Optimization of non-coding regions for a non-modified mRNA COVID-19 vaccine

The CVnCoV (CureVac) mRNA vaccine for severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) was recently evaluated in a phase 2b/3 efficacy trial in humans. CV2CoV is a second-generation mRNA vaccine containing non-modified nucleosides but with optimized non-coding regions and enhanced antigen expression. Here we report the results of a head-to-head comparison of the immunogenicity and protective efficacy of CVnCoV and CV2CoV in non-human primates. We immunized 18 cynomolgus macaques with two doses of 12 μg lipid nanoparticle-formulated CVnCoV or CV2CoV or with sham (n = 6 per group). Compared with CVnCoV, CV2CoV induced substantially higher titres of binding and neutralizing antibodies, memory B cell responses and T cell responses as well as more potent neutralizing antibody responses against SARS-CoV-2 variants, including the Delta variant. Moreover, CV2CoV was found to be comparably immunogenic to the BNT162b2 (Pfizer) vaccine in macaques. Although CVnCoV provided partial protection against SARS-CoV-2 challenge, CV2CoV afforded more robust protection with markedly lower viral loads in the upper and lower respiratory tracts. Binding and neutralizing antibody titres were correlated with protective efficacy. These data demonstrate that optimization of non-coding regions can greatly improve the immunogenicity and protective efficacy of a non-modified mRNA SARS-CoV-2 vaccine in non-human primates.

Efficacy results in humans have recently been reported for the CVnCoV (CureVac) mRNA vaccine in the phase 2b/3 HERALD trial in a population that included multiple viral variants. In this trial, the observed vaccine efficacy against symptomatic coronavirus disease 2019 (COVID-19) was approximately 48% and 53% in the overall study population and in a subgroup of participants 18–60 years of age, respectively. CV2CoV is a second-generation mRNA vaccine that incorporates modifications of non-coding regions that were selected by empiric screening for improved antigen expression. Both CVnCoV and CV2CoV are based on RNAactive technology and consist of non-chemically modified sequence-engineered mRNA without pseudouridine. Both vaccines encode the same full-length, pre-fusion stabilized severe respiratory syndrome coronavirus-2 (SARS-CoV-2) spike protein and are encapsulated in lipid nanoparticles (LNPs) with identical composition. CV2CoV has been engineered with different non-coding regions flanking the open reading frame, which have previously been shown to improve transgene expression and protection against SARS-CoV-2 in ACE2-transgenic mice. Specifically, CV2CoV includes 5’ untranslated region (UTR) HSD17B4 and 3’ UTR PSMB3 elements followed by a histone stem–loop motif and a poly(A) sequence. In the present study, we make a head-to-head comparison of the immunogenicity and protective efficacy of CVnCoV and CV2CoV against SARS-CoV-2 challenge in non-human primates.

Vaccine immunogenicity

We immunized 18 cynomolgus macaques intramuscularly with 12 μg CVnCoV, 12 μg CV2CoV or sham vaccine. The animals were primed at week 0 and were boosted at week 4. No clinical adverse effects were observed following vaccination. To assess innate immune responses, sera were isolated from all animals 24 h after the first vaccination to evaluate innate cytokine responses. CV2CoV induced higher levels of IFNα2a, IP-10 and MIP-1 than CVnCoV (P = 0.0152, P = 0.0152 and P = 0.0411, respectively).
Binding antibody responses were assessed by performing receptor-binding domain (RBD)-specific enzyme-linked immunosorbent assays (ELISAs) at multiple time points following immunization\(^{15,16}\). At week 2, binding antibody titres were detected only with CV2CoV and not with CVnCoV, with median values of 25 (range, 25–25) and 799 (range, 62–2,010) for CVnCoV and CV2CoV, respectively (Fig. 2a). One week after the week 4 boost, the binding antibody titres were increased, with median values of 55 (range, 20–302) and 131 (range, 62–578) for CVnCoV and CV2CoV, respectively. By week 8, the binding antibody titres had increased in the CVnCoV group but were still >50 times lower than those in the CV2CoV group (\(P = 0.0043\)), with median values of 214 (range, 47–1,238) and 14,827 (range, 2,133–37,079), respectively.

Neutralizing antibody responses were assessed by pseudovirus neutralization assay using the vaccine-matched SARS-CoV-2 wild-type (WT) WA1/2020 strain\(^{15–17}\). The neutralizing antibody titres followed a trend similar to that of the binding antibody titres (Fig. 2b). At week 2, neutralizing antibodies were detected only with CV2CoV and not with CVnCoV, with median values of 20 (range, 20–20) and 131 (range, 62–578) for CVnCoV and CV2CoV, respectively (Fig. 2b). One week after the week 4 boost, the neutralizing antibody titres were increased, with median values of 55 (range, 20–302) and 15,827 (range, 3,985–81,081) for CVnCoV and CV2CoV, respectively. By week 8, the neutralizing antibody titres had increased in the CVnCoV group but were still >20 times lower than those in the CV2CoV group (\(P = 0.0022\)), with median values of 196 (range, 20–405) and 4,752 (range, 414–6,793), respectively.

At week 6, the median pseudovirus neutralizing antibody titres against the D614G, B.1.1.7 (Alpha) and B.1.351 (Beta) variants for CVnCoV were 121, 101 and 189, respectively, while they were 4,962, 1,813 and 735 for CV2CoV (Fig. 2c). The median pseudovirus neutralizing antibody titres against the C.37 (Lambda), B.1.617.1 (Kappa) and B.1.617.2 (Delta) variants for CVnCoV were 516, 158 and 36, respectively, while they were 1,195, 541 and 568 for CV2CoV (Extended Data Fig. 2). The pseudovirus neutralizing antibody titres induced by CV2CoV were higher than those induced by CVnCoV for the WT (WA1/2020), D614G, B.1.1.7 (Alpha), B.1.351 (Beta), C.37 (Lambda), B.1.617.1 (Kappa) and B.1.617.2 (Delta) strains (\(P = 0.0043, 0.0087, 0.0043, 0.1320, 0.026, 0.0022\) and 0.0043, respectively). Taken together, these data show that CV2CoV induces substantially higher pseudovirus neutralizing antibody titres against SARS-CoV-2 variants than CVnCoV.

Live-virus neutralizing antibody titres\(^1\) were largely consistent with those for the pseudovirus. The live-virus neutralizing antibody responses elicited by CV2CoV were higher than those elicited by CVnCoV against the WA1/2020 and B.1.617.2 (Delta) strains (\(P = 0.0466\) and 0.0152, respectively), with similar trends for B.1.1.7 (Alpha) and B.1.351 (Beta) (\(P = 0.0628\) and 0.1450, respectively) (Fig. 2d).
The CVnCoV-immunized animals showed peak medians of 4.92 (range, 2.40–6.61) log_{10}-transformed sgRNA copies per ml in the BAL and 6.42 (range, 4.46–7.81) log_{10}-transformed sgRNA copies per swab in the nasal swab samples (Fig. 3). The CVnCoV-immunized animals exhibited peak medians of 2.90 (range, 1.70–4.64) log_{10}-transformed sgRNA copies per ml in the BAL and 3.17 (range, 2.59–5.63) log_{10}-transformed sgRNA copies per swab in the nasal swab samples (Fig. 3), with resolution of sgRNA levels in the BAL samples by day 2 in most animals and by day 4 in all animals. Overall, CVnCoV resulted in significantly lower peak viral loads than CVnCoV in both the BAL (P = 0.041) and nasal swab (P = 0.0087) samples (Fig. 4a, b).

We next evaluated the immune correlates of protection. The log_{10}-transformed ELISA and neutralizing antibody titres at week 6 were inversely correlated with the peak log_{10}-transformed sgRNA copies per ml in the BAL samples (P = 0.0008, R = −0.7148 and P = 0.0015, R = −0.6912, respectively, by two-sided Spearman rank-correlation test) (Fig. 4c, d) and with the peak sgRNA copies per nasal swab in the nasal swab samples (P = 0.0001, R = −0.8346 and P = 0.0001, R = −0.8766, respectively, by two-sided Spearman rank-correlation test) (Fig. 4d, f). Consistent with prior observations from our laboratory and others, these findings suggest that binding and neutralizing antibody titres are important correlates of protection for these SARS-CoV-2 vaccines in non-human primates. Similar correlates of protection were observed with viral loads assessed as area under the curve (Extended Data Fig. 4). Moreover, we assessed infectious virus titres by TCID_{50} assay on day 2 after challenge, which showed no detectable virus in five of six animals in the CV2CoV group (Extended Data Fig. 5).

Following challenge, we observed anamnestic binding and neutralizing antibody responses in all CVnCoV-vaccinated animals and in a subset of the CV2CoV-vaccinated animals (Extended Data Fig. 6). On day 10 after challenge, the animals were necropsied, and their lung tissues were evaluated by histopathology. Viral replication was largely resolved by day 10 in the animals vaccinated with CVnCoV and CV2CoV, and those with sham treatment had higher cumulative lung pathology scores (CVnCoV animals compared with sham controls, P = 0.0368; CV2CoV animals compared with sham controls, P = 0.0065) (Extended Data Fig. 3c). IL-4 responses were detected in both groups but were higher in the CVnCoV group (P = 0.022 and P = 0.0152, respectively) (Extended Data Fig. 3a, b). T cell responses were assessed by interferon γ (IFNγ) and interleukin (IL)-4 enzyme-linked immunosorbent spot (ELISPOT) assay using pooled spike peptides at week 6. IFNγ responses were detected in both groups but were higher in the CV2CoV group (P = 0.0065) (Extended Data Fig. 3c). IL-4 responses were not detectable, suggesting that CVnCoV and CV2CoV induce T helper type 1-biased responses (Extended Data Fig. 3d).

**Protective efficacy**

All animals were challenged at week 8 with 1.0 × 10^{5} median tissue culture infectious doses (TCID_{50}) of the SARS-CoV-2 WA1/2020 strain via the intranasal and intratracheal routes. Viral loads were assessed in bronchoalveolar lavage (BAL) and nasal swab samples collected on days 1, 2, 4, 7 and 10 following challenge by quantitative PCR with reverse transcription (RT–PCR) specific for subgenomic RNA (sgRNA). The sgRNA levels in the BAL and nasal swab samples in the sham group peaked on day 2 and largely resolved by day 10. The sham controls had peak medians of 6.02 (range, 4.62–6.81) log_{10}-transformed sgRNA copies per ml in the BAL and 7.35 (range, 5.84–8.09) log_{10}-transformed sgRNA copies per swab in the nasal swab samples on day 2 (Fig. 3). The CV2CoV-immunized animals showed peak medians of 4.92 (range, 2.40–6.61) log_{10}-transformed sgRNA copies per ml in the BAL and 6.42 (range, 4.46–7.81) log_{10}-transformed sgRNA copies per swab in the nasal swab samples (Fig. 3). The CV2CoV-immunized animals exhibited peak medians of 2.90 (range, 1.70–4.64) log_{10}-transformed sgRNA copies per ml in the BAL and 3.17 (range, 2.59–5.63) log_{10}-transformed sgRNA copies per swab in the nasal swab samples (Fig. 3), with resolution of sgRNA levels in the BAL samples by day 2 in most animals and by day 4 in all animals. Overall, CV2CoV resulted in significantly lower peak viral loads than CVnCoV in both the BAL (P = 0.041) and nasal swab (P = 0.0087) samples (Fig. 4a, b).

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Animals compared with sham controls, \( P = 0.0022 \) (Extended Data Fig. 7a). Animals in the sham group also had more lung lobes affected (Extended Data Fig. 7b) and more extensive lung lesions, with a greater proportion of lung lobes showing evidence of interstitial inflammation, alveolar inflammatory infiltrates and type II pneumocyte hyperplasia (Extended Data Fig. 7c–h). No significant eosinophilia was observed. The pathological lesions in vaccinated animals were similar to those observed for animals in the sham group (Extended Data Fig. 7i–l) but were overall fewer in number and more focal in distribution.

**Discussion**

CV2CoV elicited substantially greater humoral and cellular immune responses and provided significantly improved protective efficacy against SARS-CoV-2 challenge as compared with CVnCoV in macaques. These data suggest that optimization of non-coding elements of the mRNA backbone can substantially improve the immunogenicity and protective efficacy of mRNA vaccines. Both CVnCoV and CV2CoV contain only non-modified nucleosides with no pseudouridine or derivates, and CV2CoV has previously been shown to lead to higher antigen expression than CVnCoV in cell culture. The neutralizing antibody titres induced by CV2CoV were comparable in macaques to those reported for BNT162b2 in a prior study. As previously reported for other vaccines, the neutralizing antibody titres against certain SARS-CoV-2 variants, such as the B.1.351 (Beta) and B.1.617.2 (Delta) variants, were lower than those against the parental strain WA1/2020. Although our challenge virus in this study was SARS-CoV-2 WA1/2020, the neutralizing antibody titres elicited by CV2CoV to viral variants exceeded the values we previously reported as threshold titres for protection of (50–100)\(^{17,19,22}\). However, future studies will be required to directly assess the protective efficacy of CV2CoV against SARS-CoV-2 variants of concern in non-human primates.

CV2CoV induced both antigen-specific memory B cell responses and T cell responses. Although the correlates of protection in this study were binding and neutralizing antibody titres\(^{34,35}\), it is likely that CD8\(^+\) T cells contribute to viral clearance in tissues\(^{36,37}\). We previously reported that depletion of CD8\(^+\) T cells partially abrogated protective efficacy against SARS-CoV-2 re-challenge in convalescent macaques. Memory B cells might contribute to the durability of antibody responses\(^{38,39}\), B cell germinal centre responses and the durability of protective efficacy following CV2CoV vaccination remain to be determined. Moreover,
although this study was not specifically designed as a safety study, it is worth noting that we did not observe any adverse effects following CVnCoV or CV2CoV vaccination, nor did we observe unexpected or enhanced pathology in the vaccinated animals at necropsy.

In summary, our data show that optimization of non-coding regions in a SARS-CoV-2 mRNA vaccine can substantially improve its immunogenicity against multiple viral variants and can enhance its protective efficacy against SARS-CoV-2 challenge in macaques. The improved characteristics of CV2CoV over those of CVnCoV might translate into increased efficacy in humans; accordingly, clinical trials of CV2CoV are planned.

Online content
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The serum levels of 19 analytes that have been associated with immune regulations and were approved by the Bioqual Institutional Animal Care and Use Committee.

Cytokine analyses
The serum levels of 19 analytes that have been associated with immune response to viral infection were tested using the U-PLEX Viral Combo 1 (NP) kit (K15069L-1) obtained from Meso Scale Discovery. The 19 analytes and their detection limits (LLODs) included G-CSF (1.5 pg ml−1), IFN-gamma (7.4 pg ml−1), IL-1β (0.15 pg ml−1), IL-2 (0.06 pg ml−1), IL-5 (0.24 pg ml−1), IL-6 (0.33 pg ml−1), IL-7 (1.5 pg ml−1), IL-8 (0.15 pg ml−1), IL-9 (0.14 pg ml−1), IL-10 (0.14 pg ml−1), IL-12p70 (0.54 pg ml−1), IP-10 (0.49 pg ml−1), MCP-1 (0.74 pg ml−1), MIP-1α (7.7 pg ml−1), TNF (0.54 pg ml−1) and VEGF-A (2.0 pg ml−1). All serum samples were assayed in duplicate. The assay was performed by the Metabolism and Mitochondrial Research Core (Beth Israel Deaconess Medical Center, Boston, MA) following the manufacturer’s instructions. The assay plates were read by a Meso QuickPlex SQ 120 instrument, and the data were analysed using Discovery Workbench 4.0 software.

ELISA
RBD-specific binding antibodies were assessed by ELISA as described previously16,17. In brief, 96-well plates were coated with 1 μg ml−1 SARS-CoV-2 RBD protein (40592-VNAH, Sino Biological) in 1× DPBS and were incubated at 4°C overnight. After incubation, the plates were washed three times with wash buffer (0.05% Tween-20 in 1× DPBS) and were blocked with 350 μl casein block per well for 2–3 h at room temperature. After incubation, the block solution was discarded, and the plates were blotted dry. Serial dilutions of heat-inactivated serum diluted in casein block were added to the wells, and the plates were incubated for 1 h at room temperature. Next, the plates were washed three times and were then incubated for 1 h with a 1:1,000 dilution of anti-macaque IgG HRP (NIH NHP Reagent Program) at room temperature in the dark. The plates were then washed three more 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 of SeraCare KPL TMB Stop solution per well. The absorbance at 450 nm was recorded using a VersaMax or Omega microplate reader. The ELISA endpoint titres were defined as the highest reciprocal serum dilution that yielded an absorbance >0.2, and the log_{10} endpoint titres are reported. The immunological assays were performed with blinding.

Pseudovirus neutralization assay
SARS-CoV-2 pseudoviruses encoding a luciferase reporter gene were generated as described previously15. In brief, the packaging plasmid pSpAX2 (AIDS Resource and Reagent Program), luciferase reporter plasmid pLenti-CMV Puro-Luc (Addgene) and spike protein-encoding pcDNA3.1-SARS-CoV-2 SΔCT plasmid of variants were co-transfected into HEK293T cells by Lipofectamine 2000 (ThermoFisher Scientific). Pseudoviruses of SARS-CoV-2 variants were generated by using the WAI/2020 strain (Wuhan/WIV04/2019; GISAID accession ID, EPI_ISL_402124), the strain with a D614G mutation, the B.1.1.7 variant (GISAID accession ID, EPI_ISL_601443), the B.1.351 variant (GISAID accession ID, EPI_ISL_712096), the C37 variant (GenBank ID, QRX62290), the B.1.671.1 variant (GISAID accession ID, EPI_ISL_1384866) and the B.1.617.2 variant (GISAID accession ID, EPI_ISL_2020950). Supernatants containing the pseudotype viruses, which were purified by centrifugation and filtration with a 0.45-μm filter, were collected 48 h after transfection. To determine the neutralization activity of the plasma or serum samples from the animals studied, HEK293T-hACE2 cells were seeded in 96-well tissue culture plates at a density of 1.75 × 10^4 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 being added to the HEK293T-hACE2 cells. The cells were lysed 48 h after infection in Steady-Glo Luciferase Assay buffer (Promega) according to the manufacturer’s instructions. The 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 WAI/2020, B.1.1.7, B.1.351 and B.1.617.2 viruses were designed to encode nanoluciferase (nLuc) and were recovered via reverse genetics18. One day before the assay, Vero E6 USAMRID cells were plated at 20,000 cells per well in clear-bottomed, black-walled plates. The cells were inspected to ensure confluency on the day of the assay. The serum samples were tested at a starting dilution of 1:20 and were serially diluted threefold for up to nine dilution spots. The serially diluted serum samples were added in duplicate to the cells at 75 plaque-forming units at 37°C with 5% CO2. The cells were lysed 48 h later, and the luciferase activity was measured using Nano-Glo Luciferase Assay System (Promega) according to the manufacturer’s specifications. The 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.
B cell immunophenotyping

Fresh peripheral blood mononuclear cells were stained with Aqua live/dead dye (Invitrogen) for 20 min, washed with 2% FBS in DPBS and suspended in 2% FBS in DPBS with Fc Block (BD) for 10 min, followed by staining with monoclonal antibodies against CD45 (clone D058-1283, BV505), CD8 (clone SB1, BV601), CD19 (clone J519, BV421), CD25 (clone M-A52, BV515), CD44 (clone HCD44, BV589), CD103 (clone H-23, BV378), CD141 (clone H-8, BV615), and negative (medium only) controls were included in each assay. The plates 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 were incubated at 37 °C, 5.0% CO2, for 4 d, and the cell monolayers were visually inspected for cytopathic effects. TCID₅₀ was calculated using the Read–Muench formula.

Histopathology

At the time of fixation, lungs were suffused with 10% formalin to expand the alveoli. All tissues were fixed in 10% formalin and block-sectioned at 5 μm. The slides were baked for 30–60 min at 65 °C, deparaffinized in xylen, rehydrated through a series of graded ethanol to distilled water and then stained with haematoxylin and eosin. Blinded histopathological evaluation was performed by a board-certified veterinary pathologist (A.J.M.).

Statistical analyses

Statistical analyses were performed using GraphPad Prism (version 9.0) software (GraphPad Software), and comparisons between groups were performed using a two-tailed non-parametric Mann–Whitney U test. P values of less than 0.05 were considered as significant. Correlations were assessed by applying two-sided Spearman rank-correlation tests.

Data availability

All data are available in the manuscript and its Supplementary Information. Source data are provided with this paper.

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Author contributions

S.R., B.P., S.O.M., N.R. and D.H.B. designed the study, M.S.G., J.Y., A. Chandrashekar, N.B.M., X.H., J.L., K.M., A.M., D.R.M., R.S.B., A.C.M.B., V.G., D.H., S.P., D.S., O.S. and J.B. performed immunological and virological assays. X.L. and A.C.C. performed cytokine
analysis. L.P., D.V., Z.F., J.Y.-O., J.M., R.B., A. Cook, E.T., H.A. and M.G.L. led the clinical care of the animals. M.S.G. and D.H.B. wrote the manuscript with all co-authors.

Competing interests S.R., B.P., N.R. and S.O.M. are employees of CureVac AG, Tübingen, Germany, a publicly listed company developing mRNA-based vaccines and immunotherapeutics. Authors may hold shares in the company. S.R., B.P. and N.R. are inventors on several patents on mRNA vaccination and use thereof. The other authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41586-021-04231-6.

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

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Extended Data Fig. 1 | Innate cytokine induction following mRNA immunization (6/group). Sera isolated 24h post first injection were analyzed for a panel of 19 cytokines associated with viral infection using a U-PLEX Viral Combo kit from Meso Scale Discovery. Changes in cytokine levels above the detection limits were detectable for 9 cytokines. Each dot represents an individual animal, bars depict the median and the dotted line shows limit of detection. Statistical analysis was performed using two-tailed nonparametric Mann-Whitney test.
Extended Data Fig. 2 | Neutralizing antibody titers against variants.
Animals (6/group) were vaccinated twice with 12μg of CVnCoV or CV2CoV on d0 and d28 or remained untreated as negative controls (sham). Sera isolated on d42 (week 6) were analyzed for pseudovirus neutralizing antibody titers against C.37 (Lambda), B.1.617.1 (Kappa) and B.1.617.2 (Delta) variants. Each dot represents an individual animal, bars depict the median and the dotted line shows limit of detection.
Extended Data Fig. 3 | Memory B and T cell immune responses day 42 following immunization. PBMCs from negative control (sham), CVnCoV or CV2CoV vaccinated animals (6/group) isolated on d42 of the experiment were stained for (a) RBD and (b) Spike-specific activated memory B cells and analyzed by high-parameter flow cytometry. IFNγ responses to pooled spike peptides were analyzed via ELISPOT (c). Each dot represents an individual animal, bars depict the median and the dotted line shows limit of detection. Statistical analysis was performed using two-tailed nonparametric Mann-Whitney test. PBMC = peripheral blood mononuclear cell; SFC = spot forming cells.
Extended Data Fig. 4 | Binding and neutralizing antibody titers correlate with protection against SARS-CoV-2. Summary of area under curve (AUC) viral load values following SARS-CoV-2 challenge in BAL and nasal swab samples (6/group) (a, b); antibody correlates of protection for binding antibodies (c, d) and neutralizing antibodies (e, f). Statistical analysis was performed using two-tailed nonparametric Mann-Whitney test. Correlations was analyzed by two-sided Spearman rank-correlation test. NAbs = neutralizing antibodies, BAL = bronchoalveolar lavage NS = nasal swab.
Extended Data Fig. 5 | Infectious virus titers after SARS-CoV-2 challenge (6/group). Infectious virus titers of BAL and nasal swab samples collected 2 days post challenge were analyzed by TCID$_{50}$ assays. Each dot represents an individual animal, bars depict the median and the dotted line shows limit of detection. Statistical analysis was performed using two-tailed nonparametric Mann-Whitney test.
Extended Data Fig. 6 | Post-challenge binding and neutralizing antibody responses (6/group). Negative control (sham) or animals vaccinated on d0 and d28 of the experiment with 12 μg of CVnCoV or CV2CoV as indicated were subjected to challenge infection using 1.0×10⁵ TCID₅₀ SARS-CoV-2 via intranasal (IN) and intratracheal (IT) routes. (a) Titers of RBD binding antibodies and (b) pseudovirus neutralizing antibodies against ancestral SARS-CoV-2 strain were evaluated before (week 8) and a week after challenge infection (week 9). Each dot represents an individual animal, bars depict the median and the dotted line shows limit of detection. Statistical analysis was performed using two-tailed nonparametric Mann-Whitney test. NAbs = neutralizing antibodies.
Extended Data Fig. 7  CVnCoV and CV2CoV protect the lungs from pathological changes upon viral challenge (6/group). Eight lung lobes (4 sections from right and left, caudal to cranial) were assessed and scored (1-4) for each of the following lesions: 1) Interstitial inflammation and septal thickening 2) Eosinophilic interstitial infiltrate 3) Neutrophilic interstitial infiltrate 4) Hyaline membranes 5) Interstitial fibrosis 6) Alveolar infiltrate, macrophage 7) Alveolar/Bronchoalveolar infiltrate, neutrophils 8) Syncytial cells 9) Type II pneumocyte hyperplasia 10) Broncholar infiltrate, macrophage 11) Broncholar infiltrate, neutrophils 12) BALT hyperplasia 13) Bronchiolar/peribronchiolar inflammation 14) Perivascular, mononuclear infiltrates 15) Vessels, endothelialitis. Each feature assessed was assigned a score of 0 = no significant findings; 1 = minimal; 2 = mild; 3 = moderate; 4 = marked/severe. (a) Cumulative scores per animal (b) Cumulative scores per lung lobe. Individual animals are represented by symbols. Representative histopathology from sham vaccinated (c–h), CVnCoV vaccinated (i, j), and CV2CoV vaccinated (k, l) animals showing (c, d, inset) alveolar macrophage infiltrate, (e, f, inset) syncytial cells (arrowheads) and type II pneumocyte hyperplasia, inset (g, h, inset) bronchiolar epithelial necrosis with neutrophilic infiltrates (i) alveolar neutrophilic infiltrate and alveolar septal thickening (j) focal consolidation with inflammation composed of macrophages, neutrophils, and syncytial cells (k) focal pneumocyte hyperplasia, syncytial cells and inflammatory infiltrates (l) peribronchiolar inflammation. Statistical analysis was performed using two-tailed nonparametric Mann-Whitney test. Scale bars: 100 microns (c), 50 microns (e, g) 20 microns (i-l). BALT bronchus associated lymphoid tissue.
Reporting Summary

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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

FlowJo Version 10.6.2 was used

Data analysis

Virologic and immunologic data analysis was performed using GraphPad Prism 9.0.0 [GraphPad Software]. Specifically, cytokine data analysis was done using DISCOVERY WORKBENCH® 4.0 software.

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 Portfolio guidelines for submitting code & software for further information.

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All data are available in the manuscript and the supplementary material.
Field-specific reporting

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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=18 animals (N=6 animals for each vaccine or sham control groups). Based on our previous experience with SARS-CoV-2 in cynomolgus as well as rhesus macaques, this sample size provides sufficient power to determine differences in protective efficacy of both vaccinated groups 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. Attempts in replication were successful. |
| Randomization | Animals were balanced for age and gender and otherwise randomly allocated to groups. All other sample randomization throughout the study was random. |
| 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 |
| - Animals and other organisms | |
| - Human research participants | |
| - Clinical data | |
| - Dual use research of concern | |

Antibodies

For ELISA assay: anti-macaque IgG HRP (NIH NHP Reagent Program); for ELISPOT assay: mouse anti-human IFN-γ monoclonal antibody (BD Pharmingen), IL-4 capture monoclonal antibody (Mabtech), rabbit polyclonal anti-human IFN-γ (U-Cytech) and Streptavidin-alkaline phosphatase antibody (IFN-γ; Southern Biotechnology/IL-4 (Mabtech). For B cell ICS assay: monoclonal antibodies against CD45 (clone D05B-1283, BUV805), CD3 (clone SP34-2, APC-Cy7), CD7 (clone M-T701, Alexa700), CD123 (clone 6H6, Alexa700), CD11c (clone 3.9, Alexa700), CD20 (clone 2H7, PE-Cy5), IgA (goat polyclonal antibodies, APC), IgG (clone G18-145, BUV737), IgM (clone G20-127, BUV396), IgD (goat polyclonal antibodies, PE), CD80 (clone L307-4, BV786), CD95 (clone DX2, BV711), CD27 (clone M-T71, BUV563), CD21 (clone B-ly4, BV605), CD14 (clone M5E2, BV570) and CD138 (clone DI-101, PE-CF594).

Validation

mAbs were used according to manufacturer’s instructions and previously published methods; mAbs were validated and titrated for specificity prior to use

Eukaryotic cell lines

Policy information about [cell lines](http://cell-lines).

| Cell line source(s) | HEK293T and Vero E6 cells Commerically purchased (ATCC) |
| Authentication | Cell lines were not authenticated. |
| Mycoplasma contamination | Negative for mycoplasma |
Animals and other organisms

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

| Category                | Details                                                                 |
|-------------------------|-------------------------------------------------------------------------|
| Laboratory animals      | 18 male and female cynomolgus macaques (Macaca fascicularis), 3-20 years old |
| Wild animals            | None                                                                    |
| Field-collected samples | None                                                                    |
| Ethics oversight        | All 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). |

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