Protective efficacy of Ad26.COV2.S against SARS-CoV-2 B.1.351 in macaques

The emergence of SARS-CoV-2 variants that partially evade neutralizing antibodies poses a threat to the efficacy of current COVID-19 vaccines. The Ad26.COV2.S vaccine expresses a stabilized spike protein from the WA1/2020 strain of SARS-CoV-2, and has recently demonstrated protective efficacy against symptomatic COVID-19 in humans in several geographical regions—including in South Africa, where 95% of sequenced viruses in cases of COVID-19 were the B.1.351 variant. 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 to WA1/2020, but elicited comparable CD8 and CD4 T cell responses against the WA1/2020, B.1.351, B.1.1.7, P.1 and CAL.20C variants. B.1.351 infection of 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 macaques after 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, and some variants have also demonstrated partial evasion of natural and vaccine-elicited neutralizing antibodies. Ad26.COV2.S is a replication-incompetent human adenovirus type 26 vector that expresses a prefusion stabilized SARS-CoV-2 spike protein (S) from the Wuhan 2019 strain of SARS-CoV-2. It was previously reported that Ad26.COV2.S demonstrated protective efficacy against SARS-CoV-2 WA1/2020 challenges in hamsters and nonhuman primates, and also showed safety and immunogenicity in humans. A recent phase III efficacy trial has shown that Ad26.COV2.S provided 86%, 88% and 82% protection against severe COVID-19 disease by day 28 after vaccination in the USA, Brazil and South Africa, respectively.

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 macaques per group) as follows: groups 1 and 3 received a sham vaccine (sham control macaques), and groups 2 and 4 received a single immunization with 5 × 10^9 viral particles of Ad26.COV2.S; after 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

We assessed vaccine-induced antibody responses against the SARS-CoV-2 WA1/2020 strain as well as against B.1.351. Using a luciferase-based pseudovirus neutralizing antibody assay, we found that the median neutralizing antibody titres in macaques that received Ad26.COV2.S vaccine were less than 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 macaques at week 6 (Fig. 1a). These data show a median 4.5-fold reduction of neutralizing antibody titres against B.1.351 as compared to WA1/2020 (P = 0.0002, Wilcoxon
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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 week 0 (top panels) and week 6 (bottom panels) in macaques that received a single immunization of sham vaccine (left panels) or 5 × 10^10 viral particles of Ad26.COV2.S (right panels). **b**, RBD-specific binding antibody responses of sham control (left panels) or Ad26.COV2.S-vaccinated (right panels) macaques against WA1/2020, B.1.1.7, and B.1.351 were assessed by ELISA at week 0 (top panels) and week 6 (bottom panels). **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 at week 6. Macaques 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 the limits of quantification of the assay. *n* = 24 independent samples (12 sham and 12 Ad26.COV2.S).

**Homologous and heterologous SARS-CoV-2 challenges**

We challenged all macaques at week 6 with a 5 × 10^5 50% tissue culture infective dose (TCID_{50}) of SARS-CoV-2 WA1/2020, D614G, or B.1.351 by the intranasal and intratracheal routes. We assessed viral loads in bronchoalveolar lavage (BAL) and nasal swabs by reverse-transcription PCR (RT–PCR) specific for subgenomic mRNA (sgRNA), which is believed to be necessary for replication of virus^16,23,24. All sham control macaques were infected and showed higher median peak sgRNA of 6.16 (range of 4.93–6.80) log_{10}(sgRNA copies per ml) in BAL for B.1.351, as compared to 4.80 (range of 4.70–5.52) log_{10}(sgRNA copies per ml) for WA1/2020 (Fig. 3a). By contrast, vaccinated macaques demonstrated a median peak of 3.62 (range of 3.37–4.43) log_{10}(sgRNA copies per ml) in BAL for B.1.351, as compared with less than 1.69 (range of <1.69 to 3.23) log_{10}(sgRNA copies per ml) in BAL for WA1/2020 (Fig. 3a). Sham control macaques also showed a trend towards a higher median peak sgRNA of 5.90 (range of 4.73–6.47) log_{10}(sgRNA copies per swab) in nasal swabs for B.1.351, as compared with 5.48 (range of 4.44–6.00) log_{10}(sgRNA copies per swab) for WA1/2020 (Fig. 3b). Vaccinated macaques demonstrated a median peak of 3.57 (range of 2.41–4.21) log_{10}(sgRNA copies per swab) in nasal swabs for B.1.351, as compared with 2.64 (range of <1.69 to 3.89) log_{10}(sgRNA copies per swab) in nasal swabs for WA1/2020 (Fig. 3b).

B.1.351 led to higher peak viral loads, faster kinetics of viral replication and a longer duration of viral replication as compared with WA1/2020 in sham control macaques, which suggests 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 per ml in BAL for WA1/2020 and B.1.351, respectively, and a 2.84 and 2.33 log reduction of peak sgRNA copies per swab in nasal swabs for WA1/2020 and B.1.351, respectively (*P* = 0.0022 for both BAL and nasal swabs for both WA1/2020 and B.1.351, Wilcoxon rank-sum tests) (Fig. 4a). By day 4 after challenge, viral loads were undetectable in Ad26.COV2.S-vaccinated macaques after both WA1/2020 and B.1.351 challenge, whereas viral loads were positive in most sham control macaques for WA1/2020 and in all sham control macaques for B.1.351 (Fig. 4b). Ad26.COV2.S also provided similar robust protection against day 2 infectious virus titres, as assessed by TCID_{50} assays (Extended Data Fig. 3).
Correlates of protection

On day 10 after challenge (study week 8), sham control macaques developed both humoral and cellular immune responses, as expected (Extended Data Figs. 4–6). In sham control macaques, WA1/2020 challenge led to higher neutralizing antibody titres in sham control macaques probably reflect the early increase in humoral and cellular immune responses after challenge. The immunogenicity data. Ad26.COV2.S-vaccinated macaques developed both humoral and cellular immune responses, as expected (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 macaques developed increased humoral and cellular immune responses after challenge. The low ELISA titres in sham control macaques probably reflect the early (day 10) time point after challenge (Extended Data Fig. 4b).

Peak log_{10}(sgRNA) in BAL (Extended Data Fig. 7) and in nasal swabs (Extended Data Fig. 8) after challenge inversely correlated with log_{10} ELISA, neutralizing antibody and ELISPOT responses at week 6, which suggests 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 control macaques at necropsy on day 10 after WA1/2020 or B.1.351 challenge (Fig. 5a, b), although viral replication had largely resolved by day 10. Sham control macaques infected with WA1/2020 and B.1.351 had histopathological lesions that were 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 control macaques, as were multifocal regions of fibrosis (Fig. 5c, d, Extended Data Fig. 9). Ad26.COV2.S-vaccinated macaques 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).
It has previously been reported that Ad26.COV2.S provided robust protection against challenge with SARS-CoV-2 WA1/2020 in both rhesus macaques and hamsters\(^1\)–\(^3\). 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 several mutations (including E484K) that lead to partial evasion of natural and vaccine-elicited neutralizing antibodies.\(^1\)–\(^3\). Binding and neutralizing antibody titres were suppressed 4–5-fold against B.1.351 as compared to WA1/2020, but Fc functional antibody responses were affected less, and T cell responses were not affected at all by the SARS-CoV-2 variants. Ad26.COV2.S provided robust protection against both high-dose WA1/2020 and B.1.351 challenges. 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 III clinical trial of Ad26.COV2.S that was conducted in the USA, Latin America (Argentina, Brazil, Chile, Colombia, Mexico and Peru) and South Africa\(^4\). Robust protection was observed in all geographical regions, with similar levels of protection against severe COVID-19 disease regardless of variant, including in the USA, 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 a higher magnitude of and more prolonged viral replication in the upper and lower respiratory tracts than did WA1/2020. Nevertheless, Ad26.COV2.S provided robust protection against both viruses, although levels of virus in BAL and nasal swabs were higher after B.1.351 challenge than after WA1/2020 challenge.

No evidence of eosinophilic infiltrates or enhanced respiratory disease was observed in Ad26.COV2.S-vaccinated macaques.

**Discussion**

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**Fig. 4** | Summary of protective efficacy after SARS-CoV-2 challenge. **a, b.** Peak (a) and day 4 (b) viral loads in BAL (left) and nasal swabs (right) of sham control (sham) and Ad26.COV2.S-vaccinated (Ad26) macaques after challenge with WA1/2020 or B.1.351. Horizontal red bars reflect median values. \(P\) values reflect two-sided Wilcoxon rank-sum tests. Dotted lines reflect the limits of quantification of the assay. \(n = 24\) independent samples (12 sham and 12 Ad26.COV2.S).

**Fig. 5** | Histopathology after SARS-CoV-2 challenge. **a.** Cumulative histopathologic scoring of lung lesions from eight representative lung lobes from Ad26.COV2.S-vaccinated (Ad26) and sham control (sham) macaques on day 10 after challenge with 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 macaque, 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), bronchioalveolar infiltrate (neutrophils), epithelial syncytia, type II pneumocyte hyperplasia, bronchi infiltrate (macrophages), bronchi infiltrate (neutrophils), bronchi (hyperplasia of bronchus-associated lymphoid tissue), bronchiolar or peribronchiolar infiltrate (mononuclear cells), perivascular infiltrate (mononuclear cells) and endothelialitis. Each feature assessed was assigned a score of: 0, no substantial findings; 1, minimal; 2, mild; 3, moderate; 4, moderate to severe; 5, marked or severe. Scores were added for all lesions across all lung lobes for each macaque, for a maximum possible score of 600 for each macaque. Horizontal red lines reflect median values. \(P\) values reflect two-sided Wilcoxon rank-sum tests. **c–f.** Representative lung histopathology from at least eight evaluated tissues from sham control (c, d) and Ad26.COV2.S-vaccinated (e, f) macaques challenged with WA1/2020 (c, e) or B.1.351 (d, f) on day 10 after the challenge, showing increased alveolar macrophages and thickened alveolar septa with inflammatory infiltrates and fibrosis (c), increased alveolar macrophages and epithelial syncytia within alveolar spaces, thickened and fibrotic alveolar septa with inflammatory infiltrates, focal alveolar and perivascular inflammatory infiltrates (d), focal perivascular inflammation (e) and focal expansion of alveolar septa with inflammatory infiltrates (f). Lungs evaluated were inflated or suffused with 10% formalin. In c–f, tissues were stained with haematoxylin and eosin. Scale bars, 20 \(\mu\)m. \(n = 24\) independent samples (12 sham and 12 Ad26.COV2.S) (a, b); \(n = 4\) representative samples (2 sham and 2 Ad26.COV2.S) (c–f).
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. Several SARS-CoV-2 vaccines have previously 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 it has previously been reported that IgG was sufficient for protection against homologous SARS-CoV-2 challenge in macaques and that CD8 T cell responses also contributed to protection if antibody titres were subprotective.

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 whether Ad26.COV2.S, as well as other vaccines, protect against other SARS-CoV-2 variants of concern.

Online content
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**Methods**

No statistical methods were used to predetermine sample size. Macaques were randomized into groups. All immunological, virological and histopathological studies were performed blinded.

**Macaques and study design**

Twenty-four outbred Indian-origin adult male and female rhesus macaques (Macaca mulatta) (3–11 years old) were randomly allocated to groups. All macaques were housed at Bioqual. Macaques received a single immunization of $5 \times 10^5$ viral particles of Ad26.COV2.S ($n = 12$) or sham ($n = 12$) by the intramuscular route without adjuvant at week 0. At week 6, all macaques were challenged with $5 \times 10^6$ TCID$_{50}$ SARS-CoV-2 from strains USA-WA1/2020 (BEI Resources; NR-5225) (which was grown in VeroE6 cells and deep sequenced as previously described) 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 $S$ greater than 2.5% frequency and no mutations elsewhere in the virus at greater than 13% frequency. Virus was administered as 1 ml by the intranasal route (0.5 ml in each nare) and 1 ml by the intratracheal route. All immunological, virological and histopathological 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.

**Pseudovirus-based virus neutralization assay**

The SARS-CoV-2 pseudoviruses expressing a luciferase reporter gene were generated essentially as previously described. In brief, the packaging plasmid pSPAX2 (AIDS Resource and Reagent Program), luciferase reporter plasmid pLenti-CMV Puro-Luc (Addgene) and S 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 WAI/2020 strain (Wuhan/WIV04/2019, GISAID accession identifier EPI_ISL_402124), D614G mutation, B.1.1.7 variant (GISAID accession identifier EPI_ISL_601443) or B.1.351 variant (GISAID accession identifier EPI_ISL_712096). The supernatants containing the pseudotype viruses were collected 48 h after transfection, and were purified by centrifugation and filtration with a 0.45-μm filter. To determine the neutralization activity of the plasma or serum samples from participants, HEK293T cells expressing human ACE2 (HEK293-hACE2 cells) were seeded in 96-well tissue culture plates at a density of $7.5 \times 10^3$ cells per well overnight. Threefold 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. Forty-eight hours after infection, cells were lysed in Steady-Glo Luciferase Assay (Promega) according to the manufacturer’s instructions. SARS-CoV-2 neutralization titres were defined as the sample dilution at which a 50% reduction in relative light units (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. One day before 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 threefold 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% CO$_2$ for 1 h. After incubation, serially diluted sera and virus-only controls were added in duplicate to the cells at 75 plaque-forming units at 37 °C with 5% CO$_2$. 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). Virus neutralization titres 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 previously described. In brief, 96-well plates were coated with 0.5 μg ml$^{-1}$ RBD protein in 1× DPBS and incubated at 4 °C overnight. After incubation, plates were washed once with wash buffer (0.05% Tween 20 in 1× DPBS) and blocked with 350 μl casein block per 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, before three further washes and a 1-h incubation with a 1 μg ml$^{-1}$ 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 450 nm was recorded using a VersaMax microplate reader. For each sample, ELISA endpoint titre 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 450 nm. log$_{10}$-transformed endpoint titres are reported.

**ECLA**

ECLA plates (MesoScale Discovery SARS-CoV-2 IgG Cat No: N05CA-1; panel 7) were designed and produced with up to nine antigen spots in each well, and assays were performed essentially as previously described. The antigens included were WA1/2020, B.1.1.7, P.1, and B.1.351 and RBD. The plates were blocked with 50 μl of blocker A (1% BSA in MilliQ water) solution for at least 30 min at room temperature before adding 700 μl 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 1× 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 titres for each sample are reported as RLU’s, which were calculated as sample RLU minus blank RLU for each spot for each sample. The limit of detection was defined as 1,000 RLU for each assay.

**Fc functional antibody assays**

Fc functional profiling included the assessment of antibody-dependent monocyte phagocytosis and antibody-dependent complement deposition. In brief, fluorescent beads (LifeTechnologies) were coupled via carbboxy-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.

**IFNγ ELISPOT assay**

Pooled peptide ELISPOT assays were performed essentially as previously described. Peptide pools consisted of 15 amino acid peptides overlapping by 11 amino acids spanning the SARS-CoV-2 $S$ from the
probes as previously described23,24. A standard was generated by first copies. Viral loads were quantified from BAL fluid and nasal swabs.

The insert was in vitro-transcribed to RNA using the AmpliCap-Max T7 fragment was subsequently cloned into a pcDNA3.1+ expression plasmid. SARS-CoV-2 sgRNA assay defined as CD28+CD95+ T cells. Data were analysed with FlowJo v.9.9. Analysed by BD FACSymphony system. Central memory T cells 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 assay**

Multi-parameter pooled-peptide intracellular cytokine staining assays were performed essentially as previously described2,3,26.27. Peptide pools consisted of 15 amino acid peptides overlapping by 11 amino acids spanning the SARS-CoV-2 S from the WA1/2020 strain or variant strains. Then, 10⁶ PBMCs per well were resuspended in 100 μl of R10 medium supplemented with CD49d monoclonal antibody (1 μg ml⁻¹). Each sample was assayed with mock (100 μl of R10 plus 0.5% DMSO; background control), peptide pools (2 μg ml⁻¹), or 10 pg ml⁻¹ phorbol myristate acetate and 1 μg ml⁻¹ ionomycin (Sigma-Aldrich) (100 μl; 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 min and then stained with predetermined titres of monoclonal antibodies against CD279 (clone EH12.1, BB700), CD38 (clone SP34.2, Alexa 700), IL-2 (clone MQ1-17H12, APC) and CD3 (clone JES3-9D7, PE CY7), IL-13 (clone JES10-5A2, BV421), TNF (clone Mab11, ECD), IL-10 (clone B4G12, BV510), CD95 (clone DX2, BUV737) and CD8 (clone SK1, BUV805), for 30 min.

Cells were then washed twice with 2% FBS in DPBS buffer and incubated for 15 min with 200 μl of BD Cytofix/CytoPerm Fixation/Permeabilization solution. Cells were washed twice with 1× Perm Wash buffer (BD Perm/Wash Buffer 10× in the CytoFix/CytoPerm Fixation/Permeabilization kit diluted with MilliQ water and passed through a 0.22-μm filter) and stained with intracellularly with monoclonal antibodies against Ki67 (clone BS6, FITC), CD69 (clone TP1.S5.3, ECD), IL-10 (clone JES3-9D7, PE CY7), IL-13 (clone JES10-5A2, BV421), TNF (clone Mab11, BV650), IL-4 (clone MP4-25D2, BV711), IFNγ (clone B27; BV395), CD45 (clone DO58-1283, BV615), IL-2 (clone MQ1-17H12, APC) and CD3 (clone SP34.2, Alexa 700), for 30 min. Cells were washed twice with 1× Perm Wash buffer and fixed with 250 μl of freshly prepared 1.5% formaldehyde. Fixed cells were transferred to a 96-well round-bottom plate and analysed by BD FACSymphony system. Central memory T cells were defined as CD28+CD95+ T cells. Data were analysed with FlowJo v.9.9.

**sgRNA assay**

SARS-CoV-2 E gene sgRNA was assessed by RT–PCR using primers and probes as previously described2,3,24. A standard was generated by first synthesizing a fragment of the subgenomic E gene25. 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 1 × 10⁶ copies to 1 × 10⁴ copies. Viral loads were quantified from BAL fluid and nasal swabs.

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 min, 42 °C for 1 h, then 85 °C for 5 min. A Taqman custom gene expression assay (Thermo Fisher Scientific) was designed using the sequences targeting the E gene sgRNA25. The sequences for the custom assay were as follows, forward primer, sgleadCoV2.Fwd: CGATCTCTCTGTAGATCTGTTTCTC; E_Sarbeco.R: ATATTGACGGTAGTACCACACA, E_Sarbeco.P1 (probe): VIC-ACACTAGCCATCTCCTAGGCTC-MGB. These primers and 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 s, then 45 cycles of 95 °C for 1 s and 60 °C for 20 s. Standard curves were used to calculate sgRNA 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 A. Creanga) were plated at 25,000 cells per well in DMEM with 10% FBS and gentamicin, and the cultures were incubated at 37 °C, 5.0% CO₂. Medium 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 titre) 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 cytopathic effect. The TCID₅₀ was calculated using the Read–Muench formula.

**Histopathology**

Lungs on day 10 after SARS-CoV-2 challenge were evaluated by histopathology. At the time of fixation, lungs were suffused with 10% formalin to expand the alveoli. All tissues were fixed in 10% neutral buffered formalin to expand the alveoli. All tissues were fixed in 10% formalin and were incubated at 37 °C, 5.0% CO₂. Medium 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 titre) 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 cytopathic effect. The TCID₅₀ was calculated using the Read–Muench formula.

**Statistical analyses**

Comparisons of virological, immunological and histopathological data were 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 GraphPad Prism 8.4.2 (GraphPad Software).

**Reporting summary**

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

**Data availability**

All relevant data are available in the Article and its Supplementary Information. Any additional data are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Consortium on Pathogen Readiness (MassCPR) and the National Institutes of Health (CA260476).

Author contributions D.H.B., F.W. and R.Z. designed the study. J.Y., I.H.T., N.B.M., K.M., J.L., C.J.-O., A. Chandrashekar, T.A., E.A.B., A. Chang, S.G., V.M.G., D.L.H., F.N., J.N., S.P., O.S., D.S. and H.W. performed the immunological and virological 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. Cook, D.B.-W., E.T., H.A. and M.G.L. led the clinical care of the macaques. F.W., R.Z. and H.S. provided the vaccine. T.H., K.B. and A.I.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
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Correspondence and requests for materials should be addressed to D.H.B.

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Extended Data Fig. 1 | Live virus neutralizing antibody responses in vaccinated rhesus macaques. Live virus neutralizing antibody 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 $5 \times 10^{10}$ viral particles of Ad26.COV2.S. Macaques 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 quantification. $n = 24$ independent samples (12 sham, 12 Ad26.COV2.S).
Extended Data Fig. 2 | Binding antibody responses in vaccinated rhesus macaques by ECLA. a, b, 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 week 0 (a) and week 6 (b) in macaques that received a single immunization of sham-negative control or 5 × 10^10 viral particles of Ad26.COV2.S. Macaques 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 quantification.
Extended Data Fig. 3 | Infectious virus titres after SARS-CoV-2 challenge.
Day 2 infectious virus titres by TCID_{50} assays in BAL and nasal swabs after challenge. Horizontal red bars reflect median values. *P* values reflect two-sided Wilcoxon rank-sum tests. Dotted lines reflect assay limits of quantification. *n* = 24 independent samples (12 sham, 12 Ad26.COV2.S).
Extended Data Fig. 4 | Binding and neutralizing antibody responses in challenged rhesus macaques. 

(a) and (b) Pseudovirus neutralizing antibody assays against the SARS-CoV-2 WA1/2020, D614G, and B.1.351 variants were assessed (a) and 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 (b) on day 10 after challenge in macaques that received a single immunization of sham vaccine or $5 \times 10^{10}$ viral particles of Ad26.COV2.S. Macaques 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 quantification. $n = 24$ 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 after challenge in macaques that received a single immunization of sham vaccine or $5 \times 10^{10}$ viral particles of Ad26.COV2.S.

Macques 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 quantification. $n = 24$ 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 IFNγ ELISPOT assays on day 10 after 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 IFNγ intracellular cytokine staining assays on day 10 after 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. a, b. Correlations of log(peak sgRNA copies per ml) in BAL after challenge versus log-transformed ELISA titres, neutralizing antibody titres or ELISPOT responses to the homologous (a) or heterologous (b) challenge virus (WA1/2020, B.1.351) at week 6 after 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 nasal swabs.**

**a, b.** Correlations of log(peak sgRNA copies per ml) in nasal swabs after challenge versus log-transformed ELISA titres, neutralizing antibody titres or ELISPOT responses to the homologous (a) or heterologous (b) challenge virus (WA1/2020, B.1.351) at week 6 after 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 control macaques after SARS-CoV-2 challenge. a–f. Locally extensive moderate-to-severe lesions were observed in sham control macaques challenged with WA1/2020 (a–c) or B.1.351 (d–f), on day 10 after 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. f, Perivascular infiltrates and interstitial inflammation. At least eight tissues were assessed per macaques. Haematoxylin and eosin staining. Scale bars, 20 μm.
Extended Data Fig. 10 | Representative histopathology in Ad26.COV2.S-vaccinated macaques after SARS-CoV-2 challenge. a–f. Focal minimal-to-mild lesions were observed in Ad26.COV2.S-vaccinated macaques challenged with WA1/2020 (a–c) or B.1.351 (d–f), on day 10 after 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 eight tissues were assessed per macaque. Haematoxylin and eosin staining. Scale bar, 20 μm.
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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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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 | Methods |
|----------------------------------|---------|
| n/a Involved in the study        | n/a Involved in the study |
| ☐ ☐ Antibodies                   | ☐ ☐ ChIP-seq |
| ☐ ☐ Eukaryotic cell lines        | ☐ ☐ Flow cytometry |
| ☐ ☐ Palaeontology and archaeology| ☐ ☐ MRI-based neuroimaging |
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| ☐ ☐ Human research participants  |         |
| ☐ ☐ Clinical data                |         |
| ☐ ☐ Dual use research of concern |         |

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 C51), CD4 (clone L200, BV510), CD45 (clone 0D8-1283, BV615), CD95 (clone DX2, BV711), IFN-γ (clone B27, BV395), IL2 (clone MQ1-17H12, APC), CD3 (clone SP34.2, Alexa 700), for 800CW-conjugated goat anti-human secondary antibody (Li-COR), for rhesus IgG1, IgG2, IgG3, IgM (NIH NHP Reagent Program), mouse anti-mouse IgG-PE antibody (Southern Biotech), anti-CD107a (PE-Cy7, BD), anti-CD56 (PE-Cy7, BD), anti-MIP-1B (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: Commerially 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).
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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.