Broad betacoronavirus neutralization by a stem helix–specific human antibody

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Abstract: The spillovers of betacoronaviruses in humans and the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants highlight the need for broad coronavirus countermeasures. We describe five monoclonal antibodies (mAbs) cross-reacting with the stem helix of multiple betacoronavirus spike glycoproteins isolated from COVID-19 convalescent individuals. Using structural and functional studies, we show that the mAb with the greatest breadth (S2P6) neutralizes pseudotyped viruses from three different subgenera through the inhibition of membrane fusion, and we delineate the molecular basis for its cross-reactivity. S2P6 reduces viral burden in hamsters challenged with SARS-CoV-2 through viral neutralization and Fc-mediated effector functions. Stem helix antibodies are rare, oftentimes of narrow specificity, and can acquire neutralization breadth through somatic mutations. These data provide a framework for structure-guided design of pan-betacoronavirus vaccines eliciting broad protection.

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Broad betacoronavirus neutralization by a stem helix-specific human antibody

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The spillovers of β-coronaviruses in humans and the emergence of SARS-CoV-2 variants highlight the need for broad coronavirus countermeasures. We describe five monoclonal antibodies (mAbs) cross-reacting with the stem helix of multiple β-coronavirus spike glycoproteins isolated from COVID-19 convalescent individuals. Using structural and functional studies we show that the mAb with the greatest breadth (S2P6) neutralizes pseudotyped viruses from three different subgenera through inhibition of membrane fusion and delineate the molecular basis for its cross-reactivity. S2P6 reduces viral burden in hamsters challenged with SARS-CoV-2 through viral neutralization and Fc-mediated effector functions. Stem helix antibodies are rare, oftentimes of narrow specificity and can acquire neutralization breadth through somatic mutations. These data provide a framework for structure-guided design of pan-β-coronavirus vaccines eliciting broad protection.

A human broadly neutralizing β-coronavirus mAb targeting the conserved spike stem helix reduces SARS-CoV-2 burden in hamsters

Introduction

Coronaviruses are zoonotic pathogens responsible for several epidemics and a pandemic in the past two decades. All three highly pathogenic coronaviruses belong to the β-coronavirus genus: SARS-CoV-2 and SARS-CoV cluster within the sarbecovirus subgenus and originated in bats whereas MERS-CoV belongs to the merbecovirus subgenus and is transmitted to humans through dromedary camels. Moreover, HCoV-HKU1 and HCoV-OC43 (embecovirus subgenus, β-coronaviruses) as well as NL63 and 229E (setracovirus and davinacovirus subgenera, α-coronaviruses) are endemic and cause common colds in humans.

The coronavirus spike (S) glycoprotein promotes viral entry into host cells through an S1 subunit which engages host receptors and an S2 subunit mediating membrane fusion (1). The S1 subunit is the major target of (neutralizing) antibodies (Abs), and is more genetically variable than the S2 subunit (2–5). Accordingly, Abs binding to the S1 subunit receptor-binding domain (RBD) and N-terminal domain (NTD) exert a selective pressure resulting in the emergence of new variants (5–10). SARS-CoV-2 S comprises 22 N-linked glycans (13 and 9 of them in the S1 and S2 subunits, respectively) participating in folding as well as modulating access to host proteases and antibodies (1, 2, 11–13). MAbs binding to the S2 fusion machinery can neutralize distantly related coronaviruses (14–16) as observed for other viruses, such as influenza and HIV-1 (17, 18).

Results

Isolation of a broadly neutralizing β-coronavirus mAb from a convalescent SARS-CoV-2-exposed individual

To identify mAbs targeting highly conserved regions of the S glycoprotein, we interrogated human IgG+ memory B cells from three COVID-19 convalescent donors. Five mAbs bound to the prefusion-stabilized S ectodomain trimers of SARS-CoV and SARS-CoV-2 (sarbecovirus), MERS-CoV (merbecovirus), OC43 and HKU1 (embecovirus), but not to the human α-coronaviruses 229E and NL63 (Fig. 1A-C and Fig. S1A-B). S2P6 and S2S43 mAbs were each derived from a separate donor and use VH1-46*01 and D5-12*01 genes whereas P34D10, P34G12 and P34E3 mAbs derived from a third donor, are clonally related and use the VH3-30
gene (fig. S1A). These 5 mAbs share nucleotide sequence identities with their respective
germline genes ranging from 86 to 97% (fig. S1A), which correspond to a higher level of
mutations than that observed for RBD- and NTD-specific mAbs isolated early after SARS-CoV-2
infection or vaccination (5, 19–21). The mAbs bound to all prefusion-stabilized human-
infecting β-coronavirus S trimers (Fig. 1A-C and fig. S1B) and they all neutralize SARS-CoV-2
S VSV pseudotypes (fig.S1C).

S2P6 was selected for further in-depth characterization because it exhibited the overall greatest
cross-reactivity and neutralization breadth. S2P6 bound both prefusion and post-fusion SARS-
CoV-2 S with comparable avidities, indicating that its epitope might be (at least partially)
accessible in both conformational states (Fig. 1A-B and fig. S1D). S2P6 bound to all full-length
SARS-CoV-2 S variants tested and to 25 S glycoproteins representative of all sarbecovirus
clades (Fig. 1D and fig. S1E-F). Using surface plasmon resonance (SPR), we found that the
S2P6 Fab fragment had the highest affinity for SARS-CoV-2 S and SARS-CoV S followed by
MERS-CoV S and OC43 S with equilibrium dissociation constants (Kd) of 7, 7, 12 and 16 nM,
respectively (fig. S1G-H). S2P6 also bound to HKU1 S albeit with reduced affinity (Kd ~120
nM) (fig. S1G). Collectively, these data demonstrate that S2P6 cross-reacts with all human-
infecting β-coronaviruses.

To evaluate the neutralization potency and breadth of S2P6, we investigated its ability to inhibit
entry of authentic SARS-CoV-2 into Vero-E6 cells in the presence or absence of Tmprss2 as
this protease activates fusion with the cytoplasmic membrane in cultured lung cells (22–24).
S2P6 completely neutralized infection of Tmprss2-positive Vero-E6 cells but was less
effective in inhibiting infection of Vero-E6 cells lacking Tmprss2 (Fig. 1E), suggesting that
S2P6 binding might be reduced at endosomal pH. Indeed, the recognition of prefusion SARS-
CoV-2 S by S2P6 is pH dependent with higher binding affinity at pH 7, relative to pH 5, in both
IgG and Fab formats (fig. S1H). We subsequently assessed S2P6-mediated neutralization of
vesicular stomatitis virus (VSV) (25) pseudotyped with SARS-CoV-2 S of several variants of
concern (VOC), including B.1.1.7 (alpha), B.1.351 (beta), P.1 (gamma) and B.1.617.1 (kappa)
and observed similar potency to that found against the parental SARS-CoV-2 D614G S (Fig. 1F
and fig. S1I). Moreover, S2P6 inhibited SARS-CoV S, Pangolin Guangdong 2019 (PANG/GD)
S, MERS-CoV S and OC43 S VSV pseudotypes with IC50 values ranging from 0.02 to 17 μg/ml
(Fig. 1G). S2P6 is therefore a broadly neutralizing β-coronavirus mAb with activity against
SARS-CoV-2 and SARS-CoV-related viruses as well as members of the merbecovirus and
embeccovirus subgenera.

Peptide mapping experiments using 15-mer linear overlapping peptides revealed that all five
mAbs bound to peptides containing the SARS-CoV-2 motif F1148KEELDKYF1156 located in the
S2 subunit stem helix (Fig. 1H and fig. S2A). This region is strictly conserved in SARS-CoV,
highly conserved among other β-coronaviruses, and overlaps with the epitopes of the B6 (Fig.
1I) and 28D9 mouse mAbs (14, 15). S2P6 bound efficiently to the stem helix peptides of the five
β-coronaviruses that infect humans (albeit with a faster off-rate for HKU1) as well as of the
MERS-CoV-related bat viruses (HKU4 and HKU5) and murine hepatitis virus (MHV) (Fig. 1J
and fig. S2B). S2S43 exhibited similar overall binding than S2P6 with markedly weaker
reactivity towards the HKU1, HKU4 and HKU5 peptides, whereas the three clonally related
P34D10, P34G12, and P34E3 mAbs exhibited weaker or no binding to HKU4 and HKU5
peptides (fig. S2B).
Structural basis for S2P6 binding to the conserved S glycoprotein stem helix

To determine the molecular basis of the S2P6 neutralization breadth, we determined a cryo-EM structure of the SARS-CoV-2 S ectodomain trimer in complex with the Fab fragments of S2P6 and S2M11 (to lock the RBDS in the closed state (21)) at 4.2 Å overall resolution. The marked conformational dynamics of the region recognized by S2P6 limited the local resolution of the stem helix/Fab to approximately ~12 Å and 3D classification of the cryo-EM data revealed incomplete Fab saturation (Fig. 2A, table S1 and fig S3A-F). Our cryo-EM structure confirms that S2P6 recognizes the stem helix and suggests that the mAb disrupts its quaternary structure, which is presumed to form a 3-helix bundle in prefusion SARS-CoV-2 S (2, 3, 14). To overcome the limited resolution of the stem helix/Fab interface in the cryo-EM structure, we determined a crystal structure of the S2P6 Fab in complex with the SARS-CoV-2 S stem helix peptide (residues 1146-1159) at 2.67 Å resolution (Fig. 2B-D, fig. S4A and table S2). The peptide folds as an amphipathic α-helix resolved for residues 1146 to 1159. S2P6 buryes approximately 600 Å² upon binding to its epitope using shape-complementarity and hydrogen bonding involving complementarity determining regions (CDR) H1-H3, L1 and L3. The light chain residues Y33, Y92, G93, S94, P96, P97 and F99 as well as heavy chain residues Y33, I50, H57, T58, S59, P101, K102 and G103 form a deep groove in which the hydrophobic side of the stem helix docks through residues F1148, L1152, Y1155 and F1156. Binding specificity is provided through backbone hydrogen bonding of residues F1148\_SARS-CoV-2 and K1149\_SARS-CoV-2 with CDRH3 P101, side chain hydrogen bonding of residues E1151\_SARS-CoV-2 with CDRL1 Y33 and CDRL3 S94, D1153\_SARS-CoV-2 with CDRH1 Y33 side chains as well as Y1155\_SARS-CoV-2\_S with CDRH2 S59 through a water molecule and F1156\_SARS-CoV-2\_S with CDRH2 H57 also through a water molecule (Fig. 2C-D). The contribution of each epitope residue was validated by single substitution scan analysis with most mutations at positions 1148, 1151-1153 and 1155-1156 abolishing S2P6 binding, highlighting the importance of these residues for mAb recognition (fig. S5A). A substitution scan analysis performed on the P34D10, P34G12 and P34E3 mAbs revealed a similar pattern of key interacting residues (fig. S5A). Residue Y1155 is conservatively substituted to F1238\_MERS-CoV or W1237/1240\_HKU1/OC43 and residue D1153 is conserved in MERS-CoV and OC43 but mutated to S1235 for HKU1 (fig. S5B). The residue scan and structural results suggest that the reduced binding affinity of S2P6 for HKU1 S is at least partially due to the D1153\_SARS-CoV-2/S1235\_HKU1 substitution which is expected to dampen electrostatic interactions with the CDRH1 Y33 side chain hydroxyl (Fig. 2C-D).

Although S2P6 and B6 recognize a similar epitope (fig. S4B-C), they bind with opposite orientations of the heavy and light chains relative to the stem helix and the S2P6-bound structure resolves 3 additional C-terminal peptide residues (1156-1159) compared to the B6-bound structure (1147-1156) (fig. S4B-C). Superposition of both structures based on the stem helix reveals that B6 CDRH2 would sterically clash with H1159\_SARS-CoV-2, putatively explaining the broader cross-reactivity of S2P6 over B6 (fig. S4B-C).

To further validate our structural data, we carried out viral escape mutant selection in vitro in the presence of S2P6 using a replication-competent VSV-SARS-CoV-2 S virus (27). After two passages, virus neutralization by S2P6 was abrogated and deep sequencing revealed the emergence of five distinct resistance mutations: L1152F, D1153N/G/A and F1156L, which are
consistent with the structural data and substitution scan analysis (fig. S5A). Although these
mutations have been detected with very low frequencies in circulating SARS-CoV-2 isolates
(146 out of 1,217,814 sequences as of April 30, 2021), the subdominant immunogenicity of this
cryptic site, likely due to its limited exposure, might result in low Ab pressure limiting
accumulation of mutations in this epitope.

Stem helix-targeting mAbs inhibit S-mediated membrane fusion

The S stem helix forms a 3-helix bundle in many prefusion cryoEM structures of SARS-CoV-2 S
and SARS-CoV S although it is not fully resolved (2, 3, 11, 28–30). In contrast, the
S2P6/S2M11/SARS-CoV-2 S cryoEM structure suggests that the quaternary organization of the
stem is disrupted (Fig. 2A) consistent with S2P6 binding to the hydrophobic face of the stem
helix. This face is mostly buried through homo-oligomeric interactions in prefusion S, and may
be only transiently available for Ab binding. Although we observed S2P6 binding to post-fusion
SARS-CoV-2 S (fig. S1D), the epitope recognized is buried at the interface with the other two
protomers of the rod-shaped trimer and therefore might become accessible due to conformational
dynamics, as is the case for the pre-fusion state (fig. S4D) (14, 31–33). Based on these data, we
propose that S2P6 binding to S sterically interferes with the conformational changes leading to
membrane fusion, as observed for B6 (14) and 27D9 (15).

To validate the inferred mechanism of S2P6-mediated broad coronavirus neutralization, we first
showed that S2P6 binding did not block engagement of SARS-CoV-2 S by ACE2 using ELISA,
as expected based on the distance of its epitope from the RBD (Fig. 3A). S2P6, however,
blocked cell-cell fusion between Vero-E6 cells transfected with full-length SARS-CoV-2 S as
effectively as the S2M11 mAb which locks SARS-CoV-2 S in the closed state (21) (Fig. 3B).
We previously described mAbs targeting RBD antigenic site Ia (e.g. S2E12) and Ila (e.g.
S2X259 or S2X35) which can mimic receptor attachment and prematurely trigger fusogenic S
conformational changes (11, 20, 34). Accordingly, S2P6 at concentrations as low as 1 ng/ml
abrogated the formation of syncytia mediated by S2E12 (34). Collectively, these results suggest
that the main mechanism of S2P6 neutralization is to prevent viral entry by impeding S
fusogenic rearrangements thus inhibiting membrane fusion (Fig. 3C).
S2P6-mediated protection in hamsters is enhanced by Fc-mediated effector functions

Functions mediated by the constant (Fc)-region of Abs can contribute to in vivo protection by promoting viral clearance and anti-viral immune responses (35–38). We analyzed the ability of S2P6 to trigger activation of the Fc receptors, FcγRIIIa and FcγRIIIa, as well as to exert Fc effector functions in vitro. S2P6 promoted moderate dose-dependent FcγRIIIa and FcγRIIIa mediated signaling using a luciferase reporter assay (Fig. 4A). However, S2P6 promoted robust activation of Ab-dependent cell cytotoxicity (ADCC), to levels comparable to those observed with the S309 mAb (26), following incubation of SARS-CoV-2 S-expressing CHO-K1 target cells with human peripheral blood mononuclear cells (PBMCs) (Fig. 4B). S2P6 also triggered measurable Ab-dependent cellular phagocytosis (ADCP) activity using Cell-Trace-Violet-labelled PBMCs as phagocytic cells, and SARS-CoV-2 S-expressing CHO (CHO-S) as target cells (Fig. 4C). Finally, S2P6 did not promote complement-dependent cytotoxicity (CDC) (Fig. 4D) indicating that S2P6 Fc-mediated effector functions, but not CDC, might participate in viral control in vivo.

We evaluated the prophylactic activity of S2P6 against challenge with the prototypic (Wuhan-1 related) SARS-CoV-2 in a Syrian hamster model (39). As we previously showed that the human IgG1 Fc fragment poorly recognizes hamster FcγRs (34), as we compared S2P6 harboring a human IgG1 constant region (Hu-S2P6) with S2P6 harboring a hamster IgG2a constant region (Hm-S2P6), the latter enabling optimal interactions with hamster FcγRs. Two different doses of Hu-S2P6 or Hm-S2P6 were administered 24 hours prior to intranasal SARS-CoV-2 challenge and the lungs of the animals were assessed 4 days post-infection for viral RNA load and replicating virus. Hm-S2P6 administered at 20 mg/kg reduced lung viral RNA copies and replicating viral titers by two orders of magnitude relative to a control mAb but did not exert any antiviral effect at 2 mg/kg (Fig. 4E and fig. S6). Hm-S2P6 at 20 mg/kg reduced lung viral RNA load to levels statistically significantly lower than those observed with Hu-S2P6 at 20 mg/kg, suggesting a beneficial effect of S2P6 effector functions in vivo. Based on the comparable S2P6 neutralization potencies towards SARS-CoV-2 VOC observed in vitro (Fig. 1F), we assessed the protective efficacy of S2P6 in hamsters challenged with SARS-CoV-2 B.1.351 (beta VOC). Prophylactic administration of Hu-S2P6 at 20 mg/kg reduced replicating viral titers in the lungs (but not RNA copy numbers) by ~1.5 order of magnitude relative to the control group. Although this difference was not statistically significant, the observed efficacy against this VOC is in line with the strict conservation of the stem helix epitope in all VOC identified to date (Fig 4F).

Collectively, these findings demonstrate that Abs targeting a highly conserved epitope in the S fusion machinery can trigger Fc-mediated ADCC and ADCP in vitro and that their in vivo antiviral activity may rely on both neutralization and effector functions.

Natural infection or vaccination predominantly elicit stem helix-directed Abs of narrow specificities

To understand how frequently stem helix-specific Abs are elicited, we analysed plasma samples from pre-pandemic, COVID-19 convalescent, and COVID-19 vaccinated individuals to determine IgG binding titers to the SARS-CoV-2/SARS-CoV (SARS-CoV/-2), OC43, MERS-CoV, HKU1, HKU4 and HKU5 stem helix peptides (Fig. 5A and fig. S7). In pre-pandemic samples, we only observed binding to the HKU1 stem helix peptide, probably reflecting prior infection with this virus in the cohort. Stem helix-specific Abs were found at low frequencies in
individuals previously infected with SARS-CoV-2 or those who had received two doses of mRNA vaccines (Fig. 5A). The highest frequencies of stem helix-specific serum Abs were observed for vaccinated individuals who were previously infected (ranging from 22% to 78% against the different stem helix peptides). Overall, these data show that plasma Ab responses to the stem helix are elicited upon SARS-CoV-2 infection or vaccination although they are relatively rare. Evaluation of the prevalence of mAbs targeting other fusion machinery epitopes will reveal if this trend holds true for the overall S2 subunit or if it is a specificity of the cryptic stem helix epitope.

Next, we investigated the frequencies of stem helix-specific memory B cells among 21 convalescent and 17 vaccinated individuals using a clonal analysis based on in vitro polyclonal stimulation (41) (Fig. 5B-G and fig. S7-S9). In both cohorts, we observed frequencies of culture positive for stem helix-specific IgGs ranging from 0-2.5%, except for one individual (infected and vaccinated with a single dose of mRNA vaccine) for which 99.6% of culture supernatants were binding to the SARS-CoV-2 stem helix (fig. S9). Most SARS-CoV-2 stem helix-specific memory B cells were cross-reactive with OC43, consistent with the high sequence identity of the stem helices of these two viruses (Fig. 1H). Abs specific for the HKU1 S stem helix were found but they were not cross-reactive with other $\beta$-coronaviruses except for one convalescent and one vaccinated individuals (Fig. 5D and G). This analysis revealed a single example of cross-reactivity to all stem helix $\beta$-coronavirus peptides tested (Fig. 5E), whereas most other Abs show limited cross-reactivity among $\beta$-coronaviruses.

Broadly reactive $\beta$-coronavirus Abs enhance their binding affinity and cross-reactivity through somatic mutations

To define the ontogeny of the broadly reactive $\beta$-coronavirus mAbs presented here, we generated a panel of germline variants of S2P6, P34D10, P34E3 and P34G12. Two out of seven S2P6 heavy chain residues that are mutated relative to germline contribute to epitope recognition (Q32 and H57) whereas none of the 5 light chain mutated residues participate in S binding (Fig. 2C-D). To address the role of VH and VK somatic mutations we generated a panel of S2P6 germline variants for the heavy or the light chain, or both variable regions (VH and VK). The fully germline reverted S2P6 (unmutated common ancestor - UCA) bound to OC43 and MERS-CoV stem helix peptides (with approximately 1 order of magnitude higher EC50 compared to the mature mAb) but did not bind to SARS-CoV-2 or HKU1 stem helix peptides or spike trimers (Fig. 5H and fig. S10A-C). Somatic mutations in VH were sufficient for high avidity binding to SARS-CoV-2, whereas both VH and VK mutations were required for optimal binding to HKU1. The presence of residue G103 in CDRH3 was essential for binding to all $\beta$-coronaviruses (fig. S10A-C). These findings indicate that the S2P6 mAb likely arose in response to OC43 infection, and its specificity was broadened towards SARS-CoV-2 and HKU1 through somatic mutations selected upon natural infection with one or both of these $\beta$-coronaviruses. In contrast, analysis of UCA binding of the clonally related P34D10, P34G12 and P34E3 mAbs suggest they were likely primed by HKU1 infection, rather than OC43, and acquired cross-reactivity towards the other human $\beta$-coronaviruses primarily through somatic mutations in VH (Fig. 5I and fig. S10D-F).

Collectively, these findings demonstrate that broadly reactive $\beta$-coronavirus Abs may result from priming of virus-specific B cells gaining affinity and cross-reactivity through somatic mutations in response to heterotypic coronavirus exposures.
Discussion

The coronavirus S₂ subunit (fusion machinery) contains several conserved antigenic sites, including the fusion peptide and the heptad-repeat 2 regions (42–47). The recent identification of 4 cross-reactive mAbs targeting the stem helix in the S₂ subunit highlighted the importance of this epitope, which is conserved among β-coronavirus but not α-coronavirus S glycoproteins (14–16, 48), although none of these mAbs inhibit members of all three β-coronavirus subgenera.

Here, we identified five stem helix-specific human mAbs cross-reacting with human and animal β-coronaviruses and showed that S2P6 broadly neutralized all sarbecoviruses, merbecoviruses and embecoviruses pseudotypes evaluated through inhibition of membrane fusion. We show that a S₂ subunit-directed mAb reduces lung viral burden in hamsters challenged with SARS-CoV-2 with a key contribution of Fc-mediated effector functions, as described for some SARS-CoV-2 RBD-specific mAbs (35, 38) and influenza virus hemagglutinin stem mAbs (17, 49, 50). While this study showcases the stem helix as a target of pan-β-coronavirus Abs, the existence of other cross-reactive S₂ epitopes with neutralizing potential remains a subject of investigation. Stem helix-targeted Abs are elicited upon natural infection by endemic (OC43 or HKU1) or pandemic (SARS-CoV-2) coronaviruses as well as by COVID-19 mRNA vaccines. Similarly to the subdominance of Abs recognizing the conserved hemagglutinin stem region of influenza A viruses (49, 51), stem helix-specific Abs are present at low titers in convalescent or vaccinees plasma and at low frequencies in their memory B cell repertoires, possibly as a result of limited epitope exposure. These findings along with the moderate neutralization potency of these mAbs indicate that eliciting high enough titers of stem helix-targeted mAbs through vaccination will be a key challenge to overcome to develop pan-β-coronavirus vaccines. We propose that harnessing recent advances in computational protein design, such as epitope-focused vaccine design approaches as described for the respiratory syncytial virus fusion protein (52–54) and multivalent display as described for influenza virus (55–60) to target the S stem helix or the fusion peptide regions might enable elicitation of broad β-coronavirus immunity.

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Data and Materials Availability

The cryoEM map and coordinates have been deposited to the Electron Microscopy Databank and Protein Data Bank with accession numbers.

Supplementary Materials

Materials and Methods

Figs. S1 to S10

Tables S1 to S2

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Figure legends.
Fig. 1. The S2P6 cross-reactive mAb broadly neutralizes β-coronaviruses from three subgenera. (A) Binding avidity (EC50) of 5 mAbs to prefusion coronavirus S trimer ectodomains as determined by ELISA. (B) S2P6 ELISA binding curves showing the full titration. One representative experiment out of two is shown. (C) Cladogram of representative α- (black) and β-coronavirus (red) S glycoprotein amino acid sequences inferred via maximum likelihood analysis. (D) Flow cytometry analysis of S2P6 binding (from 10 to 0.22 μg/ml) to a panel of 26 S glycoproteins representative of all sarbecovirus clades (left) and 8 SARS-CoV-2 variants (right) displayed as EC50 values. (E) Neutralization of authentic SARS-CoV-2 by S2P6 determined using VeroE6-TMPRSS2 (left) or VeroE6 (right) cells. The S309 mAb that binds RBD site IV (26) is included for comparison. Mean ± s.d. of triplicates from one representative experiment out of three is shown. (F) S2P6–mediated neutralization of SARS-CoV-2 B.1.1.7 (alpha) S, B.1.351 (beta) S, P.1 (gamma) S and B1.617.1 (kappa) S VSV pseudotypes represented as IC50 fold change relative to wildtype (D614G) S VSV pseudotype. Individual values for each of the two replicates are shown as open circles, the mean as a colored bar and the SD as error bars. (G) S2P6–mediated neutralization of VSV pseudotyped with various β-
coronavirus S glycoproteins. Error bars indicate standard deviation of triplicates. IC50 values:
2.4 µg/ml, 1.4 µg/ml, 17.1 µg/ml, 1.3 µg/ml and 0.02 µg/ml for SARS-CoV, SARS-CoV-2, MERS-CoV, OC43 and PANG/GD, respectively. (H) Alignment of β-coronavirus stem helix amino acid sequences with the S2P6 epitope boxed. Residue numbering is shown according to SARS-CoV-2 S. The sequences of the peptides used in this study are shown in blue and N-linked glycosylation sequons are highlighted in yellow. (I) Competition assay for S2P6/B6 binding to the SARS-CoV-2/SARS-CoV stem helix peptide (herein defined as SARS-CoV/-2). B6 binding in presence of S2P6 (red line), B6 binding in absence of S2P6 (black line) and the control lacking the SARS-CoV/-2 stem helix peptide (grey line) are shown. (J) Kinetics of S2P6 binding to a panel of biotinylated β-coronavirus stem helix peptides immobilized at the surface of biolayer interferometry biosensors.

Fig. 2: Structural basis for the broad S2P6 cross-reactivity with the conserved coronavirus stem helix peptide. (A) Composite model of the S2P6-bound SARS-CoV-2 S cryoEM structure and of the S2P6-bound stem helix peptide crystal structure docked in the cryoEM map (transparent gray surface). SARS-CoV-2 S protomers are colored pink, cyan and gold, the S2P6
Fab heavy and light chains are colored purple and magenta and the S2M11 Fab heavy and light chains are colored dark and light gray, respectively. (B) Crystal structure of the S2P6 Fab (surface rendering) in complex with the SARS-CoV-2 S stem helix peptide (yellow ribbon with side chains rendered as sticks). (C-D) Ribbon diagram in two orthogonal orientations of the S2P6 Fab bound to the SARS-CoV-2 S stem helix peptide showing a conserved network of interactions. Only key interface residues and the S2P6 CDR loops are shown for clarity. Residues Q32 and H57, that are mutated during affinity maturation of the S2P6 heavy chain, are colored blue. Hydrogen bonds are indicated with dashed lines. Residues substituted in the escape mutants isolated are underlined.

**Fig. 3. S2P6 binding disrupts the stem helix bundle and sterically inhibits membrane fusion.** (A) SARS-CoV-2 S binding to ACE2 in the presence of mAb S2P6 analyzed by ELISA. S2E12 was included as a positive control. (B) S2P6 inhibition of cell-cell fusion using Vero-E6 cells transfected with SARS-CoV-2 S. S2M11 was included as a positive control. Inhibition of fusion values are normalized to the percentage of fusion without mAb (100%) and to that of fusion of non-transfected cells (0%). (C) Proposed mechanism of inhibition mediated by the S2P6 mAb. S2P6 binds to the hydrophobic core of the stem helix bundle and disrupts its quaternary structure. S2P6 binding likely prevents S2 subunit refolding from the pre- to the post-fusion state and blocks viral entry.
Fig. 4. S2P6 activates effector functions and reduces SARS-CoV-2 lung burden in Syrian hamsters. (A) NFAT-driven luciferase signal induced in Jurkat cells stably expressing FcγRIIa (V158, left) or FcγRIIa (V131, right) upon S2P6 binding to full-length wild-type SARS-CoV-2 S expressed at the surface of CHO target cells. S309 is included as positive control. RLU, relative luminescence unit. (B) mAb-mediated ADCC using SARS-CoV-2 CHO-K1 cells (genetically engineered to stably express a HaloTag-HiBit) as target cells and PBMC as effector cells. The magnitude of NK cell–mediated killing is expressed as percentage of specific lysis. (C) mAb-mediated ADCP using Cell-Trace-Violet-labelled PBMCs as a source of phagocytic cells (monocytes) and PKH67–fluorescently labeled S-expressing CHO cells as target cells. The y-axis indicates percentage of monocytes double-positive for anti-CD14 (monocyte) marker and PKH67. (D) Lysis of SARS-CoV-2 S stably transfected CHO cells by mAbs in the presence of complement. S309 was included as positive control, S309-GRLR with diminished FcγR binding capacity and an unrelated mAb (neg mAb) were used as negative controls. (E) Syrian hamsters were administered with the indicated amount of S2P6 mAb harboring either a hamster (Hm-S2P6) or a human (Hu-S2P6) constant region before intranasal challenge with prototypic SARS-CoV-2 (Wuhan-1 related). An irrelevant mAb (MGG2 against P. falciparum CSP) at 20 mg/kg was used as negative control (40). Viral RNA loads and replicating virus titers are shown on the left and right, respectively. (F) Prophylactic administration of Hu-S2P6 at 20 mg/kg in hamsters challenged with SARS-CoV-2 B.1.351 (beta) VOC. Viral RNA loads and replicating virus titers are shown on the left and right, respectively. * P<0.05, ** P<0.01 Mann-Whitney test.
Fig. 5: Stem helix-directed Abs are rare, of narrow specificities and enhance their binding affinity and cross-reactivity through somatic mutations. (A) Binding of pre-pandemic (P, n=88), COVID-19 convalescent (C, n=72), vaccinees immune (VI, n=9) and vaccinees naïve (VN, n=37) plasma Abs (diluted 1:10) to immobilized β-coronavirus stem helix peptides analyzed by ELISA. A cutoff of 0.7 was determined based on signal of pre-pandemic samples and binding to uncoated ELISA plates (horizontal dash line). The fraction of samples for which binding above the cutoff was detected is indicated. (B-G) Analysis of memory B-cell specificities for β-coronavirus stem helix peptides. Each dot represents a well containing oligoclonal B cell supernatant screened for the presence of stem helix peptide binding IgG Abs using ELISA. Samples obtained from 21 COVID-19 convalescent individuals (B-D) and 16 vaccinees (E-G). Pairwise reactivity comparison is shown for SARS-CoV-2 and OC43 (C and F) and SARS-CoV-2 and HKU1 (D and G). Cultures cross-reactive with at least three peptides