with DMEM supplemented with 10% FBS, streptomycin (100 μg/mL) and penicillin (100 U/mL). VeroE6 cells (ATCC CRL-1586) are a kidney epithelial cells from African green monkeys. VeroE6 cells were cultured at 37°C with 5% CO2 in DMEM supplemented with or without streptomycin (100 μg/mL) and penicillin (100 U/mL) and with or without 5 or 10% FBS, and with or without TPCK-trypsin. PBMC were isolated from macaque sera and cultured in RPMI1640 Glutamax+ medium (Gibco) supplemented with 10 % FBS.

Viruses
SARS-CoV-2 virus (hCoV-19/France/ IDF0372/2020 strain) was isolated by the National Reference Center for Respiratory Viruses (Institut Pasteur, Paris, France) as previously described and produced by two passages on Vero E6 cells in DMEM (Dulbecco’s Modified Eagles Medium) without FBS, supplemented with 1% P/S (penicillin at 10,000 U ml⁻¹ and streptomycin at 10,000 μg ml⁻¹) and 1 μg ml⁻¹ TPCK-trypsin at 37 °C in a humidified CO2 incubator and titrated on Vero E6 cells. Whole genome sequencing was performed as described without any modifications observed compared with the initial specimen and sequences were deposited after assembly on the GISAID EpiCoV platform under accession number ID EPI_ISL_410720.

Ethics and biosafety statement
Cynomolgus macaques (Macaca fascicularis) originating from Mauritian AAALAC certified breeding centers were used in this study. MF1-MF4, vaccinated group and MF5-MF8, control group.

| Gender | Date of birth | Age (years) | Weight at Day 0 post exposure (kg) | Developmental stage |
|--------|---------------|-------------|-----------------------------------|---------------------|
| MF1    | M             | 04/04/2017  | 3.68                              | Young adult         |
| MF2    | M             | 05/04/2017  | 3.68                              | Young adult         |
| MF3    | M             | 10/04/2017  | 3.67                              | Young adult         |

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All animals were housed in IDMIT infrastructure facilities (CEA, Fontenay-aux-roses), under BSL-2 and BSL-3 containment when necessary (Animal facility authorization #D92-032-02, Préfecture des Hauts de Seine, France) and in compliance with European Directive 2010/63/EU, the French regulations and the Standards for Human Care and Use of Laboratory Animals, of the Office for Laboratory Animal Welfare (OLAW, assurance number #A5826-01, US). The protocols were approved by the institutional ethical committee “Comité d’Ethique en Expérimentation Animale du Commissariat à l’Energie Atomique et aux Energies Alternatives” (CEtEA #44) under statement number A20-011. The study was authorized by the “Research, Innovation and Education Ministry” under registration number APAFIS#24434-2020030216532863.

Animals and study design
Cynomolgus macaques were randomly assigned in two experimental groups. The vaccinated group (n = 4) received 50 mg of SARS CoV-2 S-LV adjuvanted with 500 mg of MPLA liposomes (Polymun Scientific, Klosterneuburg, Austria) diluted in PBS at weeks 0, 4, 8 and 19, while control animals (n = 4) received no vaccination. Vaccinated animals were sampled in blood at weeks 0, 2, 4, 6, 8, 10, 12, 14, 19, 21 and 22. At week 24, all animals were exposed to a total dose of 10⁵ pfu of SARS-CoV-2 virus (hCoV-19/France/IDF0372/2020 strain; GISAID EpiCoV platform under accession number EPI_ISL_410720) via the combination of intranasal and intra-tracheal routes (0.25 mL in each nostril and 4.5 mL in the trachea, i.e., a total of 5 mL; day 0), using atropine (0.04 mg/kg) for pre-medication and ketamine (5 mg/kg) with medetomidine (0.042 mg/kg) for anesthesia. Nasopharyngeal, tracheal and rectal swabs, were collected at days 2, 3, 4, 6, 7, 10, 14 and 27 days past exposure (dpe) while blood was taken at days 2, 4, 7, 10, 14 and 27 dpe. Bronchoalveolar lavages (BAL) were performed using 50 mL sterile saline on 3 and 7 dpe. Chest CT was performed at 3, 7, 10 and 14 dpe in anesthetized animals using tiletamine (4 mg kg⁻¹) and zolazepam (4 mg kg⁻¹). Blood cell counts, haemoglobin, and haematocrit, were determined from EDTA blood using a DHX800 analyzer (Beckman Coulter).

METHODS DETAILS

Protein expression and purification
The SARS-CoV-2 S gene encoding residues 1-1208 with proline substitutions at residues 986 and 987 (“2P”), a “GSAS” substitution at the furin cleavage site (residues 682-685) a C-terminal T4 fibrin trimerization motif, an HRV3C protease cleavage site, a Twin-StrepTag and Hexa-His-tag was transiently expressed in FreeStyle293F cells (Thermo Fisher Scientific) using polyethylenimine (PEI) 1 mg/ml for transfection. Supernatants were harvested five days post-transfection, centrifuged for 30 min at 5000 rpm and filtered using 0.20 μm filters (ClearLine/C226). SARS-CoV-2 S protein was purified from the supernatant by Ni²⁺-Sepharose chromatography (Excel purification resin, Cytiva) in buffer A (50 mM HEPES pH 7.4, 200 mM NaCl) and eluted in buffer B (50 mM HEPES pH 7.4, 200 mM NaCl, 500 mM imidazole). Eluted SARS-CoV-2 S containing fractions were concentrated using Amicon Ultra (cut-off: 30 KDa) (Millipore) and further purified by size-exclusion chromatography (SEC) on a Superose 6 column (GE Healthcare) in buffer A or in PBS.

For RBD expression, the following reagent was produced under HHSN272201400008C and obtained through BEI Resources, NIAID, NIH: Vector pCAGGS containing the SARS-Related Coronavirus 2, Wuhan-Hu-1 Spike Glycoprotein Receptor Binding Domain (RBD), NR-52309. The SARS-CoV-2 S RBD domain (residues 319 to 541) was expressed in EXPI293 cells by transient transfection according to the manufacturer’s protocol (Thermo Fisher Scientific). Supernatants were harvested five days after transfection and cleared by centrifugation. The supernatant was passed through a 0.45 μm filter and RBD was purified using Ni²⁺-chromatography (HisTrap HP column, GE Healthcare) in buffer A (50 mM HEPES pH 7.4, 200 mM NaCl) and eluted in buffer B (50 mM HEPES pH 7.4, 200 mM NaCl, 500 mM imidazole). Eluted SARS-CoV-2 S containing fractions were concentrated using Amicon Ultra (cut-off: 30 KDa) (Millipore) and further purified by size-exclusion chromatography (SEC) on a Superdex 75 column (GE Healthcare) in buffer C. Protein concentrations were determined using absorption coefficients at 280 nm calculated with ProtParam (https://web.expasy.org/).

SARS-CoV-2 S crosslinking
S protein at 1 mg/ml in PBS was cross-linked with 4% formaldehyde (FA) (Sigma) overnight at room temperature. The reaction was stopped with 1 M Tris HCl pH 7.4 adjusting the sample buffer to 7.5 mM Tris/HCl pH 7.4. FA was removed by PBS buffer exchange using 30 KDa cut-off concentrators (Amicon). FA crosslinking was confirmed by separating SARS-CoV-2 FA-S on a 10% SDS-PAGE under reducing conditions.
S protein coupling to liposomes
Liposomes for conjugating S protein were prepared as described previously with modifications. Briefly, liposomes were composed of 60% of L-α-phosphatidylcholine, 4% His tag-conjugating lipid, DGS-NTA-(N2+44) and 36% cholesterol (Avanti Polar Lipids). Lipid components were dissolved in chloroform, mixed and placed for two hours in a desiccator under vacuum at room temperature to obtain a lipid film. The film was hydrated in filtered (0.22 μm) PBS and liposomes were prepared by extrusion using membrane filters with a pore size of 0.1 μm (Whatman Nuclepore Track-Etch membranes). The integrity and size of the liposomes was analyzed by negative staining-EM. For protein coupling, the liposomes were incubated overnight with FA-S or S protein in a 3:1 ratio (w/w). Free FA-S protein was separated from the FA-S-proteoliposomes (S-LVs) by sucrose gradient (5-40%) centrifugation in a SW55 rotor at 40,000 rpm for 2 h. The amount of protein conjugated to the liposomes was determined by Bradford assay and SDS-PAGE densitometry analysis comparing S-LV bands with standard S protein concentrations.

S protein thermostability
Thermal denaturation of SARS-CoV-2 S, native or FA-cross-linked was analyzed by differential scanning fluorimetry coupled to back scattering using a Prometheus NT-48 instrument (Nanotemper Technologies, Munich, DE). Protein samples were first extensively dialyzed against PBS pH 7.4, and the protein concentration was adjusted to 0.3 mg/ml. 10 μL of sample were loaded into the capillary (w/w). Free FA-S protein was separated from the FA-S-proteoliposomes (S-LVs) by sucrose gradient (5-40%) centrifugation in a SW55 rotor at 40,000 rpm for 2 h. The amount of protein conjugated to the liposomes was determined by Bradford assay and SDS-PAGE densitometry analysis comparing S-LV bands with standard S protein concentrations.

Negative stain electron microscopy
Protein samples were visualized by negative-stain electron microscopy (EM) using 3-4 μL aliquots containing 0.1-0.2 mg/ml of protein. Samples were applied for 10 s onto a mica carbon film and transferred to 400-mesh Cu grids that had been glow discharged at 20 mA for 30 s and then negatively stained with 2% (wt/vol) Uranyl Acetate (UAc) for 30 s. Data were collected on a FEI Tecnai T12 LaB6-EM operating at 120 kV accelerating voltage at 23k magnification (pixel size of 2.8 Å) using a Gatan Orius 1000 CCD Camera. Two-dimensional (2D) class averaging was performed with the software Relion 125 using on average 30–40 micrographs per sample. The 5 best obtained classes were calculated from around 6000 particles each.

Cryo-electron microscopy
Data collection
3.5 μL of sample were applied to 1.2/1.3 C-Flat (Protochips Inc) holey carbon grids and plunged frozen in liquid ethane with a Vitrobot Mark IV (Thermo Fisher Scientific) (6 s blot time, blot force 0). The sample was observed with a Glacios electron microscope (Thermo Fischer Scientific) at 200 kV. Images were recorded automatically on a K2 summit direct electron detector (Gatan Inc., USA) in counting mode with SerialEM. 124 Movies were recorded for a total exposure of 4.5 s with 40 frames per movie and a total dose of 40 e−/Å². The magnification was 36,000x (1.15 Å/pixel at the camera level). The defocus of the images was changed between −1.0 and −2.5 μm. Two different datasets have been acquired on the same grid. First, 1040 movies were recorded with stage movement between each hole and then 7518 more movies were recorded with image shifts on a 3x3 hole pattern.

3D reconstruction
The movies were drift-corrected with motioncor2. 124 The remaining image processing was performed with RELION 3.12. 125 and CTF estimation with GCTF. 126 An initial set of particles (box size of 200 pixels, sampling of 2.3 Å/pixel) was obtained by auto-picking with a Gaussian blob. After 2D classification, the best looking 2D class averages were used for a second round of auto picking. Following another 2D classification step, the particles belonging to the best looking 2D class averages were used to create an ab-initio starting 3D model which was then used to calculate a first 3D reconstruction with C3 symmetry. The 2D projections from that 3D model were then used for one last auto picking which resulted in a total of 2,582,857 particles. Following another 2D classification and a 3D classification (C1 symmetry, 5 classes) steps, a 3D map at 4.6 Å resolution was obtained from 240,777 particles. The particles were re-extracted (box size of 400 pixels, sampling of 1.15 Å/pixel). After further 3D refinement (C3 symmetry) and 3D classification (C1 symmetry, no alignment, 3 classes) steps, a final set of 126,719 particles was identified which resulted in a 3D map at 4.6 Å resolution. The final model was validated with MOLPROBITY. 127

Model refinement
The atomic model of the S protein in the closed conformation (PDB 6VXX) 11 was rigid-body fitted inside the cryo-EM density map in CHIMERA. 129 The atomic coordinates were then refined with PHENIX. 130 The refined atomic models were visually checked and adjusted (if necessary) in COOT. 131 The final model was validated with MOLPROBITY. 132 The figures were prepared with CHIMERA and CHIMERAX. 129,132 The data collection and atomic model statistics are summarized in Table S1. The atomic coordinates and the cryo-EM map have been deposited in the Protein Data Bank under the accession codes 7Q1Z and EMD-13776, respectively.
Virus quantification in NHP samples

Upper respiratory (nasopharyngeal and tracheal) and rectal specimens were collected with swabs (Viral Transport Medium, CDC, DSR-052-01). Tracheal swabs were performed by insertion of the swab above the tip of the epiglottis into the upper trachea at approximately 1.5 cm of the epiglottis. All specimens were stored between 2°C and 8°C until analysis by RT-qPCR with a plasmid standard concentration range containing an RdRp gene fragment including the RdRp-IP4 RT-PCR target sequence. SARS-CoV-2 E gene subgenomic mRNA (sgRNA) levels were assessed by RT-qPCR using primers and probes previously described: \(\text{E-S-D614G, E-S-E484K, N501Y, D614G, H655Y and T1027I in P.1 (Gamma, BR).}\)

Chest CT and image analysis

Lung images were acquired using a computed tomography (CT) system (Vereos-Ingenuity, Philips) as previously described, analyzed using INTELLISPACE PORTAL 8 software (Philips Healthcare). All images had the same window level of –300 and window width of 1,600. Lesions were defined as ground glass opacity, crazy-paving pattern, consolidation or pleural thickening as previously described. Lesions and scoring were assessed in each lung lobe blindly and independently by two persons and the final results were established by consensus. Overall CT scores include the lesion type (scored from 0 to 3) and lesion volume (scored from 0 to 4) summed for each lobe as previously described.

ELISA

Serum antibody titers specific for soluble native S glycoprotein, FA-cross-linked S (FA-S) and for RBD were determined using an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well micro titer plates were coated with 1 \(\mu\)g of S, FA-S or RBD proteins at 4°C overnight in PBS and blocked with 5% BSA for 1 h at room temperature after 3 washes with 150 \(\mu\)L PBS Tween-20 0.05%. Serum dilutions were added to each well for 2h at 37°C and plates were washed 5 times with PBS Tween. A horseradish peroxidase (HRP) conjugated goat anti-mouse H+L antibody (Invitrogen) was then added and incubated for 1h before excess Ab was washed out and HRP substrate added. Absorbance was determined at 450 nm. Antibody titers were expressed as ED50 (effective Dilution 50-values) and were determined as the serum dilution at which IgG binding was reduced by 50%. ED50 were calculated from crude data (O.D) after normalization using GraphPad Prism (version 6) "log(inhibitor) vs normalized response" function. ELISA were performed in duplicates.

Protein coupling to luminex beads

Proteins were covalently coupled to Magplex beads (Luminex Corporation) via a two-step carbodiimide reaction using a ratio of 75 \(\mu\)g SARS-CoV-2 S to 12.5 million beads. Magplex beads (Luminex Corporation) were washed with 100 mM mono-basic sodium phosphate pH 6.2 and activated for 30 min on a rotator at RT by addition of Sulfo-N-Hydroxysulfosuccinimide (Thermo Fisher Scientific). The activated beads were washed three times with 50 mM MES pH 5.0 and added to SARS-CoV-2 S protein, which was diluted in 50 mM MES pH 5.0. The coupling reaction was incubated for 3 h on a rotator at RT. The beads were subsequently washed with PBS and blocked with PBS containing 2% BSA, 3% FCS and 0.02% Tween-20 for 30 min on a rotator at RT. Finally, the beads were washed and stored in PBS containing 0.05% Sodium Azide at 4°C and used within 3 months.

Luminex assay

50 \(\mu\)L of a working bead mixture containing 20 beads per \(\mu\)L was incubated overnight at 4°C with 50 \(\mu\)L of diluted nasopharyngeal fluid. Nasopharyngeal fluids were diluted 1:20 for detection of S-specific IgG and IgA. Plates were sealed and incubated on a plate shaker overnight at 4°C. Plates were washed with TBS containing 0.05% Tween-20 (TBST) using a hand-held magnetic separator. Beads were resuspended in 50 \(\mu\)L of Goat-anti-mouse IgG-Biotin or Goat-anti monkey IgA-Biotin (Sigma Aldrich) and incubated on a plate shaker overnight at 4°C. Afterwards, the beads were washed with TBST, resuspended in 50 \(\mu\)L of Streptavidin-PE (ThermoFisher Scientific) and incubated on a plate shaker at RT for 1 h. Finally, the beads were washed with TBST and resuspended in 70 \(\mu\)L Magpix drive fluid (Luminex Corporation). The beads were agitated for a few minutes on a plate shaker at RT and then readout was performed on the MAGPIX (Luminex Corporation). Reproducibility of the results was confirmed by performing replicate runs.

Pseudovirus neutralization assay

Pseudovirus was produced by co-transfecting the pCR3 SARS-CoV-2-S\(\Delta E\) expression plasmid (Wuhan Hu-1; GenBank: MN908947.3) with the pHIV-1NL43 \(\Delta Env\)-NanoLuc reporter virus plasmid in HEK293T cells (ATCC, CRL-11268). The pCR3 SARS-CoV-2-S\(\Delta E\) expression plasmid contained the following mutations compared to the WT for the variants of concern: deletion (\(\Delta\)) of H69, V70 and Y144, N501Y, A570D, D614G, P681H, T716I, S982A and D1118H in B.1.1.7 (Alpha, UK); L18F, D80A, D21S, L242H, R246I, K417N, E484K, N501Y, D614G and A701V in B.1.351 (Beta, SA); L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y and T1027I in P.1 (Gamma, BR).

HEK293T/ACE2 cells kindly provided by Dr. Paul Bieniasz were seeded at a density of 20,000 cells/well in a 96-well plate coated with 50 \(\mu\)g/mL poly-L-lysine 1 day prior to the start of the neutralization assay. Heat-inactivated sera (1:100 dilution) were serial
diluted in 3-fold steps in cell culture medium (DMEM (Gibco), supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL) and GlutaMax (Gibco)), mixed in a 1:1 ratio with pseudovirus and incubated for 1 h at 37°C. These mixtures were then added to the cells in a 1:1 ratio and incubated for 48 h at 37°C, followed by a PBS wash and lysis buffer added. The luciferase activity in cell lysates was measured using the Nano-Glo Luciferase Assay System (Promega) and GloMax system (Turner Biosystems). Relative luminescence units (RLU) were normalized to the positive control wells where cells were infected with pseudovirus in the absence of sera. The neutralization titers (ID₅₀) were determined as the serum dilution at which infectivity was inhibited by 50%, respectively using a non-linear regression curve fit (GraphPad Prism software version 8.3). Notably, this pseudovirus neutralization assay revealed an excellent correlation with authentic virus neutralization on a panel of human convalescent sera.

### Antigen specific T cell assays using non-human primate cells

To analyze the SARS-CoV-2 protein-specific T cell, 15-mer peptides (n = 157 and n = 158) overlapping by 11 amino acids (aa) and covering the SARS-CoV-2 Spike sequence (aa 1 to 1273) were synthesized by JPT Peptide Technologies (Berlin, Germany) and used at a final concentration of 2 μg/mL.

T-cell responses were characterized by measurement of the frequency of PBMC expressing IL-2 (PerCP5.5, MQ1-17H12, BD), IL-17a (Alexa700, N49-653, BD), IFN-γ (V450, B27, BD), TNF-α (BV605, Mab11, BioLegend), IL-13 (BV711, JES10-5A2, BD), CD137 (APC, 4B4, BD) and CD154 (FITC, TRAP1, BD) upon stimulation with the two peptide pools. CD3 (APC-Cy7, SP34-2, BD), CD4 (BV510, L200, BD) and CD8 (PE-Vio770, BW135/80, Miltenyi Biotec) antibodies was used as lineage markers. One million of PBMC were cultured in complete medium (RPMI1640 Glutamax+, Gibco; supplemented with 10% FBS), supplemented with co-stimulatory antibodies (FastImmune CD28/CD49d, Becton Dickinson). The cells were stimulated with S sequence overlapping peptide pools at a final concentration of 2 μg/mL. Brefeldin A was added to each well at a final concentration of 10 μg/mL and the plate was incubated at 37°C, 5% CO2 during 18 h. Next, cells were washed, stained with a viability dye (LIVE/DEAD fixable Blue dead cell stain kit, ThermoFisher), and then fixed and permeabilized with the BD Cytofix/Cytoperm reagent. Permeabilized cell samples were stored at -80°C before the staining procedure. Antibody staining was performed in a single step following permeabilization. After 30 min of incubation at 4°C, in the dark, cells were washed in BD Perm/Wash buffer then acquired on the LSRII cytometer (Beckton Dickinson). Analyses were performed with the FlowJo v.10 software. Data are presented as the sum of each peptide pool and the non-stimulated (NS) condition was multiplied by two.

### Statistical analysis

Statistical significance between groups was performed using Graphpad Prism (v9.2.0). Differences between unmatched groups were compared using an unpaired Mann–Whitney U test (significance p<0.05), and differences between matched groups were compared using Wilcoxon signed-rank test (p<0.1). Statistical analysis of NHP gRNA and sgRNA were carried out using Mann-Whitney unpaired t-test in GraphPad Prism software (v8.3.0).