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The emergence of SARS-CoV-2 variants that partially evade neutralizing antibodies poses a threat to the efficacy of current COVID-19 vaccines[1,2]. The Ad26.COV2.S vaccine expresses a stabilized Spike protein from the WA1/2020 strain and has recently demonstrated protective efficacy against symptomatic COVID-19 in humans in multiple geographic regions, including in South Africa where 95% of sequenced viruses in COVID-19 cases were the B.1.351 variant[3]. Here we show that Ad26.COV2.S elicits humoral and cellular immune responses that cross-react with the B.1.351 variant and protects against B.1.351 challenge in rhesus macaques. Ad26.COV2.S induced lower binding and neutralizing antibodies against B.1.351 as compared with WA1/2020 but elicited CD8 and CD4 T cell responses that were comparable against WA1/2020, B.1.351, B.1.1.7, P.1, and CAL.20C variants. B.1.351 infection of sham control rhesus macaques resulted in higher levels of virus replication in bronchoalveolar lavage and nasal swabs than did WA1/2020 infection. Ad26.COV2.S provided robust protection against both WA1/2020 and B.1.351, although we observed higher levels of virus in vaccinated animals following B.1.351 challenge. These data demonstrate that Ad26.COV2.S provided robust protection against B.1.351 challenge in rhesus macaques. Our findings have important implications for vaccine control of SARS-CoV-2 variants of concern.

SARS-CoV-2 variants of concern have shown increased transmissibility and pathogenicity in humans[4,5], and certain variants have also demonstrated partial evasion of antibody responses, including natural and vaccine-elicited neutralizing antibodies[1,2,6,7]. Ad26.COV2.S is a replication-incompetent human adenovirus type 26 (Ad26) vector[8] expressing a prefusion stabilized SARS-CoV-2 Spike protein[9,10] from the Wuhan 2019 strain. We previously reported that Ad26.COV2.S demonstrated protective efficacy against SARS-CoV-2 WA1/2020 challenges in hamsters and nonhuman primates[11–13] and also showed safety and immunogenicity in humans[14,15]. Recently, a phase 3 efficacy trial showed that Ad26.COV2.S provided 86%, 88%, and 82% protection against severe COVID-19 disease by day 28 in the United States, Brazil, and South Africa, respectively[3].

We developed a B.1.351 challenge stock by expansion of a seed stock (BEI Resources; NR-54974) in Calu-3 cells (ATCC HTB-55). We immunized 24 rhesus macaques in 4 experimental groups (N=6/group) as follows: Groups 1 and 3 received a sham vaccine, and Groups 2 and 4 received a single immunization with $5x10^{10}$ viral particles (vp) Ad26.COV2.S; following vaccination, Groups 1 and 2 were challenged with the original SARS-CoV 2 strain WA1/2020, and Groups 3 and 4 were challenged with the SARS-CoV-2 variant B.1.351.

Ad26.COV2.S Immunogenicity and Cross-Reactivity Against Variants

Following vaccination, we assessed antibody responses against the SARS-CoV-2 WA1/2020 strain as well as against B.1.351. Using a luciferase-based pseudovirus neutralizing antibody (NAb) assay[12,16–18], the median NAb titers in animals that received Ad26.COV2.S vaccine were <20 at week 0 and were 693, 561, and 155 against the WA1/2020, D614G, and B.1.351 strains, respectively, in Ad26.COV2.S vaccinated animals at week 6 (Fig. 1a). These data show a median 4.5-fold reduction of NAb titers against B.1.351 as compared with WA1/2020 (P=0.0002, Wilcoxon rank-sum test). Live virus neutralizing antibody assays[19]

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showed a greater reduction of NAb titers against B.1.351 (Extended Data Fig. 1).

Median receptor binding domain (RBD)-specific ELISA titers in animals that received Ad26.COV2.S vaccine were <25 at week 0 and were 4050, 3186, and 805 against the WA1/2020, B.1.1.7, and B.1.351 strains, respectively, in Ad26.COV2.S vaccinated animals at week 6 (Fig. 1b). These data show a median 5.0-fold reduction of RBD-specific ELISA titers against B.1.351 as compared with WA1/2020 (P<0.0001, Wilcoxon rank-sum test). An electrochemiluminescence assay (ECLA)[20] was also used to evaluate Spike (S)- and RBD-specific binding antibody responses to WA1/2020, B.1.1.7, P.1., and B.1.351 (Extended Data Fig. 2). Similar to the ELISA titers, median RBD-specific ECLA responses were reduced against P.1 and B.1.351 as compared with WA1/2020 at week 6, whereas less of an effect was observed with S-specific ECLA responses. Antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent complement deposition (ADCD) responses[21] showed more comparable responses against WA1/2020 and B.1.351 (Fig. 1c).

S-specific cellular immune responses were assessed by pooled peptide IFN-γ ELISPOT assays in peripheral blood mononuclear cells (PBMC) at week 4. ELISPOT responses were comparable to the WA1/2020, B.1.351, B.1.1.7, P.1, and CAL.20C strains, with no evidence of decreased responses against the variants (Fig. 2a). S-specific CD8+ and CD4+ T cell responses were also evaluated by multiparameter intracellular cytokine staining assays at week 6 (Supplementary Fig. 1). IFN-γ CD8+ and CD4+ T cell responses were comparable to WA1/2020, B.1.351, B.1.1.7, P.1, and CAL.20C (Fig. 2b). Similarly, IFN-γ central memory CD28+CD95+ CD4+ and CD8+ T cell responses were comparable across these variants (Fig. 2c). These data show that S-specific cellular immune responses were comparable to these SARS-CoV-2 variants.

Protective Efficacy Against Homologous and Heterologous SARS-CoV-2 Challenge

We challenged all animals at week 6 with $5x10^5$ TCID50 SARS-CoV-2 WA1/2020[12,16,17,22] or B.1.351 by the intranasal (IN) and intratracheal (IT) routes. We assessed viral loads in bronchoalveolar lavage (BAL) and nasal swabs (NS) by RT-PCR specific for subgenomic mRNA (sgRNA), which is believed to measure replicating virus[16,23,24]. All sham controls were infected and showed higher median peak sgRNA of 6.16 (range 4.93-6.80) log_{10} sgRNA copies/ml in BAL for B.1.351 as compared with 4.80 (range 4.70-5.52) log_{10} sgRNA copies/ml for WA1/2020 (Fig. 3a). In contrast, vaccinated animals demonstrated a median peak of 3.62 (range 3.37-4.43) log_{10} sgRNA copies/ml in BAL for B.1.351 as compared with <1.69 (range <1.69-3.23) log_{10} sgRNA copies/ml in BAL for WA1/2020 (Fig. 3a). Sham controls also showed a trend towards a higher median peak sgRNA of 5.90 (range 4.73-6.47) log_{10} sgRNA copies/swab in NS for B.1.351 as compared with 5.48 (range 4.44-6.00) log_{10} sgRNA copies/swab for WA1.2020 (Fig. 3b). Vaccinated animals demonstrated a median peak of 3.57 (range 2.41-4.21) log_{10} sgRNA copies/swab in NS for B.1.351 as compared with 2.64 (range <1.69-3.89) log_{10} sgRNA copies/swab in NS for WA1/2020 (Fig. 3b).

B.1.351 led to higher peak viral loads, faster kinetics of viral replication, and longer duration of viral replication as compared with WA1/2020 in sham animals, suggesting that B.1.351 is a more stringent challenge in the macaque model. Ad26.COV2.S provided robust protection against peak viral replication for both strains, including a 3.13 and 2.54 log reduction of peak sgRNA copies/ml in BAL for WA1/2020 and B.1.351, respectively, and a 2.84 and 2.33 log reduction of peak sgRNA copies/swab in NS for WA1/2020 and B.1.351, respectively (P=0.0022 for both BAL and NS for both WA1/2020 and B.1.351, Wilcoxon rank-sum tests; Fig. 4a). By day 4 following challenge, viral loads were undetectable in Ad26.COV2.S vaccinated animals following both WA1/2020 and B.1.351 challenge, whereas viral loads were still positive in most sham controls for WA1/2020 and in all sham controls for B.1.351 (Fig. 4b). Ad26.COV2.S also provided similar robust protection against day 2 infectious virus titers assessed by TCID50 assays (Extended Data Fig. 3).

Immune Responses Following Challenge and Immune Correlates of Protection

On day 10 following challenge (study week 8), sham controls developed both humoral and cellular immune responses, as expected (Extended Data Figs. 4-6). In sham controls, WA1/2020 challenge led to higher NAb titers to WA1/2020 than to B.1.351, whereas B.1.351 challenge led to higher NAb titers to B.1.351 than to WA1/2020 (Extended Data Fig. 4a), and cellular responses were comparable across all strains regardless of the challenge virus (Extended Data Fig. 6), consistent with the vaccine immunogenicity data. Ad26.COV2.S vaccinated animals developed increased humoral and cellular immune responses following challenge. The low ELISA titers in sham controls likely reflect the early day 10 timepoint following challenge (Extended Data Fig. 4b),

Peak log_{10} sgRNA in BAL (Extended Data Fig. 7) and in NS (Extended Data Fig. 8) following challenge inversely correlated with log_{10} ELISA, NAb, and ELISPOT responses at week 6, suggesting that both antibody and T cell responses correlate with protection. Correlations were slightly stronger for immune responses against the homologous challenge virus as compared with the heterologous challenge virus.

Histopathology

Ad26.COV2.S vaccinated macaques demonstrated reduced lung histopathology compared with sham animals at necropsy on day 10 following WA1/2020 and B.1.351 challenge (Fig. 5a, b), although viral replication had largely resolved by day 10. Sham animals infected with WA1/2020 and B.1.351 had histopathologic lesions consistent with previous reports[16], including focal to locally extensive interstitial pneumonia with neutrophilic and mononuclear interstitial infiltrates, alveolar syncytia, and increased numbers of alveolar macrophages. Perivascular inflammation and type II pneumocyte hyperplasia were prominent features in both groups of sham animals, as were multifocal regions of fibrosis (Fig. 5c, d; Extended Data Fig. 9). Ad26.COV2.S vaccinated animals had only rare lesions, predominantly small and focal regions of interstitial inflammation and rare syncytia in isolated lung lobes (Fig. 5e, f; Extended Data Fig. 10). No evidence of eosinophilic infiltrates or enhanced respiratory disease was observed in vaccinated animals.

Discussion

We previously reported that Ad26.COV2.S provided robust protection against challenge with SARS-CoV-2 WA1/2020 in both rhesus macaques and hamsters[11–13]. In this study, we show that Ad26.COV2.S induced cross-reactive antibody and T cell responses against SARS-CoV-2 variants of concern, including the B.1.351 variant, which has multiple mutations including E484K that lead to partial evasion of natural and vaccine-elicited neutralizing antibodies[1,2,6,7]. Binding and neutralizing antibody titers were suppressed 4-5-fold against B.1.351 as compared with WA1/2020, but Fc functional antibody responses were impacted less, and T cell responses were not impacted at all by the SARS-CoV-2 variants, presumably due to sequence similarities of the Spike proteins. Ad26.COV2.S provided robust protection against both high-dose WA1/2020 and B.1.351 challenge. These data have important implications for the potential utility of current vaccines and inform boosting strategies against SARS-CoV-2 variants of concern.

Our data are consistent with findings in humans in a recent phase 3 clinical trial of Ad26.COV2.S that was conducted in the United States, Latin America, and South Africa[3]. Robust protection was observed in all geographic regions, with similar levels of protection against severe COVID-19 disease regardless of variants, including in the U.S., in Brazil where 69% of cases with sequence data were the P.2 variant, and in

South Africa where 95% of cases with sequence data were the B.1.351 variant. In the current study in macaques, B.1.351 infection led to higher magnitude and more prolonged viral replication in the upper and lower respiratory tracts than did WA1/2020. Ad26.COV2.S provided robust protection against both viruses, although levels of virus in BAL and NS were higher following B.1.351 challenge than following WA1/2020 challenge.

To the best of our knowledge, this is the first report of a SARS-CoV-2 vaccine evaluated for efficacy against a SARS-CoV-2 variant of concern in macaques. Various SARS-CoV-2 vaccines have been reported to protect against homologous WA1/2020 challenges but have not yet been reported against B.1.351 challenges. Our study does not define mechanistic correlates of protection against SARS-CoV-2 variants, but we reported previously that IgG was sufficient for protection against SARS-CoV-2 challenge in macaques, but that CD8 T cell responses also contributed to protection if antibody titers were subprotective[22].

In conclusion, Ad26.COV2.S induced cross-reactive humoral and cellular immune responses and provided robust protection against the heterologous SARS-CoV-2 variant B.1.351 in rhesus macaques. Future studies will determine if Ad26.COV2.S as well as other vaccines protect against other SARS-CoV-2 variants of concern.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-021-03732-8.

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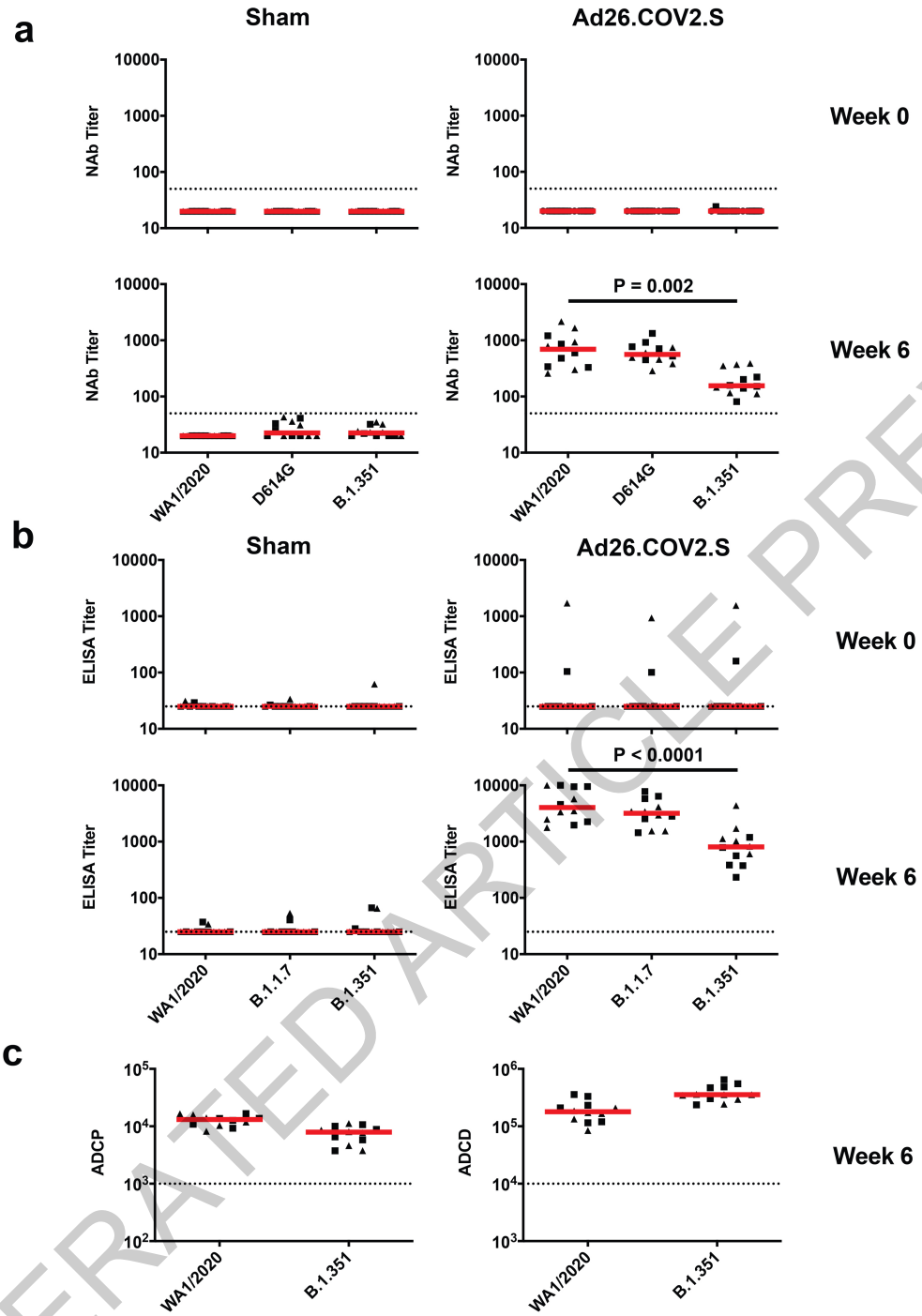

Fig. 1 | Antibody responses in vaccinated rhesus macaques. (**a**) Pseudovirus neutralizing antibody (NAb) assays against the SARS-CoV-2 WA1/2020, D614G, and B.1.351 variants were assessed at weeks 0 and 6 in macaques that received a single immunization of sham vaccine or $5x10^{10}$ vp Ad26.COV2.S. (**b**) RBD-specific binding antibody responses against WA1/2020, B.1.1.7, and B.1.351 were assessed by ELISA. (**c**) Antibody-dependent cellular phagocytosis (ADCP; phagocytic score) and antibody-dependent complement deposition (ADCD; mean fluorescence intensity) were evaluated against WA1/2020 and B.1.351. Animals that eventually were challenged with WA1/2020 (triangles) or B.1.351 (squares) are depicted. Horizontal red bars reflect median responses. P-values reflect two-sided Wilcoxon rank-sum tests. Dotted lines reflect assay limits of quantitation. n=24 independent samples (12 sham, 12 Ad26.COV2.S).

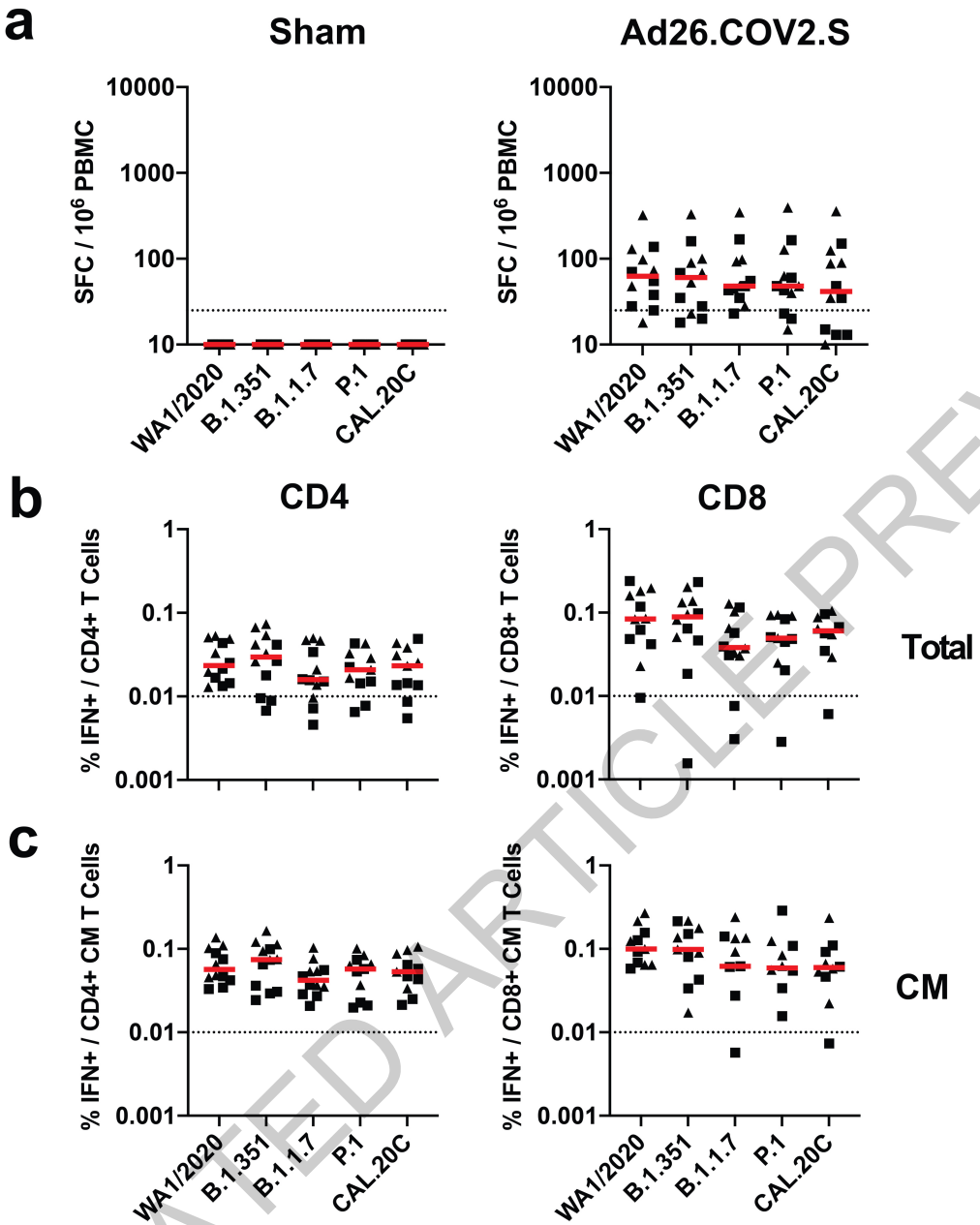

Fig. 2 | T cell responses in vaccinated rhesus macaques. Cellular immune responses to pooled S peptides were assessed by (**a**) IFN-γ ELISPOT assays at week 4 and (**b, c**) IFN-γ intracellular cytokine staining (ICS) assays at week 6 to WA1/2020, B.1.351, B.1.1.7, P.1, and CAL.20C variants. ICS assays show IFN-γ responses in (**b**) CD4+ and CD8+ T cells and (**c**) CD28+CD95+ CD4+ and CD8+ central memory (CM) T cells. Animals that eventually were challenged with WA1/2020 (triangles) or B.1.351 (squares) are depicted. Horizontal red bars reflect median responses. Dotted lines reflect assay limits of quantitation. n=24 independent samples (12 sham, 12 Ad26.COV2.S).

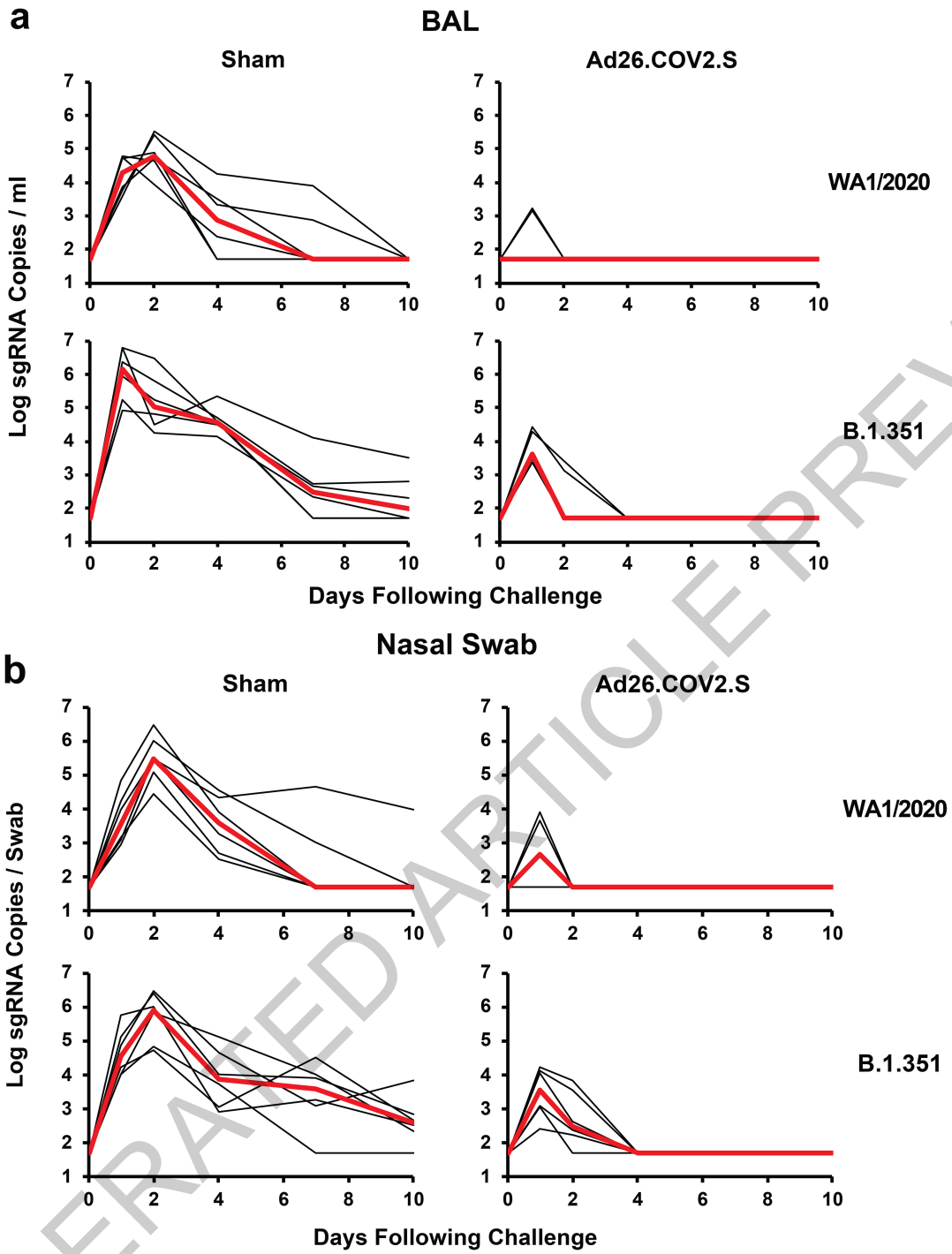

Fig. 3 | Protective efficacy following SARS-CoV-2 challenge. Rhesus macaques were challenged by the intranasal and intratracheal routes with $5x10^5$ TCID50 SARS-CoV-2 WA1/2020 or B.1.351. (**a**) Log_{10} sgRNA copies/ml (limit of quantification 50 copies/ml) are shown in bronchoalveolar lavage (BAL) following challenge. (**b**) Log_{10} sgRNA copies/swab (limit of quantification 50 copies/swab) are shown in nasal swabs (NS) following challenge. Red lines reflect median values. n=24 independent samples (12 sham, 12 Ad26.COV2.S).

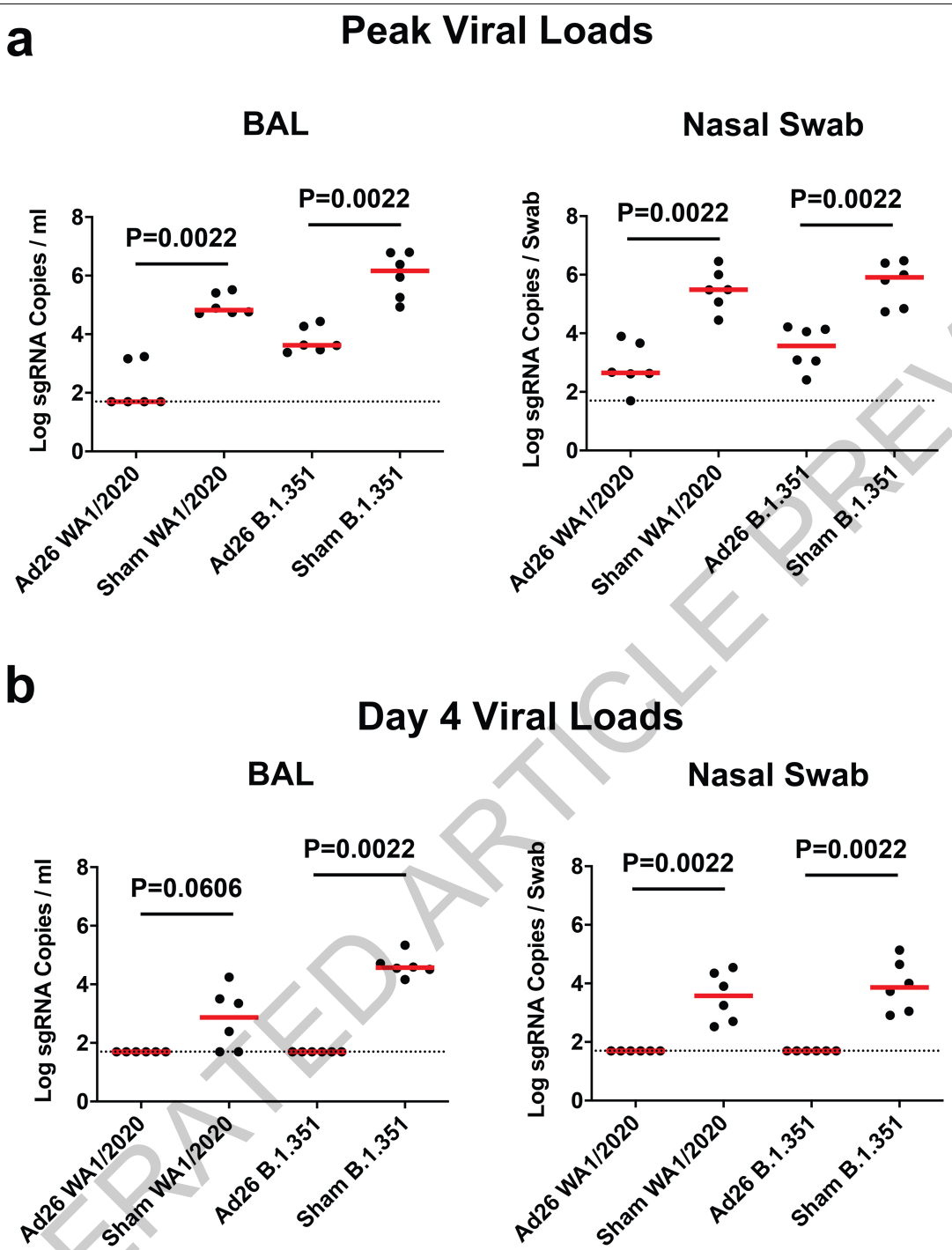

Fig. 4 | Summary of protective efficacy following SARS-CoV-2 challenge. (**a**) Peak and (**b**) day 4 viral loads in bronchoalveolar lavage (BAL) and nasal swabs (NS) following challenge. Horizontal red bars reflect median values. P-values reflect two-sided Wilcoxon rank-sum tests. Dotted lines reflect assay limits of quantitation. n=24 independent samples (12 sham, 12 Ad26.COV2.S).

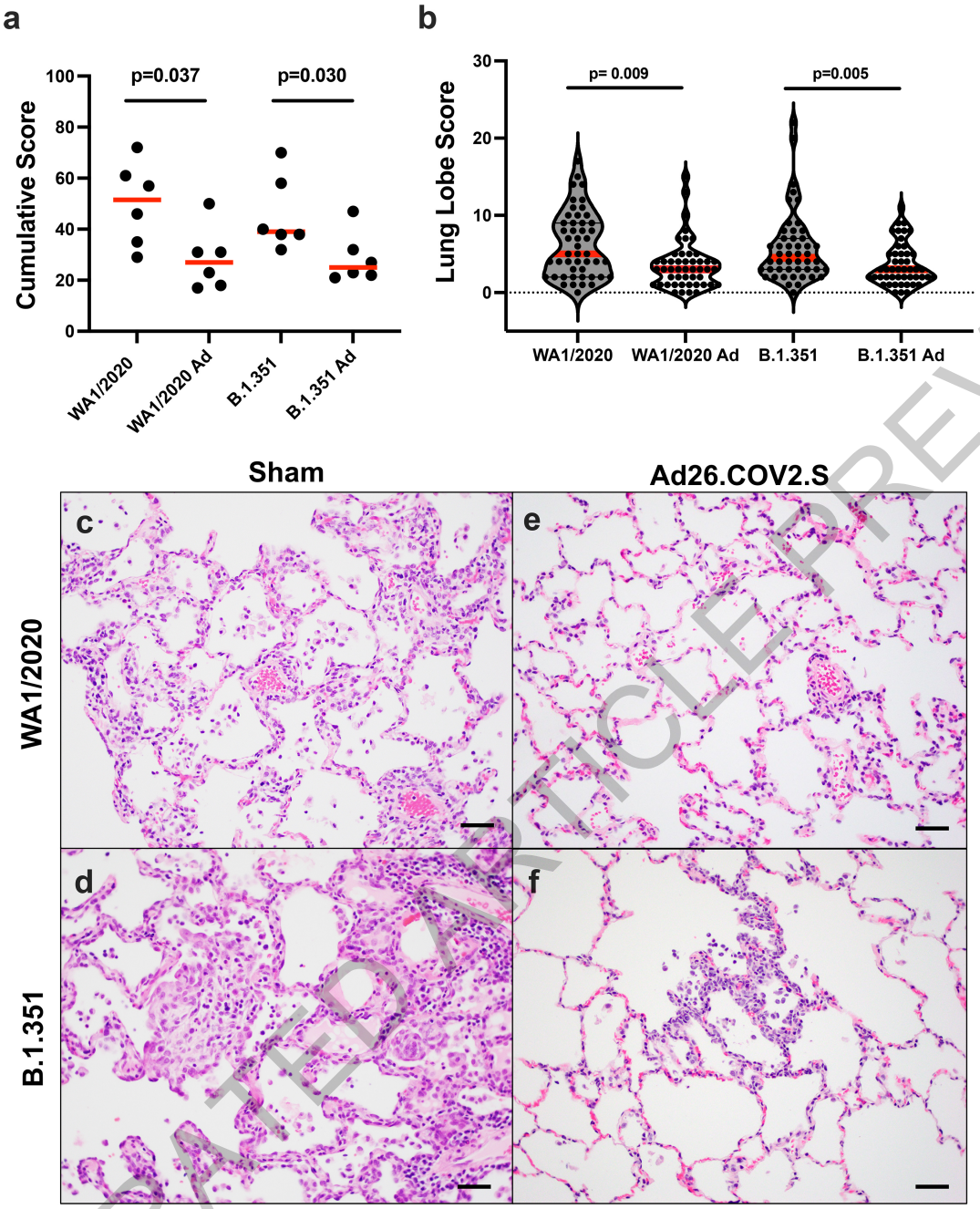

Fig. 5 | Histopathology following SARS-CoV-2 challenge. (**a**) Cumulative histopathologic scoring of lung lesions from 8 representative lung lobes from vaccinated and sham animals on day 10 following SARS-CoV-2 challenge with either the WA1/2020 or B.1.351 SARS CoV-2 variants. (**b**) Eight representative samples from cranial, middle, and caudal lung lobes from the left and right lungs were evaluated from each animal and were scored independently for each of the following lesions: interstitial inflammation and septal thickening, interstitial infiltrate (eosinophils), interstitial infiltrate (neutrophils), hyaline membranes, interstitial fibrosis, alveolar infiltrate (macrophages), bronchoalveolar infiltrate (neutrophils), epithelial syncytia, type II pneumocyte hyperplasia, bronchi infiltrate (macrophages), bronchi infiltrate (neutrophils), bronchi (BALT hyperplasia), bronchiolar/peribronchiolar infiltrate (mononuclear cells), perivascular infiltrate (mononuclear cells), and endothelialitis. Each feature assessed was assigned a score of 0=no significant findings; 1=minimal; 2= mild; 3=moderate; 4=moderate-severe; 5= marked/severe. Scores were added for all lesions across all lung lobes for each animal for a maximum possible score of 600 for each monkey. Horizontal red lines reflect median values. P-values reflect two-sided Wilcoxon rank-sum tests. Representative lung histopathology from at least 8 evaluated tissues from sham (**c, d**) and Ad26.COV2.S vaccinated (**e, f**) animals challenged with either WA1/2020 (**c, e**) or B.1.351 (**d, f**) on day 10 following SARS-CoV-2 challenge, showing (**c**) increased alveolar macrophages and thickened alveolar septa with inflammatory infiltrates and fibrosis, (**d**) increased alveolar macrophages and epithelial syncytia within alveolar spaces, thickened and fibrotic alveolar septa with inflammatory infiltrates, focal alveolar, and perivascular inflammatory infiltrates, (**e**) focal perivascular inflammation, and (**f**) focal expansion of alveolar septa with inflammatory infiltrates. Lungs evaluated were inflated/suffused with 10% formalin. (**c-f**) Hematoxylin and eosin (H&E). Scale bars = 20 microns. n=24 independent samples (12 sham, 12 Ad26.COV2.S) (**a, b**); n=4 representative samples (2 sham, 2 Ad26.COV2.S) (**c-f**).

Methods

Animals and study design

24 outbred Indian-origin adult male and female rhesus macaques (*Macaca mulatta*) ages 3-11 years old were randomly allocated to groups. All animals were housed at Bioqual, Inc. (Rockville, MD). Animals received a single immunization of $5x10^{10}$ viral particles (vp) Ad26.COV2.S (N=12) or sham (N=12) by the intramuscular route without adjuvant at week 0. At week 6, all animals were challenged with $5x10^5$ TCID50 SARS-CoV-2 from strains USA-WA1/2020 (BEI Resources; NR-5228), which was grown in VeroE6 cells and deep sequenced as described previously[16], or B.1.351 (BEI Resources; NR-54974). The B.1.351 stock was grown in Calu-3 cells and was deep sequenced, which confirmed the expected sequence identity with no mutations in the Spike protein greater than >2.5% frequency and no mutations elsewhere in the virus at >13% frequency. Virus was administered as 1 ml by the intranasal (IN) route (0.5 ml in each nare) and 1 ml by the intratracheal (IT) route. All immunologic, virologic, and histopathologic studies were performed blinded. Animal studies were conducted in compliance with all relevant local, state, and federal regulations and were approved by the Bioqual Institutional Animal Care and Use Committee (IACUC).

Pseudovirus-based virus neutralization assay

The SARS-CoV-2 pseudoviruses expressing a luciferase reporter gene were generated essentially as described previously[12,16–18]. Briefly, the packaging plasmid psPAX2 (AIDS Resource and Reagent Program), luciferase reporter plasmid pLenti-CMV Puro-Luc (Addgene), and spike protein expressing pcDNA3.1-SARS CoV-2 SΔCT of variants were co-transfected into HEK293T cells by lipofectamine 2000 (ThermoFisher). Pseudoviruses of SARS-CoV-2 variants were generated by using WA1/2020 strain (Wuhan/WIV04/2019, GISAID accession ID: EPI_ISL_402124), D614G mutation, B.1.1.7 variant (GISAID accession ID: EPI_ISL_601443), or B.1.351 variant (GISAID accession ID: EPI_ISL_712096). The supernatants containing the pseudotype viruses were collected 48 h post-transfection, which were purified by centrifugation and filtration with 0.45 μm filter. To determine the neutralization activity of the plasma or serum samples from participants, HEK293T-hACE2 cells were seeded in 96-well tissue culture plates at a density of 1.75×10^4 cells/well overnight. Three-fold serial dilutions of heat inactivated serum or plasma samples were prepared and mixed with 50 μL of pseudovirus. The mixture was incubated at 37°C for 1 h before adding to HEK293T-hACE2 cells. 48 h after infection, cells were lysed in Steady-Glo Luciferase Assay (Promega) according to the manufacturer's instructions. SARS-CoV-2 neutralization titers were defined as the sample dilution at which a 50% reduction in relative light unit (RLU) was observed relative to the average of the virus control wells.

Live virus neutralization assay

Full-length SARS-CoV-2 WA1/2020, B.1.351, and B.1.1.7, viruses were designed to express nanoluciferase (nLuc) and were recovered via reverse genetics[19]. One day prior to the assay, Vero E6 USAMRID cells were plated at 20,000 cells per well in clear bottom black walled plates. Cells were inspected to ensure confluency on the day of assay. Serum samples were tested at a starting dilution of 1:20 and were serially diluted 3-fold up to nine dilution spots. Serially diluted serum samples were mixed in equal volume with diluted virus. Antibody-virus and virus only mixtures were then incubated at 37 °C with 5% CO2 for one hour. Following incubation, serially diluted sera and virus only controls were added in duplicate to the cells at 75 PFU at 37 °C with 5% CO2. Twenty four hours later, the cells were lysed, and luciferase activity was measured via Nano-Glo Luciferase Assay System (Promega) according to the manufacturer specifications. Luminescence was measured by a Spectramax M3 plate reader (Molecular Devices, San Jose, CA). Virus neutralization titers were defined as the sample dilution at which a 50% reduction in RLU was observed relative to the average of the virus control wells.

ELISA

WA1/2020, B.1.1.7, and B.1.351 RBD-specific binding antibodies were assessed by ELISA essentially as described previously[12,16,17]. Briefly, 96-well plates were coated with 0.5μg/ml RBD protein in 1X DPBS and incubated at 4 °C overnight. After incubation, plates were washed once with wash buffer (0.05% Tween 20 in 1 X DPBS) and blocked with 350 μL Casein block/well for 2-3 h at room temperature. After incubation, block solution was discarded and plates were blotted dry. Serial dilutions of heat-inactivated serum diluted in casein block were added to wells and plates were incubated for 1 h at room temperature, prior to three further washes and a 1 h incubation with a 1μg/ml dilution of anti-macaque IgG HRP (Nonhuman Primate Reagent Resource) at room temperature in the dark. Plates were then washed three times, and 100 μL of SeraCare KPL TMB SureBlue Start solution was added to each well; plate development was halted by the addition of 100 μL SeraCare KPL TMB Stop solution per well. The absorbance at 450nm was recorded using a VersaMax microplate reader. For each sample, ELISA endpoint titer was calculated in Graphpad Prism software, using a four-parameter logistic curve fit to calculate the reciprocal serum dilution that yields an absorbance value of 0.2 at 450nm. Log10 endpoint titers are reported.

Electrochemiluminescence assay (ECLA)

ECLA plates (MesoScale Discovery SARS-CoV-2 IgG Cat No: N05CA-1; Panel 7) were designed and produced with up to 9 antigen spots in each well, and assays were performed essentially as described previously[20]. The antigens included were WA1/2020, B.1.1.7, P.1, and B.1.351 S and RBD. The plates were blocked with 50 uL of Blocker A (1% BSA in MilliQ water) solution for at least 30 m at room temperature shaking at 700 rpm with a digital microplate shaker. During blocking the serum was diluted 1:5,000 in Diluent 100. The plates were then washed 3 times with 150 μL of the MSD kit Wash Buffer, blotted dry, and 50 μL of the diluted samples were added in duplicate to the plates and set to shake at 700 rpm at room temperature for at least 2 h. The plates were again washed 3 times and 50 μL of SULFO-Tagged anti-Human IgG detection antibody (MesoScale Discovery) diluted to 1x in Diluent 100 was added to each well and incubated shaking at 700 rpm at room temperature for at least 1 h. Plates were then washed 3 times and 150 μL of MSD GOLD Read Buffer B was added to each well and the plates were read immediately after on a MESO QuickPlex SQ 120 machine. MSD titers for each sample was reported as Relative Light Units (RLU) which were calculated as Sample RLU minus Blank RLU for each spot for each sample. The limit of detection was defined as 1000 RLU for each assay.

Fc functional antibody assays

Fc functional profiling included the assessment of antibody dependent monocyte phagocytosis (ADCP) and antibody dependent complement deposition (ADCD)[21]. Briefly, fluorescent beads (LifeTechnologies) were coupled via carboxy-coupling, and plasma were added, allowing immune complex formation, excess antibodies were washed away, followed by the addition of THP1 monocytes, primary neutrophils, or guinea pig complement, individually, respectively. The level of phagocytosis and complement deposition was assessed by flow cytometry.

IFN-γ enzyme-linked immunospot (ELISPOT) assay

Pooled peptide ELISPOT assays were performed essentially as described previously[12,16,17]. Peptide pools consisted of 15 amino acid peptides overlapping by 11 amino acids spanning the SARS-CoV-2 Spike protein from the WA1/2020 strain or variant strains. ELISPOT plates were coated with mouse anti-human IFN-γ monoclonal antibody from BD Pharmigen at 5 μg/well and incubated overnight at 4 °C. Plates were washed with DPBS wash buffer (DPBS with 0.25% Tween20), and blocked with R10 media (RPMI with 10% heat inactivated FBS with 1% of 100x

penicillin-streptomycin) for 1-4 h at 37 °C. SARS-CoV-2 peptides (21st Century Biochemicals; the variants peptides contain the WT backbone) were prepared & plated at a concentration of 1 µg/well, and 200,000 cells/well were added to the plate. The peptides and cells were incubated for 18-24 h at 37 °C. All steps following this incubation were performed at room temperature. The plates were washed with ELISPOT wash buffer (11% 10x DPBS and 0.3% Tween20 in 1L MilliQ water) and incubated for 2 h with Rabbit polyclonal anti-human IFN-γ Biotin from U-Cytech (1 µg/mL). The plates were washed a second time and incubated for 2 h with Streptavidin-alkaline phosphatase from Southern Biotech (2 µg/mL). The final wash was followed by the addition of Nitor-blue Tetrazolium Chloride/5-bromo-4-chloro 3'indolyphosphate p-toludine salt (NBT/BCIP chromagen) substrate solution for 7 min. The chromagen was discarded and the plates were washed with water and dried in a dim place for 24 h. Plates were scanned and counted on a Cellular Technologies Limited Immunospot Analyzer.

Intracellular cytokine staining (ICS) assay

Multiparameter pooled peptide ICS assays were performed essentially as described previously[12,16,17]. Peptide pools consisted of 15 amino acid peptides overlapping by 11 amino acids spanning the SARS-CoV-2 Spike protein from the WA1/2020 strain or variant strains. 10^6 PBMCs/well were re-suspended in 100 µL of R10 media supplemented with CD49d monoclonal antibody (1 µg/mL). Each sample was assessed with mock (100 µL of R10 plus 0.5% DMSO; background control), peptide pools (2 µg/mL), or 10 pg/mL phorbol myristate acetate (PMA) and 1 µg/mL ionomycin (Sigma-Aldrich) (100µL; positive control) and incubated at 37 °C for 1 h. After incubation, 0.25 µL of GolgiStop and 0.25 µL of GolgiPlug in 50 µL of R10 was added to each well and incubated at 37 °C for 8 h and then held at 4 °C overnight. The next day, the cells were washed twice with DPBS, stained with Near IR live/dead dye for 10 mins and then stained with predetermined titers of mAbs against CD279 (clone EH12.1, BB700), CD38 (clone OKT10, PE), CD28 (clone 28.2, PE CY5), CD4 (clone L200, BV510), CD95 (clone DX2, BUV737), CD8 (clone SK1, BUV805), for 30 min. Cells were then washed twice with 2% FBS/DPBS buffer and incubated for 15 min with 200µL of BD CytoFix/CytoPerm Fixation/Permeabilization solution. Cells were washed twice with 1X Perm Wash buffer (BD Perm/WashTM Buffer 10X in the CytoFix/CytoPerm Fixation/ Permeabilization kit diluted with MilliQ water and passed through 0.22µm filter) and stained with intracellularly with mAbs against Ki67 (clone B56, FITC), CD69 (clone TP1.55.3, ECD), IL10 (clone JES3-9D7, PE CY7), IL13 (clone JES10-5A2, BV421), TNF-α (clone Mab11, BV650), IL4 (clone MP4-25D2, BV711), IFN-γ (clone B27; BUV395), CD45 (clone D058-1283, BUV615), IL2 (clone MQ1-17H12, APC), CD3 (clone SP34.2, Alexa 700), for 30 min. Cells were washed twice with 1X Perm Wash buffer and fixed with 250µL of freshly prepared 1.5% formaldehyde. Fixed cells were transferred to 96-well round bottom plate and analyzed by BD FACSymphony system. Central memory T cells were defined as CD28+CD95+ T cells. Data were analyzed with FlowJo v9.9.

Subgenomic RNA assay

SARS-CoV-2 E gene subgenomic RNA (sgRNA) was assessed by RT-PCR using primers and probes as previously described[23,24]. A standard was generated by first synthesizing a gene fragment of the subgenomic E gene[23]. The gene fragment was subsequently cloned into a pcDNA3.1+ expression plasmid using restriction site cloning (Integrated DNA Technologies). The insert was in vitro transcribed to RNA using the AmpliCap-Max T7 High Yield Message Maker Kit (CellScript). Log dilutions of the standard were prepared for RT-PCR assays ranging from $1x10^{10}$ copies to $1x10^{-1}$ copies. Viral loads were quantified from bronchoalveolar lavage (BAL) fluid and nasal swabs (NS). RNA extraction was performed on a QIAcube HT using the IndiSpin QIAcube HT Pathogen Kit according to manufacturer's specifications (Qiagen). The standard dilutions and extracted RNA samples were reverse transcribed using SuperScript VILO Master Mix (Invitrogen) following the cycling conditions described by the manufacturer, 25 °C for 10 Minutes, 42 °C for 1 Hour then 85 °C for 5 Minutes. A Taqman custom gene expression assay (Thermo Fisher Scientific) was designed using the sequences targeting the E gene sgRNA[23]. The sequences for the custom assay were as follows, forward primer, sgLeadCoV2.Fwd: CGATCTCTTGTAGATC TGTTCTC, E_Sarbeco_R: ATATTGCAGCAGTACGCACACA, E_Sarbeco_P1 (probe): VIC-ACACTAGCCATCCTTACTGCGCTTCG-MGB. These primers/probes were equally reactive for both variants. Reactions were carried out in duplicate for samples and standards on the QuantStudio 6 and 7 Flex Real-Time PCR Systems (Applied Biosystems) with the thermal cycling conditions, initial denaturation at 95 °C for 20 seconds, then 45 cycles of 95 °C for 1 second and 60 °C for 20 seconds. Standard curves were used to calculate subgenomic RNA copies per ml or per swab; the quantitative assay sensitivity was 50 copies per ml or per swab.

TCID50 assay

Vero TMPRSS2 cells (obtained from Adrian Creanga, Vaccine Research Center-NIAID) were plated at 25,000 cells/well in DMEM with 10% FBS and gentamicin, and the cultures are incubated at 37 °C, 5.0% CO2. Media was aspirated and replaced with 180 µL of DMEM with 2% FBS and gentamicin. Serial dilution of samples as well as positive (virus stock of known infectious titer) and negative (medium only) controls were included in each assay. The plates are incubated at 37 °C, 5.0% CO2 for 4 days. Cell monolayers were visually inspected for CPE. The TCID50 was calculated using the Read-Muench formula.

Histopathology

Lungs on day 10 following SARS-CoV-2 challenge were evaluated by histopathology. At time of fixation, lungs were suffused with 10% formalin to expand the alveoli. All tissues were fixed in 10% formalin and blocks sectioned at 5 µm. Slides were incubated for 30-60 min at 65 °C then deparaffinized in xylene and rehydrated through a series of graded ethanol to distilled water. Sections were stained with hematoxylin and eosin (H&E). Blinded evaluation and scoring was performed by a board-certified veterinary pathologist (A.J.M.).

Statistical analyses

Comparisons of virologic, immunologic, and histopathologic data was performed using GraphPad Prism 8.4.2 (GraphPad Software). Comparison of data between groups was performed using two-sided Wilcoxon rank-sum tests. Correlation analyses were performed using two-sided Spearman rank-correlation tests. P-values of less than 0.05 were considered significant.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

All data are available in the manuscript and the supplementary material. Source data are provided with this paper.

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Author contributions D.H.B., F.W., and R.Z. designed the study. J.Y., L.H.T., N.B.M., K.M., J.L., C.J.D., A.C., T.A., E.A.B., A.C., S.G., V.M.G., D.L.H., F.N., J.N., S.P., O.S., D.S., and H.W. performed the immunologic and virologic assays. D.R.M. and R.S.B. performed the live virus neutralization assays. C.A. and G.A. performed the Fc functional antibody assays. L.P., D.V., Z.F., R.B., A.C.,

D.B.W., E.T., H.A., and M.G.L. led the clinical care of the animals. F.W., R.Z., and H.S. provided the vaccine. T.H., K.B., and A.J.M. led the histopathology. D.H.B. wrote the paper with all co-authors.

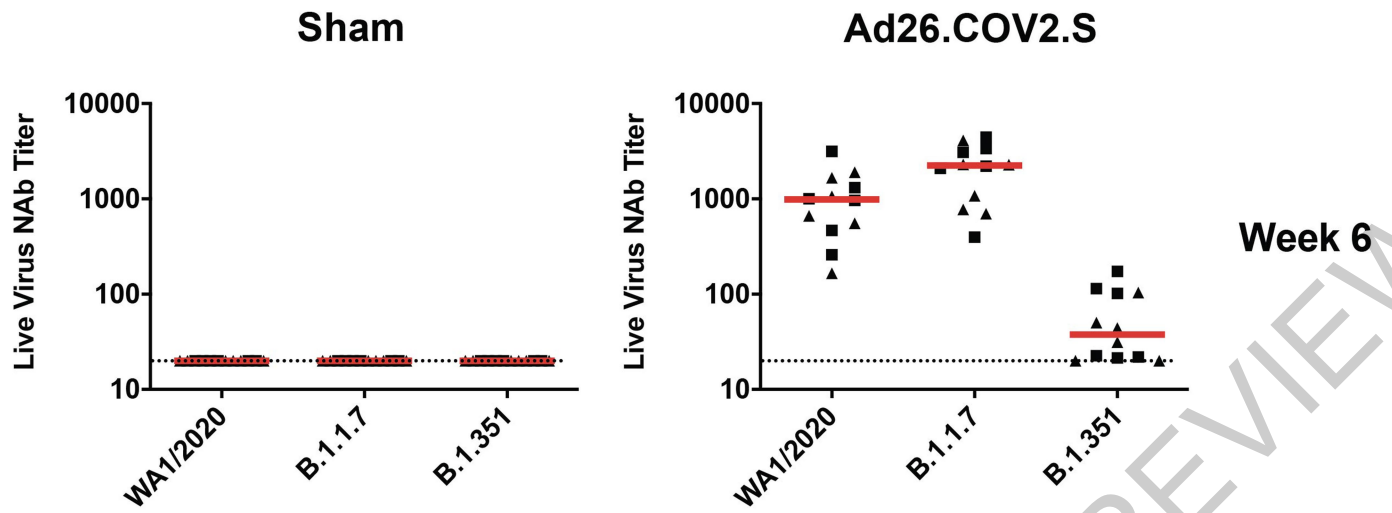
Competing interests D.H.B., R.Z., F.W. and H.S are co-inventors on provisional vaccine patents (63/121,482; 63/133,969; 63/135,182). R.Z., F.W., and H.S. are employees of Janssen Vaccines & Prevention BV and may hold stock in Johnson & Johnson.

Additional information

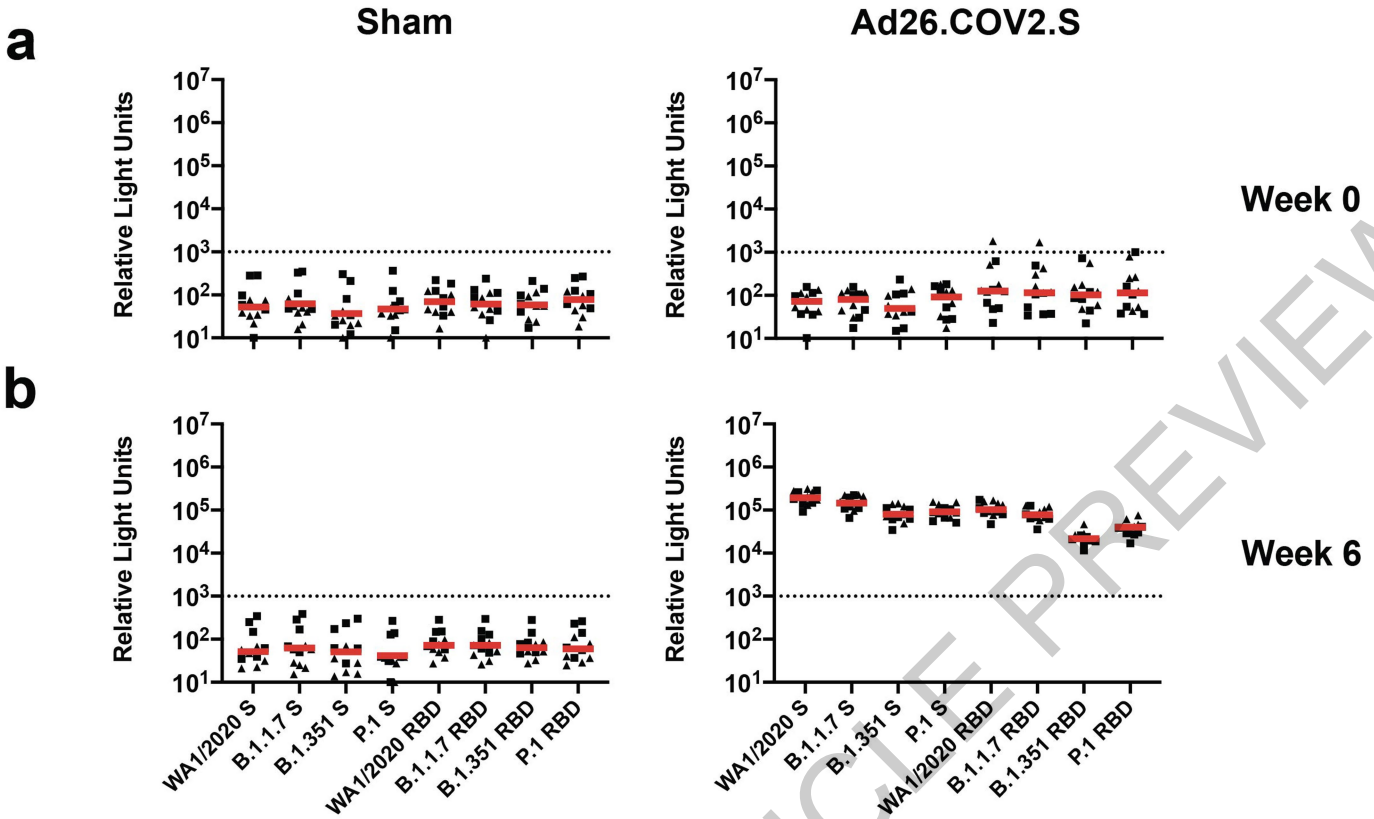
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41586-021-03732-8.

Correspondence and requests for materials should be addressed to D.H.B.

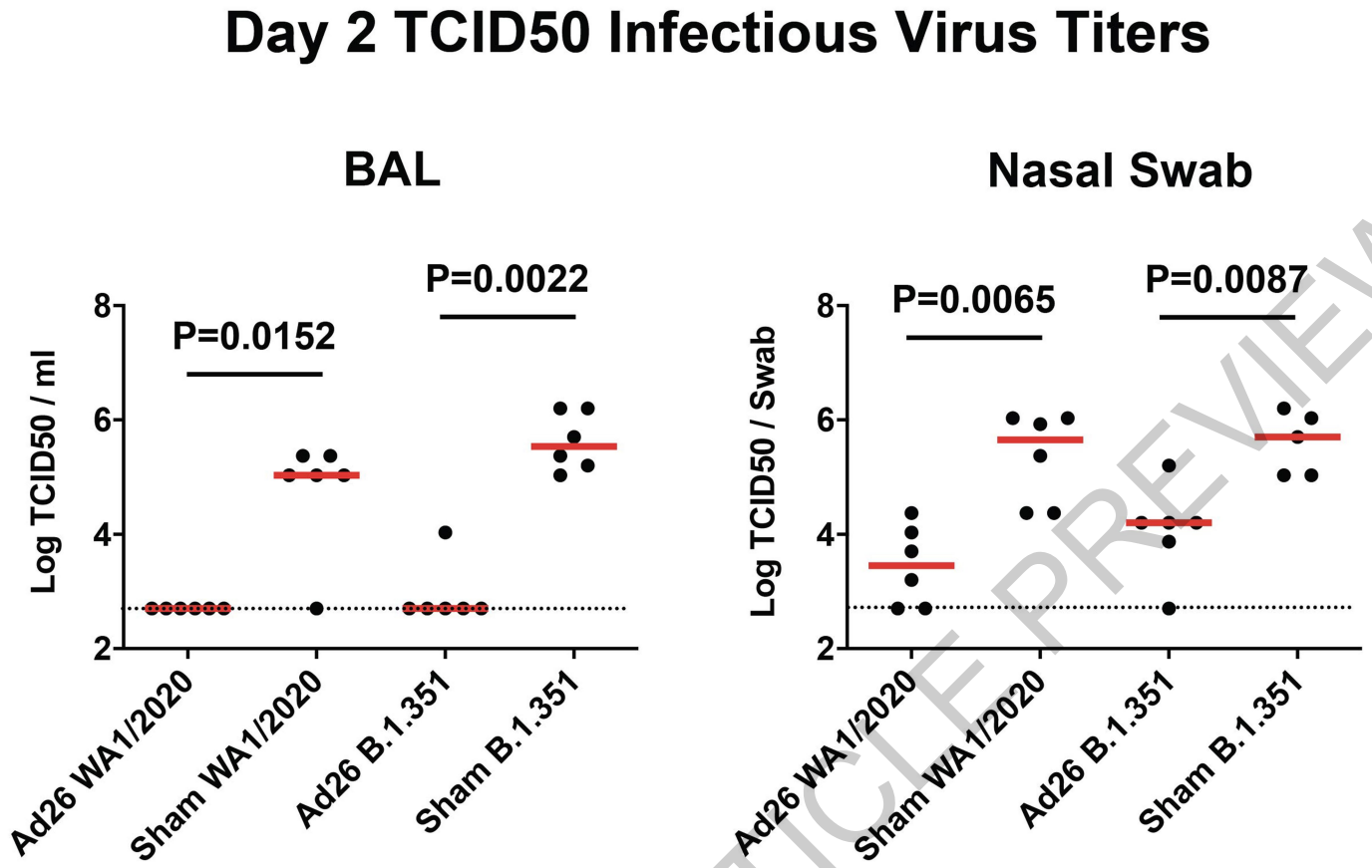
Peer review information *Nature* thanks Wolfgang Baumgärtner, Andrianus Boon and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.



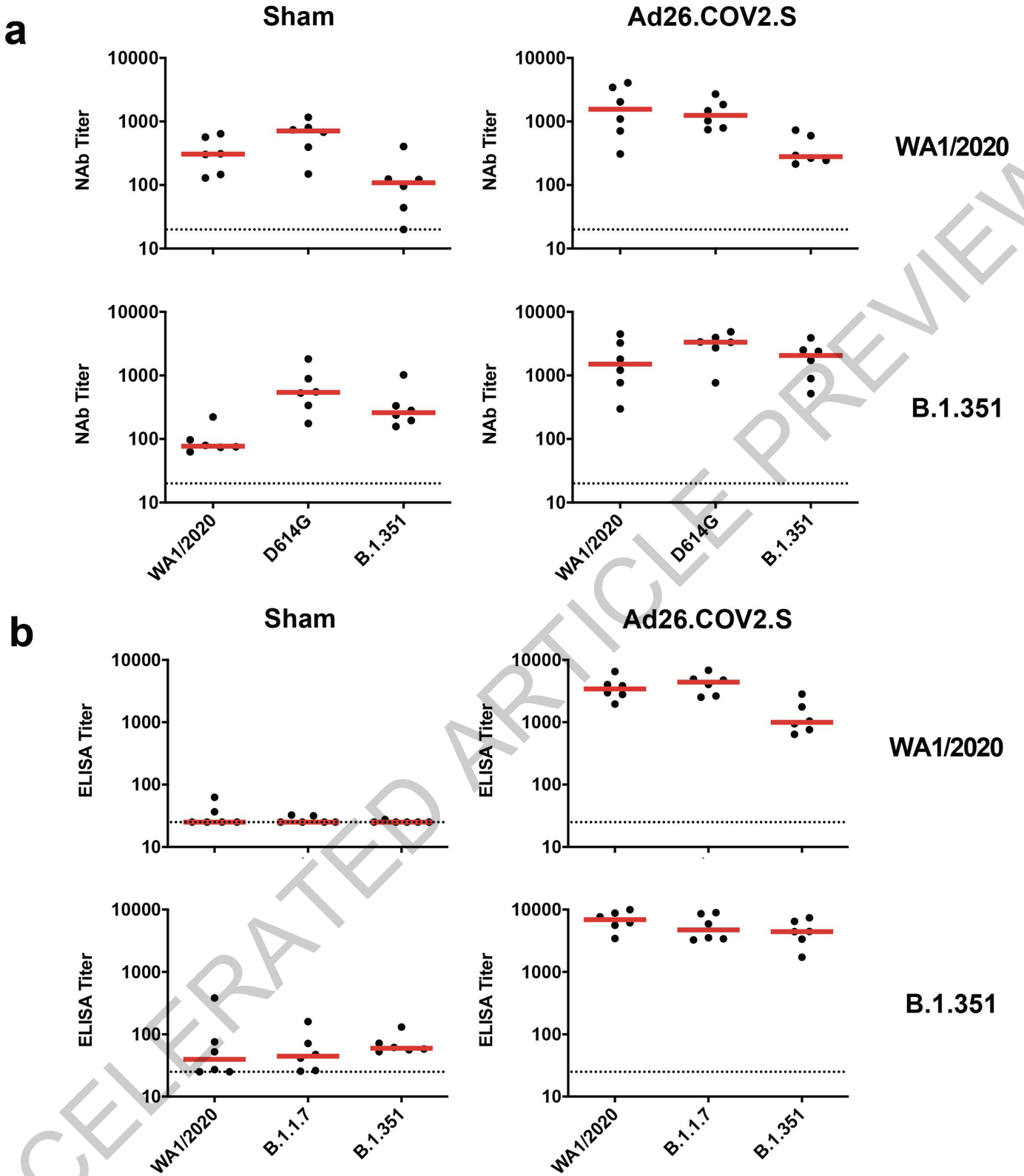
Extended Data Fig. 1 | Live virus neutralizing antibody responses in vaccinated rhesus macaques. Live virus neutralizing antibody (NAb) responses against the SARS-CoV-2 WA1/2020, B.1.1.7, and B.1.351 variants were assessed at week 6 in macaques that received a single immunization of sham vaccine or $5x10^{10}$ vp Ad26.COV2.S. Animals that eventually were challenged with WA1/2020 (triangles) or B.1.351 (squares) are depicted. Horizontal red bars reflect median responses. Dotted lines reflect assay limits of quantitation. n=24 independent samples (12 sham, 12 Ad26.COV2.S).



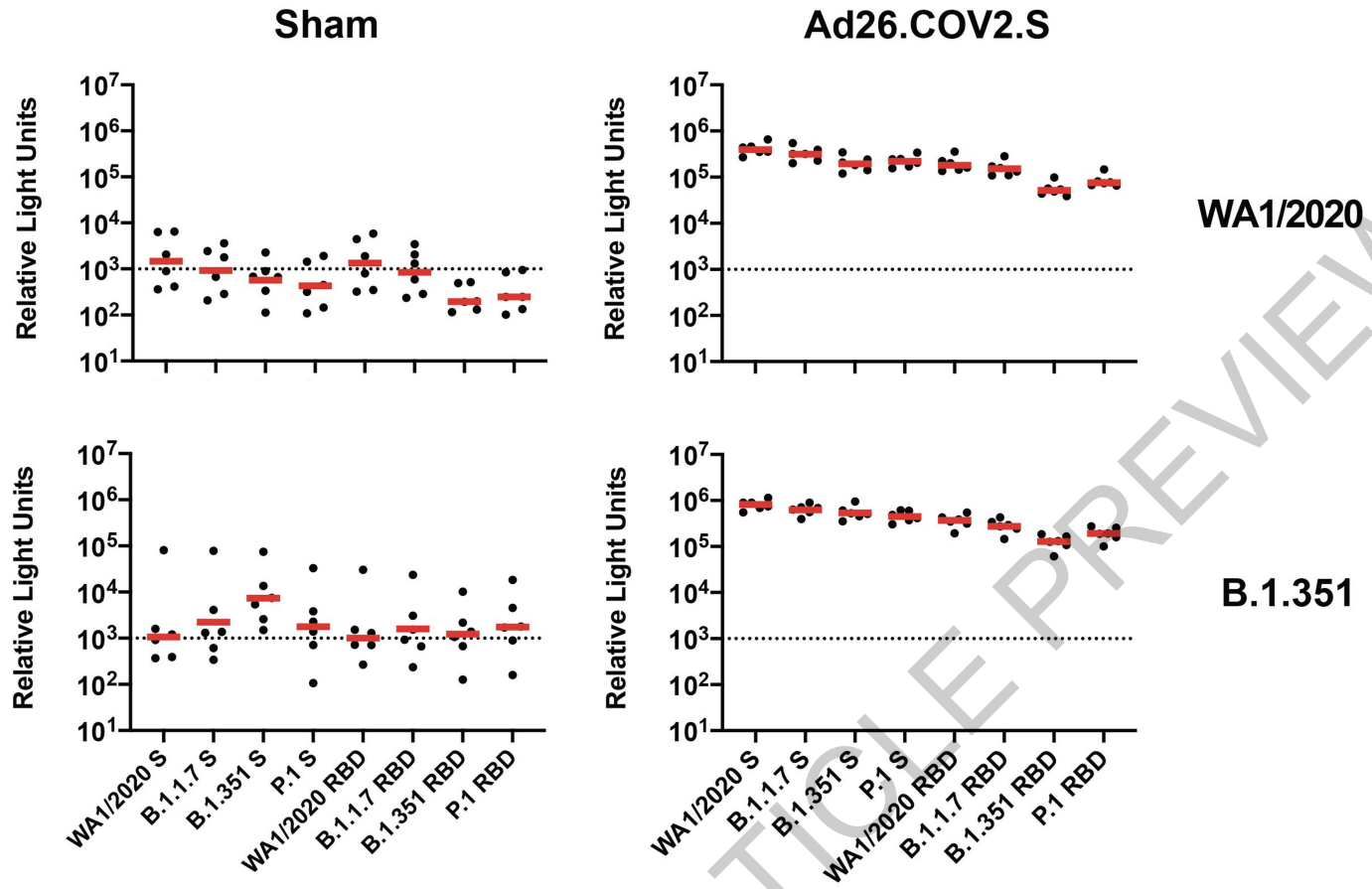
Extended Data Fig. 2 | Binding antibody responses in vaccinated rhesus macaques by ECLA. S- and RBD-specific binding antibody responses against the SARS-CoV-2 WA1/2020, B.1.1.7, B.1.351, and P.1 variants were assessed by ECLA at (**a**) week 0 and (**b**) week 6 in monkeys that received a single immunization of sham negative controls or $5x10^{10}$ vp Ad26.COV2.S. Animals that eventually were challenged with WA1/2020 (triangles) or B.1.351 (squares) are depicted. Horizontal red bars reflect median responses. Dotted lines reflect assay limits of quantitation.



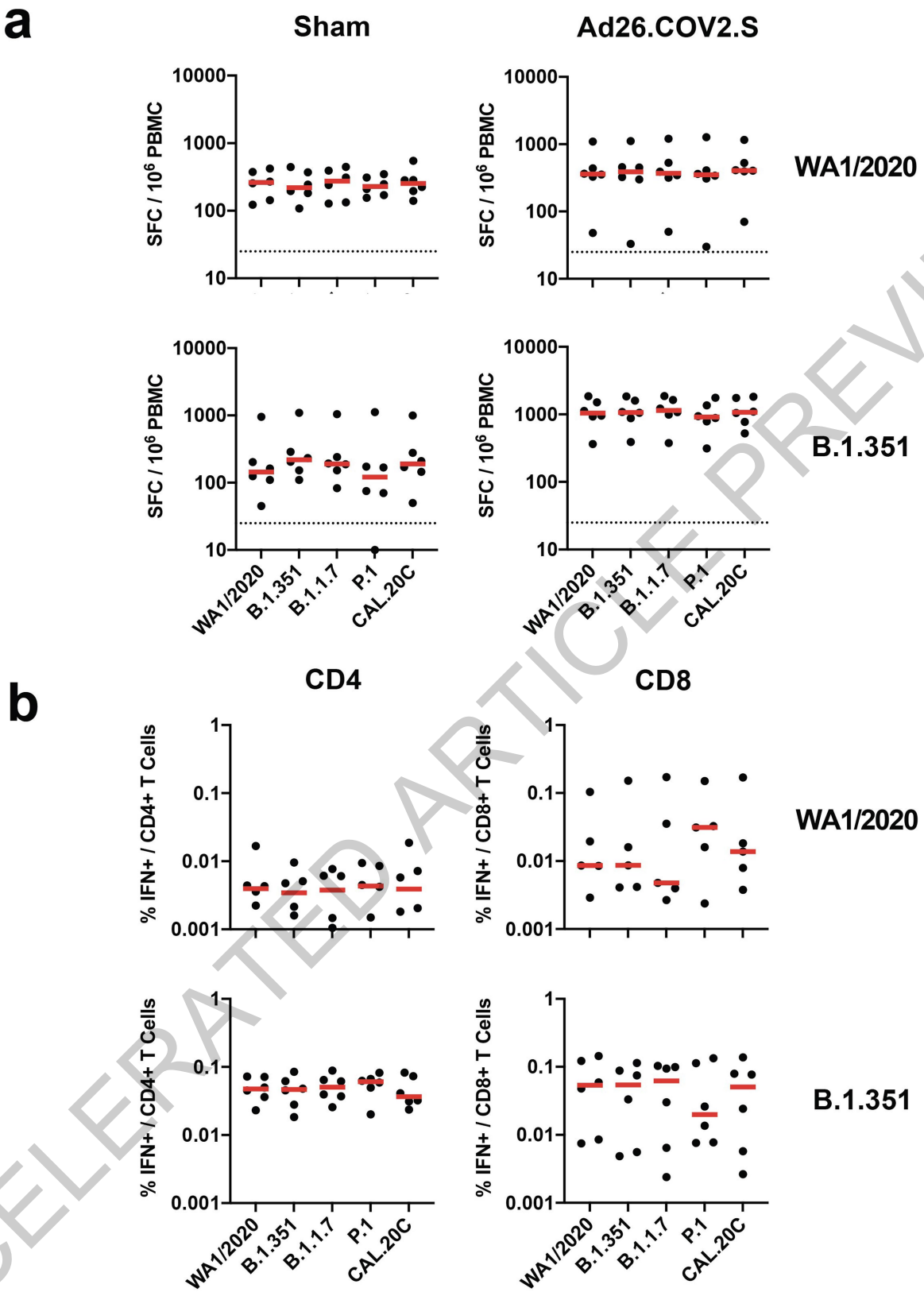
Extended Data Fig. 3 | Infectious virus titers following SARS-CoV-2 challenge. Day 2 infectious virus titers by TCID50 assays in bronchoalveolar lavage (BAL) and nasal swabs (NS) following challenge. Horizontal red bars reflect median values. P-values reflect two-sided Wilcoxon rank-sum tests. Dotted lines reflect assay limits of quantitation. n=24 independent samples (12 sham, 12 Ad26.COV2.S).



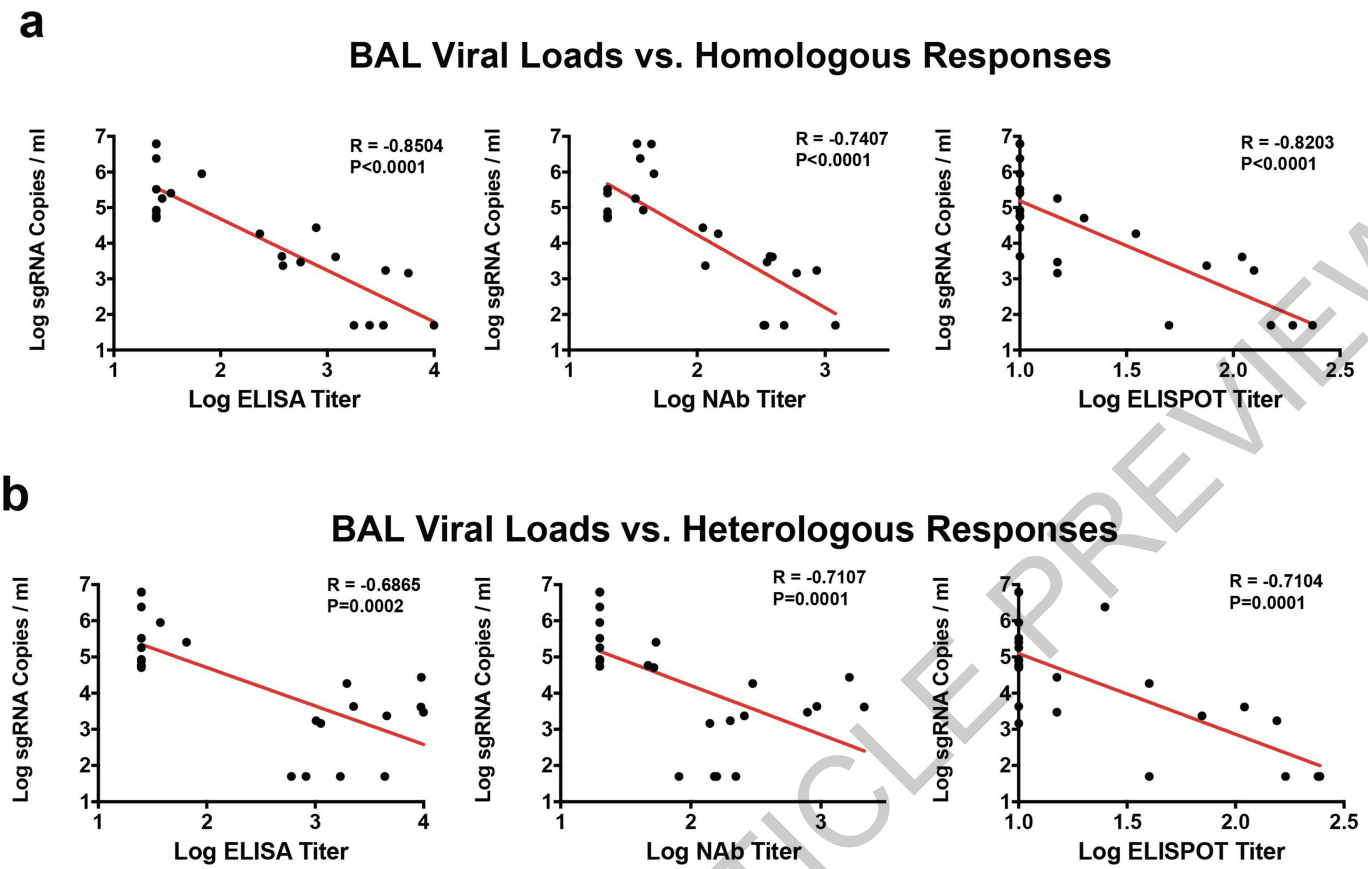
Extended Data Fig. 4 | Binding and neutralizing antibody responses in challenged rhesus macaques. (**a**) Pseudovirus neutralizing antibody (NAb) assays against the SARS-CoV-2 WA1/2020, D614G, and B.1.351 variants were assessed and (**b**) RBD-specific binding antibody responses against the SARS-CoV-2 WA1/2020, B.1.1.7, and B.1.351 variants were assessed by ELISA on day 10 following challenge in macaques that received a single immunization of sham vaccine or $5x10^{10}$ vp Ad26.COV2.S. Animals that were challenged with WA1/2020 or B.1.351 are shown in separate graphs. Horizontal red bars reflect median responses. Dotted lines reflect assay limits of quantitation. n=24 independent samples (12 sham, 12 Ad26.COV2.S).



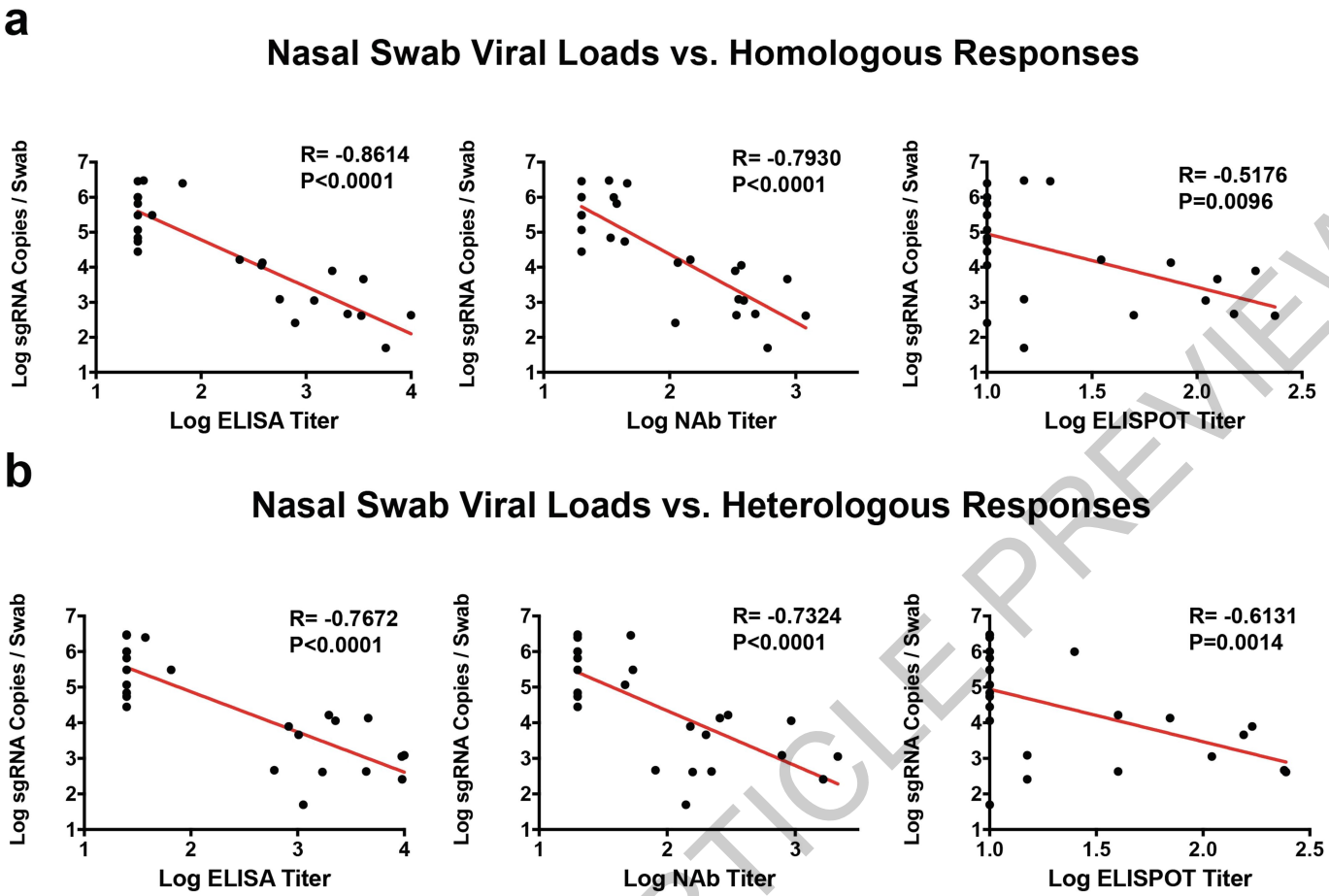
Extended Data Fig. 5 | Binding antibody responses in challenged rhesus macaques by ECLA. S- and RBD-specific binding antibody responses against the SARS-CoV-2 WA1/2020, B.1.1.7, B.1.351, and P.1 variants were assessed by ECLA on day 10 following challenge in macaques that received a single immunization of sham vaccine or $5x10^{10}$ vp Ad26.COV2.S. Animals that were challenged with WA1/2020 or B.1.351 are shown in separate graphs. Horizontal red bars reflect median responses. Dotted lines reflect assay limit of quantitation. n=24 independent samples (12 sham, 12 Ad26.COV2.S).



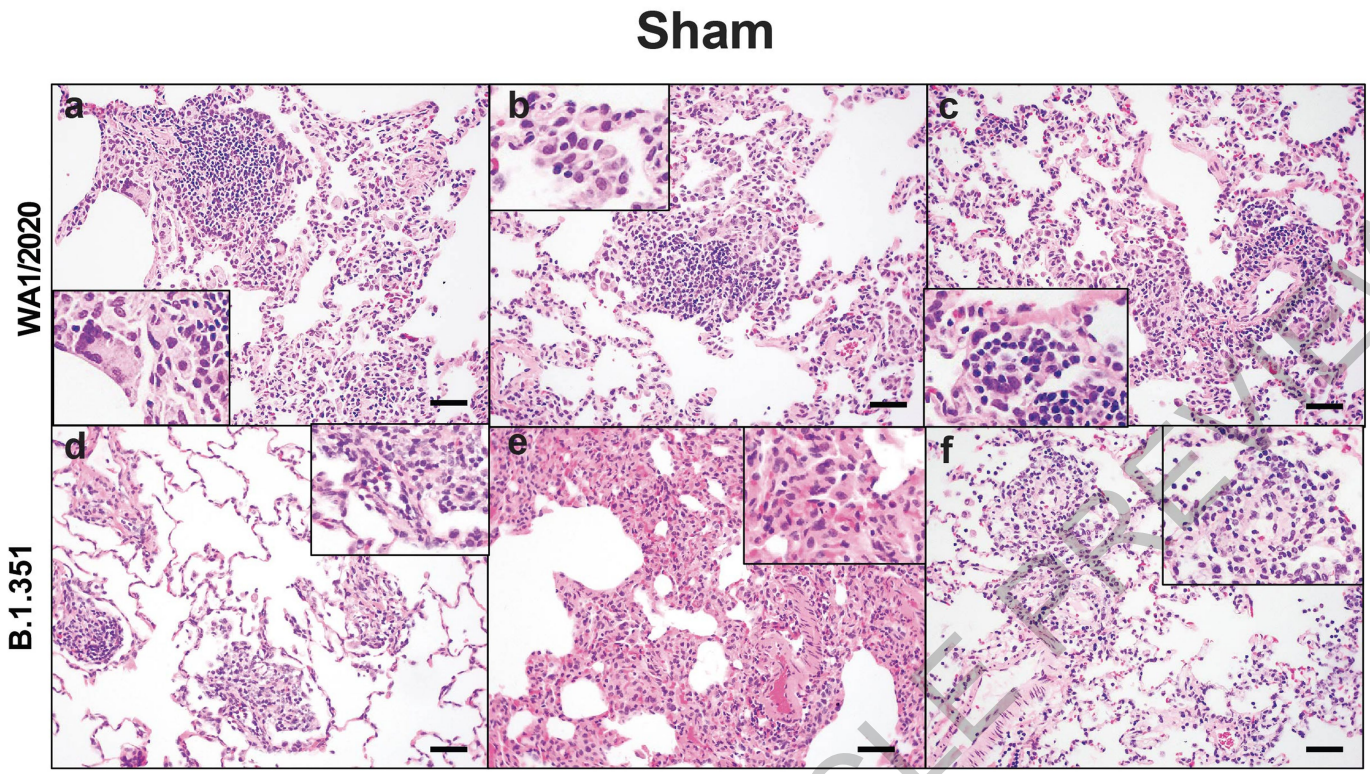
Extended Data Fig. 6 | T cell responses in vaccinated rhesus macaques by ELISPOT assays. (**a**) Cellular immune responses to pooled S peptides were assessed by IFN-γ ELISPOT assays on day 10 following challenge to WA1/2020, B.1.351, B.1.1.7, P.1, and CAL.20C variants. (**b**) CD4+ and CD8+ T cell responses to pooled S peptides were assessed by IFN-γ intracellular cytokine staining (ICS) assays on day 10 following challenge to WA1/2020, B.1.351, B.1.1.7, P.1, and CAL.20C variants. Horizontal red bars reflect median responses. n=24 independent samples (12 sham, 12 Ad26.COV2.S).



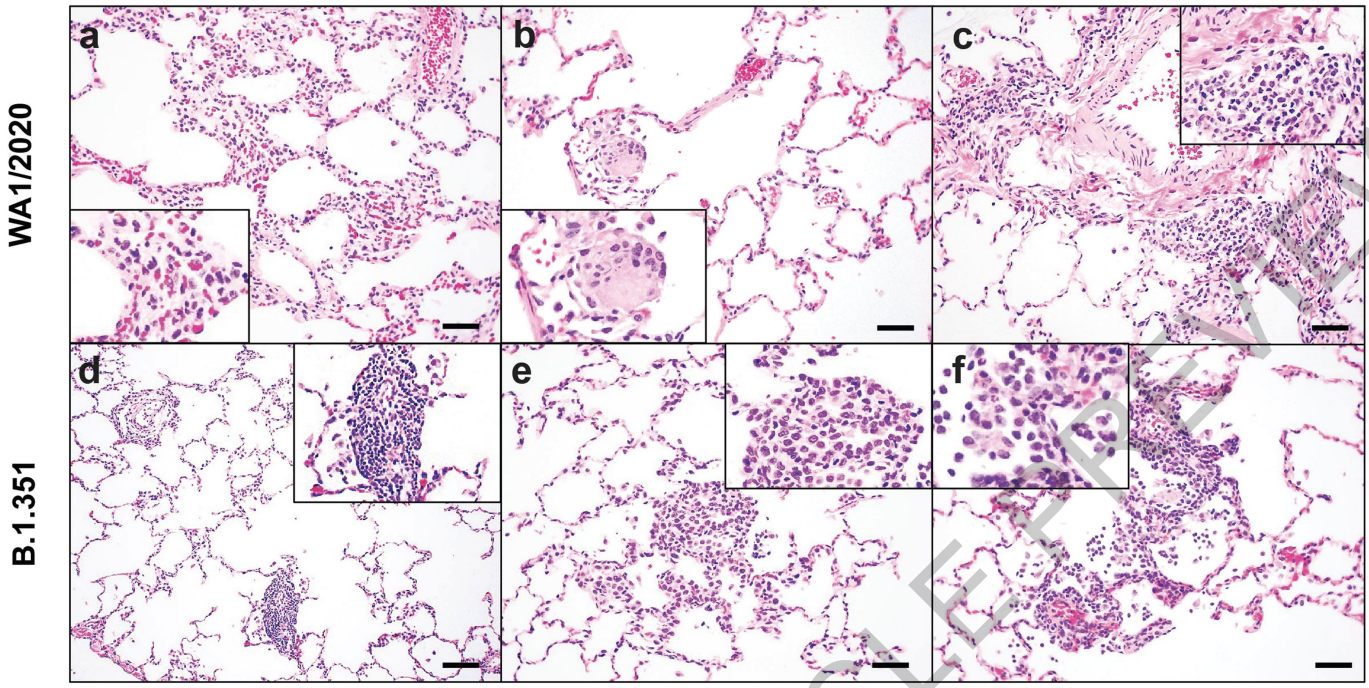
Extended Data Fig. 7 | Correlates of protection in BAL. Correlations of log peak sgRNA copies/ml in BAL following challenge vs. log ELISA titers, log NAb titers, or log ELISPOT responses to the (**a**) homologous or (**b**) heterologous challenge virus (WA1/2020, B.1.351) at week 6 following vaccination. Red lines reflect the best linear fit relationship between these variables. P and R values reflect two-sided Spearman rank-correlation tests.



Extended Data Fig. 8 | Correlates of protection in NS. Correlations of log peak sgRNA copies/ml in nasal swabs following challenge vs. log ELISA titers, log NAb titers, or log ELISPOT responses to the (**a**) homologous or (**b**) heterologous challenge virus (WA1/2020, B.1.351) at week 6 following vaccination. Red lines reflect the best linear fit relationship between these variables. P and R values reflect two-sided Spearman rank-correlation tests.



Extended Data Fig. 9 | Representative histopathology in sham animals following SARS-CoV-2 challenge. Locally extensive moderate to severe lesions were observed in sham vaccinated monkeys challenged with WA1/2020 (**a**-**c**) and B.1.351 (**d**-**f**) on day 10 following SARS-CoV-2 challenge. (**a**) Syncytia, lymphoid proliferation, and locally extensive interstitial inflammation; (**b**) type II pneumocyte hyperplasia and lymphoid proliferation; (**c**) perivascular alveolar infiltrates and interstitial inflammation; (**d**) alveolar macrophage infiltrates; (**e**) severe mononuclear alveolar infiltrates and pneumocyte hyperplasia; and (**f**) perivascular infiltrates and interstitial inflammation. At least 8 tissues were assessed per animal. Hematoxylin and eosin. Scale bar = 20 microns.



Extended Data Fig. 10 | Representative histopathology in Ad26.COV2.S vaccinated animals following SARS-CoV-2 challenge. Focal minimal to mild lesions were observed in Ad26.COV2.S vaccinated monkeys WA1/2020 (**a-c**) and B.1.351 (**d-f**) on day 10 following SARS-CoV-2 challenge. (**a**) Interstitial inflammation; (**b**) syncytia; (**c**) perivascular neutrophilic infiltrates; (**d**) perivascular mononuclear inflammation; (**e**) type II pneumocyte hyperplasia; (**f**) alveolar macrophage infiltrates. At least 8 tissues were assessed per animal. Hematoxylin and eosin. Scale bar = 20 microns.

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Reporting Summary

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☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
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☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	QuantStudio 6 was used to collect sgRNA data.
Data analysis	Analysis of virologic and immunologic data was performed using GraphPad Prism 8.4.2 (GraphPad Software). Flow cytometry data was analyzed with FlowJo v9.9.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size includes N=24 vaccinated animals (N=6 animals/group; Mercado et al Nature 2020). Based on our experience with SARS-CoV-2 in rhesus macaques, this sample size can differentiate large differences in protective efficacy compared with the sham controls.
Data exclusions	No data were excluded.
Replication	Virologic and immunologic measures were performed in duplicate. Technical replicates were minimally different. All attempts at replication were successful.
Randomization	Animals were balanced for age and gender and otherwise randomly allocated to groups.
Blinding	All immunologic and virologic assays were performed blinded.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	For ELISA and ELISPOT assays anti-macaque IgG HRP (NIH NHP Reagent Program), rabbit polyclonal anti-human IFN-γ (U-Cytech); for ICS assays mAbs against CD279 (clone EH12.1, BB700), CD38 (clone OKT10, PE), CD28 (clone 28.2, PE CY5), CD4 (clone L200, BV510), CD45 (clone D058-1283, BUV615), CD95 (clone DX2, BUV737), CD8 (clone SK1, BUV805), Ki67 (clone B56, FITC), CD69 (clone TP1.55.3, ECD), IL10 (clone JES3-9D7, PE CY7), IL13 (clone JES10-5A2, BV421), TNF-α (clone Mab11, BV650), IL4 (clone MP4-25D2, BV711), IFN-γ (clone B27; BUV395), IL2 (clone MQ1-17H12, APC), CD3 (clone SP34.2, Alexa 700) (BD); for 800CW-conjugated goat-anti-human secondary antibody (Li-COR); anti-rhesus IgG1, IgG2, IgG3, IgA, IgM (NIH NHP Reagent Program); tertiary goat anti-mouse IgG-PE antibody (Southern Biotech), anti-CD107a (PE-Cy7, BD), anti-CD56 (PE-Cy7, BD), anti-MIP-1β (PE, BD), mouse anti-human IFN-γ monoclonal antibody (BD), Streptavidin-alkaline phosphatase antibody (Southern Biotech), CD49d (BD), sulfo-tagged anti-human IgG (MesoScale Discovery).
Validation	all mAbs used according to manufacturer's instructions and were titrated prior to use

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	None
Authentication	Commerically purchased (ATCC) and evaluated in control experiments prior to use
Mycoplasma contamination	Negative for mycoplasma

Commonly misidentified lines (See ICLAC register)	None were utilized

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	24 outbred Indian-origin adult male and female rhesus macaques (Macaca mulatta), 3-11 years old
Wild animals	None
Field-collected samples	None
Ethics oversight	Bioqual IACUC

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Isolated PBMC
Instrument	BD FACSymphony
Software	FlowJo v9.9
Cell population abundance	No sorting was performed
Gating strategy	See gating strategy in Supplementary Figure 1

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.