Comprehensive Mapping of Binding Hot Spots of SARS-CoV-2 RBD-specific Neutralizing Antibodies for Tracking Immune Escape Variants

Chunyan Yi

State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences

Xiaoyu Sun

State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences

Yixiao Lin

Shanghai Public Health Clinical Center, Shanghai Medical College, Fudan University

Chenjian Gu

Shanghai Institute of Infectious Diseases and Biosecurity, Shanghai Medical College, Fudan University

Longfei Ding

Shanghai Public Health Clinical Center, Shanghai Medical College, Fudan University

Xiao Lu

State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences

Zhuo Yang

State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences

Yaguang Zhang

State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences

Liyan Ma

State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences

Wangpeng Gu

State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences

Aidong Qu

Shanghai Institute of Biological Products Co., Ltd

Xu Zhou

Shanghai Institute of Biological Products Co., Ltd

Xiuling Li

Shanghai Institute of Biological Products Co., Ltd

Jianqing Xu

Shanghai Public Health Clinical Center, Shanghai Medical College, Fudan University

Zhiyang Ling

State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences

Youhua Xie

Shanghai Institute of Infectious Diseases and Biosecurity, Shanghai Medical College, Fudan University

Hongzhou Lu

Shanghai Public Health Clinical Center, Shanghai Medical College, Fudan University

Bing Sun (✉ bsun@sibs.ac.cn)

State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences

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Abstract

The receptor-binding domain (RBD) variants of SARS-CoV-2 could impair antibody-mediated neutralization of the virus by host immunity; thus, prospective surveillance for such antibody escape mutants is urgently needed. Here, we comprehensively profiled four antigenic sites of the RBD and mapped the binding hot spots for a panel of RBD-specific monoclonal antibodies isolated from COVID-19 convalescents, especially dominant VH3-53/3–66 antibodies, which are valuable indicators of antigenic changes in the RBD. We further demonstrated that several natural mutations, namely, K417N, F486L, N450K, L452R, E484K, F490S and R346S, significantly decreased the neutralizing activity of multiple human monoclonal antibodies and of human convalescent plasma obtained in the early stage of the COVID-19 pandemic. Of note, among the natural escape mutations, L452R enhanced ACE2 binding affinity, indicating that it potentially increased virulence. Overall, the in-depth maps may have far-reaching value for surveillance of SARS-CoV-2 immune escape variants and guidance of vaccine design.

Main

Coronavirus disease 2019 (COVID-19), caused by the newly emerging severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)¹, has spread extensively worldwide². As of April 2021, the global cases of COVID-19 had surpassed 130 million, resulting in more than 2.8 million deaths according to the World Health Organization. During the past year, great achievements have been made in scientific research, especially with regard to the development of vaccines and antibody therapies^{3,4}. The receptor-binding domain (RBD) of the spike (S) protein that mediates viral entry by binding with the human cell surface protein angiotensin-converting enzyme 2 (ACE2)⁵ is the dominant target of most neutralizing antibodies (NAbs) and vaccines^{3,6}.

However, RBD-specific NAbs face a formidable foe. Molecular epidemiology studies have demonstrated that the RBD is highly variable; in particular, the immunodominant receptor-binding motif (RBM) is the most divergent region, and the variations allow the virus to evade the antibody response^{7,8}. Several studies have shown that SARS-CoV-2 had a low genetic barrier to RBD-specific NAb resistance since a variety of independent escape mutations can easily arise in the vesicular stomatitis virus (VSV)-SARS-CoV-2 chimera system under antibody pressure^{9,10}. Therefore, tracking mutations in the RBD region, which could potentially impact COVID-19 progression and treatment strategies, is vital^{8,11}.

To better understand the viral emergence and evolution, various viral genomes have been sequenced during the COVID-19 pandemic¹². Notably, a recent increase in variants with less susceptible to vaccines and increased infectivity caused great concern¹¹. The mutations are featuring RBD. Among the SARS-CoV-2 variants of concern, B.1.351 (RBD-K417N/E484K/N501Y) discovered in South Africa and P.1 (RBD-K417T/E484K/N501Y) discovered in Brazil have been demonstrated to have high potential to reduce the efficacy of some vaccines and NAbs^{13–15}. The RBD mutation E484K has been associated with resistance to many NAbs^{10,16}. In addition, the key residues in the RBD at which amino acid substitutions are

associated with immune escape remain unclear. Such information would provide an effective early warning to prevent the rapid and extensive spread of dangerous new SARS-CoV-2 variants. Typically, only a few interacting residues make energetic contributions to antigen-antibody binding¹⁷; these residues are called “hot spots”^{18,19}. Identification of such hot spot residues recognized by NAb, especially dominant NAb, is important for monitoring antibody-based immune escape.

To gain a broad picture of the protective antigenic sites in the RBD, we performed global alanine scanning using a number of RBD-specific NAb isolated from COVID-19 convalescents, including several broadly reactive NAb. Furthermore, we assessed the impacts of natural mutations at key positions on antibody escape and ACE2 binding function. Our study provides a clear experimental reference for monitoring the immune escape of NAb. Additionally, the highly potent NAb described here represent new opportunities for the prevention and treatment of COVID-19.

Results

Isolation and characterization of RBD-specific NAb from COVID-19 convalescent donors.

To elucidate the degree to which SARS-CoV-2 will adapt to evade NAb, we first generated RBD-specific NAb by screening a cohort of 36 convalescent patients infected with early-circulating SARS-CoV-2 strains during January-March 2020 and selected four donors with high titres of plasma RBD-binding antibodies and NAb against a SARS-CoV-2 pseudovirus (Extended Data Fig. 1). We then conducted single-cell PCR experiments to generate human monoclonal antibodies (mAb) from memory B cells using the SARS-CoV-2 RBD as the bait. Ninety-three RBD-specific antibodies were identified (Fig. 1a and Supplementary Table 1). More than 95% of the RBD-specific mAb did not bind to the denatured form of RBD, indicating that the epitopes targeted by RBD-specific antibodies induced by natural infection are highly dependent on conformation. Furthermore, approximately 12-24% of isolated mAb from a given donor showed cross-reactivity with SARS-CoV; these findings suggested that cross-reactive mAb could be induced by natural infection, as the SARS-CoV-2 and SARS-CoV RBDs share 76% amino acid identity²⁰.

Remarkably, 50-72% of the mAb from each donor exhibited detectable pseudovirus SARS-CoV-2 neutralization ($IC_{50} < 5 \mu g/ml$), with 12-48% design-ated high-potency mAb ($IC_{50} \leq 50 ng/ml$) (Fig. 1b and Supplementary Table 1). Expectedly, 25-64% of the mAb from each donor had measurable receptor-blocking activity ($IC_{50} < 5 \mu g/ml$), suggesting that most of the RBD-specific NAb protect against virus infection via mechanisms that block attachment to the cellular receptor ACE2. More importantly, three antibodies potently neutralized the SARS-CoV-2 pseudovirus with an IC_{50} of 14-25 ng/ml and moderately neutralized the SARS-CoV pseudovirus with an IC_{50} of 0.9-5 $\mu g/ml$. They efficiently blocked binding of the SARS-CoV-2 and SARS-CoV RBDs to ACE2 (Extended Data Fig. 2a-c and Table 1), unlike a previously described broadly reactive NAb, S309, which was independent of receptor binding inhibition²². As shown in Fig. 1d and Supplementary Table 2, the NAb were nearly unrestricted in the germline gene repertoire. Among these heavy chains, 73% originated from IGHV3 and IGHV4. We also observed that RBD-binding

NABs were strongly biased towards IGHV3-53/3-66, consistent with the findings of previous studies^{4,23,24}, and suggest that they play an important role in the humoral immune response to SARS-CoV-2 infection²⁵.

Finally, we verified that the 19 selected mAbs could efficiently neutralize authentic SARS-CoV-2 infection with IC₅₀ values from 20 ng/ml to 1.8 µg/ml, and we found that several NABs exhibited neutralizing activity comparable to that of CB6, which is in clinical²⁶. The 19 NABs were used as probes to search for the binding determinants of RBD-specific NABs due to their high affinity for the SARS-CoV-2 RBD and potent neutralization of SARS-CoV-2 (Table 1 and Supplementary Fig. 1).

Mapping and characterization of protective antigenic sites and antibody binding hot spots on RBD.

To define the epitopes recognized by selected RBD-specific NABs, we first performed competitive binding experiments. Three well-described mAbs targeting independent epitopes, CB6, CR3022 and S309, were used as controls^{22,26,27}. Our panel of NABs could be classified into 5 groups (Table 2). The group 1 and group 2 antibodies competed with CB6, while the group 4 antibodies competed with S309. The three cross-reactive antibodies in group 5 competed with CR3022. The antibodies in group 2 and group 3 may have larger footprints than those in the other groups because they competed with antibodies from two of the other groups. Interestingly, 4/5 of the group 1 antibodies utilized VH3-53/3-66 and had short CDRH3 lengths of 9-13 amino acids (Extended Data Table 1).

To obtain a comprehensive view of the antigenic sites on the SARS-CoV-2 RBD and the determinants of RBD NAb recognition, we performed global RBD alanine scanning mutagenesis (at nearly 190 RBD amino acid positions) with a panel of 17 mAbs derived from the five groups. Functional epitope mapping identified 33 binding determinants for NABs and defined four major antigenic sites (1-4) targeted by RBD-specific NABs based on their structural locations and epitope competition results. Site 1 and Site 2 overlap with ACE2 binding sites, while Site 3 and Site 4 are located outside the sites (Fig. 2a, b and Supplementary Table 3). Some antibodies bind to only one site, whereas others contact more.

The group 1 antibodies mainly bound to site 1. We demonstrated that some mutations, namely, N417A, F456A, N460A, A475V, F486A, and N487A, led to less binding for multiple group 1 antibodies. To verify whether these residues were crucial for the dominant VH3-53/3-66 germline antibodies, we selected eleven VH3-53/3-66 germline antibodies, including CB6 and B38 antibodies whose structures have previously been characterized^{26,28}, as probes (Extended Data Fig. 3a). We demonstrated that most of the VH3-53/3-66 NAb prototypes were sensitive to mutations at F456 and N487 in different extent—while N417, N460, A475 and F486 were also involved in the contact of some VH3-53/3-66 NABs (Fig. 2c). The heavy chains of CB6 and B38 use a similar structural mode for epitope recognition (Extended Data Fig. 3b). The conserved germline-encoded CDRH1 and CDRH2 together with the distinct CDRH3 contribute to tight contact with the core epitope on the RBD formed by N417, F456, N460, A475 and N487 residues within antigenic site 1, suggesting that mutations at these positions may give rise to resistance to VH3-53/3-66-prototype NABs. With regard to the site 2 binding region, we observed that substitutions at shared positions (N450, L452, E484 and F490) reduced the binding of NABs in groups 2 and 3 to the RBD. In

addition, R346 within antigenic site 3 showed favourable interactions with three mAbs from group 3 and group 4. Antibodies in group 5 distinctly preferentially bound to the residues within site 4 (Fig. 2a). We further investigated whether the identified residues involved in antibody binding also influence ACE2 binding (Fig. 2a). As expected, substantial loss of ACE2 binding was caused by mutations in surface residues, namely, F456A, F486A, N487A, Y505A and Y449A, each of which is involved in ACE2 binding according to structural analysis^{5,29}. Two core region mutations distant from the ACE2 binding surface, N343A and W436A, also resulted in loss of ACE2 binding.

We analyzed the relationship between neutralization potency and each antigenic site (Fig. 2d and Supplementary Table 4). Over 70% of the highly potent NAbs targeted antigenic sites 1 and 2; thus, antigenic sites 1 and 2 are the prime targets of SARS-CoV-2 neutralizing antibodies. Furthermore, SARS-CoV-2 and SARS-CoV cross-reactive mAbs mainly targeted antigenic sites 3 and 4 (Fig. 2e), indicating that these two sites are conserved exposed sites, consistent with the findings of previous studies^{22,27}. Taken together, the results have implications for the design of SARS-CoV-2 vaccines, and the binding hot spots of RBD-specific NAbs identified here will support direct and intentional monitoring of immune escape mutants.

The residues essential for RBD folding and antigen conformation are evolutionarily conserved among sarbecoviruses.

Our landscape of mapping data also demonstrated that mutations at approximately 20% of the positions (38 of 190 positions) led to substantial loss of binding for nearly all NAbs as well as recombinant hACE2 (Extended Data Table 2). Most of these residues were buried within the RBD core structure, thus were likely to facilitate RBD folding. Our data also revealed the importance of disulfide bonds in the RBD. (Fig. 3a). Furthermore, these residues were highly conserved across clade 1, 2 and 3 sarbecoviruses^{30,31}, including human and animal isolates (Fig. 3b). The fact that the unchanged residues are so evolutionarily conserved means that they play vital roles and that their preservation is necessary for virus survival. Additionally, point mutations can strongly affect protein stability, which may in turn affect protein function, as illustrated by studies on other viruses^{19,32}. The top 17 destabilizing mutations predicted by the two structure-based methods MAESTRO³³ and DUET³⁴ showed high free energy change ($\Delta\Delta G$) values and low average antibody binding percentages. Mutations resulting in improper RBD folding should be considered in determining functional epitope of antibodies by alanine scanning. Based on structural analysis of the RBD-CB6 complex and the RBD-B38 complex, the conserved Y421 residue is part of the epitope of these complexes because it forms hydrogen bonds with G54 in the CDRH2 of CB6 and B38 (Extended Data Fig. 3b). Collectively, the data suggest that some residues may have low mutational tolerance, so targeting these positions with antibodies could limit viral escape.

Natural substitutions of antibody binding hot spots confer resistance to multiple NAbs.

To investigate the residue polymorphism of each antigenic site, we computed the sequence variability at positions that were binding determinants for selected mAbs (Extended Data Fig. 4a, b). The data showed

that site 1 and site 2 were more polymorphic than site 3 and site 4. Mutations were more frequently introduced in the positions with variable sequences between SARS-CoV-2 and SARS-CoV such that multiple sites were replaced by the same residues or by residues with similar biophysical properties at the corresponding positions of SARS-CoV RBD. Additionally, residues at positions 417, 475, 484, 452, 490 and 346, which are key recognition sites for multiple NABs, were highly polymorphic. In contrast, some conserved residues that were proven to be critical for ACE2 binding by alanine scanning, such as N487, Y505, Y449, W436 and N343 (Fig. 2a), had limited variability, suggesting that these residues have a low inherent tolerance for mutations.

To explore the impacts of natural mutations on NAb binding, we constructed and expressed RBDs with single-amino acid substitutions that are present in circulating human isolates of SARS-CoV-2. As shown in Fig. 4a and Supplementary Table 5, for residues at which several alternate amino acids with different side chains were selected, the different substitutions did not contribute equally to NAb binding. For example, alanine scanning revealed that the F456A mutation caused loss of binding of VH3-53/3-66 NABs, but the natural F456L variation did not result in resistance to VH3-53/3-66 NABs. We also observed that L452R rather than L452M led to substantial loss of binding to 24-34L, 25-F8, 24-12K, and 28-15L. E484A and E484K rather than E484D resulted in resistance to 26-34L, 24-12K and 25-F8. F490V, F490S, and F490L each resulted in strong resistance to 26-34L, 25-F8 and 24-12K. P337R instead of P337S conferred resistance to S309, and R346S instead of R346T caused significant loss of binding of 24-12K, 28-15L and 25-C9. V382E rather than V382L reduced the binding activity of 28-26K and CR3022 in group 5. Collectively, the data suggest that different properties of amino acid substitutions, including hydrophobicity, polarity and charge, might determine resistance in terms of requirements for interactions with mAbs.

We further purified 17 mammalian cells expressed RBD single-point mutants and measured their binding activity with a panel of NABs from the five groups (Fig. 4b). The fold changes in the EC_{50} values compared to those of the wild-type RBD were investigated, and the results were consistent with those of our preliminary mutational scan. Moreover, the molecular mechanisms of the effects of the mutations on three well-characterized mAbs, CB6, S309 and CR3022, were well explained by the structures (Fig. 4c-e). For example, replacement of K417 with Asn (N) greatly weakened CB6 binding affinity by disrupting a strong salt bridge between K417 in the SARS-CoV-2 RBD and the CB6 CDRH3. However, E340K and K378N disrupted the key hydrogen bonds with S309 and CR3022 respectively.

To further investigate whether the binding escape mutants exhibited NAb resistance, we constructed a panel of 17 SARS-CoV-2 pseudovirus variants, including 16 single RBD mutants, to examine their impacts on the neutralization potency of the 12 NABs mentioned above (Fig. 4f). Since the dominant S sequence variant seen in clinical isolates is D614G, all the SARS-CoV-2 pseudovirus variants we constructed were coupled with the D614G variant³⁵. As expected, in agreement with the EC_{50} value results (in which the RBD substitutions resulted in high EC_{50} values for NABs), the pseudovirus variants correspondingly conferred resistance to NABs with high IC_{50} values. Notably, the most frequent RBD variants seen in

clinical isolates, N501Y and S477N, remained similarly sensitive to the majority of the selected NABs; only 24-1L failed to neutralize N501Y. Substitutions responsible for major antigenic escape were in antigenic site 1 (K417N, F486L), antigenic site 2 (N450K, E484K, L452R, F490S) and antigenic site 3 (R346S). Using our immune escape mapping strategy, we identified a natural mutant, E340K, in the circulating virus that conferred resistance to a broadly reactive NAB, S309, and five mutants that resulted in resistance to CB6. These findings could inform the therapeutic use of these antibodies in clinical studies. **Expectedly**, the South African variant (RBD-K417N/E484K/N501Y) facilitated resistance to a somewhat wider range of NABs than single mutations, which conferred complete resistance to five highly potent NABs targeting two major antigenic clusters 1 and 2. However, the site 3-targeting antibodies 25-C9 and S309 and the site 4-targeting antibody 28-26K retained their ability to neutralize the B.1.351 pseudovirus.

Impacts of RBD mutations on ACE2 binding affinity.

To investigate how the antigenic escape residues identified by our study affect the RBD-ACE2 interaction, the binding affinities of eighteen mutated RBDs to ACE2 were analysed with BIAcore 8K (Supplementary Fig. 2). As shown in Fig. 5a, the majority of the mutants retained or even exhibited enhanced hACE2 binding. N501Y, L452R and S477N mutants exhibited high affinity for ACE2 and exhibited 9.24- to 14.66-fold higher binding affinity than wild-type RBD. These data provide a reasonable explanation for the high frequencies of the three mutations in clinical sequencing data (Extended data Fig. 4). Unlike N501Y, which induced tighter binding with ACE2³⁶, S477N and L452R occurred at sites that were likely not in the ACE2 contact region (Fig. 5b). It is possible that the mutations altered the charge within the flexible loop region of the RBM, creating a more favourable environment for binding. Notably, the key antibody escape mutations K417N, N450K, E484K, F490S and R346S had limited effects on ACE2 binding affinity with fold changes between 0.4 and 2.5, suggesting that they were not accompanied by loss of fitness. Despite of the strengthened binding to ACE2 caused by the substitution N501Y, the affinity of the South African B.1.351 variant for ACE2 was only 2.8-fold higher than that of the wild-type RBD, as it was balanced out by two additional mutations (K417N and E484K). Overall, our current data are likely to be useful for understanding the evolutionary mechanism that governs the emergence of viral escape mutants.

The key natural mutations were able to escape neutralization by COVID-19 convalescent donors.

To examine whether pseudoviruses with the key antigenic escape mutations conferred resistance to convalescent plasma from the first wave of SARS-CoV-2 infection in early 2020, nine out of 36 convalescent plasma samples (2, 6, 23, 24, 25, 26, 27, 28 and 32) were selected, and they exhibited different degrees of S-binding, receptor-blocking and neutralizing activity (Extended Data Table 3). The resistance profile of each human convalescent plasma sample was distinct, possibly because of the different repertoires of antigenic sites on the RBD targeted by polyclonal antibodies (Fig. 6a, b). Markedly, K417N, F486L, L452R, E484K and R346S resulted in resistance to at least six plasma samples, as the NAb titres were approximately 2-4 times lower than those for the wild type, indicating that NABs targeting these key residues were enriched in human convalescent plasma. On the other hand, both F490S and N450K resulted in resistance to neutralization by plasma samples 6 and 24; in particular, the NAb titre of

plasma sample 24 against F490S was reduced by 4.6 times. N501Y and S477N, two of the most abundant RBD mutations, provide resistance to a very limited number of human convalescent plasma, indicating that they may not pose a threat to human immune protection against natural infection, in agreement with the conclusions of previous studies^{10,37,38}. Finally, we demonstrated that compared with the wild-type residues, the N501Y, K417N, and E484K mutations in the B.1.351-variant pseudovirus dramatically reduced the neutralizing ability of all the plasma samples, with 2.1- to 7.4 -fold reductions in NAb titres, which are consistent with previous studies¹⁵. In addition, this combination of mutations resulted in more resistance than single mutations. Overall, these data suggest that circulating viruses with single mutations at antigen binding hot spots could be resistant to neutralization by human convalescent plasma but that no single amino acid mutation can enable robust escape. The evolution of co-mutations at distinct major antigenic sites is worthy of considerable attention.

Discussion

Functional mapping analysis revealed that the majority of RBD-specific NAb target four antigenic sites, site 1, site 2, site 3 and site 4, which are consistent with the four structurally defined regions⁴. Molecular epidemiology analysis demonstrated that the immunodominant sites 1 and 2 are the most divergent regions, suggesting that variability in these sites is the main factor contributing to RBD antigenic evolution. In contrast, sites 3 and 4, which are located in the core region, are more conserved and present a high genetic barrier to immune resistance, providing strong support for the development of antibodies and vaccine candidates that preserve these two conserved antigenic sites.

Several binding hot spots that tend to be targeted by RBD-specific NAb have been identified by alanine scanning: K417, F456, A475, F486, N487, N450, R452, E484, F490 and R346. Some of the hot spots have also been reported as the key binding determinants for NAb by other groups^{9,39}. Thus, the antigenic maps could be useful for monitoring viral escape of the majority cluster of RBD-targeting antibodies. Our comprehensive escape mutation map based on the natural substitutions of the binding hot spots not only confirms the widely circulating strains carrying important immune escape RBD mutations such as K417N, E484K and L452R, but also facilitate the discovery of new immune escape-enabling mutations that occur at low frequencies such as F486L, N450K, F490S and R346S which could potentially impact disease severity and influence treatment strategies in the future. We found that the natural mutant K417N was resistant to several highly potent NAb derived from VH3-53/3-66, including the clinically approved CB6³⁹. In addition, we found that circulating viruses evolved different substitutions at the same positions to evade the immune response at two hot spots, including E484 and F490. Our findings and those of previous studies suggest that the B.1.351 variant with combined K417N/E484K/N501Y substitutions exhibits considerably enhanced resistance to numerous NAb and polyclonal antibodies in human convalescent plasma¹³⁻¹⁵. SARS-CoV-2 variants carrying antigenic escape-enabling mutations at multiple residues may be resistant to vaccines or antibody-based therapeutics. Thus, Special attention should be paid to the accumulation of co-mutations at distinct major antigenic sites during evolution.

Comparison of the hACE2 binding affinity between RBD mutants demonstrated that some antigenic escape-enabling mutations preserved or even enhanced hACE2 binding, which implies that these mutations are not accompanied by loss of fitness and are more likely to occur and spread quickly under immune pressure. The substitution L452R was particularly notable, as it also conferred resistance to multiple NABs and human convalescent plasma. Therefore, circulating SARS-CoV-2 variants with L452R in the RBD region might be more infectious and less susceptible to NABs and vaccines than variants without this mutation. Our previous study has also demonstrated that the SARS-CoV-2 RBD carrying L452K exhibits enhanced ACE2 binding. In contrast, the SARS-CoV RBD with K439 (452) L exhibits decreased ACE2 binding, indicating the involvement of a similar mechanism²⁰. Co-mutations are needed to balance the fitness cost of antigenic change, as has been described for other viruses^{40,41}. Given the high mutation rate of this virus, additional combinations of mutations that are compatible with viral fitness or are associated with enhanced viral resistance are likely to arise⁷. Future work that combines investigations of antigenic mutations and receptor binding affinity-affecting mutations should be performed to analyse antigenic changes, and research on viral fitness evolution is needed to monitor emerging high-risk strains.

Our comprehensive antigenic maps of RBD targeting by a panel of representative NABs provides valuable information for monitoring of the clinical consequences of SARS-CoV-2 evolution. Additionally, the reagents described here, particularly the three new broadly reactive NABs, offer new options for antibody-based therapeutics, and provide a useful set of tools for evaluation of vaccines that are currently under investigation.

Methods

Recombinant proteins

To generate recombinant human ACE2-hFc, a DNA fragment encoding the extracellular domain of human ACE2 (residues S19 to S740) was fused in-frame with an N-terminal human IgE signal peptide and a C-terminal human IgG1 Fc. There is a flexible “GSGGGG” linker between ACE2 and human IgG1 Fc. The recombinant hACE2 protein was expressed by ExpiCHO-S™ cells and purified with Protein A (MabSelect Prism A, GE Healthcare) followed by size exclusion chromatography using a Superdex 200 10/300 column (GE Healthcare).

To generate recombinant RBD mutants, the DNA sequences encoding the SARS-CoV-2 RBD soluble fragments encompassing amino acids 319-591 of the S protein were fused in-frame with an N-terminal human IgE signal peptide and a C-terminal 8× His tag. The RBD mutants and wild-type RBD were produced transiently in ExpiCHO-S™ mammalian cells (Invitrogen). The proteins were purified by metal affinity chromatography using a His Trap Excel column (GE) and dialyzed against phosphate-buffered saline (PBS). The following S proteins expressed either from baculovirus or mammalian expression systems were all utilized for the binding activity assay: SARS-CoV-2 S-ECD-His (Sino Biological, Cat.No. 40589-V08B1) and SARS-CoV RBD-His (Sino Biological, Cat. No. 40150-V08B2).

Isolation, cloning, expression and purification of RBD-specific mAbs

This study was approved by the Ethics Review Board of the Shanghai Public Health Clinical Center, Fudan University. Human mAbs were generated from human memory B cells by single-cell RT-PCR as previously described⁴². Briefly, peripheral blood mononuclear cells (PBMCs) were stained with Percp-CY5.5-CD4 (BD, Cat. No.560650), Percp-CY5.5-CD14 (BD, Cat. No. 562692), Percp-CY5.5-CD8 (BD, Cat. No. 565310), FITC-CD19 (BD, Cat. No. 555412), APC-IgG (BD, Cat. No. 550931), and biotinylated RBD-streptavidin-SA BV421 before CD19+IgG+RBD+ single B cells were sorted into 96-well plates containing lysis buffer. The VH, VK and VL variable genes were amplified by RT-PCR and nested PCR using cocktails of specific primers and then cloned into expression vectors⁴³. The gene sequence analysis of mAbs were performed by IMGT and IgBLAST. The S309, CR3022, CB6 and B38 VH and VL sequences were synthesized and cloned into expression vectors (Shanghai Generay Biotech Co., Ltd). The plasmids encoding the paired heavy chain and light chain were co-transfected into ExpiCHO™ cells according to the manufacturer's instructions (Thermo Fisher Scientific, Cat. No. A29129). After 7 days, the antibodies were purified from the ExpiCHO™ cell supernatants using Protein A.

ELISA.

To determine the binding activities of the antibodies or plasma, recombinant protein (0.5 µg/ml in 100 µl/well) was captured in a 96-well plate overnight, and the plate was blocked with 2% bovine serum albumin (BSA) in PBS-Tween 20 (PBST) for 2 h. The antibodies or plasma samples were serially diluted in PBST and incubated in the wells for 2 h. The samples were washed three times, and an anti-human Fc HRP antibody (Sigma-Aldrich) was used to detect the binding affinity. The absorbance at 450 nm was recorded by a plate reader (Bio-tek). To assess the reactivity of SARS-CoV-2 RBD-specific antibodies to the denatured RBD, ELISA was performed as mentioned above. The RBD was denatured with 0.1% SDS, 50 mM DTT and a metal water bath at 100°C for 5 min. Anti-HCV 8D6 IgG1 was used as an isotype control⁴⁴.

For the receptor blocking assay, hACE2-FC (5µg/ml) was coated onto microplates. The isolated antibodies or plasma samples were serially diluted and incubated with the biotinylated RBD (200 ng/ml) for 1 h and then added to the wells after washing and blocking. HRP-conjugated streptavidin was used as the detection antibody. The following procedure was the same as mentioned above. The percentage of binding inhibition was calculated as the percent reduction in RBD binding to hACE2-FC from the value in the absence of the antibody.

For antibody competition assays, excessive amounts of primary antibodies (50 µg/ml) were added to the recombinant RBD pre-coated plates at a concentration of 0.5 µg/ml, and the plates were incubated for 1 h at 37°C. Biotin-labelled secondary detection mAbs (1 µg/ml) were then added to the plates. The plates were washed after 2 h of incubation at 37°C, and binding of the detection antibodies was detected with HRP-conjugated streptavidin.

RBD mutagenesis and binding measurements

The DNA sequences encoding the SARS-CoV-2 RBD soluble fragments encompassing amino acids 319-589 of the S protein were fused in-frame with an N-terminal human IgE signal peptide and a C-terminal human IgG1 Fc and 8× His tag. Global alanine scanning of the RBD was performed using site-directed mutagenesis of RBD residues (330-521) to alanine (natural mutations for alanine residues). Substitutions of the residues at the antigenic sites selected for mutagenesis were based on the 2019 Novel Coronavirus Resource (2019nCoV-R) released by the China National Center for Bioinformation (<https://bigd.big.ac.cn/ncov/protein>). Site-directed mutagenesis was induced with a commercialized KOD-Plus mutagenesis kit (TOYOBO). All of the mutations were confirmed by DNA sequence analysis (Biosune). The resulting plasmids were transfected into ExpiCHO-S™ cells in 12-well plates. The supernatants were harvested 96 h after transfection. Sandwich ELISA was performed to measure individual E2 protein expression in the cell supernatant. In brief, a mouse anti-6× His tag mAb was used to coat plates; 20-, 100-, 500-fold dilutions of cell supernatant were added; and the proteins were captured by the anti-6× His tag mAb. Serially diluted purified RBD-FC-8× His (2-fold dilutions from 500 ng/ml) was used as a standard, and detection was performed with HRP-conjugated goat anti-hFc (Sigma). The concentration of the sample was calculated according to a standard curve generated from the serial dilution data. Another ELISA was performed to analyse the relative binding activities of these RBD mutants for RBD-specific antibodies and ACE2; wild-type RBD was used as a control. Then, 300 ng/ml concentrations of the RBD mutants were incubated with plates pre-coated with 500 ng of antibodies or 500 ng of ACE2-FC, and detection was performed with an HRP-conjugated mouse anti-6× His mAb. The signals produced by binding of the mutants to the mAbs were compared to those produced by binding of the wild type.

Biolayer interferometry (BLI) analysis of RBD and antibody binding affinity

Binding affinity (KD) analysis was conducted by BLI at 25°C using an Octet Red 96 system (ForteBio, Inc.) as previously described. mAbs (20 µg/ml) were captured on an anti-human IgG-Fc (AHC)-coated biosensor surface for 5 min. The baseline interference was then read for 60 s in kinetics buffer (KB: 1× PBS, 0.01% BSA, and 0.02% Tween-20), after which the sensors were immersed into wells containing recombinant RBD diluted in KB for 300 s (association phase). The sensors were then immersed in KB for the indicated times (dissociation phase) for up to 900 s. The mean K_{on} , K_{off} and apparent KD values were calculated using an equation globally fitted to a 1:1 binding kinetic model with an R^2 value of ≥ 0.95 .

Surface plasmon resonance (SPR) analysis of RBD mutants and ACE2-FC

The affinity of RBD mutants for ACE2-FC was measured using SPR. Purified ACE2-FC was captured on a Protein A sensor chip using a BIAcore 8K chip (GE Healthcare). RBD samples at the following concentrations were prepared and injected at 100 µl/min: 0 nM, 6.25 nM, 12.5 nM, 25 nM, 50 nM, 100 nM, and 200 nM. The BIAcore chip was regenerated between each cycle with regeneration buffer containing 10 mM glycine (pH 1.5). The generated binding curves were used to extract the kinetics of the rate of RBD binding to ACE2-FC. Due to the slow off rate of this interaction, separate extended dissociation off rate

experiments were performed. The data were double reference-subtracted and fit using a 1:1 binding model.

Pseudovirus neutralization assay

For pseudovirus variants, individual mutations associated with RBD antibody escape were introduced into a full-length SARS-CoV S plasmid using homologous recombination. All of the mutations were confirmed by DNA sequence analysis (Biosune). SARS-CoV, SARS-CoV-2, and SARS-CoV-2 pseudovirus variants were produced as previously described. Briefly, plasmids encoding the full-length S gene and pNL4-3.luc.RE were cotransfected into HEK 293T cells in 10 cm dishes with Lipofectamine 3000 Transfection Reagent. The medium was replaced after 6 h, and the virus supernatants were collected 48 h after transfection. HEK 293T-hACE2 cells were plated into 96-well plates one day prior. The pseudovirus titre was measured with HIV p24 ELISA kits. The virus was then diluted in DMEM with 10% FBS, mixed with an equal volume of serially diluted antibodies or plasma (preheated at 56°C for 30 min to inactivate complement) and incubated for 1 h at 37°C. The mixtures were transferred to HEK 293T-hACE2 cells. The cells were incubated at 37°C for 48 h and then subjected to cell lysis and a luciferase activity assay (Promega). The percent neutralization was calculated by comparing the luciferase value of the antibody-containing samples to those of the untreated controls. The neutralization curves were fit by nonlinear regression using GraphPad Prism software.

Authentic virus neutralization assay

An authentic virus neutralization assay was performed in the BSL-3 laboratory of Fudan University in compliance with the guidelines of the laboratory biosafety manual. The SARS-CoV-2 clinical isolate nCoV-SH01 (GenBank: MT121215.1) was amplified in Vero E6 cells and used for authentic virus neutralization⁴⁴. Live SARS-CoV-2 virus (100 TCID₅₀, 50 µl) was mixed with 50 µl of three-fold serially diluted RBD-specific NAb and incubated at 37°C for 1 h. The cellular supernatant was discarded, and the antibody-virus mixture was transferred into Vero E6 cells and allowed absorption for 1 h at 37 °C. Inoculums were then removed before adding the overlay media (100 µl DMEM with 2% FBS and 1% carboxymethylcellulose). After incubation for 3-4 days at 37°C in a humidified atmosphere with 5% CO₂, the plates were inspected under an inverted optical microscope. Then overlays were removed, cells were fixed with 4% paraformaldehyde solution for 10 min and stained with crystal violet. The numbers of SARS-CoV-2 foci were calculated and wells with individual plaques were used to determine the virus titer. The neutralization curves were fit by nonlinear regression using GraphPad Prism software.

Structure analysis

Structural figures were generated using the PyMOL Molecular Graphics System (Version 2.0, Schrödinger, LLC). The programs MAESTRO and DUET were used to predict the stability of RBD alanine mutants based on the RBD structure (Protein Data Bank [PDB] ID code 7C01).

Data availability

The structures used for analysis and the amino acid sequences of the heavy chain and light chain of control mAbs were 6M0J (SARS-CoV-2 RBD/hACE2), 7C01 (SARS-CoV-2 RBD/CB6), 6WPT (SARS-CoV-2 S/S309), 7A5S (SARS-CoV-2 S/CR3022) and 7BZ5 (SARS-CoV-2 RBD/B38), which were downloaded from the National Center for Biotechnology Information (NCBI) PDB. The mutation information of the SARS-CoV-2 S protein was generated from CNCB-NGDC/the 2019nCoV-R (<https://bigd.big.ac.cn/ncov/protein>)^{45,46}. All consensus full-length, nonredundant polyprotein sequences of clade 1, 2 and 3 sarbecoviruses, including human and animal isolates, are available from the GISAID database (<https://www.gisaid.org>) and the NCBI database. The sequences were aligned and analysed using ClustalX.

Declarations

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These authors contributed equally: Chunyan Yi, Xiaoyu Sun, Yixiao Lin & Chenjian Gu

Corresponding author: Bing Sun, Youhua Xie, Jianqing Xu & Zhiyang Ling

Author contributions: B.S., H.Z.L., Y.H.X. and Z.Y.L. initiated, designed and supervised the study. C.Y.Y. and X.Y.S. designed and performed all the experiments. Y.X.L. collected the blood sample and conducted human serum-related experiments. L.F.D. contributed to the isolation of spike-specific memory B cells. C.J.G. contributed to the authentic virus neutralization experiments. X.L., Y.G.Z., L.Y.M. and W.P.G. provided materials and helped with some experiments during this study. A.D.Q., X.Z. and X.L.L. prepared the recombinant hACE2 protein. B.S., Z.Y.L., C.Y.Y. and X.Y.S. analysed the data. Z.Y. performed structural analysis. J.Q.X. provided valuable suggestions. Z.Y.L., C.Y.Y., X.Y.S., and B.S. wrote the paper.

Ethics declarations

Competing interests: The authors declare no competing interests.

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Tables

Tables 1-2 are available in the Supplementary Files.

Figures

Figure 1

Characterization of SARS-CoV-2 natural infection-induced RBD-specific mAbs. **a**, Antibody binding activity (EC₅₀) with native and denatured SARS-CoV-2 RBD as well as native RBD of SARS-CoV was measured by ELISA. Upper panel, four ranges per donor; lower panel, percentage of mAbs from each donor with the indicated EC₅₀ range. N.B., non-binding activity. **b**, SARS-CoV-2 pseudo-virus neutralization potency (IC₅₀) (left). Percentage of antibodies with indicated neutralization potencies (right). N.N., non-neutralization. Results are derived from a single experiment performed in triplicate. **c**, ACE2 blocking activity (IC₅₀) (left). Percentage of antibodies with indicated receptor blocking potencies (right). For (b) and (c), N.N., non-neutralization or non-blocking activity. The data represent one representative experiment of two independent experiments. **d**, Distribution of heavy-chain variable (VH) germline genes of RBD-specific NAb.

Figure 2

Determination and characterization of antibody-escaped antigenic sites on RBD. a, Mapping of binding sites of a panel of RBD-specific NABs by global alanine scanning. 33 amino acid positions were identified in four antigenic sites (1-4) on RBD as main targets for RBD-specific NABs. Degree of binding reduction was defined as percentage by OD450 of each mutant relative to OD450 of RBD wild type and is represented as a heatmap from white (reduction) to blue (no impact). The data are representative of at least two independent experiments. b, Location of four distinct antigenic sites on the RBD region (PDB ID: 6M0J). The color-coding scheme is described as follows: Site 1 (Orange), Site 2 (green), Site 3 (slate blue), Site 4 (Red); ACE2 (wheat). Top and down are shown from different angles. The key hot spots targeted by NABs are shown. c, key residues for VH3-53/3-66 dominant NABs recognition. Binding fold change was calculated as follows: EC50 of RBD mutant / EC50 of RBD wildtype. d, Antibodies are grouped according to neutralization potency and colored by the usage frequency of each antigenic sites. Each antibody was tested for competition with a panel of known antibodies and assigned to an antigenic site based on the competition profile. e, Percentage of SARS-CoV-2 and SARS-CoV crossing reactive

antibodies targeting each antigenic site. The data represent one representative experiment of two independent experiments.

Figure 3

The mutations that impair global folding of RBD are evolutionally conserved. a, The residues of which Alanine substitution impaired RBD antigenic conformation and ACE2 binding ability are shown in a surface representation (PDB ID: 6M0J). Mutants located in core region are highlighted in green and RBM are in cyan. Yellow sticks indicate disulfide bridges. b, Conservation of residues that are essential for RBD folding across clades of Sarbecoviruses. Human and animal SARS-related coronaviruses were classified by clades. SARS-CoV-2 genome sequences (n=364,409) retrieved from GISAID and Genbank on January 19th 2021 (n=11,839) and human SARS-CoV genome sequences (n=200) from Genbank were used to annotate variants of the spike glycoprotein. Dashes indicate identity to SARS-CoV-2 consensus residues. Variants found in at least two sequences are parenthesized. c, Top predicated destabilizing mutants by structural analysis. The score of MAESTRO and DUET is for predicted impact on stability from the RBD structure (PDB ID: 7C01). Average percent binding versus wild-type RBD for conformation-dependent RBD antibodies are shown.

Figure 4

The impacts of natural mutations at antigenic sites on binding and neutralizing activities of RBD-specific NAb. a, Binding activity of RBD natural mutants with a panel of NAb was evaluated by ELISA. Degree of binding reduction was defined as percentage by OD450 of each mutant relative to OD450 of RBD wildtype is represented as a heatmap from white (reduction) to blue (no impact). The data are representative of at least two independent experiments. b, Binding of 17 purified mammalian expressed RBD single point mutants and South Africa variants B.1.351 with a panel of NAb. Binding fold change

was calculated as follows: EC50 of RBD mutant / EC50 of RBD wildtype. N.B., not binding. c, Details of the structural interaction between CB6 and RBD (PDB ID: 7C01) d, Details of the structural of interaction between S309 and RBD (PDB ID: 6WPT). e, Details of the structural interaction between CR3022 and RBD (PDB ID:7A5S). For c-e, polar interactions are indicated by yellow dashed lines. Cyan, heavy chain; light blue, light chain; Grey, RBD. Key residues on RBD highlighted in orange sticks; Key residues on heavy chain highlighted in cyan sticks. f, Neutralization of 17 pseudo-virus variants by a panel of RBD-specific NAbs. Neutralization fold change was calculated as follows: IC50 of pseudo-virus variants / IC50 of the wild type. IC50 values were calculated from three independent experiments. The data represent one representative experiment of two independent experiments.

Figure 5

The binding affinity between antibody escape mutants and ACE2. a, Affinity measurement of purified RBD mutants for binding to immobilized ACE2-FC by Surface plasmon resonance (SPR). The Kon and Koff were determined by BIAcore and the KD were computed as Koff / Kon. Neutralization fold change was calculated as follows: KD value of RBD wild type / KD value of RBD mutants. b, The residues that are important for resistance to antibodies are presented on the interface of the ACE2 and RBD. The position of mutants that enhance ACE2 binding affinity are highlighted in red (affinity fold change ≥ 2.5); comparable to wildtype are in orange (affinity fold change between 2.5-0.4); reduce ACE2 binding affinity are in slate blue (affinity fold change ≤ 0.4). The data represent one representative experiment of two independent experiments.

Figure 6

Effect of natural mutations on neutralizing activity of convalescent plasma. a, Neutralization potency of nine convalescent plasma against pseudo-virus variants. The data are presented as the highest plasma dilution giving a $\geq 50\%$ inhibition of pseudo-typed virus infection (NT₅₀). NT₅₀ values were calculated from three independent experiments. b, The neutralizing antiserum titer against pseudo-typed virus RBD variants decrease relative to wildtype pseudo-typed virus. Neutralization fold change was calculated as follows: NT₅₀ of the wild type / NT₅₀ of pseudo-virus variants. The data represent one representative experiment of two independent experiments.

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