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Neutralizing antibodies targeting the receptor binding domain (RBD) of the SARS-CoV-2 Spike (S) are among the most promising approaches against coronavirus disease 2019 (COVID-19)^{1,2}. We developed a bispecific, IgG1-like molecule (CoV-X2) based on two antibodies derived from COVID-19 convalescent donors, C121 and C135³. CoV-X2 simultaneously binds two independent sites on the RBD and, unlike its parental antibodies, prevents detectable S binding to Angiotensin-Converting Enzyme 2 (ACE2), the virus cellular receptor. Furthermore, CoV-X2 neutralizes SARS-CoV-2 and its variants of concern, as well as the escape mutants generated by the parental monoclonals. In a novel animal model of SARS-CoV-2 infection with lung inflammation, CoV-X2 protects mice from disease and suppresses viral escape. Thus, simultaneous targeting of non-overlapping RBD epitopes by IgG-like bispecific antibodies is feasible and effective, combining into a single molecule the advantages of antibody cocktails.

The COVID-19 pandemic prompted an unprecedented effort to develop effective countermeasures against SARS-CoV-2. Pre-clinical data and phase III clinical studies indicate that monoclonal antibodies (mAbs) could be effectively deployed for prevention or treatment during the viral symptoms phase of the disease^{1,2}. Cocktails of two or more mAbs are preferred over a single antibody for increased efficacy and prevention of viral escape. However, this approach requires increased manufacturing costs and volumes, which are problematic at a time when the supply chain is under pressure to meet the high demand for COVID-19 therapeutics, vaccines and biologics in general⁴. Cocktails also complicate formulation^{5,6} and hinder novel strategies like antibody delivery by viral vectors or by non-vectored nucleic acids^{7,8}. Instead, multispecific antibodies embody the advantages of a cocktail within a single molecule.

To this avail, we employed structural information⁹ and computational simulations to design bispecifics that would simultaneously bind to (i) independent sites on the same RBD and (ii) distinct RBDs on a S trimer. Out of several designs evaluated by atomistic Molecular

Dynamics simulations, 4 were produced and CoV-X2 was the most potent neutralizer of SARS-CoV-2 pseudovirus, with half-maximal inhibitory concentration (IC₅₀) = 0.04 nM (5.8 ng/mL) (Extended Data Fig. 1). CoV-X2 is a human-derived, CrossMab-format IgG1-like bispecific antibody¹⁰ resulting from the combination of the Fragment antigen binding (Fab) of mAbs C121 and C135, two potent SARS-CoV-2 neutralizers³. Structural predictions showed that CoV-X2, but not its parental monoclonals, can bind bivalently to all RBD conformations on the S trimer, preventing ACE2 access (Fig. 1a and Extended Data Fig. 2)¹¹.

CoV-X2 bound with low nanomolar affinity to RBD, S trimer, and to several mutants, including the naturally occurring variants B.1 (D614G in S protein), B.1.1.7 (N501Y in RBD) and B.1.351 (K417N, E484K and N501Y in RBD)^{12,13}, and the escape mutants of the parental mAbs¹⁴ (Extended Data Figs. 3–5).

CoV-X2 also bound to pre-formed C121/RBD and C135/RBD complexes, thus confirming that both of its arms are functional (Fig. 1b, c). Next, an avidity assay by Surface Plasmon Resonance (SPR) was used to experimentally confirm the computational prediction that CoV-X2

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can simultaneously engage two sites on the same RBD (Methods, Fig. 1d and Extended Data Fig. 6). Avidity occurs when IgGs bind bivalently to antigens, resulting in slower dissociation rates (k_d) (Extended Data Fig. 6a). Accordingly, C121 and C135 IgG showed avidity at high antigen concentrations due to inter-molecular binding of adjacent RBDs; at lower antigen concentrations the dissociation rate was instead faster since inter-molecular binding was prevented by the increased distance between RBD molecules, resulting in loss of avidity. Intra-molecular avidity is not possible for C121 and C135 since a single epitope is available on each RBD molecule. By contrast, CoV-X2 maintained avidity even at low antigen concentrations, indicating bivalent, intra-molecular binding (Fig. 1d and Extended Data Fig. 6). ELISA assays were then performed to evaluate the ability of CoV-X2 to inhibit the binding of recombinant ACE2 to the S trimer (Fig. 1e, f). In line with the structural information⁹, C135 did not affect the ACE2/S interaction. C121, which occupies the ACE2 binding site on the RBD, prevented ACE2 binding but only partially. By contrast, ACE2 binding was not detected in the presence of CoV-X2, suggesting a synergistic effect by the two moieties composing the bispecific.

To assess the neutralizing ability of CoV-X2 *in vitro*, we first used SARS-CoV-2 pseudoviruses¹⁵. The bispecific neutralized pseudovirus carrying wild-type SARS-CoV-2 S at sub-nanomolar concentrations ($IC_{50} = 0.04$ nM (5.8 ng/mL); $IC_{90} = 0.3$ nM (44 ng/mL)), which was similar or better than the parental IgGs and >100-fold better IC_{50} than the parental Fabs (Fig. 1g). CoV-X2 remained effective against pseudoviruses bearing escape mutations that made them resistant to the individual mAbs (Fig. 1g)¹⁴ and against a pseudovirus with RBD mutations found in the B.1.351 variant (first reported in South Africa, $IC_{50} = 1.3$ nM (191 ng/mL); Extended data Fig. 5). To confirm CoV-X2 efficacy, we performed plaque reduction neutralization assays with infectious virus. CoV-X2 efficiently neutralized: SARS-CoV-2 ($IC_{50} = 0.9$ nM); the D614G variant first appearing in Europe (B.1, $IC_{50} = 0.2$ nM); the B.1.1.7 variant first observed in the United Kingdom ($IC_{50} = 0.2$ nM); the P.1 variant first isolated in Brazil ($IC_{50} = 2.1$ nM) and B.1.351 first isolated in South Africa ($IC_{50} = 12$ nM; Fig. 1h). The latter two have almost identical mutations in the RBD, the only difference being N vs. T at position 417, which does not interact with CoV-X2. Nonetheless, neutralization of B.1.351 was lower, suggesting either some conformational differences in the RBD or long-range effects deriving from other mutations in the S protein. A similar behavior is seen with the wild-type sequence (D614), which has lower neutralization than G614 even if no other difference is present; a plausible explanation is that G614 makes the CoV-X2 epitopes more accessible by favoring the RBD 'up' conformation.¹⁶ We conclude that the *in vitro* binding and neutralizing properties of CoV-X2 make it preferable over its parental antibodies.

To assess the clinical potential of CoV-X2, we investigated its ability to protect animals from infection and disease. We first developed a novel mouse model in which human ACE2 (hACE2) is expressed by upper and lower respiratory tract cells upon inhalation of a modified Adeno Associated Virus (AAV-hACE2, see Methods, Fig. 2 and Extended Data Fig. 7).

This approach enables rapid production of large cohorts of animals and has the advantage of being applicable to wild-type and mutant mouse colonies, independently of age and gender. Moreover, since AAV vectors are only weakly immunogenic and cytotoxic, the system allows for prolonged expression of hACE2^{17–20} (Extended Data Fig. 7). SARS-CoV-2 infection of ACE2 humanized mice results in progressive weight loss, respiratory pathology and disease requiring culling on day 8 post infection (dpi, Fig. 2a–c and Extended Data Fig. 7).

To evaluate the protective effect of antibodies, hACE2 mice were treated either one day before SARS-CoV-2 infection (150 µg; Fig 2d, e, g, h) or 12 hours after challenge (250 µg; Fig 2d, f) and monitored over time. Upon intranasal infection with 1×10^4 pfu of SARS-CoV-2 (SARS-CoV-2/human/Czech Republic/951/2020), isotype control (antibody against E-protein of Zika virus) treated animals showed weight

loss starting at 3 dpi, and by 8 dpi most animals had lost approximately 25–30% of their body weight reaching humane endpoint (Fig. 2e, f). Infectious virus could be recovered from the lungs (Fig. 2g), viral RNA was detected also in the spleen (Fig. 2h) but not in the heart (data not shown). Lung pathology resembled severe COVID-19 in humans²¹ and was characterized by Diffuse Alveolar Damage (DAD; 50–80% of tissue area), alveolar replacement with infiltrates of immune cells and fibroblasts, thickened septa and infiltrations by activated macrophages with foamy cytoplasm (Fig. 2j). By contrast, animals treated with CoV-X2 maintained their body weight ($P < 0.0001$ at 4–8 dpi when compared to isotype; Fig. 2e; P values between all groups in Extended Data Table 1), had reduced viral RNA burden (Fig. 2g, h) and displayed neither macro- nor histopathological changes (DAD <5–10%, Fig. 2i, j). While at 2 dpi infectious virus was detected in all animals treated with either C121 (n=5), C135 (n=5) or control (n=8), it was only detected at low levels in one out of 5 mice receiving CoV-X2 (Fig 2g). At 5 dpi infectious virus could be readily recovered from controls (5 of 6), but only from 1 out of 10 animals treated with CoV-X2 (Fig 2g). Furthermore, CoV-X2 was protective also when administered 12 hours after SARS-CoV-2 challenge (Fig. 2d, f). Since none of the CoV-X2 treated mice exhibited symptoms at any time, we conclude that CoV-X2 protects mice from infection and disease.

Since monotherapy with C121 or C135 mAbs leads to virus escape *in vitro*¹⁴, we treated hACE2 mice with the individual antibodies and sequenced the virus. Only wild-type RBD sequences were obtained from controls (n=10). Instead, the virus in mice treated with C121 selected for a mutation resulting in E484D (5 of 5 mice that were analyzed at 8 dpi). C121 escape mutations at E484 were previously observed *in vitro*¹⁴ and changes at this residue (present also in the B.1.351 and P.1 variants) reduce neutralization by human sera by more than 10-fold²². E484D affects intermolecular H-bonds at the core of the C121/RBD interface and it is suggested to increase the RBD affinity for ACE2²³. Virus with D484 is pathogenic, since 7 out of 9 mice treated with C121 developed disease (Fig. 2e) and only D484 virus was found in their lungs. In contrast, and unlike the *in vitro* results¹⁴, no virus evasion or pathology was observed in mice treated with C135 (n=5; Fig. 2e and data not shown). In CoV-X2 treated animals, even though no infectious virus was retrieved (8 dpi, n=13) and no symptoms ever noticed, low levels of residual viral RNA could be detected in some animals after 40 cycles of PCR amplification: in 6 of 13 animals the virus sequence was wild-type and in 2 mice overlapping sequencing traces were consistent with coexistence of wild-type and D484. Thus, in those 2 of 13 animals with D484 CoV-X2 remained protective even if the mutation diluted the effective antibody concentration, presumably leaving only the C135 moiety active.

Monoclonal antibodies targeting the SARS-CoV-2 S are in advanced clinical trials and show promise against COVID-19¹². Concomitant use of multiple antibodies is preferred for increased efficacy and added resistance against viral evasion. Indeed, the virus can escape pressure by a single antibody *in vitro* and, as shown here, also in animals. Moreover, RBD mutations threatening the efficacy of single monoclonals have already been detected in virus circulating in minks and humans¹⁴, including mutations at the C121 and C135 epitopes (Extended Data Fig. 8). One disadvantage of antibody cocktails is the requirement for twice or more the development and production capacity than for single mAbs, which is a significant challenge in light of the augmented demand due to COVID-19 related vaccines and therapeutics on top of the need to maintain production of biologics for other diseases.⁴

Multispecific antibodies offer the advantages of cocktails in a single molecule. Indeed, we have shown that the CoV-X2 bispecific is more effective than the related monoclonals at inhibiting ACE2 binding; it has sub-nanomolar IC_{50} against a broader array of viral sequences; and it protects animals from SARS-CoV-2 even when C121, its potent parental mAb, fails due to the insurgence of viral escape. C135, the other parental mAb, did not generate escape in our animal experiment but readily generated them *in vitro*¹⁴. CoV-X2 is expected to be more

resistant to viral escape compared to monoclonals. Indeed, we have shown that CoV-X2 binds and neutralizes mutants not recognized by its parental mAbs as well as variants of concern that recently emerged in United Kingdom¹², South Africa¹³ and Brazil²⁴.

CoV-X2, unlike other multispecifics²⁵, is a fully human IgG-like molecule. As such, it has favorable developability and could be further engineered to alter effector functions. For example, the Fragment crystallizable (Fc) of CoV-X2 was already modified to modulate its interaction with Fc receptors and complement (LALA-PG mutations)²⁶ without affecting its antigen-binding properties. The LALA modification prevents Antibody Dependent Enhancement (ADE) of flavivirus infection²⁷ and it may be a desirable modification also in the context of SARS-CoV-2, since cellular and animal experiments with coronaviruses, including SARS-CoV^{28,29}, support the possibility of ADE. Other modifications, like LS²⁶ for increased half-life, are easily achievable. Finally, CoV-X2 is human-derived and produced in a format (CrossMab) already shown to be safe in clinical trials³⁰, which further supports its developability. Thus, IgG-like bispecifics are worth adding to the arsenal employed to combat SARS-CoV-2 and its plausible future mutations.

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/s41586-021-03461-y>.

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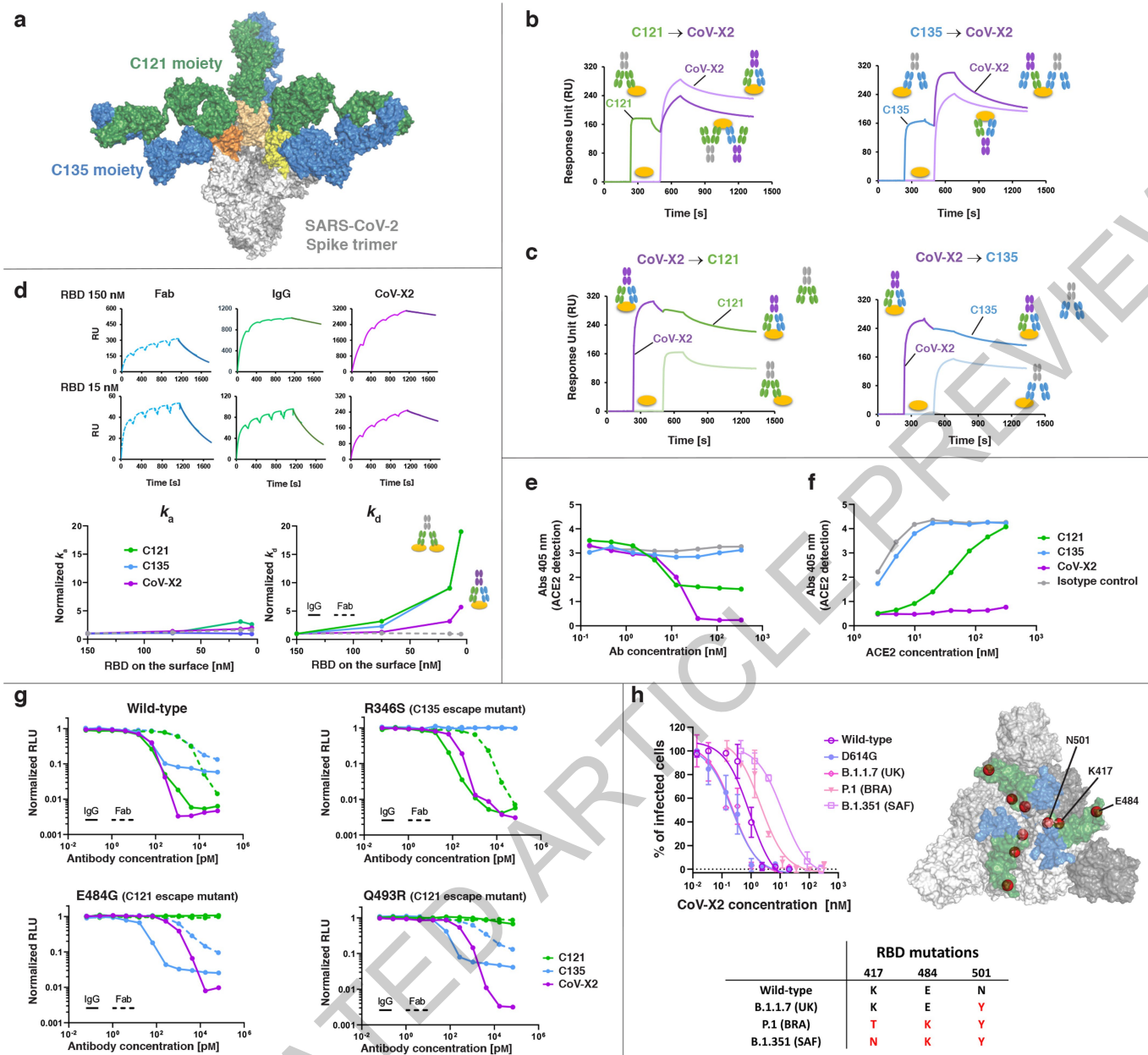


Fig. 1 | Biochemical and *in vitro* neutralizing properties of CoV-X2 are superior to its parental mAbs. **a**, Computational simulations predict bivalent binding of CoV-X2 to all three RBDs on the S trimer (see also Extended Data Fig. 2). Green and blue are C121 and C135 moieties, respectively; RBDs are in shades of yellow/orange. **b**, **c**, SPR demonstrates that both arms of CoV-X2 are functional. In **(b)**, immobilized RBD complexed with the indicated mAb (first antibody) binds to CoV-X2 (second antibody). In **(c)**, the RBD/CoV-X2 complex prevents binding by the single mAbs. Shaded colors are controls (second antibody only). **d**, Both arms of CoV-X2 bind simultaneously to the RBD since, contrary to the monoclonals, avidity is retained at decreasing RBD concentrations. On top, representative SPR traces indicating the different dissociations of antibodies (or Fab) binding to RBD immobilized at different concentrations on the SPR chip (see also Extended Data Fig. 6). At the bottom, plots of the normalized k_a and k_d values obtained with different concentrations of immobilized RBD. Increasing normalized dissociation rate (k_d) values indicate loss of avidity. **e**, **f**, CoV-X2 fully prevents ACE2 binding to S trimer in

ELISA. ACE2 binding to antibody/S trimer complexes is measured either with increasing concentration of the indicated antibody and constant ACE2 (**e**), or at constant antibody concentration with increasing ACE2 (**f**). Mean of two replicates is shown. **g**, CoV-X2 neutralizes SARS-CoV-2 pseudovirus and escape mutants of its parental mAbs. Normalized relative luminescence (RLU) for cell lysates after infection with nanoluc-expressing SARS-CoV-2 pseudovirus in the presence of increasing concentrations of antibodies. Wild-type SARS-CoV-2 pseudovirus (left) is shown alongside three escape mutants generated in the presence of C121 or C135¹⁴. Dashed lines are parental Fabs. Mean of two independent experiments with two replicates each. **h**, Neutralization of SARS-CoV-2 isolates with sequences corresponding to viruses first isolated in China (wild-type), Italy (D614G), United Kingdom (UK; B.1.1.7), Brazil (BRA; P.1) and South Africa (SAF; B.1.351). Mean of three experiments with standard deviation is shown. RBD residues mutated in the variants are indicated in the table and as red spheres on the S trimer structure, where the epitope of C135 (blue) and C121 (green) are shown.

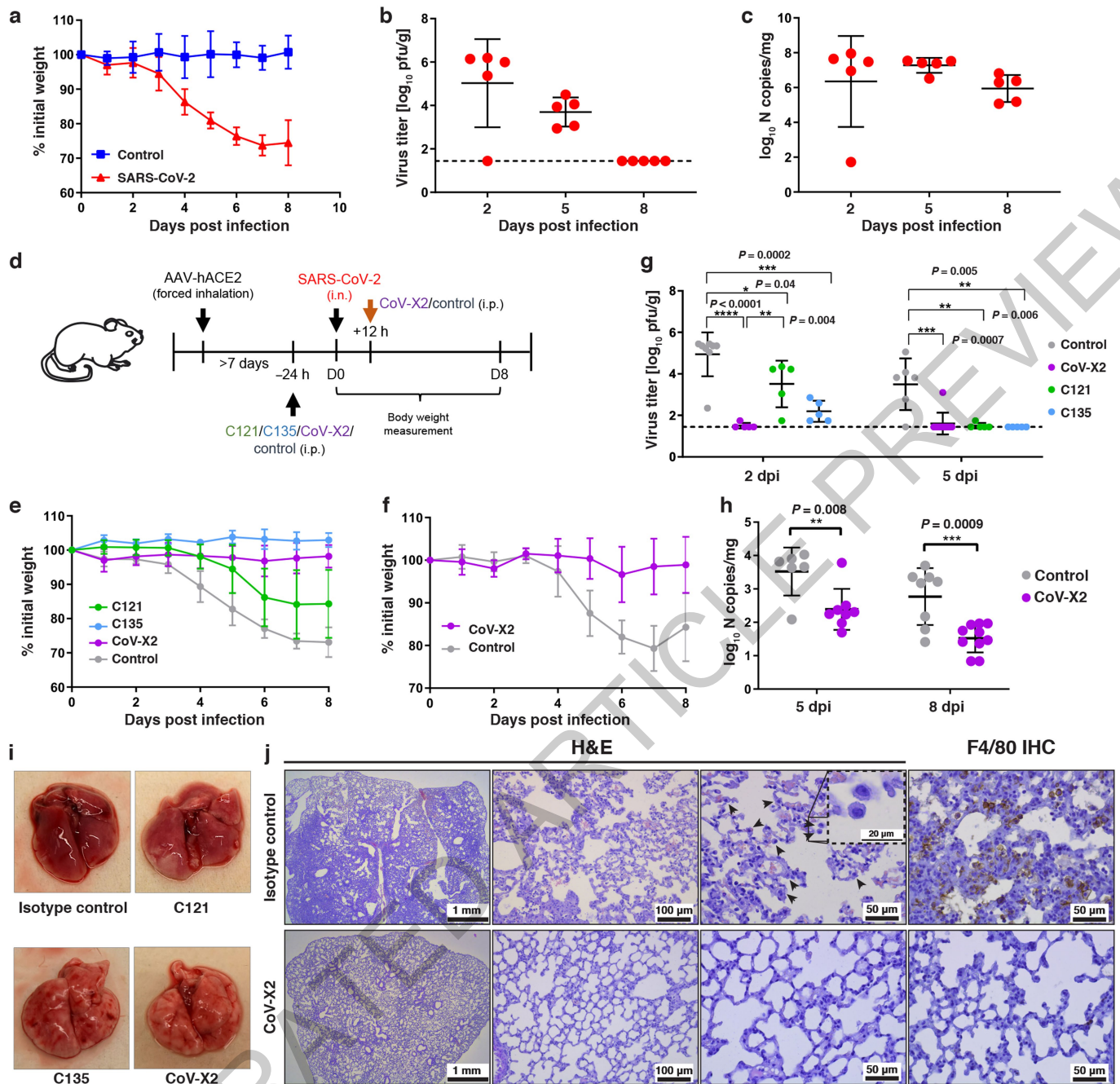


Fig. 2 | CoV-X2 protects AAV-hACE2-transduced mice against SARS-CoV-2 disease. **a**, Loss of body weight over time in SARS-CoV-2 infected mice. 13 to 15 weeks old C57Bl/6NCR1 wild-type female mice were transduced with AAV-hACE2 by forced inhalation, which provides delivery of viral particles to both upper and lower respiratory tract. After >7 days, mice were either infected with SARS-CoV-2 (1×10^4 pfu) or received vehiculum by the intranasal route. Weight was monitored daily for 8 days (SARS-CoV-2, $n = 5$; control, $n = 4$). Mean with standard deviation is shown. **b**, Kinetic of viral burden in the lungs from SARS-CoV-2-infected mice ($n = 5$) by plaque assays. Mean with standard deviation; the dashed line indicates the limit of detection. **c**, Kinetic of viral RNA levels in lung samples from SARS-CoV-2-infected mice ($n = 5$) by RT-qPCR. Mean with standard deviation. **d**, Schematic of the experimental layout. Wild-type mice were transduced with AAV-hACE2 by forced inhalation. After >7 days, the mice were inoculated intraperitoneally (i.p.) with antibodies either one day before (-24 h; black arrow) or 12 hours after (+12 h; red arrow) being infected intranasally (i.n.) with SARS-CoV-2 (1×10^4 pfu). Changes in body weight

were monitored daily in mice treated 24 hours before infection (**e**, C121, $n = 9$; C135, $n = 5$; CoV-X2, $n = 13$; isotype control, $n = 10$) or 12 hours after infection (**f**, CoV-X2 $n = 4$; control $n = 10$). Mean with standard deviation is shown. **g**, Lung viral burden by plaque assay at 2 dpi (isotype control $n = 8$; CoV-X2 $n = 5$; C121 $n = 5$; C135 $n = 5$) and 5 dpi (isotype control $n = 6$; CoV-X2 $n = 10$; C121 $n = 5$; C135 $n = 5$). The dashed line indicates the limit of detection; mean with standard deviation. P value was calculated with two-tailed Student's *t* test. **h**, Spleen viral RNA levels by RT-qPCR at 5 and 8 dpi (gray: isotype control, $n = 6$ or 8 as indicated by dots; purple: CoV-X2, $n = 8$ or 10 as indicated by dots). Mean with standard deviation. P value was calculated with two-tailed Student's *t* test. **i**, Photographs of lungs collected from infected mice (8 dpi). **j**, Histopathology and F4/80 immunohistochemistry (IHC). Hematoxylin and Eosin-stained (H&E) sections of paraffin-embedded lungs from infected mice (8 dpi). Arrowheads point to foamy macrophages. F4/80 IHC shows abundant macrophage infiltration in lungs of mice treated with isotype control but not with CoV-X2. Each image is representative of two separate experiments ($n = 3$ to 5 mice per group).

Protein expression and purification

To express SARS-CoV-2 S protein, a codon optimized gene encoding residues 1–1208 (GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>): MN908947) with proline substitutions at residues 986 and 987, a “GSAS” substitution at the furin cleavage site (residues 682–685), a C-terminal T4 fibrin trimerization motif and a C-terminal 8x HisTag was synthesized and cloned into the mammalian expression vector pcDNA3.1(+) by Genscript. Codon-optimized nucleotide sequences encoding SARS-CoV-2 RBD (residues 319–591; GenBank: MN908947) with a C-terminal 8x HisTag was likewise obtained from Genscript. Human ACE2 fused to the Fc region of mouse IgG at the C-terminal was also synthesized and cloned into the mammalian expression vector pcDNA3.1(+). All proteins were produced by transient PEI transfection in Expi293F™ cells (ThermoFisher), purified from the cell supernatants 6 days after transfection by HiTrap™ Chelating HP (Cytiva), analyzed by SDS-PAGE and dynamic light scattering (DLS) on a DynaPro NanoStar (Wyatt Technology, software Dynamics v7.1.7.16). RBD and S trimer mutations were introduced in the above plasmids by gene-synthesis (Genscript) and purified as above. All proteins underwent quality control and biophysical characterization to ensure functionality, stability, lack of aggregation and batch to batch reproducibility.

mAb production. Plasmid containing the coding sequence for the production of monoclonal antibodies C121, C135, and C144 were prepared as previously described³. These mAbs were produced by transient PEI transfection in Expi293F™ cells (ThermoFisher), purified from the cell supernatants 6 days after transfection by HiTrap™ Protein A HP (Cytiva) and HiLoad Superdex 200 16/60 column (Cytiva).

Design, expression and purification of bispecific antibodies. Bispecific antibodies in the single-chain Fv format (CoV-scb1 and CoV-scb2) were designed from the sequences of the variable regions of monoclonal antibodies C144/C135 (CoV-scb1) and C121/C135³ (CoV-scb2) following the method we previously described³¹, N-terminal signal peptides (residues 1–19; UniProt (<https://www.uniprot.org/>): P01743) and a C-terminal 6x HisTag were added. The constructs were synthesized and subcloned into the mammalian expression vector pcDNA3.1(+) by Genscript. The single-chain bispecifics were produced by transient PEI transfection in Expi293F™ cells (ThermoFisher), purified from the cell supernatants 6 days after transfection by HiTrap™ Chelating HP (Cytiva) and HiLoad Superdex 75 16/60 column (Cytiva).

Bispecific antibodies in the CrossMab format were designed from the sequences of the variable regions of antibodies C144/C135 (CoV-X1) and C121/C135³ (CoV-X2). Light and heavy chains constant regions sequences (UniProt P01834 and UniProt P01857) were added. The CrossMAbs were designed, as previously described,¹⁰ with CH1-CL crossover in the C135 moiety. Four constructs, one for each light chain (LC(C144), LC(C121), and LC^{CH1-CL}(C135)) and heavy chain (HC(C144), HC(C121), and HC^{CH1-CL}(C135)) were synthesized and subcloned into the mammalian expression vector pcDNA3.1(+) by Genscript. Signal peptides were included at the N-terminal of the variable sequences for expression purposes (residues 1–19; UniProt P01743 for the heavy chains and residues 1–20; UniProt P06312 for the light chains). The antibodies were produced by PEI transient transfection, plasmids ratio 1:1:1:1, in Expi293F™ cells (ThermoFisher), purified from the cell supernatants 6 days after transfection by HiTrap™ Protein A HP (Cytiva), and HiLoad Superdex 200 16/60 column (Cytiva).

All antibodies underwent quality control and biophysical characterization to ensure functionality, stability, lack of aggregation and batch to batch reproducibility.

Computational modelling

CoV-scb1 and CoV-scb2 were modelled starting from the variable fragment of available experimental structures of the parental antibodies (PDB (<https://www.rcsb.org/>) ID: 7K8X for C121, 7K8Z for C135 and 7K90 for C144); the connecting linkers were manually added (Pymol); stability and feasibility of the bispecific constructs on the S-trimer were manually and visually investigated according to structural biology considerations.

CoV-X1 and CoV-X2 were assembled starting from the experimental structures of individual Fabs antibodies in complex with SARS-CoV-2 (PDB ID: 7K8X for C121, 7K8Z for C135 and 7K90 for C144). The Fc moiety was manually placed in proximity of the C-terminus of each Fab heavy chains; CH1 domains were then connected to the Fc using the ALMOST toolkit³², thus obtaining the full antibody structures.

The S trimer coordinates were obtained from available experimental structures; loop regions not present in the structure were modelled using the I-TASSER suite³³. PDB ID 6VXX served as a basis for the ‘three down’ conformation; PDB ID 6VYB for the “one RBD up” conformation; PDB ID 7A93 for the “two RBD up” conformation. Conformations not directly available (e.g. trimer with “three RBD up”) were obtained by structural alignment and repetition of the appropriate conformation in S monomer structures (e.g. monomeric “RBD up”) using the PyMol software suite³⁴.

All the combinations of S trimer (three RBD down, one up, two up and three up) and antibodies (bispecifics formed by C121, C135, C144) were subjected to 400 ns or 200 ns of fully atomistic Molecular Dynamics simulations (MD) to obtain energetically favorable and stable conformations using GROMACS.³⁵ Calculations were performed on the CINECA-Marconi100 supercomputer.

The system was initially set up and equilibrated through standard MD protocols: proteins were centered in a triclinic box, 0.2 nm from the edge, filled with SPCE water model and 0.15 M Na⁺Cl⁻ ions using the AMBER99SB-ILDN protein force field. Energy minimization was performed in order to let the ions achieve a stable conformation. Temperature and pressure equilibration steps, respectively at 310 K and 1 Bar, of 100 ps each were completed before performing the full MD simulations with the above-mentioned force field. MD trajectory files were analyzed after removal of Periodic Boundary Conditions. The stability of each simulated complex was verified by root mean square deviation and visual analysis.

Surface Plasmon Resonance (SPR)

The antibodies binding properties were analyzed at 25 °C on a Biacore™ 8K instrument (GE Healthcare) using 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% Tween-20 as running buffer.

SARS-CoV-2 RBDs or full S protein, and their mutants, were immobilized on the surface of CM5 chips (Cytiva) through standard amine coupling. Increasing concentration of antibodies (3.12, 6.25, 12.5, 25, and 50 nM) were injected using a single-cycle kinetics setting; analyte responses were corrected for unspecific binding and buffer responses. Curve fitting and data analysis were performed with Biacore™ Insight Evaluation Software v2.0.15.12933.

Competition experiments were performed to access the ability of COV-X2 to bind its target with both arms. A low amount of RBD (5 nM) was immobilized on the surface of a CM5 chip through standard amine coupling. C121 or C135 antibodies were injected at high concentration (1.5 μM) to saturate the corresponding binding sites on the RBD; CoV-X2 (200 nM) was subsequently injected. The same experimental setting was performed with a different injection order as a control: COV-X2 (1.5 μM) injected to saturate RBD binding sites and subsequent injection of C121 or C135 (200 nM).

Analysis and comparison of kinetics parameters at different RBD concentrations were performed as following. RBD was immobilized on the surface of a CM5 chip at 5, 15, 75 and 150 nM. Increasing

concentrations of antibodies (3.12, 6.25, 12.5, 25, 50 nM) were injected using a single-cycle kinetics setting; analyte responses were corrected for unspecific binding and buffer responses. Curve fitting and data analysis were performed as above.

Binding inhibition of hACE2

Enzyme-linked immunosorbent assays (ELISA) were used to investigate the antibodies ability to inhibit the binding of S to hACE2. Each experiment was performed in duplicate, reporting the mean of the two replicates; error bars represent the standard deviation of the measured values. 96-well ELISA plates were coated at 4 °C with 37 nM S protein, washed and blocked with PBS+10% FCS. Antibodies were then added either at constant saturating concentration (160 nM) or at different dilutions (starting from 340 nM and serially diluted 1 to 3), see main text and figures) and incubated 1 h at 25 °C; after washing, hACE-mFc was added either at constant saturating concentration (160 nM) or at different dilutions (starting from 340 nM and serially diluted 1 to 2) and left 1 h at 25 °C. After further washing, bound hACE was detected using standard protocols with goat anti-mouse IgG coupled to alkaline phosphatase (dilution 1:500 SouthernBiotech). ELISA plates were measured with the reader software Gen5 Version 1.11.5, BioTek Instruments, Inc. Data were analyzed with Microsoft Excel 2016 and GraphPad Prism Version 8.4.2.

SARS-CoV-2 pseudotyped reporter virus and pseudotyped virus neutralization assay

A panel of plasmids expressing RBD-mutant SARS-CoV-2 spike proteins in the context of pSARS-CoV-2-S_{Δ19} have been described previously^{14,36}. The mutant KEN (K417N+E484K+N501Y) was constructed in the context of a pSARS-CoV-2-S_{Δ19} variant with a mutation in the furin cleavage site (R683G). The IC₅₀ of these pseudotypes were compared to a wildtype SARS-CoV-2 spike sequence carrying R683G. Generation of SARS-CoV-2 pseudotyped HIV-1 particles was performed as previously described^{13,15}.

The neutralization activity of the bispecific and monoclonal antibodies was measured as previously reported^{3,15}. In brief, fourfold serially diluted antibodies were incubated with SARS-CoV-2 pseudotyped virus for 1 h at 37 °C. The mixture was subsequently incubated with 293T_{ACE2} cells for 48 h; the cells were washed twice with PBS and lysed with Luciferase Cell Culture Lysis 5× reagent (Promega). Nanoluc Luciferase activity in lysates was measured using the Nano-Glo Luciferase Assay System (Promega) with Modulus II Microplate Reader User interface (TURNER BioSystems). The obtained relative luminescence units, indicative of virus presence, were normalized to those derived from cells infected with SARS-CoV-2 pseudotyped virus in the absence of antibodies. The half-maximal inhibitory concentration (IC₅₀) was determined using four-parameter nonlinear regression (GraphPad Prism).

SARS-CoV-2 virus neutralization assay

The neutralizing activity of CoV-X2 against SARS-CoV-2 wild-type (first isolated in China), D614G (B.1, first isolated in Italy), B.1.1.7 (first isolated in UK), P.1 (first isolated in Brazil) and B.1.351 (first isolated in South Africa) was investigated by plaque reduction neutralization tests following the protocol reported in³⁷. Briefly, 50 µL of antibody, starting from a concentration of 12 µg/mL or 190 µg/mL in a serial threefold dilution, were mixed in a flat bottom tissue culture microtiter plate (COSTAR, 14 Corning Incorporated, NY 14831, USA) with an equal volume of 100 TCID₅₀ of infectious virus that was isolated from COVID-19 patients, sequenced, titrated and incubated at 33 °C in 5% CO₂. After 1 h, 3x10⁴ (100 µL) VERO E6 cells (VERO C1008, Vero 76, clone 18 E6, Vero E6; ATCC® CRL-1586™) were added to each well. After three days of incubation, cells were stained with Gram's crystal violet solution (Merck) plus 5% formaldehyde 40% m/v (Carlo Erba S.p.A.) for 30 min. Microtiter plates were then washed in water. Wells were analysed to evaluate the degree of cytopathic effect (CPE) compared to untreated control. Each

experiment was performed in triplicate. The half-maximal inhibitory concentration (IC₅₀) was determined using three-parameter nonlinear regression (GraphPad Prism).

AAV-hACE2 vector design

Plasmid design and construction. The AAV transfer plasmid expressing human angiotensin-converting enzyme 2 (AAV-hACE2) was created by replacing the GFP sequence with hACE2 cDNA obtained from the HEK293 cell line (#CRL-1573™, ATCC®, mycoplasma-free, population doubling lower than 13) in a pAAV-GFP plasmid (#AAV-400, Cellbiolabs). HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, #D5796, Sigma Aldrich Ltd.) supplemented with 10% FBS (#10082139, Gibco) and 1% penicillin-streptomycin (#XC-A4122100, BioSera). Cells were kept at 37 °C under 5% CO₂ atmosphere and 95% humidity.

To obtain the hACE2 sequence, total RNA was isolated from a confluent 10 cm² plate of HEK293 cells using RNeasy Mini Kit (Qiagen, #74104) according to the manufacturer's protocol and reverse-transcribed with M-MLV Reverse Transcriptase (Promega, #M1701). The generated cDNA was used as a template for PCR amplification with a pair of primers (hACE2_Fw: 5'-ATGTCAAGCTCTTCCTGG 3', hACE2_Rv: 5'-CTAAAAGGAGGTCTGAACATC 3') specific for hACE2 (NM_021804.3) using Phusion® High-Fidelity DNA Polymerase (NEB, #M0530S). The PCR product was separated in a 1% agarose gel (SeaKem LE AGAROSE; East Port, #50004); the band of appropriate size (2418 bp) was extracted using NucleoSpin® Gel and PCR Clean-up (Takara, #740609) according to the manufacturer protocol. The extracted product was treated with DreamTaq Green DNA Polymerase (Thermo Scientific™, #EP0711) in the presence of dATPs to add 3'A overhangs to the PCR product. The product was then sub-cloned into pGEM-T Easy Vector (Promega, #A1360). Proper insertion of the product was assessed by HindIII (Thermo Scientific™, #ER0501) and SacI (Thermo Scientific™, #ER1132) double-digestion control. Kozak sequence and Spel recognition site were added at the 5' and 3' end of the hACE2 cDNA PCR product, respectively, by amplification with a specific pair of primers (hACE2_Kozak_Fw: 5'-CAGGGGACGATGTCAAGCTCTTCCTGG-3', hACE2_Spe_Rv: 5'-ACTAGTGATCTAAAAGGAGGT-3') using Phusion® High-Fidelity DNA Polymerase (NEB, #M0530S). The amplified product was separated in 1% agarose gel (SeaKem LE AGAROSE; East Port, #50004), extracted with NucleoSpin® Gel and PCR Clean-up (Takara, #740609) according to the manufacturer's protocol. The extracted hACE2 sequence was PCR amplified with a pair of primers with microhomology arms (hACE2_IF_Fw: 5'-TTCGAACATCGATTGCAGGGGACGATGTCAAG-3', hACE2_Spe_IF_Rv: 5'-GCGTGCTCGAGGCACTAGTGATCTAAAAGGAGGT-3') and Phusion® High-Fidelity DNA Polymerase (NEB, #M0530S), and was subsequently purified from agarose gel with NucleoSpin® Gel and PCR Clean-up (Takara, #740609).

The GFP sequence in the pAAV-GFP control vector (#AAV-400, Cellbiolabs) was excised by double-digestion using EcoRI (Thermo Scientific™, #ER0271)/HindIII (Thermo Scientific™, #ER0501) restriction enzymes and replaced with the hACE2 sequence flanked by microhomology arms using the In-Fusion cloning system (In-Fusion HD Cloning Kit, Takara Bio Europe, #639648) according to the manufacturer's protocol. Proper insertion and presence of the Spel recognition site were confirmed by double-digestion using HindIII (Thermo Scientific™, #ER0501)/MluI (Thermo Scientific™, #ER0562) and Spel (Thermo Scientific™, #ER1252), respectively. The generated AAV-hACE2 vector was sequenced (Eurofins Genomics) using the following primers: CMV_Fw: 5'-AAATGGGCGGTAGGCGTG-3', seq_hACE2_start1: 5'-TGGAGATCTGAGGTCCG-3', seq_hACE2_start2: 5'-TCTTCCTCCCACAGCTCCT-3', seq_hACE2_1: 5'-CAGTTGATTGAAGATGTGGA-3', seq_hACE2_2: 5'-AGAAGTGGAGGTGGATG-3', seq_hACE2_3: 5'-AGAAGTGAAGTTGAAAAGG-3'. The produced hACE2 sequence was 100% identical to the reference hACE2 sequence (NM_021804.3).

Transfection of Neuro-2a cells. Neuro-2a cells (CCL-131TM, ATCC[®], mycoplasma-free, population doubling lower than 10) used for validation of AAV-hACE2 function were cultured in Dulbecco's modified Eagle's medium (DMEM, #D5796, Sigma Aldrich Ltd.) supplemented with 10% FBS (#10082139, Gibco) and 1% penicillin-streptomycin (#XC-A4122100, BioSera) and kept at 37 °C under 5% CO₂ atmosphere and 95% humidity. Three 60 mm² plates were seeded each with 4x10⁵ cells and transfected the next day with the AAV-hACE2 vector using Lipofectamine[®] 2000 Transfection Reagent (Invitrogen, #11668027) according to the manufacturer's protocol for the 6-well plate transfection. Transfection with pAAV-GFP control vector (#AAV-400, Cellbiolabs) and non-transfected HEK293 cells were used as negative controls. After 48 h, cells were harvested for Western Blotting in RIPA buffer, supplemented with AEBSF protease inhibitor (AppliChem GmbH, #A1421) and cComplete[™] Mini Protease Inhibitor Cocktail (Sigma Aldrich Ltd., #O4693124001). Expression of hACE2 in Neuro-2a was compared to the non-transfected HEK293 cells.

AAV-hACE2 particles production

AAV293 cells transfection. The AAV293 cell line (Cellbiolabs, #AAV-100, mycoplasma-free, population doubling lower than 8) used for AAV production was cultured in Dulbecco's modified Eagle's medium (DMEM, #D5796, Sigma Aldrich Ltd.) supplemented with 10% FBS (#10082139, Gibco), 1% penicillin-streptomycin (#XC-A4122100, BioSera) and 1% NEAA (#M7145, Sigma Aldrich Ltd.). Cells were kept at 37 °C under 5% CO₂ atmosphere and 95% humidity. A day before transfection, 6.5x10⁶ cells were seeded on 15 cm² cultivation plates to reach 80-90% confluency on the day of transfection. Vectors pHelper (#340202, Cell BioLabs), AAV Rep/Cap 2/9n (#112865, Addgene) and AAV-hACE2 were used for transfection in equimolar ratio. The total amount of DNA (28 µg/plate) diluted in 1 mL/plate of DMEM medium was mixed with Linear Polyethylenimine Hydrochloride, M.W. 40000 PEI (#24765-1, Polysciences) in 1:2.7 ratio. After 20 min of incubation at RT, the transfection mixture was added to a cultivation plate with FBS-reduced medium (DMEM supplemented with 1% FBS) in a drop-wise manner. After 5 hours of incubation at 37 °C under 5% CO₂ atmosphere, the medium was removed and replaced with fresh complete growth medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin).

AAV293-hACE2 harvest. 3 days post-transfection, both cell medium and cells were harvested for AAV particles isolation. These procedures were adapted and modified from Zolotukhin, S., et al.³⁸. The medium was collected into 50 mL centrifuge tubes and cells were washed twice with 5 mL of PBS. Subsequently, cells were scraped in 1 mL of PBS and centrifuged at 1000 g for 10 min at 4 °C. The supernatant was added to the previously collected cell medium; the cell pellet was kept on ice during subsequent processing. The medium was centrifuged at 3200 g for 15 min at 4 °C and the supernatant was then filtered into a sterile glass bottle using 0.22 µm PES membranes. PEG-8000 (#V3011, Promega) was added to the media in a glass bottle in a 1:4 ratio. The mixture was stirred slowly at 4 °C for 1 hour and then incubated overnight at 4 °C without stirring to allow full virus precipitation. The following day, the medium was centrifuged at 2800 g for 20 min at 4 °C and the pellet resuspended in 10 mL of PBS solution with 0.001% Pluronic[™] F-68 Non-ionic Surfactant (#24040032, Gibco) and 200 mM NaCl (#S5886, Sigma Aldrich Ltd.)

and sonicated at 50% amplitude with 4x1 s on/15 min off pulses on ice. The cell lysate was centrifuged at 3200 g for 15 min at 4 °C. Subsequently, 50 U/mL of Benzonase[®] Nuclease (#E1014-25KU, Sigma Aldrich Ltd.) were added to the viral suspension to degrade any residual DNA. After incubation for 1 hour at 37 °C, the viral suspension was centrifuged at 2400 g for 10 min at 4 °C and the clarified supernatant was further purified.

AAV-hACE2 purification by iodixanol gradient ultracentrifugation. A gradient consisting of 15% iodixanol (in 1 M NaCl, 2.7 mM MgCl₂, 2 mM KCl in phosphate buffer), 25% iodixanol (in 2.7 mM MgCl₂, 2 mM KCl, and 0.001% phenol red in PBS), 40% iodixanol (in 2.7 mM MgCl₂ and 2 mM KCl in PBS), and 0.002% phenol red (#P3532, Sigma Aldrich Ltd.) in 60% iodixanol (OptiPrep[™] Density Gradient Medium, #D1556, Sigma Aldrich Ltd.) was prepared in QuickSeal tubes according to Zolotukhin et al., 1999³⁸. 5 mL of clarified viral supernatant were carefully added on the top of the gradient and the rest of the tube was filled up with PBS. Ultracentrifugation was carried out at 350'000 g for 90 min in a pre-cooled T70i rotor at 10 °C. After ultracentrifugation, ~750 µL fractions were collected from the 40% iodixanol phase using an 18G needle puncturing the QuickSeal tube at the interface of the 60% and 40% iodixanol.

AAV-hACE2 purity validation and buffer exchange. The purity of the collected fractions from 40% iodixanol containing AAV-hACE2 particles was assessed by SDS-PAGE. 10 µL of each collected fraction were mixed with 3.5 µL of 4x Laemmli Buffer and loaded to 4–20% Mini-PROTEAN[®] TGX[™] Precast Protein Gel (#4561096, Bio-Rad). The gel were shortly washed in dH₂O and stained with silver according to the manufacturer's protocol (Pierce Silver Stain Kit, #10096113, Thermo Scientific[™]).

Selected AAV-hACE2 fractions were pooled and concentrated using Amicon Ultra-0.5 Centrifugal Filter Unit (molecular weight cut-off 100 kDa). First, Amicon filter membranes were activated by incubation with 0.1% Pluronic[™] F-68, 0.01% Pluronic[™] F-68 and 200 mM NaCl, followed by 0.001% of Pluronic[™] F-68 in PBS and centrifugation at 1900 g for 5 min at 4 °C. Pooled fractions with AAV-hACE2 particles were loaded onto activated Amicon filter membranes and centrifuged at 2600 g for 5 min at 4 °C. The membranes were washed several times with 0.001% Pluronic[™] F-68 in PBS (centrifugation at 2600 g for 8 min at 4 °C) until the residual iodixanol was completely removed from the solution. To elute and concentrate the viral suspension, the membranes were covered with ~ 5 mL of formulation buffer and incubated for 5 min at RT. Amicon filters were centrifuged at 2600 g at 4 °C for approx. 1.5 min until ~ 0.5 mL of the formulation buffer with AAV-hACE2 particles were left. The eluate was transferred into sterile 1.5 mL tubes, quantified and stored at 4 °C for up to 2 weeks for short term *in vivo* application or at –80 °C for long term storage.

AAV-hACE2 titration by qPCR. The protocol for quantification and determination of the number of genome-containing particles of AAV-hACE2 was adapted from Aurnhammer et al.³⁹, using qPCR. Purified AAV-hACE2 particles were treated with DNase I (#EN0521, Thermo Scientific[™]) to eliminate contaminating plasmid DNA. Serial dilutions of a AAV-hACE2 viral suspension were used as template in two separate reactions, one detecting viral ITR sequences (ITR_Fw: 5'-GGAACCCCTAGTGATGGAGTT-3', ITR_Rv: 5'-CGGCCTCAGTGAGCGA-3') and the second hACE2 (hACE2_Fw: 5'-CCATTGGTCTTCTGTACCCG-3', hACE2_Rv: 5'-AGACCATCCACCTCCACTTCTC-3'). Data analysis was performed using the LightCycler[®] 480 Software, Version 1.5. AAV concentration (the number of viral genomes in 1 µL of AAV sample) was determined by comparison to standard curves of defined concentrations of AAV-hACE2 vector. Each qPCR run was performed in triplicate; six serial dilutions of the AAV-hACE2 vector were used as positive controls and standards

Mouse experiments

This study was carried out in strict accordance with the Czech national law and guidelines on the use of experimental animals and protection of animals against cruelty (Animal Welfare Act No. 246/1992 Coll.). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institute of Parasitology and of the Departmental

Expert Committee for the Approval of Projects of Experiments on Animals of the Academy of Sciences of the Czech Republic (permit 82/2020).

Application of AAV-hACE2 viral particles to mice. 13-15 weeks old C57Bl/6Ncrl female mice were anesthetized by intraperitoneal injection of ketamine/xylazine (0.1 mg/g; Biopharm)/(0.01 mg/g; Bioveta). Viral particles containing AAV-hACE2 were diluted to a final concentration of 4×10^9 GC in 40 μ L of PBS. This volume was applied to mice by forced inhalation. The tip of the nose was gently clipped with tweezers and the tongue gently pulled out. After the mouse started breathing through the oral cavity, 40 μ L of viral suspension were applied by a 200 μ L pipette tip into the oral cavity and inhaled by the mouse through the trachea into the lungs.

To access whether hACE2 was expressed in lungs, lung tissue was harvested and analyzed by Western Blot in RIPA buffer, supplemented with AEBF protease inhibitor (AppliChem GmbH, #A1421) and cOmplete™ Mini Protease Inhibitor Cocktail (Sigma Aldrich Ltd., #04693124001) 1-, 2- and 4-weeks post application. Expression of hACE2 in AAV-hACE2 transduced mice was compared to non-treated C57Bl/6Ncrl mice. Histone H3 antibody (Cat. No.: ab1791, Abcam, lot: GR3237685-2; 1:1000 dilution) was used as a loading control.

Mouse infection. SARS-CoV-2 (strain SARS-CoV-2/human/Czech Republic/951/2020, isolated from a clinical sample at the National Institute of Health, Prague, Czech Republic), kindly provided by Dr. Jan Weber, Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic, was used for mouse infection. The virus was passaged in Vero E6 cells five times before its use in this study.

At least 7 days post application of the AAV-hACE2 virus particles, mice were infected intranasally with SARS-CoV-2 (1×10^4 pfu) in a total volume of 50 μ L DMEM. Mice were monitored and weighted daily over an 8-day period. Treated mice were injected intraperitoneally with either 150 μ g of antibodies 24 hours prior to the infection or 250 μ g 12 hours post-infection. Animals were sacrificed at the indicated times post-infection and their tissues harvested for analysis.

Measurement of the viral burden. Tissues were weighed and homogenized using Precellys 24 (Bertin Technologies) and prepared as 20% (wt/vol) suspension in DMEM medium containing 10% newborn calf serum. The homogenates were clarified by centrifugation at 5000 g and the supernatant medium was used for plaque assay and viral RNA isolation.

Plaque assays were performed in Vero E6 cells (ATCC CRL-1586; mycoplasma-free) grown at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; #LM-D1112/500, Biosera) supplemented with 10% fetal bovine serum (#FB-1001G/500; Biosera), and 100 U/mL penicillin, 100 μ g/mL streptomycin (Antibiotic Antimycotic Solution; #A5955; Sigma), and 1% L-glutamine (#XC-T1755/100; Biosera) using a modified protocol originally developed by De Madrid and Porterfield.⁴⁰ Briefly, serial dilutions of virus were prepared in 24-well tissue culture plates and cells were added to each well ($0.6 - 1.5 \times 10^5$ cells per well). After 4 h the suspension was overlaid with 1.5% (wt/vol) carboxymethyl-cellulose (#C4888; Sigma) in DMEM. Following a 5-day incubation at 37 °C and 5% CO₂, plates were washed with phosphate-buffered saline and the cell monolayers were stained with naphthol blue black (#195243; Sigma). The virus titer was expressed as pfu/mL.

RNA was isolated from tissue homogenates using the QIAmp Viral RNA mini kit (#52906; Qiagen) following manufacturer's instructions.

Viral RNA was quantified using EliGene COVID19 Basic a RT (#90077-RT-A; Elisabeth Pharmacon) according to the manufacturer's protocol. A calibration curve was constructed from four dilutions of a sample quantified using Quanty COVID-19 kit (#RT-25; Clonit) according to the recommendations from the manufacturer. All real-time PCR reactions were performed using a LightCycler 480 (Roche).

For sequencing, isolated RNA was used as a template for one-step RT-PCR (Qiagen OneStep RT-PCR Kit; #210212; Qiagen) with primers specific for SARS-CoV-2 RBD sequence (SARS-CoV-2_seq_FW: 5'-GCACTTGACCCTCTCTCAGAAAC-3'; SARS-CoV-2_seq_RV: 5'-GACTCAGTAAGAACACCTGTGCC-3'). The reaction mixture (final volume 25 μ L) contained 5 μ L of QIAGEN OneStep RT-PCR Buffer, 1 μ L of dNTP mix, 5 μ L of 5x Q-Solution, 1 μ L of QIAGEN OneStep RT-PCR Enzyme Mix, 6 μ L of RNase-free water, 1.5 μ L of each primer (stock concentration, 0.01 mM), and 4 μ L of template RNA. The cycling conditions were as follows: Reverse transcription (30 min at 50 °C), Initial PCR activation (15 min at 95 °C), 3-step cycling: 40 cycles of 94 °C for 30 s, 52.6 °C for 30 s, and 72 °C for 1 min, followed by Final extension (10 min at 72 °C). The PCR products were visualized in a 1.7% Agarose gel in Tris-acetate-EDTA buffer. The amplified DNA was purified by using Wizard® SV Gel and PCR Clean-Up System (#A9285; Promega) according to the recommendations of the manufacturer.

The purified DNA was prepared for sequencing (Sanger method) by a commercial service (Eurofins Genomics) with the following conditions (final volume 17 μ L): 15 μ L of PCR product with a concentration of 5 ng/ μ L and 2 μ L of primer with a concentration of 10 μ M. The sequencing data was analyzed using BioEdit Sequence Alignment Editor, version 7.2.0.

Histology and immunohistochemistry. Lungs were fixed in 4% PFA. Tissues were processed using a Leica ASP6025 automatic vacuum tissue processor and embedded in paraffin using a Leica EG1150 H+C embedding station. 2 μ m sections were prepared using a Leica RM2255 rotary microtome and sections were stained with hematoxylin and eosin using Leica ST5020 + CV5030 stainer and coverslipper.

To assess the presence of macrophages, a rabbit anti-mouse F4/80 monoclonal antibody (D2S9R XP^R Rabbit mAb, Cat. #70076, Cell Signaling Technology, USA, Lot 5, RRID AB_2799771) was used at 1:800 dilution as primary antibody. The histological sections (thickness 4–5 μ m) were deparaffinized in a Multistainer Leica ST5020 (Leica Biosystems). Antigens were retrieved by heating the slides in citrate buffer pH 6 (Zytomed – Systems, Germany). Endogenous peroxidase was neutralized with 3% H₂O₂. Sections were incubated for 1 hour at RT with a 1:800 dilution of the primary antibody. After washing they were incubated with anti-rabbit secondary antibody conjugated with HRP (Zytomed, Germany, Cat. # ZUC 032-100, Lot A0880-4; no dilution). Staining of the sections was developed with a diaminobenzidine substrate kit (DAKO - Agilent) and sections were counterstained with Harris Hematoxylin (Sigma Aldrich – Merck) in a Multistainer Leica.

Biosafety statement

All work with infectious SARS-CoV-2 was performed in BSL3 facilities at the Institute of Parasitology, Biology Centre of the Czech Academy of Science, Ceske Budejovice, and Veterinary Research Institute, Brno, Czech Republic, using appropriate powered air purifying, positive pressure respirators and protective equipment.

Reporting summary

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

Data availability

The authors declare that data supporting the findings of this study are available within the paper and its supplementary information. All other data are available from the corresponding author upon reasonable request. Published data were taken from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>), UniProt (<https://www.uniprot.org/>), Protein Data Bank, PDB (<https://www.rcsb.org/>) and ViPR database (<https://www.viprbrc.org/>). Source data are provided with this paper.

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Author contributions R.D.G. M.Pe., L.S., F.Mu., J.C.L., F.Ma, D.M., C.I., E.P., S.D.G., M.Pa., F.B., D.M., S.Gi., C.O.B, F.B., J.C.S, F.G, S.Ga, designed and carried out experiments and analyzed results, produced plasmids, antibodies and viral proteins. P.N., T.M., J.H., V.H, B.M., N.P., A.F., J.T., V.I., M.Pa., D.Z., P.B., I.B., P.S., D.R., performed animal experiments and analyzed the results. L.V, D.F.R., D.R., Q.P.H., A.P., L.C., P.J.B., M.C.N., P.D.B., T.H. conceived and designed study and experiments and analyzed the results. P.N., T.M., R.N., O.P., J.P., J.R., R.S. conceived and designed the mouse model. L.V., D.F.R., D.R, R.D.G. wrote the manuscript with input from all co-authors.

Competing interests In connection with this work the Institute for Research in Biomedicine has filed a provisional patent application on which L.V. is inventor (PCT/EP2020/085342). The Rockefeller University has filed a provisional patent application on coronavirus antibodies on which D.F.R. and M.C.N. are inventors.

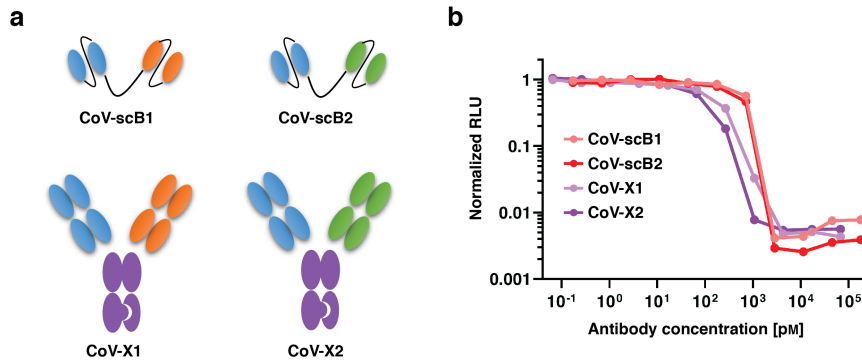
Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41586-021-03461-y>.

Correspondence and requests for materials should be addressed to D.F.R., D.R. or L.V.

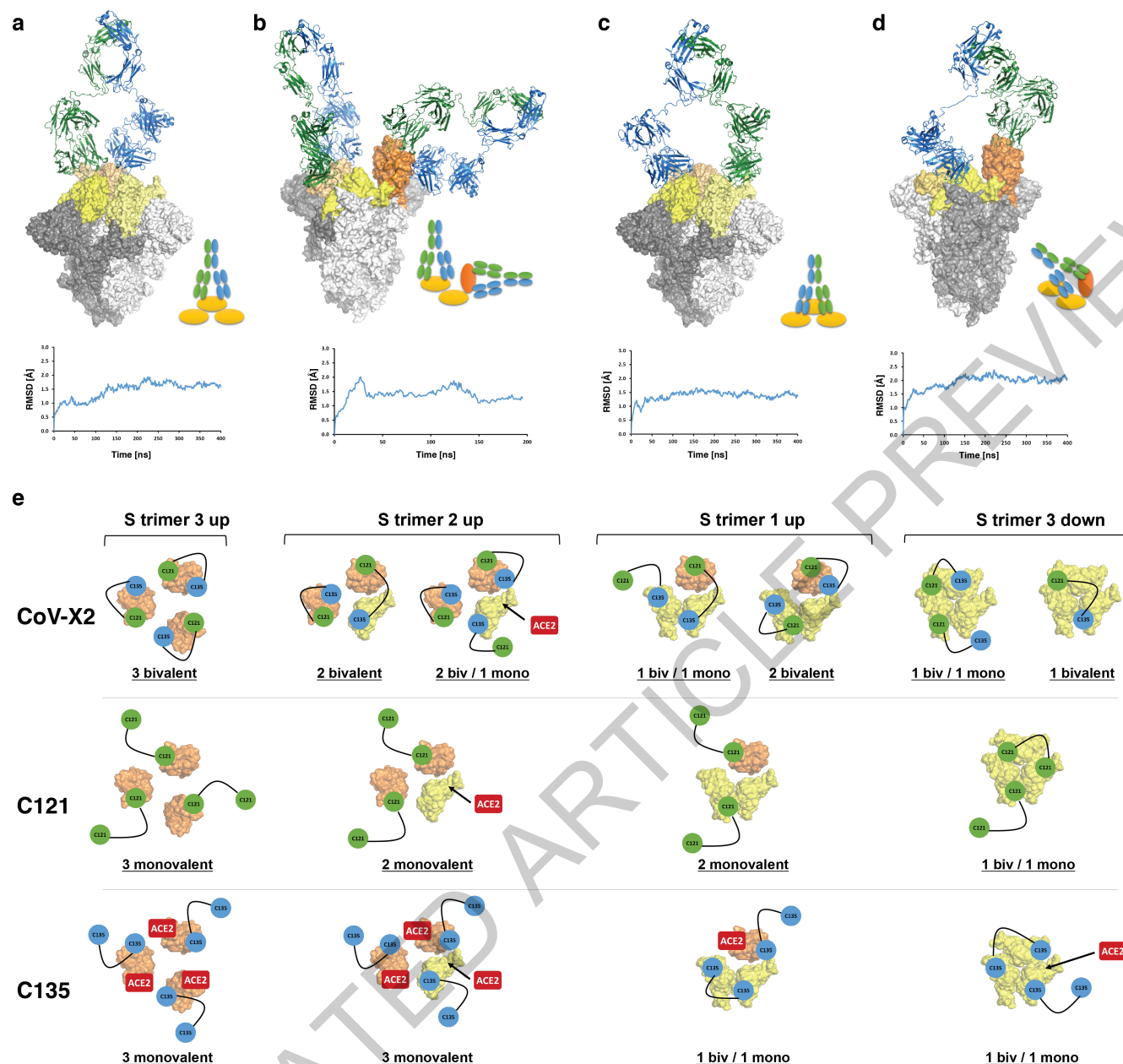
Peer review information *Nature* thanks Stanley Perlman and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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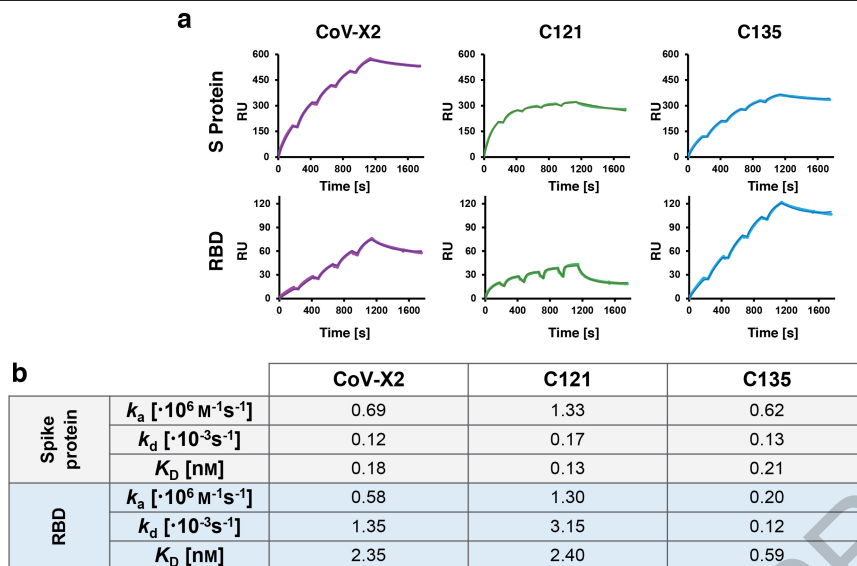
Extended Data Fig. 1 | Neutralization of SARS-CoV-2 pseudovirus by bispecific antibodies. **a**, Schematic representation of the 4 bispecific constructs; two in scFv format and two as IgG-like CrossMAb with knob-in-hole. The parental monoclonals forming the bispecifics are color-coded (C135 blue, C144 orange, C121 green; Fc region in purple). **b**, All 4 constructs neutralize

SARS-CoV-2 pseudovirus *in vitro* at sub-nanomolar concentrations (IC_{50} : 0.13, 0.04, 0.74 and 0.53 nM for CoV-X1, CoV-X2, CoV-scB1 and CoV-scB2, respectively). Normalized relative luminescence values, which correlate to infection, are reported versus antibody concentration, as detailed in Schmidt *et al.*¹⁵. Mean of two experiments is shown.

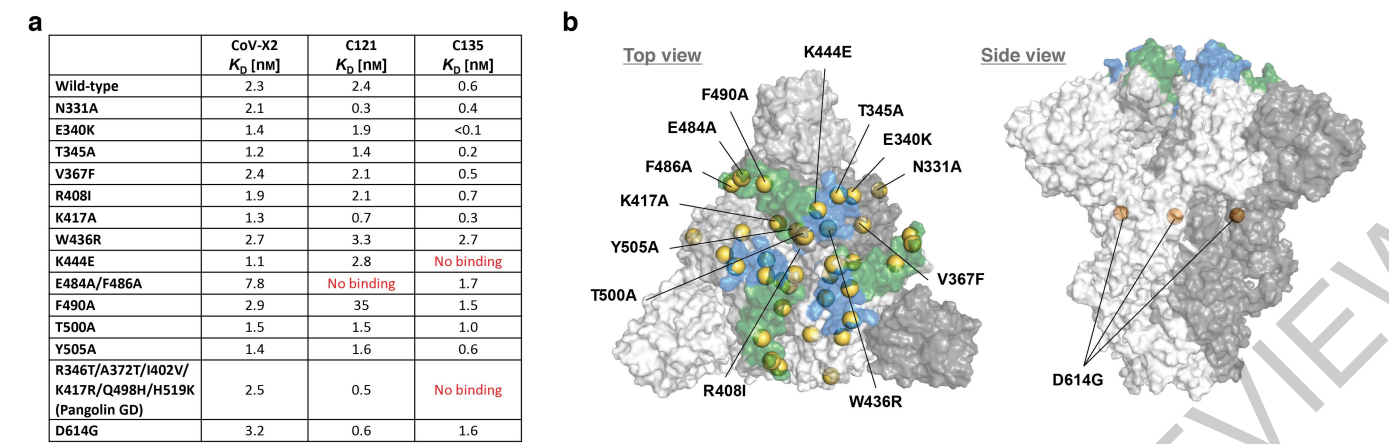


Extended Data Fig. 2 | CoV-X2 engages its epitopes on all RBD conformations on the S trimer. **a–d**, Molecular Dynamics (MD) simulations of the complex between the CoV-X2 bispecific and S trimers with RBD in either all down, all up or mixed up/down conformations show that CoV-X2 can engage a single RBD with both arms (**a,b**), two adjacent RBDs in the down conformation (**c**), and two RBDs in the up/down conformation (**b,d**). The complexes were subjected to up to 400 ns of fully atomistic MD simulations to assess feasibility and stability of the bound conformations. Root-mean-squared deviations (RMSD) values are shown to indicate structural stability. S trimer is in shades of grey, RBDs in yellow (down conformation) and orange (up), the C121 and C135 moieties of CoV-X2 are in green and blue, respectively. **e**, Schematic

representation of the computationally predicted binding modes of CoV-X2, C121 IgG and C135 IgG on the S trimer, colored as in **a–d**. Antibodies are represented by connected circles; ACE2 is in red on the RBD if it can bind directly to a given conformation; it has an arrow pointing to the RBD if ACE2 binding is achieved after an allowed switch to the up conformation. For example, in the 3-up conformation (left), CoV-X2 can engage all the RBDs with bivalent binding, whereas C121 and C135 can only achieve monovalent binding. C135 binding does not prevent interaction with ACE2. The situation is similar in the other S conformations (2-up 1-down, 2-down 1-up and 3-down), with only the bispecific achieving bivalent interaction and preventing ACE2 access in all conformations.

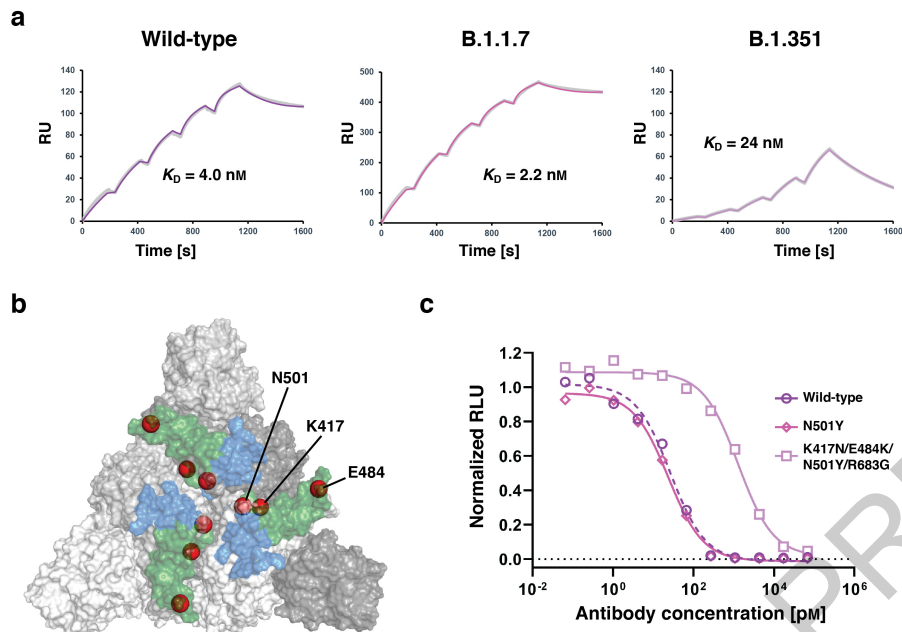


Extended Data Fig. 3 | CoV-X2 and its parental mAbs bind recombinant, isolated RBD and S trimer with low nanomolar affinity. a, Representative SPR traces from which the data in **(b)** was derived. **b,** Kinetic parameters for the binding of C121 IgG, C135 IgG, and CoV-X2 to S trimer and RBD.



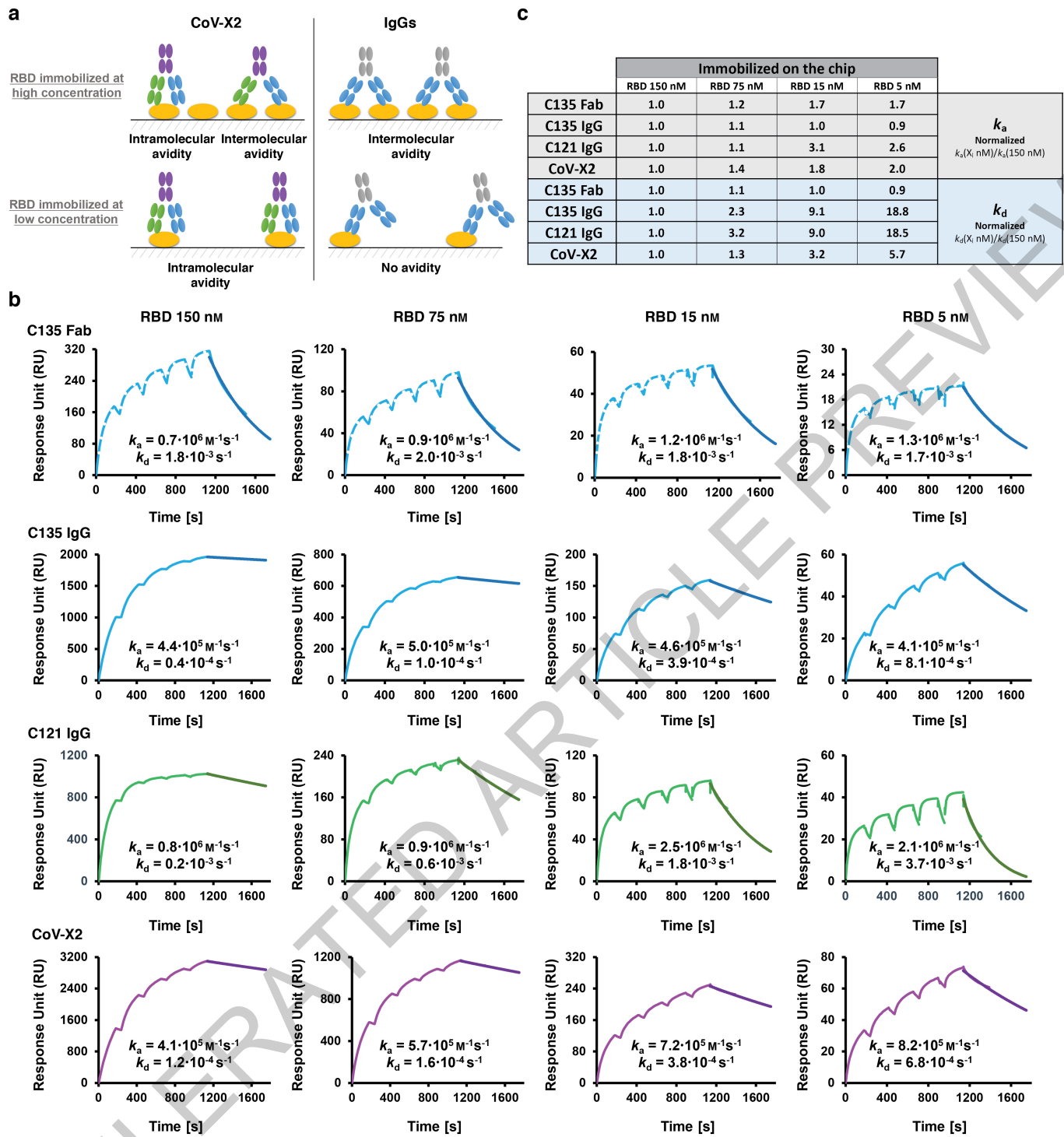
Extended Data Fig. 4 | CoV-X2 binds with low-nanomolar affinity to S protein mutants, including some that are not recognized by the parental mAbs C121 and C135. a, SPR-derived binding affinities of CoV-X2, C121 IgG and

C135 IgG to several S trimer mutants. **b**, Mutations tested in (a) are indicated by yellow spheres on the surface representation of the S trimer. The epitopes of C121 (green) and C135 (blue) are shown.



Extended Data Fig. 5 | Efficacy of CoV-X2 against B.1.1.7 and B.1.351 variants. **a**, SPR traces showing binding of CoV-X2 to the RBD corresponding to wild-type, B.1.1.7 (also known as UK) and B.1.351 (also known as South African) variants of SARS-CoV-2. **b**, Residues mutated in the variants are shown as red spheres on the surface representation of the S trimer. The epitopes of C121

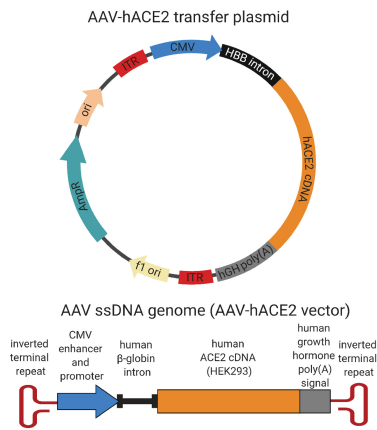
(green) and C135 (blue) are shown. **c**, Neutralization of SARS-CoV-2 pseudoviruses expressing wild-type, N501Y and K417N/E484K/N501Y/R683G (corresponding to South African mutants in the RBD, see Figure 1h) S protein by CoV-X2. Mean of two experiments is shown.



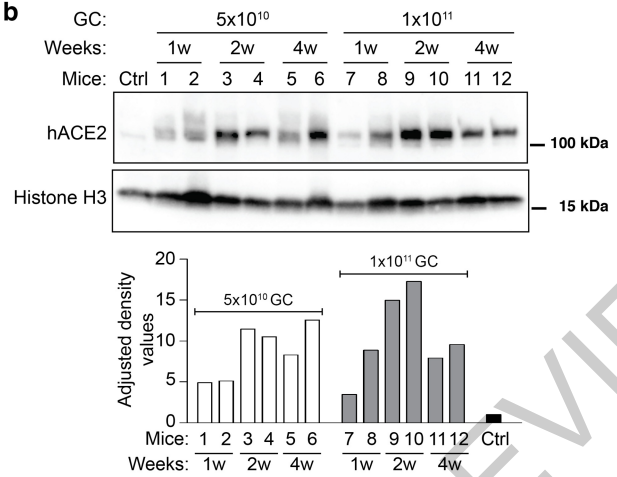
Extended Data Fig. 6 | SPR-based avidity assays confirm that CoV-X2 can engage bivalently on a single RBD. **a**, CoV-X2 and monoclonal IgGs (C121 or C135) have different binding modes available when high or low quantities of RBD are immobilized on the surface of the SPR chip. mAbs have avidity effects at high RBD concentrations due to intermolecular binding, which results in slower dissociation rate (k_d), but not at low RBD concentrations, since bivalent binding to a single RBD is impossible. In contrast, the bispecific has avidity at both high and low concentrations, since bivalent binding to its two epitopes on a single RBD is possible. k_a is not affected by avidity. **b**, Experimental

confirmation that CoV-X2 engages bivalently on a single RBD. SPR traces used to determine k_a and k_d of mAbs, Fab and bispecific at different concentrations of immobilized RBD (see Fig. 1d) are shown. **c**, Table summarizing the SPR results plotted in Fig. 1d. k_a and k_d were normalized against the values at the highest RBD concentration. k_a and Fab k_d were unaffected by the RBD concentration, as expected. k_d became faster for the monoclonals (loss of avidity) but less so for the bispecific (avidity maintained due to simultaneous binding to two sites on a single RBD).

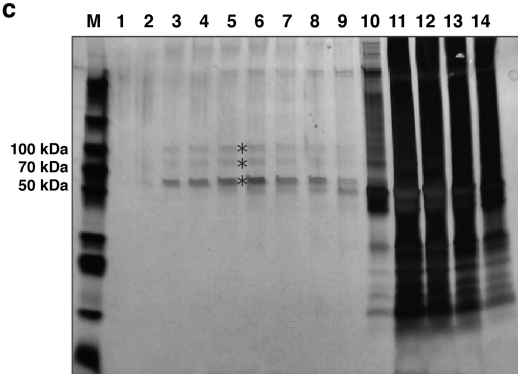
a



b



c

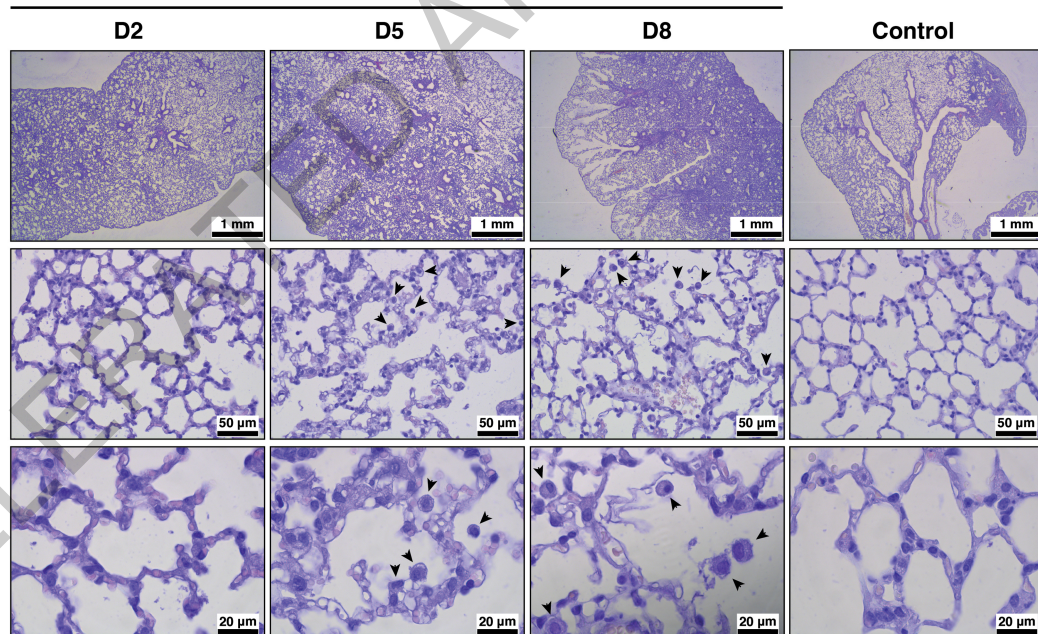


d

AAV-hACE2 dilutions	Ct1	Ct2	Ct3	Average Ct	No. of GC (log)	No. of GC	No. of GC counted from dilution
3125000x	25.63	25.68	25.70	25.67	3.526	3.36E+03	1.05E+10
625000x	24.70	24.60	24.70	24.67	3.874	7.48E+03	4.67E+09
125000x	22.84	22.69	22.83	22.79	4.526	3.36E+04	4.20E+09
25000x	20.65	20.61	20.86	20.71	5.248	1.77E+05	4.43E+09
5000x	18.33	18.43	18.25	18.34	6.070	1.18E+06	5.88E+09
1000x	16.15	16.00	16.05	16.07	6.858	7.21E+06	7.21E+09
Average No. of GC/reaction				GC/ μ l			PBS (to 40 μ l)
AAV-hACE2				6.15E+09			38.37

e

SARS-CoV-2 infection



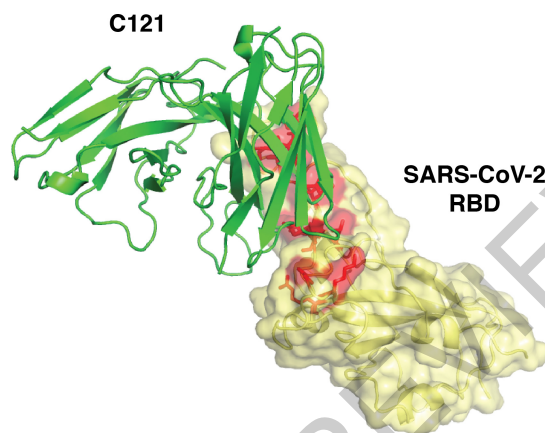
Extended Data Fig. 7 | See next page for caption.

Extended Data Fig. 7 | Generation of the new AAV-hACE2-transduced mouse model for COVID-19. **a**, Diagram of the AAV-hACE2 plasmid and corresponding Adeno Associated viral vector. **b**, Western blot analysis detecting hACE2 expression in the lungs of one non-transduced control mouse (Ctrl) and 12 mice transduced with two different doses of AAV-hACE2 viral particles (5×10^{10} or 1×10^{11} genome copies (GC)). Lung tissue was collected 1, 2, or 4 weeks (w) post transduction. Histone H3 was used as control for quantification (bottom). Quantitative analysis represents normalized data from membrane images (top, see also Supplementary Fig. 1), and was performed using ImageJ. Representative data from two independent experiments are shown. **c**, Preparation of concentrated AAV-hACE2. AAV-hACE2 plasmid was co-transfected with pHelper and AAV Rep/Cap 2/9n vectors into 293AAV cells (see Methods). In order to increase viral titers, viral particles from both cell lysate and PEG-precipitated growth medium were ultracentrifuged in discontinuous iodixanol gradient. The silver-stained SDS-PAGE gel shows 14 consecutive fractions: 1-9 represent enriched AAV fractions used for experiments, whereas fractions 10-14 are contaminated with proteinaceous cell debris. Iodixanol was chosen as a density gradient medium due to its low

toxicity *in vivo* and its easy removal by ultrafiltration. M is protein marker, * are AAV capsid proteins VP1, VP2, and VP3. Representative data from two independent experiments are shown. **d**, The amount of AAV particles was estimated by qRT-PCR. The number of genome copies (GC) expressed as log was calculated from a standard curve. From one 15 cm^2 dish, $75 \mu\text{l}$ with 2.0×10^{12} GC/ml were prepared, which is sufficient for hACE2 humanization of 37 mice. **e**, Kinetic of lung histopathology in SARS-CoV-2 infected ACE2 humanized mice. Hematoxylin and Eosin-stained sections showed inflammatory infiltrates composed of lymphocytes, macrophages, neutrophils, and fibroblasts replacing the alveoli. The size of the affected areas increased over time (area of diffuse alveolar damage: control <5-10%, 2 dpi <10-30%, 5 dpi 20-80%, 8 dpi 50-90%). Alveolar septa were thickened in areas close to infiltrates. In samples collected at 5 and 8 dpi, an increased number of activated macrophages with foamy cytoplasm (black arrowheads) was seen. AAV-hACE2 transduced, SARS-CoV-2 uninfected mice were used as control and showed no significant pathology. Each image is representative of two separate experiments (n = 3 to 5 mice per group).

a

wt	mutant	Strain Name	wt	mutant	Strain Name
Arg403	Lys	/USA/VA-DCLS-0630/2020 /USA/VA-DCLS-0439/2020 /AUS/VIC1787/2020	Gly485	Arg	/AUS/VIC1829/2020 /AUS/VIC1960/2020 /AUS/VIC1693/2020 /AUS/VIC1660/2020 /AUS/VIC1683/2020 /AUS/VIC1588/2020 /AUS/VIC1565/2020 /AUS/VIC1611/2020 /AUS/VIC1812/2020 /AUS/VIC2023/2020
Lys444	Asn	/AUS/VIC4515/2020			
Gly446	Asp	/USA/FL-BPHL-2211/2020			
	Val	/USA/MN-MDH-1430/2020 /AUS/VIC913/2020 /AUS/VIC6087/2020 /AUS/VIC9542/2020			
		Gln			/USA/VA-DCLS-1404/2020
Leu452	Arg	/USA/CA-CZB-12872/2020	Cys488	Arg	/IRN/COVID19-IRVSH4/2020
	Met	/BHR/340798279_S5_L001/2020 /USA/CA-CZB-1043/2020 /IND/906/2020	Phe490	Leu	/AUS/VIC10024/2020 /AUS/VIC766/2020
		Phe	/AUS/VIC10121/2020 /AUS/VIC5196/2020		Val
Leu455	Phe		Pro491	His	/IND/906/2020
Val483	Ala	/USA/WA-UW-6527/2020 /USA/WA-UW-1587/2020 /USA/WA-RML-2/2020 /USA/WA-RML-6/2020 /USA/WA-RML-5/2020 /USA/UT-03764/2020	Ser494	Pro	/AUS/VIC10121/2020 /AUS/VIC5196/2020
		Phe			/AUS/VIC2139/2020 /USA/MA-UW-629/2020
		Gln			/USA/WA-UW-6527/2020 /USA/CA-CZB-4047/2020 /USA/CA-CZB-11677/2020 /USA/CA-CZB-6994/2020 /USA/CA-CZB-11010/2020 /USA/MI-MDHHS-SC20047/2020 /USA/CA-CZB-12810/2020 /AUS/VIC9505/2020
					Lys
	Glu484	Ala		/AUS/VIC2139/2020 /USA/MA-UW-629/2020	
Gln		/USA/UT-UPLH-2009538/2020 /IND/GBRC278a/2020 /USA/SEARCH-1462-SAN/2020			
		Lys			
	Ala	/USA/VA-DCLS-1615/2020			

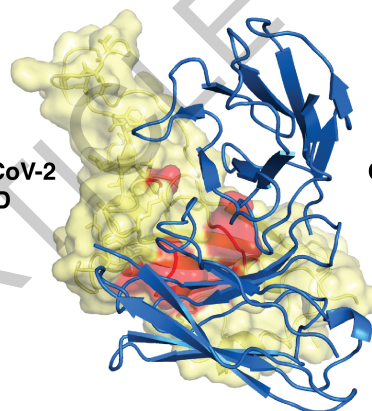


b

wt	mutant	Strain Name
Phe342	Leu	England/01_1/29
Ala344	Ser	/USA/WA-S2278/2020 /USA/WA-S2530/2020
	Thr	/IND/GBRC431a/2020
	Val	/AUS/VIC10958/2020
Thr345	Ser	/USA/WA-S1049/2020
	Ile	/PER/covper051/2020
Arg346	Thr	/IND/GBRC333/2020
Trp436	Thr	/IND/GBRC333/2020
Asn439	Lys	/USA/IL-UW/799/2020
Asn440	Lys	/HKG/Case5138/2020
Leu441	Ile	/USA/FL-BPHL-0297/2020
Asn450	Lys	/IND/906/2020

SARS-CoV-2 RBD

C135



Extended Data Fig. 8 | Natural SARS-CoV-2 variants in the C121 and C135 epitopes. Summary of naturally occurring mutations in the C121 (a) or C135 (b) epitopes reported in circulating SARS-CoV-2 (as of January 1, 2021). The location of the mutated residues is shown in red on the RBD structure. C121 and

C135 variable regions are in green and blue (PDB ID: 7K8X and 7K8Z respectively). All the variants were taken from ViPR database (<https://www.viprbrc.org/>).

Extended Data Table 1 | Summary of the P values for the mouse protection experiment

	C121	C135	CoV-X2	Isotype control
C121	—	P<0.0001	P<0.0001	P<0.01
C135	P<0.0001	—	P>0.05	P<0.0001
CoV-X2	P<0.0001	P>0.05	—	P<0.0001
Isotype control	P<0.01	P<0.0001	P<0.0001	—

Statistical comparison of body weight differences in animals treated with the individual monoclonal antibodies (C121 or C135), the CoV-X2 bispecific or isotype control at 8 dpi (related to Fig. 2e). P values were determined with the one-way ANOVA test. Comparison of the entire curves (Fig. 2e) by the One Sample Wilcoxon Test or by the ANOVA followed by Turkey-Kramer post-test reveals that the isotype control treated group is statistically different from any of the other groups (CoV-X2, $p=0.0159$; C135, $p=0.0043$; C121, $p=0.0010$).

Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- ☒ ☐ A description of all covariates tested
- ☒ ☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ 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)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ 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

SPR data were collected with Biacore 8K Control Software v2.0.15.12933.
ELISA plates were measured with the reader software Gen5 Version 1.11.5, BioTek Instruments, Inc.
DLS measurements were performed using Dynamics v7.1.7.16, Wyatt Technology Corp.
The building of bispecific antibody was performed using ALMOST toolkit version 1 and Pymol 2.4.
The Molecular Dynamics simulations were performed using Gromacs 2020.2.
LightCycler® 480 Software, Version 1.5
Modulus II Microplate Reader User interface version 2.1.0 by TURNER BioSystems
I-TASSER Suite 5.1
ImageJ Version 1.53h

Data analysis

SPR data were analyzed with Biacore Inside Evaluation Software v2.0.15.12933.
DLS data were analyzed using Dynamics v7.1.7.16 (Wyatt Technology Corp.)
Data from ELISA and mouse experiments were analyzed with GraphPad Prism Version 8.4.2.
The 3D structures were analyzed using Pymol 2.4.
The MD trajectories were analyzed using Gromacs 2020.2
Microsoft Excel 2016
BioEdit Sequence Alignment Editor, version 7.2.0.

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.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The authors declare that data supporting the findings of this study are available within the paper and its supplementary information files. All other data are available from the corresponding author upon reasonable request. Published data were taken from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>), UniProt (<https://www.uniprot.org/>), Protein Data Bank, PDB (<https://www.rcsb.org/>) and ViPR database (<https://www.viprbrc.org/>).

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

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

Sample size	No sample-size calculation was performed. The sample sizes were chosen based on experience and previously published papers (e.g., Zost et al., Nature. 2020 Aug;584(7821):443-449. doi: 10.1038/s41586-020-2548-6; Hassan et al., Cell. 2020 Aug 6;182(3):744-753.e4. doi: 10.1016/j.cell.2020.06.011.). Details about groups and sample sizes for mouse virus challenge studies are provided in the manuscript and figure legends.
Data exclusions	No data were excluded.
Replication	Experiments successfully repeated at least twice.
Randomization	The mice were randomly assigned to cages and the cages were then randomized into groups.
Blinding	Blinding was not relevant to this study, except lung pathology evaluation. The readouts of all experiments, except lung pathology evaluation, could be assessed objectively. Mouse weight loss was determined using body weight measurement as a readout, and plaque assay or RT-qPCR was used to quantify viral burden. For lung study pathology, H&E stained tissue sections were scored by an experienced histopathologist blinded to the compositions of the groups.

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

Primary antibodies used:
 - In this study we used and analyzed antibodies C121, C135 and C144 against SARS-CoV-2 (in house production).
 - Anti-Zika virus monoclonal antibody Z021 (Robbiani et al, Cell 2017) used as isotype control
 - Anti-Histone H3 antibody, Cat. No.: ab1791 (Abcam), lot: GR3237685-2, dilution: 1:1000
 - Anti-ACE2 antibody, Cat. No.: ab15348 (Abcam), lot: GR3333640-7, dilution: 1:1000
 - F4/80 (D2S9R) XP® Rabbit mAb (Cat.#70076) Cell Signaling Technology, USA, Lot 5, RRID AB_2799771, dilution 1:800

Following secondary antibodies were used:

- Goat Anti-Mouse IgG, Human ads-AP, Cat.-No.: 1030-04 (SouthernBiotech, dilution 1:500)
- Goat Anti-Human IgG-AP, Cat.-No.: 2040-04 (SouthernBiotech, dilution 1:500)
- HRP-Polymer anti-Rabbit (Ready-to-use), Zytomed, Germany, Cat. # ZUC 032-100, Lot A0880-4 (no dilution)
- Goat Anti-Rabbit IgG H&L (HRP), Cat. No.: ab205718 (Abcam), lot: GR3269880-1, dilution: 1:10000

Validation

The human monoclonal antibody Z021, which binds to the Envelope Domain III of the Zika virus, was previously reported and validated (PMID: 31413072).
No validation statements for the other antibodies that are commercially available.

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)

Expi293F cells (Thermo Fisher Scientific)
293 [HEK-293] cell line (CRL-1573™) (ATCC®)
293AAV cell line (AAV-100) (Cell Biolabs, Inc)
Neuro-2a cell line (CCL-131™) (ATCC®)
Vero E6 (CRL-1586™) (ATCC®)
293TAce2 (derived from 293T as reported in Robbiani et al. Nature, DOI: 10.1038/s41586-020-2456-9)

Authentication

Not authenticated after purchase

Mycoplasma contamination

In situ analysis never detected Mycoplasma infection.

Commonly misidentified lines
(See [ICLAC](#) register)

No commonly misidentified cell line have been used in this study.

Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

Laboratory animals

Mice C57Bl/6NCrI (females, 13-15 weeks old). Mice were housed in individually ventilated cages (Green line, Tecniplast, Italy) in the controlled environment with a 12/12 hour light-dark cycle, 20-22 °C and 45-65% humidity. Animals were fed the standard chow diet (1314 Altromin, Germany) ad libitum. Drinking water was filtered by reverse osmosis and chlorinated to 1.5 ppm. Water bottles were changed weekly.

Wild animals

The study did not involve wild animals.

Field-collected samples

The study did not involve field-collected samples.

Ethics oversight

The experiments were approved by the Committee on the Ethics of Animal Experiments of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Institute of Molecular Genetics of the Czech Academy of Sciences, and of the Departmental Expert Committee for the Approval of Projects of Experiments on Animals of the Academy of Sciences of the Czech Republic (permits 82/2020 and 101/2020).

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