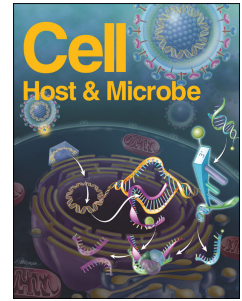


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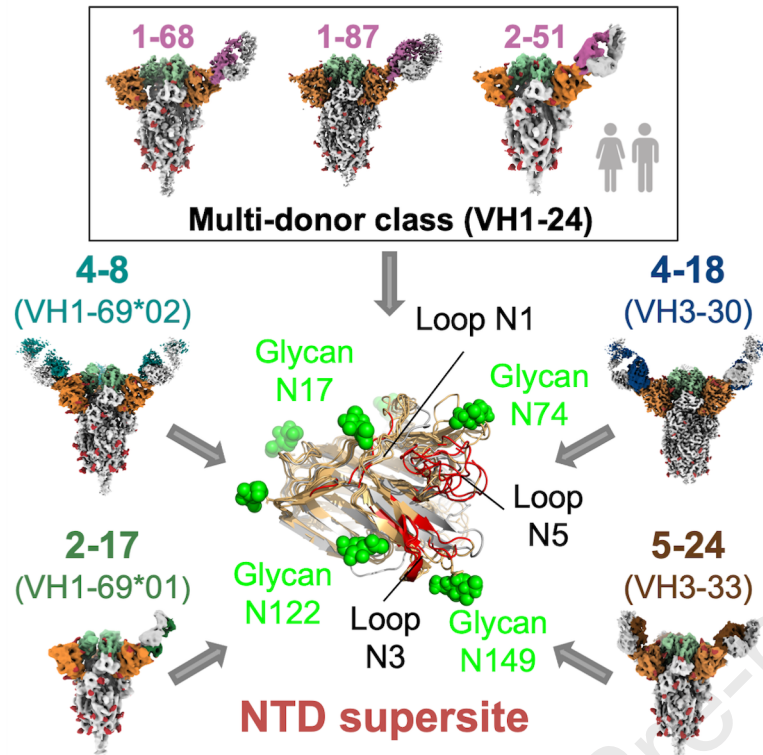
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Summary

Numerous antibodies that neutralize SARS-CoV-2 have been identified, and these generally target either the receptor-binding domain (RBD) or the N-terminal domain (NTD) of the viral spike. While RBD-directed antibodies have been extensively studied, far less is known about NTD-directed antibodies. Here we report cryo-EM and crystal structures for seven potent NTD-directed neutralizing antibodies in complex with spike or isolated NTD. These structures defined several antibody classes, with at least one observed in multiple convalescent donors. The structures revealed all seven antibodies target a common surface, bordered by glycans *N17*, *N74*, *N122*, and *N149*. This site – formed primarily by a mobile β -hairpin and several flexible loops – was highly electropositive, located at the periphery of the spike, and the largest glycan-free surface of NTD facing away from the viral membrane. Thus, in contrast to neutralizing RBD-directed antibodies that recognize multiple non-overlapping epitopes, potent NTD-directed neutralizing antibodies appear to target a single supersite.

Keywords: COVID-19, SARS-CoV-2, neutralizing antibody, N-terminal domain, multi-donor antibody, antibody class.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent for Coronavirus Disease 2019 (COVID-19), emerged in 2019, rapidly establishing an ongoing worldwide pandemic with tens of millions infected and over one million dead (Callaway et al., 2020; Cucinotta and Vanelli, 2020; Dong et al., 2020). In response, an unprecedented global effort to develop vaccines and therapeutics is well underway. One promising approach is the identification of SARS-CoV-2-neutralizing antibodies, which could be used as therapeutic or prophylactic agents. Analysis of such antibodies can reveal viral sites of vulnerability to antibody neutralization, which can help guide the development of vaccines or therapeutics (Burton and Walker, 2020). The primary target for neutralizing antibodies is the viral spike protein, a trimeric type I viral fusion machine (Walls et al., 2020; Wrapp et al., 2020b) that binds virus to the ACE2 receptor on host cells (Benton et al., 2020; Yan et al., 2020; Zhou et al., 2020) and mediates fusion between the viral and cell membranes. The spike protein is comprised of two subunits: the S1 subunit comprising the N-terminal domain (NTD), the receptor-binding domain (RBD) and several other subdomains, and the S2 subunit that mediates virus–cell membrane fusion (Walls et al., 2020; Wrapp et al., 2020b).

The majority of SARS-CoV-2 neutralizing antibodies so far identified target RBD (Brouwer et al., 2020; Cao et al., 2020; Chen et al., 2020; Chi et al., 2020; Ju et al., 2020; Liu et al., 2020b; Pinto et al., 2020a; Robbiani et al., 2020; Rogers et al., 2020; Seydoux et al., 2020; Wang et al., 2020a; Wrapp et al., 2020a; Wu et al., 2020; Zeng et al., 2020; Zost et al., 2020). Structural studies (Barnes et al., 2020a; Barnes et al., 2020b; Liu et al., 2020a; Wang et al., 2020b; Yuan et al., 2020b) and binding competition experiments (Liu et al., 2020a), have revealed neutralizing antibodies to recognize RBD at multiple distinct sites, and further revealed

multi-donor RBD-directed antibody classes that appear to be elicited with high frequency in the human population (Barnes et al., 2020b; Robbiani et al., 2020; Wu et al., 2020; Yuan et al., 2020b) as well as in mice with a humanized immune system (Hansen et al., 2020). Neutralization for many RBD-directed antibodies can be explained by interference with RBD-ACE2 interaction, and/or impeding the ability of RBD to adopt the “up” conformation (Barnes et al., 2020b; Liu et al., 2020a; Yuan et al., 2020b) required for ACE2 binding (Benton et al., 2020).

NTD-directed neutralizing antibodies targeting the MERS betacoronavirus have been extensively characterized (Chen et al., 2017; Pallesen et al., 2017; Wang et al., 2018; Zhou et al., 2019). For SARS-CoV-2, three cryo-EM structures have been reported for NTD-directed neutralizing antibodies: 4A8 (Chi et al., 2020), FC05 (Wang et al., 2021) and CM25 (Voss et al., 2020) in complex with SARS-CoV-2 spike, the last two reported near the time of submission of this paper. NTD-directed antibodies have also been observed in electron microscopy (EM) analyses of antibodies from the sera of convalescent donors (Barnes et al., 2020b; Brouwer et al., 2020), and a low-resolution structure of a very potent antibody 4-8 has been reported (Liu et al., 2020a). This report was also notable for the identification of multiple NTD-neutralizing antibodies with potencies rivaling those of the best RBD-directed neutralizing antibodies.

Here we describe cryo-EM and crystal structures for seven potentially neutralizing antibodies in complex with either SARS-CoV-2 spike or NTD. We analyzed the genetic basis of recognition for each of the seven antibodies, and further clustered them into antibody classes with similar genetics and modes of recognition. We also analyzed the antibody angles of approach and their recognized epitope. Remarkably, all seven antibodies targeted a single glycan-free surface of NTD, defining an NTD-antigenic supersite. We propose that all potentially neutralizing NTD-directed SARS-CoV-2 neutralizing antibodies might target this site.

Results

NTD-directed SARS-CoV-2 neutralizing antibodies

Prior studies have identified SARS-CoV-2-neutralizing antibodies that are S1-directed, but do not recognize RBD (Brouwer et al., 2020; Kreer et al., 2020; Rogers et al., 2020; Seydoux et al., 2020; Zost et al., 2020). Other studies have further delineated recognition and shown such antibodies to recognize NTD (Chi et al., 2020; Liu et al., 2020a; Zost et al., 2020). We identified a total of 17 published NTD antibodies, confirmed by the ELISA binding and competition data (Figure S1). We found that they derived from only 9 VH genes, with antibodies originating from five genes (VH1-24, VH1-69, VH3-30, VH1-8, and VH4-39) evident in multiple donors. While these observations were sparse, they raised the possibility that NTD-directed neutralizing responses in different individuals could involve the convergent development of similar antibodies.

Structures for seven such antibodies in complex with SARS-CoV-2 spike or isolated NTD are presented below, grouped by VH gene.

NTD-directed neutralizing antibodies derived from VH1-24 represent a multi-donor class

Four NTD-directed neutralizing antibodies identified from convalescent donors – antibodies 1-68 and 1-87 derived from donor ‘1’, and 2-51 derived from donor ‘2’ (Liu et al., 2020a) and antibody 4A8 from a third donor (Chi et al., 2020) all of which were encoded by the *VH1-24* gene (**Figure 1A**). In addition to utilizing the same VH gene, three of these antibodies, 1-68, 1-87, and 4A8 utilized an identical set of heavy chain-antibody genes – VH1-24, D6-19, and JH6 – and showed significant similarity in their heavy chain third-complementarity-

determining regions (CDR H3s), each of which was 21 amino acids in length (**Figure S2**).

Antibody 2-51 also utilized VH1-24, but utilized different D and J genes, D6-13 and JH4, encoding a shorter CDR H3 region of only 14 amino acids. These VH1-24-derived antibodies utilized four different VL-genes; 1-87, 2-51, and 1-69 utilized lambda light chains VL2-14, VL2-8, and VL2-18, respectively, while 4A8 utilized kappa light chain VK2-24.

We determined cryo-EM structures for the spike complexes with antibodies 1-68, 1-87, and 2-51 at overall resolutions of 3.8 Å, 3.63 Å, and 3.71 Å, respectively (**Figure 1B, Figure S3A and Table S1**). We also produced a locally refined cryo-EM map around the antibody:spike interface for 1-87 at 3.83 Å resolution, which allowed construction and refinement of an atomic model (**Figure 1C**). However, resolution in the antibody:spike interface region was blurred by domain motions for antibodies 2-51 and 1-68. We therefore produced crystals for 2-51 in complex with NTD, which provided an x-ray structure at 3.65 Å resolution (**Figure 1D and Table S2**).

Cryo-EM reconstructions of the VH1-24-derived 1-68, 1-87, and 2-51 antibodies each show a single Fab bound to the NTD of one subunit of the trimeric spike (**Figure 1B**). All antibodies target, with similar angle of approach, a single region on NTD – the loop region furthest from the spike-trimer axis. Moreover, the epitope and angle of approach for antibodies 1-68, 1-87 and 2-51 appear similar to those of antibody 4A8 (Chi et al., 2020), also derived from the VH1-24 gene.

Chi et al. (2020) defined the NTD loops in the 4A8-binding region as N1-N5 (corresponding to residue stretches 14 to 26, 67 to 79, 141 to 156, 177 to 186, and 246 to 260, respectively), and we adopt this nomenclature here. We note that the region defined as the N3 loop corresponds to a β -hairpin that includes both β -strands that form a stem region and a short

loop that connects them. The N1-N5 loops are disordered in most structures of spike, but some of these loops become ordered in antibody complexes. The structure of antibody 1-87 in complex with spike reveals almost all interactions to be mediated through heavy chain, with heavy chain accounting for 1057 Å² buried surface area and light chain 126 Å². The 19-residue CDR H3 loop provides the predominant interaction (**Figure 1C, middle panel**), with additional contributions mainly from CDR H1 (**Figure 1C, right panel**). CDR H3, which inserts between the N3 and N5 loops of NTD (**Figure 1C, left panel**) includes several hydrophobic residues (Ile96_{HC}, Val98_{HC}, Ile99_{HC}, Pro100_{bHC}, and Tyr100_{hHC}) which interact with aromatic residues in N3 including Tyr145_{NTD} and Trp152_{NTD}, and with hydrophobic residues of N5. Residues Ser100_{dHC} and Asp101_{HC} in CDR H3 also form hydrogen bonds with Trp152_{NTD} and Gly252_{NTD} respectively; the N-terminal glutamine residue of the heavy chain is also involved in hydrogen bonds with Ser254_{NTD} and Ser255_{NTD} in N5. Residues in CDR H1 form a network of hydrogen bonds involving positively charged residues from N3, Lys147_{NTD}, and N5, Arg246_{NTD}, with interactions by the side chains CDR H1 residues Tyr27_{HC} and Glu31_{HC}. Two additional VH1-24-gene specific glutamic acid residues – Glu53_{HC} in CDR H2 (**Figure S4A**), and framework residue Glu71_{HC} each participate in salt bridges with NTD. The only interaction mediated by the light chain is a hydrophobic interaction between Tyr49_{LC} in CDR L2 and Pro251_{NTD} in N5.

The crystal structure of antibody 2-51 in complex with NTD reveals recognition remarkably similar to that of 1-87. The 14-residue CDR H3 loop of 2-51 inserts between the N3 and N5 loops of NTD (**Figure 1E, left panel**) with additional interactions from CDR H1. CDR H3, includes three aromatic residues (Trp96_{HC}, Tyr98_{HC}, and Tyr102_{HC}), which interact with aromatic residues in N3 including Tyr145_{NTD} and Trp152_{NTD}, and with hydrophobic residues of N5 including Tyr248_{NTD}, Leu249_{NTD}, and Pro251_{NTD} (**Figure 1E, middle panel**). The heavy

chain Gln1_{HC} residue is involved in hydrogen bonds with Thr250_{NTD} and Pro251_{NTD} in N5, similar to the hydrogen-bonding pattern observed for 1-87. The only interaction mediated by the light chain is a hydrogen bond between Ser56_{LC} in CDR L2 and Asp253_{NTD} in N5. Residues of CDR H1 form a network of hydrogen bonds nearly identical to the network formed in the 1-87 crystal structure (**Figure 1E, right panel**). Comparison of the 1-87 and 2-51 structures reveals a comparable level of similarity with VH1-24-derived antibody 4A8.

Overall, their common derivation from a common gene and highly similar recognition show the VH1-24 antibodies to define a multi-donor antibody class. Overall, the interaction is dominated by conserved contacts in CDR H1, along with hydrophobic interactions mediated by CDR H3. The VH1-24 gene restriction is explained partly by the conserved interactions of CDR H1 including those of the VH1-24-specific Glu31, and VH1-24-specific residues Glu53 and Glu71 (**Figure S5A**).

NTD-directed neutralizing antibodies derived from VH3-30 and VH3-33 genes show distinct recognition

Three neutralizing antibodies directed against NTD have been reported that derived from the highly similar VH genes VH3-30 and VH3-33. The high similarity of these genes, which encode only two amino acid differences between them (**Figure 2A**), raised the possibility that these antibodies might represent a multi-donor class despite their derivation from two distinct genes. We therefore determined cryo-EM structures for spike complexes with antibodies derived from each gene: antibody 4-18 from VH3-30 and antibody 5-24 from VH3-33 at 2.97 and 3.93 Å resolutions, respectively (**Figure 2B-C, Figure S3B-C, and Table S1**).

The cryo-EM structure of antibody 4-18 in complex with spike reveals an epitope that overlaps the VH1-24 antibodies, but with a significantly different overall mode of recognition. Overall, interactions are primarily mediated by CDR H2 and CDR L3, with additional contributions from CDRs H3, L1, and L2 (**Figure 2D, left panel; Figure S4B**). While CDR H3 inserts between NTD loops N3 and N5 like VH1-24 antibodies, the light chain CDR L3 binds adjacent to this region and also forms interactions with the NTD N1 loop. CDR H2 mediates extensive hydrogen bonding and hydrophobic interactions with NTD (**Figure 2D, middle panel**). CDR H2 residues Ser55_{HC} and Asn56_{HC} form hydrogen bonds with both backbone and side chain of Asn17_{NTD} at the base of the NTD N17 glycan in the N1 region. CDR H2 residues Ser52_{HC} and His58_{HC} also form hydrogen bonds with Tyr248_{NTD} in N5. Hydrophobic interactions are observed for CDR H2 residue Val50_{HC} with Leu249_{NTD} and Tyr52_{aHC} with Pro251_{NTD} in N5. In the light chain, Tyr95_{bLC} from the CDR L3 loop hydrogen bonds with glycan N17 (**Figure 2D, right panel**) within the N-terminal region, which is typically disordered in ligand-free spikes.

Despite containing multiple aromatic residues in CDR H3, for the most part these residues do not form substantial hydrophobic interactions with residues from NTD, with exceptions Tyr98_{HC} and Tyr100_{HC}, which bury 98 Å² and 205 Å² accessible surface area in the interface, respectively (**Figure S4B, left panel**). Rather, the primary interactions mediated by CDR H3 are hydrogen bonds, including from the backbone carbonyl of Tyr98_{HC} with the backbone amide of NTD Ser247_{NTD} and a hydrogen bond from the side chain hydroxyl of Tyr100_{HC} with the side chains from NTD residues Glu156_{NTD} and Arg158_{NTD}.

The structure of VH3-33-derived antibody 5-24 (**Figure 2C**) in complex with spike reveals targeting of an overlapping epitope in NTD, but with overall recognition mediated by

CDR H3 with additional contributions from CDR H1, but without the involvement of CDR L3 as seen for antibody 4-18 (**Figure 2E, left panel**). Also different from 4-18, four aromatic residues in the CDR H3 region of antibody 5-24 make extensive hydrophobic contacts with NTD loop N5 and the stem of the N3 β -hairpin (**Figure 2E, middle panel**), distinct from the hydrogen bond-dominated recognition observed in 4-18; recognition by other CDRs is also different.

Overall, while they target overlapping regions in NTD, recognition by VH3-33-derived antibody 5-24 is substantially different from that mediated by VH3-30-derived antibody 4-18. These dissimilarities show that, despite their derivation from highly similar VH genes, the NTD-directed neutralizing antibodies from VH3-30 and VH3-33 are not members of a single antibody class. Structural analysis showed that Ser52_{HC} from VH3-30 forms a hydrogen bond with Tyr248_{NTD}. Substitution of Ser52_{HC} with the VH3-33-encoded Trp would lead to significant clashes with residues in CDR H2 and NTD loops N1 and N5 (**Figure S4C**), which could abolish the interaction between 4-18 and NTD. This suggests that the VH3-33 antibodies containing Trp52_{HC} cannot recognize NTD through a binding mode similar to VH3-30 antibody 4-18.

NTD-directed neutralizing antibodies derived from the VH1-69 gene appear to comprise both reproducible and distinct classes

Of the 17 currently characterized NTD-directed or likely NTD-directed neutralizing antibodies (**Figure S1A**), three – antibodies 2-17 and 4-8 (Liu et al., 2020a) and antibody COV2-2676 (Zost et al., 2020) derived from the VH1-69 gene, with antibodies 2-17 and 4-8 deriving from the VH1-69*01 and VH1-69*02 alleles, respectively (**Figures 3A**). Further, the CDR L3 regions of 2-17 and COV2-2676 showed high similarity (**Figure 3E**). To understand the recognition of these VH1-69-derived antibodies we determined cryo-EM structures for spike

complexes with the two most potent: antibodies 2-17 and 4-8 with IC_{50} potencies of 0.007 and 0.009 $\mu\text{g/mL}$.

Single-particle cryo-EM data for antibody 4-8 yielded a 3D reconstruction at 3.25 Å resolution (**Figure 3B, Figure S3D and Table S1**), however, like antibody 2-17 (**Figure 3C, Figure S3E and Table S1**), and as reported previously (Liu et al., 2020a), high mobility of the bound Fab blurred the interface region. We used local refinement with particle subtraction to obtain a high-quality reconstruction for the 4-8 interface with spike (**Figure 3D**). Like the VH1-24-derived antibodies, CDR H3 binds between the NTD N3 and N5 loops, but in a distinctive way; CDR H3 dominates the interface and its approach to the N3/N5 region is nearly orthogonal to that observed for CDR H3 in the VH1-24 class antibodies (**Figure 3D, middle panel**). Recognition by other CDRs is also distinct from the other NTD-directed neutralizing antibodies (**Figure 3D, right panel; Figure S4D**)

We also collected single-particle cryo-EM data for antibody 2-17, yielding a 3D reconstruction at 4.47 Å resolution (**Figure 3C, Figure S3E and Table S1**). Despite the poor resolution of the 2-17 cryo-EM maps, we were able to locally refine the interface region and model the antibody in complex with NTD as C α chains.

We compared the heavy and light chain epitope footprints on NTD for antibodies 4-18 (**Figure 3B, right panel**) and 2-17 (**Figure 3C, right panel**). Notably, the orientation of the heavy and light chains between 2-17 and 4-8 were rotated ~90 degrees from each other, indicating different modes of recognition, and showing that they are of different classes.

We next asked whether other NTD-directed neutralizing antibodies derived from VH1-69 heavy chain might be members of the 2-17 or 4-8 classes. Analysis of light chains indicated antibodies 2-17 and COV2-2676, derived from genes KV3-15 and KV3-11, respectively, to be

remarkably similar in their light chain CDR L3 regions suggesting that 2-17 and COV2-2676 might be of the same class. Overall, we observed NTD-directed neutralizing antibodies from VH1-69 to form at least two classes, with similarity of light chains suggesting antibodies 2-17 and COV2-2676 might represent a reproducible class observed in two different donors.

Functional requirements for somatic hypermutation (SHM)

Sequence analyses showed that all NTD-directed antibodies accumulate somatic hypermutations in their paratope regions (**Figures 1A, 2A, and 3A**). To help understand characteristics of the antibody precursors, we reverted the paratope-region somatic hypermutations observed in seven NTD-directed antibodies (1-87, 1-68, 2-51, 4-18, 5-24, 4-8, and 2-17) to their respective germline residues. Overall, these germline-reverted antibodies showed substantially reduced binding affinities and neutralization potencies (**Figure S6**). For the VH1-24 multi-donor antibody class, antibodies 1-87, 1-68, and 2-51 shared two convergent somatic hypermutations (T30I and G55A/V) in heavy chain, reversion of which showed significantly (~4-20-fold) reduced binding affinity (**Figure S6A-B**). For antibodies 4-18 and 5-24, derived from VH3-30 and VH3-33, respectively, reversion of SHMs in combination in each antibody nearly abolished neutralization (**Figure S6C**). For the two VH1-69-derived antibodies 2-17 and 4-8, neutralization was improved by SHMs by ~20- and ~8-fold, respectively. Thus, all of the NTD-directed potently neutralizing antibodies we tested required affinity maturation to achieve high binding affinity and high potency. The VH3-30 and VH3-33 antibodies were more sensitive to SHMs than the antibodies derived from other VH genes. Nonetheless, antibodies corresponding to the initial recombinants, with reversion of all paratope SHMs in combination, could still bind to spike with apparent IgG K_{DS} ~2-70 nM, suggesting that precursor B cells of

the NTD antibodies are likely to be efficiently activated by spike binding. Gene-specific substitution profiles (Sheng et al., 2017) showed that the observed SHMs are each generated by the somatic hypermutation machinery with high frequencies (**Figures 1A, 2A, and 3A**), suggesting that requirements for SHM are unlikely to present a significant barrier to antibody development.

NTD-directed potently neutralizing antibodies have similar angles of approach

To gain an overall understanding of the angle of antibody approach to the spike by these NTD-directed potently neutralizing antibodies, we determined their angles of approach around a latitudinal axis to define freedom between viral and host cell membranes, and around a longitudinal axis to define freedom within the plane of the membrane. Relative to the viral spike, the latitudinal axis is perpendicular to the trimer axis, and the longitudinal axis is parallel to this axis. Latitudinal and longitudinal approach angles among the NTD-neutralizing antibodies were similar – with antibodies approaching spike with antigen-combining surface oriented towards the viral membrane (**Figure 4A-C**).

We also analyzed the heavy-light chain orientations in the complexes with spike (**Figure 4D**). Here the NTD-directed antibodies differed, with three of the antibodies, 4-18 from VH3-30 and 2-17 and 4-8 from VH1-69, showing heavy and light chain angles of approach that differed from the other five antibodies. Thus, while the heavy/light orientation relative to spike could differ substantially, lesser differences were observed in latitudinal and longitudinal angles of approach, with all NTD-neutralizing antibodies approaching spike from “above” with their antigen-binding surfaces oriented towards the viral membrane.

NTD-directed antibodies induce conformational changes in NTD and spike

To gain insight into the impact of antibody recognition on the conformation of NTD, we superimposed antibody-spike or antibody-NTD complexes onto the NTD domain, and examined the structural alteration in NTD versus NTD in the ligand-free spike (PDB: 6ZGE), calculating the per-residue C α movement between bound and ligand-free (**Figure 5A**), which ranged as high as 16-18 Å for most of the NTD-directed antibodies, though 4-8 (10.1 Å) and 5-24 (11.7 Å) were somewhat lower. The largest structural change occurred in the N3 β -hairpin, although the mobile N1 and N5 loops also showed large deviations (**Figure 5B**). In general, the regions of NTD that moved were contacted by antibody (**Figure 5C**), indicating the conformational changes were a direct consequence of antibody binding. In addition to the conformational change induced in NTD, we observed other changes in spike. Notably, the 4-18 antibody bound spike was substantially better ordered than the other NTD-bound spikes (achieving a nominal cryo-EM resolution of 2.97 Å, which was ~1 Å better than most of the other complexes). Examination of the 4-18 bound spike indicated almost 40° rotation in the central S2 triple helical bundle (**Figure S4E**).

Overall, binding of NTD-directed antibodies induced substantial structural rearrangements, not only in recognized loops but also of the N3- β -hairpin. The higher immunogenicity observed with flexible regions likely stems from increased accessibility due to lower glycan-coverage as well as the ability of these regions to assume distinct conformations required for diverse antibodies to bind, with the recognized site on NTD apparently exemplifying this effect.

The NTD supersite

To define the spike surface recognized by potent NTD-directed neutralizing antibodies, we analyzed the epitopes for all eight of the NTD-directed neutralizing antibodies with defined structures, the seven described in this study as well as antibody 4A8, described previously (Chi et al., 2020) (**Table S3**). These ranged in potency from remarkably potent 2-17 and 2-51 antibodies with IC_{50} of 0.007 $\mu\text{g/mL}$ to the neutralizing, but substantially less potent 4A8 with IC_{50} of 0.39 $\mu\text{g/mL}$; all eight of these antibodies recognize overlapping epitopes on NTD (**Figure 6A**). Antigenic sites containing epitopes for genetically diverse antibodies have been widely denoted as antigenic ‘supersites’ in prior studies involving influenza virus and HIV antibodies (Kong et al., 2013; Kumar et al., 2020; Lee and Wilson, 2015; Longo et al., 2016; Moyo et al., 2020; Zhou et al., 2015; Zhou et al., 2014), we define the spike surface recognized by at least two antibodies, from different classes, of these eight potent NTD-directed antibodies, as the NTD supersite (**Figure 6B and Tables S4-S5**).

The NTD supersite was located at the periphery of the spike, distal from the 3-fold axis, and facing away from the viral membrane. This surface was surrounded by four glycans, *N17*, *N74*, *N122*, and *N149*, and nominally ‘glycan free’, although molecular dynamics simulations with fully-glycosylated spike indicated some glycan-coverage, though less than adjacent regions more proximal to the spike 3-fold (**Figure 6C**).

To gain insight into the structural features of the NTD supersite, we first analyzed the distribution of epitopes versus potency, but did not observe substantial variation in potency over the NTD supersite (**Figure 6D, left panel**). In addition, we measured Fab affinity to spike (**Table S6**), but found no correlation with potency. Electrostatic surface analysis revealed the supersite to have strong positive electrostatic potential (**Figure 6D, middle panel**), while recognizing antibodies had complementary strong electronegative potential (**Figure S7**).

With respect to recognized conformation, we compared the ligand-free conformation of the supersite versus its antibody-bound conformation; the recognized β -strands at the center of the epitope were displaced ~ 3 Å, with loops N1, N3 and N5 moving substantially more, up to 18 Å (**Figure 6D, right panel**). With respect to correlation with potency, we observed the magnitude of induced NTD conformational change to trend inversely with potency. This is not surprising in that the requirement for conformational change is likely to lower the energy of binding. Notably, antibodies that induced larger conformational changes were also more electronegative, potentially providing an explanation for the observation that increasing negative charge trended with reduced potency.

Overall, the NTD supersite comprised a structurally plastic surface, formed primarily by the N3 β -hairpin and including other flexible regions such as the N1 and N5 loops. This surface was also both glycan-free and highly electropositive – and facing away from the electronegative viral membrane.

NTD supersites in other betacoronaviruses

To understand the generality of the single-NTD supersite that we observe for SARS-CoV-2, we examined the recognition of NTD-directed neutralizing antibodies targeting other betacoronaviruses. Searches of the PDB found only two NTD-directed antibodies targeting other betacoronaviruses: these two antibodies, G2 (Wang et al., 2018) and 7d10 (Zhou et al., 2019), both neutralized MERS and targeted overlapping glycan-free surfaces on NTD facing away from the viral membrane (**Figure 7A**). The epitopes for both of these antibodies partially overlapped the analogous surface comprising the NTD-supersite in SARS-CoV-2, but were more centrally located and more proximal to the spike 3-fold.

Quantification of the average number of proximal glycan atoms indicated lower glycan density over the NTD-supersite than over the equivalent surfaces recognized by G2 and 7d10 antibodies. The epitopes recognized by these two antibodies also showed substantially less conformational mobility. Thus, potent NTD-directed neutralizing antibodies targeting SARS-CoV-2 preferentially recognized a less glycosylated, more flexible region than the analogous surfaces recognized by NTD-directed antibodies neutralizing MERS.

Since the MERS and SARS-CoV-2 directed antibodies both targeted glycan-free sites on NTD facing away from the viral membrane, we sought to understand the properties of the equivalent surfaces of other coronavirus spikes. We calculated the sequence divergence of other betacoronaviruses and mapped this to the NTD surface, which showed high diversity in sequence (**Figure 7B**). We modeled the sequence-predicted glycans on the HKU1 spike structure (PDB: 5I08) and examined the location of glycans on HKU1 NTD (**Figure 7C**). Notably glycan N171 was observed to be directly the region of overlap between the equivalent positions of the SARS-CoV-2 supersite, 7d10 and G2 epitopes. Thus, the presence of glycans may impact the presence or absence of NTD-sites of vulnerability in betacoronaviruses.

Discussion

Antibodies directed to NTD and to RBD can neutralize with high potency (less than 0.01 $\mu\text{g/mL}$ IC_{50}). While RBD shows many non-overlapping sites of vulnerability to antibody (Barnes et al., 2020a; Brouwer et al., 2020; Lv et al., 2020; Pinto et al., 2020b; Yuan et al., 2020a), NTD appears to contain only a single site of vulnerability to neutralization. As discussed above, one reason for this may be the high glycan-density on NTD, with 8 *N*-linked glycans in ~300 residues, a density of one glycan per ~40 residues, and few glycan-free surfaces that can be

easily recognized by the immune system. A second reason may be the restricted approach angle which we observed for all known NTD-directed neutralizing antibodies, including the seven reported here, which all approach spike from “above”. We note in this context that competition analysis indicates other NTD-directed antibodies capable of recognizing spike and forming a separate competition group to be non-neutralizing (Liu et al., 2020) – and the other large surface on NTD that is exposed on spike faces toward the viral membrane. This surface is mostly glycan-free, and antibodies binding to it would be required to approach from “below”. Thus, unlike RBD, where neutralizing antibodies appear to have diverse approach angles, the presence of only a single- NTD site of vulnerability may relate to the requirement to approach from “above.”

In addition to satisfying requirements stemming from the restricted approach angle, the higher relative prevalence of NTD-supersite-directed antibodies is likely to stem from increased immunogenicity due to both the lower relative glycan density of the supersite, as well as the flexible nature of the N3 hairpin and N5 loop primary recognition regions, and their ability to assume distinct conformations that allow for recognition by diverse antibodies. In the case of the multi-donor VH1-24 antibody class, which arises from the most prevalent VH-gene utilized (**Figure S1A**), two additional NTD-directed neutralizing class members have recently been identified, FC05 and CM25 (Voss et al., 2020; Wang et al., 2021). We found VH1-24 to be the most negatively charged human VH gene (**Figure S5B**). Such negative electrostatic potential complements the highly electropositive nature of the NTD supersite that we observe here (**Figure 6D**). Thus, multiple factors including epitope glycosylation and flexibility, restrictions on approach angle, and paratope charge complementarity can contribute to the prevalence of antibodies targeting the NTD supersite.

Although the approach to the spike from “above” observed for all NTD-directed neutralizing antibodies is consistent with a neutralization mechanism based on steric hindrance of spike interaction with ACE2 receptor at the cell membrane, there is currently no evidence for competition between NTD-directed antibodies and ACE2 (Liu et al., 2020a). A plausible alternative model would be for antibody recognition of the NTD supersite to impede spike function in mediating fusion of virus and host cell membranes. Indeed, protease-resistance analysis of MERS spike in complex with MERS NTD-directed neutralizing antibody 7d10 showed that 7d10 binding prevented increased protease sensitivity associated with the prefusion-to-postfusion transition (Zhou et al., 2019). A conformational stabilization mechanism could also explain how an antibody that binds only one subunit per spike trimer could achieve effective neutralization. Further studies will be required to understand mechanisms of neutralization for antibodies that recognize the NTD-supersite.

With respect to vaccine implications, our results clearly identify the NTD site of vulnerability most likely to elicit neutralizing antibodies. There are many ways that this information can be incorporated into vaccine design, including the inclusion of NTD along with RBD in vaccine formulations, the multivalent display of the NTD supersite on nanoparticle immunogens, and epitope-focusing through the creation of scaffolds displaying the N3- β -hairpin and other regions of recognized by NTD-directed neutralizing antibodies.

With respect to the therapeutic potential of NTD-directed antibodies, these target a site that is remote from those targeting RBD sites and thus should provide complementary neutralization to RBD-directed antibodies and require distinct escape pathways. The fact that all, or a great majority, of NTD-neutralizing antibodies target a single site, however, suggests there may be little utility to utilizing combinations of NTD-directed neutralizing antibodies.

Finally, new mutant SARS-CoV-2 strains, particularly those emerged in the UK and South Africa (strains B.1.1.7 and B.1.351, respectively) are concerning due to increased transmissibility, and these strains escape most NTD-directed neutralizing antibodies. B.1.1.7 includes NTD deletion mutations $\Delta 69-70$ and $\Delta 144$, and strain B.1.351 includes NTD mutations $\Delta 242-244$ and R246I. Consistent with our findings, the mutated positions including 144, 242-244, and 246 are all within the NTD supersite. While the deletion at 69-70 is outside of the supersite, it forms part of the hairpin N2 loop of NTD, its deletion could significantly impact the conformation of the NTD supersite. Notably, only three residues were shared among the eight NTD-directed neutralizing antibody epitopes analyzed here: Y144, R246, and L249 (**Table S5**). Interestingly, two of these three residues are the exact residues mutated in emerging variants of concern ($\Delta 144$ and R246I), and L249 is likely affected by $\Delta 242-244$. Thus, the flipside of a single supersite is that variation of the supersite may induce resistance against most of the antibodies targeting the site – and be selected for among emerging variants.

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the US Department of Energy, Basic Energy Sciences, Office of Science, under contract number W-31-109-Eng-38. Support for this work was provided by the Intramural Research Program of the Vaccine Research Center, National Institute of Allergy and Infectious Diseases (NIAID). Support for this work was also provided by Samuel Yin, Pony Ma, Peggy & Andrew Cherng, Bii Biosciences, Jack Ma Foundation, JBP Foundation, Carol Ludwig, and Roger & David Wu, COVID-19 Fast Grants, the Self Graduate Fellowship Program, and NIH grants DP5OD023118, R21AI143407, and R21AI144408. Some of this work was performed at the Columbia University Cryo-EM Center at the Zuckerman Institute, and some at the Simons Electron Microscopy Center (SEMC) and National Center for Cryo-EM Access and Training (NCCAT) located at the New York Structural Biology Center, supported by grants from the Simons Foundation (SF349247), NYSTAR, and the NIH National Institute of General Medical Sciences (GM103310).

Author Contributions

GC led the determination of 5 cryo-EM structures shown in Fig. 1-3, YG provided sequence analyses defining classes in Fig. 1-3, TZ led analysis for Fig. 4-7, JG carried out map refinement and model building for 4-8 and 2-17 structures with spike, ML provided glycan-masking analysis for supersite, correlations for supersite properties and threading of diverse spikes, MR led determination of 1 cryo-EM structure, ER led crystal structure determination of 2-51 with isolated NTD, JY provided antibodies for structural analysis, FB provided spike, mutant spikes and stabilized spikes, JB provided almost all Fabs, YH, LL, MSN and PW carried out antibody reversion analyses, YH and MSN performed authentic virus neutralization, PSK determined SPR affinity measurements, RR assisted with glycan density calculations, ASO provided spike, REA provided MSD trajectories for

glycosylated spike, BZ provided antibodies, GYC supervised informatics analysis and assisted with antibody structural threading and docking, DDH supervised IgG production and reversion analysis, ZS supervised sequence analysis, PDK supervised structural analyses, and LS supervised structural determinations and led the overall project.

Declaration of Interests

DDH, YH, JY, LL, MSN and PW are inventors of a patent describing some of the antibodies reported on here.

Main Figure Titles and Legends

Figure 1. NTD-directed neutralizing antibodies derived from the VH1-24 gene define a multi-donor antibody class.

- (A) Sequence alignment of VH1-24-derived NTD-directed antibodies showing paratope residues, somatic hypermutations, and gene-specific substitution profile (GSSP) showing somatic hypermutation probabilities for VH1-24 gene. Antibody positions are assigned using the Kabat scheme, the CDRs are assigned by IMGT scheme. Paratope residues are highlighted by underscoring and colored by interaction types. Amino acids in GSSP are colored by chemical property.
- (B) Cryo-EM reconstructions for spike complexes with antibodies 1-87, 1-68, and 2-51. NTD is shown in orange, RBD in green, glycans in red, with antibody heavy chains in magenta and light chains in gray.
- (C) Expanded view of 1-87 interactions with NTD showing overall interface (left panel), recognition by CDR H3 (middle panel), and recognition by CDR H1 (right panel). NTD regions N3 (residues 141-156) and N5 (residues 246-260) are colored in shades of orange; CDR H1, H2, H3 are colored in shades of magenta; CDR L1, L2, and L3 are colored in shades of gray. Nitrogen atoms are colored in blue, oxygen atoms in red; hydrogen bonds (distance $<3.2 \text{ \AA}$) are represented as dashed lines.
- (D) Crystal structure of antibody 2-51 complexed with NTD, colored as in (B).
- (E) Expanded view of 2-51 interactions with NTD showing overall interface (left panel), recognition by CDR H3 (middle panel), and recognition by CDR H1 (right panel), colored as in (C).

See also Figures S1-S7, Table S1 and Table S2.

Figure 2. NTD-directed neutralizing antibodies derived from the closely related VH3-30 and VH3-33 genes show distinct binding modes.

- (A) Sequence alignment of VH3-30-derived (4-18) and VH3-33-derived (5-24) NTD-directed antibodies showing paratope residues, somatic hypermutations, and gene-specific substitution profile (GSSP) showing positional somatic hypermutation probabilities for VH3-30 gene. Substitutions between VH3-30 and VH3-33 germline genes are highlighted in green.
- (B) Cryo-EM reconstruction for spike complex with antibody 4-18 from two orthogonal views; NTD is shown in orange, RBD in green, glycans in red, with antibody heavy chain in blue and light chain in gray.
- (C) Cryo-EM reconstruction for spike complex with antibody 5-24 from two orthogonal views; NTD is shown in orange, RBD in green, glycans in red, with antibody heavy chain in brown and light chain in gray.
- (D) Expanded view of 4-18 interactions with NTD showing the overall interface (left panel), recognition in CDR H2 (middle panel), and recognition in CDR L3 (right panel). NTD regions N1 (residues 14-26), N3 (residues 141-156) and N5 (residues 246-260) are shown in shades of orange; CDR H1, H2, H3 are shown in shades of blue; CDR L1, L2, and L3 are shown in shades of gray.
- (E) Expanded view of 5-24 interactions with NTD showing the overall interface (left panel), recognition in CDR H3 (middle panel), and recognition in CDR H1 (right panel), colored as in (D) except for CDR H1, H2, H3, which are colored in shades of brown.

See also Figure S1, Figure S3, Figure S6 and Table S1.

Figure 3. NTD-directed neutralizing antibodies derived from the closely related VH1-69*01 and VH1-69*02 genes show distinct binding modes.

- (A) Sequence alignment for VH1-69*01-derived (2-17) and VH1-69*02-derived (4-8) NTD-directed antibodies showing somatic hypermutations and paratope residues, with gene-specific substitution profile (GSSP) showing positional somatic hypermutation probabilities for VH1-69. Residues that differ between VH1-69*01 and VH1-69*02 alleles are highlighted in green.
- (B) Cryo-EM reconstruction for spike complex with antibody 4-8; NTD is shown in orange, RBD in green, glycans in red, with antibody heavy chain in teal and light chain in gray. Heavy and light chain footprint on NTD (right panel).
- (C) Cryo-EM reconstruction for spike complex with antibody 2-17 (left panel); NTD is shown in orange, RBD in green, glycans in red, with antibody heavy chain in dark green and light chain in gray. Heavy and light chain footprint on NTD (right panel, NTD shown with template-based modeling).
- (D) Expanded view of 4-8 interactions with NTD showing the overall interface (left panel), recognition in CDR H3 (middle panel), and recognition in CDR L2 (right panel). NTD regions N1 (residues 14-26), N3 (residues 141-156) and N5 (residues 246-260) are shown in shades of orange; CDR H1, H2, H3 are shown in shades of teal; CDR L1, L2, and L3 are shown in shades of gray.
- (E) Sequence alignment of light chain of VH1-69-derived antibodies showing diverse germline gene usage (2-17 and COV2-2676 utilizes kappa light chain, 4-8 utilizes lambda light chain), IGKV3-15*01 is used as reference. Paratope residues of 4-8 are colored as in (A).

See also Figure S1, Figure S3, Figure S6 and Table S1.

Figure 4. Angles of approach for NTD-directed neutralizing antibodies.

- (A) Overall approach of NTD-directed neutralizing antibodies to spike with angles defined with red arrows. Three-fold axis is indicated by a black triangle. Antibodies are represented by long axes of the Fabs and colored by heavy chain colors defined in Figures 1-3.
- (B) Latitudinal and longitudinal angles of approach.
- (C) Angles of recognition for antibodies grouped by VH-gene. Notably, only those from VH1-24 show a consistent orientation.
- (D) Heavy-light chain orientations show graphically (left) and quantitatively (right).

Figure 5. NTD-directed antibodies induce conformational changes in NTD and spike.

- (A) Conformational changes in NTD induced by binding of neutralizing antibodies. Antibody-bound NTDs are shown in cartoon representation and colored by per residue $C\alpha$ movements compared to unliganded NTD. Antibodies are shown in gray cartoon. Major NTD loops interacting with antibodies are labeled.
- (B) Sequence of NTD highlighting antibody contact and conformational change. Epitope residues for each antibody are marked with a number representing $C\alpha$ movements (\AA) from unliganded NTD, the symbol “X” indicates movement 10 \AA and above. Potential glycosylation sites on NTD are highlighted in green (distances are shown for antibodies with sufficiently resolved interfaces; antibody 2-17 was only at 4.4 \AA , and the interface of antibody 1-68 showed extensive mobility).

(C) Epitope regions on NTD (red) and their conformational change. Glycans on NTD are shown as green spheres.

Figure 6. A structurally plastic antigenic supersite in the distal-loop region of NTD revealed by comparison of antibodies derived from the four multi-donor classes.

- (A) Epitopes of NTD-targeting antibodies colored by potency (cryo-EM structures were of sufficient resolution to define all epitopes, except for 2-17 and 1-68, which utilized polyAla-based template modeling and homology modeling respectively). Epitope residues are listed in Table S3.
- (B) The supersite of vulnerability on NTD. Supersite residues are listed in Table S5.
- (C) Glycan coverage of the spike. The NTD supersite is surrounded by glycans at *N17*, *N74*, *N122* and *N149*.
- (D) NTD structural properties and antibody potency. Epitope surfaces of different antibodies were overlaid onto NTD with shades of red representing potency (left). Electrostatic potential on NTD (middle). Structural variation of NTD bound by NTD-directed antibodies (right). See also Figure S1, Figure S6, Figure S7 and Table S3-S6.

Figure 7. NTD supersite on MERS betacoronaviruses.

- (A) Epitopes of MERS NTD antibodies target a site closer to the trimer axis. Borders of epitopes of antibody G2 and 7d10 are colored teal and cyan, respectively. SARS-CoV-2 NTD supersite is shown as red boundary line. Glycans are shown as green spheres.
- (B) Spike sequence entropy between betacoronaviruses.
- (C) NTD of HKU1 spike is substantially glycosylated.

STAR★METHODS

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RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Lawrence Shapiro (lss8@columbia.edu).

Materials Availability

Expression plasmids generated in this study for expressing SARS-CoV-2 proteins and antibody mutants will be shared upon request.

Data and Code Availability

The cryo-EM structures and the crystallographic structure are in the process of being deposited to the Electron Microscopy Data Bank (EMDB) and the Protein Data Bank (RCSB PDB).

Cryo-EM structural models and maps of NTD-directed antibodies in complex with SARS-CoV-2 spike have been deposited in the PDB and EMDB for antibodies 1-87 (PDB:7L2D, EMDB: EMD-23125), 2-17 (PDB: 7LQW, EMDB: EMD-23490), 4-8 (PDB: 7LQV, EMDB: EMD-23489), 4-18 (PDB:7L2E, EMDB: EMD-23126) and 5-24 (PDB: 7L2F, EMDB: EMD-23127); cryo-EM maps have been deposited for antibodies 1-68 (EMDB: EMD-23150) and 2-51 (EMDB: EMD-231251). The crystallographic structure of antibody 2-51 in complex with SARS-CoV-2 spike NTD has been deposited in the PDB with accession code 7L2C.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines

FreeStyle 293-F (cat# R79007), Expi293F cells (cat# A14635) were from Thermo Fisher Scientific. HEK293S GnTI- (cat# CRL-3022), HEK 293T/17 (cat# CRL-11268), I1 mouse hybridoma (cat# CRL-2700) and Vero E6 cells (cat# CRL-1586) were from ATCC.

FreeStyle 293-F cells and were cultured in serum-free FreeStyle 293 Expression Medium (Gibco, cat# 12338026) at 37 °C, 10% CO₂, 115 rpm. HEK 293S GnTI- cells were cultured in FreeStyle 293 Expression Medium at 37 °C, 10% CO₂, 115 rpm. Expi293F cells were cultured in Expi293 Expression Medium (Gibco, cat# A14635) at 37 °C, 8% CO₂, 125 rpm. HEK 293T/17 cells and Vero E6 cells were cultured in 10% Fetal Bovine Serum (FBS, Gibco cat# 16140071)

supplemented Dulbecco's Modified Eagle Medium (DMEM, ATCC cat# 30-2002) at 37 °C, 5% CO₂. Cell lines were not specifically authenticated.

METHOD DETAILS

Protein samples expression and purification

The SARS-CoV-2 S2P and HexaPro spike variant constructs were produced as described in Wrapp et al., 2020 and in Hsieh et al., 2020 respectively. They were expressed in Human Embryonic Kidney (HEK) Freestyle 293-F cells (Invitrogen) in suspension culture using serum-free media (Invitrogen) and transfected into HEK293 cells using polyethyleneimine (Polysciences). Cell growths were harvested four days after transfection, and the secreted proteins were purified from supernatant by nickel affinity chromatography using Ni-NTA IMAC Sepharose 6 Fast Flow resin (GE Healthcare) followed by size exclusion chromatography on a Superdex 200 column (GE Healthcare) in 10 mM Tris, 150 mM NaCl, pH 7.4.

The N-terminal domain of SARS-CoV-2 spike (NTD, residues 1-330) was cloned into the pVRC-8400 mammalian expression plasmid, with a C-terminal 6X-His-tag cleavable by HRV-3C protease. The NTD construct was transiently transfected into HEK293 GnTI- cells suspension culture in serum-free media using polyethyleneimine. Four days after transfection, the secreted protein was purified using Ni-NTA IMAC Sepharose 6 Fast Flow resin followed by size exclusion chromatography on a Superdex 200 column in 10 mM Tris, 150 mM NaCl, pH 7.4. Fractions containing NTD were combined and 1% (w/w) HRV-3C protease (Thermo fisher) was added to remove the C-terminal His-tag, followed by incubation for 24 hrs at 4 °C. Inverse IMAC using Ni-NTA resin was then performed to purify NTD from the His-tag and residual

uncleaved protein. Enzymatic deglycosylation of NTD was carried out by adding 2.5 μ L Endo Hf (NEB) per 20 μ g of NTD and incubating for 24 hrs at 25 °C; a second round of SEC was performed to remove excess Endo Hf and to exchange buffer in 10 mM Tris, 150 mM NaCl, pH 7.4. Protein purity was analyzed by SDS-PAGE at every step.

NTD-directed monoclonal antibodies 1-68, 1-87, 2-17, 2-51, 4-8, 4-18 and 5-24 were expressed and purified as described in (Liu et al., 2020a). Fabs fragments were produced by digestion of IgGs with immobilized papain at 37 °C for 3 hrs in 50 mM phosphate buffer, 120 mM NaCl, 30 mM cysteine, 1 mM EDTA, pH 7. The resulting Fabs were either purified from Fc by affinity chromatography on protein A (1-68, 1-87, 2-17, 2-51 and 4-8) or used as Fab/Fc mixture (4-18 and 5-24). Fab purity was analyzed by SDS-PAGE; all Fabs were buffer-exchanged into 10 mM Tris, 150 mM, pH 7.4 for crystallization and cryo-EM experiments.

Antibody mutagenesis

For each antibody, variable genes were optimized for human cell expression and synthesized by GenScript. VH and VL were inserted separately into plasmids (gWiz or pcDNA3.4) that encoding the constant region for heavy chain and light chain. Monoclonal antibodies were expressed in Expi293F (ThermoFisher, A14527) by co-transfection heavy chain and light chain expressing plasmids using polyethylenimine (PEI, Linear, MV~25,000, Polysciences, Inc. Cat. No. 23966) and culture in 37 °C degree shaker at 125 rpm and 8% CO₂. Supernatants were collected on day 5, antibodies were purified by rProtein A Sepharose (GE, 17-1279-01) affinity chromatography.

Antibody gene mutations were introduced by QuikChange II site directed mutagenesis kit (Agilent, Cat. No. 200524).

Antibody Fab binding affinity measurement by surface plasmon resonance

SPR binding assays for Fabs were performed using a Biacore T200 biosensor, equipped with a Series S CM5 chip, in a running buffer of 10 mM HEPES pH 7.4, 150 mM NaCl, 0.1 mg/mL BSA and 0.01% (v/v) Tween-20 at 25 °C.

HexaPro Spike was captured through its C-terminal his-tag over an anti-his antibody surface. These surfaces were generated using the His-capture kit (Cytiva, MA) according to the instructions of the manufacturer, resulting in approximately 10,000 RU of anti-his antibody over each surface. HexaPro was captured over a single flow cell at a capture level of 500-800RU with Fabs with higher KDs (2-17 and 4-18) requiring higher capture levels. An anti-his antibody surface was used as a reference flow cell to remove bulk shift changes from the binding signal.

Fabs were tested using a three-fold dilution series ranging from 2.96-240 nM, except for Fabs 4-18 and 2-17, which were analyzed at concentrations of 8.88-720 nM. The association and dissociation rates were each monitored for 120 s and 600 s respectively, at 50 μ L/min. The bound HexaPro/Fab complex was regenerated from the anti-his antibody surface using a 10s pulse of 15 mM H_3PO_4 at a flow rate of 100 μ L/min, followed by a 60 s buffer wash at the same flow rate. Each Fab was tested in order of increasing protein concentration, in duplicate. Blank buffer cycles were performed by injecting running buffer instead of Fab to remove systematic noise from the binding signal. The data was processed and fit to 1:1 single cycle model using the Scrubber 2.0 (BioLogic Software). For each parameter reported, the number in brackets represents the error of the fit in the last significant figure.

Full IgG binding affinity measurements by surface plasmon resonance

The mammalian expression vector that encodes the ectodomain of the SARS-CoV-2 S trimer for full IgG binding affinity measurement was kindly provided by Dr. Jason McLellan (Wrapp et al., 2020b). SARS-CoV-2 S trimer expression vector was transiently transfected into Expi293 cells using 1 mg/mL of polyethylenimine (Polysciences). Five days post transfection, the S trimer was purified using Strep-Tactin XT Resin (Zymo Research).

The binding affinities of full IgG antibodies to SARS-CoV-2 spike protein were determined using surface plasmon resonance (SPR) and a BIAcore T200 instrument (GE Healthcare) at 25 °C. The anti-his antibody was first immobilized onto two different flow cells of a CM5 sensorchip (BR100030, Cytiva) surface using the His Capture Kit (28995056, Cytiva) according to the manufacturer's protocol. The His-tagged SARS-CoV-2 spike protein was then injected and captured on flow cells 2. Flow cells 1 was used as the negative control. A three-fold dilution series of antibodies with concentrations ranging from 300 nM to 1.2 nM were injected over the sensor surface for 30 s at a flow rate of 10 μ L/minute. The dissociation was monitored for 300 s and the surface was regenerated with 10 mM Glycine pH 1.5 (BR100354, Cytiva). The running and sample buffer is 10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% P-20 (HBS-EP+ buffer, BR100826, Cytiva). The resulting data were fit to a 1:1 binding model using Biacore Evaluation Software and were plotted using Graphpad.

Pseudoviruses neutralization assays

Recombinant Indiana VSV (rVSV) expressing SARS-CoV-2 spikes were generated as previously described. HEK293T cells were grown to 80% confluency before transfection with pCMV3-SARS-CoV-2-spike (kindly provided by Dr. Peihui Wang, Shandong University, China) using FuGENE 6 (Promega). Cells were cultured overnight at 37 °C with 5% CO₂. The next day,

medium was removed and VSV-G pseudo-typed Δ G-luciferase (G* Δ G-luciferase, Kerafast) was used to infect the cells in DMEM at a MOI of 3 for 1 hr before washing the cells with 1X DPBS three times. DMEM supplemented with anti-VSV-G antibody (I1, mouse hybridoma supernatant from CRL-2700; ATCC) was added to the infected cells and they were cultured overnight as described above. The next day, the supernatant was harvested and clarified by centrifugation at 300g for 10 min and aliquots stored at -80 °C.

Neutralization assays were performed by incubating pseudoviruses with serial dilutions antibodies, and scored by the reduction in luciferase gene expression. In brief, Vero E6 cells were seeded in a 96-well plate at a concentration of 2×10^4 cells per well. Pseudoviruses were incubated the next day with serial dilutions of the test samples in triplicate for 30 mins at 37 °C. The mixture was added to cultured cells and incubated for an additional 24 hrs. The luminescence was measured by Britelite plus Reporter Gene Assay System (PerkinElmer). IC₅₀ was defined as the dilution at which the relative light units were reduced by 50% compared with the virus control wells (virus + cells) after subtraction of the background in the control groups with cells only. The IC₅₀ values were calculated using non-linear regression in GraphPad Prism.

Authentic SARS-CoV-2 microplate neutralization

An end-point dilution assay in a 96-well plate format was performed to measure the neutralization activity of purified mAbs. In brief, each antibody was serially diluted (5-fold dilutions) starting at 50 μ g/mL. Triplicates of each mAb dilution were incubated with SARS-CoV-2 at an MOI of 0.1 in EMEM with 7.5% inactivated fetal calf serum (FCS) for 1 hour at 37 °C. Post incubation, the virus-antibody mixture was transferred onto a monolayer of Vero E6 cells grown overnight. The cells were incubated with the mixture for 70 hours. Cytopathic effect

(CPE) of viral infection was visually scored for each well in a blinded fashion by two independent observers. The results were then converted into percentage neutralization at a given mAb concentration, and the averages \pm SEM were plotted using a five-parameter dose-response curve in GraphPad Prism v8.0.

Antibody gene assignments and genetic analyses

The 17 SARS-COV-2 neutralizing antibodies were collected from seven publications. We annotated these antibodies using IgBLAST-1.16.0 with the default parameters (Ye et al., 2013). For antibodies which have cDNA sequences deposited, the V and J genes were assigned using SONAR version 2.0 (<https://github.com/scharch/sonar/>) with germline gene database from IMGT (Lefranc, 2008; Schramm et al., 2016). For each antibody, the N-addition, D gene, and P-addition regions were annotated by IMGT V-QUEST (Brochet et al., 2008). To identify somatic hypermutations, each antibody sequence was aligned to the assigned germline gene using MUSCLE v3.8.31 (Edgar, 2004). Somatic hypermutations were identified from the alignment. In addition, the analysis of single cell antibody repertoire sequencing data of SARS-CoV-2 patient 2 from (Liu et al., 2020a), showed that 29 of the 38 unique transcripts assigned to IGLV2-14*01 share nucleotide mutations G156T and T165G. These mutations lead to amino acid mutations E50D and N53K. Both nucleotide mutations are also observed in 82 of 90 unique IGLV2-14 transcripts from patient 1 of the same study. Because these transcripts having different VJ recombination and paired with different heavy chain genes, the chances that the two convergent mutations are the results of somatic hypermutation are very low. Thus, we suspect that both donors contain a new IGLV2-14 gene allele (IGLV2-14*0X), which was deposited to European

Nucleotide Archive (ENA) with project accession numbers: PRJEB31020. Light chain of 1-87 was assigned to the IGLV2-14*0X allele.

Cryo-EM samples preparation

Samples for cryo-EM grids preparation were produced by mixing purified SARS-CoV-2 S2P spike (final trimer concentration of 0.33 mg/mL) with NTD-directed Fabs in a 1:9 molar ratio, followed by incubation on ice for 1 hr. The final buffer for 1-87, 4-18 and 5-24 complexes was 10 mM sodium acetate, 150 mM NaCl, pH 4.5; the final buffer for 1-68, 2-17, 2-51 and 4-8 complexes was 10 mM sodium acetate, 150 mM NaCl, pH 5.5. n-Dodecyl β -D-maltoside (DDM) at a final concentration of 0.005% (w/v) was added to the mixtures to prevent aggregation during vitrification. Cryo-EM grids were prepared by applying 2 μ L of sample to a freshly glow-discharged carbon-coated copper grid (CF 1.2/1.3 300 mesh); the sample was vitrified in liquid ethane using a Vitrobot Mark IV with a wait time of 30 s and a blot time of 3 s.

Cryo-EM data collection, processing and structure refinement

Cryo-EM data were collected using the Leginon software (Suloway et al., 2005) installed on a Titan Krios electron microscope operating at 300 kV, equipped with a Gatan K3-BioQuantum direct detection device. The total dose was fractionated for 3 s over 60 raw frames or 2 s over 40 raw frames. Motion correction, CTF estimation, particle extraction, 2D classification, ab initio model generation, 3D refinements and local resolution estimation for all datasets were carried out in cryoSPARC 2.15 (Punjani et al., 2017); particles were picked using Topaz (Bepler et al., 2019). Symmetry expansion and focused classification in RELION 3.1 (Scheres, 2012) was used for S2P spike complex with 2-51. The particle orientation distribution was assessed by angular

distribution plots for all EM structures. Whenever a preferred orientation issue was identified, we calculated the sphericity values and the Fourier shell correlation (FSC) curves using the 3D FSC Processing Server (Tan et al., 2017). The interface between NTD and the Fab was locally refined by using a mask that included NTD and the variable domains of the Fab; symmetry-expanded particles in C3 were used in the local refinement for S2P spike complexes with 4-18 and 5-24. The 4-8 interface was locally refined following particle subtraction without symmetry expansion, since it lacked C3 symmetry, using a mask over the same region. Particle subtraction did not improve the density in the local refinement of the 2-17 interface and was therefore not used in the final map refinement. The density at the interface was well-defined for S2P spike complexes with 1-87, 4-8, 4-18 and 5-24, providing structural details of antibody binding to NTD. The 2-17 interface density allowed for fitting of the main chain but did not provide enough detail to confidently fit side chains, which were left out of the model in that region.

SARS CoV-2 S2P spike density was modeled using PDB entry 6VXX (Walls et al., 2020), as initial template. The RBDs and were initially modeled using PDB entry 7BZ5 (Wu et al., 2020); the NTDs were initially modeled using PDB entry 6ZGE (Wrobel et al., 2020). The initial models for all Fab variable regions were obtained using the SAbPred server (Dunbar et al., 2016).

Automated and manual model building were iteratively performed using real space refinement in Phenix (Adams et al., 2004) and Coot (Emsley and Cowtan, 2004) respectively. Geometry validation and structure quality assessment were performed using EMRinger (Barad et al., 2015) and Molprobity (Davis et al., 2004). Map-fitting cross correlation (Fit-in-Map tool) and figures preparation were carried out using PyMOL and UCSF Chimera (Pettersen et al., 2004) and Chimera X (Pettersen et al., 2021). A summary of the cryo-EM data collection, reconstruction and refinement statistics is shown in Table S1.

X-ray crystallography sample preparation, data collection, structure solution and refinement

Purified SARS-CoV-2 spike N-terminal domain (NTD) and 2-51 Fab were mixed at a 1:1 molar ratio and incubated at 4 °C for 1 hr; the Fab-NTD complex was purified by SEC on a Superdex 200 column in buffer 10 mM Tris, 150 mM NaCl, pH 7.4. Fractions containing the complex were combined and concentrated to a total protein concentration of 7.5 mg/mL for crystal screening by the sitting drop vapor diffusion method at 25 °C. Diffracting crystals of NTD in complex with 2-51 Fab grew in 0.16 M calcium acetate, 0.08 M sodium cacodylate, 14.4% PEG 8000, 20% glycerol, pH 6.5. For data collection, crystals were cryo-protected by briefly soaking in reservoir solution supplemented with 35% (v/v) glycerol before flash-cooling in liquid nitrogen. X-ray diffraction data was collected to 3.44 Å resolution at 100 K from a single flash-cooled crystal on beamline 24ID-C at the Advanced Photon Source (APS) at Argonne National Laboratory. Diffraction data were processed with XDS (Kabsch, 2010) and scaled using AIMLESS (Evans and Murshudov, 2013) from the CCP4 software suite (Winn et al., 2011). Molecular replacement was performed with Phaser (McCoy, 2007) using the structure of NTD in complex with 4A8 (extracted from the whole spike-Fab structure from PDB entry 7C2L) as search model. Structure refinement was performed with a 3.65 Å high-resolution cutoff using Phenix refine (Adams et al., 2010) and PDB-redo (Joosten et al., 2014) alternated with manual model building using Coot. The Molprobit server was used for geometry validation and structure quality assessment. A summary of the X-ray crystallography data collection and refinement statistics is shown in Table S2.

Calculation of antibody angle of approach

The angles of antibody approach to the NTD of SARS-CoV-2 spike were calculated with UCSF Chimera (Pettersen et al., 2014). To define the latitudinal and longitudinal access of an antibody to the viral spike, we first defined two reference axes: the 3-fold axis of the spike trimer and a line perpendicular to the 3-fold axis and passing through the C α atom of Trp104 located in the hydrophobic core of the NTD. We then defined the axis of antibody as the long axis of the Fab. The latitudinal access, which describes the freedom between the viral and host cell membranes, was defined as the angle between the antibody axis and the 3-fold axis; the longitudinal access, which describes the freedom within the plane of the membrane, was defined as the angle between the antibody axis and the other reference axis. The angles between two axes were calculated with the built-in function of UCSF Chimera, and expressed in 0-to-180-degree scale for the latitudinal angles and 0-to-360-degree scale counter-clockwise to show the longitudinal angles. To compare the relative orientations of antibody heavy and light chains, we used a vector going from the center of heavy chain variable domain to that of the light chain variable domain.

Glycan analysis

To estimate the effect of glycan shield on protein surface residue, we used an in-house algorithm, GLYCO, to quantify the number of glycan atoms associated with each residue. Briefly, the number of glycan atoms per protein surface residue was counted within 34 Å radius distance cutoff, while the glycans do not provide the shielding effect were excluded in the calculation. This analysis was performed on each trajectory of molecular dynamics simulation (Casalino et al., 2020) to average the glycan atom counts for each residue.

Interface definition and net charge computation

The residues of each antibody paratope and epitope were obtained by running PDBePISA (Krissinel and Henrick, 2007), with the default parameters. Cryo-EM structures were of sufficient resolution to define all epitopes, except for 1-68 and 2-17. The epitope of 1-68 was obtained by docking the structure of the homolog antibody 1-87 in complex with NTD in the locally refined EM map of 1-68:NTD and real-space refinement was used to orient the residues of 1-68 that differed from 1-87. The missing side chains of 2-17 were added by CHARMM-GUI (Jo et al., 2008) and the structure was combined with NTD to produce a model for the complex; the NTD structure was obtained by template-based Swiss modeling (Waterhouse et al., 2018). The hydrophobic interaction residues among antibodies were defined by the buried surface area (BSA) that large than 20 \AA^2 . The antigen contact region for germline genes were adopt from (Sela-Culang et al., 2013), which includes additional interactions not accounted for in the CDRs. The number of charged residues were counted and the summation of their charges was used to quantify the net charge of selected residues.

C α distance calculation

The C α distances per residue between superimposed ligand-free and antibody-bound NTD were calculated by Python 3.8. The 8 NTD-antibody complexes (NTD-5-24, NTD-4-8, NTD-2-17, NTD-4-18, NTD-1-87, NTD-4A8, NTD-2-51, NTD-1-68) were used as antibody bound structures, and the averaged C α distances per residue of 8 complexes were analyzed.

RMSD calculation

The root mean square deviation (RMSD) of antibody epitopes was calculated by PyMOL 2.4 between two superimposed NTDs – ligand-free and antibody-bound structures. The identical ligand-free and antibody-bound NTDs described in C α distance analysis were used in the calculation. Only matching atoms with the same name and number in both epitopes were included in the calculation. The epitope residues of antibodies were obtained by running FreeSASA (Mitternacht, 2016) with probe radius 1.4 Å. The residues with non-zero surface area difference between NTD and NTD-antibody complex structures were selected as protein epitope residues to the corresponding antibodies.

Sequence entropy of betacoronavirus spike

Human coronavirus reference amino acid sequences of OC43 (UniProt ID: P36334), HKU1 (UniProt ID: Q5MQD0), SARS (UniProt ID: P59594), and MERS (UniProt ID: W5ZZF5), as well as the initial SARS-CoV-2 (GenBank: QHO60594.1) sequence reported in Washington state were aligned using MAFFT software with default parameters (Kato et al., 2002). Subsequently, we used the R bio3d package's function Conserv with default parameters to estimate sequence conservation at all alignment position

QUANTIFICATION AND STATISTICAL ANALYSIS

The statistical analyses for the pseudovirus and authentic virus neutralization assessments were performed using GraphPad Prism for calculation of mean value and SEM for each data point (see Figures S1 and S6). The SPR data were fitted using Biacore Evaluation Software (see Figure S6), and Scrubber (see Table S6). Cryo-EM data were processed and analyzed using

cryoSPARC and Relion (see Figure S3). Cryo-EM and crystallographic structural statistics were analyzed using Phenix, Molprobit, EMringer and Chimera (see Tables S1 and S2). Statistical details of experiments are described in Method Details or Figure Legends.

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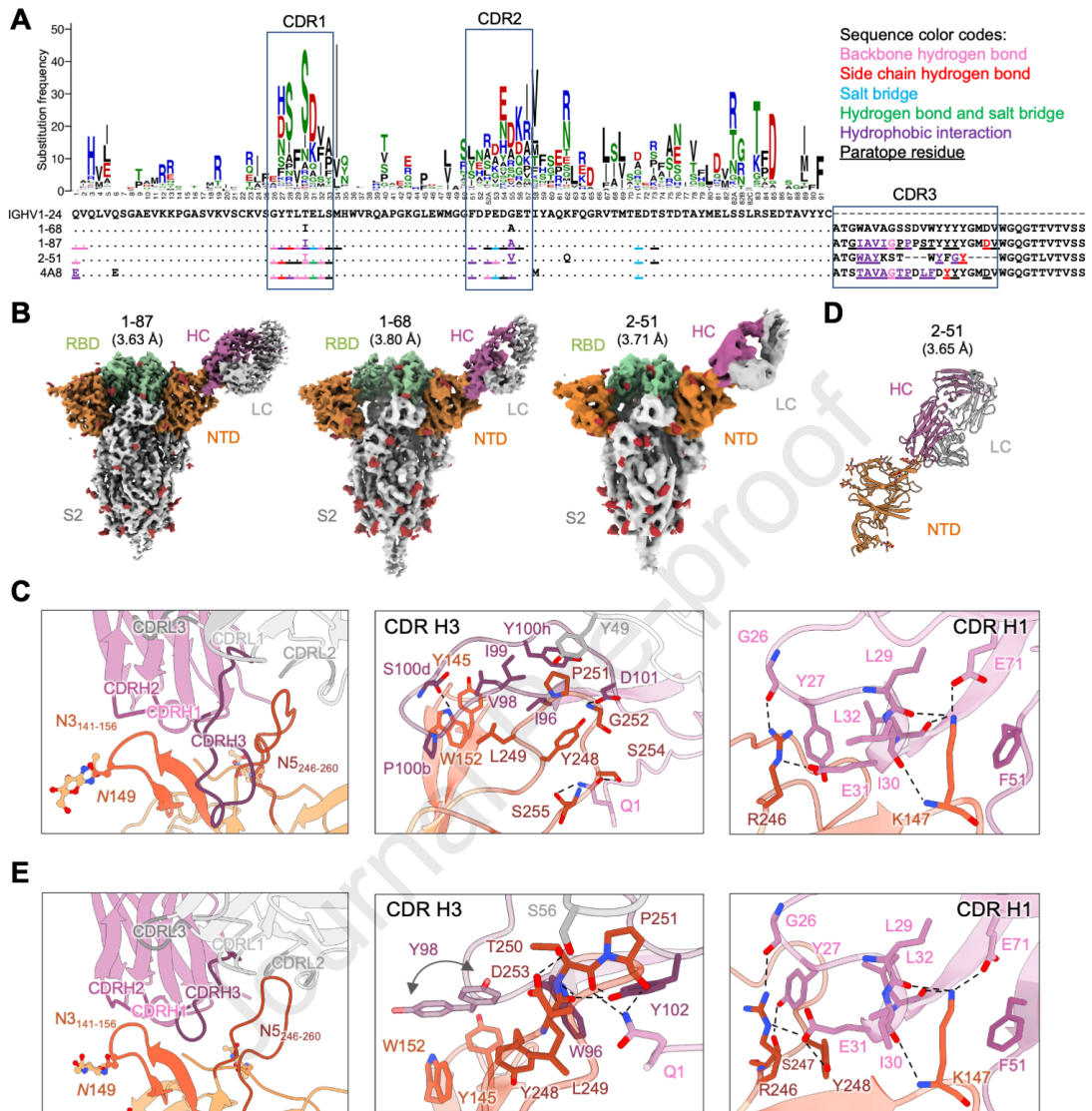
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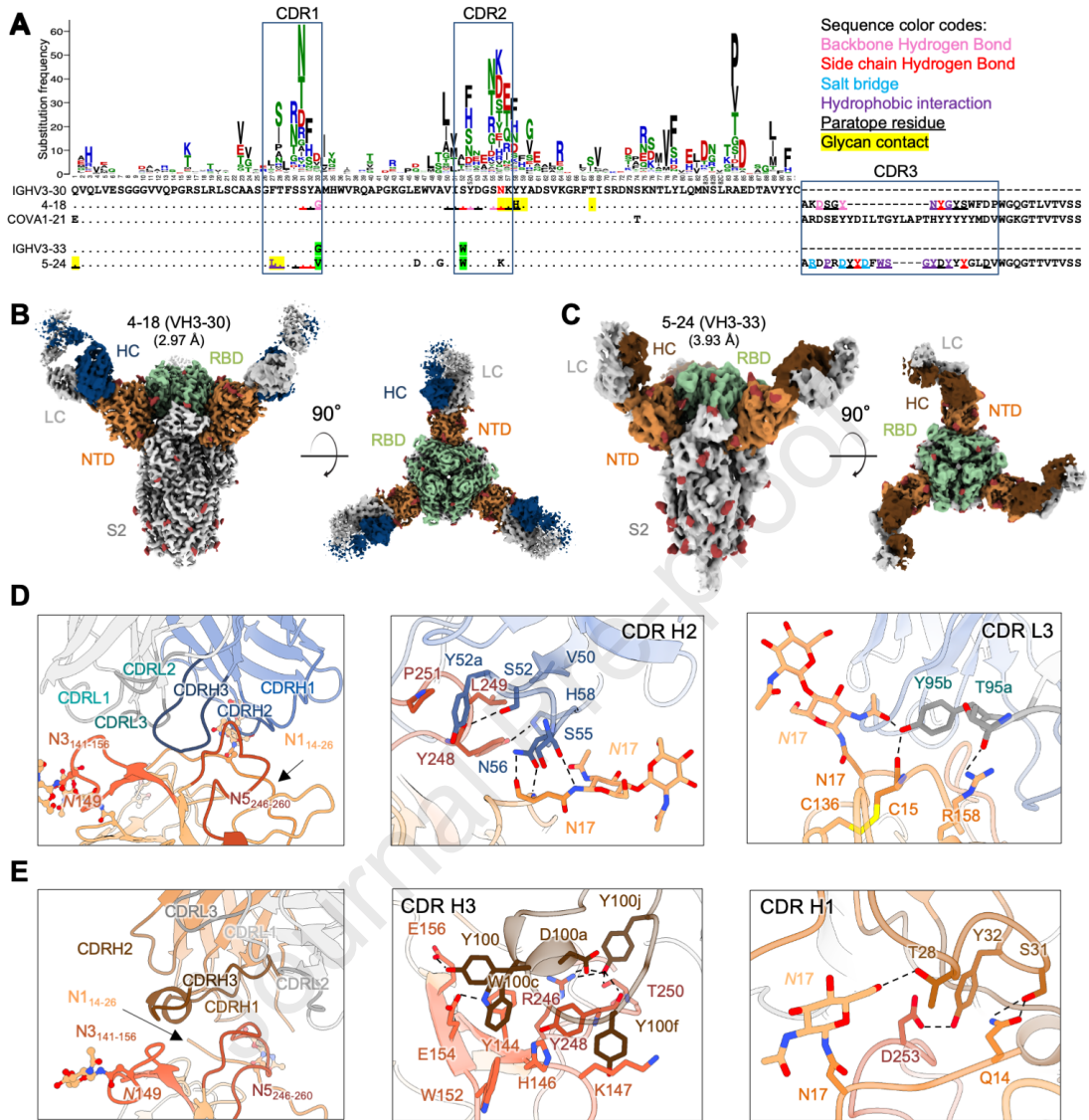
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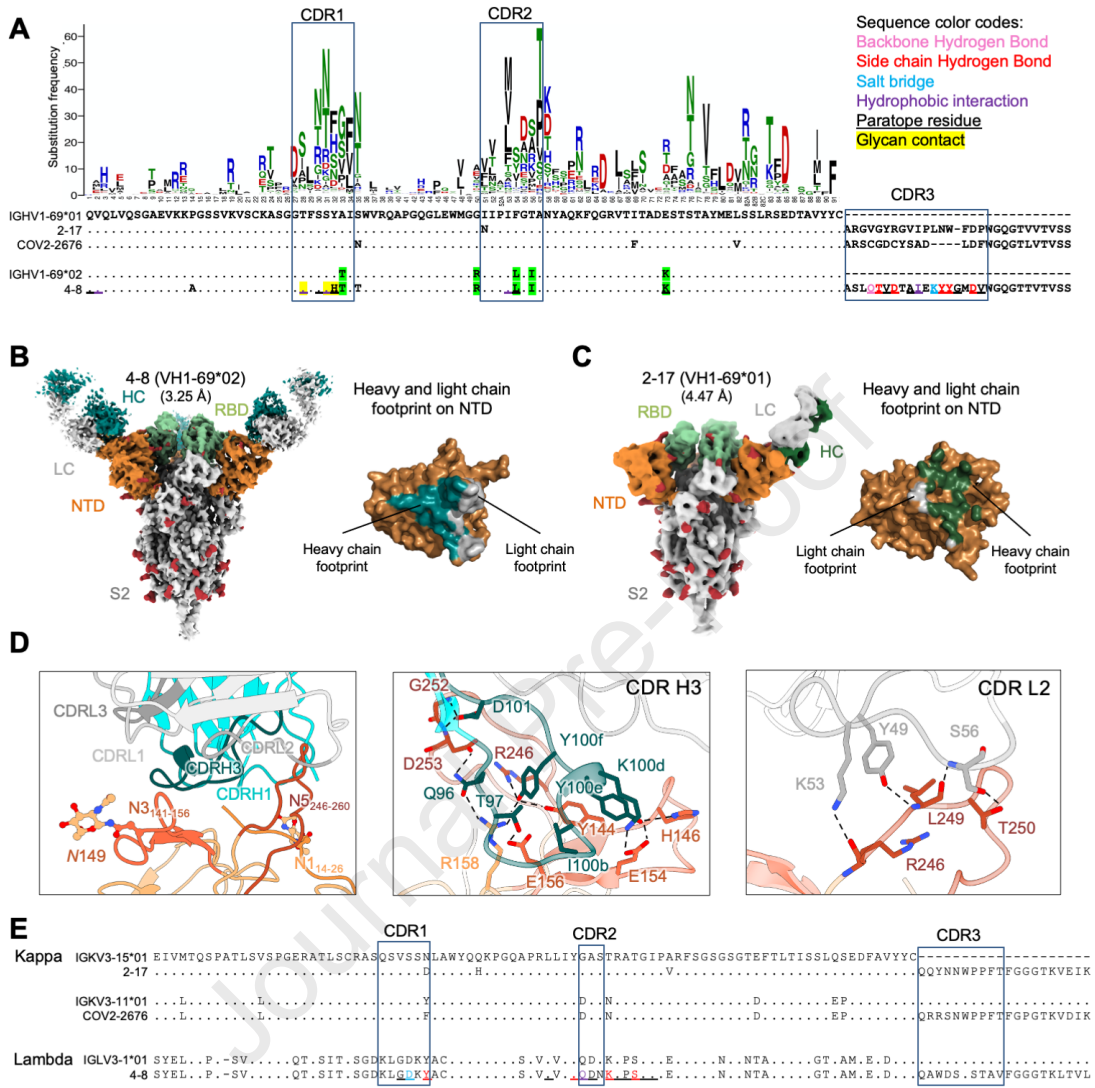
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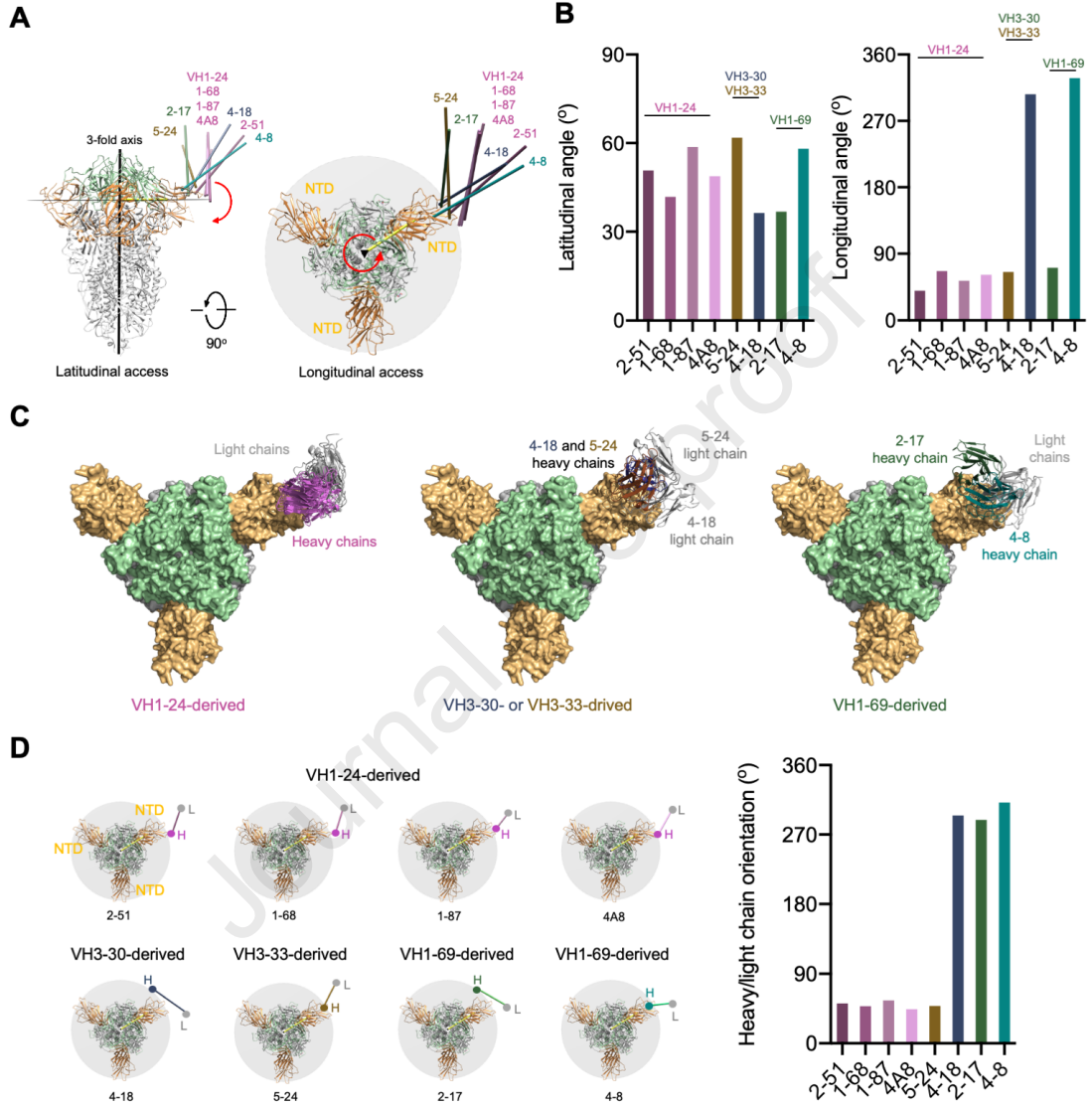
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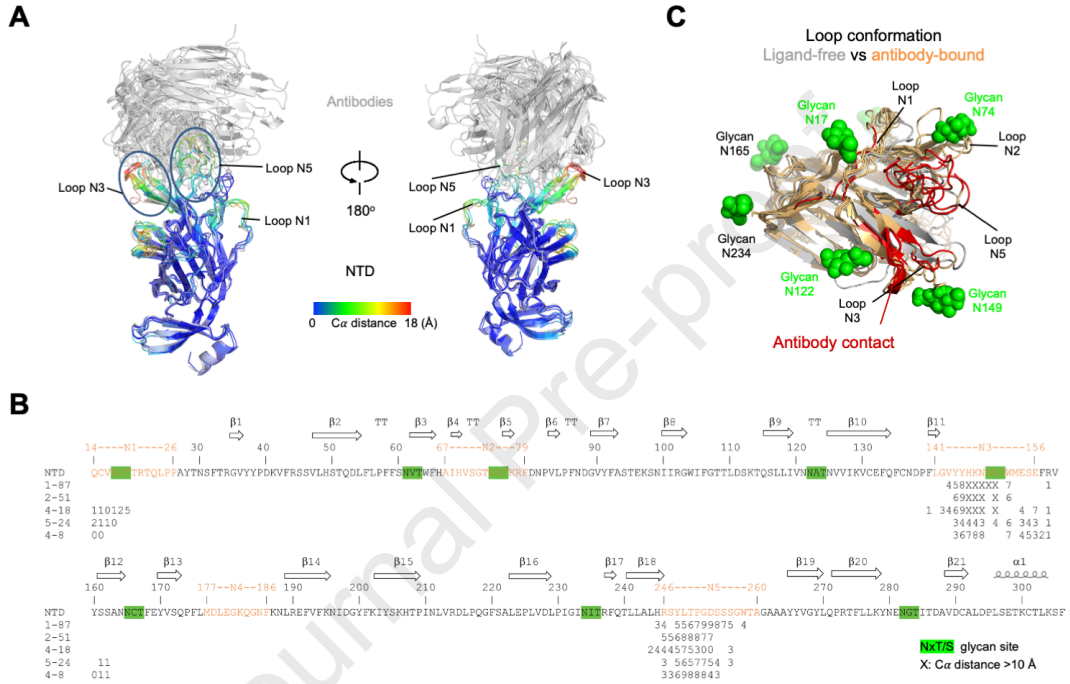
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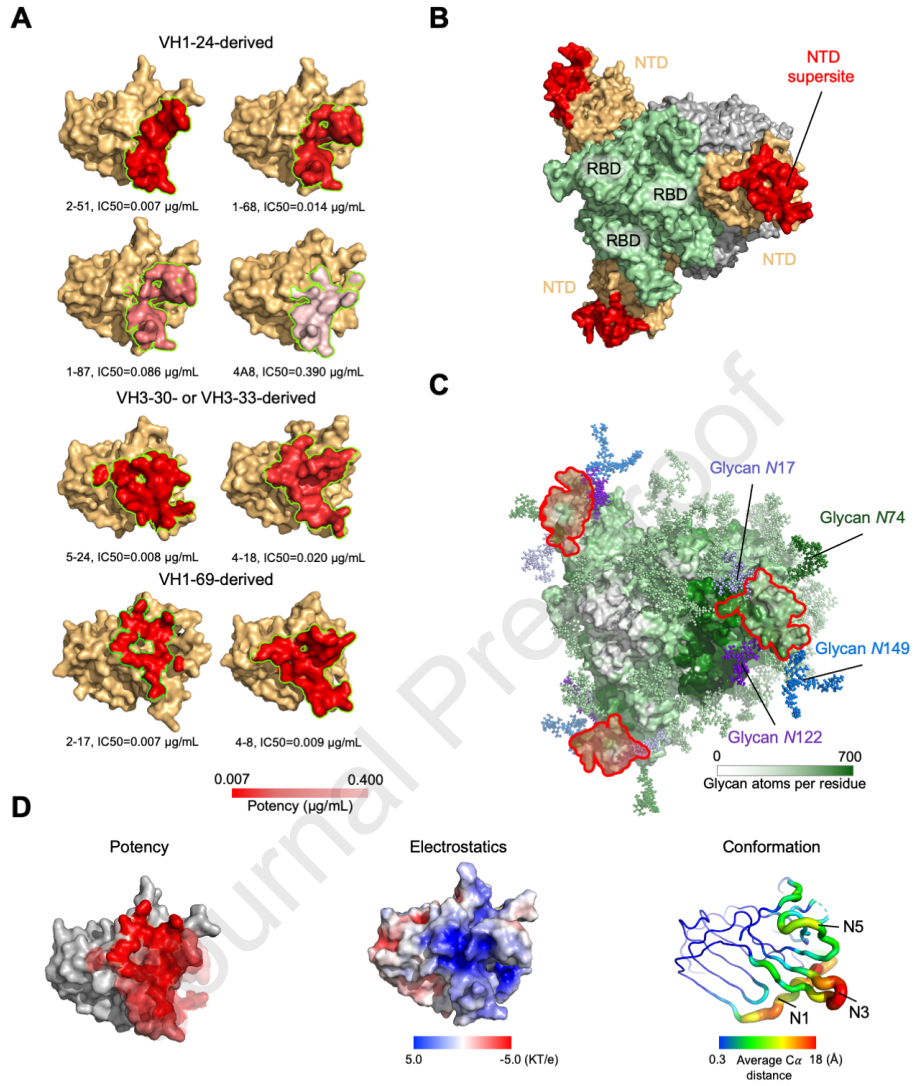


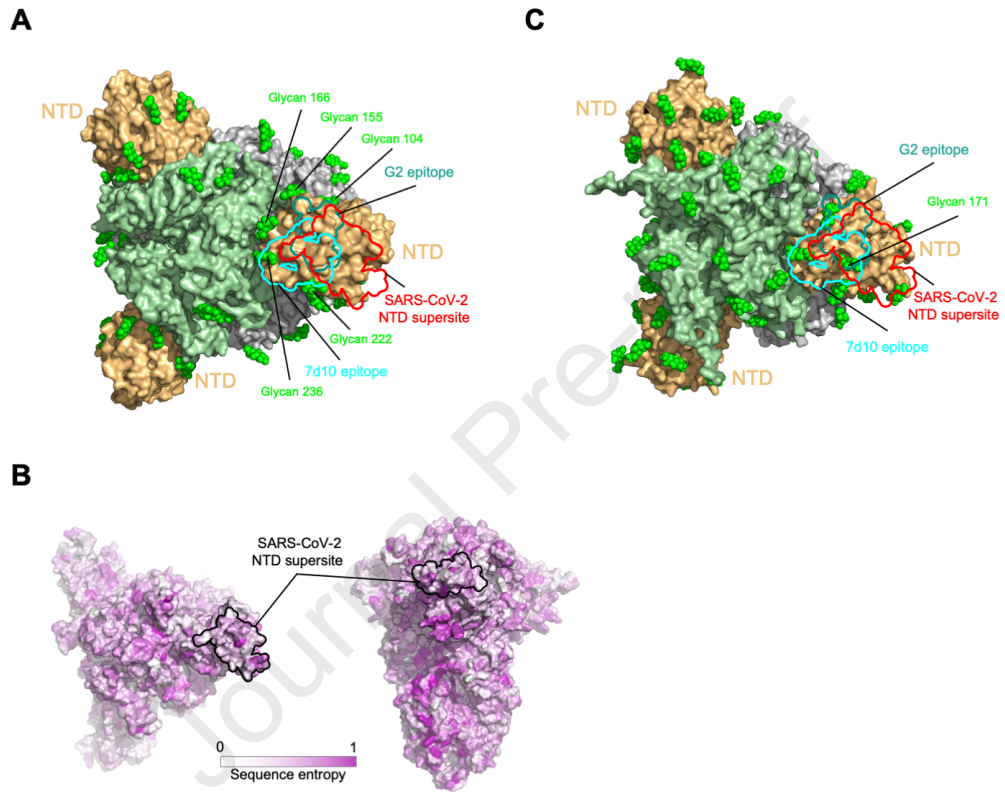












Highlights

- Structures of seven NTD-directed neutralizing antibody complexes with spike or NTD
- Structures define distinct recognition classes, one observed in multiple donors
- Supersite is glycan free, electropositive, with mobile β -hairpin and flexible loops
- Most potent NTD-directed neutralizing antibodies may target this supersite

eTOC blurb

Cerutti et al. report structural analysis of seven potent neutralizing antibodies targeting the N-Terminal domain of SARS-CoV-2 spike. All antibodies recognize a common glycan-free, electropositive surface comprised of a mobile β -hairpin and flexible loops. While RBD-directed antibodies recognize non-overlapping epitopes, these findings indicate NTD-directed antibodies predominantly target a single supersite.