

1 **Bispecific antibody neutralizes circulating SARS-CoV-2 variants, prevents**
2 **escape and protects mice from disease**

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41 **Summary**

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

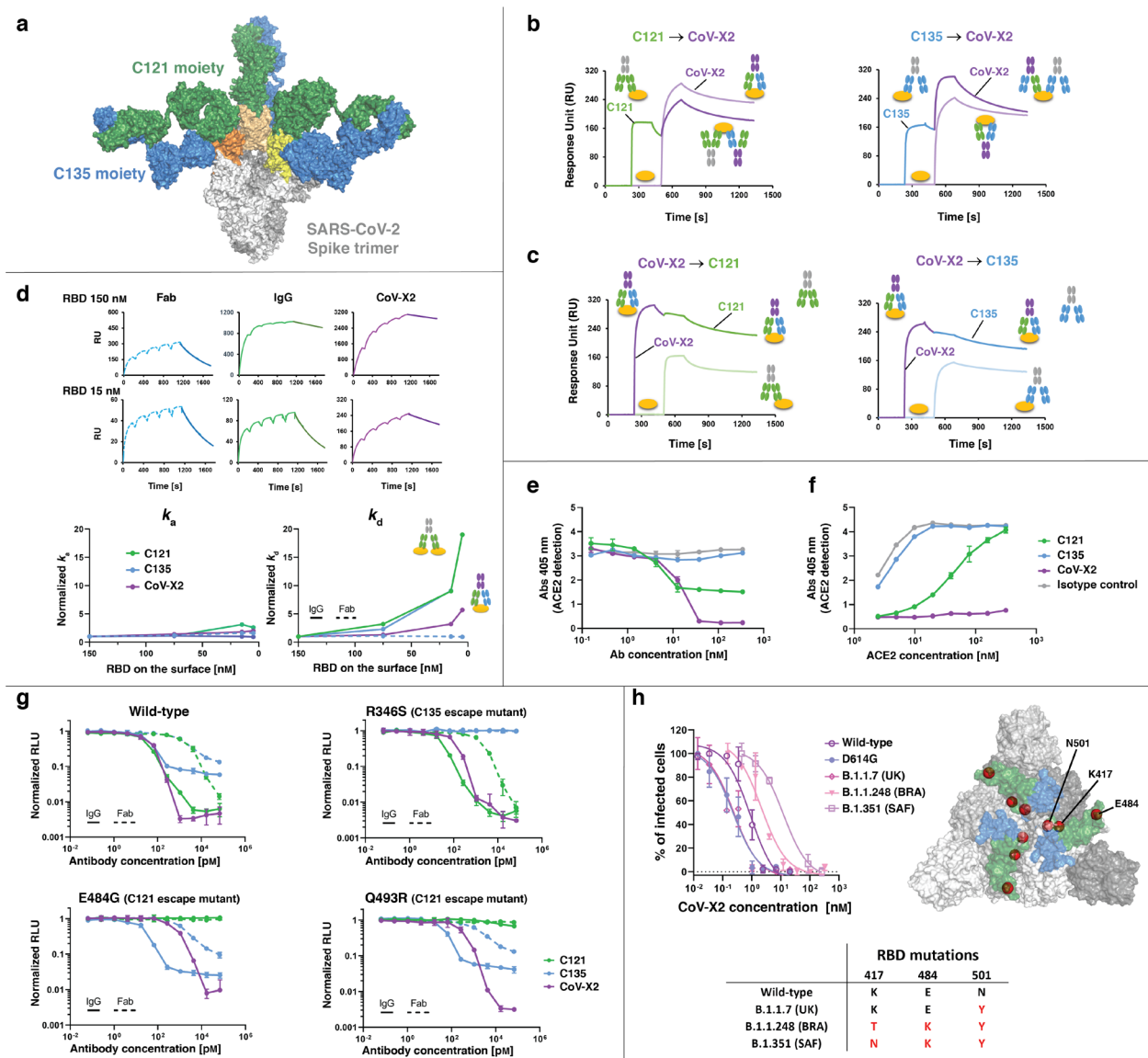
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54 The COVID-19 pandemic prompted an unprecedented effort to develop effective countermeasures
55 against SARS-CoV-2. Pre-clinical data and phase III clinical studies indicate that monoclonal
56 antibodies (mAbs) could be effectively deployed for prevention or treatment during the viral
57 symptoms phase of the disease^{1,2}. Cocktails of two or more mAbs are preferred over a single
58 antibody for increased efficacy and prevention of viral escape. However, this approach requires
59 increased manufacturing costs and volumes, which are problematic at a time when the supply chain
60 is under pressure to meet the high demand for COVID-19 therapeutics, vaccines and biologics in
61 general⁴. Cocktails also complicate formulation^{5,6} and hinder novel strategies like antibody
62 delivery by viral vectors or by non-vectored nucleic acids⁷⁻⁹. Instead, multispecific antibodies
63 embody the advantages of a cocktail within a single molecule.

64 To this avail, we employed structural information¹⁰ and computational simulations to
65 design bispecifics that would simultaneously bind to (i) independent sites on the same RBD and
66 (ii) distinct RBDs on a S trimer. Out of several designs evaluated by atomistic Molecular Dynamics
67 simulations, 4 were produced and CoV-X2 was the most potent neutralizer of SARS-CoV-2
68 pseudovirus, with half-maximal inhibitory concentration (IC₅₀) = 0.04 nM (5.8 ng/mL) (Extended
69 Data Fig.1). CoV-X2 is a human-derived, CrossMAB-format IgG1-like bispecific antibody¹¹
70 resulting from the combination of the Fragment antigen binding (Fab) of mAbs C121 and C135,
71 two potent SARS-CoV-2 neutralizers³. Structural predictions showed that CoV-X2, but not its
72 parental monoclonals, can bind bivalently to all RBD conformations on the S trimer, preventing
73 ACE2 access (Fig.1a and Extended Data Fig.2)¹².

74 CoV-X2 bound with low nanomolar affinity to RBD, S trimer, and to several mutants,
75 including the naturally occurring variants B.1 (D614G in S protein), B.1.1.7 (N501Y in RBD) and

76 B.1.351 (K417N, E484K and N501Y in RBD)^{13,14}, and the escape mutants of the parental mAbs¹⁵
 77 (Extended Data Figs.3-5).



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

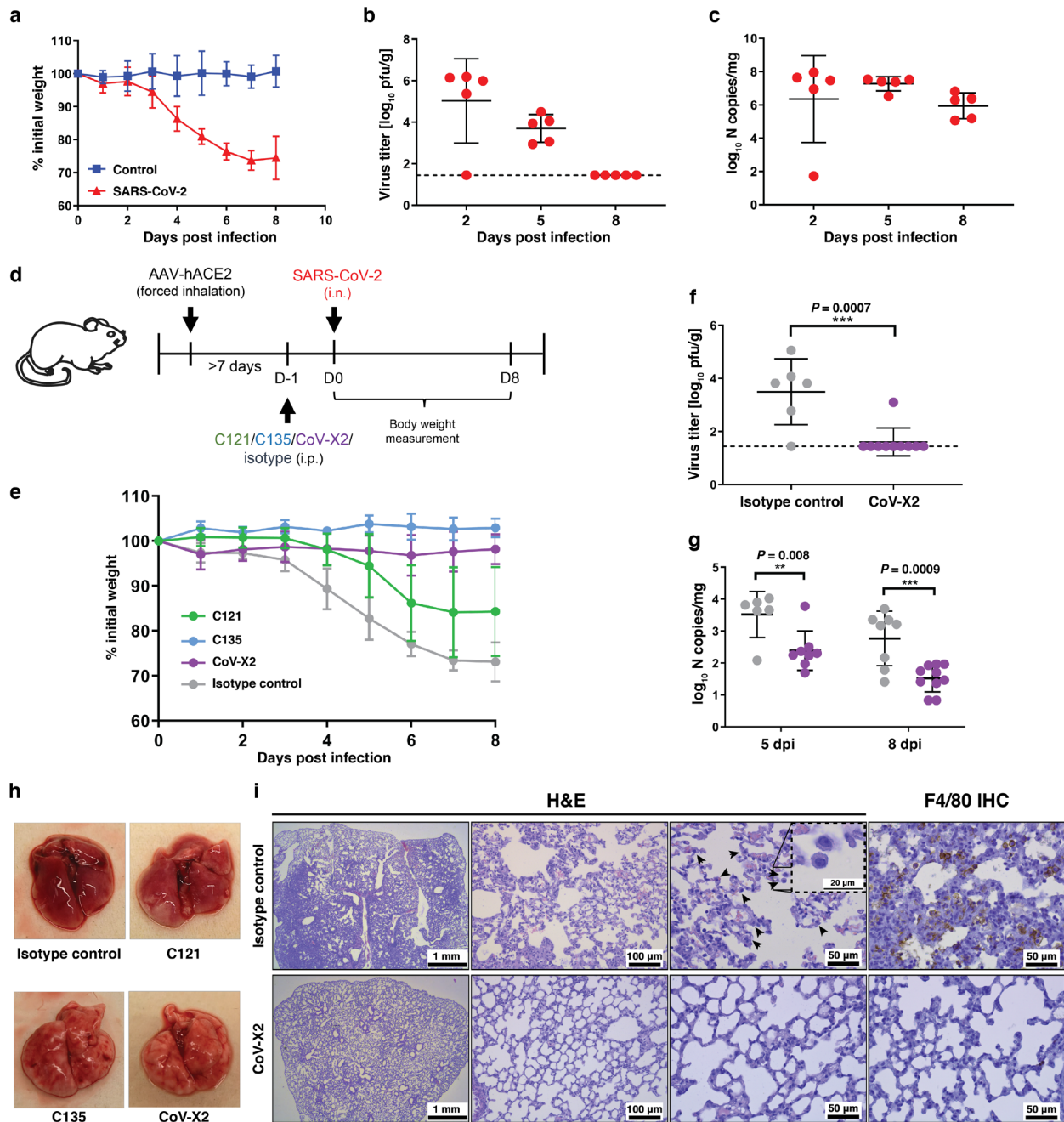
91 antibody/S trimer complexes is measured either with increasing concentration of the indicated antibody and
92 constant ACE2 (e), or at constant antibody concentration with increasing ACE2 (f). Mean with standard
93 deviation of two experiments is shown. g, CoV-X2 neutralizes SARS-CoV-2 pseudovirus and escape
94 mutants of its parental mAbs. Normalized relative luminescence (RLU) for cell lysates after infection with
95 nanoluc-expressing SARS-CoV-2 pseudovirus in the presence of increasing concentrations of antibodies.
96 Wild-type SARS-CoV-2 pseudovirus (left) is shown alongside three escape mutants generated in the
97 presence of C121 or C135¹⁵. Dashed lines are parental Fabs. Mean with standard deviation; one of two
98 independent experiments. h, Neutralization of SARS-CoV-2 isolates with sequences corresponding to
99 viruses first isolated in China (wild-type), Italy (D614G), United Kingdom (UK; B.1.1.7), Brazil (BRA;
100 B.1.1.248) and South Africa (SAF; B.1.351). RBD residues mutated in the variants are indicated in the
101 table and as red spheres on the S trimer structure, where the epitope of C135 (blue) and C121 (green) are
102 shown.

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105 CoV-X2 also bound to pre-formed C121/RBD and C135/RBD complexes, thus confirming
106 that both of its arms are functional (Fig.1b,c). Next, an avidity assay by Surface Plasmon
107 Resonance (SPR) was used to experimentally confirm the computational prediction that CoV-X2
108 can simultaneously engage two sites on the same RBD (Methods, Fig.1d and Extended Data Fig.6).
109 Avidity occurs when IgGs bind bivalently to antigens, resulting in slower dissociation rates (k_d)
110 (Extended Data Fig.6a). Accordingly, C121 and C135 IgG showed avidity at high antigen
111 concentrations due to inter-molecular binding of adjacent RBDs; at lower antigen concentrations
112 the dissociation rate was instead faster since inter-molecular binding was prevented by the
113 increased distance between RBD molecules, resulting in loss of avidity. Intra-molecular avidity is
114 not possible for C121 and C135 since a single epitope is available on each RBD molecule. By
115 contrast, CoV-X2 maintained avidity even at low antigen concentrations, indicating bivalent, intra-
116 molecular binding (Fig.1d and Extended Data Fig.6). ELISA assays were then performed to
117 evaluate the ability of CoV-X2 to inhibit the binding of recombinant ACE2 to the S trimer
118 (Fig.1e,f). In line with the structural information¹⁰, C135 did not affect the ACE2/S interaction.
119 C121, which occupies the ACE2 binding site on the RBD, prevented ACE2 binding but only
120 partially. By contrast, ACE2 binding was not detected in the presence of CoV-X2, suggesting a
121 synergistic effect by the two moieties composing the bispecific.

122 To assess the neutralizing ability of CoV-X2 *in vitro*, we first used SARS-CoV-2
123 pseudoviruses¹⁶. The bispecific neutralized pseudovirus carrying wild-type SARS-CoV-2 S at sub-
124 nanomolar concentrations ($IC_{50} = 0.04$ nM (5.8 ng/mL); $IC_{90} = 0.3$ nM (44 ng/mL)), which was
125 similar or better than the parental IgGs and >100-fold better IC_{50} than the parental Fabs (Fig.1g).
126 CoV-X2 remained effective against pseudoviruses bearing escape mutations that made them
127 resistant to the individual mAbs (Fig.1g)¹⁵ and against a pseudovirus with RBD mutations found
128 in the B.1.351 variant (first reported in South Africa, $IC_{50} = 1.3$ nM (191 ng/mL); Extended data
129 Fig. 5). To confirm CoV-X2 efficacy, we performed plaque reduction neutralization assays with
130 infectious virus. CoV-X2 efficiently neutralized: SARS-CoV-2 ($IC_{50} = 0.9$ nM); the D614G variant
131 first appearing in Europe (B.1, $IC_{50} = 0.2$ nM); the B.1.1.7 variant first observed in the United
132 Kingdom ($IC_{50} = 0.2$ nM); the B.1.1.248 variant first isolated in Brazil ($IC_{50} = 2.1$ nM) and B.1.351
133 first isolated in South Africa ($IC_{50} = 12$ nM; Fig.1h). The latter two have almost identical mutations
134 in the RBD, the only difference being N vs. T at position 417, which does not interact with
135 CoV-X2. Nonetheless, neutralization of B.1.351 was lower, suggesting either some
136 conformational differences in the RBD or long-range effects deriving from other mutations in the
137 S protein. A similar behavior is seen with the wild-type sequence (D614), which has lower
138 neutralization than G614 even if no other difference is present; a plausible explanation is that G614
139 makes the CoV-X2 epitopes more accessible by favoring the RBD ‘up’ conformation.¹⁷ We
140 conclude that the *in vitro* binding and neutralizing properties of CoV-X2 make it preferable over
141 its parental antibodies.

142 To assess the clinical potential of CoV-X2, we investigated its ability to protect animals
143 from infection and disease. We first developed a novel mouse model in which human ACE2

144 (hACE2) is expressed by upper and lower respiratory tract cells upon inhalation of a modified
 145 Adeno Associated Virus (AAV-hACE2, see Methods, Fig.2 and Extended Data Fig.7).



146

147 **Fig.2 | CoV-X2 protects AAV-hACE2-transduced mice against SARS-CoV-2 disease.** **a**, Loss of body
 148 weight over time in SARS-CoV-2 infected mice. 13 to 15 weeks old C57Bl/6NcrJ wild-type female mice
 149 were transduced with AAV-hACE2 by forced inhalation, which provides delivery of viral particles to both
 150 upper and lower respiratory tract. After >7 days, mice were either infected with SARS-CoV-2 (1×10^4 pfu)
 151 or received vehiculum by the intranasal route. Weight was monitored daily for 8 days (SARS-CoV-2, n =
 152 5; control, n = 4). Mean with standard deviation is shown. **b**, Kinetic of viral burden in the lungs from

153 SARS-CoV-2-infected mice by plaque assays. Mean with standard deviation; the dashed line indicates the
154 limit of detection. **c**, Kinetic of viral RNA levels in lung samples from SARS-CoV-2-infected mice by RT-
155 qPCR. Mean with standard deviation. **d**, Schematic of the experimental layout. Wild-type mice were
156 transduced with AAV-hACE2 by forced inhalation. After >7 days, mice were inoculated intraperitoneally
157 (i.p) with 150 µg of antibodies. One day later, the mice were infected intranasally (i.n.) with SARS-CoV-2
158 (1×10^4 pfu). **e**, Changes in body weight upon infection were monitored daily in antibody-treated mice
159 (C121, n=9; C135, n=5; CoV-X2, n=13; isotype control, n=10). Mean with standard deviation is shown. **f**,
160 Lung viral burden by plaque assay at 5 dpi (isotype control, n=6; CoV-X2, n=10). The dashed line indicates
161 the limit of detection; mean with standard deviation. P value was calculated with two-tailed Student's t test.
162 **g**, Spleen viral RNA levels by RT-qPCR at 5 and 8 dpi (gray: isotype control; purple: CoV-X2). Mean with
163 standard deviation. P value was calculated with two-tailed Student's t test. **h**, Photographs of lungs collected
164 from infected mice (8 dpi). **i**, Histopathology and F4/80 immunohistochemistry (IHC). Hematoxylin and
165 Eosin-stained (H&E) sections of paraffin-embedded lungs from infected mice (8 dpi). Arrowheads point to
166 foamy macrophages. F4/80 IHC shows abundant macrophage infiltration in lungs of mice treated with
167 isotype control but not with CoV-X2.

168

169 This approach enables rapid production of large cohorts of animals and has the advantage of being
170 applicable to wild-type and mutant mouse colonies, independently of age and gender. Moreover,
171 since AAV vectors are only weakly immunogenic and cytotoxic, the system allows for prolonged
172 expression of hACE2¹⁸⁻²¹ (Extended Data Fig.7). SARS-CoV-2 infection of ACE2 humanized
173 mice results in progressive weight loss, respiratory pathology and disease requiring culling on day
174 8 post infection (dpi, Fig.2a–c and Extended Data Fig.7).

175 To evaluate the protective effect of antibodies, hACE2 mice were treated with antibody
176 (150 µg) one day before SARS-CoV-2 challenge and monitored over time (Fig.2d–i). Upon
177 intranasal infection with 1×10^4 pfu of SARS-CoV-2 (SARS-CoV-2/human/Czech
178 Republic/951/2020), isotype control treated animals showed weight loss starting at 3 dpi, and by
179 8 dpi most animals had lost approximately 25–30% of their body weight reaching humane endpoint
180 (Fig.2e). Infectious virus could be recovered from the lungs (Fig.2f), viral RNA was detected also
181 in the spleen (Fig.2g) but not in the heart (data not shown). Lung pathology resembled severe
182 COVID-19 in humans²² and was characterized by Diffuse Alveolar Damage (DAD; 50-80% of
183 tissue area), alveolar replacement with infiltrates of immune cells and fibroblasts, thickened septa

184 and infiltrations by activated macrophages with foamy cytoplasm (Fig.2i). In contrast, animals
185 treated with CoV-X2 maintained their body weight ($P < 0.0001$ at 4–8 dpi when compared to
186 isotype; Fig.2e; P values between all groups in Extended Data Table1), had reduced viral RNA in
187 the spleen (Fig.2g) and displayed neither macro- nor histopathological changes (DAD $< 5-10\%$,
188 Fig.2h,i). While infectious virus could be readily recovered from controls (5 of 6), it was only
189 recovered from 1 out of 10 CoV-X2 treated animals at 5 dpi (Fig. 2f) and could not be recovered
190 from any of 13 animals at 8 dpi (data not shown). Since none of the CoV-X2 treated mice exhibited
191 symptoms at any time, we conclude that CoV-X2 protects mice from infection and disease.

192 Since monotherapy with C121 or C135 mAbs leads to virus escape *in vitro*¹⁵, we treated
193 hACE2 mice with the individual antibodies and sequenced the virus. Only wild-type RBD
194 sequences were obtained from controls (n=10). Instead, the virus in mice treated with C121
195 selected for a mutation resulting in E484D (5 of 5 mice that were analyzed at 8 dpi). C121 escape
196 mutations at E484 were previously observed *in vitro*¹⁵ and changes at this residue (present also in
197 the B.1.351 and B.1.1.248 variants) reduce neutralization by human sera by more than 10-fold²³.
198 E484D affects intermolecular H-bonds at the core of the C121/RBD interface and it is suggested
199 to increase the RBD affinity for ACE2²⁴. Virus with D484 is pathogenic, since 7 out of 9 mice
200 treated with C121 developed disease (Fig.2e) and only D484 virus was found in their lungs. In
201 contrast, and unlike the *in vitro* results¹⁵, no virus evasion or pathology was observed in mice
202 treated with C135 (n=5; Fig.2e and data not shown). In CoV-X2 treated animals, even though no
203 infectious virus was retrieved (8 dpi, n=13) and no symptoms ever noticed, low levels of residual
204 viral RNA could be detected in some animals after 40 cycles of PCR amplification: in 6 of 13
205 animals the virus sequence was wild-type and in 2 mice overlapping sequencing traces were
206 consistent with coexistence of wild-type and D484. Thus, in those 2 of 13 animals with D484 CoV-

207 X2 remained protective even if the mutation diluted the effective antibody concentration,
208 presumably leaving only the C135 moiety active. Finally, CoV-X2 was protective also when
209 administered 12 hours after SARS-CoV-2 challenge (Extended Data Fig.8)

210 Monoclonal antibodies targeting the SARS-CoV-2 S are in advanced clinical trials and
211 show promise against COVID-19^{1,2}. Concomitant use of multiple antibodies is preferred for
212 increased efficacy and added resistance against viral evasion. Indeed, the virus can escape pressure
213 by a single antibody *in vitro* and, as shown here, also in animals. Moreover, RBD mutations
214 threatening the efficacy of single monoclonals have already been detected in virus circulating in
215 minks and humans²⁵, including mutations at the C121 and C135 epitopes (Extended Data Fig.9).
216 One disadvantage of antibody cocktails is the requirement for twice or more the development and
217 production capacity than for single mAbs, which is a significant challenge in light of the
218 augmented demand due to COVID-19 related vaccines and therapeutics on top of the need to
219 maintain production of biologics for other diseases.⁴

220 Multispecific antibodies offer the advantages of cocktails in a single molecule. Indeed, we
221 have shown that the CoV-X2 bispecific is more effective than the related monoclonals at inhibiting
222 ACE2 binding; it has sub-nanomolar IC₅₀ against a broader array of viral sequences; and it protects
223 animals from SARS-CoV-2 even when C121, its potent parental mAb, fails due to the insurgence
224 of viral escape. C135, the other parental mAb, did not generate escape in our animal experiment
225 but readily generated them *in vitro*¹⁵. CoV-X2 is expected to be more resistant to viral escape
226 compared to monoclonals. Indeed, we have shown that CoV-X2 binds and neutralizes mutants not
227 recognized by its parental mAbs as well as variants of concern that recently emerged in United
228 Kingdom¹³, South Africa¹⁴ and Brazil²⁶.

229 CoV-X2, unlike other multispecifics²⁷, is a fully human IgG-like molecule. As such, it has
230 favorable developability and could be further engineered to alter effector functions. For example,
231 the Fragment crystallizable (Fc) of CoV-X2 was already modified to modulate its interaction with
232 Fc receptors and complement (LALA-PG mutations)²⁸ without affecting its antigen-binding
233 properties. The LALA modification prevents Antibody Dependent Enhancement (ADE) of
234 flavivirus infection^{29,30} and it may be a desirable modification also in the context of SARS-CoV-
235 2, since cellular and animal experiments with coronaviruses, including SARS-CoV³¹⁻³³, support
236 the possibility of ADE. Other modifications, like LS²⁸ for increased half-life, are easily achievable.
237 Finally, CoV-X2 is human-derived and produced in a format (CrossMab) already shown to be safe
238 in clinical trials³⁴, which further supports its developability. Thus, IgG-like bispecifics are worth
239 adding to the arsenal employed to combat SARS-CoV-2 and its plausible future mutations.

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326

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344

345 **Author contributions**

346 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,
347 F.B., J.C.S, F.G, S.Ga, designed and carried out experiments and analyzed results, produced
348 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.,
349 D.Z., P.B., I.B., P.S., D.R., performed animal experiments and analyzed the results. L.V, D.F.R.,
350 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

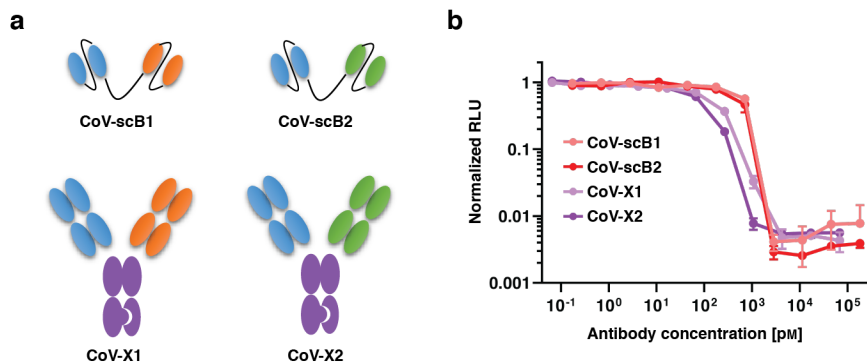
351 experiments and analyzed the results. P.N., T.M., R.N., O.P., J.P., J.R., R.S. conceived and
352 designed the mouse model. L.V., D.F.R., D.R, R.D.G. wrote the manuscript with input from all
353 co-authors.

354 **Competing interests**

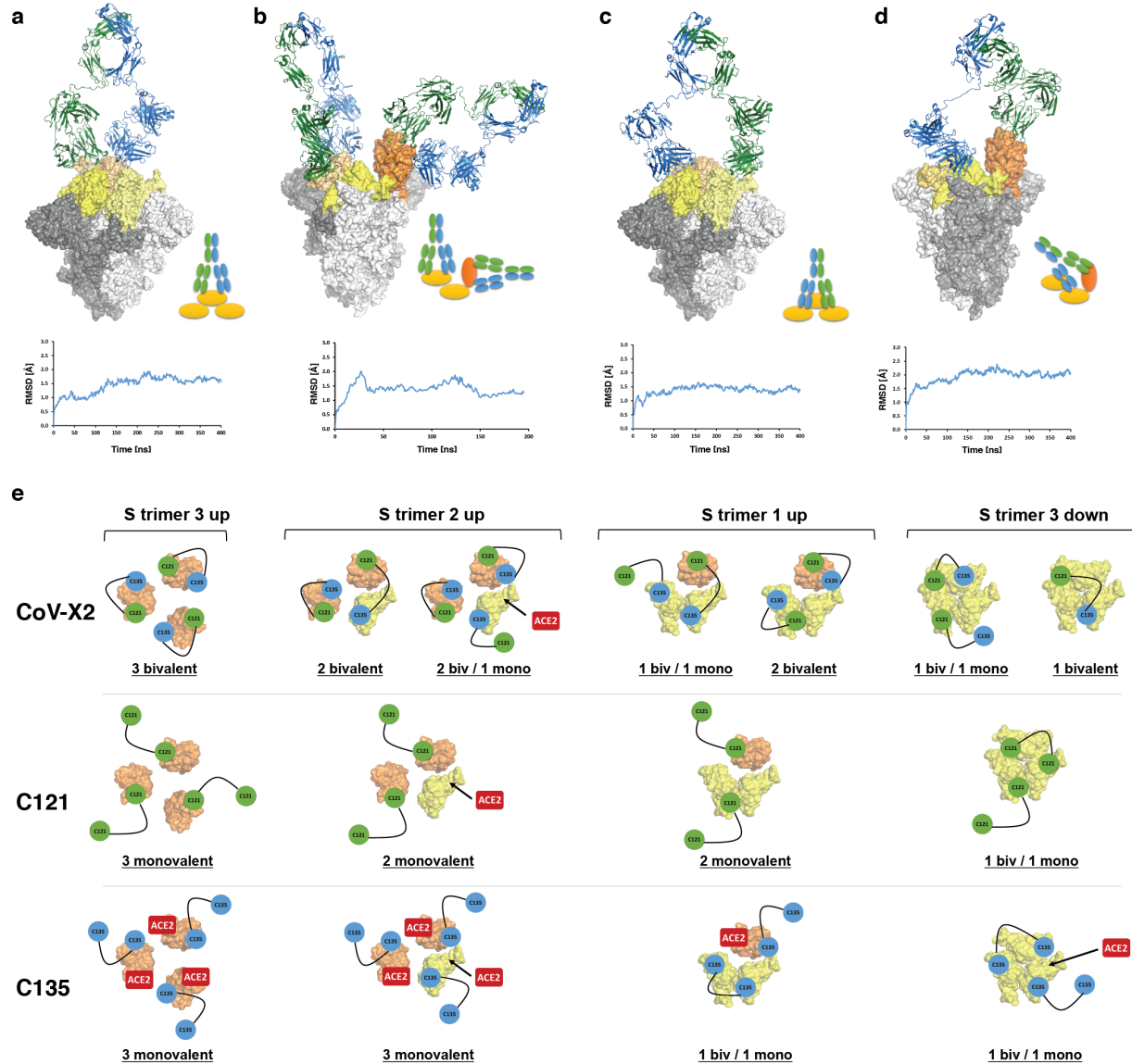
355 In connection with this work the Institute for Research in Biomedicine has filed a provisional
356 patent application on which L.V. is inventor (PCT/EP2020/085342). The Rockefeller University
357 has filed a provisional patent application on coronavirus antibodies on which D.F.R. and M.C.N.
358 are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

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360 **Extended data figures**



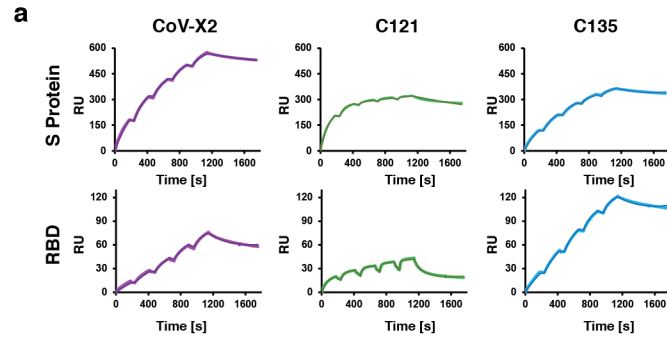
361 **Extended Data Fig.1 | Neutralization of SARS-CoV-2 pseudovirus by bispecific antibodies. a,**
362 **Schematic representation of the 4 bispecific constructs; two in scFv format and two as IgG-like CrossMab**
364 **with knob-in-hole. The parental monoclonals forming the bispecifics are color-coded (C135 blue, C144**
365 **orange, C121 green; Fc region in purple). b, All 4 constructs neutralize SARS-CoV-2 pseudovirus *in vitro***
366 **at sub-nanomolar concentrations (IC₅₀: 0.13, 0.04, 0.74 and 0.53 nM for CoV-X1, CoV-X2, CoV-scB1 and**
367 **CoV-scB2, respectively). Normalized relative luminescence values, which correlate to infection, are**
368 **reported versus antibody concentration, as detailed in Schmidt *et al.*¹⁶. Mean with standard deviation is**
369 **shown, representative of two independent experiments.**



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371 **Extended Data Fig.2 | CoV-X2 engages its epitopes on all RBD conformations on the S trimer. a–d,**
 372 Molecular Dynamics (MD) simulations of the complex between the CoV-X2 bispecific and S trimers with
 373 RBD in either all down, all up or mixed up/down conformations show that CoV-X2 can engage a single
 374 RBD with both arms (a,b), two adjacent RBDs in the down conformation (c), and two RBDs in the up/down
 375 conformation (b,d). The complexes were subjected to up to 400 ns of fully atomistic MD simulations to
 376 assess feasibility and stability of the bound conformations. Root-mean-squared deviations (RMSD) values
 377 are shown to indicate structural stability. S trimer is in shades of grey, RBDs in yellow (down conformation)
 378 and orange (up), the C121 and C135 moieties of CoV-X2 are in green and blue, respectively. e, Schematic
 379 representation of the computationally predicted binding modes of CoV-X2, C121 IgG and C135 IgG on the
 380 S trimer, colored as in a–d. Antibodies are represented by connected circles; ACE2 is in red on the RBD if
 381 it can bind directly to a given conformation; it has an arrow pointing to the RBD if ACE2 binding is
 382 achieved after an allowed switch to the up conformation. For example, in the 3-up conformation (left),
 383 CoV-X2 can engage all the RBDs with bivalent binding, whereas C121 and C135 can only achieve
 384 monovalent binding. C135 binding does not prevent interaction with ACE2. The situation is similar in the

385 other S conformations (2-up 1-down, 2-down 1-up and 3-down), with only the bispecific achieving bivalent
386 interaction and preventing ACE2 access in all conformations.

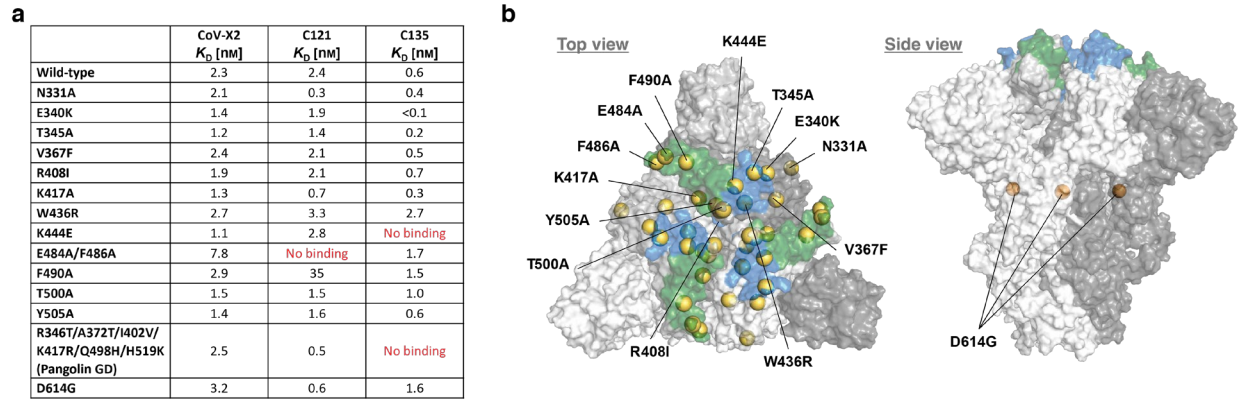


b

		CoV-X2	C121	C135
Spike protein	k_a [$\cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$]	0.69	1.33	0.62
	k_d [$\cdot 10^{-3} \text{ s}^{-1}$]	0.12	0.17	0.13
	K_D [nM]	0.18	0.13	0.21
RBD	k_a [$\cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$]	0.58	1.30	0.20
	k_d [$\cdot 10^{-3} \text{ s}^{-1}$]	1.35	3.15	0.12
	K_D [nM]	2.35	2.40	0.59

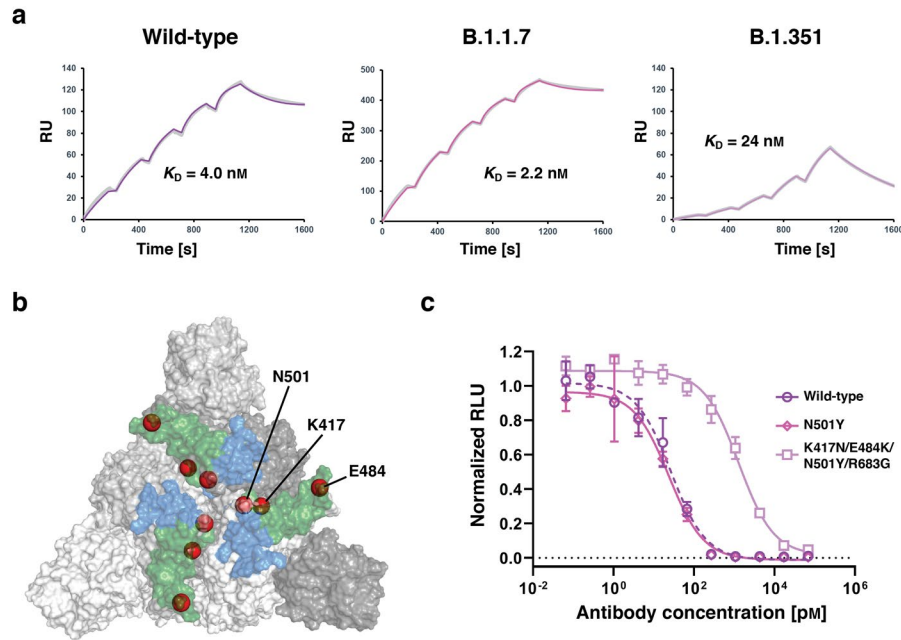
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388 **Extended Data Fig.3 | CoV-X2 and its parental mAbs bind recombinant, isolated RBD and S trimer**
 389 **with low nanomolar affinity. a,** Representative SPR traces from which the data in (b) was derived. **b,**
 390 Kinetic parameters for the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



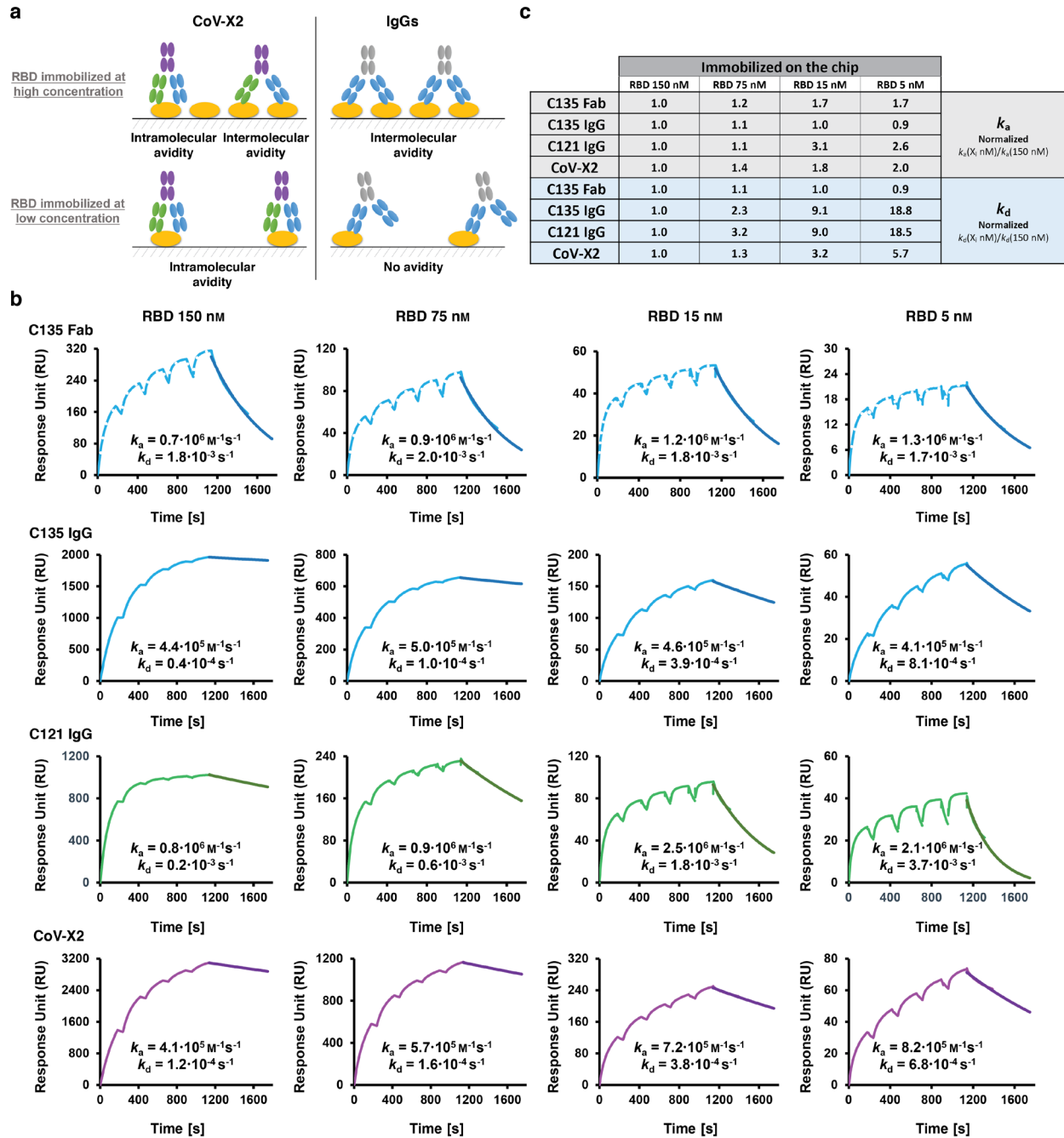
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392 **Extended Data Fig.4 | CoV-X2 binds with low-nanomolar affinity to S protein mutants, including**
 393 **some that are not recognized by the parental mAbs C121 and C135. a, SPR-derived binding affinities**
 394 **of CoV-X2, C121 IgG and C135 IgG to several S trimer mutants. b, Mutations tested in (a) are indicated**
 395 **by yellow spheres on the surface representation of the S trimer. The epitopes of C121 (green) and C135**
 396 **(blue) are shown.**



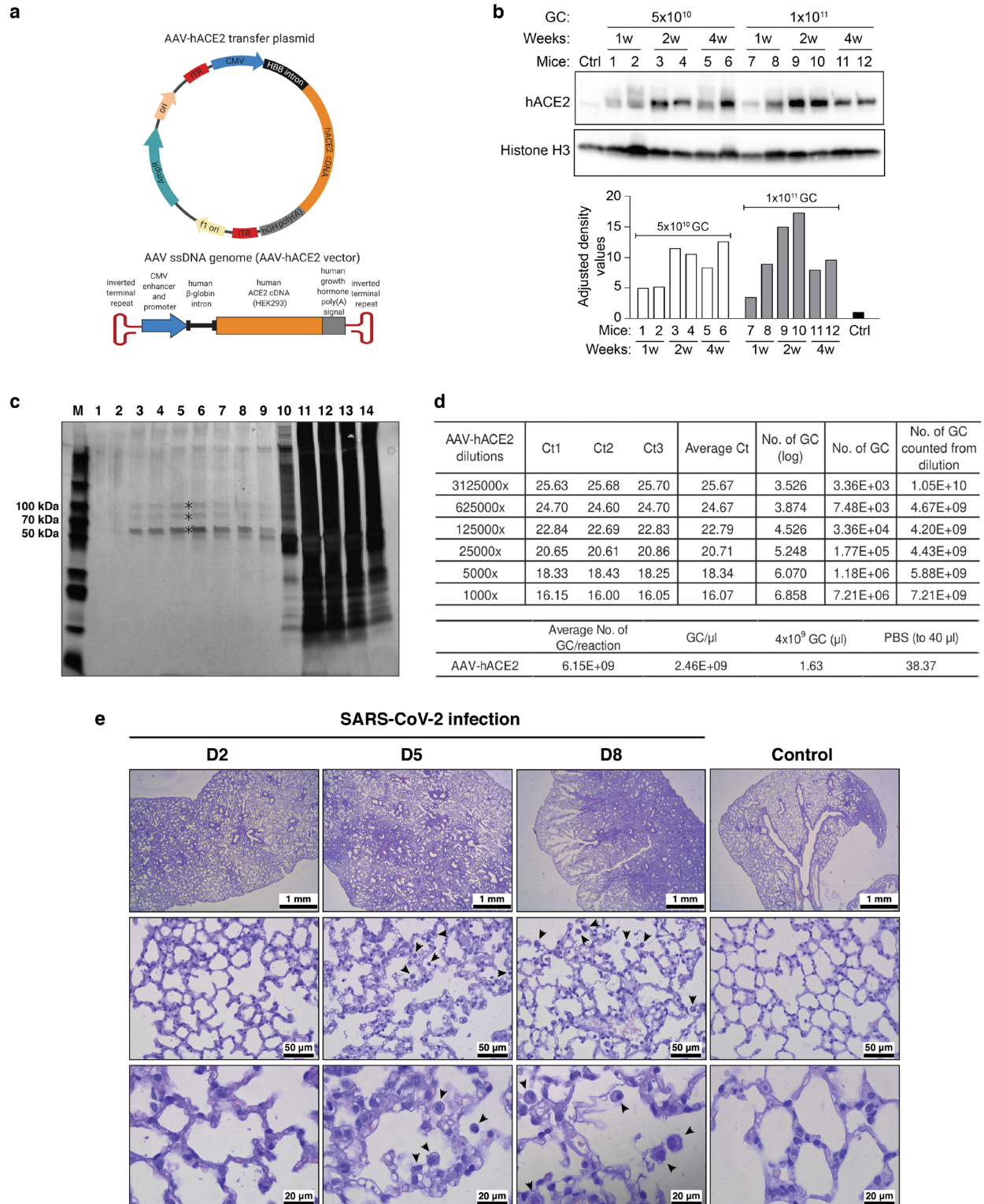
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398 **Extended Data Fig.5 | Efficacy of CoV-X2 against B.1.1.7 and B.1.351 variants.** **a**, SPR traces showing
399 binding of CoV-X2 to the RBD corresponding to wild-type, B.1.1.7 (also known as UK) and B.1.351 (also
400 known as South African) variants of SARS-CoV-2. **b**, Residues mutated in the variants are shown as red
401 spheres on the surface representation of the S trimer. The epitopes of C121 (green) and C135 (blue) are
402 shown. **c**, Neutralization of SARS-CoV-2 pseudoviruses expressing wild-type, N501Y and
403 K417N/E484K/N501Y/R683G (corresponding to South African mutants in the RBD, see Figure 1h) S
404 protein by CoV-X2.
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 407 **Extended Data Fig.6 | SPR-based avidity assays confirm that CoV-X2 can engage bivalently on a**
 408 **single RBD.** **a**, CoV-X2 and monoclonal IgGs (C121 or C135) have different binding modes available
 409 when high or low quantities of RBD are immobilized on the surface of the SPR chip. mAbs have avidity
 410 effects at high RBD concentrations due to intermolecular binding, which results in slower dissociation rate
 411 (k_d), but not at low RBD concentrations, since bivalent binding to a single RBD is impossible. In contrast,
 412 the bispecific has avidity at both high and low concentrations, since bivalent binding to its two epitopes on
 413 a single RBD is possible. k_a is not affected by avidity. **b**, Experimental confirmation that CoV-X2 engages
 414 bivalently on a single RBD. SPR traces used to determine k_a and k_d of mAbs, Fab and bispecific at different
 415 concentrations of immobilized RBD (see Fig.1d) are shown. **c**, Table summarizing the SPR results plotted
 416 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

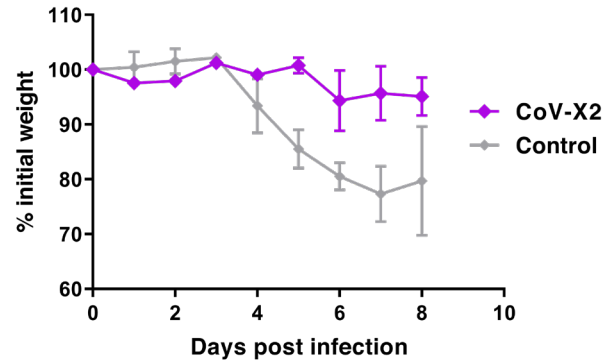
417 unaffected by the RBD concentration, as expected. k_d became faster for the monoclonals (loss of avidity)
418 but less so for the bispecific (avidity maintained due to simultaneous binding to two sites on a single RBD).



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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

425 quantification (bottom). **c**, Preparation of concentrated AAV-hACE2. AAV-hACE2 plasmid was co-
426 transfected with pHelper and AAV Rep/Cap 2/9n vectors into 293AAV cells (see Methods). In order to
427 increase viral titers, viral particles from both cell lysate and PEG-precipitated growth medium were
428 ultracentrifuged in discontinuous iodixanol gradient. The silver-stained SDS-PAGE gel shows 14
429 consecutive fractions: 1-9 represent enriched AAV fractions used for experiments, whereas fractions 10-
430 14 are contaminated with proteinaceous cell debris. Iodixanol was chosen as a density gradient medium
431 due to its low toxicity *in vivo* and its easy removal by ultrafiltration. M is protein marker, * are AAV capsid
432 proteins VP1, VP2, and VP3. **d**, The amount of AAV particles was estimated by qRT-PCR. The number of
433 genome copies (GC) expressed as log was calculated from a standard curve. From one 15 cm² dish, 75 μ l
434 with 2.0×10^{12} GC/ml were prepared, which is sufficient for hACE2 humanization of 37 mice. **e**, Kinetic of
435 lung histopathology in SARS-CoV-2 infected ACE2 humanized mice. Hematoxylin and Eosin-stained
436 sections showed inflammatory infiltrates composed of lymphocytes, macrophages, neutrophils, and
437 fibroblasts replacing the alveoli. The size of the affected areas increased over time (area of diffuse alveolar
438 damage: control <5-10%, 2 dpi <10-30%, 5 dpi 20-80 %, 8 dpi 50-90%). Alveolar septa were thickened in
439 areas close to infiltrates. In samples collected at 5 and 8 dpi, an increased number of activated macrophages
440 with foamy cytoplasm (black arrowheads) was seen. AAV-hACE2 transduced, SARS-CoV-2 uninfected
441 mice were used as control and showed no significant pathology.

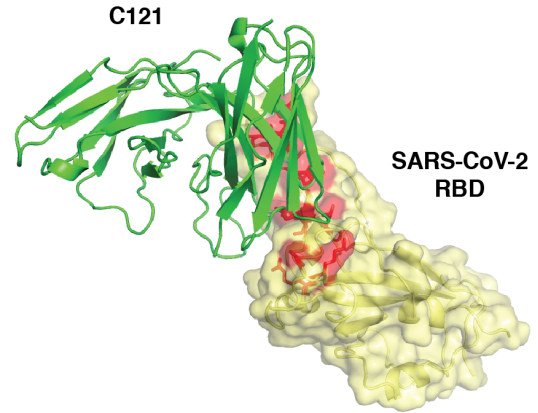


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Extended Data Fig.8 | Post-exposure administration of CoV-X2 protects SARS-CoV-2 infected mice from disease. Animals were infected intranasally with 10^4 pfu of SARS-CoV-2 and treated with 250 $\mu\text{g}/\text{mouse}$ of either isotype control antibody (n=3) or CoV-X2 (n=2) 12 hours later. Weight loss and pathological signs were apparent in control but not in CoV-X2 treated animals.

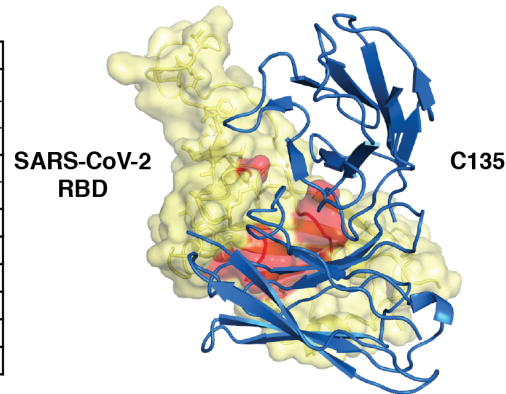
a

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			
Leu452	Gln	/USA/VA-DCLS-1404/2020	Cys488	Arg	/IRN/COVID19-IRVSH4/2020
	Arg	/USA/CA-CZB-12872/2020	Phe490	Leu	/AUS/VIC10024/2020 /AUS/VIC766/2020
	Met	/BHR/340798279_55_L001/2020 /USA/CA-CZB-1043/2020 /IND/906/2020		Pro491	His
Leu455	Phe	/AUS/VIC10121/2020 /AUS/VIC5196/2020	Gln493	Leu	/USA/WI-UW-371/2020
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	/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
		Phe			/AUS/VIC2139/2020 /USA/MA-UW-629/2020
		Glu484			Gln
	Lys				/USA/UT-QDX-1869/2020 /BHR/340859913_511/2020 /USA/IL-UW-379/2020
	Ala	/USA/VA-DCLS-1615/2020		Leu	/AUS/VIC5057/2020



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



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449 **Extended Data Fig.9 | Natural SARS-CoV-2 variants in the C121 and C135 epitopes.** Summary of
 450 naturally occurring mutations in the C121 (a) or C135 (b) epitopes reported in circulating SARS-CoV-2
 451 (as of January 1, 2021). The location of the mutated residues is shown in red on the RBD structure. C121
 452 and C135 variable regions are in green and blue (PDB ID: 7K8X and 7K8Z respectively).
 453

	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	–

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Extended Data Table 1 | Summary of the P values for the mouse protection experiment. 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 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, C135, or C121; P<0.05).