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The recent emergence of SARS-CoV-2 variants of concern (VOC)^{1–10} and the recurrent spillovers of coronaviruses^{11,12} in the human population highlight the need for broadly neutralizing antibodies that are not affected by the ongoing antigenic drift and that can prevent or treat future zoonotic infections. Here, we describe a human monoclonal antibody (mAb), designated S2X259, recognizing a highly conserved cryptic receptor-binding domain (RBD) epitope and cross-reacting with spikes from all sarbecovirus clades. S2X259 broadly neutralizes spike-mediated entry of SARS-CoV-2 including the B.1.1.7, B.1.351, P.1, B.1.427/B.1.429 VOC, as well as a wide spectrum of human and potentially zoonotic sarbecoviruses through inhibition of ACE2 binding to the RBD. Furthermore, deep-mutational scanning and *in vitro* escape selection experiments demonstrate that S2X259 possesses an escape profile limited to the single substitution G504D. We show that prophylactic and therapeutic administration of S2X259 protects Syrian hamsters against challenge with the prototypic SARS-CoV-2 and the B.1.351 VOC, suggesting this mAb is a promising candidate for the prevention and treatment of emergent variants and zoonotic infections. Our data unveil a key antigenic site targeted by broadly-neutralizing antibodies and will guide the design of pan-sarbecovirus vaccines.

SARS-CoV-2 has resulted in a global pandemic causing over 182 million infections and more than 3 million fatalities as of July 2021. SARS-CoV-2 genetic drift has resulted in emerging variants of concern (VOC) characterized by higher transmissibility, immune evasion and disease severity relative to the ancestral isolate^{1–10}. Countermeasures, such as vaccines and therapeutics, are needed to cope with SARS-CoV-2 evolution and to protect against future sarbecovirus spillovers.

The sarbecovirus spike glycoprotein (S) mediates viral entry into host cells, represents the main target of neutralizing antibodies (Abs) and is the focus of vaccine design¹¹. S comprises an S₁ subunit, which recognizes host cell receptors, and an S₂ subunit that promotes viral-cell membrane fusion^{12,13}. The S₁ subunit includes the receptor-binding domain (RBD), which in the case of SARS-CoV and SARS-CoV-2 interacts with angiotensin-converting enzyme 2 (ACE2) to allow virus entry^{12,14–16}.

A highly conserved region on sarbecovirus RBDs, designated antigenic site II¹⁷, has been shown to elicit SARS-CoV and SARS-CoV-2 cross-neutralizing Abs^{18,19}. However, site II becomes accessible only when at least two RBDs in the spike trimer adopt an open conformation and thus has limited immunogenicity^{17,19}. Here, we describe a site II-targeting mAb designated S2X259 that possesses exceptional neutralization breadth within the sarbecovirus subgenus, including all VOC, and an escape profile limited to a sole and rare substitution. In addition, we show that prophylactic and therapeutic administration of S2X259 protects Syrian hamsters against SARS-CoV-2 challenges with prototypic virus and B.1.351 VOC. Our findings identify S2X259 as a promising countermeasure to protect against SARS-CoV-2 antigenic drift as well as potentially new zoonotic infections and highlight the importance of RBD site II for pan-sarbecovirus vaccine design.

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Discovery of a broadly neutralizing sarbecovirus mAb

We identified one mAb from the memory B cells of a COVID-19 convalescent individual, designated S2X259 (Table S1), which cross-reacted with 25 out of 26 S glycoproteins representative of all sarbecovirus clades^{18,20} (Fig. 1a and Extended Data Fig. 1a-c). No binding, however, was observed to embecovirus S (HCoV-HKU1 and HCoV-OC43) or merbecovirus S (MERS-CoV) glycoproteins (Extended Data Fig. 1a, b).

The cross-reactivity of S2X259 within the sarbecovirus subgenus was further confirmed using ELISA with a panel of 12 RBDs (Fig. 1b). S2X259 bound tightly to all RBDs tested (EC₅₀ < 32 ng/ml) with the exception of two bat strains ZC45 (EC₅₀ = 539 ng/ml) and Anlong-112 (EC₅₀ = 1281 ng/ml) (Fig. 1b). The broad recognition of S from the divergent bat Asian-related (clade 2) and non-Asian bat (clade 3) clades highlights the exceptional breadth of S2X259 in comparison with previously reported mAbs^{17,21,22} (Extended Data Fig. 1d). Surface plasmon resonance (SPR) indicated that the S2X259 Fab fragment bound to clade 1a, 1b and clade 3 RBDs with nano- to picomolar affinities, and to clade 2 RBDs with micro- to nanomolar affinities. Of note, the affinity of S2X259 for the Pangolin-Guanxi (P-GX) RBD was 10 to 100-fold lower compared to other clade 1b RBDs (Extended Data Fig. 2a). The S2X259 Fab recognized both the SARS-CoV-2 prefusion-stabilized S ectodomain trimer and the recombinant RBD with picomolar affinities albeit with a slower on-rate for binding to S presumably due to limited epitope exposure^{12,13} (Extended Data Fig. 2a). Finally, S2X259 binding was unaffected by several point mutants or constellation of mutations identified in circulating clinical isolates (K417V, N439K, Y453F, E484K) and in the B.1.1.7 (N501Y), B.1.351 (K417N/E484K/N501Y), B.1.427/B.1.429 (L452R), and P.1 (K417T/E484K/N501Y) lineage RBDs (Fig. 1b and Extended Data Fig. 2b).

We next evaluated the neutralization potency of S2X259 using authentic SARS-CoV-2 Nluc as well as murine leukemia virus (MLV) and vesicular stomatitis virus (VSV) pseudotyping systems. S2X259 potently neutralized authentic SARS-CoV-2, SARS-CoV-2 S MLV and SARS-CoV-2 S VSV with IC₅₀s of 144 ng/mL, 87 ng/mL and 213 ng/mL, respectively (Fig. 1c-d). We subsequently evaluated S2X259-mediated neutralization against VSV pseudotypes harbouring SARS-CoV-2 S from the B.1.1.7, B.1.351, P.1, B.1.427/B.1.429, B.1.617.1 and other lineages as well as the N439K and Y453F single mutations^{5,23-25}. S2X259 neutralized all of these mutants with similar potency, in line with the identical binding affinities determined using RBD mutants (Fig. 1d-e and Extended Data Fig. 2b and 3a, b and Table S2). Furthermore, S2X259 neutralized also a broad panel of ACE2-utilizing sarbecoviruses with comparable potencies, including SARS-CoV-2 Pangolin Guangdong (P-GD), RaTG13, SARS-CoV, WIV1, WIV16 and RsSHC014 (Fig. 1f-g and Table S2). In line with the SPR data, S2X259 neutralized P-GX S VSV pseudotypes but with reduced potency compared to SARS-CoV-2 (Fig. 1g and Extended Data Fig. 2a and Table S2).

Structural basis for broad sarbecovirus neutralization by S2X259 mAb

3D classification of the cryo-electron microscopy (cryoEM) data showed the presence of SARS-CoV-2 S ectodomain trimers saturated with three S2X259 Fabs bound to open RBDs swung out to various extent for which we determined a consensus structure at 3.2 Å resolution (Fig. 2a-b, Extended Data Fig. 4 and Table S3). We used local refinement to determine a 3.7 Å resolution map of the region corresponding to the S2X259 variable domains and RBD. In parallel, we crystallized the S2X259 Fab-bound SARS-CoV-2 RBD in the presence of the S2H97 Fab²⁶ and determined a 2.65 Å resolution X-ray structure (Fig. 2c-e and Table S4).

S2X259 recognizes a glycan-free, cryptic epitope within antigenic site IIa which was previously defined based on the non-clonally related S2X35 mAb isolated from the same donor¹⁷. S2X259 binding requires opening of two RBDs to grant access to the Fab in the context of the S trimer (Fig. 2a-b). S2X259 contacts the RBD using both heavy and

light chains, which respectively contribute two thirds and one third of the -950 Å² paratope surface buried upon binding. Specifically, S2X259 uses complementary determining regions (CDRs) H1-H3, L1 and L3 to contact RBD residues 369-386, which form two α-helices and an intervening β-strand belonging to the structurally conserved RBD β-sheet, as well as the residues 404-411 and 499-508 which form a continuous surface made up of an α-helix and a loop followed by an α-helix, respectively.

The S2X259 epitope is conserved in circulating SARS-CoV-2 isolates and does not comprise prevalent RBD mutations, such as S477N, N439K or L452R. S2X259 also circumvents residues 417 and 484, and contacts the backbone of residue N501 but not its side chain, explaining the high-affinity binding to the B.1.1.7, B.1.351 and P.1 RBDs and potent neutralization of pseudotyped VOC viruses (Fig. 1c, e-f and Extended Data Fig. 2b). S2X259 broad neutralization results from the conservation of its epitope among sarbecoviruses and from the angle of approach of the Fab that circumvents the SARS-CoV N357 glycan present in all sarbecoviruses, except SARS-CoV-2 (SARS-CoV-2 N370) (Extended Data Fig. 5). The SARS-CoV-2 and P-GX RBDs differ at only two positions, G504N and Y508F, and the structural data indicate that the former substitution would likely dampen mAb binding through steric hindrance as indicated by *in vitro* neutralization data (Fig. 1g).

S2X259 binding to the RBD does not affect or prevent recognition by site I or site IV-targeting mAbs (Extended Data Fig. 6a,b), which represent the majority of mAbs currently approved or in the clinic^{17,22,27,28}. Competition binding assays suggest that serum Abs directed to the RBD antigenic site II are subdominant in convalescent and vaccinated individuals as compared to Abs directed toward site I (Extended Data Fig. 6c). Therefore, S2X259 can be used in combination with other site I or site IV mAbs to maximize breadth against currently circulating and emerging SARS-CoV-2 variants as well as against future sarbecovirus infections from the animal reservoirs.

S2X259 viral escape is limited to a single substitution in the epitope

Using deep-mutational scanning (DMS) with a yeast-display RBD mutant library²⁹, we exhaustively mapped mutations that escape S2X259 binding. Strikingly, binding was strongly reduced by only a restricted number of amino acid substitutions, compared to previously described neutralizing mAbs²⁶, with mutations at RBD site 504 yielding the most marked reduction in binding (Fig. 3a and Extended Data Fig. 7a-c). Indeed, substitution of G504 with most other amino acids reduced S2X259 binding compared to the wildtype (Wuhan-1) RBD, highlighting the importance of this residue for mAb recognition.

To further explore the functional significance of the DMS data, we passaged a replication competent VSV-SARS-CoV-2 S chimera^{30,31} in Vero cells in the presence of S2X259 mAb. The selective pressure imposed by S2X259 led to the emergence of viral mutants harbouring the G504D mutation, which was the sole mutation present in each of the 18 neutralization-resistant plaques sampled. This substitution did not impair viral fitness of the VSV-SARS-CoV-2 S chimera (Extended Data Fig. 7d-e and Table S5). In the same conditions, S2X35¹⁷, which also targets antigenic site IIa, selected for 6 escape mutants at 5 different positions (Y369S, K378E, D405N, V503D/G, G504D/S)²⁶ (Table S5).

The G504D substitution is very rare as it has been detected in only 27 out of 1,345,708 (0.002%) SARS-CoV-2 genome sequences available on GISAID as of May 2021 (Fig. 3b). Furthermore, in agreement with the DMS results, S2X259 efficiently neutralized VSV pseudoviruses harbouring S mutations located in S2X259-contact residues and found with high frequency in human isolates (Fig. 3b and Extended Data Fig. 7f). In the same analysis, we confirmed the loss of S2X259 neutralizing activity against the G504D mutant. S2X259 CDRL1 and CDRL3 contact G504 or surrounding residues, explaining that substituting a glycine with any other residue prevents mAb binding via steric hindrance along with

potential disruption of the interactions with RBD residue D405, which is in close proximity to G504 (Extended Data Fig. 7g).

These data also support the hypothesis proposed above for the markedly reduced affinity and neutralization potency of S2X259 against P-GX pseudovirus which harbours the G504N mutation but maintains an aspartate at position 405 (Fig. 1g, Extended Data Figs. 2a, 7f and 8). However, S2X259 binding to clade 2 S bearing G504D/E mutations (e.g., Shanxi2011 or YN2013) was not abrogated (Fig. 1b) possibly due to substitution at the SARS-CoV-2 equivalent position 405 with a serine and to substantial deletions in the RBM (Extended Data Fig. 8).

The finding that the only escape mutation for S2X259 is the rare G504D substitution suggests this mAb might have a high barrier for the emergence of SARS-CoV-2 escape mutants, which might prove essential during the next stages of the pandemic.

S2X259 protects Syrian hamsters from SARS-CoV-2 challenge

In line with structural data indicating a clashing between S2X259 and ACE2 for binding to the RBD (Extended Data Fig. 9a), we found that S2X259 efficiently blocked binding of SARS-CoV-2 and SARS-CoV RBDs to human ACE2 (Extended Data Fig. 9b).

In addition, S2X259 binding efficiently promoted shedding of the S₁ subunit from cell-surface-expressed full-length SARS-CoV-2 S, as previously shown with other RBD-specific mAbs^{17,32–35} but not with S2M11 which locks S in the prefusion closed state²¹ (Extended Data Fig. 9c). The efficient S₁ shedding induced by S2X259 might be responsible for the lack of FcγRIIIa and FcγRIIIa activation, which we used as a proxy for Ab-dependent cellular phagocytosis (ADCP) and Ab-dependent cellular cytotoxicity (ADCC), respectively (Extended Data Fig. 9d–e). Indeed, performing the same assays using target cells expressing an uncleavable pre-fusion stabilized SARS-CoV-2 S protein (unable to release the S₁ subunit) we confirmed the ability of S2X259 mAb to induce activation of FcγRIIIa and FcγRIIIa in these conditions (Extended Data Fig. 9f–g).

These data show that the primary mechanism of S2X259-mediated neutralization of SARS-CoV-2 and other sarbecoviruses relies on blocking viral attachment to host cell receptors through competitive inhibition of ACE2 binding. Premature triggering of S₁ subunit shedding could inactivate viruses before encountering target cells albeit reducing activation of S2X259 mediated effector functions.

Next, we evaluated the prophylactic activity of S2X259 against challenge with the prototypic (Wuhan-1 related) SARS-CoV-2 in a Syrian hamster model¹⁶. S2X259 was administered at 1, 5 and 25 mg/kg via intraperitoneal injection before intranasal challenge and the lungs of the animals were collected 4 days later for the quantification of viral RNA and replicating virus. Despite the lack of Fc-dependent effector functions *in vitro* and their proposed contribution to *in vivo* protection against SARS-CoV-2^{37,38}, administration of S2X259 at 1 mg/kg reduced the amount of viral RNA and infectious virus detected in the lungs by approximately 2 and 2.5 orders of magnitude, respectively, compared to hamsters receiving a control mAb (Fig. 4a–b). Furthermore, complete abrogation of virus replication was observed in the lungs of the animals administered with S2X259 at 5 and 25 mg/kg relative to the control group (Fig. 4b). Therapeutic administration (post-exposure) of S2X259 at 5 mg/kg reduced the amounts of viral RNA detected by 3 logs and abrogated viral replication in the lungs of all animals but one (Fig. 4c–d). Animals administered with S2X259 at 10 or 20 mg/kg displayed a further -1 log reduction of viral RNAs with complete abrogation of viral replication (Fig. 4c–d). In both prophylactic and therapeutic settings, viral replication in the lungs detected at day 4 inversely correlated with serum mAb concentration measured at the time of infection or at day 4 post-infection in prophylactic and therapeutic settings, respectively (Extended Data Fig. 10a, b).

We next assessed the prophylactic activity of S2X259 against B.1.351 SARS-CoV-2 challenge³⁹. S2X259 at 1 and 4 mg/kg or in combination

with S309 (each mAb at 1 mg/kg) significantly decreased the amount of viral RNA detected in the lungs relative to hamsters receiving a control mAb (Fig. 4e). Moreover, S2X259 at 4 mg/kg or in combination with S309 (each mAb at 1 mg/kg) completely abrogated SARS-CoV-2 B.1.351 virus replication in most of the animals (Fig. 4f). As was the case for the prototypic SARS-CoV-2, the amounts of infectious virus at day 4 detected in the group administered with only S2X259 also inversely correlated with serum mAb concentration measured at the time of infection (Extended Data Fig. 10c). Moreover, animals receiving the mAb cocktail appeared to benefit from the additive contribution of the S309 mAb (Fig. 4f and Extended Data Fig. 10d, e).

Discussion

The large number of mutations in the S glycoprotein of SARS-CoV-2 VOC^{4,23,25} has been shown to abrogate or reduce the neutralization potency of several mAbs and vaccine-elicited Abs^{5–10,40}. Moreover, the detection of a large pool of sarbecoviruses in bats and other mammals across multiple continents suggests that future cross-species transmission events are likely to occur^{41–43}. However, approved SARS-CoV-2 therapeutics and vaccines predominantly target or elicit immunity against immunodominant but highly variable epitopes and therefore have a limited efficacy against antigenic drift and genetically distinct strains with zoonotic potential^{40,44}.

Strategies for eliciting broadly neutralizing and protective sarbecovirus Abs targeting the RBD have recently been proposed^{18,40} and previous work by our group and others described mAbs now in the clinic with extended coverage within clade 1 sarbecoviruses^{20,22}. The recent results from a Phase III study demonstrating efficacy (i.e., 85% reduction of hospitalizations or deaths) of the cross-reactive derivative of the S309 mAb (sotrovimab) paves the way for the development of additional neutralizing mAbs targeting conserved RBD epitopes that may broaden the coverage across sarbecoviruses and increase the barrier to resistance.

Here, we identified the S2X259 mAb that targets the RBD antigenic site II and uniquely cross-reacts with all SARS-CoV-2 VOC evaluated as well as with 30 spike trimers or RBDs from all four sarbecovirus clades. S2X259 broadly neutralizes pseudoviruses harbouring spikes from the B.1.1.7, B.1.351, P.1, B.1.427/B.1.429 and B.1.617.1 lineages and from other representative members of SARS-CoV-2 (clade 1b) and SARS-CoV (clade 1a) clades. Of note, S2X259 neutralizes WIV1 S and RsSHC014 S (clade 1a) pseudotyped virus that were previously suggested to be potential zoonotic threats to human health^{41,42}. Moreover, S2X259 also recognizes S and RBDs from bat Asian (clade 2) and non-Asian (clade 3) sarbecovirus clades. The coverage of clade 2 might be particularly important as Letko et al.¹⁶ showed that exogenous protease addition to pseudoviruses from this clade resulted in detectable S-mediated entry in human cells. Furthermore, although clade 2 and 3 sarbecoviruses have not been reported to bind human ACE2, mutation or recombination events could lead to human ACE2 binding and entry^{16,45}. Overall, these findings indicate that S2X259 is a promising candidate for clinical development and could be stockpiled as part of a pandemic preparedness toolbox.

Based on the growing body of data demonstrating that Abs targeting the SARS-CoV-2 RBD account for most neutralizing activity in COVID-19 convalescent patient sera^{17,46}, we propose that RBD-based vaccines could better promote elicitation of high titres of S2X259-like neutralizing Abs due to the enhanced accessibility of this antigenic site compared to S-based vaccines^{40,47}. Our serological competition data confirmed that the Ab responses against the conserved site targeted by S2X259 mAb is subdominant in convalescent donors as well as in individuals receiving S-based mRNA vaccines. We anticipate these data will guide future efforts to develop SARS-CoV-2 vaccines overcoming the emergence of variants as well as pan-sarbecovirus vaccines.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-021-03817-4>.

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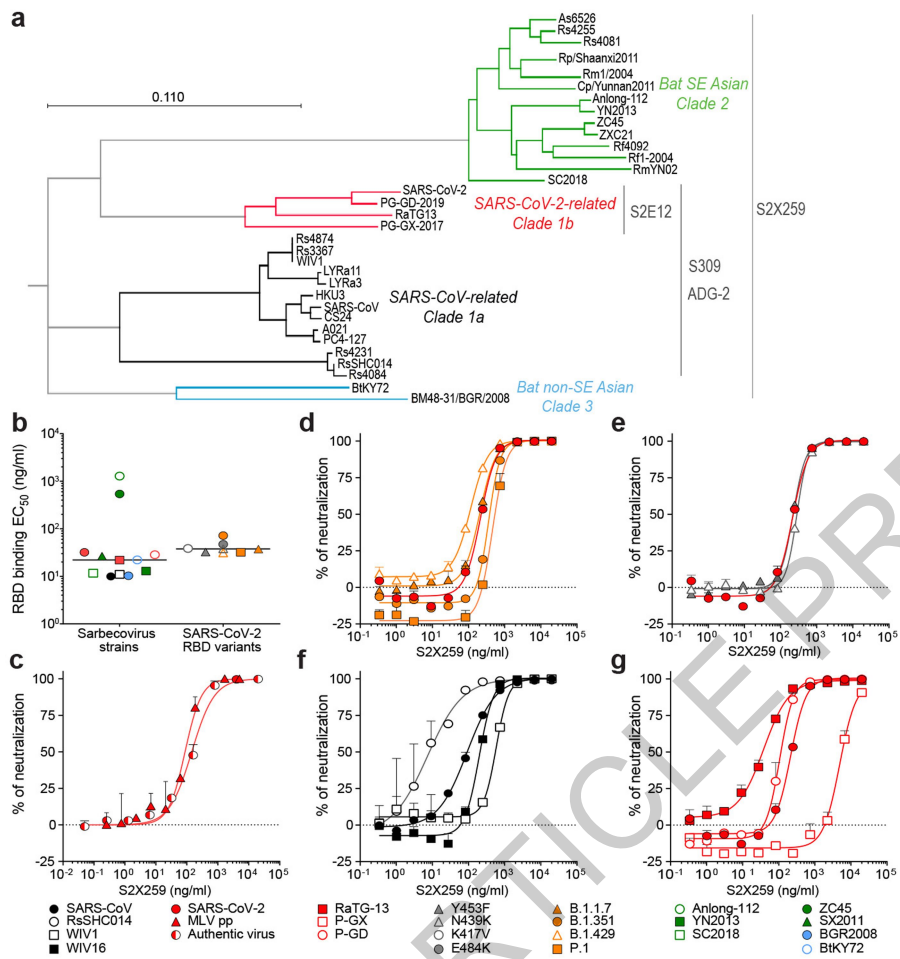


Fig. 1 | Identification of a broadly neutralizing sarbecovirus mAb.

a, Phylogenetic tree of sarbecovirus RBDs constructed via maximum likelihood analysis of amino acid sequences retrieved from GISAID and GenBank. Cross-reactivity within the sarbecovirus subgenus for S2E12²¹, S309²², and ADG-2²⁰ is included for comparison. **b**, S2X259 binding to RBDs representative of the different sarbecovirus clades and SARS-CoV-2 variants as measured by ELISA. **c**, S2X259-mediated neutralization of SARS-CoV-2 Nluc authentic virus

and SARS-CoV-2 S MLV-pseudotyped virus (MLV-pp). **d-e**, S2X259-mediated neutralization of VSV pseudotypes harbouring SARS-CoV-2 S from isolates representing the B.1.1.7, B.1.351, P.1 and B.1.429 VOC (d) as well as single RBD mutants (e). **f-g**, S2X259-mediated neutralization of VSV pseudotypes harbouring SARS-CoV-related (clade 1a, f) or SARS-CoV-2-related (clade 1b, g) S glycoproteins. *n* = 2 independent experiments. Error bars indicate standard deviation of duplicates or triplicates.

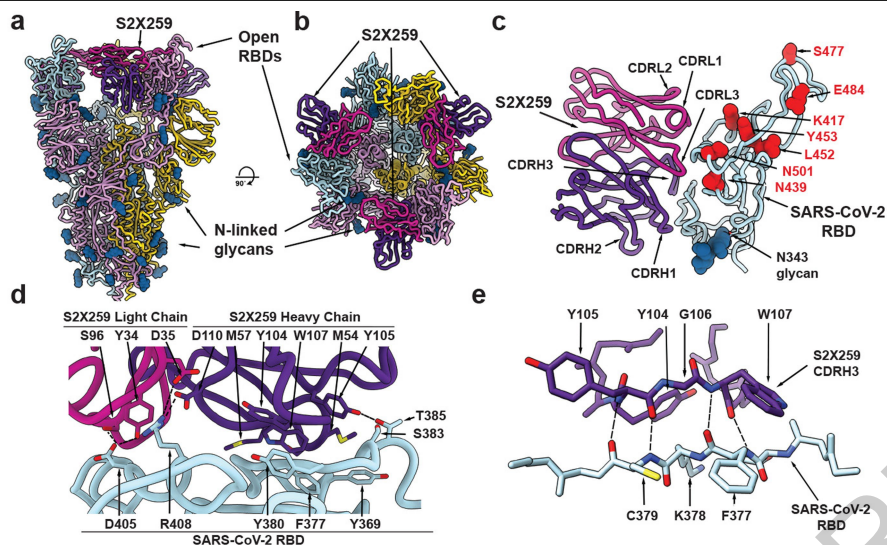


Fig. 2 | The S2X259 broadly neutralizing sarbecovirus mAb recognizes RBD antigenic site II. a-b, CryoEM structure of the prefusion SARS-CoV-2 S ectodomain trimer with three S2X259 Fab fragments bound to three open RBDs viewed along two orthogonal orientations (PDB 7RA8 and PDB 7RAL). **c,** The S2X259 binding mode involving contacts with multiple RBD regions. Residues corresponding to prevalent RBD mutations are shown as red spheres

(PDB 7M7W). **d-e,** Close-up views showing selected interactions formed between S2X259 and the SARS-CoV-2 RBD. In panels a-e, each SARS-CoV-2 S protomer is coloured distinctly (cyan, pink and gold) whereas the S2X259 light and heavy chain variable domains are coloured magenta and purple, respectively. N-linked glycans are rendered as blue spheres in panels a-c.

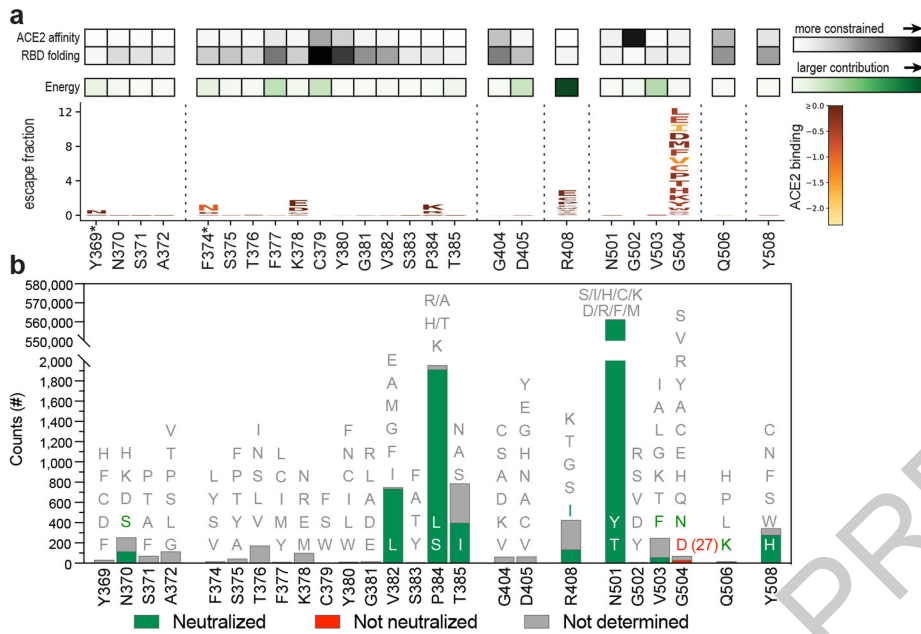


Fig. 3 | S2X259 is resilient to a broad spectrum of escape mutations.
a, Complete mapping of mutations reducing S2X259 binding using yeast-displayed RBD and deep mutational scanning (DMS). Mean mutation effect on ACE2 affinity, RBD folding, and contribution to S2X259 binding of substitutions at each position in the S2X259 epitope is reported. * mutations

introducing a N-linked glycosylation sites that may not be tolerated in full spike. **b**, Frequency of mutants within the S2X259 epitope based on SARS-CoV-2 genome sequences available on GISAID as of May 2021. S2X259 neutralizing activity against selected mutations is reported.

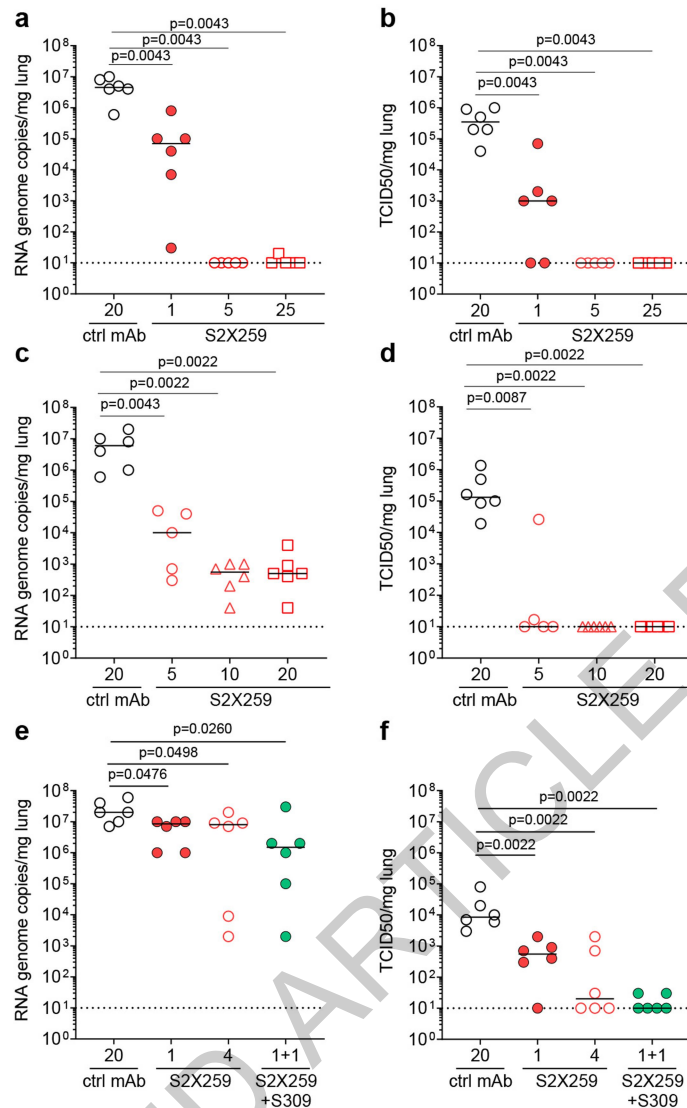


Fig. 4 | S2X259 protects hamsters challenged with prototypic and B.1.351 SARS-CoV-2. **a-b**, Viral RNA loads (a) and replicating virus titres (TCID50) (b) in the lungs of Syrian hamsters at 4 days post-intranasal infection with prototypic SARS-CoV-2 following prophylactic administration of S2X259 mAb at 1 (n=6 animals), 5 (n=5 animals), and 25 (n=5 animals) mg/kg. **c-d**, Viral RNA loads (c) and replicating virus titres (TCID50) (d) in the lungs of Syrian hamsters after therapeutic administration of S2X259 at 5 (n=5 animals), 10 (n=6 animals), and

20 (n=6 animals) mg/kg 24h after infection with Wuhan-related SARS-CoV-2. **e-f**, Quantification of viral RNA loads (e) and replicating virus titres (TCID50) (f) in the lungs of Syrian hamsters 4 days post intranasal challenge with B.1.351 SARS-CoV-2 VOC following prophylactic administration of S2X259 at 1 mg/kg (n=6 animals), 4 mg/kg (n=6 animals), and in combination with S309 (1+1 mg/kg, n=6 animals). Data from one independent experiment are presented. Two-tailed Mann-Whitney test was used for statistical analysis of significance.

Methods

Cell lines

Cell lines used in this study were obtained from ATCC (HEK293T, Vero and Vero-E6), ThermoFisher Scientific (ExpiCHO cells, FreeStyle™ 293-F cells and Expi293F™ cells) or were generated via lentiviral transduction (ExpiCHO-S, HEK293T-ACE2). None of the cell lines used were authenticated. Cell lines were routinely tested for mycoplasma contamination.

Sample donors

Samples from a SARS-CoV-2 recovered individual, designated as donor X (male, 52 years-old), was obtained 75 days after symptoms onset under study protocols approved by the local Institutional Review Boards (Canton Ticino Ethics Committee, Switzerland). Samples from additional nine SARS-CoV-2 convalescent and nine vaccinated subjects were obtained under study protocols approved by the Canton Ticino Ethics Committee, Switzerland and the Ethical committee of Luigi Sacco Hospital, Milan, Italy. For recovered individual samples were collected between 14 and 75 days after symptoms onset, for vaccinees samples were collected between 13 and 20 days after the second dose of mRNA-based SARS-CoV-2 vaccine. All donors provided written informed consent for the use of blood and blood components (such as PBMCs, sera or plasma).

Isolation of peripheral blood mononuclear cells (PBMCs), plasma and sera

PBMCs were isolated from blood draw performed using tubes pre-filled with heparin, followed by Ficoll density gradient centrifugation. PBMCs were either used freshly along SARS-CoV2 Spike protein specific memory B cells sorting or stored in liquid nitrogen for later use. Sera were obtained from blood collected using tubes containing clot activator, followed by centrifugation and stored at -80 °C.

B-cell isolation and recombinant S2X259 mAb production

Starting from freshly isolated PBMCs or upon cells thawing, B cells were enriched by staining with CD19 PE-Cy7 and incubation with anti-PE beads, followed by positive selection using LS columns. Enriched B cells were stained with anti-IgM, anti-IgD, anti-CD14 and anti-IgA, all PE labelled, and perfusion SARS-CoV-2 S with a biotinylated avi tag conjugated to Streptavidin Alexa-Fluor 647 (Life Technologies). SARSCoV-2 S-specific IgG+ memory B cells were sorted by flow cytometry via gating for PE negative and Alexa-Fluor 647 positive cells. Sorted IgG+ memory B cells were seeded in 384-well microtiter plates on a monolayer of mesenchymal stromal cells at 0.5 cells/well in the presence CpG2006, IL-2, IL-6 and IL-21, as previously described⁴⁹. After 7 days, the supernatants were screened for binding to SARS-CoV-2 RBD and pre-fusion S as well as for neutralization activity using a high-throughput VSV SARS-CoV-2 S based microneutralization assay. Selection of the antibody of interest in secondary screening was based on reactivity to a panel of sarbecovirus RBDs as well as on the neutralizing activity. Antibody VH and VL sequences were obtained by RT-PCR and mAbs were expressed as recombinant human Fab fragment or as IgG1 (G1m3 allotype). ExpiCHO cells were transiently transfected with heavy and light chain expression vectors as previously described²².

Affinity purification was performed on ÄKTA Xpress FPLC (Cytiva) operated by UNICORN software version 5.11 (Build 407) using HiTrap Protein A columns (Cytiva) for full length human and hamster mAbs and CaptureSelect CHI-XL MiniChrom columns (ThermoFisher Scientific) for Fab fragments, using PBS as mobile phase. Buffer exchange to the appropriate formulation buffer was performed with a HiTrap Fast desalting column (Cytiva). The final products were sterilized by filtration through 0.22 µm filters and stored at 4 °C.

Sarbecovirus sequence analysis and SARS-CoV-2 mutant counts

Alignment and phylogenetic tree of the strains within the sarbecovirus subgenus was generated using MEGA X and CLC Main work bench

21.0.3 (Qiagen). The following sequences were retrieved from GISAID and NCBI: A021 (AAV97986.1). The viral sequences were obtained from GISAID EpiCoV project (<https://www.gisaid.org/>). Analysis was performed on sequences submitted to GISAID up to April 2nd, 2021. The spike protein sequences were either obtained directly from the protein dump provided by GISAID or, for the latest submitted sequences that were not incorporated yet in the protein dump at the day of data retrieval, from the genomic sequences with exonerate⁵⁰ 2.2.4.0--haf93ef1_3 (<https://quay.io/repository/biocontainers/exonerate?tab=tags>) using protein to DNA alignment with parameters *-m protein2dna --refine full --minintron 999999 --percent 20* and using accession YP_009724390.1 as a reference. Multiple sequence alignment of all human spike proteins was performed with mafft⁵¹ 7.475--h516909a_0 (<https://quay.io/repository/biocontainers/mafft?tab=tags>) with parameters *--auto --reorder --keeplength --addfragments* using the same reference as above. Spike sequences that contained >10% ambiguous amino acid or that were < than 80% of the canonical protein length were discarded. A total of 923,686 sequences were used for analysis. Variants were then extracted as compared to the reference with R 4.0.2 (<https://www.r-project.org/>) using Biostrings 2.56.0.

Binding to cell surface expressed Sarbecovirus S proteins by Flow Cytometry

ExpiCHO-S cells were seeded at 6 x 10⁶ cells/mL in a volume of 5 mL in a 50 mL bioreactor. Spike encoding plasmids HKU3 (QND76020.1), Rs3367 (AGZ48818.1), YN2013 (AIA62330.1), Rs4874 (ATO98205.1), Rs4255 (ATO98193.1), Rs4231 (ATO98157.1), Rs4084 (ATO98132.1), ZXC21 (AVP78042.1), ZC45 (AVP78031.1), Rp/Shaanxi2011 (AGC74165.1), Rm1/2004 (ABD75332.1), Rf1-2004 (ABD75323.1), BM48-31/BGR/2008 (YP_003858584.1), RaTG13 (QHR63300.2), PC4-127 (AAU93318.1), SARS-CoV2 (YP_009724390.1), LYRa3 (AHX37569.1), Cp/Yunnan2011 (AGC74176.1), SARS-CoV Urbani (AAP13441.1), As6526 (ATO98108.1), BtKY72 (APO40579.1), RmYN02 (EPI_ISL_412977), Pangolin_Guangdong-2019 (EPI_ISL_410721), Pangolin-Guanxi-2017 (EPI_ISL_410539), HKU3-12 (ADE34812.1) were diluted in cold OptiPRO SFM (Life Technologies, 12309-050), mixed with ExpiFectamine CHO Reagent (Life Technologies, A29130) and added to the cells. Transfected cells were then incubated at 37 °C with 8% CO₂ with an orbital shaking speed of 250 RPM (orbital diameter of 25 mm) for 42 hours. Transiently transfected ExpiCHO cells were harvested and washed two times in wash buffer (PBS 2% FBS, 2 mM EDTA). Cells were counted and distributed into round bottom 96-well plates (Corning, 3799) and incubated with S2X259 mAb serial dilutions from 50 µg/ml. Alexa Fluor647-labelled Goat Anti-Human IgG secondary Ab (Jackson ImmunoResearch, 109-606-098) was prepared at 2 µg/ml and added onto cells after two washing steps. Cells were then washed twice and resuspended in wash buffer for data acquisition at ZE5 cytometer (Biorad).

Recombinant protein production

Wild-type SARS-CoV-2 RBD (with N-terminal signal peptide and 'ETGT', and C-terminal 8xHis-tag) was expressed in Expi293F cells at 37 °C and 8% CO₂ in the presence of 10 µM kifunensine. Transfection was performed using the ExpiFectamine 293 Transfection Kit (Thermo Fisher Scientific). Cell culture supernatant was collected four days after transfection and supplemented with 10x PBS to a final concentration of 2.5x PBS (342.5 mM NaCl, 6.75 mM KCl and 29.75 mM phosphates). For crystallization, wild-type SARS-CoV-2 RBD was purified using a 5 mL HisTALON superflow cartridge (Takara Bio) followed by size exclusion chromatography using a Superdex 200 Increase 10/300 GL column pre-equilibrated in 20 mM Tris-HCl pH 7.5, 150 mM NaCl. RBD was deglycosylated with EndoH and mixed with a 1.3-fold molar excess of S2X259 Fab and S2H97 Fab. The complex was purified on a Superdex 200 Increase 10/300 GL column pre-equilibrated with 20 mM Tris-HCl pH 7.5, 150 mM NaCl. SARS-CoV-2 S hexaprotein, used for cryo-EM single particle studies, was expressed and purified as described before²¹.

Enzyme-linked immunosorbent assay (ELISA)

96 half area well-plates (Corning, 3690) were coated over-night at 4 °C with 25 µl of sarbecoviruses RBD proteins WIV1 (AGZ48831.1), Anlong-112 (ARI44804.1), YN2013 (AIA62330.1), SC2018 (QDF43815.1), ZC45 (AVP78031.1), Rp/Shaanxi2011 (AGC74165.1), BM48-31/BGR/2008 (YP_003858584.1), RaTG13 (QHR63300.2), SARS-CoV2 (YP_009724390.1), SARS-CoV Urbani (AAP13441.1), BtKY72 (APO40579.1), Pangolin_Guangdong-2019 (EPI_ISL_410721) and SARS-CoV-2 RBD mutants, prepared at 5 µg/ml in PBS pH 7.2. Plates were then blocked with PBS 1% BSA (Sigma-Aldrich, A3059) and subsequently incubated with mAb serial dilutions for 1 h at room temperature. After 4 washing steps with PBS 0.05% Tween 20 (PBS-T) (Sigma-Aldrich, 93773), goat anti-human IgG secondary antibody (Southern Biotech, 2040-04) was added and incubated for 1 h at room temperature. Plates were then washed 4 times with PBS-T and 4-NitroPhenyl phosphate (pNPP, Sigma-Aldrich, 71768) substrate was added. After 30 min incubation, absorbance at 405 nm was measured by a plate reader (Biotek) and data plotted using Prism GraphPad 9.1.0.

S2X259 blockade-of-binding to RBD

S2X259 and S2H14 mAbs were biotinylated using EZ-Link™ NHS-PEG solid phase biotinylation kit (ThermoFisher Scientific) and binding to RBD tested to set optimal concentration to be used in the assay after sample desalting using Zeba™ Spin Desalting Columns (ThermoFisher Scientific). Half area 96 well-plates were coated over-night at 4 °C with SARS-CoV-2 RBD-mouse Fc Tag diluted at 1 µg/ml in PBS. After a blocking step with Blocker Casein (ThermoFisher Scientific), serial plasma dilutions in Blocker Casein were incubated 30 min at room temperature. Biotinylated S2X259 or S2H14 were added at a concentration achieving 80% of maximal binding and the mixture were incubated for 30 min at room temperature. Alkaline-phosphatase conjugated streptavidin (Jackson ImmunoResearch) was diluted at 0.5 µg/ml in Blocker Casein and added on plates previously washed 4 times with PBS 0.05% Tween 20. After 45 min incubation, plates were washed and 4-NitroPhenyl phosphate substrate incubated from 1h at room temperature. Absorbance at 405 nm was measured and percentage of inhibition was calculated as follows: $(1 - (OD \text{ sample} - OD \text{ neg ctr}) / (OD \text{ pos ctr} - OD \text{ neg ctr})) \times 100$.

MLV-based pseudotyped virus production and neutralization

To generate SARS-CoV-2 S murine leukemia virus pseudotyped virus, HEK293T cells were seeded in 10-cm dishes in DMEM supplemented with 10% FBS. The next day cells were transfected with a SARS-CoV-2 S glycoprotein-encoding plasmid harbouring the D19 C-terminal truncation⁵², an MLV Gag-Pol packaging construct and the reporter vector pTG-Luc, using the X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's instructions. Cells were then incubated at 37 °C with 5% CO₂ for 72 h. Supernatant was harvested and cleared from cellular debris by centrifugation at 400 x g, and stored at -80 °C.

For neutralization assays, Vero E6 cells were seeded into white 96-well plates (PerkinElmer) at 20,000 cells/well and cultured overnight at 37 °C with 5% CO₂ in 100 µl DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The next day, MLV-SARS-CoV-2 pseudovirus was activated with 10 µg/ml TPCK treated-Trypsin (Worthington Biochem) for 1 h at 37 °C. Recombinant antibodies at various concentrations were incubated with activated pseudovirus for 1 h at 37 °C. The Vero E6 cells were then washed with DMEM, and 50 µl of pseudovirus/mAbs mixes were added and incubated for 2 h at 37 °C with 5% CO₂. After incubation, 50 µl of DMEM containing 20% FBS and 2% penicillin/streptomycin were added and the cells were incubated 48 h at 37 °C with 5% CO₂. Following these 48 h of infection, culture medium was removed from the cells and 50 µl/well of Bio-Glo (Promega) diluted 1:2 with PBS with Ca²⁺Mg²⁺ (Thermo Fisher) was added to the cells and incubated in the dark for 15 min before reading on a Synergy HI Hybrid

Multi-Mode plate reader (Biotek). Measurements were done in duplicate, RLU values were converted to percentage of neutralization and plotted with a nonlinear regression curve fit in GraphPad Prism 9.1.0.

VSV-based pseudotype virus production and neutralization assay

SARS-CoV-2 S (CAD0240757.1), RaTG13 S (QHR63300.2), Pangolin-Guangdong S (QLR06867.1), Pangolin-Guanxi S (QIA48623.1), SARS-CoV S (YP_009825051.1), WIV1 S (AGZ48831.1), WIV16 S (ALK02457.1), RsSHCO14 S (AGZ48806.1), the VOC B.1.429 S (QTC60823) and the VOCs, N437K, Y453F, B.1.1.7 S, B.1.351 S and P.1 S with their corresponding mutations inserted in the SARS-CoV-2 S (CAD0240757.1) backbone were used to pseudotype VSV. Pseudotyped viruses were prepared using 293T cells seeded in 10-cm dishes. Briefly, cells in DMEM supplemented with 10% FBS, 1% PenStrep were transfected with the plasmid encoding for the corresponding S glycoprotein using lipofectamine 2000 (Life Technologies) following manufacturer's indications. One day post-transfection, cells were infected with VSV (G*ΔG-luciferase) and after 2 h, infected cells were washed four times with DMEM before adding medium supplemented with anti-VSV-G antibody (1I- mouse hybridoma supernatant diluted 1 to 50, from CRL-2700, ATCC). Particles were harvested 18 h post-inoculation, clarified from cellular debris by centrifugation at 2,000 x g for 5 min and concentrated 10 times using a 30 kDa cut off membrane and used for neutralization experiments, aliquoted and frozen at -80 °C until use in neutralization experiments.

For neutralization, stable 293T cells expressing ACE2⁵³ in DMEM supplemented with 10% FBS, 1% PenStrep were seeded at 40,000 cells/well into clear bottom white walled 96-well plates and cultured overnight at 37 °C. Twelve-point 3-fold serial dilutions of the corresponding mAb were prepared in DMEM and pseudotyped VSV viruses were added 1:1 to each mAb dilution in the presence of anti-VSV-G antibody from 1I- mouse hybridoma supernatant diluted 50 times. After 45 min incubation at 37 °C, 40 µl of the mixture was added to the cells and 2 h post-infection, 40 µl DMEM was added to the cells. After 17-20 h 50 µl/well of One-Glo-EX substrate (Promega) was added to the cells and incubated in the dark for 5-10 min prior reading on a Varioskan LUX plate reader (ThermoFisher). Measurements were done in duplicate with two independent productions of pseudotyped viruses and RLU values were converted to percentage of neutralization and plotted with a nonlinear regression curve fit in GraphPad Prism 9.1.0.

Generation of VSV SARS-CoV-2 S pseudotypes from a selection of circulating variants

Replication defective VSV pseudovirus expressing SARS-CoV-2 spike protein were generated as previously described⁵⁴ with some modifications. Plasmids encoding the SARS-CoV-2 S glycoprotein corresponding to the VOC used to produce SARS-CoV-2-VSV were obtained using a multisep overlap extension PCR protocol⁵⁷. Briefly, the mutations of the different VOC lineages were encoded on each primer pair used to amplify sequential, overlapping fragments of the SARS-CoV-2 S encoding DNA sequence from a pCDNA_SARS-CoV-2_S_D19 plasmid encoding the C terminally truncated Spike protein that has been shown to be better expressed at the cell³⁰. The amplified overlapping fragments were separated on agarose gel and purified using Illustra GFX™ PCR DNA and Gel Band Purification Kit (Merck KGaA). The purified overlapping fragments were joined by performing 10 cycles of PCR without the addition of primers, followed by 30 PCR cycles using a single pair of external primers carrying 5' overhangs homologous to the vector backbone. The final PCR product was then cloned into the pCDNA3 vector using the Takara In-fusion HD cloning kit following manufacturer's instructions.

Lenti-X 293T cells (Takara, 632180) were seeded in 10-cm dishes at a density of 5x10⁶ cells/cm² and the following day transfected with 10 µg of spike expression plasmid with TransIT-Lenti (Mirus, 6600) according to the manufacturer's instructions. One day post-transfection, cells

were infected with VSV-luc (VSV-G) (Kerafast, EH1020-PM) for 1 h, rinsed three times with PBS, then incubated for an additional 24 h in complete media at 37 °C. The cell supernatant was clarified by centrifugation, filtered (0.45 µm), aliquoted, and frozen at -80 °C.

For VSV pseudovirus neutralization assays, Vero-E6 cells were grown in DMEM supplemented with 10% FBS and seeded into CulturePlate-96, White Opaque 96-well Microplate (PerkinElmer) at a density of 2×10^4 cells per well. The next day, mAbs were serially diluted in pre-warmed complete media, mixed at 1:1 ratio with pseudovirus and incubated for 1 h at 37 °C in round bottom polypropylene plates. Media from cells was aspirated and 50 µL of virus-mAb complexes were added to cells and then incubated for 1 h at 37 °C. An additional 100 µL of prewarmed complete media was then added on top of complexes and cells incubated for an additional 16-24 h. Conditions were tested in duplicate wells.

Virus-mAb-containing media was then aspirated from cells and 50 µL of a 1:2 dilution of SteadyLite Plus (Perkin Elmer) in PBS with Ca^{2+} / Mg^{2+} was added to cells. Plates were incubated for 10 min at room temperature and then analyzed on the Synergy H1 Hybrid Multi-Mode reader (Biotek). Measurements were done in two independent experiments and RLU values were converted to percentage of neutralization and plotted with a nonlinear regression curve fit with an upper constraint equal to 100 and a lower constraint equal to 0 in Prism (Version 9.1.0). IC50 values across biological replicates are presented as arithmetic mean \pm standard deviation.

Neutralization of SARS-CoV-2 B.1.351 pseudotyped VSV by S2X259/S309 antibody cocktail

Vero E6 cells were plated into white 96-well plates (PerkinElmer) at 20'000 cells/well and cultured overnight at 37 °C 5% CO₂ in 100 µL culture medium (DMEM 10% FBS 1% penicillin/streptomycin). The next day, S2E12 and S309 antibodies were serially diluted in infection medium (DMEM 10% heat inactivated FBS 1% penicillin/streptomycin) and mixed in a matrix format before incubation with SARS-CoV-2 B.1.351 pseudovirus (MOI 0.1) at 37 °C 5% CO₂ for 1 hour. Cell culture medium was aspirated and 50 µL of mAbs/pseudovirus mix added onto cells. After incubation at 37 °C 5% CO₂ for 1 hour, 100 µL of infection medium was added to cell and incubation continued for the following 20 hours. For results read out, medium was removed and 50 µL/well of Steadylite PLUS (PerkinElmer) 1:2 diluted in PBS + Ca^{2+} / Mg^{2+} was added and incubated 15 min at room temperature in the dark. Luminescence signals were measured using a Synergy H1 reader (Biotek) with 1 sec integration time. Data were analyzed using MacSynergy II and synergy plots obtained with 99.9% confidence were used for graphical elaboration with GraphPad Prism 9.1.0.

Neutralization of authentic SARS-CoV-2-Nluc virus

Neutralization of authentic SARS-CoV-2 by entry-inhibition assay was determined using SARS-CoV-2-Nluc, an infectious clone of SARS-CoV-2 (based on strain 2019-nCoV/USA_WA1/2020) which encodes nanoluciferase in place of the viral ORF7 and demonstrated comparable growth kinetics to wildtype virus⁵⁵. Vero E6 cells were seeded into black-walled, clear-bottom 96-well plates at 2×10^4 cells/well and cultured overnight at 37 °C. The next day, 9-point 4-fold serial dilutions of mAbs were prepared in infection media (DMEM + 10% FBS). SARS-CoV-2-Nluc was diluted in infection media at a final MOI of 0.01 PFU/cell, added to the mAb dilutions and incubated for 30 minutes at 37 °C. Media was removed from the Vero E6 cells, mAb-virus complexes were added and incubated at 37 °C for 24 hours. Media was removed from the cells, Nano-Glo luciferase substrate (Promega) was added according to the manufacturer's recommendations, incubated for 10 minutes at room temperature and the luciferase signal was quantified on a VICTOR Nivo plate reader (Perkin Elmer).

Affinity determination by Surface Plasmon Resonance (SPR)

SPR binding measurements were performed using a Biacore T200 instrument, running buffer was Cytiva HBS-EP+ (pH 7.4), and all

measurements were performed at 25 °C. For Fab binding, anti-AviTag pAb (for capturing S proteins) or StrepTactin XT (for capturing RBDs) were covalently immobilized on CM5 chips. S2X259 Fab concentrations were 11, 33, 100, and 300 nM run as single-cycle kinetics. Double reference-subtracted data were fit to a 1:1 binding model using Biacore Evaluation software, which yields an "apparent KD" for the S-binding data because the kinetics also reflect S conformational dynamics. For SARS-CoV-2 S the dissociation rate was too slow to fit, so the $K_{D,app}$ is reported as an upper limit. The K_D above 1 µM is approximate and was determined from a fit where R_{max} was set to a constant based on theoretical R_{max} .

Affinity determination using bio-layer interferometry

Biotinylated RBD (WT, N501Y, K417N-E484K-N501Y, or K417T-E484K-N501Y) were immobilized at 5 ng/µL in undiluted 10X Kinetics Buffer (Pall) to SA sensors until a load level of 1.1 nm. A 1:3 dilution series of Fab in undiluted kinetics buffer starting at 10 nM was used for 600 seconds association prior to 600 second dissociation to determine protein-protein affinity. The data were baseline subtracted and the plots fitted using the Pall FortéBio/Sartorius analysis software (version 12.0). Data were plotted in Prism 9.1.0.

Competition assay by Biolayer Interferometry (BLI)

BLI was used to assess S2X259 competition with S309 and S2E12 using an Octet Red96 (FortéBio). All reagents were prepared in kinetics buffer (PBS 0.01% BSA) at the indicated concentrations. His-tagged SARS-CoV-2 RBD was prepared at 8 µg/ml and loaded on pre-hydrated anti-penta-HIS biosensors (Sartorius) for 2.5 min. Biosensors were then moved into a solution containing S2X259 mAb and association recorded for 7 min. A second association step was subsequently performed into S2X259 (as control), S309 and S2E12 mAbs solutions at 20 µg/ml and recorded for 7 min. Response values were exported and plotted using GraphPad Prism 9.1.0.

Cell-surface mAb-mediated S₁ shedding

CHO cells stably expressing wild-type SARS-CoV-2 S were resuspended in wash buffer (PBS 1% BSA, 2 mM EDTA) and treated with 10 µg/mL TPCK-trypsin (Worthington Biochem) for 30 min at 37 °C. Cells were then washed and distributed into round bottom 96-well plates (90,000 cells/well). S2X259 was added to cells at 15 µg/mL final concentration for 180 min at 37 °C. Cells were collected at different time points (5, 30, 60, 120 and 180), washed with wash buffer at 4 °C, and incubated with 1.5 µg/mL secondary goat anti-human IgG, Fc fragment specific (Jackson ImmunoResearch) on ice for 20 min. Cells were washed and resuspended in wash buffer and analyzed with ZE5 FACS (Bio-rad).

Measurement of Fc-effector functions

S2X259-dependent activation of human FcγRIIIa was performed with a bioluminescent reporter assay. ExpiCHO cells transiently expressing full-length wild-type SARS-CoV-2 S (target cells) or full-length prefusion stabilized SARS-CoV-2 S, which harbours the 2P mutation and S1/S2 furin cleavage site mutation (RRARS to SGAG) as previously described¹², were incubated with different amounts of mAbs. After a 15-minute incubation, Jurkat cells stably expressing FcγRIIIa receptor (V158 variant) or FcγRIIa receptor (H131 variant) and NFAT-driven luciferase gene (effector cells) were added at an effector to target ratio of 6:1 for FcγRIIIa and 5:1 for FcγRIIa. Signaling was quantified by the luciferase signal produced as a result of NFAT pathway activation. Luminescence was measured after 20 hours of incubation at 37 °C with 5% CO₂ with a luminometer using the Bio-Glo-TM Luciferase Assay Reagent according to the manufacturer's instructions (Promega).

In vivo mAb testing using a Syrian hamster model

KU LEUVEN R&D has developed and validated a SARS-CoV-2 Syrian Golden hamster infection model^{36,39}. The SARS-CoV-2 Wuhan (BetaCov/

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Belgium/GHB-03021/2020-EPIISL109407976|2020-02-03) and B.1.351 (hCoV10519/Belgium/regal-1920/2021; EPI_ISL_896474, 2021-01-11) isolates used in this study, were recovered from nasopharyngeal swabs taken from a RT-qPCR confirmed asymptomatic patient who returned from Wuhan, China in February 2020 and from a patient with respiratory symptoms returned to Belgium in January 2021, respectively. A close relatedness with the prototypic Wuhan-Hu-1 2019 SARS-CoV-2 and with B.1.351 lineage was confirmed by sequencing and phylogenetic analysis. Infectious viruses were isolated by serial passaging on Vero E6 cells and passage 6 for SARS-CoV-2 Wuhan and passage 2 for B.1.351 viruses were used for the study. The titre of the virus stock was determined by end-point dilution on Vero E6 cells by the Reed and Muench method⁵⁶. This work was conducted in the high-containment A3 and BSL3+ facilities of the KU Leuven Rega Institute (3CAPS) under licenses AMV 30112018 SBB 219 2018 0892 and AMV 23102017 SBB 219 20170589 according to institutional guidelines.

Syrian hamsters (*Mesocricetus auratus*) were purchased from Janvier Laboratories and were housed per two in ventilated isolator cages (IsoCage N Biocontainment System, Tecniplast) with ad libitum access to food and water and cage enrichment (wood block). Housing conditions and experimental procedures were approved by the ethical committee of animal experimentation of KU Leuven (license P065-2020). Hamsters were randomized and the sample size was chosen based on prior experience with this animal model. In order to evaluate the prophylactic efficacy 6-10 week-old female hamsters were administered by intraperitoneal injection with S2X259 mAb at 1, 4, 5 and 25 mg/kg or a combination of S2X259/S309 mAbs (1 mg/kg + 1 mg/kg) 48 hours before intranasal infection with 1.89×10⁶ TCID₅₀ in 50 µl inoculum. Blood samples were collected before infection and serum was obtained for PK analysis. To assess the therapeutic efficacy 6-8 weeks old Syrian hamsters were administered by intraperitoneally (i.p.) with Isotype control Ab (20 mg/kg) or S2X259 mAb (at 20, 10 or 5 mg/kg) 24h after infection with SARS-CoV-2. Blood samples were collected at end-point sacrifice and serum was obtained for PK analysis. Hamsters were monitored for appearance, behaviour and weight. At day 4 post infection hamsters were euthanized by intraperitoneal injection of 500 µL Dolethal (200 mg/mL sodium pentobarbital, Vétoquinol SA). Lungs were collected, homogenized using bead disruption (Precellys) in 350 µL RLT buffer (RNeasy Mini kit, Qiagen) and centrifuged (10,000 rpm, 5 minutes, 4 °C) to pellet the cell debris. RNA was extracted using a NucleoSpin kit (Macherey-Nagel) according to the manufacturer's instructions. RT-qPCR was performed on a LightCycler96 platform (Roche) using the iTaq Universal Probes One-Step RTqPCR kit (BioRad) with N2 primers and probes targeting the nucleocapsid³⁶. Standards of SARS-CoV-2 cDNA (IDT) were used to express viral genome copies per mg tissue or per mL serum. To quantify infectious SARS-CoV-2 particles, endpoint titrations were performed on confluent Vero E6 cells in 96-well plates. The hamster derived samples for RNA quantification and viral load titration were run by technicians who were blinded to treatment group of the analysed samples. Viral titres were calculated by the Reed and Muench method⁵⁶ and were expressed as 50% tissue culture infectious dose (TCID₅₀) per mg tissue.

Evaluation of escape mutants via deep mutational scanning (DMS)

A previously described deep mutational scanning approach⁵⁷ was used to identify RBD mutations that escape S2X259 binding. Briefly, duplicate libraries containing virtually all possible amino acid changes compatible with ACE2 binding and RBD folding within the Wuhan-Hu-1 SARS-CoV-2 RBD sequence were expressed on the surface of yeast^{29,57}. Libraries were labelled at 59 ng/mL S2X259 antibody (the EC90 for binding to yeast-displayed SARS-CoV-2 RBD determined in isogenic pilot binding experiments), and fluorescence-activated cell sorting (FACS) was used to select RBD+ cells that exhibit reduced antibody binding as previously described^{57,58}. Libraries were sequenced before

and after selection to determine per-mutation escape fractions as previously described⁵⁸. Experiments were performed in duplicate with independently generated mutant libraries, and we report the average mutant escape fraction across the duplicates. Raw escape fractions are available on GitHub: https://github.com/jbloomlab/SARS-CoV-2-RBD_MAP_Vir_mAbs/blob/main/results/supp_data/s2X259_raw_data.csv. Detailed steps of analysis and code for the deep mutational scanning selections are available on GitHub: https://github.com/jbloomlab/SARS-CoV-2-RBD_MAP_Vir_mAbs.

Selection of SARS-CoV-2 monoclonal antibody escape mutants (MARMS)

VSV-SARS-CoV-2 chimera was used to select for SARS-CoV-2 monoclonal antibody resistant mutants (MARMS) previously described^{30,31}. Briefly, MARMS were recovered by plaque isolation on Vero cells with the indicated mAb in the overlay. The concentration of mAb in the overlay was determined by neutralization assays at a multiplicity of infection (MOI) of 100. Escape clones were plaque-purified on Vero cells in the presence of mAb, and plaques in agarose plugs were amplified on MA104 cells with the mAb present in the medium. Viral stocks were amplified on MA104 cells at an MOI of 0.01 in Medium 199 containing 2% FBS and 20 mM HEPES pH 7.7 (Millipore Sigma) at 34 °C. Viral supernatants were harvested upon extensive cytopathic effect and clarified of cell debris by centrifugation at 1,000 x g for 5 min. Aliquots were maintained at -80 °C. Viral RNA was extracted from VSV-SARS-CoV-2 mutant viruses using RNeasy Mini kit (Qiagen), and S was amplified using OneStep RT-PCR Kit (Qiagen). The mutations were identified by Sanger sequencing (GENEWIZ). Their resistance was verified by subsequent virus infection in the presence or absence of antibody. Briefly, Vero cells were seeded into 12 well plates for overnight. The virus was serially diluted using DMEM and cells were infected at 37 °C for 1h. Cells were cultured with an agarose overlay in the presence or absence of mAb at 34 °C for 2 days. Plates were scanned on a biomolecular imager and expression of eGFP is shown at 48 hours post-infection.

Crystallization, data collection, structure determination and analysis

Crystals of the SARS-CoV-2-RBD-S2X259-S2H97 Fab complex were obtained at 20 °C by sitting drop vapor diffusion. A total of 200 nL complex at 5.7 mg/ml were mixed with 200 nL mother liquor solution containing 0.12 M Monosaccharides mix, 20% (v/v) Ethylene glycol, 10% (w/v) PEG 8000, 0.1 M Tris (base)/bicine pH 8.5, 0.02 M Sodium chloride, 0.01 M MES pH 6 and 3% (v/v) Jeffamine ED-2003. Crystals were flash frozen in liquid nitrogen. Data were collected at Beamline 9-2 of the Stanford Synchrotron Radiation Lightsource facility in Stanford, CA. Data were processed with the XDS software package⁵⁹ for a final dataset of 2.65 Å in space group P21. The RBD-S2X259-S2H97 Fab complex structure was solved by molecular replacement using phaser⁶⁰ from a starting model consisting of SARS-CoV-2 RBD (PDB ID: 7JX3) and homology models of the S2X259 and S2H97 Fabs built using the Molecular Operating Environment (MOE) software package from the Chemical Computing Group (<https://www.chemcomp.com>). Several subsequent rounds of model building and refinement were performed using Coot⁶¹, ISOLDE⁶², Refmac5⁶³, and MOE (<https://www.chemcomp.com>), to arrive at a final model for the ternary complex.

CryoEM sample preparation, data collection and data processing

Recombinantly expressed and purified fab S2X259 and SARS-CoV-2 S hexaprotein were incubated at 1 mg/ml with a 1.2 molar excess of fab at 4 °C during 1 hr. Three microliters of the complex mixture were loaded onto freshly glow discharged R2/2 UltrAuFoil grids (200 mesh) or lacey grids covered with a thin layer of manually added carbon, prior to plunge freezing using a vitrobot MarkIV (ThermoFisher Scientific) with a blot force of 0 and 7-7.5 sec blot time (for the UltrAuFoil grids) or with a

blot force of -1 and 2.5 sec blot time (for the lacey thin carbon grids) at 100% humidity and 21 °C.

Data were acquired on a FEI Titan Krios transmission electron microscope operated at 300 kV and equipped with a Gatan K2 Summit direct detector and Gatan Quantum GIF energy filter, operated in zero-loss mode with a slit width of 20 eV. Automated data collection was carried out using Leginon⁶⁴ at a nominal magnification of 130,000x with a super-resolution pixel size of 0.525 Å. The dose rate was adjusted to 8 counts/pixel/s, and each movie was fractionated in 50 frames of 200 msec. Two datasets were collected from UltrAuFoil grids with the stage tilted 30° and 55° to circumvent particle preferential orientation. The third dataset was collected on lacey grids covered with a thin layer of carbon. The three datasets, with a total of 6786 micrographs, were collected with a defocus range comprised between -0.8 and -2 µm. For each dataset, movie frame alignment, estimation of the microscope contrast-transfer function parameters, particle picking and extraction were carried out using Warp⁶⁵. Particle images were extracted with a box size of 800 pixels² and binned to 400 yielding a pixel size of 1.05 Å. The three datasets were merged and two rounds of reference-free 2D classification were performed using cryoSPARC⁶⁶. Subsequently, one round of 3D classification with 50 iterations, using PDB 6VXX as initial model, was carried out using Relion^{67,68} without imposing symmetry. 3D refinements were carried out using non-uniform refinement⁶⁹. Particle images from each dataset were subjected to Bayesian polishing⁷⁰ using Relion before merging them to perform another round of non-uniform refinement in cryoSPARC followed by per-particle defocus refinement and again non-uniform refinement. To improve the density of the S/S2X259 interface, the particles were symmetry-expanded and subjected to a Relion focus 3D classification without refining angles and shifts using a soft mask encompassing the RBD and S2X259 variable domains. Particles belonging to classes with the best resolved local density were selected and subjected to local refinement using cryoSPARC. Local resolution estimation, filtering, and sharpening were carried out using CryoSPARC. Reported resolutions are based on the gold-standard Fourier shell correlation (FSC) of 0.143 criterion and Fourier shell correlation curves were corrected for the effects of soft masking by high-resolution noise substitution⁷¹.

CryoEM model building and analysis

UCSF Chimera⁷² and Coot⁶⁴ were used to fit atomic models (PDB 6VXX or PDB 6VYB) into the cryoEM maps and the Fab variable domains were manually built. S2E12 was built in the locally refined map and subsequently validated using the Fab crystal structure. Models were refined and relaxed using Rosetta using both sharpened and unsharpened maps^{73,74}. Validation used Phenix⁷⁵, MolProbity⁷⁶ and Privateer⁷⁷. Figures were generated using UCSF ChimeraX⁷⁸.

Material availability

Materials generated in this study can be available on request and may require a material transfer agreement (MTA). The SARS-CoV-2 RBD mutant libraries (#1000000172) and unmutated parental plasmid (#166782) are available on Addgene

Reporting summary

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

Data availability

All datasets generated and information presented in the study are available from the corresponding author on reasonable request. CryoEM structure data and model are available with accession code PDB 7RA8 (EMD-24347) and PDB 7RAL (EMD-24365). The X-ray structure data and model has been deposited with accession code PDB 7M7W for RBD-S2H97-S2X259. Interactive escape maps and structural

visualizations can be found at: https://jbloomlab.github.io/SARS-CoV-2-RBD_MAP_Vir_mAbs/. Raw Illumina sequencing data from deep mutational scanning experiments are available on NCBI SRA, BioSample SAMN18315604 (SARS-CoV-2 mutant selection data). Complete table of deep mutational scanning antibody escape fractions is provided on GitHub: https://github.com/jbloomlab/SARS-CoV-2-RBD_MAP_Vir_mAbs/blob/main/results/supp_data/all_antibodies_raw_data.csv.

Code availability

Repository containing all code, analysis, and summary notebooks for the analysis of the SARS-CoV-2 deep mutational scanning escape selections available on GitHub: https://github.com/jbloomlab/SARS-CoV-2-RBD_MAP_Vir_mAbs

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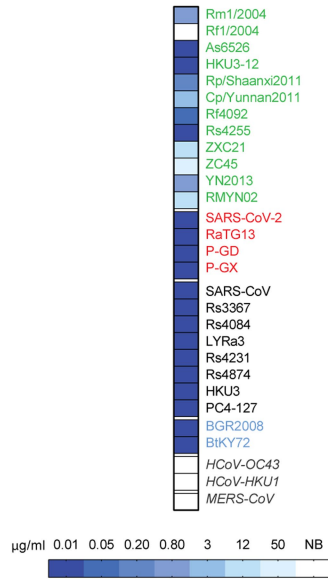
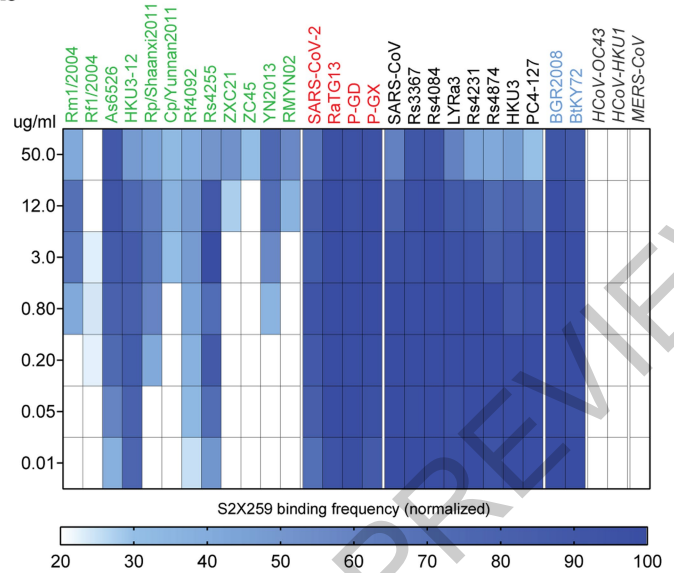
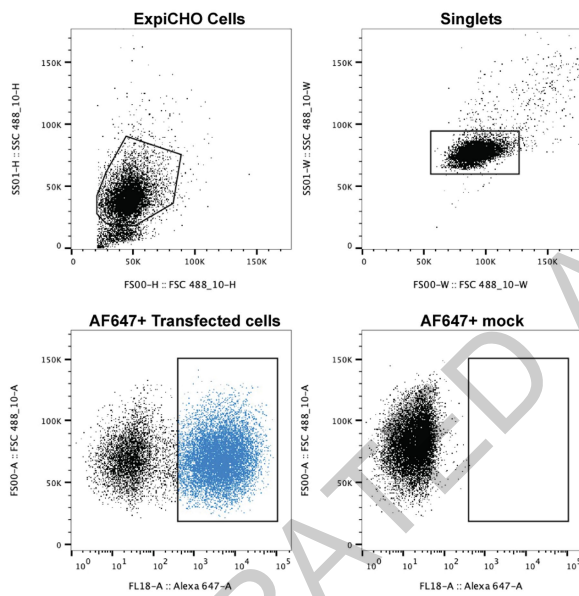
Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41586-021-03817-4>.

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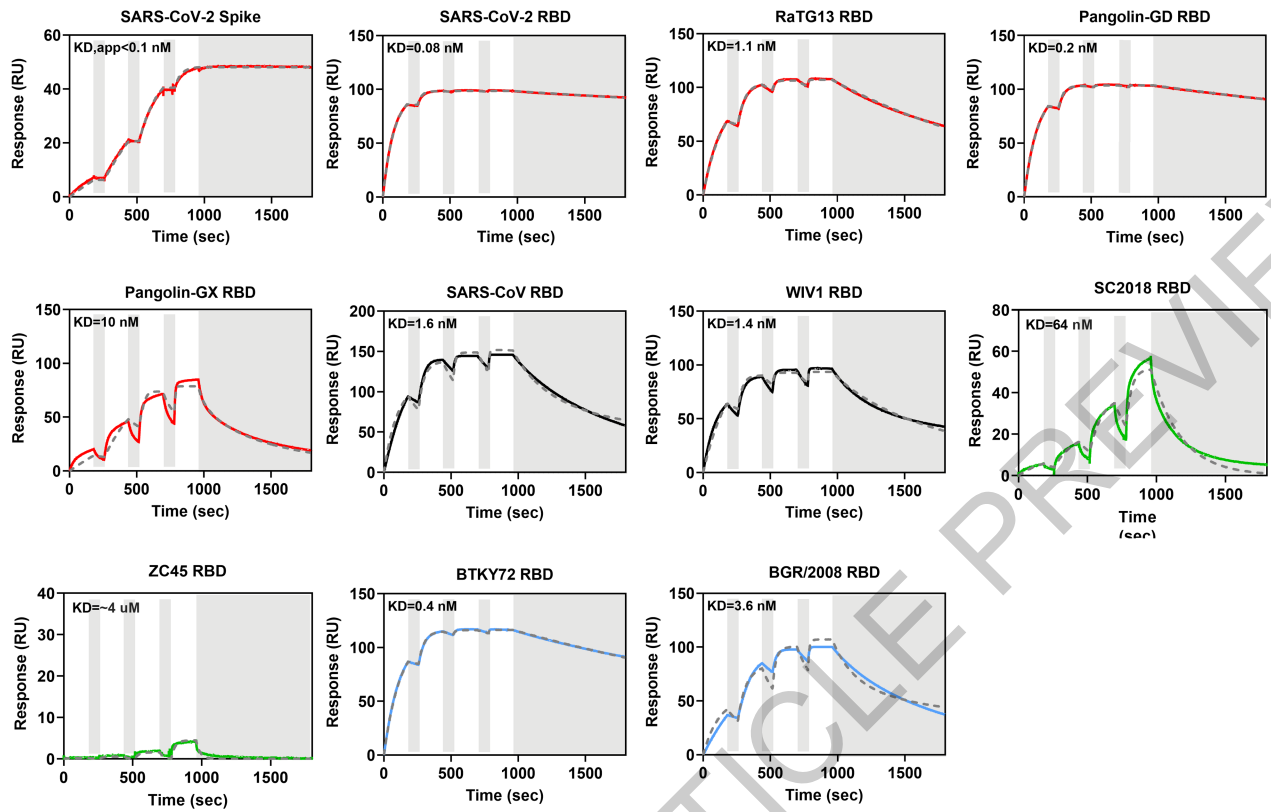
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SARS-CoV	9.97	25.58		15.52
WIV1	11.04			17.06
SARS-CoV-2	31.96	28.20	5.82	10.92
RatG13	21.88	36.81	7.50	17.93
PangGD	28.45	33.00	6.91	15.88
Anlong112	1281.00			
YN2013	12.86	348.20		
SC2018	11.63			
SX2011	26.50			239.80
ZC45	539.00			
BiKY72	22.18	27.24		
BGR2008	10.28	89.56		22.09

EC50 ng/ml: 1-50, 50-1000, 1000-5000, No binding

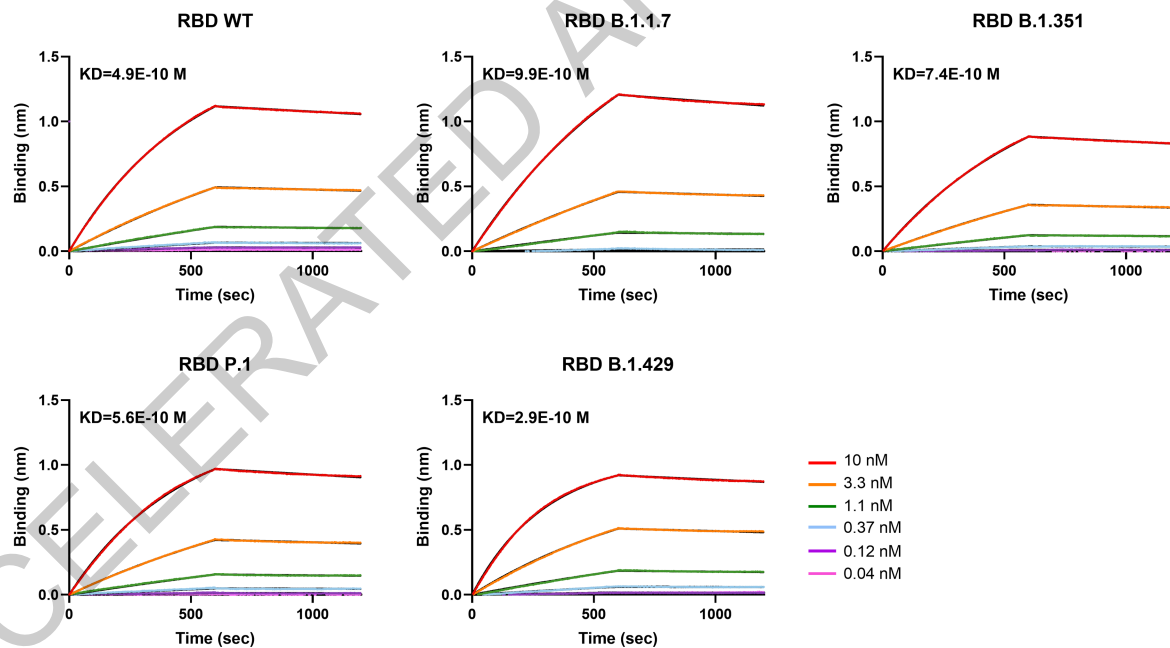
Extended Data Fig. 1 | Breadth of S2X259 mAb across sarbecovirus subgenus. **a**, Flow cytometry analysis of S2X259 cross-reactivity with a panel of 29 glycoproteins representative of sarbecovirus clades 1a, 1b, 2 and 3. The colours represent the lowest concentration of mAb at which binding was observed. **b**, FACS binding of S2X259 to a panel of sarbecovirus S glycoproteins transiently expressed in ExpiCHO cells. Results represent the binding

frequency normalized using the ratio between MFIs for S2X259 and an expression control mAb⁴⁸. **c**, FACS gating strategy used to assess binding of S2X259 or of the expression control mAb. **d**, ELISA binding of site I-targeting S2E12²¹, site II-targeting S2X259 and S2X35¹⁷, and site IV-targeting S309²² mAbs to RBDs spanning the different clades of the sarbecovirus subgenus. $n=2$ independent experiments.

a

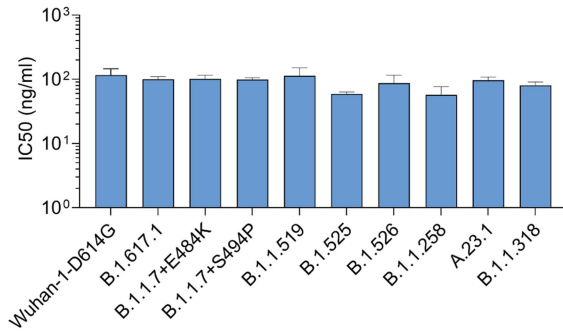
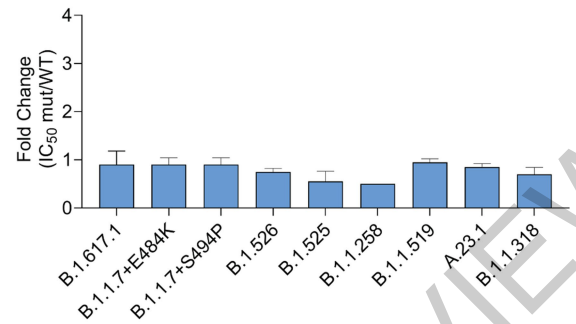


b



Extended Data Fig. 2 | S2X259 Fab binding to recombinant sarbecovirus RBDs, prefusion SARS-CoV-2 S ectodomain trimer and RBD variants. **a**, S or RBD antigens were captured on the sensor chip surface and binding to S2X259 Fab at 11, 33, 100, and 300 nM was monitored successively, in single-cycle kinetics format, by surface plasmon resonance. All data have been fit to a 1:1 binding model (black dashed line) and the equilibrium dissociation constant (K_D) is reported. For the S-binding data, we report an apparent K_D ($K_{D,app}$) since

kinetics are affected by conformational dynamics between open and closed RBD states. The colouring scheme matches the phylogenetic tree in Figure 1a. **b**, Bi-layer interferometry binding analysis of the S2X259 Fab to wildtype or VOC SARS-CoV-2 biotinylated RBDs immobilized at the surface of SA biosensors. The data are coloured according to the key and fits to a 1:1 binding model is shown as black lines. Equilibrium dissociation constants (K_D) are reported above each plot.

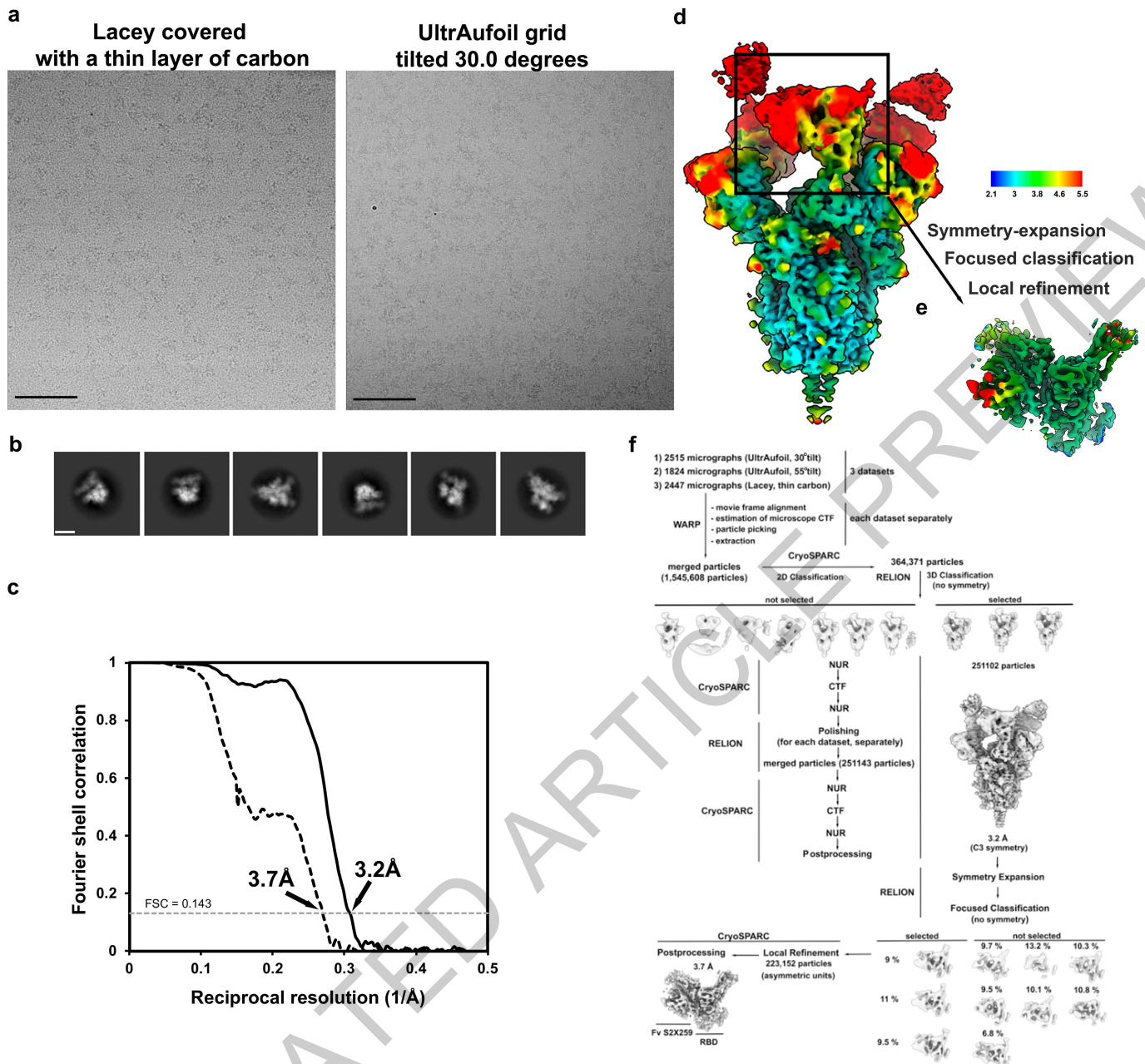
a**b**

	NTD	RBD	S1/S2	S2
B.1.1.7 (UK)	69-70del/144-del	N501Y/±(E484K or S494P)	A570D/D614G/P681H	T716I/S982A/D1113H
B.1.351 (South Africa)	L18F/D80A/D215G/R246I/±242-244del	K417N/E484K/N501Y	D614G	A701V
P.1 (Brazil)	L18F/T20N/P26S/D138Y/R190S	K417T/E484K/N501Y	D614G/H655Y	T1027I/V1167F
B.1.427/B.1.429 (California)	S13I/W152C	L452R	D614G	
B.1.1.258 (Scotland)	69-70del	N439K	D614G	
B.1.525 (Nigeria)	Q52R/A67V/69-70del/144del	E484K	D614G/Q677H	F888L
B.1.526 (New York)	L5F/T95I/D253G	E484K	D614G	A701V
A.23.1 (Liverpool)	R102I/F157L	V367F/E484K	Q613H/P681H	
B.1.1.318	T95I/144del	E484K	D614G/P681H	D796H
B.1.298 (Denmark)*	69-70del	Y453F	D614G	
B.1.617.1 (India)	E154K/T95I/G142D	L452R/E484Q	D614G/P681R	Q1071H or H1101D
B.1.1.519 (Mexico/US)		T478K	D614G/P681H	T732A

Extended Data Fig. 3 | S2X259 neutralizing activity is not affected by mutations present in circulating SARS-CoV-2 VOCs and recent lineages. a-b, Neutralization by S2X259 of SARS-CoV-2 S VSV pseudotyped circulating

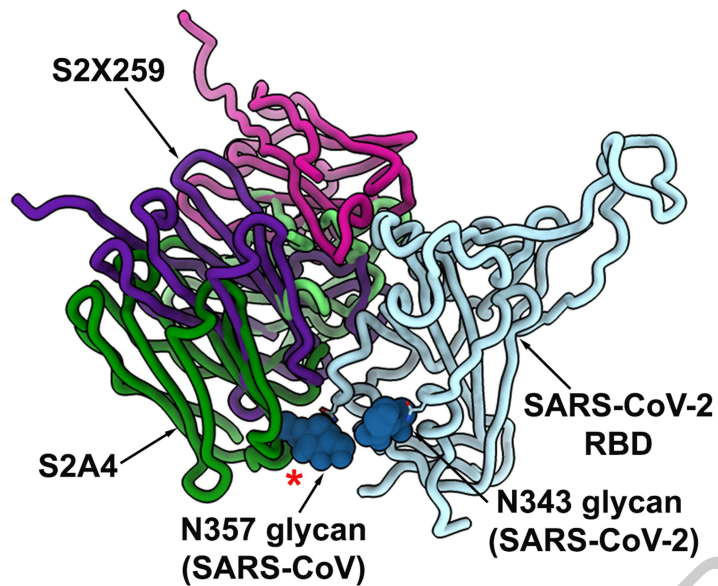
variants shown as IC50 (a) and IC50 fold change as compared to Wuhan-1-D614G (b). Schematic of the SARS-CoV-2 S and the mutation landscape in each variant is shown. Del, deletion. * Tested as single RBD mutant in panels a-b.

Article



Extended Data Fig. 4 | CryoEM data processing and validation of S2X259-bound SARS-CoV-2 S. **a-b**, Representative electron micrographs (a) and class averages (b) of SARS-CoV-2 S in complex with the S2X259 Fab. Scale bar of the micrograph: 500 Å. Scale bar of the class averages: 100 Å. **c**, Gold-standard Fourier shell correlation curves for the S trimer bound to three S2X259 Fabs (solid black line) and the locally refined reconstruction of

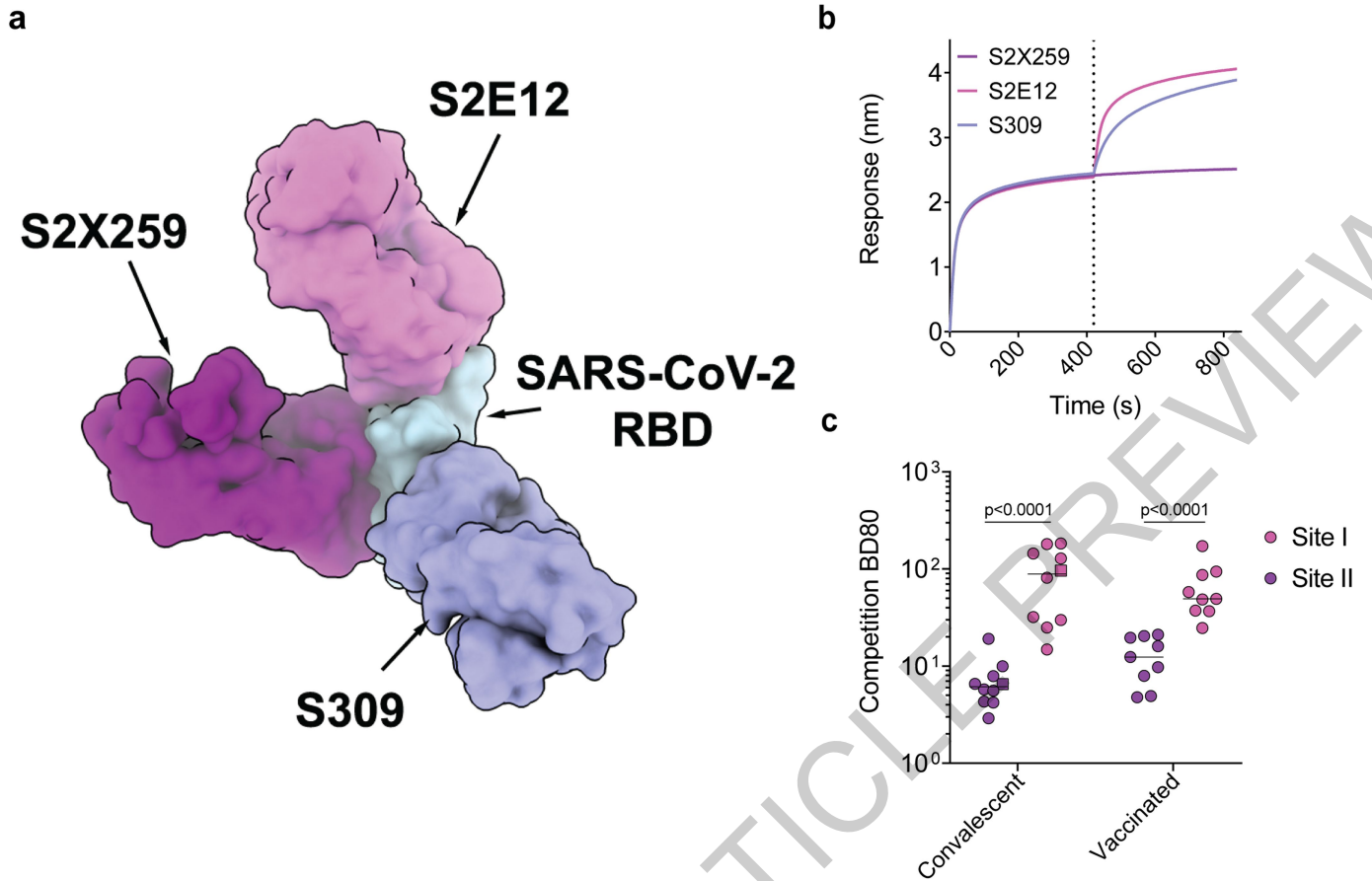
the RBD/S2X259 variable domains (dashed black line). The 0.143 cut-off is indicated by a horizontal dashed grey line. **d-e**, Local resolution map for the open S trimer bound to three S2X259 Fabs (d) and the locally refined reconstruction of the RBD/S2X259 variable domains (e). **f**, CryoEM data processing flow-chart.

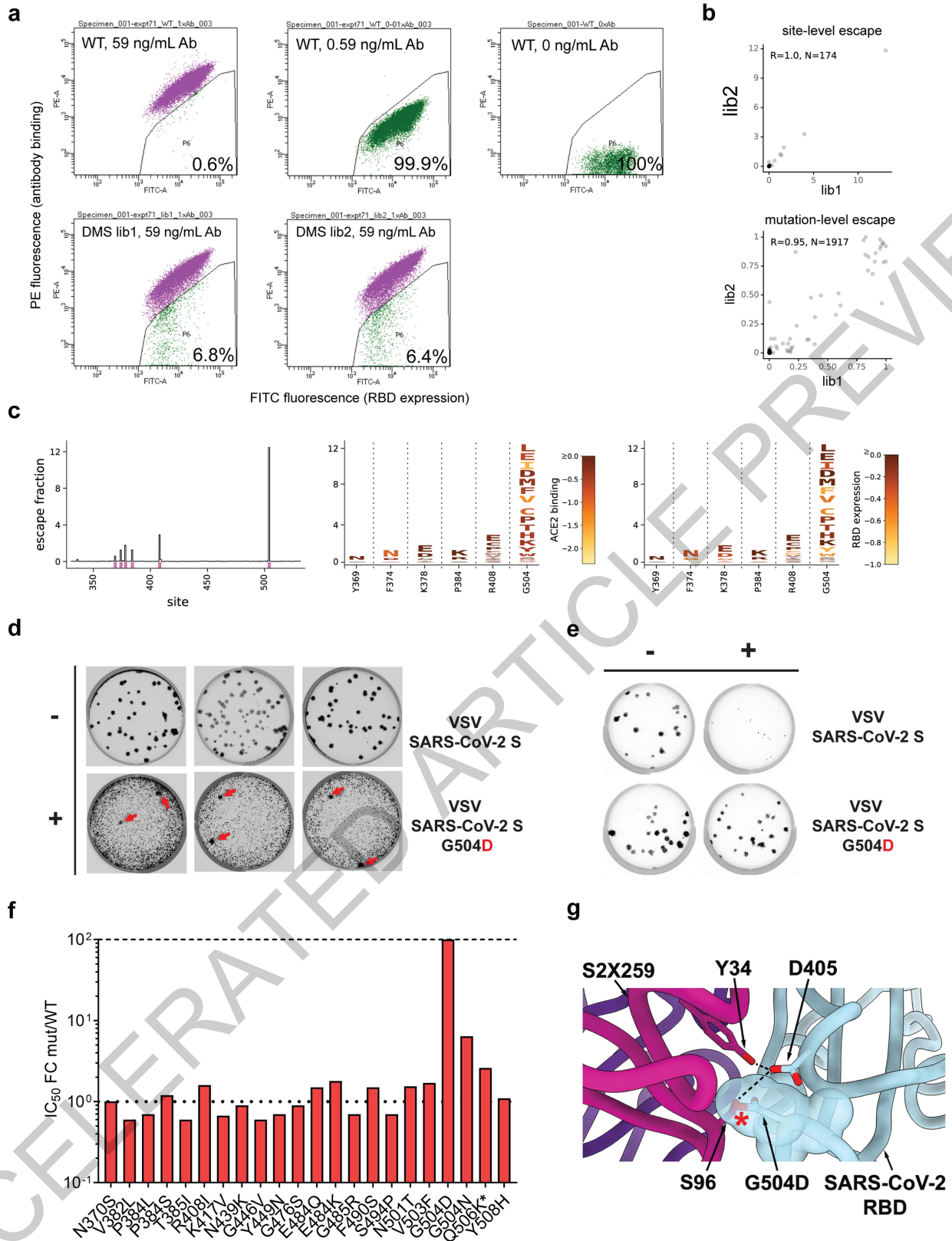


Extended Data Fig. 5 | The S2X259 angle of approach for binding to the SARS-CoV-2 RBD allows to circumvent the SARS-CoV N357 glycan present in all sarbecovirus RBDs except SARS-CoV-2. Ribbon diagram showing a superimposition of the S2X259-bound and S2A4-bound (PDB 7JVA) SARS-CoV-2 RBD¹⁷. The SARS-CoV glycan at position N357 was modelled based on the

S230-bound SARS-CoV S structure (PDB 6NB6³³) and is predicted to sterically hinder S2A4 binding (red star) but not S2X259. The mAb light and heavy chains are coloured magenta and purple (S2X259) or light and dark green (S2A4). N-linked glycans are rendered as blue spheres.

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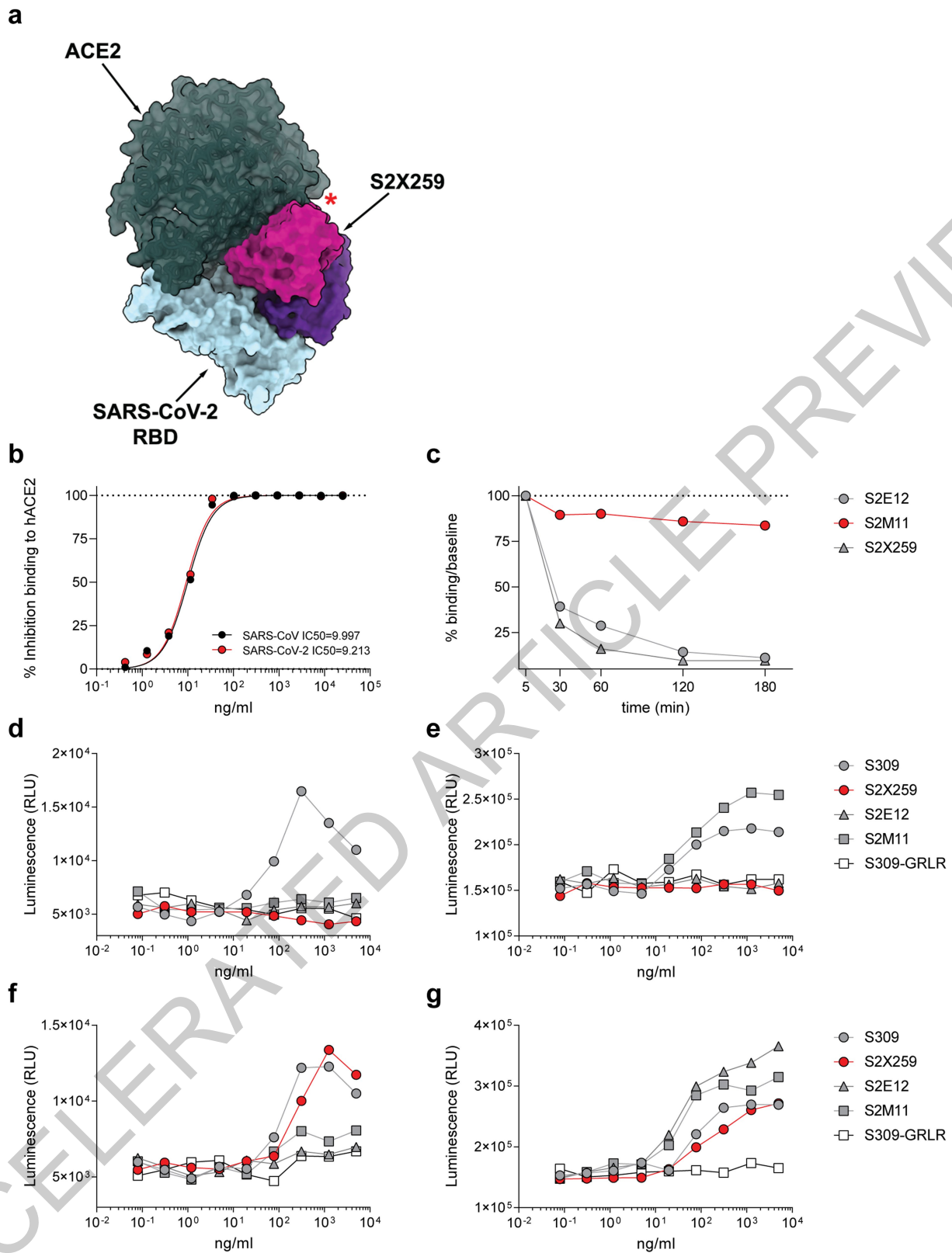
Extended Data Fig. 7 | See next page for caption.

Article

Extended Data Fig. 7 | S2X259 has a high barrier for the emergence of resistance mutants. **a**, FACS gates used in DMS to select escape variants. Top row: yeast controls expressing unmutated SARS-CoV-2 RBD labelled at relevant S2X259 concentrations for setting of selection gates. Bottom row: fraction of cells in SARS-CoV-2 mutant libraries falling into the antibody-escape bin. **b**, Correlation in site-level (top, sum of escape fractions for mutations at a site) and mutation-level (bottom) escape between independently generated and assayed RBD mutant libraries. **c**, Line plot of escape mutants along all positions in the SARS-CoV-2 RBD (left). Pink lines indicate sites that escape S2X259 binding illustrated at the mutation-level in logoplots (right). In logoplots, the height of a letter scales with its escape fraction. Letters are coloured according to their deleterious consequences for ACE2 binding (middle) or RBD expression (right) as determined in prior DMS experiments²⁹. **d**, Plaque assay

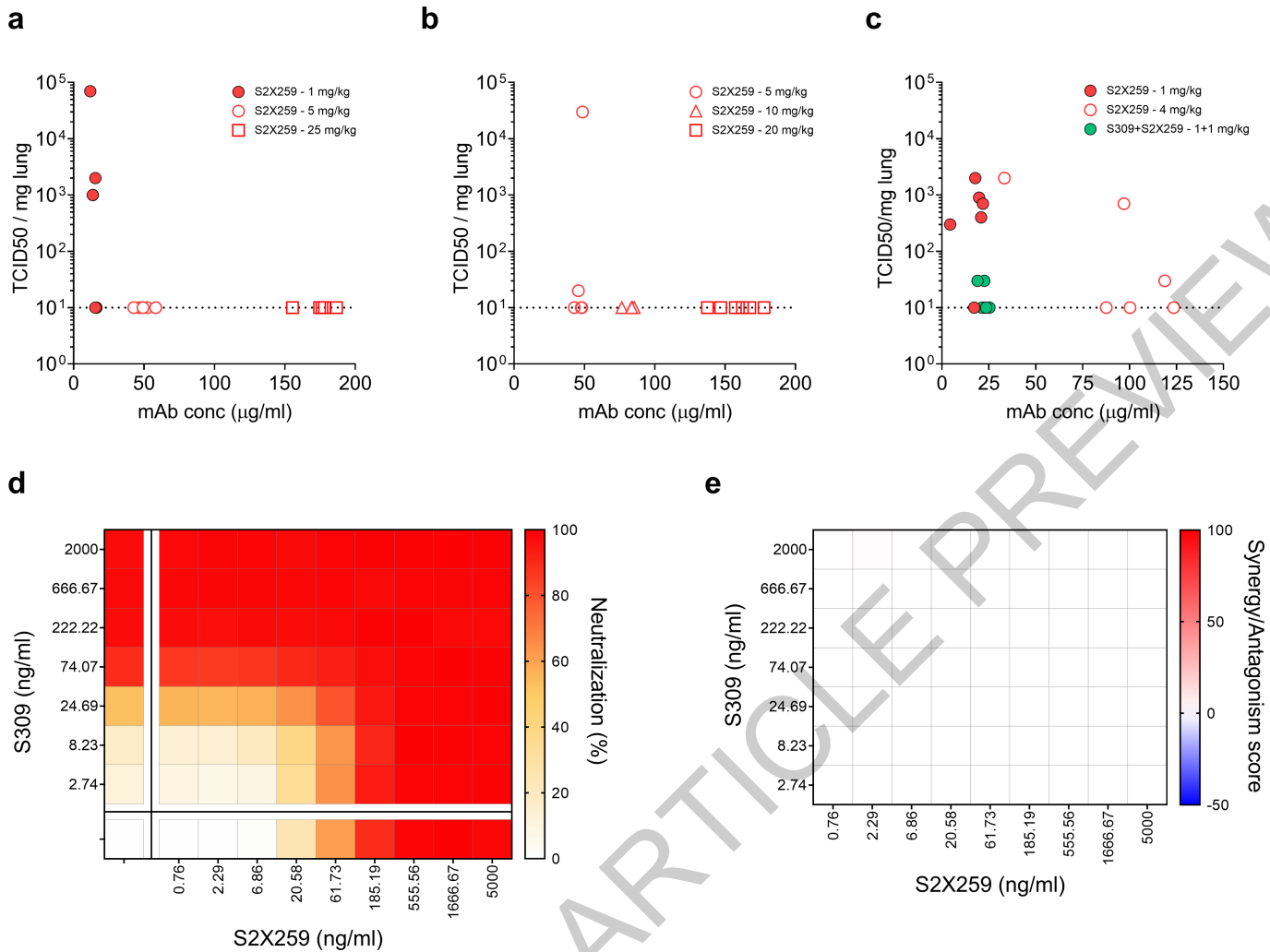
using VSV-SARS-CoV-2 chimeric virus on Vero cells with no mAb (-) or S2X259 (+) in the overlay to isolate escape mutants (red arrow). Data are representative of three independent experiments. **e**, Plaque assays performed to validate the VSV-SARS-CoV-2 G504D mutant in Vero cells in the presence (+) or absence (-) of S2X259 in the overlay. Representative image of two independent experiments is shown. **f**, S2X259 in vitro neutralizing activity against SARS-CoV-2 S VSV pseudotyped mutants. For each mutant the fold change of the IC₅₀ geometric mean vs SARS-CoV-2 S D614G is reported. *Q506K displayed a 10-fold reduction in viral entry in comparison to the other mutants. Results from two independent experiments are reported. **g**, Zoomed-in view of the S2X259/RBD interface showing that the G504D substitution would disrupt mAb binding due to steric hindrance (indicated with a red star).

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Extended Data Fig. 9 | Inhibition of ACE2 engagement, S₁ subunit shedding and activation of FcγRIIa and FcγRIIIa *in vitro*. **a**, S2X259 (purple/pink) and ACE2 (dark green) bind partially overlapping binding sites on the SARS-CoV-2 RBD (blue). **b**, Pre-incubation of serial dilutions of S2X259 with SARS-CoV-2 (red) or the SARS-CoV (black) RBDs prevents binding to immobilized human ACE2 (hACE2) ectodomain in ELISA. **c**, mAb-mediated S₁ subunit shedding from cell-surface expressed SARS-CoV-2S as determined by flow-cytometry. S2E12 was included as positive control whereas S2M11 was included as negative

control. **d-e**, NFAT-driven luciferase signal induced in Jurkat cells stably expressing FcγRIIa H131 (a) variant or FcγRIIIa V158 (b) variant by S2X259 binding to full-length wild-type SARS-CoV-2S on ExpiCHO target cells. **f-g**, NFAT-driven luciferase signal induced in Jurkat cells stably expressing FcγRIIa H131 (c) or FcγRIIIa V158 (d) variants by S2X259 binding to uncleavable full-length pre-fusion stabilized SARS-CoV-2S (unable to release the S₁ subunit) transiently expressed in ExpiCHO cells. SE12, S2M11, S309, S309-GRLR mAbs are included as controls.



Extended Data Fig. 10 | Correlation between mAbs concentration and infectious virus in vivo and in vitro neutralizing activity of S2X259/S309 antibody cocktail. a-c, Infectious virus titers in the lungs at 4 days post-infection plotted as a function of serum mAb concentrations before infection (day 0) with prototypic SARS-CoV-2 (a) and SARS-CoV-2 B.1.351 (c) in prophylactic setting or at 4 days post-infection with prototypic SARS-CoV-2 (b)

in therapeutic setting. **d-e,** SARS-CoV-2 B.1.351 VSV-based pseudotypes neutralization (d) and synergy score (e) measured combining S2X259 and S309 mAbs. Synergy was calculated using MacSynergyII (Synergy score: 0.98, Antagonism score: 0). Results represent four replicates of one independent experiment.

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Statistics

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

n/a Confirmed

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection

As described in Methods:

- * protein purification monitored by UNICORN software version 5.11
- * SPR binding assays collected and analyzed using Biacore Evaluation and Biacore Insight software
- * FACSDiva (version 6.1.3) and Everest 3.0 software for FACS data collection and cell sorting
- * Identification of escape mutants in virological assay was performed by Sanger sequencing (GENEWIZ)

Data analysis

Repository containing all code, analysis, and summary notebooks for the analysis of the SARS-CoV-2 deep mutational scanning escape selections available on GitHub: https://github.com/jbloomlab/SARS-CoV-2-RBD_MAP_Vir_mAbs. This code uses packages and versions as described in the Methods for various steps of computation.

Binding, Neutralization assays analyzed using PRISM (Version 9.1.0) as described in Methods.

Crystallography data analysis performed with XDS (VERSION Jan 31, 2020 BUILT=20200417), Coot (v. 0.9.5), ChimeraX (v. 1.1)/ISOLDE (v. 1.1), Refmac5 (v. 5.8.0267), and MOE (v. 2019.0102)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

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

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

- All datasets generated and information presented in the study are available from the corresponding author on reasonable request.
- CryoEM structure data and model are available with accession code PDB 7RA8 (EMD-24347) and PDB 7RAL (EMD-24365)
- The X-ray structure data and model has been deposited with accession code PDB 7M7W for RBD-S2H97-S2X259.
- Interactive escape maps and structural visualizations can be found at: https://jbloomlab.github.io/SARS-CoV-2-RBD_MAP_Vir_mAbs/.
- Raw Illumina sequencing data from deep mutational scanning experiments are available on NCBI SRA, BioSample SAMN18315604 (SARS-CoV-2 mutant selection data).
- Complete table of deep mutational scanning antibody escape fractions is provided on GitHub: https://github.com/jbloomlab/SARS-CoV-2-RBD_MAP_Vir_mAbs/blob/main/results/supp_data/all_antibodies_raw_data.csv.

Field-specific reporting

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

- Life sciences Behavioural & social sciences Ecological, evolutionary & environmental sciences

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

Life sciences study design

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

Sample size	For in vivo studies the sample size was chosen based on prior experience with this animal model.
Data exclusions	Lack of detection of the human monoclonal antibody in the hamster sera at 48 post-administration (prophylactic setting) or at 4 days post-infection (therapeutic setting) was considered a criteria to identify animals that were not properly administered and to exclude them from the analysis
Replication	Experimental assays were performed in biological duplicate or triplicate (or more) according to or exceeding standards in the field. We conducted all neutralization and antibody functional assays in biological duplicate, triplicate, or more, as indicated in relevant figure legends. In all cases, representative figure displays were appropriately replicated.
Randomization	Hamsters were randomized to S2X259 and control treatment. For other experiments, randomization was not a relevant feature as we were applying a uniform set of techniques across a panel of candidate monoclonal antibodies
Blinding	The hamster derived samples for RNA quantification and viral load titration were run by technicians who were blinded to treatment group of the analyzed samples

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

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| <input type="checkbox"/> | <input checked="" type="checkbox"/> Eukaryotic cell lines |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Palaeontology and archaeology |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> Animals and other organisms |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> Human research participants |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Clinical data |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Dual use research of concern |

Methods

- | n/a | Involved in the study |
|-------------------------------------|--|
| <input checked="" type="checkbox"/> | <input type="checkbox"/> ChIP-seq |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> Flow cytometry |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> MRI-based neuroimaging |

Antibodies

Antibodies used

For antibody isolation, B cells were stained with CD19 PE-Cy7 (BD Biosciences 557835), anti-IgM (BioLegend 314508), anti-IgD (BD Bioscience 555779), anti-CD14 (BD Bioscience 562691), and anti-IgA (Southern Biotech 2050-09), and spike labeled with streptavidin Alexa-Fluor 647 (Life Technologies S21374)

- * For FACS during deep mutational scanning, we used PE-conjugated goat anti-human-IgG (Jackson ImmunoResearch 109-115-098) and FITC-conjugated chicken anti-Myc (Immunology Consultants Lab, CYMC-45F).
- * For VSV pseudotyped neutralization assays, media was supplemented with anti-VSV-G antibody (I1-mouse hybridoma supertant, ATCC CRL-2700)
- * For SPR, immobilization was performed with anti-AviTag pAb (Genscript Cat # A00674-40) and goat anti-human IgG Fc pAb, (Southern Biotech Cat # 2014-01)
- * For RBD ELISA, antibody binding was detected via goat anti-human IgG (Southern Biotech, 2040-04)
- * For S1 shedding and binding FACS analysis, we used AF647-conjugated goat anti-human-IgG (Jackson ImmunoResearch 109-606-098)
- * For antibody blockade of ACE2 binding, RBD was detected via goat anti-mouse IgG (Southern Biotech 1030-04)
- * For detection of circulating mAb levels in hamster experiments, an anti-human LS mutation antibody was custom-made by BioRad AbD Serotec, and anti-human CH2 antibody from BioRad cat# MCA5748G clone R10Z8E9
- * Monoclonal antibodies (S309 and S2X259) were produced in-house using recombinant protein purification as described in the Methods. S2H97_GL and S2E12_GL were produced by ATUM.

All the commercial antibodies used in the study have been indicted with supplier name, catalog number. The monoclonal antibodies used as controls or for combination experiments were previously described by our group.

Validation

We discovered and describe S2X259 monoclonal antibody in this study. Target validation of this antibody was performed with multiple binding assays, in addition to cryoEM and x-ray crystallography structures were determined. Reactivity of primary antibodies listed above is based on the information on manufacturer's homepages.

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)

Cell lines used in this study were obtained from ATCC (HEK293T, Vero and Vero-E6), ThermoFisher Scientific (Expi CHO cells, FreeStyle™ 293-F cells and Expi293F™ cells) or were generated via lentiviral trasduction (Expi CHO-S, HEK293T-ACE2).

Authentication

None of the cell lines used were authenticated

Mycoplasma contamination

Cell lines were tested for mycoplasma contamination

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

No commonly misidentified cell lines were used in the study

Animals and other organisms

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

Laboratory animals

6-10 week-old female Syrian hamsters (*Mesocricetus auratus*) were Syrian hamsters (*Mesocricetus auratus*) were purchased from Janvier Laboratories and used in the study

Wild animals

The study did not involve wild animals

Field-collected samples

No field collected samples were used in the study

Ethics oversight

This work was conducted in the high-containment A3 and BSL3+ facilities of the KU Leuven Rega Institute (3CAPS) under licenses AMV 30112018 SBB 219 2018 0892 and AMV 23102017 SBB 219 20170589 according to institutional guidelines. Housing conditions and experimental procedures were approved by the ethical committee of animal experimentation of KU Leuven (license P065-2020).

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

Human research participants

Policy information about [studies involving human research participants](#)

Population characteristics

Participants were identified by prior SARS-CoV-2 infection for PBMC isolation. Blood drawn from donor S2X (male, 52 years-old) was obtained at day 75 after symptoms onset. For recovered individual samples were collected between 14 and 75 days after symptoms onset, for vaccinees samples were collected between 13 and 20 days after the second dose of mRNA-based SARS-CoV-2 vaccine.

Recruitment

Patients were recruited on the basis of prior SARS-CoV-2 infection or vaccination in the hospital or outpatient setting. Patients were healthy volunteers who donated blood after being informed about the study. The only exclusion criteria used were HIV or other debilitating disease, but other information about diagnosis and treatment was not collected.

Ethics oversight

Study protocols for antibody isolation were approved by the local Institutional Review Board (Canton Ticino Ethics

Ethics oversight

Committee, Switzerland), and all donors provided written informed consent for the use of blood and blood components. Study protocols for serological assays were approved by the local Institutional Review Boards relevant for each of three cohorts of samples (Canton Ticino Ethics Committee, Switzerland, the Ethical Committee of Luigi Sacco Hospital, Milan, Italy, and WCG North America, Princeton, NJ, USA). All donors provided written informed consent for the use of blood and blood components (such as PBMCs, sera or plasma) and were recruited at hospitals or as outpatients.

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

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Yeast were induced for cell-surface expression, incubated with the primary antibody from the panel, and secondary labeled with anti-Myc and anti-Fc fluorescent conjugates as described in the Methods.

Instrument

FACSAria II and ZE5 BioRad

Software

FACSDiva and Everest 3.0

Cell population abundance

We work with homogenous yeast libraries in our deep mutational scanning experiments, therefore cell population abundance is not a detail of our approach.

Gating strategy

Yeast expressing unmutated SARS-CoV-2 are labeled at 1x and 0.01x the concentration used for library labeling. Gates are drawn on the basis of these controls to standardize selection stringency across experiments. Gating schemes are shown in Extended Data Fig. 1c and 7a

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.