

## Review

# The development of neutralizing antibodies against SARS-CoV-2 and their common features

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Edited by Haian Fu

**Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a worldwide severe coronavirus disease 2019 (COVID-19) pandemic since December 2019. There is a great demand for effective therapies for the prevention and treatment of COVID-19. Developing therapeutic neutralizing antibodies (NAbs), which could block viral infection, is such a promising approach, as NAbs have been successfully applied to the treatment of other viral infections. The recent advances of antibody technology have greatly accelerated the discovery of SARS-CoV-2 NAbs, and many of which are now actively tested in clinical trials. Here, we review the approaches applied for SARS-CoV-2 NAb development, and discuss the emerging technologies underlining the antibody discovery. We further summarize the common features of these antibodies including the shared neutralizing epitopes and sequence features.**

**Keywords:** COVID-19, SARS-CoV-2, neutralizing antibody, epitope, RBD

A novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a worldwide severe coronavirus disease 2019 (COVID-19) pandemic since December 2019 (Chan et al., 2020; Huang et al., 2020). The COVID-19 pandemic has been reported to affect over 57.8 million people and cause more than 1300000 deaths in nearly all countries by November 22, 2020 (World Health Organization, 2020). Thus, there is a great demand for effective therapies for the prevention and treatment of COVID-19. Therapeutic neutralizing antibodies (NAbs), which could block viral infection, may be such a promising approach, as NAbs have been successfully applied to the treatment of respiratory syncytial virus infection and showed great potential for the treatment of other viral infections (Walker and Burton, 2018). The advances of antibody technology have greatly accelerated the discovery of SARS-CoV-2 NAbs, and many of which are now actively tested in clinical trials (by the time this review is finalizing, Regeneron's antibody

cocktail therapy has received Emergency Use Authorization from the US Food and Drug Administration). Here, we review the approaches applied for SARS-CoV-2 NAb development and discuss the emerging technologies underlining the antibody discovery. We further summarize the common features of these antibodies including the shared recognition epitopes and sequence features.

### Antibody diversification *in vivo* and antibody discovery

A typical natural antibody composes of two identical heavy chains and two identical light chains, which is coded by immunoglobulin (*Ig*) heavy chain (*IgH*) and light chain (*IgL*) genes, respectively. The N-terminal of *IgH* and *IgL* contain variable region sequences that are responsible for antigen recognition. The diversity of *Ig* variable region (*IgV*) is achieved by programmed DNA lesions and error-prone repair at *Ig* gene loci (Alt et al., 2013). During the development of B cell, V(D)J recombination assembles the numerous V, D, and J gene segments into a variable region exon, forming the first step of antibody diversification. Upon antigen stimulation, B cells undergo another round of diversification by somatic hypermutation (SHM), whereby mutations and small insertions and deletions (indels) are introduced into the variable region exon (Yeap and Meng,

Received August 31, 2020. Revised November 23, 2020. Accepted November 25, 2020.

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2019). B cells with mutations that increase binding affinity to the antigen are selected in a process called affinity maturation (Alt et al., 2013). Thus, the diversity in an antibody repertoire, contributed by V(D)J recombination and/or SHM processes from previous immune responses, ensures an effective antibody-mediated immunity upon exposure to a novel pathogen.

The inventions of hybridoma technology (Kohler and Milstein, 1975), humanized antibody (Kinashi et al., 1986), and phage surface display (Smith, 1985) have made antibody the most widely used protein reagents in both biomedical research and therapy. In the past few years, emerging new technologies have again boosted the antibody discovery process, including paired antibody gene cloning from single B cells (Tiller et al., 2008), improved memory B-cell sorting and culturing (Huang et al., 2013; Corti and Lanzavecchia, 2014), single-cell RNA Sequencing (Tang et al., 2009), antibody-humanized mouse models (originally proposed by Alt et al., 1985), and the discovery of single-domain antibody (Hamers-Casterman et al., 1993).

#### Approaches to develop SARS-CoV-2 NAbs

Within a few months after the pandemic begins, many research groups have successfully isolated SARS-CoV-2 NAbs from COVID-19 convalescent patients (Rogers et al., 2020; Seydoux et al., 2020; Shi et al., 2020; Wu et al., 2020b). A commonality in the approaches used by these groups includes the enrichment of specific B cells using SARS-CoV-2 spike (S) protein, since antibodies targeting the spike may block entry of the virus into the host cell. The SARS-CoV-2 S protein that forms a trimeric complex at the virus membrane is divided into two regions, the S1 region that mediates virus attachment to the host cell and the S2 region that triggers virus fusion (Wrapp et al., 2020b). The receptor-binding domain (RBD) located in the S1 region is responsible for directly binding to the host cell receptor, angiotensin-converting enzyme 2 (ACE2). In this context, both S1 and S-RBD fragments were used to label the human B cells in fluorescence-activated cell sorting-based single-cell sorting. Several potent NAbs that were discovered using such approaches include B38 (Wu et al., 2020b), CB6 (Shi et al., 2020), CC12.1 (Rogers et al., 2020), C121 (Robbiani et al., 2020), and CV30 (Seydoux et al., 2020). To search for antibodies that have broader neutralizing activities against several coronavirus strains, peripheral blood mononuclear cells (PBMCs) from SARS-CoV convalescent patients were used and the resulting antibodies, e.g. ADI-55689, can neutralize both SARS-CoV-2 and SARS-CoV (Pinto et al., 2020; Wec et al., 2020). Along the same line, Epstein-Barr virus transformation-based single B-cell culture (Traggiai et al., 2004) also yielded a cross-reactive NAb, S309, from SARS-CoV convalescent patients (Pinto et al., 2020).

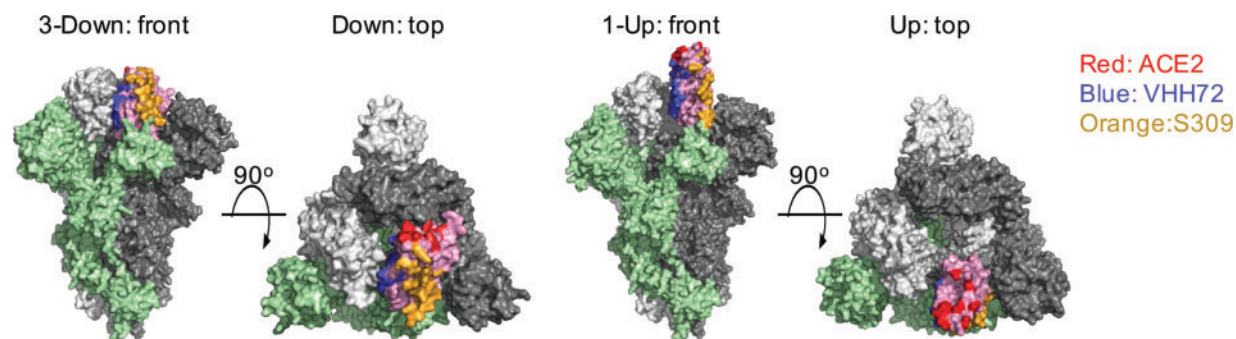
High-throughput single-cell 5'RNA-Seq further facilitates the antibody discovery. In this context, 10× Genomics technology was applied to identify paired heavy and light chain V(D)J

sequences from tens of thousands of single B cells. Two independent groups have applied bioinformatic pipelines and picked potential binders. Their data revealed that ~50% of the selected antibodies show positive binding with the S protein (Cao et al., 2020; Liu et al., 2020), and the single-cell 5'RNA-Seq approach leads to the identification of NAb clone BD-368-2 and #2-4 (Cao et al., 2020; Liu et al., 2020).

Antibody gene-humanized mouse models can also be used efficiently to isolate SARS-CoV-2 NAbs. Hansen et al. (2020) immunized genetically humanized 'VelocImmune' (VI) mice (Macdonald et al., 2014) with SARS-CoV-2 S protein-coding plasmids, then boosted with RBD protein, and cloned antibody gene from immunized spleens with fluorescent-labelled S-RBD-activated cell sorting. Several NAbs with half maximal inhibitory concentrations (IC50s) at picomolar levels were obtained by using this method. Similarly, Wang et al. (2020) used their H2L2 mouse model-derived hybridoma cells to select SARS-CoV-2-specific antibodies and yielded an NAb named 47D11 (Wang et al., 2020). The use of these mouse models is limited, as only a few pharmaceutical companies have these mice.

Another method that was used successfully to isolate SARS-CoV-2 NAbs is by combining synthetic or combinatorial antibody library with phage display. Several synthetic human IgH-derived single-domain antibody libraries were applied. In these antibody libraries, particular human IgH was first selected as the single-domain antibody scaffold, e.g. V<sub>H</sub>3-66\*01 (Wu et al., 2020a), V<sub>H</sub>3-23, V<sub>H</sub>3-7, or V<sub>H</sub>4-34 (Sun et al., 2020), and then CDRs from naïve antibody library are grafted to the specific backbone to generate a large single-domain antibody library. The pools of antibodies were then subjected to phage display with S1 or RBD proteins, and the binders were identified. Similarly, single-domain antibody libraries derived from camelids (the V region is named as nanobody) were also used. In this context, the camelids were immunized with SARS-CoV-2 S1 or S-RBD proteins, and their PBMCs were collected and the antibody genes were subjected to phage display (Hanke et al., 2020; Nieto et al., 2020). Interestingly, llama immunized with SARS-CoV and MERS-CoV S proteins were reported to generate cross-neutralizing antibodies against SARS-CoV-2, and VHH72 was identified to react with both SARS-CoV and SARS-CoV-2 (Hurlburt et al., 2020). The single-domain antibody can be further diversified by random mutagenesis, which leads to the identification of two NAbs H11-H4 and H11-D4 (Huo et al., 2020). In addition, several synthetic single-domain antibody libraries like Sybody (Zimmermann et al., 2018) were also quickly applied to find SARS-CoV-2 NAbs (Custódio et al., 2020; Li et al., 2020).

In the midst of the pandemic, the process of SARS-CoV-2 NAb discovery was greatly accelerated. Single B-cell cloning from COVID-19 convalescent patients proved to be the most feasible approach, which is further advanced by the single-cell RNA-seq (Cao et al., 2020) or single-cell analysis technologies (Zost et al., 2020). However, patient samples are generally a limitation to biomedical researchers and higher biosafety levels are required to handle PBMC samples with such a contagious



**Figure 1** Structures of SARS-CoV-2 S protein trimer complex and representative receptor/antibody recognition interfaces. Left: S protein trimer complex with three RBD ‘down’ conformation; right: S protein trimer complex with a single RBD ‘up’ conformation. One ‘up’ RBD is labelled pink in two states, and the ACE2 receptor and antibody interface are labelled in different colors. The structures are retrieved from PDB: 6VXX and PDB: 6VYB.

disease. Antibody-humanized mouse models avoid the use of patient-derived samples and allow rational design of immunogens, but the models are restricted within several pharmaceutical companies and the mouse immunization process requires weeks of time. Phage or other display techniques potentially provide a rapid antibody discovery approach, and several candidates are at preclinical status (Ejemel et al., 2020; Lou et al., 2020; Schoof et al., 2020). As revealed by the summary of COVID-19 antibody therapeutics (Yang et al., 2020), most of the therapeutic antibodies in pre-clinical or clinical trials are obtained from COVID-19 convalescent patients (Supplementary Table S1; Li et al., 2020).

### Neutralizing epitopes on SARS-CoV-2 S protein

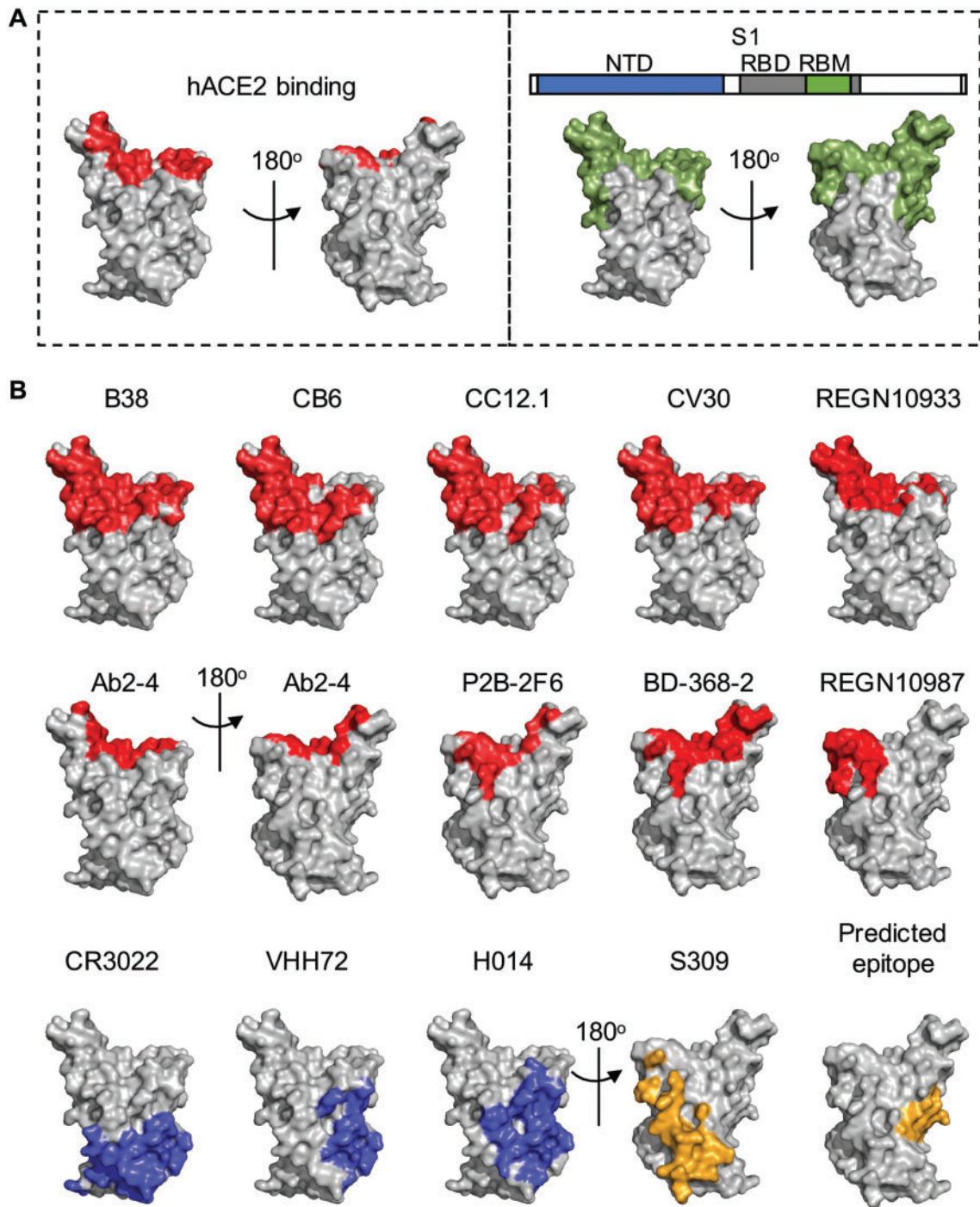
Both SARS-CoV and SARS-CoV-2 use the host ACE2 protein as its entry receptor (Li et al., 2003; Walls et al., 2020) and the affinity of S protein–ACE2 interaction contributes to the efficiency of viral transmission. Upon binding of ACE2, the S-RBD region could perform a hinge-like conformation movements, switching to an ‘up’ conformation from a ‘down’ conformation, expose the S1 subunit, and transit the S-trimer to a post-fusion conformation (Walls et al., 2020; Wrapp et al., 2020b).

Multiple assays were applied to evaluate the neutralizing activity of potential NABs. Pseudovirus assay is the most frequently used approach, in which the envelope glycoprotein in another virus vector is replaced with different forms of SARS-CoV-2 S proteins. This approach circumvents the use of authentic SARS-CoV-2 virus. Pseudovirus is used to screen or verify neutralizing activities without the need for higher biosafety facilities. So far, human immunodeficiency virus-, vesicular stomatitis virus-, and murine leukemia virus-based pseudoviruses were applied to test the neutralizing activity (Cao et al., 2020; Pinto et al., 2020; Robbani et al., 2020; Rogers et al., 2020; Shi et al., 2020; Wec et al., 2020). Although many studies (Wec et al., 2020) have reported the high correlation of neutralizing activity obtained from pseudovirus and authentic virus assays, cautions should be taken to evaluate the specific IC<sub>50</sub> in each case. Neutralization activity tested on pseudovirus or authentic

viruses may be different. In this regard, IC<sub>50</sub> of antibodies evaluated in the pseudovirus system may be 2- to 100-fold lower than that in the authentic SARS-CoV-2. Most of the abovementioned antibodies were tested with authentic SARS-CoV-2 isolates with IC<sub>50</sub> at 0.1–410 ng/ml (Supplementary Table S1). Beyond the cellular assays, protection of infected animal from death, severe symptom, or lung injury can further indicate potential prophylaxis or treatment application of NABs. A few animal models including transgenic mouse (Lv et al., 2020; Wu et al., 2020b), hamster (Cao et al., 2020; Rogers et al., 2020), and rhesus macaque (Shi et al., 2020; Zost et al., 2020) were applied. So far, nearly all the reported SARS-CoV-2 NABs bind to the S1 region. There is only one exception among the hundreds of reported SARS-CoV-2 NABs. Clone 0304-3H3, which binds to S2 fragment at nanomolar kilodaltons, inhibited authentic SARS-CoV-2 cellular infection but failed to neutralize pseudotyped SARS-CoV-2 (Chi et al., 2020).

Epitopes of many SARS-CoV-2 NABs completely or partially overlap with ACE2-binding interface, and thus the NABs are able to competitively bind to S-RBD and block its interaction with host receptor. By applying structural analysis, NABs, including B38 (Wu et al., 2020b), BD368-2 (Cao et al., 2020), C105 (Barnes et al., 2020), CC12.1, CC12.3 (Rogers et al., 2020), CV30 (Seydoux et al., 2020), CB6 (Shi et al., 2020), P2B-2F6 (Ju et al., 2020), REGN10933, REGN10987 (Hansen et al., 2020), COV2-2196, and COV2-2130 (Zost et al., 2020) were revealed to bind to the receptor-binding motif (RBM) of RBD (Figure 1). Two closely related nanobodies derived from llama, H11-D4 and H11-H4, also block the same RBM region (Huo et al., 2020). Moreover, B38, CB6, CV30, CC12.1, and CC12.3 share nearly identical epitope, which can only be accessed in the ‘up’ conformation, with most residues overlapping with ACE2-interacting surface (Figure 1; Seydoux et al., 2020; Shi et al., 2020; Wu et al., 2020a; Yuan et al., 2020a). BD368-2 and P2B-2F6 shared partially overlapping epitope thus able to bind to RBD in both ‘up’ and ‘down’ positions, and this interaction could lead to the clash with S-RBD–ACE2 binding (Cao et al., 2020; Ju et al., 2020). Similarly, REGN10933 and REGN10987 (Hansen et al., 2020) and COV2-2196 and

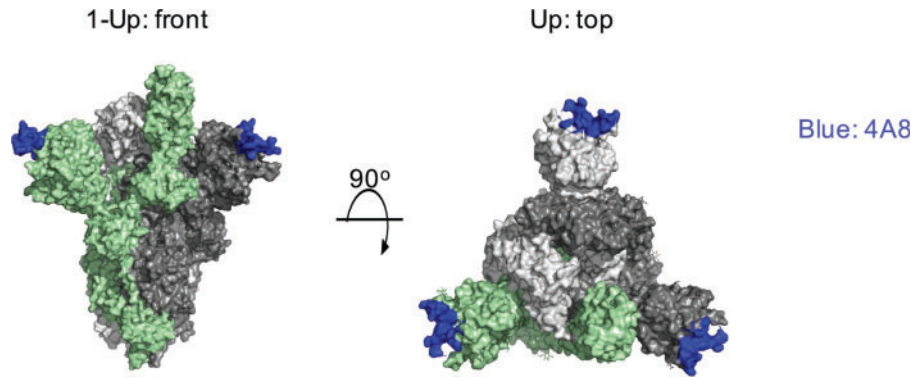




**Figure 2** Summary of neutralizing epitopes in S-RBD of SARS-CoV-2 NAbs. (A) 3D surface model for S-RBD structure showing the ACE2 interface (left) and schematic of SARS-CoV-2 S1 primary structure (right). (B) 3D surface models for S-RBD with neutralizing epitopes of indicated NAbs as labelled. Residues in the interface or epitopes are marked in different colors. The red residues locate in RBM region, while the blue or orange residues locate in non-RBM region. The SARS-CoV-2 S-RBD structure is from PDB: 6M0J. A predicted epitope based on S-RBD mutagenesis study is also shown.

COV2-2130 (Zost et al., 2020) represent two pairs of antibodies recognizing non-overlapping epitopes in RBM region. It is of note that the neutralizing epitopes in RBM region vary in the accessibility in the trimer S protein complex (Figure 1). In this

context, a recent study has categorized the RBM-antibodies into Class 1 (binding to an RBM epitope only exposed in the 'up' conformation, Figure 1) and Class 2 (binding to an RBM epitope exposed in the 'up' and 'down' conformation, Figure 1).



**Figure 3** 3D surface model for SARS-CoV-2 S protein trimer complex with an NTD-NAB. The complex structure with 4A8 NAB is from PDB: 7C2L.

These two epitopes support simultaneously binding of two NABs to S-RBD and thus serve as the basis for antibody cocktail treatments (Baum et al., 2020; Zost et al., 2020).

Besides the ACE2-interacting surface (RBM region, Figure 2A), more epitopes with neutralizing effect have recently been revealed. The epitope of CR3022 is located away from the ACE2-interacting surface and close to the N-terminal domain (NTD) of S1 region (Figure 2B). This binding may destabilize the prefusion state of the spike (Huo et al., 2020; Yuan et al., 2020b). VHH-72 interacts with S-RBD in S-RBD's 'down' conformation (Figure 2B), which traps the trimeric S protein to stay in the 'up' conformation (Wrapp et al., 2020a). Humanized H014 antibody recognizes a similar epitope as VHH-72 (Lv et al., 2020). The S309 antibody binds to another epitope away from the ACE2-interacting surface (Figure 2B) and potentially causes S-trimer cross-linking, steric hindrance, or aggregation of virions (Pinto et al., 2020). Strikingly, some non-RBM NABs show cross-neutralizing activity against both SARS-CoV and SARS-CoV-2, e.g. CR3022, VHH-72 (Wrapp et al., 2020a), H014 (Lv et al., 2020), and S309 (Pinto et al., 2020). Similarly, a non-RBM NAB 47D11 has cross-neutralizing activity, although the exact neutralizing epitope in RBD is still unknown (Wang et al., 2020).

Epitopes with neutralizing effect on the NTD of S protein were also indicated by several laboratories (Brouwer et al., 2020; Chi et al., 2020; Liu et al., 2020). Structural analyses of NAB 4A8 (Chi et al., 2020), FC05 (Zhang et al., 2020), and 4-8 (Liu et al., 2020) reveal a similar NTD epitope as revealed by a MERS-CoV NAB (Figure 3; Zhou et al., 2019). A recent preprint study revealed that combination of RBD- and NTD-targeting antibodies leads to a dramatic synergetic effect in neutralizing (Zhang et al., 2020). These NTD antibodies may cause conformation change of the S protein and block its interaction with ACE2. However, the exact neutralizing mechanism needs further investigation. Other mechanisms including blocking viral membrane fusion are also attracting possibilities.

During the transmission of SARS-CoV-2, naturally occurring variants in S protein are reported and many mutations are located in the RBD region, which may influence the reaction with

NABs (Li et al., 2020). In this context, escape mutants were easily selected upon single NAB treatment in the *in vitro* replicating pseudovirus assay (Baum et al., 2020). Deep mutation scanning was used to identify the amino acid residues affecting S protein stability and ACE2 interaction and yielded an unmutable map of S-RBD (Starr et al., 2020). The authors found a surface patch that is important for S protein stability (Figure 2B) and revealed the possibility that the surface patch could be an ideal epitope.

#### Sequence features of SARS-CoV-2 NABs

Convergent antibody responses to SARS-CoV-2 were well documented (Robbiani et al., 2020; Yuan et al., 2020a), as many studies reported that highly potent NABs frequently use a few  $V_H$  genes. In a study of analysis for IgH V usage in nearly 300 SARS-CoV-2 S-RBD binders,  $V_H3-53$  gene segment was highly enriched followed by  $V_H1-2$ ,  $V_H3-9$ ,  $V_H3-30$ , and  $V_H1-46$  (Yuan et al., 2020a). In another independent study, similar over-represented  $V_H$  usage was reported (Robbiani et al., 2020).  $V_H3-66$  and  $V_H3-53$  germlines have only 2-amino acid difference, and many highly potent NABs convergently use the  $V_H3-53/V_H3-66$  gene segment and show very similar recognition epitope, e.g. B38 (Wu et al., 2020b), CC12.1/CC12.3 (Rogers et al., 2020), CV30 (Seydoux et al., 2020), and C105 (Barnes et al., 2020) use  $V_H3-53$  gene segment, and BD-368-2 (Cao et al., 2020) and CB6 (Shi et al., 2020) use  $V_H3-66$  gene segment. Meanwhile, these  $V_H3-53/V_H3-66$ -derived heavy chains pair with various light chains, e.g.  $V_K1-9$ ,  $V_K3-20$ ,  $V_K1-39$ , and  $V_\lambda2-08$ . Structural analysis has revealed that an NY motif in CDRH1 and an SGGs motif in CDRH2 are important for RBD binding in  $V_H3-53$  germline (Yuan et al., 2020a).

The sequences of the reported panel of SARS-CoV-2 NABs are close to their germline sequence with limited mutations (Brouwer et al., 2020; Ju et al., 2020; Kreer et al., 2020; Rogers et al., 2020; Seydoux et al., 2020). NABs against Ebola (Davis et al., 2019) and MERS-CoV (Ying et al., 2015) were also reported to have low SHM rates, indicating that the immune system is quickly activated upon virus infection. The precursor

sequences of SARS-CoV-2 NAbs are also reported to be found in healthy donor (Kreer et al., 2020), indicating that human antibody repertoires can clear the virus quickly upon infection. The changes in germline sequence may have a great influence on affinity between antibody and S-RBD protein. CV30, which shows only 2-amino acid difference from the germline sequence have increased binding affinity by ~100-fold (Hurlburt et al., 2020).

The S-RBD-binding antibodies show a normal distribution of CDRH3 length from 5 to 30 amino acids, with an average length of 15 residues (Kreer et al., 2020; Zost et al., 2020). However, the CDRH3 region in isolated NAbs varies, as many V<sub>H</sub>3-53/V<sub>H</sub>3-66-derived NAbs harbor a shorter CDRH3, with a length between 9 and 12 residues (Brouwer et al., 2020), while NAbs derived from some other V<sub>H</sub> family have CDRH3 length >20 amino acids (Ju et al., 2020). Whether CDRH3 length has direct correlation to neutralizing activities of SARS-CoV-2 antibodies is still unknown.

### Perspective

Technological advances have greatly accelerated the discovery of SARS-CoV-2 NAbs. Some NAbs are now already being tested in clinical trials. However, there are still several scientific questions to be answered in the near future.

- i. Some of the NAbs have the potential to neutralize both SARS-CoV and SARS-CoV-2. Whether it is possible to identify broad NAbs against more SARS-related coronaviruses, or even the whole *Betacoronavirus* family members?
- ii. If broader NAbs can be obtained, what is the conserved isotope and underlying neutralizing mechanism? Can we rationally design/isolate such antibodies, and what can we learn to assist vaccine design?
- iii. Can we prepare a pool of NAbs based on the sequences of SARS-related coronaviruses and the epitopes with neutralizing effect, which can be immediately pulled out for passive immunotherapy in the next coronavirus disease outbreak?

### Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

### Acknowledgments

We thank Dr Bo Liu for suggestions.

### Funding

Work in the authors' labs was supported by the National Key R&D Program of China (2017YFA0506700), the National Natural Science Foundation of China (81622022 and 31970880 to F.-L.M.; 31722020, 81671634, and 81861138014 to L.-S.Y.),

Shanghai Jiao Tong University COVID-19 grant to L.-S.Y., and Shanghai Science and Technology Committee (20490760200) to F.-L.M.

**Conflict of interest:** none declared.

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