

Potently neutralizing human antibodies that block SARS-CoV-2 receptor binding and protect animals

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The COVID-19 pandemic is a major threat to global health for which there are only limited medical countermeasures, and we lack a thorough understanding of mechanisms of humoral immunity^{1,2}. From a panel of monoclonal antibodies (mAbs) targeting the spike (S) glycoprotein isolated from the B cells of infected subjects, we identified several mAbs that exhibited potent neutralizing activity with IC₅₀ values as low as 0.9 or 15 ng/mL in pseudovirus or wild-type (*wt*) SARS-CoV-2 neutralization tests, respectively. The most potent mAbs fully block the receptor-binding domain of S (S_{RBD}) from interacting with human ACE2. Competition-binding, structural, and functional studies allowed clustering of the mAbs into defined classes recognizing distinct epitopes within major antigenic sites on the S_{RBD}. Electron microscopy studies revealed that these mAbs recognize distinct conformational states of trimeric S protein. Potent neutralizing mAbs recognizing unique sites, COV2-2196 and COV2-2130, bound simultaneously to S and synergistically neutralized authentic SARS-CoV-2 virus. In two murine models of SARS-CoV-2 infection, passive transfer of either COV2-2196 or COV2-2130 alone or a combination of both mAbs protected mice from severe weight loss and reduced viral burden and inflammation in the lung. These results identify protective epitopes on the S_{RBD} and provide a structure-based framework for rational vaccine design and the selection of robust immunotherapeutic cocktails.

The S protein of SARS-CoV-2 is the molecular determinant of viral attachment, fusion, and entry into host cells³. The cryo-EM structure of a prefusion-stabilized trimeric S protein ectodomain (S2P_{ecto}) for SARS-CoV-2 reveals similar features to that of the SARS-CoV S protein⁴. This type I integral membrane protein and class I fusion protein possesses an N-

terminal subunit (S1) that mediates binding to receptor and a C-terminal subunit (S2) that mediates virus–cell membrane fusion. The S1 subunit contains an N-terminal domain (S_{NTD}) and a receptor-binding domain (S_{RBD}). SARS-CoV-2 and SARS-CoV, which share approximately 78% sequence identity in their genomes¹ both use human angiotensin-converting enzyme 2 (hACE2) as an entry receptor⁵⁻⁷. Previous studies of human immunity to other high-pathogenicity zoonotic betacoronaviruses including SARS-CoV⁸⁻¹² and Middle East respiratory syndrome (MERS)¹³⁻²² showed that Abs to the viral surface spike (S) glycoprotein mediate protective immunity. The most potent S protein-specific mAbs appear to neutralize betacoronaviruses by blocking attachment of virus to host cells by binding to the region on S_{RBD} that directly mediates engagement of the receptor. It is likely that human Abs have promise for use in modifying disease during SARS-CoV-2 infection, when used for prophylaxis, post-exposure prophylaxis, or treatment of SARS-CoV-2 infection²³. Many studies including randomized controlled trials evaluating convalescent plasma and one trial evaluating hyperimmune immunoglobulin are ongoing, but it is not yet clear whether such treatments can reduce morbidity or mortality²⁴.

We isolated a large panel of SARS-CoV-2 S protein-reactive mAbs from the B cells of two individuals who were previously infected with SARS-CoV-2 in Wuhan China²⁵. A subset of those antibodies bound to the receptor-binding domain of S (S_{RBD}) and exhibited neutralizing activity in a rapid screening assay with authentic SARS-CoV-2²⁵. Here, we defined the antigenic landscape of SARS-CoV-2 and determined which sites of S_{RBD} are the target of protective mAbs. We tested a panel of 40 anti-S human mAbs we previously pre-selected by a rapid neutralization screening assay in a quantitative focus reduction neutralization test (FRNT) with SARS-CoV-2

strain WA1/2020. These assays revealed the panel exhibited a range of half-maximal inhibitory concentration (IC_{50}) values, from 15 to over 4,000 ng/mL (visualized as a heatmap in Fig. 1a, values shown in Extended Data Table 1, and full curves shown in Extended Data Fig. 1). We hypothesized that many of these S_{RBD} -reactive mAbs neutralize virus infection by blocking S_{RBD} binding to hACE2. Indeed, most neutralizing mAbs we tested inhibited the interaction of hACE2 with trimeric S protein directly (Fig. 1a, Extended Data Fig. 2). Consistent with these results, these mAbs also bound strongly to a trimeric S ectodomain ($S2P_{ecto}$) protein or monomeric S_{RBD} (Fig. 1a, Extended Data Fig. 3). We evaluated whether $S2P_{ecto}$ or S_{RBD} binding or hACE2-blocking potency predicted binding neutralization potency independently, but none of these measurements correlated with neutralization potency (Fig. 1b-d). However, each of the mAbs in the highest neutralizing potency tier ($IC_{50} < 150$ ng/mL) also revealed strongest blocking activity against hACE2 ($IC_{50} < 150$ ng/mL) and exceptional binding activity ($EC_{50} < 2$ ng/mL) to $S2P_{ecto}$ trimer and S_{RBD} (Fig. 1e). Representative neutralization curves for two potently neutralizing mAbs designated COV2-2196 and COV2-2130 are shown in (Fig. 1f). Potent neutralization was confirmed using pseudovirus neutralization assays, which revealed far more sensitive neutralization phenotypes than *wt* virus and demonstrated a requirement for the use of live virus assays for assessment of mAb potency (Fig. 1g). Both of these mAbs bound strongly to $S2P_{ecto}$ trimer and fully blocked hACE2 binding (Fig. 1h-i).

We next defined the major antigenic sites on S_{RBD} for neutralizing mAbs by competition-binding analysis. We first used a biolayer interferometry-based competition assay with a minimal S_{RBD} domain to screen for mAbs that competed for binding with the potently neutralizing mAb COV2-2196 or a recombinant version of the previously described SARS-CoV mAb CR3022, which

recognizes a conserved cryptic epitope^{10,26}. We identified three major groups of competing mAbs (Fig. 2a). The largest group of mAbs blocked COV2-2196 but not rCR3022, while some mAbs were blocked by rCR3022 but not COV2-2196. Two mAbs, including COV2-2130, were not blocked by either reference mAb. Most mAbs competed with hACE2 for binding, suggesting that they bound near the hACE2 binding site of the S_{RBD}. We used COV2-2196, COV2-2130, and rCR3022 in an ELISA-based competition-binding assay with trimeric S2P_{ecto} protein and also found that S_{RBD} contained three major antigenic sites, with some mAbs likely making contacts in more than one site (Fig. 2b). Most of the potently neutralizing mAbs directly competed for binding with COV2-2196.

Since COV2-2196 and COV2-2130 did not compete for binding to S_{RBD}, we assessed if these mAbs synergize for virus neutralization, a phenomenon previously observed for SARS-CoV mAbs¹⁰. We tested combination responses (see dose-response neutralization matrix, Fig. 2c) in the FRNT using SARS-CoV-2 and compared these experimental values with the expected responses calculated by synergy scoring models²⁷. The comparison revealed that the combination of COV2-2196 + COV2-2130 was synergistic (with a synergy score of 17.4, where any score of >10 indicates synergy). The data in Fig. 2c shows the dose-response synergy matrix and demonstrates that a combined mAb dose of 79 ng/mL in the cocktail (16 ng/mL of COV2-2196 and 63 ng/mL of COV2-2130) had the same activity as 250 ng/mL of each individual mAb (see Fig. 2c). This finding shows that using a cocktail the dose of each mAb can be reduced by more than three-fold to achieve the same potency of virus neutralization *in vitro*.

We next defined the epitopes recognized by representative mAbs in the two major competition-binding groups that synergize for neutralization. We performed mutagenesis studies of the S_{RBD} using alanine or arginine substitution to determine critical residues for binding of neutralizing mAbs (Extended Data Fig. 4). Loss of binding studies revealed F486A or N487A as critical residues for COV2-2196 and N487A as a critical residue for COV2-2165, which compete with one another for binding, and likewise mutagenesis studies for COV2-2130 using K444A and G447R mutants defined these residues as critical for recognition (Fig. 3a). Previous structural studies have defined the interaction between the S_{RBD} and hACE2 (Fig. 3b)²⁸. Most of the interacting residues in the S_{RBD} are contained within a 60-amino-acid linear peptide that defines the hACE2 recognition motif (Fig. 3c). We next tested binding of human mAbs to this minimal peptide and found that potent neutralizing members of the largest competition-binding group including COV2-2196, COV2-2165, and COV2-2832 recognized this peptide (Fig. 3c), suggesting these mAbs make critical contacts within the hACE2 recognition motif.

We used negative-stain electron microscopy of $S2P_{ecto}$ trimer/Fab complexes to structurally determine the epitopes for these mAbs. The potentially neutralizing antibodies COV2-2196 and COV2-2165 bound to the hACE2 recognition motif of S_{RBD} and recognized the ‘open’ conformational state of the $S2P_{ecto}$ trimer (Fig. 3d). The mode of engagement of these two antibodies differed, however, as the binding pose and the angle relative to the spike ‘body’ for one was different compared to the other. COV2-2130, which represents the second competition-binding group, bound to the RBD in the $S2P_{ecto}$ trimer in the ‘closed’ position (Fig. 3d). Since COV2-2196 and COV2-2130 did not compete for binding, we attempted to make complexes of both Fabs bound at the same time to the $S2P_{ecto}$ trimer. We found that both Fabs bound

simultaneously when the S2P_{ecto} trimer was in the open position, indicating that COV2-2130 can recognize the S_{RBD} in both conformations (Fig. 3e). Overlaying the two-Fab complex with the structure of the RBD:CR3022 complex²⁶, we observed that these antibodies bind to three distinct sites on S_{RBD}, as predicted based on our competition-binding studies (Fig. 3f).

Next, we tested the prophylactic efficacy of COV2-2196 or COV2-2130 monotherapy or a combination of COV2-2196 + COV2-2130 in a newly developed SARS-CoV-2 infection model in BALB/c mice in which hACE2 is expressed in the lung after intranasal adenovirus (AdV-hACE2) transduction. In this relatively stringent disease model, we also administered a single dose of anti-Ifnar1 antibody to augment virus infection and pathogenesis, which results in a disseminated interstitial pneumonia (A. Hassan and M. Diamond, submitted for publication). We passively transferred a single dose of mAb COV2-2196 (10 mg/kg), COV2-2130 (10 mg/kg), a combination of COV2-2196 + COV2-2130 (5 mg/kg each), or an isotype control mAb (10 mg/kg) to AdV-hACE2-transduced mice one day before intranasal challenge with 4 x 10⁵ PFU of SARS-CoV-2. Prophylaxis with COV2-2196 or COV2-2130 or their combination prevented severe SARS-CoV-2-induced weight loss through the first week of infection (Fig. 4a). Viral RNA levels were reduced significantly at 7 dpi in the lung and distant sites including the heart and spleen (Fig. 4b). The expression of interferon gamma (INF-g), IL-6, CXCL10 and CCL2 cytokine and chemokine genes, which are indicators of inflammation, also was reduced in the lung of treated mice at 7 dpi—the peak of the disease (Fig. 4c).

We also tested COV2-2196 or COV2-2130 or their combination for prophylactic efficacy in an immunocompetent model using a mouse-adapted (MA) SARS-CoV-2 virus²⁹ (Fig. 4d). *In vitro*

tests showed that the IC₅₀ values for neutralization were comparable for the *wt* and MA SARS-CoV-2 viruses for these mAbs (data not shown). Each of the mAb treatments delivered at a dose of 200 µg/mouse (~ 8 mg/kg) reduced viral RNA levels up to 10⁵-fold at 2 dpi in the lung when compared to the isotype control group (Fig. 4e, left). Concordantly, all animals from COV2-2196 and COV2-2196 + COV2-2130 treatment group and 8 of 10 animals from COV2-2130 treatment no longer had infectious virus at 2 dpi in the lung as measured by plaque titer of lung tissue (Fig. 4e, right). Collectively, these results in mice suggested that COV2-2196 or COV2-2130 alone or in combination are promising candidates for treatment or prevention of COVID-19.

Here, we defined the antigenic landscape for a large panel of highly potent mAbs against SARS-CoV-2. These detailed studies and the screening studies that identified this panel of mAbs from a larger panel of hundreds²⁵ demonstrate that although diverse human neutralizing antibodies are elicited by natural infection with SARS-CoV-2, only a small subset of those mAbs are of high potency (IC₅₀<50 ng/mL against live SARS-CoV-2 virus), and therefore, suitable for therapeutic development. Biochemical and structural analysis of these potent mAbs defined three principal antigenic sites of vulnerability to neutralization by human mAbs elicited by natural infection with SARS-CoV on the S_{RBD}. Representative mAbs from the two most potent antigenic sites were shown to synergize *in vitro* and protect as an *in vivo* cocktail. This finding reveals critical features of effective humoral immunity to SARS-CoV-2 and suggests that the role of synergistic neutralization activity in polyclonal responses should be explored further. Moreover, as SARS-CoV-2 continues to circulate, population immunity elicited by natural infection may start to select for antigenic variants that escape from the selective pressure of neutralizing antibodies, reinforcing the need to target multiple epitopes of S protein in vaccines or immunotherapeutics.

210 The common S gene variants across the globe reported to date are located at positions D614G,
 211 V483A, L5F, Q675H, H655Y and S939F³⁰, far away from the amino acid variants at residues
 212 486 or 487 identified in our mutation studies for the lead mAbs studied here. Rationally-selected
 213 therapeutic cocktails like the one described here might offer even greater resistance to SARS-
 214 CoV-2 escape. These studies set the stage for preclinical evaluation and development of the
 215 identified mAbs as candidates for use as COVID-19 immunotherapeutics in humans.
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Data availability. The EM maps have been deposited at the Electron Microscopy Data Bank with accession codes EMBD 21965 (S2P_{ecto} apo), EMD-21974 (S2P_{ecto} + Fab COVs-2165), EMD-21975 (S2P_{ecto} + Fab COVs-2196), EMD-21976 (S2P_{ecto} + Fab COVs-2130) and EMD-21977 (S2P_{ecto} + Fab COV2-2196 + Fab COV2-2130). Materials reported in this study will be made available but may require execution of a Materials Transfer Agreement.

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Competing interests. R.S.B. has served as a consultant for Takeda and Sanofi Pasteur on issues related to vaccines. M.S.D. is a consultant for Inbios, Vir Biotechnology, NGM Biopharmaceuticals, Eli Lilly, and is on the Scientific Advisory Board of Moderna, a past recipient of unrelated research grant from Moderna and a current recipient of an unrelated research grant Emergent BioSolutions.

J.E.C. has served as a consultant for Sanofi and is on the Scientific Advisory Boards of CompuVax and Meissa Vaccines, is a recipient of previous unrelated research grants from Moderna and Sanofi and is Founder of IDBiologics, Inc. Vanderbilt University has applied for patents concerning SARS-CoV-2 antibodies that are related to this work. AstraZeneca has filed patents for materials/findings related to this work. J.J.S., K.R., Y.-M.L., and N.L.K. are employees of AstraZeneca and currently hold AstraZeneca stock or stock options. All other authors declared no competing interests.

Additional information

Supplementary information is available for this paper.

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REFERENCES

1. Zhou, P., *et al.* A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* **579**, 270-273 (2020).
2. Zhu, N., *et al.* A novel coronavirus from patients with pneumonia in China, 2019. *N Engl J Med* **382**, 727-733 (2020).
3. Pillay, T.S. Gene of the month: the 2019-nCoV/SARS-CoV-2 novel coronavirus spike protein. *J Clin Pathol* (2020).
4. Wrapp, D., *et al.* Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* **367**, 1260-1263 (2020).
5. Wan, Y., Shang, J., Graham, R., Baric, R.S. & Li, F. Receptor recognition by the novel Coronavirus from Wuhan: an Analysis Based on Decade-Long Structural Studies of SARS Coronavirus. *J Virol* **94**(2020).
6. Hoffmann, M., *et al.* SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* **181**, 271-280 e278 (2020).
7. Li, W., *et al.* Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* **426**, 450-454 (2003).

288

289 8. Sui, J., *et al.* Potent neutralization of severe acute respiratory syndrome (SARS)
290 coronavirus by a human mAb to S1 protein that blocks receptor association. *Proc Natl*
291 *Acad Sci U S A* **101**, 2536-2541 (2004).

292

293 9. ter Meulen, J., *et al.* Human monoclonal antibody as prophylaxis for SARS coronavirus
294 infection in ferrets. *Lancet* **363**, 2139-2141 (2004).

295

296 10. ter Meulen, J., *et al.* Human monoclonal antibody combination against SARS coronavirus:
297 synergy and coverage of escape mutants. *PLoS Med* **3**, e237 (2006).

298

299 11. Zhu, Z., *et al.* Potent cross-reactive neutralization of SARS coronavirus isolates by human
300 monoclonal antibodies. *Proc Natl Acad Sci U S A* **104**, 12123-12128 (2007).

301

302 12. Rockx, B., *et al.* Structural basis for potent cross-neutralizing human monoclonal antibody
303 protection against lethal human and zoonotic severe acute respiratory syndrome
304 coronavirus challenge. *J Virol* **82**, 3220-3235 (2008).

305

306 13. Chen, Z., *et al.* Human neutralizing monoclonal antibody inhibition of Middle East
307 respiratory syndrome coronavirus replication in the common marmoset. *J Infect Dis* **215**,
308 1807-1815 (2017).

309

14. Choi, J.H., *et al.* Characterization of a human monoclonal antibody generated from a B-cell specific for a prefusion-stabilized spike protein of Middle East respiratory syndrome coronavirus. *PLoS One* **15**, e0232757 (2020).
15. Niu, P., *et al.* Ultrapotent human neutralizing antibody repertoires against Middle East respiratory syndrome coronavirus from a recovered patient. *J Infect Dis* **218**, 1249-1260 (2018).
16. Wang, L., *et al.* Importance of neutralizing monoclonal antibodies targeting multiple antigenic sites on the Middle East respiratory syndrome coronavirus spike glycoprotein to avoid neutralization escape. *J Virol* **92**(2018).
17. Wang, N., *et al.* Structural definition of a neutralization-sensitive epitope on the MERS-CoV S1-NTD. *Cell Rep* **28**, 3395-3405 e3396 (2019).
18. Zhang, S., *et al.* Structural definition of a unique neutralization epitope on the receptor-binding domain of MERS-CoV spike glycoprotein. *Cell Rep* **24**, 441-452 (2018).
19. Corti, D., *et al.* Prophylactic and postexposure efficacy of a potent human monoclonal antibody against MERS coronavirus. *Proc Natl Acad Sci U S A* **112**, 10473-10478 (2015).
20. Jiang, L., *et al.* Potent neutralization of MERS-CoV by human neutralizing monoclonal antibodies to the viral spike glycoprotein. *Sci Transl Med* **6**, 234ra259 (2014).

21. Tang, X.C., *et al.* Identification of human neutralizing antibodies against MERS-CoV and their role in virus adaptive evolution. *Proc Natl Acad Sci U S A* **111**, E2018-2026 (2014).
22. Ying, T., *et al.* Exceptionally potent neutralization of Middle East respiratory syndrome coronavirus by human monoclonal antibodies. *J Virol* **88**, 7796-7805 (2014).
23. Jiang, S., Hillyer, C. & Du, L. Neutralizing antibodies against SARS-CoV-2 and other human coronaviruses. *Trends Immunol* **41**, 355-359 (2020).
24. Valk, S.J., *et al.* Convalescent plasma or hyperimmune immunoglobulin for people with COVID-19: a rapid review. *Cochrane Database Syst Rev* **5**, CD013600 (2020).
25. Zost, S.J., *et al.* Rapid isolation and profiling of a diverse panel of human monoclonal antibodies targeting the SARS-CoV-2 spike protein. *bioRxiv*, 2020.2005.2012.091462 (2020).
26. Yuan, M., *et al.* A highly conserved cryptic epitope in the receptor binding domains of SARS-CoV-2 and SARS-CoV. *Science* **368**, 630-633 (2020).
27. Ianevski, A., He, L., Aittokallio, T. & Tang, J. SynergyFinder: a web application for analyzing drug combination dose-response matrix data. *Bioinformatics* **33**, 2413-2415 (2017).

28. Lan, J., *et al.* Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature* **581**, 215-220 (2020).

29. Dinno, K.H., *et al.* A mouse-adapted SARS-CoV-2 model for the evaluation of COVID-19 medical countermeasures. *bioRxiv*, 2020.2005.2006.081497 (2020).

30. Laha, S., *et al.* Characterizations of SARS-CoV-2 mutational profile, spike protein stability and viral transmission. *bioRxiv*, 2020.2005.2003.066266 (2020).

Figure Legends:

Figure 1. Functional characteristics of neutralizing SARS-CoV-2 mAbs.

a. Heatmap of mAb neutralization activity, hACE2 blocking activity, and binding to either trimeric S2Pecto protein or monomeric SRBD. MAbs are ordered by neutralization potency (highest at the top, lowest at the bottom). Dashed lines indicate the 13 antibodies with a neutralization IC50 value lower than 150 ng/mL for wt virus. IC50 values are visualized for viral neutralization and hACE2 blocking, while EC50 values are visualized for binding. A recombinant form of the cross-reactive SARS-CoV SRBD mAb CR3022 is shown as a positive control, while the anti-dengue mAb 2D22 is shown as a negative control. Data are representative of at least 2 independent experiments, each performed in technical duplicate. No inhibition indicates an IC50 value of >10,000 ng/mL, while no binding indicates an EC50 value of >10,000 ng/mL.

b-d. Correlation of hACE2 blocking, S2Pecto trimer binding, or SRBD binding of mAbs with their neutralization activity. R2 values are shown for linear regression analysis of log-transformed values. Purple circles indicate mAbs with a neutralization IC50 value lower than 150 ng/mL.

e. Correlation of hACE2 blocking and S2Pecto trimer binding. R2 values are shown for linear regression analysis of log-transformed values.

f. Neutralization curves for COV2-2196 and COV2-2130 in a neutralization assay against authentic SARS-CoV-2 virus. Calculated IC50 values are denoted on the graph. Error bars denote the standard deviation of each point. Data are representative of at least 2 independent experiments, each performed in technical duplicate.

g. Neutralization curves for COV2-2196 and COV2-2130 in a pseudovirus neutralization assay. Error bars denote the standard deviation of each point. Values shown are technical duplicates from a single experiment. Calculated IC50 values from a minimum of 6 experiments are denoted on the graph.

h. hACE2 blocking curves for COV2-2196, COV2-2130, and the non-blocking SARS-CoV mAb rCR3022 in the hACE2 blocking ELISA. Calculated IC50 values are denoted on the graph. Error bars denote the standard deviation of each point. Values shown are technical triplicates from a representative experiment repeated twice.

i. ELISA binding of COV2-2196, COV2-2130, and rCR3022 to trimeric S2Pecto. Calculated EC50 values are denoted on the graph. Error bars denote the standard deviation of each point. Values shown are technical triplicates from a representative experiment repeated twice.

Figure 2. Epitope mapping of mAbs by competition-binding analysis and synergistic neutralization by a pair of mAbs.

a. Left: biolayer interferometry-based competition binding assay measuring the ability of mAbs to prevent binding of reference mAbs COV2-2196 and rCR3022 to RBD fused to mouse Fc (RBD-mFc) loaded onto anti-mouse Fc biosensors. Values in squares are % of binding of the reference mAb in the presence of the competing mAb relative to a mock-competition control. Black squares denote full competition (<33% of binding relative to no-competition control), while white squares denote no competition (>67% of binding relative to no-competition control). Right: biolayer interferometry-based competition binding assay measuring the ability of mAbs to prevent binding of hACE2. Values denote % binding of hACE2, normalized to hACE2 binding in the absence of competition. Red color denotes competition of mAb with hACE2.

b. Competition of neutralizing mAb panel with reference mAbs COV2-2130, COV2-2196, or rCR3022. Reference mAbs were biotinylated and binding of reference mAbs to trimeric S2Pecto was measured in the presence of saturating amounts of each mAb in a competition ELISA. ELISA signal for each reference mAb was normalized to the signal in the presence of the non-binding anti-dengue mAb 2D22. Black denotes full competition (<25% binding of reference mAb), grey denotes partial competition (25-60% binding of reference mAb), and white denotes no competition (>60% binding of reference mAb).

c. Neutralization dose-response matrix of wild-type SARS-CoV-2 by COV2-2196 and COV2-2130. Axes denote the concentration of each mAb. Experiment was performed in technical triplicate. Shown is a representative experiment that was performed in technical triplicate. % neutralization for each combination of mAbs is shown in each square. A white-to-red heatmap

denotes 0% neutralization to 100% neutralization, respectively. **d.** Synergy map calculated based on the SARS-CoV-2 neutralization in the above panel. Red color denotes areas where synergistic neutralization was observed, and a black box denotes the area of maximal synergy between the two mAbs.

Figure 3. Epitope identification and structural characterization of mAbs.

a. Identification of critical contact residues by alanine and arginine mutagenesis. Top: binding of COV2-2130 (gold), COV2-2165 (maroon) or COV2-2196 (dark purple) to wild-type (wt) or mutant SRBD constructs measured by biolayer interferometry. Shown on y-axis is the response normalized to the signal observed for binding to wt SRBD. Bottom: representative binding curves for COV2-2196 to wt or SRBD constructs with critical contact residues mutated.

b. Crystal structure of SARS-CoV-2 (blue) and hACE2 (green) (PDB (6M0J)). The hACE2 recognition motif is colored orange. Critical contact residues for COV2-2130 are shown as gold spheres, while critical contact residues for COV2-2196 are shown as purple spheres.

c. ELISA binding of mAbs to the 60-amino-acid hACE2 recognition motif. r2D22, an anti-dengue mAb, is shown as a negative control. Bottom: structure of hACE2 recognition motif in orange with COV2-2196 critical contact residues shown in purple.

d. Single-Fab:S2Pecto trimer complexes visualized by negative-stain electron microscopy for COV2-2130 (gold), COV2-2165 (maroon), or COV2-2196 (dark purple). The RBD is shown in blue and the S N-terminal domain (NTD) is shown in red. Electron density is shown in grey. Trimer state (open or closed) is denoted for each complex. Representative 2D class averages for each complex are shown at the bottom (box size 128 pixel).

e. COV2-2130 and COV2-2196 Fabs in complex with S2Pecto trimer. Simultaneous binding of COV2-2130 (gold) and COV2-2196 (purple) Fabs to S2Pecto trimer. Electron density is shown in grey. Trimer state (open or closed) is denoted. Representative 2D class averages for the complexes are shown at the bottom (box size 128 pixels). All images were made with Chimera.

f. Competition-binding analysis visualized on S2Pecto trimer. The CR3022 crystal structure was docked into the double-Fab:S2Pecto trimer structure. CR3022 is shown in cyan. Bottom: a quantitative Venn diagram notes the number of mAbs in each competition group and the overlap between groups.

Figure 4. Protective efficacy of neutralizing human mAbs against SARS-CoV-2 infection.

a. SARS-CoV-2 challenge model. Ten to eleven-week-old BALB/c mice (two experiments of 4-5 mice per group) were treated with anti-Ifnar1 mAb and transduced with AdV-hACE2 via the i.n. route one day later. After four days, mice were treated via the i.p. route with 200 µg of mAbs CoV2-2196, -2130, or combination (1:1 ratio) or isotype control mAb. One day later, SARS-CoV-2 was inoculated via the i.n. route. Tissues were harvested at 7 dpi for analysis (c, d).

b. Body weight change of mice in panel a. (two-way ordinary ANOVA with Tukey's post-test: **** $P < 0.0001$).

c. Viral burden in the lung, spleen and heart was measured by RT-qPCR: Kruskal-Wallis ANOVA with Dunn's post-test (*, $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). The dashed line indicates the assay limit of detection.

d. Cytokine and chemokine gene expression was measured by qPCR analysis. Kruskal-Wallis ANOVA with Dunn's post-test (*, $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

e. MA-SARS-CoV-2 challenge model. Twelve-week-old BALB/c mice (n=10) were inoculated with 105 PFU of MA-SARS-CoV-2 via the i.n. route. Body weight change of mice is shown.

f. Viral burden in the lung was measured at 2 dpi by RT-qPCR (left) or plaque assay (right) from (e): Kruskal-Wallis ANOVA with Dunn's post-test (***) $P < 0.001$, **** $P < 0.0001$).

Supplementary Information

Extended Data Figure. 1. SARS-CoV-2 neutralization curves for mAb panel. Neutralization of authentic SARS-CoV-2 by human mAbs. Mean $\pm$ SD of technical duplicates is shown. Data represent one of two or more independent experiments

Extended Data Figure 2. Inhibition curves for mAb inhibition of S2P_{ecto} binding to hACE2. Blocking of hACE2 binding to S2P_{ecto} by anti-SARS-CoV-2 neutralizing human mAbs. Mean $\pm$ SD of triplicates of one experiment is shown. Antibodies CR3022 and 2D22 served as controls.

Extended Data Figure. 3. ELISA binding of anti-SARS-CoV-2 neutralizing human mAbs to trimeric S_{RBD}, S2P_{ecto}, or SARS-CoV S2P_{ecto} antigen. Mean $\pm$ SD of triplicates and representative of two experiments are shown. Antibodies CR3022 and 2D22 served as controls.

Extended Data Figure. 4. Mapping of mAb critical contact residues by alanine and arginine mutagenesis and biolayer interferometry.

a. *Left*: Response values for mAb binding to *wt* or mutant S_{RBD} constructs normalized to *wt*.

Asterisks denote residues where increased dissociation of mAb was observed, likely indicating

492 the residue is proximal to mAb epitope. *Right:* full response curves for mAb association and
493 dissociation with *wt* or mutant S_{RBD} constructs.

494 **b.** Structure of the RBD highlighting the critical contact residues for several mAbs and their
495 location on the structure.

496

Figure 1

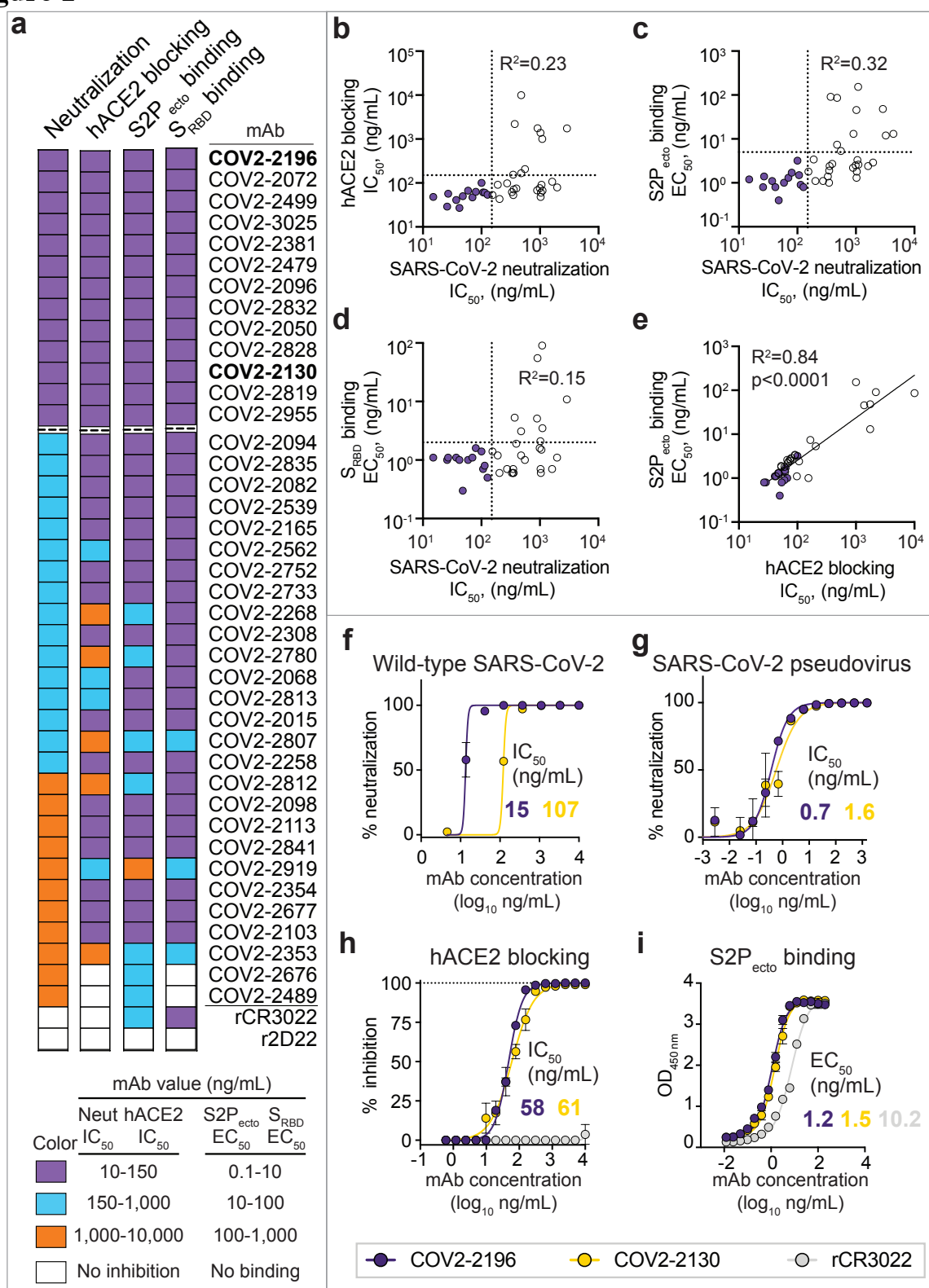


Figure 3

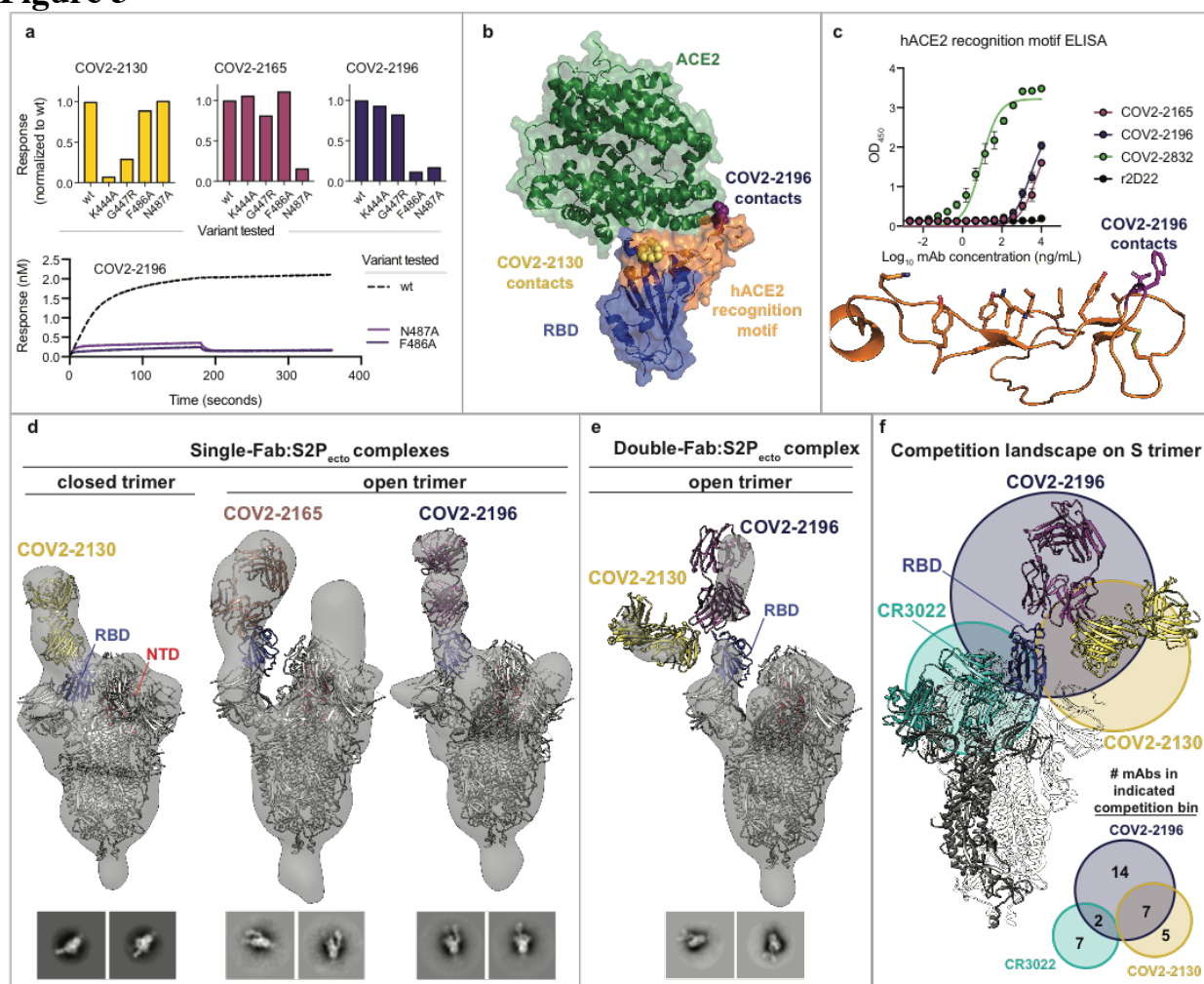
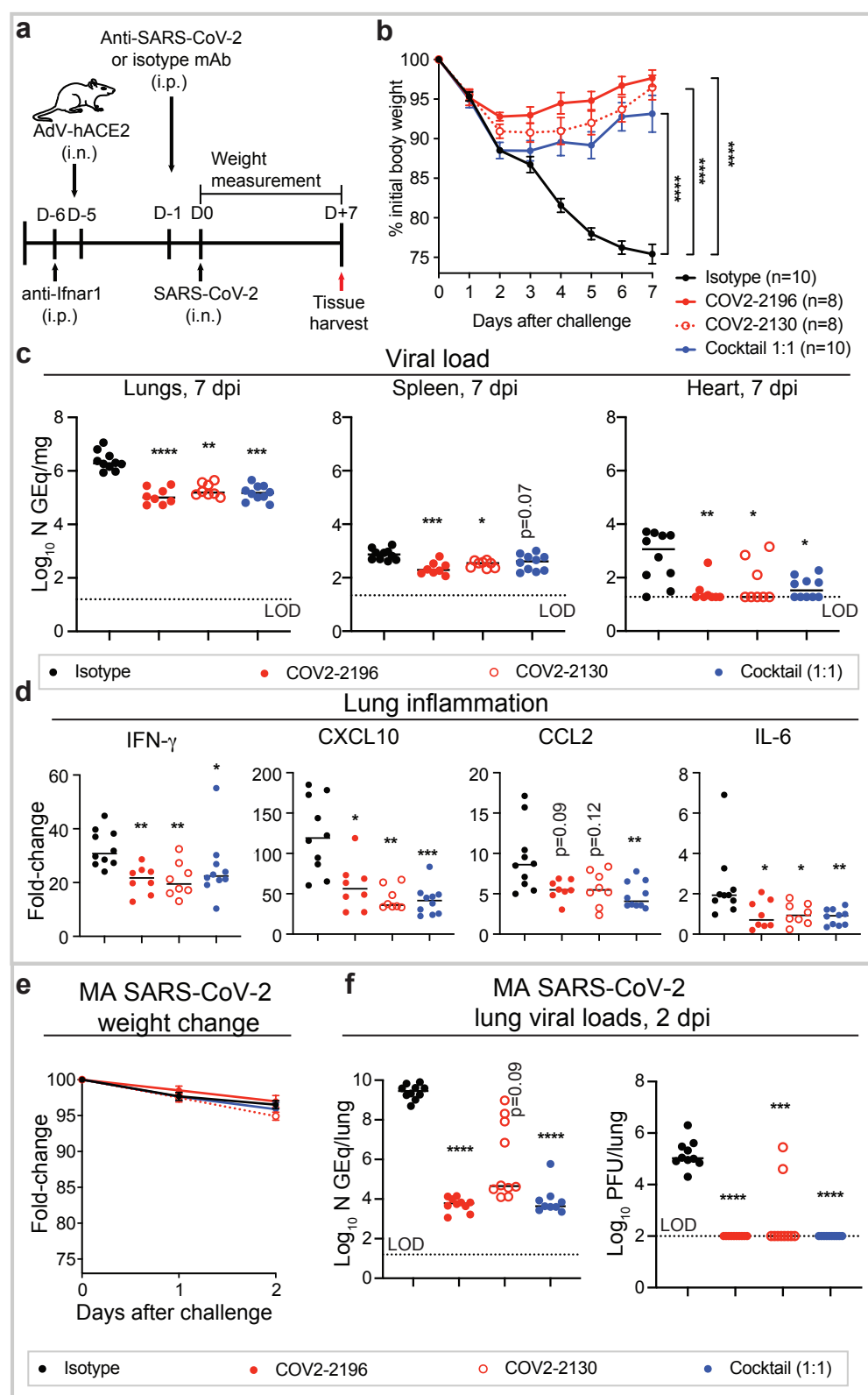
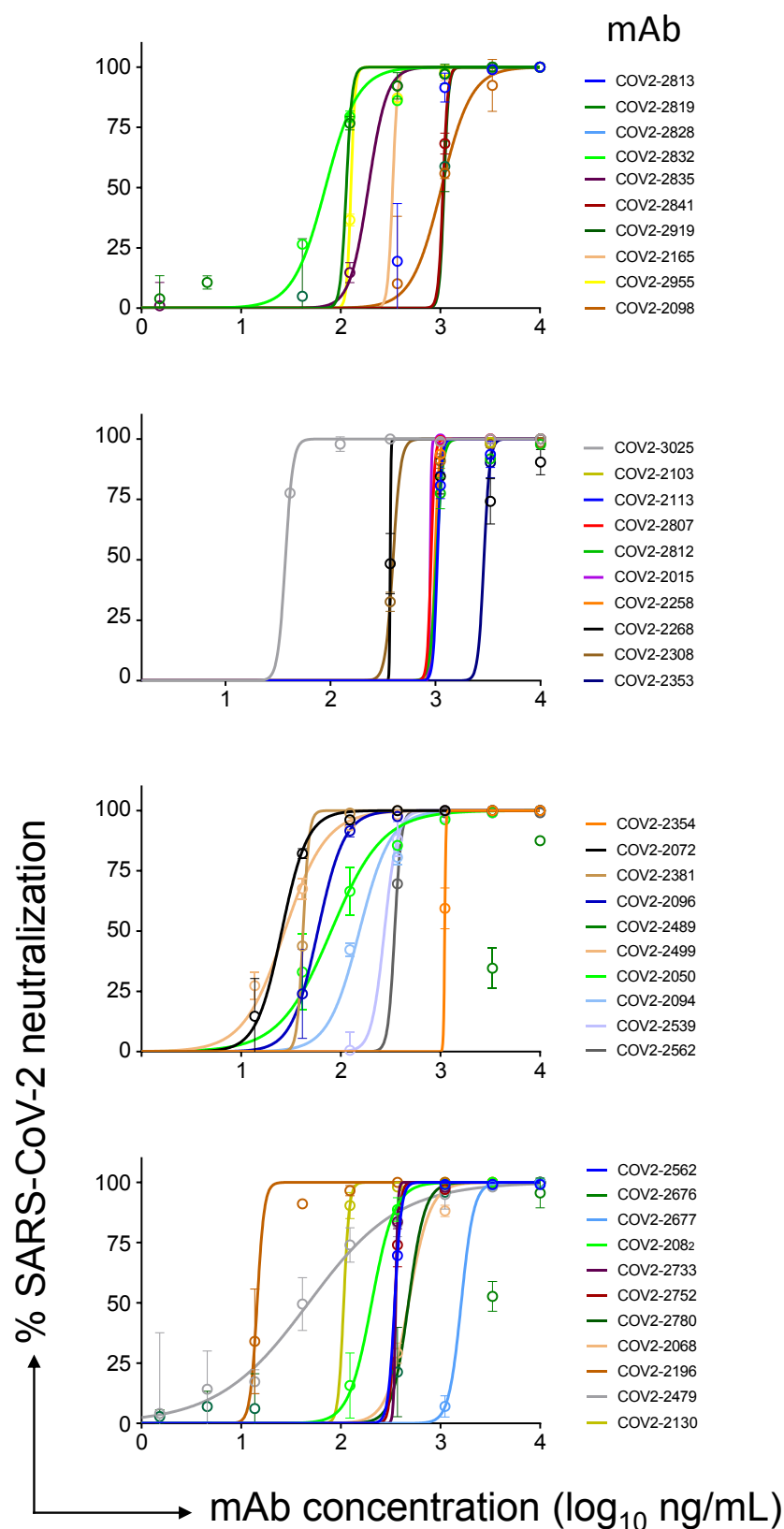


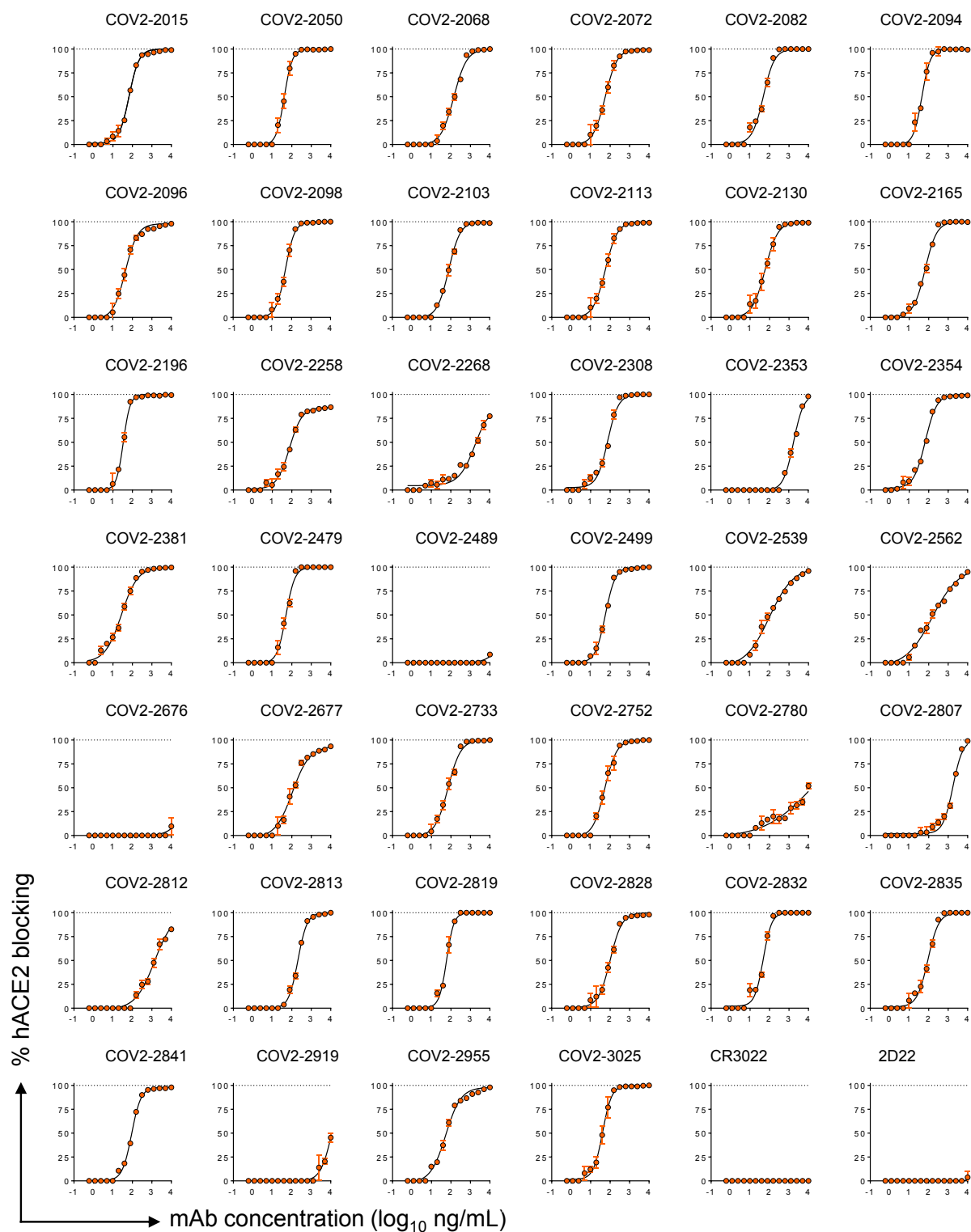
Figure 4



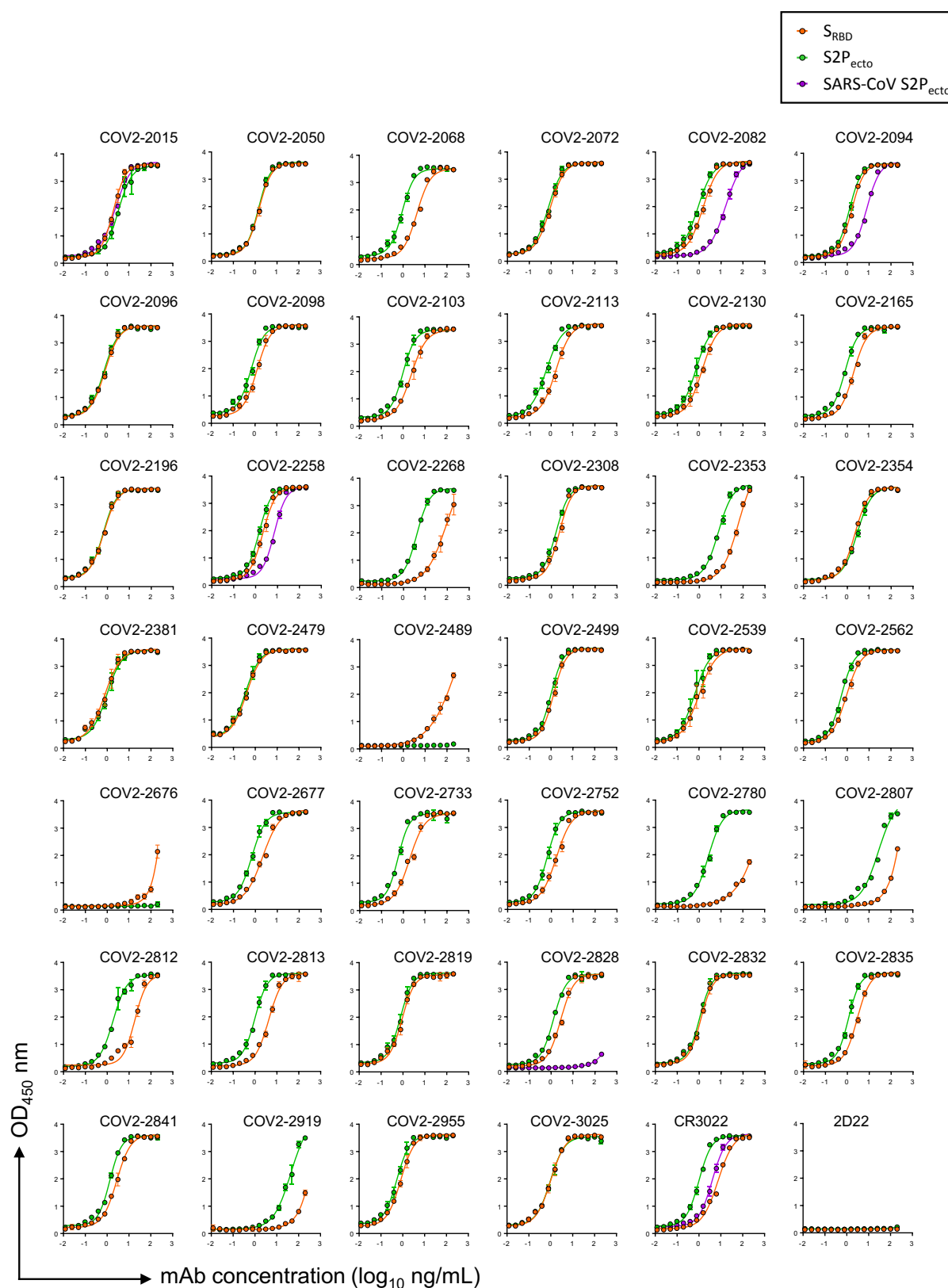
Extended Data Figure 1



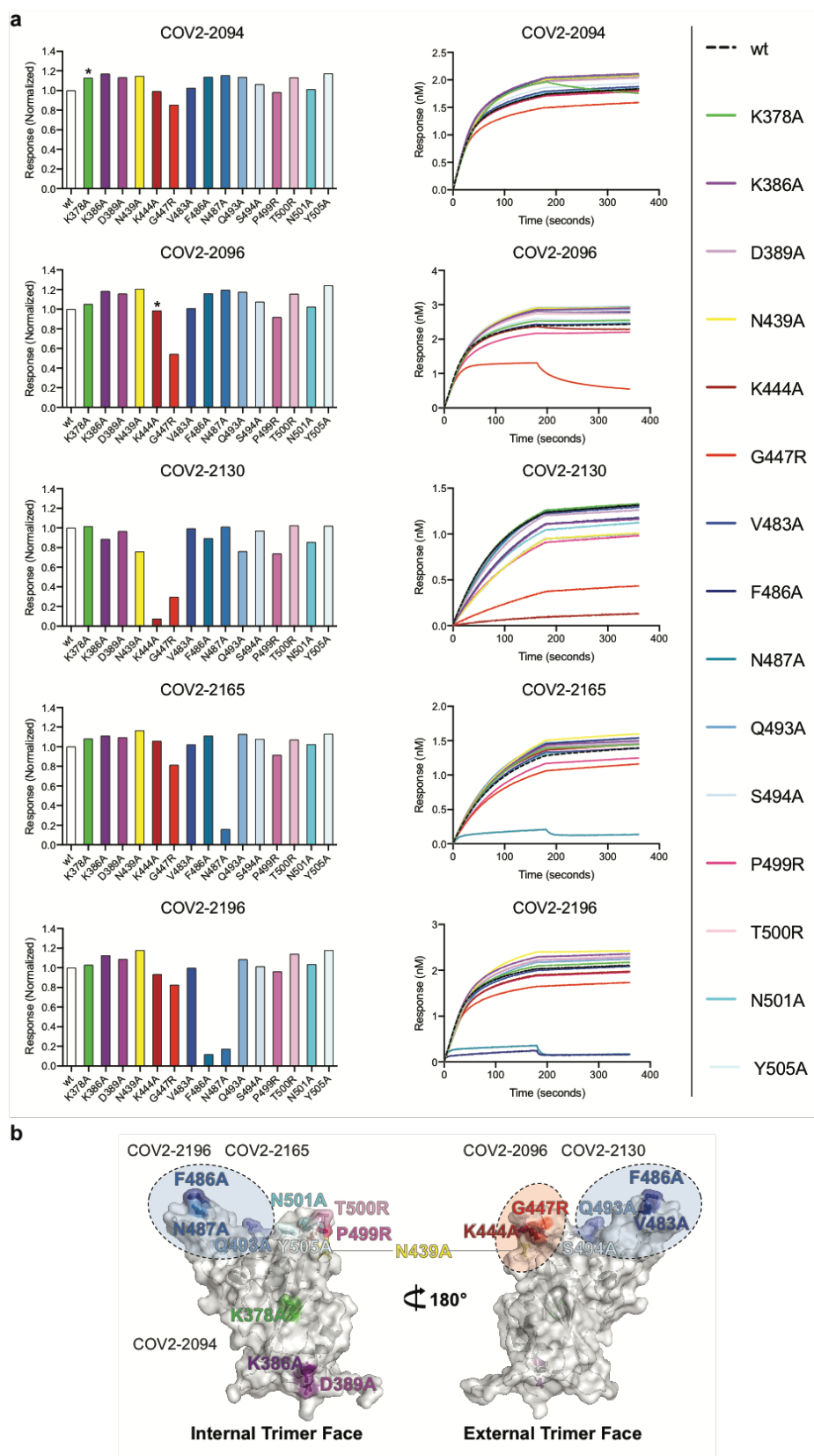
Extended Data Figure 2



Extended Data Figure 3



Extended Data Figure 4



Extended Data Table 1. Neutralization IC₅₀, hACE2 blocking IC₅₀, and EC₅₀ values for binding to S2P_{ecto} or S_{RBD} antigens for mAb panel

MAB	Neutralization IC₅₀, ng/mL	hACE2 blocking IC₅₀, ng/mL	S2P_{ecto} binding EC₅₀, ng/mL	S_{RBD} binding EC₅₀, ng/mL	SARS-CoV S2P_{ecto} binding EC₅₀, ng/mL
COV2-2015	892	68	2.2	5.1	2.7
COV2-2050	80	63	1.7	1.6	—
COV2-2068	478	166	7.4	1.2	—
COV2-2072	26	29	0.8	1.0	—
COV2-2082	204	43	1.1	0.6	19.9
COV2-2094	154	53	1.8	1.4	11.7
COV2-2096	59	67	1.0	1.0	—
COV2-2098	1,029	48	1.3	0.7	—
COV2-2103	1,969	79	2.9	1.1	—
COV2-2113	1,041	60	1.8	0.6	—
COV2-2130	107	61	1.5	0.7	—
COV2-2165	332	62	1.4	0.6	—
COV2-2196	15	48	1.2	1.1	—
COV2-2258	989	76	2.5	1.6	11.2
COV2-2268	371	2,198	91.2	5.3	—
COV2-2308	394	75	2.7	1.9	—
COV2-2353	2,891	1,750	48.0	10.9	—
COV2-2354	1,105	67	2.6	3.5	—
COV2-2381	42	27	0.8	1.0	—
COV2-2489	4,378	—	13.0	—	—
COV2-2479	48	50	0.4	0.3	—
COV2-2499	27	57	1.4	1.1	—
COV2-2539	274	98	1.1	0.7	—
COV2-2562	348	154	1.0	0.6	—
COV2-2676	3,247	—	12.0	—	—
COV2-2677	1,618	107	2.4	0.7	—
COV2-2733	356	71	2.4	0.6	—
COV2-2752	349	53	1.9	0.7	—
COV2-2780	478	10,000	86.0	3.1	—
COV2-2807	907	1,753	13.0	55.1	—
COV2-2812	1,020	1,387	45.4	2.1	—

COV2-2813	555	206	5.3	1.0	—
COV2-2819	114	60	0.9	0.8	—
COV2-2828	100	100	3.2	1.4	338
COV2-2832	70	47	1.3	1.1	—
COV2-2835	190	91	3.4	1.2	—
COV2-2841	1,065	91	3.3	1.5	—
COV2-2919	1,091	1,000	153.0	90.3	—
COV2-2955	127	54	0.8	0.5	—
COV2-3025	37	41	1.1	1.1	—
rCR3022	—	—	10.2	1.1	5.2
r2D22	—	—	—	—	—

— : indicates no detectable binding or neutralization at the highest tested concentration

Online Methods

Antibodies. The human antibodies studied in this paper were isolated from blood samples from two subjects in North America with previous laboratory-confirmed symptomatic SARS-CoV-2 infection that was acquired in China. The original clinical studies to obtain specimens after written informed consent were previously described¹ and had been approved by the Institutional Review Board of Vanderbilt University Medical Center and the Research Ethics Board of the University of Toronto. The subjects (a 56-year-old male and a 56-year-old female) are a married couple and residents of Wuhan, China who traveled to Toronto, Canada, where PBMCs were obtained by leukopheresis 50 days after symptom onset. The antibodies were isolated using diverse tools for isolation and cloning of single antigen-specific B cells and the antibody variable genes encoding monoclonal antibodies¹.

Cell culture. Vero E6 (CRL-1586, American Type Culture Collection (American Type Culture Collection, ATCC), Vero CCL81 (ATCC), HEK293 (ATCC), and HEK293T (ATCC) were maintained at 37°C in 5% CO₂ in Dulbecco's minimal essential medium (DMEM) containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 10 mM HEPES pH 7.3, 1 mM sodium pyruvate, 1× non-essential amino acids, and 100 U/mL of penicillin–streptomycin. Vero-furin cells were obtained from T. Pierson (NIH) and have been described previously². Expi293F cells (ThermoFisher Scientific, A1452) were maintained at 37°C in 8% CO₂ in Expi293F Expression Medium (ThermoFisher Scientific, A1435102). ExpiCHO cells (ThermoFisher Scientific, A29127) were maintained at 37°C in 8% CO₂ in ExpiCHO Expression Medium (ThermoFisher Scientific, A2910002). Mycoplasma testing of Expi293F and ExpiCHO cultures was performed on a monthly basis using a PCR-based mycoplasma detection kit (ATCC, 30-1012K).

Viruses. SARS-CoV-2 strain 2019 n-CoV/USA_WA1/2020 was obtained from the Centers for Disease Control and Prevention (a gift from Natalie Thornburg). Virus was passaged in Vero CCL81 cells and titrated by plaque assay on Vero E6 cells. All work with infectious SARS-CoV-2 was approved by the Washington University School of Medicine or UNC-Chapel Hill Institutional Biosafety Committees and conducted in approved BSL3 facilities using appropriate powered air purifying respirators and personal protective equipment.

Recombinant antigens and proteins. A gene encoding the ectodomain of a prefusion conformation-stabilized SARS-CoV-2 spike (S2P_{ecto}) protein was synthesized and cloned into a DNA plasmid expression vector for mammalian cells. A similarly designed S protein antigen with two prolines and removal of the furin cleavage site for stabilization of the prefusion form of S was reported previously³. Briefly, this gene includes the ectodomain of SARS-CoV-2 (to residue 1,208), a T4 fibrin trimerization domain, an AviTag site-specific biotinylation sequence, and a C-terminal 8x-His tag. To stabilize the construct in the prefusion conformation, we included substitutions K986P and V987P and mutated the furin cleavage site at residues 682-685 from RRAR to ASVG. This recombinant spike 2P-stabilized protein (designated here as S2P_{ecto}) was isolated by metal affinity chromatography on HisTrap Excel columns (GE Healthcare), and protein preparations were purified further by size-exclusion chromatography on a Superose 6 Increase 10/300 column (GE Healthcare). The presence of trimeric, prefusion conformation S protein was verified by negative-stain electron microscopy¹. For electron microscopy with S and Fabs, we expressed a variant of S2P_{ecto} lacking an AviTag but containing a C-terminal Twin-Strep-tag, similar to that described previously³. Expressed protein was isolated by metal affinity chromatography on HisTrap Excel columns (GE Healthcare), followed by further purification on a StrepTrap HP column (GE Healthcare) and size-exclusion chromatography on

TSKgel G4000SW_{XL} (TOSOH). To express the S_{RBD} subdomain of SARS-CoV-2 S protein, residues 319-541 were cloned into a mammalian expression vector downstream of an IL-2 signal peptide and upstream of a thrombin cleavage site, an AviTag, and a 6x-His tag. RBD protein fused to mouse IgG1 Fc domain (designated RBD-mFc), was purchased from Sino Biological (40592-V05H). For epitope mapping by alanine scanning, SARS-CoV-2 RBD (residues 334-526) or RBD single mutation variants were cloned with an N-terminal CD33 leader sequence and C-terminal GSSG linker, AviTag, GSSG linker, and 8xHisTag. Spike proteins were expressed in FreeStyle 293 cells (Thermo Fisher) and isolated by affinity chromatography using a HisTrap column (GE Healthcare), followed by size exclusion chromatography with a Superdex200 column (GE Healthcare). Purified proteins were analyzed by SDS-PAGE to ensure purity and appropriate molecular weights.

Electron microscopy (EM) stain grid preparation, imaging and processing of SARS-CoV-2

S2P_{ecto} protein or S2P_{ecto}/Fab complexes. To perform EM imaging, Fabs were produced by digesting recombinant chromatography-purified IgGs using resin-immobilized cysteine protease enzyme (FabALACTICA, Genovis). The digestion occurred in 100 mM sodium phosphate, 150 mM NaCl pH 7.2 for ~16 hrs at RT. In order to remove cleaved Fc and intact IgG, the digestion mix was incubated with CaptureSelect Fc resin (Genovis) for 30 min at RT in PBS buffer. If needed, the Fab was buffer exchanged into Tris buffer by centrifugation with a Zeba spin column (Thermo Scientific).

For screening and imaging of negatively-stained (NS) SARS-CoV-2 S2P_{ecto} protein in complex with human Fabs, the proteins were incubated for ~1 hr and approximately 3 µL of the sample at concentrations of about 10 to 15 µg/mL was applied to a glow discharged grid with continuous

carbon film on 400 square mesh copper EM grids (Electron Microscopy Sciences). The grids were stained with 0.75% uranyl formate (UF)⁴. Images were recorded on a Gatan US4000 4k × 4k CCD camera using an FEI TF20 (TFS) transmission electron microscope operated at 200 keV and control with SerialEM⁵. All images were taken at 50,000× magnification with a pixel size of 2.18 Å/pix in low-dose mode at a defocus of 1.5 to 1.8 μm. Total dose for the micrographs was ~25 to 38 e⁻/Å². Image processing was performed using the cryoSPARC software package⁶. Images were imported, and particles were CTF estimated. The images then were denoised and picked with Topaz⁷. The particles were extracted with a box size of 256 pixels and binned to 128 pixels. 2D class averages were performed and good classes selected for *ab-initio* model and refinement without symmetry. For EM model docking of SARS-CoV-2 S2P_{ecto} protein, the closed model (PDB: 6VXX) was used in Chimera⁸ for docking to the EM map (see also Extended Data Table 2 for details). For the SARS-CoV-2 S2P_{ecto}/Fab COV2-2165 and SARS-CoV-2 S2P_{ecto}/Fab COV2-2165 complexes, the open model of SARS-CoV-2 (PDB: 6VYB) and Fab (Fab: 12E8) was used in Chimera for docking to the EM maps (see also Extended Data Table 2 for details). For the SARS-Cov-2 S2P_{ecto}/Fab COV2-2130 complex, the closed model and Fab (PDB: 12E8) were used in Chimera for docking to the EM map (see also Extended Data Table 2 for details). All images were made with Chimera.

MAb production and purification. Sequences of mAbs that had been synthesized (Twist Bioscience) and cloned into an IgG1 monocistronic expression vector (designated as pTwist-mCis_G1) were used for mammalian cell culture mAb secretion. This vector contains an enhanced 2A sequence and GSG linker that allows simultaneous expression of mAb heavy and light chain genes from a single construct upon transfection⁹. We previously described microscale expression of

mAbs in 1 mL ExpiCHO cultures in 96-well plates¹. For larger scale mAb expression, we performed transfection (1 to 300 mL per antibody) of CHO cell cultures using the Gibco™ ExpiCHO™ Expression System and protocol for 50 mL mini bioreactor tubes (Corning) as described by the vendor. Culture supernatants were purified using HiTrap MabSelect SuRe (Cytiva, formerly GE Healthcare Life Sciences) on a 24-column parallel protein chromatography system (Protein BioSolutions). Purified mAbs were buffer-exchanged into PBS, concentrated using Amicon® Ultra-4 50KDa Centrifugal Filter Units (Millipore Sigma) and stored at 4°C until use.

ELISA binding assays. Wells of 96-well microtiter plates were coated with purified recombinant SARS-CoV-2 S protein or SARS-CoV-2 S_{RBD} protein at 4°C overnight. Plates were blocked with 2% non-fat dry milk and 2% normal goat serum in DPBS containing 0.05% Tween-20 (DPBS-T) for 1 hr. The bound antibodies were detected using goat anti-human IgG conjugated with HRP (horseradish peroxidase) (Southern Biotech) and TMB (3,3',5,5'-tetramethylbenzidine) substrate (Thermo Fisher Scientific). Color development was monitored, 1N hydrochloric acid was added to stop the reaction, and the absorbance was measured at 450 nm using a spectrophotometer (Biotek). For dose-response assays, serial dilutions of purified mAbs were applied to the wells in triplicate, and mAb binding was detected as detailed above. Half-maximal effective concentration (EC₅₀) values for binding were determined using Prism v8.0 software (GraphPad) after log transformation of mAb concentration using sigmoidal dose-response nonlinear regression analysis.

RBD minimal hACE2 recognition motif peptide binding ELISA. Wells of 384-well microtiter plates were coated with 1 µg/mL streptavidin at 4°C overnight. Plates were blocked with 0.5% BSA in DPBS containing 0.05% Tween-20 (DPBS-T) for 1 hr. Plates were washed 4x

with 1x PBST and 2 µg/mL biotinylated-ACE2 binding motif peptide (cat. # LT5578, from LifeTein, LLC) was added to bind streptavidin for 1 hr at RT. Purified mAbs were diluted in blocking buffer, added to the wells, and incubated for 1 hr at RT. The bound antibodies were detected using goat anti-human IgG conjugated with HRP (horseradish peroxidase) (cat. # 2014-05, Southern Biotech) and TMB (3,3',5,5'-tetramethylbenzidine) substrate (ThermoFisher Scientific). Color development was monitored, 1N hydrochloric acid was added to stop the reaction, and the absorbance was measured at 450 nm using a spectrophotometer (Biotek). For dose-response assays, serial 3-fold dilutions starting at 10 µg/mL concentration of purified mAbs were applied to the wells in triplicate, and mAb binding was detected as detailed above.

Analysis of binding of antibodies to variant RBD proteins with alanine or arginine point mutations. Biolayer light interferometry (BLI) was performed using an Octet RED96 instrument (ForteBio; Pall Life Sciences) and wild-type RBD protein or a mutant RBD protein with a single amino acid change at defined positions to alanine or arginine. Binding of the RBD proteins were confirmed by first capturing octa-His-tagged RBD wild-type or mutant protein from a 10 µg/mL (≈200 nM) solution onto Penta-His biosensors for 300 sec. The biosensor tips then were submerged in binding buffer (PBS/0.2% Tween 20) for a 60 sec wash, followed by immersion in a solution containing 150 nM of mAb for 180 sec (association), followed by a subsequent immersion in binding buffer for 180 sec (dissociation). Response for each RBD mutant protein was normalized to that of the wild-type RBD protein.

Focus reduction neutralization test (FRNT). Serial dilutions of mAbs were incubated with 10² FFU of SARS-CoV-2 for 1 hr at 37°C. The mAb–virus complexes were added to Vero E6 cell

culture monolayers in 96-well plates for 1 hr at 37°C. Subsequently, cells were overlaid with 1% (w/v) methylcellulose in Minimum Essential Medium (MEM) supplemented to contain 2% heat-inactivated FBS. Plates were fixed 30 hrs later by removing overlays and fixed with 4% PFA in PBS for 20 min at room temperature. The plates were incubated sequentially with 1 µg/mL of rCR3022 anti-S antibody¹⁰ and horseradish-peroxidase (HRP)-conjugated goat anti-human IgG in PBS supplemented with 0.1% (w/v) saponin (Sigma) and 0.1% bovine serum albumin (BSA). SARS-CoV-2-infected cell foci were visualized using TrueBlue peroxidase substrate (KPL) and quantitated on an ImmunoSpot 5.0.37 Macro Analyzer (Cellular Technologies). Data were processed using Prism software version 8.0 (GraphPad). IC₅₀ values were determined by nonlinear regression analysis using the Prism software.

Generation of S protein pseudotyped lentivirus. Suspension 293 cells were seeded and transfected with a third-generation HIV-based lentiviral vector expressing luciferase along with packaging plasmids encoding for the following: SARS-CoV-2 spike protein with a C-terminal 19 amino acid deletion, Rev, and Gag-pol. Medium was changed 16 to 20 hrs after transfection, and the supernatant containing virus was harvested 24 hrs later. Cell debris was removed by low-speed centrifugation, and the supernatant was passed through a 0.45 µm filter unit. The pseudovirus was pelleted by ultracentrifugation and resuspended in PBS for a 100-fold concentrated stock.

Pseudovirus neutralization assay. Serial dilutions of mAbs were prepared in a 384-well microtiter plate and pre-incubated with pseudovirus for 30 minutes at 37°C, to which 293 cells that stably express human ACE2 were added. The plate was returned to the 37°C incubator, and then 48 hrs later luciferase activity measured on an EnVision 2105 Multimode Plate Reader (Perkin Elmer) using the Bright-Glo™

Luciferase Assay System (Promega), according to manufacturer's recommendations. Percent inhibition was calculated relative to pseudovirus-alone control. IC₅₀ values were determined by nonlinear regression using the Prism software version 8.1.0 (GraphPad). The average IC₅₀ value for each antibody was determined from a minimum of 3 independent experiments.

Measurement of synergistic neutralization by an antibody combination. Synergy was defined as higher neutralizing activity mediated by a cocktail of two mAbs when compared to that mediated by individual mAbs at the same total concentration of antibodies *in vitro*. To assess if two mAbs synergize in a cocktail to neutralize SARS-CoV-2, we used a previously reported approach to quantitate synergy¹¹. To evaluate the significance of the beneficial effect from combining mAbs, the observed combination responses (dose-response matrix) were compared with the expected responses calculated by means of synergy scoring models¹¹. Virus neutralization was measured in a conventional focus reduction neutralization test (FRNT) assay using wild-type SARS-CoV-2 and Vero E6 cell culture monolayers. Individual mAbs COV2-2196 and COV2-2130 were mixed at different concentrations to assess neutralizing activity of different ratios of mAbs in the cocktail. Specifically, each of seven-fold dilutions of mAb COV2-2130 (starting from 500 ng/mL) was mixed with each of the nine dilutions of mAb COV2-2196 (starting from 500 ng/mL) in a total volume of 50 μ L of per each condition and then incubated with 50 μ L of live SARS-CoV-2 in cell culture medium (RPMI-1640 medium supplemented with 2% FBS) before applying to confluent Vero E6 cells grown in 96-well plates. The control values included those for determining dose-response of the neutralizing activity measured separately for the individual mAb COV2-2196 or COV2-2130, which were assessed at the same doses as in the cocktail. Each measurement was performed in duplicate. We next calculated

percent virus neutralization for each condition and then calculated the synergy score value, which defined interaction between these two mAbs in the cocktail. A synergy score of less than -10 indicates antagonism, a score from -10 to 10 indicates an additive effect, and a score greater than 10 indicates a synergistic effect¹¹.

MAB quantification. Quantification of purified mAbs was performed by UV spectrophotometry using a NanoDrop spectrophotometer and accounting for the extinction coefficient of human IgG.

Competition-binding analysis through biolayer interferometry. Anti-mouse IgG Fc capture biosensors (FortéBio 18-5089) on an Octet HTX biolayer interferometry instrument (FortéBio) were soaked for 10 minutes in 1x kinetics buffer (Molecular Devices 18-1105), followed by a baseline signal measurement for 60 seconds. Recombinant SARS-CoV-2 RBD fused to mouse IgG1 (RBD-mFc, Sino Biological 40592-V05H) was immobilized onto the biosensor tips for 180 seconds. After a wash step in 1x kinetics buffer for 30 seconds, the reference antibody (5 µg/mL) was incubated with the antigen-containing biosensor for 600 seconds. Reference antibodies included the SARS-CoV human mAb CR3022 and COV2-2196. After a wash step in 1x kinetics buffer for 30 seconds, the biosensor tips then were immersed into the second antibody (5 µg/mL) for 300 seconds. Maximal binding of each antibody was normalized to a buffer-only control. Self-to-self blocking was subtracted. Comparison between the maximal signal of each antibody was used to determine the percent binding of each antibody. A reduction in maximum signal to <33% of un-competed signal was considered full competition of binding for the second antibody in the presence of the reference antibody. A reduction in maximum signal to between 33 to 67% of un-competed was

considered intermediate competition of binding for the second antibody in the presence of the reference antibody. Percent binding of the maximum signal >67% was considered absence of competition of binding for the second antibody in the presence of the reference antibody.

hACE-2 inhibition analysis. Wells of 384-well microtiter plates were coated with purified recombinant SARS-CoV-2 S2P_{ecto} protein at 4°C overnight. Plates were blocked with 2% non-fat dry milk and 2% normal goat serum in DPBS-T for 1 hr. For screening assays, purified mAbs from microscale expression were diluted two-fold in blocking buffer starting from 10 µg/mL in triplicate, added to the wells (20 µL/well), and incubated for 1 hr at ambient temperature. Recombinant human hACE2 with a C-terminal FLAG tag protein was added to wells at 2 µg/mL in a 5 µL/well volume (final 0.4 µg/mL concentration of ACE2) without washing of antibody and then incubated for 40 min at ambient temperature. Plates were washed, and bound ACE2 was detected using HRP-conjugated anti-FLAG antibody (Sigma) and TMB substrate. ACE2 binding without antibody served as a control. The signal obtained for binding of the ACE2 in the presence of each dilution of tested antibody was expressed as a percentage of the ACE2 binding without antibody after subtracting the background signal. For dose-response assays, serial dilutions of purified mAbs were applied to the wells in triplicate, and mAb binding was detected as detailed above. Half-maximal inhibitory concentration (IC₅₀) values for inhibition by mAb of S2P_{ecto} protein binding to ACE2 was determined after log transformation of antibody concentration using sigmoidal dose-response nonlinear regression analysis (Prism software, GraphPad Prism version 8.0).

ACE2 blocking assay using biolayer interferometry biosensor. Anti-mouse IgG biosensors on an Octet HTX biolayer interferometry instrument (FortéBio) were soaked for 10 minutes in 1x

kinetics buffer, followed by a baseline signal measurement for 60 seconds. Recombinant SARS-CoV-2 RBD fused to mouse IgG1 (RBD-mFc, Sino Biological 40592-V05H) was immobilized onto the biosensor tips for 180 seconds. After a wash step in 1x kinetics buffer for 30 seconds, the antibody (5 µg/mL) was incubated with the antigen-coated biosensor for 600 seconds. After a wash step in 1x kinetics buffer for 30 seconds, the biosensor tips then were immersed into the ACE2 receptor (20 µg/mL) (Sigma-Aldrich SAE0064) for 300 seconds. Maximal binding of ACE2 was normalized to a buffer-only control. Percent binding of ACE2 in the presence of antibody was compared to ACE2 maximal binding. A reduction in maximal signal to <30% was considered ACE2 blocking.

High-throughput competition-binding analysis. Wells of 384-well microtiter plates were coated with purified recombinant SARS-CoV-2 S protein at 4°C overnight. Plates were blocked with 2% BSA in DPBS containing 0.05% Tween-20 (DPBS-T) for 1 hr. Micro-scale purified unlabeled mAbs were diluted ten-fold in blocking buffer, added to the wells (20 µL/well) in quadruplicates, and incubated for 1 hr at ambient temperature. A biotinylated preparation of a recombinant mAb based on the variable gene sequence of the previously described mAb CR3022¹² and also newly identified mAbs COV2-2096, -2130, and -2196 that recognized distinct antigenic regions of the SARS-CoV-2 S protein were added to each of four wells with the respective mAb at 2.5 µg/mL in a 5 µL/well volume (final 0.5 µg/mL concentration of biotinylated mAb) without washing of unlabeled antibody and then incubated for 1 hr at ambient temperature. Plates were washed, and bound antibodies were detected using HRP-conjugated avidin (Sigma) and TMB substrate. The signal obtained for binding of the biotin-labeled reference antibody in the presence of the unlabeled tested antibody was expressed as a percentage of the binding of the reference antibody alone after subtracting the

background signal. Tested mAbs were considered competing if their presence reduced the reference antibody binding to less than 41% of its maximal binding and non-competing if the signal was greater than 71%. A level of 40–70% was considered intermediate competition.

Binding analysis of mAbs to alanine or arginine RBD mutants. Biolayer light interferometry was performed using an Octet RED96 instrument (ForteBio; Pall Life Sciences). Binding was confirmed by first capturing octa-His-tagged RBD mutants 10 µg/mL ($\approx$ 200 nM) onto Penta-His biosensors for 300 s. The biosensors then were submerged in binding buffer (PBS/0.2% TWEEN 20) for a wash for 60 sec followed by immersion in a solution containing 150 nM of mAbs for 180 sec (association), followed by a subsequent immersion in binding buffer for 180 sec (dissociation). Response for each RBD mutant was normalized to that of wild-type RBD.

Mouse experiments using human *hACE2*-transduced mice. Animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at the Washington University School of Medicine (assurance number A3381–01). Virus inoculations were performed under anesthesia that was induced and maintained with ketamine hydrochloride and xylazine, and all efforts were made to minimize animal suffering.

BALB/c mice were purchased from Jackson Laboratories (strain 000651). Female mice (10–11-week-old) were given a single intraperitoneal injection of 2 mg of anti-Ifnar1 mAb (MAR1-5A3¹³, Leinco) one day before intranasal administration of 2.5×10^8 PFU of AdV-hACE2. Five days after AdV transduction, mice were inoculated with 4×10^5 PFU of SARS-CoV-2 by the intranasal route.

Anti-SARS-CoV-2 human mAbs or isotype control mAbs were administered 24 hours prior to SARS-CoV-2 inoculation. Weights were monitored on a daily basis, and animals were sacrificed at days 5 or 7 post-infection, and tissues were harvested.

Measurement of viral burden. Tissues were weighed and homogenized with zirconia beads in a MagNA Lyser instrument (Roche Life Science) in 1 ml of DMEM media supplemented with 2% heat-inactivated FBS. Tissue homogenates were clarified by centrifugation at 10,000 rpm for 5 min and stored at -80°C . RNA was extracted using MagMax mirVana Total RNA isolation kit (Thermo Scientific) and a Kingfisher Flex 96 well extraction machine (Thermo Scientific). TaqMan primers were designed to target a conserved region of the N gene using SARS-CoV-2 (MN908947) sequence as a guide (L Primer: ATGCTGCAATCGTGCTACAA; R primer: GACTGCCGCCTCTGCTC; probe: /56-FAM/TCAAGGAAC/ZEN/AACATTGCCAA/3IABkFQ/). To establish an RNA standard curve, we generated concatenated segments of the N gene in a gBlocks fragment (IDT) and cloned this into the PCR-II topo vector (Invitrogen). The vector was linearized, and *in vitro* T7-DNA-dependent RNA transcription was performed to generate materials for a quantitative standard curve.

Cytokine and chemokine mRNA measurements. RNA was isolated from lung homogenates at 7 dpi as described above. cDNA was synthesized from DNase-treated RNA using the High-Capacity cDNA Reverse Transcription kit (Thermo Scientific) with the addition of RNase inhibitor, following the manufacturer's protocol. Cytokine and chemokine expression was determined using TaqMan Fast Universal PCR master mix (Thermo Scientific) with commercial primers/probe sets specific for *IFN γ* (IDT: Mm.PT.58.41769240), *IL*

6 (Mm.PT.58.10005566), *CXCL10* (Mm.PT.58.43575827), *CCL2* (Mm.PT.58.42151692 and results were normalized to *GAPDH* (Mm.PT.39a.1) levels. Fold change was determined using the $2^{-\Delta\Delta C_t}$ method comparing anti-SARS-CoV-2 specific or isotype control mAb-treated mice to naïve controls.

Mouse experiments using wild-type mice. Animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at the UNC Chapel Hill School of Medicine (NIH/PHS Animal Welfare Assurance Number is D16-00256 (A3410-01)). Virus inoculations were performed under anesthesia that was induced and maintained with ketamine hydrochloride and xylazine, and all efforts were made to minimize animal suffering.

Mouse adapted SARS-CoV-2 (MA-SARS-CoV-2) virus. The virus was generated as described previously¹⁴. Virus was propagated in Vero E6 cells grown in DMEM with 10% Fetal Clone II and 1% Pen/Strep. Virus titer was determined by plaque assay. Briefly, virus was serially diluted and inoculated onto confluent monolayers of Vero E6 cells, followed by agarose overlay. Plaques were visualized on day 2 post-infection after staining with neutral red dye.

Wild-type mice. 12-month-old BALB/c mice from Envigo were used in experiments. Mice were acclimated in the BSL3 for at least 72 hrs prior to start of experiments. At 6 hrs prior to infection, mice were prophylactically treated with 200 µg of human monoclonal antibodies via intraperitoneal injection. The next day, mice were anesthetized with a mixture of ketamine and

xylazine and intranasally infected with 10^5 PFU of MA-SARS-CoV-2 diluted in PBS. Daily weight loss was measured, and at two days post-infection mice were euthanized by isoflurane overdose prior to tissue harvest.

Plaque assay of lung tissue homogenates. The lower lobe of the right lung was homogenized in 1 mL PBS using a MagnaLyser (Roche). Serial dilutions of virus were titrated on Vero E6 cell culture monolayers, and virus plaques were visualized by neutral red staining at two days after inoculation. The limit of detection for the assay is 100 PFU per lung.

Quantification and statistical analysis. The descriptive statistics mean $\pm$ SEM or mean $\pm$ SD were determined for continuous variables as noted. Technical and biological replicates are described in the figure legends. In the mouse studies, the comparison of weight change curves was performed using repeated measurements two-way ANOVA with Tukey's post-test using Prism v8.0 (GraphPad). Viral burden and gene expression measurements were compared using Kruskal-Wallis ANOVA with Dunn's post-test using Prism v8.0 (GraphPad). Synergy score and dose-response matrix analysis were performed using a web application SynergyFinder¹¹.

References for Online Methods

- 1 Zost SJ, G. P., Chen RE, Case JB, Reidy JX, Trivette A, Nargi RS, Sutton RE, Suryadevara N, Chen EC, Binshtein E, Shrihari S, Ostrowski M, Chu HY, Didier JE, MacRenaris KW, Jones T, Day S, Myers L, Lee FE-H, Nguyen DC, Sanz I, Martinez DR, Baric RS, Thackray LB, Diamond MS, Carnahan RH, Crowe JE Jr. Rapid isolation

and profiling of a diverse panel of human monoclonal antibodies targeting the SARS-CoV-2 spike protein. *bioRxiv* 2020.05.12.091462; doi: <https://doi.org/10.1101/2020.05.12.091462> (2020)

2 Mukherjee, S. *et al.* Enhancing dengue virus maturation using a stable furin over-expressing cell line. *Virology* **497**, 33-40, doi:10.1016/j.virol.2016.06.022 (2016).

3 Wrapp, D. *et al.* Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* **367**, 1260-1263, doi:10.1126/science.abb2507 (2020).

4 Ohi, M., Li, Y., Cheng, Y. & Walz, T. Negative staining and image classification - Powerful tools in modern electron microscopy. *Biol. Proced. Online* **6**, 23-34, doi:10.1251/bpo70 (2004).

5 Mastronarde, D. N. Automated electron microscope tomography using robust prediction of specimen movements. *J. Struct. Biol.* **152**, 36-51, doi:10.1016/j.jsb.2005.07.007 (2005).

6 Punjani, A., Rubinstein, J. L., Fleet, D. J. & Brubaker, M. A. cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nat. Methods* **14**, 290-296, doi:10.1038/nmeth.4169 (2017).

370 7 Bepler, T., Noble, A. J., and Berger, B. Topaz-Denoise: general deep denoising models
371 for cryoEM. *bioRxiv*. doi:10.1101/838920 (2019).
372

373 8 Pettersen, E. F. *et al.* UCSF Chimera--a visualization system for exploratory research and
374 analysis. *J. Comput. Chem.* **25**, 1605-1612, doi:10.1002/jcc.20084 (2004).
375

376 9 Chng, J. *et al.* Cleavage efficient 2A peptides for high level monoclonal antibody
377 expression in CHO cells. *MAbs* **7**, 403-412, doi:10.1080/19420862.2015.1008351 (2015).
378

379 10 Yuan, M. *et al.* A highly conserved cryptic epitope in the receptor binding domains of
380 SARS-CoV-2 and SARS-CoV. *Science* **368**, 630-633, doi:10.1126/science.abb7269
381 (2020).
382

383 11 Ianevski, A., He, L., Aittokallio, T. & Tang, J. SynergyFinder: a web application for
384 analyzing drug combination dose-response matrix data. *Bioinformatics* **33**, 2413-2415,
385 doi:10.1093/bioinformatics/btx162 (2017).
386

387 12 ter Meulen, J. *et al.* Human monoclonal antibody as prophylaxis for SARS coronavirus
388 infection in ferrets. *Lancet* **363**, 2139-2141, doi:10.1016/S0140-6736(04)16506-9 (2004).
389

390 13 Sheehan, K. C. *et al.* Blocking monoclonal antibodies specific for mouse IFN-alpha/beta
391 receptor subunit 1 (IFNAR-1) from mice immunized by in vivo hydrodynamic
392 transfection. *J. Interferon Cytokine Res* **26**, 804-819 (2006).

393

394 14 Dinnon KH, *et al.* A mouse-adapted SARS-CoV-2 model for the evaluation of COVID-
395 19 medical countermeasures. *bioRxiv* 2020.05.06.081497;
396 doi: <https://doi.org/10.1101/2020.05.06.081497> (2020).

397

398