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Broad strategies for neutralizing SARS-CoV-2 and other human coronaviruses with monoclonal antibodies

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Antibody therapeutics and vaccines for coronavirus disease 2019 (COVID-19) have been approved in many countries, with most being developed based on the original strain of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 has an exceptional ability to mutate under the pressure of host immunity, especially the immune-dominant spike protein of the virus, which is the target of both antibody drugs and vaccines. Given the continuous evolution of the virus and the identification of critical mutation sites, the World Health Organization (WHO) has named 5 variants of concern (VOCs): 4 are previously circulating VOCs, and 1 is currently circulating (Omicron). Due to multiple mutations in the spike protein, the recently emerged Omicron and descendent lineages have been shown to have the strongest ability to evade the neutralizing antibody (NAb) effects of current antibody drugs and vaccines. The development and characterization of broadly neutralizing antibodies (bNAbs) will provide broad strategies for the control of the sophisticated virus SARS-CoV-2. In this review, we describe how the virus evolves to escape NAbs and the potential neutralization mechanisms that associated with bNAbs. We also summarize progress in the development of bNAbs against SARS-CoV-2, human coronaviruses (CoVs) and other emerging pathogens and highlight their scientific and clinical significance.

antibody therapy, broad neutralizing antibody, human coronaviruses, SARS-CoV-2

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Introduction

The application of antibodies in humans was reported more than 130 years ago (Behring, 1890). Emil Adolf von Behring and other researchers were the first to show that, through blood plasma or serum, antibodies could be transferred from one person or animal to another person, who then obtained immune protection (Kaufmann, 2017). In 1901, the Nobel Prize in Physiology or Medicine was granted to Emil Adolf von Behring "for his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and death (Raju, 1998)." Since then, the clinical use of immunoglobulin products containing polyclonal neutralizing antibodies (NAbs) has been extended to prevent and treat multiple infectious diseases and toxins, including influenza A viruses, rabies virus, hepatitis B virus (HBV), Cytomegalovirus (CMV) and tetanus (Mair-Jenkins et al., 2015; Nicholls et al., 1983). After many decades of work, scientists have defined the chemical properties and structure of antibodies (Edelman, 1973), developed a method to generate monoclonal antibody (mAb) and determined the genetic

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principle for the generation of antibody diversity (Tonegawa, 1983).

The landmark technology of mAb development, as the foundation of the antibody drug industry, was invented by Georges Köhler and César Milstein in 1975 (Köhler and Milstein, 1975). Approximately 10 years later, in 1986, the first licenced mouse mAb Orthoclone OKT3 (muromonab-CD3) was approved to prevent kidney transplant rejection (Emmons and Hunsicker, 1987). In 1998, the first antiviral mAb drug, Palivizumab was licenced by the FDA, although polyclonal antibody products have been used to prevent viral infections or treat toxin-related diseases for many years (Sandritter, 1999). Palivizumab is a humanized mAb for the prevention of respiratory syncytial virus (RSV) in high-risk infants. Compared wtih polyclonal products, Palivizumab provides an increased batch-to-batch stability and a reduced risk of blood-borne pathogen infection with approximately 50-fold enhanced potency (Graham and Ambrosino, 2015). These advantages are also the reasons why mAb drugs are replacing polyclonal antibody products against rabies virus (de Melo et al., 2022), HBV (Cerino et al., 2019) and other pathogens or toxins. Overall, achievements in bioscience, medical science, and bioengineering have accelerated the clinical development of antibody drugs targeting TNF-α, EGFR, CD20, HER2, PD-1/PD-L1 and emerging new molecules (Carter and Lazar, 2018). Thus far, a large number of patients with autoimmune diseases, cancer, and other conditions have benefited from antibody therapies (Chan and Carter, 2010).

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is responsible for coronavirus disease 2019 (COVID-19), has been a tremendous threat to human society and health. Since the emergence of this novel coronavirus (CoV) at the end of 2019, antibody drugs against it have been developed (Corti et al., 2021). The spike (S) protein mediates viral entry and serves as a dominant target of most NAbs and vaccines. However, SARS-CoV-2 has an exceptional ability to mutate and evade human immunity, and some clinically approved antibody drugs are losing their ability to neutralize newly emerging variants to varying degrees (Cameroni et al., 2022; Cao et al., 2022a; Yi et al., 2022).

Broadly neutralizing antibodies (bNAbs) that target conserved epitopes of functional domains of viruses have the potential to provide universal protection. The concept of bNAbs has been proven to be useful against many viruses, including influenza A viruses, Human Immunodeficiency Virus (HIV), Hepatitis C virus (HCV), and RSV (Burton et al., 2012).

In this review, we focus on SARS-CoV-2 variants and mutations within the S protein that are targets if current SARS-CoV-2 antibody therapies. Furthermore, we summarize research on bNAbs against SARS-CoV-2 variants, the genus *Betacoronavirus*, and even the subfamily *Orthocoronavirinae*. Finally, we discuss the potential clinical application of bNAbs together with vaccines and small molecular drugs to combat SARS-CoV-2 variants or emerging CoVs in the future.

Antibody drugs played an important role in combating COVID-19 at the early stage of the pandemic (Focosi et al., 2022); however, the newly emerged Omicron variant and derivatives are able to escape most of the antibodies that have been approved or are in clinical trials (Cao et al., 2022a). The role of antibodies in the current SARS-CoV-2 pandemic and potential new CoV pandemic remains unresolved. We propose and examine evidence for broad strategies for combating CoVs by antibodies to answer the following questions: How are human CoVs neutralized by antibodies? How does SARS-CoV-2 evolve to escape NAbs? Do bNAbs against human CoVs exist? In addition, we sought to describe how the COVID-19 pandemic has accelerated the discovery and development of specific antibody therapies and the clinical-related design of SARS-CoV-2 antibodies.

Human coronaviruses and SARS-CoV-2 variants

CoVs are enveloped positive-sense single-stranded RNA viruses (V'kovski et al., 2021). In the past 20 years, 3 highly pathogenic CoVs that infect humans have emerged: severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002–2003 (Dimitrov, 2003), Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 (Lu et al., 2013), and SARS-CoV-2 first reported in 2019 (Li et al., 2020). In addition, 4 human CoVs have been identified that cause seasonal mild respiratory tract infections similar to the "common cold" in humans: HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1. HCoV-229E and HCoV-OC43 have long been known to circulate in humans, whereas HCoV-NL63 and HCoV-HKU1 were identified after the outbreak of SARS-CoV (Hamre and Procknow, 1966; Tyrrell and Bynoe, 1965; van der Hoek et al., 2004; Woo et al., 2005). The receptors for human CoVs vary and include angiotensin-converting enzyme 2 (ACE2) for SARS-CoV-2, SARS-CoV and HCoV-NL63, dipeptidyl peptidase-4 (DPP4) for MERS-CoV, and aminopeptidase N and sialic acids (APN) for HCoV-229E (Lu et al., 2013; Tortorici et al., 2019; Wong et al., 2017; Wu et al., 2009) (Figure 1A).

The COVID-19 pandemic caused by SARS-CoV-2 is considered among the deadest pandemics in the past century (Morens and Fauci, 2020). According to statistics from the World Health Organization (WHO), over 600 million infections and about 6.5 million deaths due to COVID-19 worldwide have been reported as of early September 2022. The first genome sequence of SARS-CoV-2 was released in January 2020 (Wu et al., 2020) and became the reference for the development of vaccines, therapeutic antibodies, and

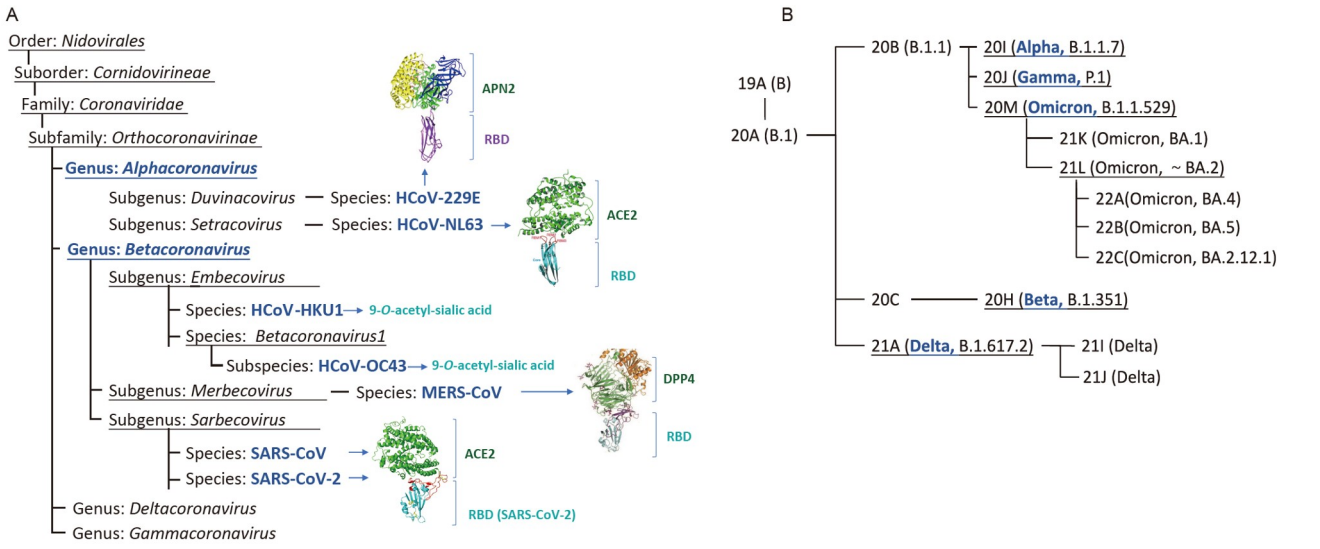

Figure 1 Human CoVs and SARS-CoV-2 variants. A, Human CoVs and their receptors. Based on the 10th International Committee on Taxonomy of Viruses (ICTV) report, CoVs are classified under the realm *Riboviria*, order *Nidovirales*, suborder *Cornidovirineae*, family *Coronaviridae*, subfamily *Orthocoronavirinae*. *Orthocoronavirinae* is further divided into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. Regarding CoVs that can infect humans, HCoV-229E and HCoV-NL63 from the genus *Alphacoronavirus* belong to the subgenera *Duvinacovirus* and *Setracovirus*, respectively; both HCoV-OC43 and HCoV-HKU1 from the genus *Betacoronavirus* belong to the subgenus *Embecovirus*; MERS-CoV belongs to *Merbecovirus*; and both SARS-CoV and SARS-CoV-2 belong to *Sarbecovirus*. The receptors for the human CoVs are shown. B, Classifications and geographic distribution of SARS-CoV-2 VOCs. Nextstrain clade naming strategy (flat "year-letter" names), WHO label (VOCs) and Pango Lineage naming are shown.

other antivirals. However, genetic lineages of SARS-CoV-2 have been emerging and circulating around the world since the beginning of the COVID-19 pandemic (du Plessis et al., 2021). In the first few months of the pandemic, a modest rate of mutation frequency was observed, possibly due to the relatively low population of infected individuals, which did not result in strong immune pressure. Several studies have revealed some mutations in the receptor binding domain (RBD) that may enhance ACE2 binding affinity, alter immunogenicity or facilitate the evasion of NAbs, which should have been particularly considered at the early stage of the COVID-19 pandemic (Starr et al., 2020; Zhou et al., 2020).

In late 2020, considering the emergence of variants that posed an increased risk to global public health, the WHO characterized variants of interest (VOIs) and variants of concern (VOCs) to prioritize global monitoring and research and ultimately inform the ongoing response to the pandemic (Konings et al., 2021) (Figure 1B). One SARS-CoV-2 VOC has been demonstrated to be associated with one or more of the following changes: an increase in transmissibility or detrimental change in COVID-19 epidemiology; an increase in virulence or change in clinical disease presentation; or a decrease in effectiveness of public health and social measures or available diagnostics, vaccines, and therapeutics. The Omicron and variants include B.1.1.529, BA.1, BA.2, BA.2.12.1, BA.4, BA.5 and descendent lineages are currently circulating (https://www.who.int/activities/tracking-SARS-CoV-2-variants).

S protein is the main target of NAbs

The S protein consists of two functional subunits, S1 and S2. The S1 subunit is responsible for binding to the receptor and has four domains: the amino-terminal (N-terminal) domain (NTD), the RBD and two carboxy-terminal (C-terminal) domains (CTD1 and CTD2). The RBD has two subdomains: a core structure and an extended loop named the receptor-binding motif (RBM) that forms the contact surface with ACE2. The S2 subunit is responsible for the fusion of the virus with a host cell and includes major domains of the fusion peptide (FP), heptad repeat 1 (HR1) and heptad repeat 2 (HR2). In addition, two proteolytic cleavage sites have been identified: the S1/S2 site between these two subunits and the S2′ site followed by the FP in the S2 subunit (Starr et al., 2020) (Figure 2A–E). The S proteins of other CoVs have a similar structure (Lu et al., 2013; Wu et al., 2009).

The first step of entry is attachment of the virus to the host cell, as mediated by specific binding of the RBD to the cellular receptor (Figure 2F). The expression and tissue distribution of the receptor subsequently influence viral tropism and pathogenicity. The major NAb epitopes of the S protein are located in the RBD, which is immunodominant and is the target of 90% of the neutralizing antibodies present in SARS-CoV-2 immune sera (Piccoli et al., 2020). Although overall RBD-specific serum IgG titres wane with a half-life of 49 days, NAb titres and avidity increase over time for some individuals, consistent with affinity maturation (Piccoli et al., 2020). The antigen of most approved SARS-CoV-2

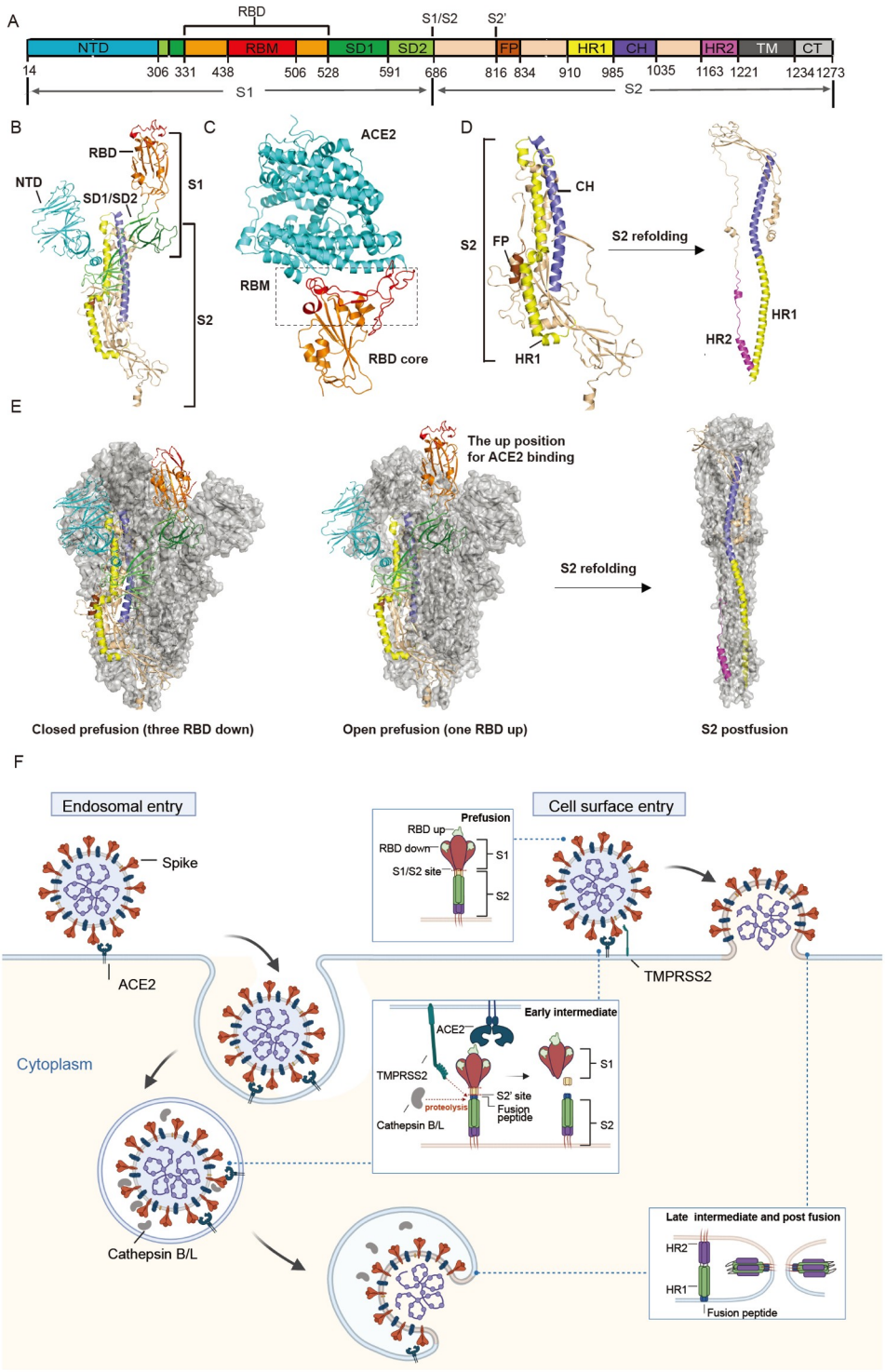

Figure 2 Structure of the SARS-CoV-2 S protein and the invasion mechanism involved in its function. A–D, Functional domains of the S protein (SARS-CoV-2). A, Numbering of the SARS-CoV-2 S protein; the domains and proteolytic sites are annotated. B, The structure of the S protein. C, Interaction between the RBM of the RBD and the cellular receptor ACE2. D, S2 structure. Left structure, prefusion; right structure, post fusion. E, Confirmations of the structures of the S protein. Left structure, closed prefusion with three RBDs "down"; middle structure, open prefusion structure with one RBD "up" and two RBDs "down"; right structure, postfusion S2. F, Invasion of a host cell by SARS-CoV-2 via endosome-dependent and direct fusion. S1/S2 is cleaved before a virus starts to invade a host cell. After binding of the virus to the cell surface ACE2, S2′ proteolysis is a key step in initiating the conformational change of S2, which causes fusion and release of the viral genome. Two entry routes have been observed depending on the abundance of membrane TMPRSS2. The dynamics of the S protein during invasion include a prefusion conformation with one or more RBDs "up", early intermediate conformations after receptor binding and S2′ proteolysis, late intermediate conformation that facilitate membrane fusion, and finally, the post fusion conformation. The image in F was created using BioRender.com.

vaccines are S protein with modifications to stabilize the structure or retain the prefusion conformation, while the inactivated vaccines also contain full-length wild type S protein (Creech et al., 2021). These vaccines potentialy provide protection by inducing the production of RBD-specific antibodies, while the NTD might also induce protective immunity (Amanat et al., 2021; Gao et al., 2020; Wang et al., 2021d).

After receptor binding, a CoV invades the host cell via two mechanisms, cell surface entry (Hoffmann et al., 2020) or endosomal entry (Zhou et al., 2020) pathways, which result in fusion of the viral membrane and the host cell membrane. Two S protein proteolysis events are typically necessary: one at the boundary of S1-S2 subunits and another at the S2′ site of the S2 subunit. In the case of SARS-CoV-2, the S1/S2 boundary is cleaved during the maturation process before the release of virions from an infected cell, but the S2′ site is cleaved at the target cell following ACE2 binding. Binding of the S protein to ACE2 induces conformational changes in the S1 subunit and exposes the S2′ cleavage site. The S2′ site is cleaved by different proteases. In the presence of transmembrane serine protease 2 (TMPRSS2), S2′ cleavage occurs at the cell surface. If the target cell expresses insufficient TMPRSS2 or if a virus-ACE2 complex does not encounter TMPRSS2, the virus-ACE2 complex will be internalized via endocytosis into endolysosomes, where S2′ cleavage is performed by cathepsins B/L in the acidic environment of that organelle (Ou et al., 2020). In both entry pathways, cleavage of the S2′ site exposes the fusion peptide (FP) and dissociates S1 from S2. This dissociation subsequently induces dramatic conformational changes in the S2 subunit (especially in HR1), propelling the fusion peptide forward into the target membrane and initiating membrane fusion. Fusion between the viral and cellular membranes forms a fusion pore through which the viral RNA is released into the host cell cytoplasm for replication (Jackson et al., 2022) (Figure 2F).

Overall, the development of NAbs has focused on the mechanisms of SARS-CoV-2 entry into host cells, including the binding of the S protein to its receptor ACE2 and subsequent membrane fusion mediated by the S protein, which are also shared by other CoVs (Corti et al., 2021; Li et al., 2022b; Li et al., 2021). As SARS-CoV-2 is phylogenetically closely related to SARS-CoV and shares some identical amino acids within the RBD, antibodies derived for neutralizing SARS-CoV have been tested, with a few of them showing cross-neutralizing activity against SARS-CoV-2 (Pinto et al., 2020; Rappazzo et al., 2021). Furthermore, great efforts have been directed at developing potent and specific antibodies. Some monoclonal human antibodies targeting the S2' site and fusion peptide or the stem helix of S2 exhibit broad neutralizing activity *in vitro* and *in vivo* against SARS-CoV-2 and other human CoVs, which will be discussed in detail below.

Different conformational states of the S protein

The S protein is structurally flexible and dynamic during the cellular entry processes of CoVs (Cai et al., 2020; Turoňová et al., 2020). The conformations include prefusion, early intermediate, late intermediate and post fusion. The prefusion conformation is characterized by an intact trimer structure with a cleaved S1/S2 site and RBDs in "up" or "down" states (Benton et al., 2020). Binding of the S protein to the receptor induces the exposure and proteolytic cleavage of the S2′ site, thus triggering the exposure of the fusion peptide and dissociation of S1 from the S protein trimer (early intermediate conformation) (Yu et al., 2022). Release of S1 enables a conformational change of the S2 subunit and insertion of FP into the host cell membrane (late intermediate conformation), which leads to fusion of the host and viral membranes (post-fusion conformation) (Costello et al., 2022) (Figure 2E, F). Antibodies against CoVs have been identified to target different conformations of the S protein and neutralize the virus by interfering with the key processes of invasion.

Platforms for generating SARS-CoV-2 antibodies

The COVID-19 pandemic accelerated the discovery and development of specific antibody therapies. SARS-CoV-2 antibodies have been isolated from two major sources: human or transgenic mice with integrated human immunoglobulin (Ig) loci (Jakobovits et al., 2007). Recent progress in B-cell screening technology for antibody discovery has accelerated rapid isolation of potent SARS-CoV-2 NAbs. Human donors who recovered from infection or were immunized with the COVID-19 vaccine with potent serum neutralizing activities may be selected as B-cell donors.

High-throughput screening of antigen-specific antibody-secreting cells (ASCs) or antigen-specific memory B cells is an essential step in the discovery of therapeutic antibody sequences. In general, traditional procedures, including phage display, hybridoma followed by humanization and EBV-immortalized human ASCs, are time-consuming (Brekke and Sandlie, 2003), but recent advances in flow cytometry, advanced microfluidics platforms, microengraving and nanowell-based technologies have enabled highly multiplexed single-cell separations or secretion detection (Figure 3).

The protocol for generating fully human mAbs specific to a vaccinating antigen was published in 2009 (Smith et al., 2009), describing a method centred on single-cell RT-PCR. Using this technology, many researchers including us successfully developed fully human bNAbs against influenza A virus, HCV, RSV and SARS-CoV-2 (Sun et al., 2022a; Wang et al., 2016; Yi et al., 2021a; Yi et al., 2021b). In addition, the

antibody cocktail BRII-198/BRII-196 was approved by the National Medical Products Administration (NMPA) of China on December 8, 2021, and the antibodies were obtained by Fluorescence activated Cell Sorting (FACS) plus single-cell RT-PCR technology (Ju et al., 2020).

During the COVID-19 pandemic, the Berkeley Lights Beacon Optofluidic system was used for the high-throughput isolation of fully human antibodies against SARS-CoV-2, with which antibody sequences were obtained in several days. Briefly, B cells were loaded onto an OptoSelect chip, and assays were performed to select single plasma B cells secreting SARS-CoV-2-specific antibodies. Antigen-reactive B cells were exported to 96-well plates preloaded with lysis buffer, followed by single-cell sequencing (Fu et al., 2021; Zost et al., 2020b). The components of the human antibody cocktail Evusheld were isolated from convalescent patients with COVID-19, with contributions from the Berkeley Lights Beacon Optofluidic system (Zost et al., 2020b).

Additionally, the recent development of high-throughput single-cell RNA and VDJ sequencing of B-cell receptor repertoires using 10× Chromium has outperformed single-cell RT-PCR in terms of B-cell screening throughput (Lv et al., 2020; Shiakolas et al., 2022; Zost et al., 2020b). Many antibodies, including the bNAbs BD55-5840 and BD-55-5514, have been obtained using this technology (Cao et al., 2022b)

The epitope landscape and escape maps of RBD

The technique of single B-cell antibody cloning and antibody sequencing allows researchers to rapidly isolate and analyse a large number of antigen-specific mAbs from PBMCs of SARS-CoV-2-infected or vaccinated subjects (Table 1). Numerous studies have been performed to isolate full-length S protein- or RBD-specific mAbs from convalescent patients with COVID-19 (Corti et al., 2021; Shrestha et al., 2021). Consistent with the important function of the RBD, the majority of SARS-CoV-2 NAbs with high potency identified to date target the RBD (Table 1). Various antigenic sites have been described by performing epitope mapping of several

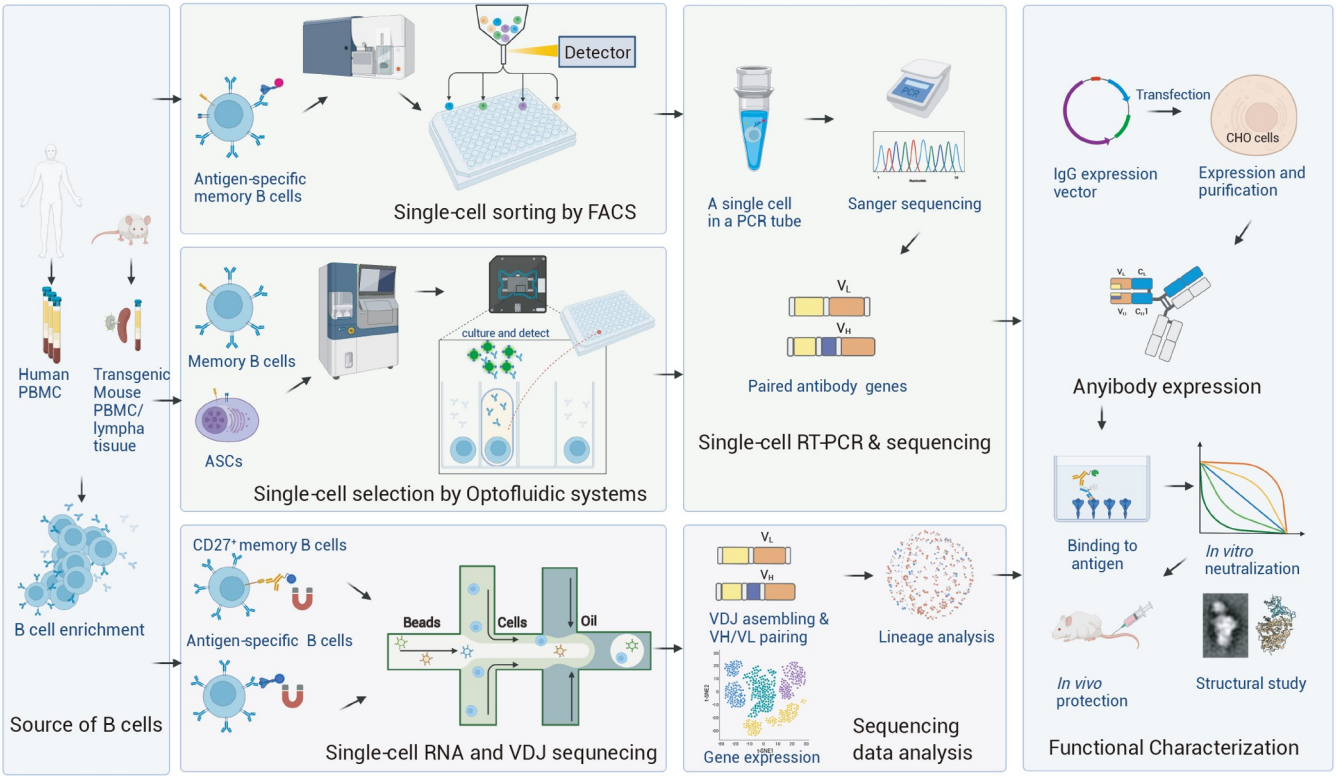

Figure 3 Schematic of platforms for generating SARS-CoV-2 antibodies. The first step is to identify a source of B cells from suitable infected or vaccinated donors or humanized animals. Blood or lymphatic tissue from humans or transgenic mice is collected, and peripheral blood mononuclear cells (PBMCs) or lymphocytes are separated, followed by the enrichment of B cells (optional). The second step is to identify and isolate antigen-specific B cells and obtain paired heavy- and light-chain sequences. In general, 3 routes are used: flow cytometry sorting of antigen-specific memory B cells into separate PCR tubes followed by single-cell RT-PCR and sequencing; loading ASCs, including plasma cells or *in vitro*-cultured memory B cells, into an Optofluidic system (Beacon, Berkeley Lights) for functional screening followed by exporting positive B cells for single-cell RT-PCR and sequencing; isolating memory B cells using immunomagnetic selection and loading them into a Chromium device (10×Genomics) followed by RNA and VDJ library preparation and sequencing. The third step is antibody expression and functional characterization. Mammalian cells (CHO and HEK293 cells) are usually used for recombinant expression of antibodies followed by binding (ELISA, Octet or Biacore), *in vitro* and *in vivo* functional evaluations and structural studies. The figure was created using BioRender.com.

Table 1 Antibodies with broadly neutralizing activity against SARS-CoV-2 variants and sarbecoviruses[a)]

Breadth	Name	Target	Epitope	Neutralizing mechanism	VH germline	VL germline	Source	Platform	Fc	PDB ID	Reference
SARS-COV-2 early strains	LY-CoV016 (CB6)	RBD	Class 1	Receptor blocking	IGHV3-66	IGK1-39	Convalescent COVID-19 patients	RBD bait, single B-cell cloning	LALA	7C01	(Shi et al., 2020)
	LYCoV555	RBD	Class 2	Receptor blocking	IGHV1-69	IGKV1-39	COVID-19 patients	Single B-cell sequencing	WT	7KMG	(Jones et al., 2021)
	REGN 10987 (Imdevimab)	RBD	Class 3	Receptor blocking	IGHV3-30	IGLV2-14	Humanized mice and convalescent patients	S bait, single B-cell cloning	WT	6XDG	(Baum et al., 2020)
	REGN10933 (Casirivimab)	RBD	Class 2	Receptor blocking	IGHV3-11	IGKV1-33	Humanized mice and convalescent patients	S bait, single B-cell cloning	WT	6XDG	(Baum et al., 2020)
	COV2-2196 (AZD8895)	RBD	Class 2	Receptor blocking	IGHV1-58	IGLV3-20	COVID-19 patients	RBD bait, single B-cell cloning and sequencing	TM/YTE	7L7E	(Dong et al., 2021)
	COV2-2130 (AZD1061)	RBD	Class 3	Receptor blocking	IGHV3-15	IGKV4-1	COVID-19 patients	RBD bait, single B-cell cloning and sequencing	TM/YTE	7L7E	(Dong et al., 2021)
	4-18	NTD	Site 1 supersite	Conformational change inhibition	IGHV3-30	IGLV3-25	COVID-19 patients	S trimer bait, single B-cell sequencing	WT	7L2E	(Cerutti et al., 2021b)
	5-7	NTD	Hydrophobic pocket	n	IGHV1-46	IGKV1-9	COVID-19 patients	S trimer bait, single B-cell sequencing	WT	7RW2	(Cerutti et al., 2021a)
SARS-CoV-2 cross-variants	BRII-196 (P2C-1F11)	RBD	Class 1	Receptor blocking	IGHV3-66	IGKV3-20	Convalescent COVID-19 patients	RBD bait, single B-cell cloning	WT	7CDI	(Ju et al., 2020)
	BRII-198 (P2B-1G5)	RBD	Class 3	Receptor blocking	n	n	Convalescent COVID-19 patients	RBD bait, single B-cell cloning	WT	n	(Ju et al., 2020)
	LY-CoV1404	RBD	Class 3	Receptor blocking	IGHV2-5	IGLV2-14	COVID-19 patients	Microfluidic screening, single B-cell sequencing	WT	7MMO	(Westendorf et al., 2022)
	25-C4	RBD	Class 1	Receptor blocking	IGHV3-53	IGKV3-20	Convalescent COVID-19 patients	RBD bait, single B-cell cloning	WT	n	(Yi et al., 2022)
	XGv347	RBD	Class 2	Receptor blocking	IGHV1-58	IGKV3-20	CoronaVac (Sinovac) inactivated virus vaccinee	Single memory B cell isolation and sequencing	WT	7WEC	(Wang et al., 2022a)
	S3H3	SD1	3 loops of SD1	Block the release of S1 from S2	IGHV1-61	IGKV3-12	Mouse immunization	Hybridoma	WT	7WKA	(Hong et al., 2022)
Sarbecoviruses	S309	RBD	Class 3	Receptor blocking independent	IGVH1-18	IGVK3-20	SARS convalescent patients	EBV-immortalized memory B cells.	LS	6WPT	(Pinto et al., 2020)
	BD55-5840	RBD	Class 3	Receptor blocking	n	n	SARS convalescent patients received 2 doses of CoronaVac and 1 dose of ZF2001	Single B-cell sequencing	WT	n	(Cao et al., 2022b)
	ADG-2	RBD	Class 1/4	Receptor blocking	IGHV3-21	IGLV1-40	2003 SARS survivor	Single B-cell cloning	WT	n	(Rappazzo et al., 2021)
	S2X259	RBD	Class 2	Receptor blocking	IGHV1-69	IGLV1-40	Convalescent COVID-19 patients	Single B-cell culture	WT	7RA8	(Tortorici et al., 2021)
	S2K146	RBD	Class 1	Receptor blocking	IGHV3-43	IGLV1-44	Convalescent COVID-19 patients	Single B-cell culture	WT	7TAT	(Park et al., 2022)
	XG014	RBD	Class 3	Locks RBD in the non-functional "down" conformation	IGHV5-51	IGLV1-51	Convalescent COVID-19 patients	Single B-cell culture	WT	7V2A	(Liu et al., 2022c)
	BD-55-5514	RBD	Class 1/4	Receptor blocking	n	n	SARS convalescent patients received 2 doses of CoronaVac and 1 dose of ZF2001	Single B-cell sequencing	WT	n	(Cao et al., 2022b)
	H014	RBD	Class 4	Receptor blocking	IGHV1S135	IGKV5-43	Immunized mice	Phage-display	WT	7CAC	(Lv et al., 2020)
	10-40	RBD	Class 4	Receptor blocking	IGHV4-39	IGLV6-57	Convalescent COVID-19 patients	B.1.351 S trimer, single B-cell cloning	WT	7SD5	(Liu et al., 2022a)
	28-26K	RBD	Class 4	Receptor blocking	IGHV3-21	IGKV1-5	Convalescent COVID-19 patients	Memory B-cell	WT	n	(Yi et al., 2022; Yi et al., 2021a)

a) RBD, receptor binding domain; VH, heavy chain variable domain; VL, light chain variable domain; IGHV, immunoglobulin heavy chain variable region genes; IGKV, immunoglobulin kappa variable genes; IGLV, immunoglobulin lambda variable genes; EBV, Epstein-Barr virus; n, not available. Note: Due to space limitations, some research papers are not cited.

RBD-directed NAbs. Piccoli et al. grouped antibodies into 6 epitopes termed Ia, Ib, IIa, IIb, IIc and IV (Piccoli et al., 2020); Barnes et al. categorized NAbs against RBD into four classes (class 1–class 4) based on a structural analysis and the mechanisms of neutralization (Barnes et al., 2020). We also classified 4 antigenic sites within RBD (site 1–site 4) by competing assays of a series of NAbs and global alanine scanning, which showed consistency with the 4-class scheme (Barnes et al., 2020; Yi et al., 2021a). Some antibodies bind to only one site, whereas others contact more sites. Site 1 and site 2 within the RBM and overlapping with the ACE2 contact surface are major targets of highly potent ACE2-blocking NAbs. However, the immunodominant RBM is the most divergent region, which is the main factor contributing to resistance to NAbs targeting sites 1 and 2 (Figure 4A). In contrast, antigenic sites 3 and 4 are mainly located in the non-RBM region, which is relatively conserved in sarbecovirus, comprising SARS-CoV-2, SARS-CoV, and other animal viruses predominantly of bat origin (Shrestha et al., 2021). Some NAbs targeting site 3 and site 4 have been shown to exhibit broad neutralization.

In the context of the prefusion trimeric S protein, the RBD can adopt two conformational states, described as “up” and “down”, with the former conformation exposing the RBM for ACE2 interaction (Figure 2E). Most class 1 antibodies, in particular the VH3-53/3-66 public clonotype, can only access their epitope in the RBD “up” position; conversely, site 2-targeting antibodies and site 3-targeting antibodies bind both the RBD “up” and “down” positions. Site 4 antibodies usually target a cryptic epitope that faces the interior of the S protein on the “up” RBD (Barnes et al., 2020).

As the RBD is the main target of NAbs and vaccines, mutations that affect its antigenicity are of particular importance. Indeed, extensive efforts have been expended to track immune escape mutations and anticipate and facilitate a timely response to new variants that might alter the efficacy of neutralizing mAbs and polyclonal neutralizing responses from natural infection or vaccine immunization (Muecksch et al., 2022; Prévost and Finzi, 2021; Wang et al., 2022a) (Figure 4B, C). In accordance with epidemiological data, we utilized the natural substitutions of the binding hot spots of RBD-specific NAbs targeting different antigenic sites to elucidate the degree to which SARS-CoV-2 adapts to evade NAb binding and neutralization and identified mutations that must be considered (Yi et al., 2021a). Liu et al. generated escape variants present in SARS-CoV-2 VOCs *in vitro* by culturing a VSV-SARS-CoV-2 chimeric virus in the presence of a panel of anti-RBD NAbs (Liu et al., 2022b). Alternatively, *in vitro* selection of SARS-CoV-2 escape variants under the pressure of NAbs and deep mutational scanning (DMS) experiments with yeast cells expressing all possible amino acid substitutions in the RBD have been performed to understand the escape mechanism of SARS-CoV-2 (Greaney et al., 2021; Liu et al., 2021). Interestingly, these complementary approaches produced some consistent results. For example, the substitutions E484K and K417N/T have been identified as important escape mutations using different strategies (Greaney et al., 2021; Jangra et al., 2021; Prévost and Finzi, 2021) (Figure 4C).

Several distinct mechanisms by which mutations affect the antigenic properties of SARS-CoV-2 have been identified, including substitutions of epitope residues, glycan shielding, allosteric structural effects, deletions and insertions, and transformation of entry pathways (Harvey et al., 2021). The primary escape mechanism is a change in the biophysical properties of epitope residues that directly diminishes antibody binding. Typically, only a few interacting residues have energetic contributions to antigen antibody binding, which are called “hot spots” (Weiss et al., 2000). Structural analyse and experimental mutation scanning strategies provide valuable information on the key residues involved in antibody-antigen interactions. Our previous study showed that at least 33 amino acid positions within four independent antigenic sites (1 to 4) of the RBD are valuable indicators of antigenic changes based on global RBD alanine scanning with a panel of 19 selected NAbs (Yi et al., 2021a). Several studies have also identified key determinants for multiple NAbs targeting each antigenic site: site 1 (K417, F456, N460, A475, F486, N487 and Q493), site 2 (G446, N450, L452, E484, and F490), site 3 (N343, T345, and R346) and site 4 (S371, S373, and K378). Substitutions at these positions confer resistance to multiple NAbs (Cao et al., 2022a; Iketani et al., 2022). The valuable information that K417 is essential for prototype VH3-53/3-66 NAbs (class 1) recognition and facilitates the prediction of the potential significance of the K417N or K417T mutation present in the B.1.351, P.1 and B.1.1.529 lineages (Figure 4C). Mutation at K417 explains why early developed LY-CoV016 (CB6) failed to neutralize the B.1.351, P.1 and B.1.1.529 lineages (Figure 4B). Additionally, the class 2 antibodies REGN10933, COV2-2196 (ADZ8895) and LY-CoV555 are sensitive to the E484 mutations present in B.1.351, B.1.525, B.1.1.526, P.1, B.1.617.1 and B.1.1.529 (Figure 4B, C). Amino acid substitutions outside or near an epitope footprint may also affect antibody binding through allosteric structural effects. The E406W mutation located in nonoverlapping RBD epitopes facilitates escape from both REGN-COV-2 (Casirivimab and Imdevimab, administered together) and Cilgavimab (AZD1061)-mediated neutralization. This observation suggests that this mutation might disrupt the conformation of antigenic sites while retaining detectable binding to dimeric human ACE2 (Starr et al., 2021).

Most notably, the substantially mutated Omicron variants show resistance to most previously isolated mAbs and polyclonal plasma from natural infection or vaccination (Cao et al., 2022a; Yi et al., 2022) (Figure 4B). On the one hand,

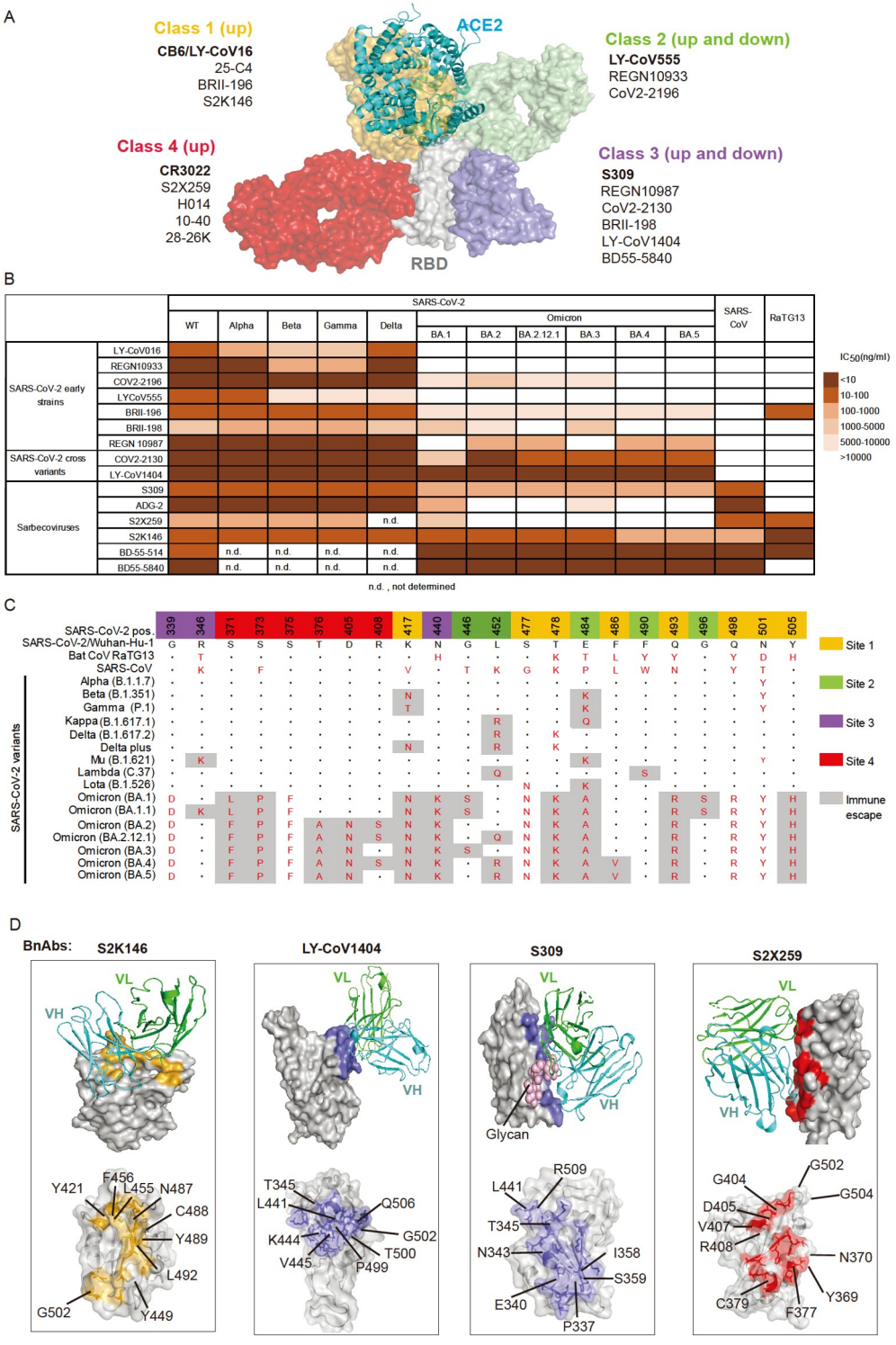

Figure 4 bNAbs targeting the SARS-CoV-2 RBD. A, Structures of previously reported antibodies (bold), representing frequently observed SARS-CoV-2 NAb classes 1 to 4, are overlaid on the ACE2 structure, with additional representative SARS-CoV-2 NAbs listed. The colour-coding scheme is as follows: class 1 (orange, PDB: 7C01), class 2 (green, PDB: 7KMG), class 3 (slate blue, PDB: 6WPT), class 4 (red, PDB: 6W41); ACE2 (Cyan, 6M0J), and RBD (grey). B, Neutralizing breadth of SARS-CoV-2 RBD-targeting NAbs, as derived from published data. The colour gradient used in the table indicates the neutralizing potency of each antibody against different SARS-CoV-2 variants and different sarbecoviruses. n,d, not determined in the published papers. See Table 2 for references. C, RBD sequence alignment of the mutations in SARS-CoV-2 variants compared with the SARS-CoV-2 initial strain Wuhan-Hu-1. The positions are highlighted according to the RBD antigenic site recognized. Immune escape mutations are shown in grey. D, Structure of four groups of bNAbs targeting the RBD. Fab regions are shown as cyan cartoons for the heavy chain (HC) and green cartoons for the light chain (LC), and the RBD orientation is fixed in the upwards state shown on the S trimer (upper panel; see Table 1 for PDB accession numbers). The epitope footprints of antibodies against the RBD are coloured according to the RBD antigenic site recognized. The stem of the N343 glycan is shown as a light pink sphere. The conserved epitope residues involved in the interaction with different mAbs are labelled (lower panel).

Omicron variants not only carry previously identified immune escape mutations found in other VOCs, such as substitutions at the K417 and E484 positions, but they have also evolved new resistant mutations at all four antigenic sites, comprising antigenic site 1 (K417N, Q493R and Y505H), site 2 (G446S, E484A, G496S), site 3 (N440K) and site 4 (S371L and S373P) (Figure 4C). Moreover, the L452 Q/R, D405N, R408S and F486V mutations carried by BA.2/BA.4/BA.5 sublineages would further undermine the neutralization by NAbs isolated previously and the humoral immunity elicited by BA.1 infection. Furthermore, substitutions of S371-T376 in the non-RBM region result in resistance to multiple NAbs via allosteric structural effects (Cao et al., 2022b). In summary, SARS-CoV-2 has the exceptional ability to mutate and evade the human immune system, which highlights the importance of developing second-generation bNAbs targeting more conserved regions in the RBD or the S2 subunit.

Potent bNAbs target the RBD

The substantially mutated Omicron variant and its sublineages have been shown to have the ability to escape most existing RBD-directed NAbs, including several approved therapeutics (Figure 4B, Table 1). A small number of NAbs targeting the evolutionarily conserved non-RBM epitopes of the RBD (site 3 and site 4) or recognizing the conserved receptor binding residues within the RBM through ACE2 molecular mimicry overcome the Omicron antigenic shift and exhibit cross-variant neutralization. We summarize at least four groups of RBD-directed NAbs with cross-variants or pan-sarbecovirus neutralization (Figure 4D, Table 1**)**. An understanding of the characteristics that confer broad neutralization of these mAbs is key to developing therapeutic antibodies and designing universal vaccines against SARS-CoV-2 variants and even future zoonotic sarbecoviruses.

The first group is ACE2 mimicry NAbs targeting evolutionarily conserved ACE2-binding residues in antigenic site1. Despite the genetic divergence and plasticity of the RBM, which is implicated in immune evasion, these RBM-NAbs overcome the mutational plasticity of the RBM. Young-Jun Park et al. developed a human mAb, designated S2K146, that broadly neutralizes sarbecoviruses using ACE2 as the entry receptor (Park et al., 2022) (Figure 4D). Structural and functional studies have shown that the epitope footprint of S2K146 on the SARS-CoV-2 RBD highly resembles that of the ACE2 receptor, with 18 of 24 epitope residues shared with the ACE2 binding site. This allows the antibody to potently inhibit receptor attachment. Based on deep mutational scanning of a yeast displayed RBD mutant library, S2K146 binding was reduced by only a restricted number of amino acid substitutions. Furthermore, none of the individual mutations that affected S2K146 binding are present in VOCs, especially in the recently identified Omicron variant. Thus, S2K146-mediated sarbecovirus neutralization relies on competitively inhibiting receptor attachment. Nevertheless, recent studies have shown that S2K146 is sensitive to BA.4/BA.5, which carry an additional F486V mutation, with a 10-fold reduction in neutralization capacity but neutralization is retained (Cao et al., 2022b; Iketani et al., 2022) (Figure 4B). Coincidentally, we also identified a cross-variant antibody named 25-C4 that targets the conserved key residues F456 and N487, which are critical for ACE2 binding (Yi et al., 2022). Thus, conserved ACE2-binding residues present a vulnerabke site for developing broad solutions against SARS-CoV-2 variants.

The second group is ACE2-blocking mAbs targeting conserved site 3. A recently authorized ACE2-blocking mAb, LY-CoV1404 (Bebtelovimab, class 3), displays remarkably potent neutralizing activity against all known SARS-CoV-2 variants, including Omicron and its subvariants, although it is unable to neutralize SARS-CoV (Westendorf et al., 2022). LY-CoV1404 was isolated from a convalescent patient with COVID-19 using high-throughput B-cell screening technology. A structural analysis revealed that LY-CoV1404 targets a large epitope footprint comprising T345, R346, N439-N450, and Q498-Q506, part of which overlaps the ACE2-interacting site of the S protein that is accessible in both the “up” and “down” conformations of the RBD (Figure 4D). The structural location of the epitope covers canonical site 3 and partial site 2. Except for residues N439 and N501, the epitope residues of LY-CoV1404 are highly conserved based on epidemiological data. However, the most common mutations N439K and N501Y at these positions do not affect the binding and neutralizing activity of LY-CoV1404.

Another group of antibodies in class 3 are non-ACE2-blocking NAbs with broad sarbecovirus neutralization activity, such as S309 and 47D11 (Pinto et al., 2020; Wang et al., 2020). S309 was isolated from a SARS survivor and targets a site distal to the RBM, comprising the N343 glycan that is highly conserved among sarbecoviruses (Figure 4D). The epitope of 47D11 is located in a conserved hydrophobic pocket near glycan N343 (Fedry et al., 2021). 47D11 was shown to impair S protein-mediated syncytium formation in SARS-CoV and SARS-CoV-2, indicating that it may function as a fusion inhibitor (Wang et al., 2020). S309 may exert a similar effect. S309 has broad neutralizing activity against multiple sarbecoviruses, including SARS-CoV-2 VOCs. Furthermore, Fc-engineered S309 recruits effector mechanisms, including antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP), leading to increased neutralization activity. The mAb VIR-7831 derived from the parent antibody S309 has been used to treat mild to moderate COVID-19. Yunlong Cao

et al. recently developed a bNAb named SA58 (BD55-5840, class 3) isolated from convalescent patients diagnosed with SARS who received the SARS-CoV-2 vaccine that displays high potency against all Omicron subvariants and the sarbecoviruses SARS-CoV, Pangolin-GD and RaTG13 (Cao et al., 2022b). The cryo-EM structures of SA58 in complex with BA.1 S protein reveal that an N-linked glycan on N343 is involved in interaction with SA58, which is similar to S309. The conserved N343 glycan is critically recognized by multiple class 3 antibodies. G339, E340, T345 and particularly R346 are also critical for SA58 binding. Another human antibody derived from a convalescent individual previously infected with SARS-CoV-2 named XG014, broadly neutralizes sarbecoviruses, including SARS-CoV-2 and its circulating variants, SARS-CoV and bat SARSr-CoV WIV1. XG041 targets a region that partially overlaps with S309 including the conserved N343 glycan, while the former interacts with 2 RBDs simultaneously and blocks all RBDs in the "down" conformation (Liu et al., 2022c; Zhou et al., 2021).

The final group of broadly RBD-directed antibodies mainly bind to site 4, a highly conserved, cryptic epitope outside the RBM. A panel of site 4-specific mAbs has been reported to have wide neutralizing breadth against sarbecoviruses, including DH1047, S2X259, ADG-2, 2-36, COVA1-16, 28-26K/25-F7/25-D9, H014 and 10-40 (Liu et al., 2020; Liu et al., 2022a; Lv et al., 2020; Rappazzo et al., 2021; Yi et al., 2021a). Cryo-EM characterization of the SARS-CoV-2 S trimer in complex with several mAbs, including H014, ADG-2, S2X259 and 10-40, have indicated that this epitope is only accessible when the RBD is in the open conformation (Figure 4D). A functional study revealed that this class of NAbs mediates neutralization by blocking access of SARS-CoV-2 and SARS-CoV RBDs to human ACE2. Structural data suggest competition between the mAbs and ACE2 for binding to the open RBD. The conserved RBD residues 369-386, 404-411 and 501-508 are involved in the antibody interaction. However, recent studies have reported that the newly emerged Omicron and its subvariants possess resistance to several NAbs of this class (ADG-2, DH1047, 10-40 and S2X259), with an at least a 10-fold reduction in neutralization, which may be due to changes at 371-376, R405 and R408 (Iketani et al., 2022) (Figure 4D). Nevertheless, Yunlong Cao et al. screened an antibody named SA55 (BD55-5514, class 1/4) with high potency and breadth against all Omicron variants and other sarbecoviruses, suggesting that it represents a good therapeutic drug candidate (Cao et al., 2022b).

Characterization and molecular mechanism of NAbs directed against the NTD and SD-1

The NTD is also a target of neutralizing mAbs, some of which have potencies rivalling those of the excellent RBD-specific NAbs (Ju et al., 2020; Liu et al., 2022a). The epitopes of a panel of potently neutralizing NTD-directed antibodies isolated from patients with COVID-19 have been extensively characterized in structural studies. Notably, most of the NTD-directed NAbs from diverse lineages target a single antigenic supersite. This site, located at the periphery of the S protein, is the largest glycan-free surface on the NTD facing away from the viral membrane and is bordered by glycans N17, N74, N122, and N149 (Figure 5A). The surface is also highly electropositive and primarily formed by a mobile β-hairpin and several flexible loops (Liu et al., 2022a). In addition, Gabriele Cerutti et al. discovered a second site of neutralization vulnerability in the SARS-CoV-2 NTD outside of the antigenic supersite according to the cryo-EM structure of an NTD-recognizing antibody named 5-7 in complex with the SARS-CoV-2 S protein (Cerutti et al., 2021a). Antibody 5-7 recognizes an exposed hydrophobic pocket between the two sheets of the NTD β sandwich, which was previously identified as the binding site for hydrophobic molecules (Figure 5A). The molecular mechanism of NTD-directed NAbs has not been clearly elucidated. Because NTD-directed NAbs do not block S recognition by the ACE2 receptor, a plausible alternative mechanism is inhibition of the conformational changes required for fusion of the virus and host cell membranes. Indeed, the MERS NTD-directed NAb 7d10 decreases the protease sensitivity involved in the prefusion-to-post fusion transition, indicating that NTD-directed NAbs may prevent conformational changes in the S protein before exposure of protease binding sites and the fusion peptide (Zhou et al., 2019).

Nonetheless, NTD-specific NAbs were shown to have a low genetic barrier to resistance due to the rapid emergence of escape mutants under selective pressure *in vitro*. Remarkably, both distinct classes of NTD NAbs, 4-18 and 5-7, suffer from significantly reduced or abolished neutralizing activity against most of the currently circulating VOCs due to substantial changes within or near the antibody binding sites (Cerutti et al., 2021a; Wang et al., 2021c). Deletions in the NTD have appeared repeatedly in the evolution of SARS-CoV-2 and have been reported to cause substantial changes in NTD antigenicity. The deletion mutations Δ69-70, Δ144-146, Δ210, and Δ243-244 and substitutions of epitope residues confer robust resistance to NTD-directed NAbs via allosteric structural effects or epitope alteration. Thus, NTD-directed NAbs only neutralize early strains. Overall, the hypervariable antigenic sites of the NTD may function as an "immunological decoy" to evade the immune system. Thus, the low cross-variant neutralizing activity of NTD-directed NAbs may limit their potential for clinical application.

Moreover, the unique cross-variant NAb S3H3 generated from S trimer-immunized mice has been reported to recognize the S1 subdomain (SD1), which has rarely been described. The structures of the Omicron prefusion S trimer

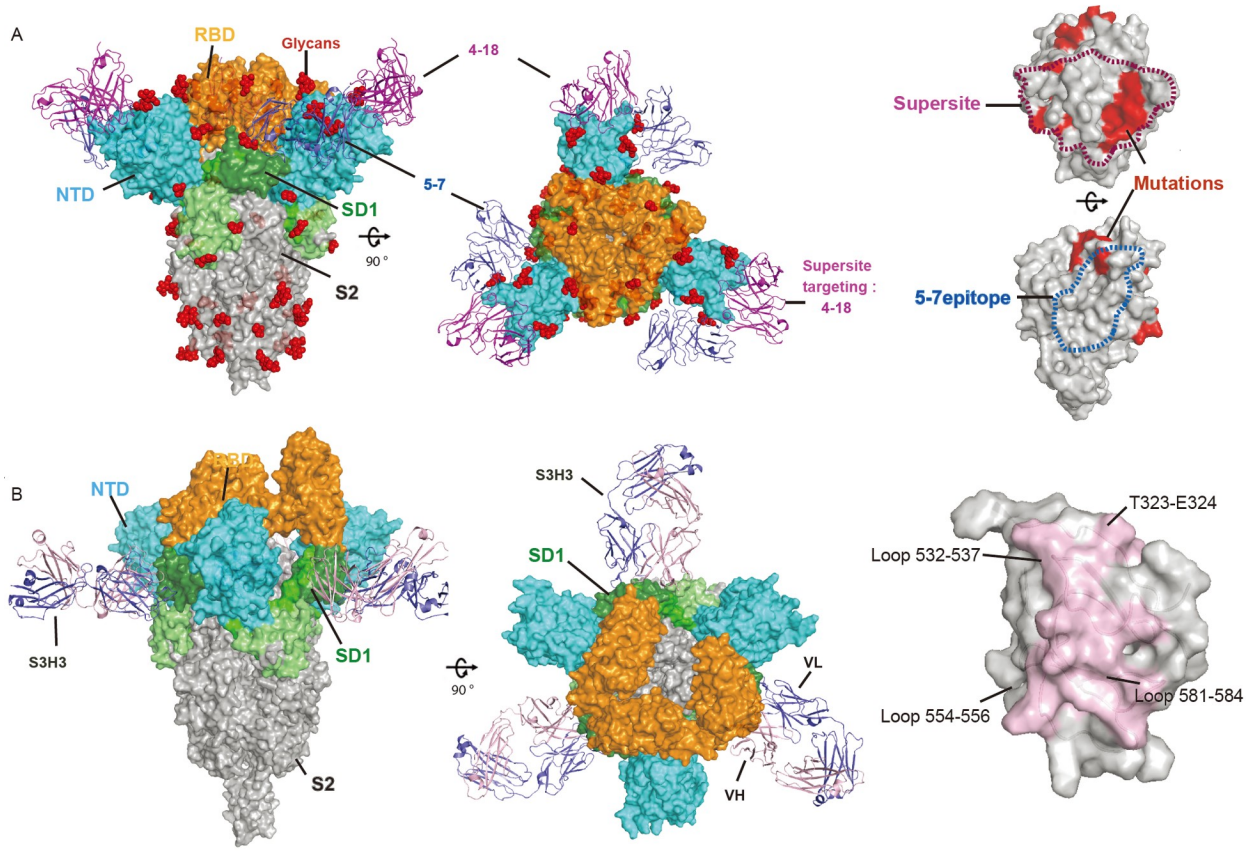

Figure 5 Antibodies targeting the NTD and SD1 of the SARS-CoV-2 S protein. A, The two antigenic sites of the NTD targeted by NAbs. Cryo-EM reconstruction of 4-18 Fab and 5-7 in complex with the S trimer. Side and top views are shown in the left and middle panels. Notably, 4-18 represents one of the NAbs targeting the NTD supersite (magenta); 5-7 targets a distinct epitope near the supersite and is shown in slate. Left panels show the footprint and residue variation for the supersite and 5-7 epitope on the NTD surface. The epitope footprints are represented as dots with supersites in magenta and 5-7 epitopes in slate. Mutations present in SARS-CoV-2 circulating variants are highlighted in red. B, Cryo-EM analyses of the prefusion Omicron S ectodomain trimer with three SD1-directed S3H3 Fab fragments. Side and top views are shown in the left and middle panels, with the heavy chain and light chain of S3H3 Fab presented in light pink and light blue, respectively. The structural elements involved in the interaction between S3H3 Fab and SD1 are labelled.

in complex with the Fab of S3H3 show that this antibody recognizes several discontinuous parts, including T323-E324 and the three loops (loop532-537, loop554-556 and loop581-584) of SD1 (Hong et al., 2022) (Figure 5B**)**. The SD1 region recognized by S3H3 is highly conserved among SARS-CoV-2 variants, thus conferring the cross-neutralization capacity of S3H3 towards different SARS-CoV-2 variants, including the highly mutated Omicron. The binding of S3H3 to the S trimer may inhibit viral entry by blocking the release of S1 from S2 required for the fusion process, indicating the complexity of the fusion machinery of SARS-CoV-2. SD-1 antibodies bind outside of the ACE-2 binding region and hence provide the potential for synergistic effects when combined with mAbs that interfere with ACE2 binding (Xu et al., 2022).

S2 subunit-targeting bNAbs and the challenges in developing pancoronavirus vaccines and antivirals

The CoV S2 subunit is closely involved in the fusion mechanism between the cell membrane and virus particles. Proteases, such as cathepsin in the endosome or TMPRSS2 at the cell surface and even furin and trypsin, have been implicated in cleavage of the S1/S2 site and S2′ site, facilitating the exposure of the fusion peptide to initiate membrane fusion (Jackson et al., 2022). The S2 subunit is an attractive antibody target, as it is more conserved among different CoV genera than RBD and NTD regions, which should be a higher genetic barrier to resistance under selection pressure. Indeed, the S2 subunit is significantly less mutated than the RBD region of SARS-CoV-2 VOCs (van der Straten et al., 2022; Vitiello et al., 2022).

Although some RBD- and NTD-targeting mAbs have been shown to have broad neutralizing activities, their breadth is limited to sarbecoviruses within betacoronavirus, including SARS-CoV-2 variants (Rappazzo et al., 2021; Tortorici et al., 2021). Notably, S2-targeting antibodies widen the breadth to cover betacoronaviruses or even a diverse group of CoVs across all four genera. Several antibodies with a broad spectrum that target the stem helix within the peptide

$_{1140}$PLQPELDSFKEELDKYFKNHTS$_{1161}$ (namely, downstream from the connector domain and before the HR2 region) of the S protein, which is much more conserved among betacoronaviruses, including SARS-CoV-2 variants, have been described (Sauer et al., 2021; Tortorici et al., 2021; Wang et al., 2021a; Zhou et al., 2022) (Figure 6A–C and Table 2). The structures of the Fab-stem helix peptide complexes, together with mechanistic analyses, reveal that these antibodies neutralize the virus by preventing S2 subunit refolding from the pre- to postfusion states and subsequently block virus entry by inhibiting membrane fusion. Their neutralizing breadth varies within betacoronavirus: 28D9 neutralizes merbecoviruses (Wang et al., 2021a), B6 neutralizes merbecoviruses and embecoviruses (Sauer et al., 2021), and S2P6 and CC40.8 neutralize all betacoronaviruses, including SARS-CoV-2 VOCs (Tortorici et al., 2021; Zhou et al., 2022) (Figure 6A). The lack of cross-neutralization of both 28D9 and B6 for SARS-like viruses might be attributed to the distinct structures of the stem helix in the S trimer of different CoVs. Although S2P6, CC40.8 and B6 recognize a similar epitope, they bind with distinct orientations to the stem helix region, and their detailed interactions vary. For example, superimposition of structures based on the stem helix region reveals that B6 HCDR2 sterically clashes with residue H1159 of SARS-CoV-2, possibly explaining the broader cross-reactivity of S2P6 than B6. Notably, this epitope is expected to be partially buried in the centre of a three-helix bundle in the S2 subunit. The possible detailed neutralization mechanism is that the antibody disrupts the S2 stem three-helix bundle and recognizes an intermediate state of the S trimer. A common feature of these mAbs is their lower neutralization IC50 values in the μg mL^{-1} range compared with some potent RBD mAbs in the ng mL^{-1} range. Moreover, S2P6 and CC40.8 have no addi-

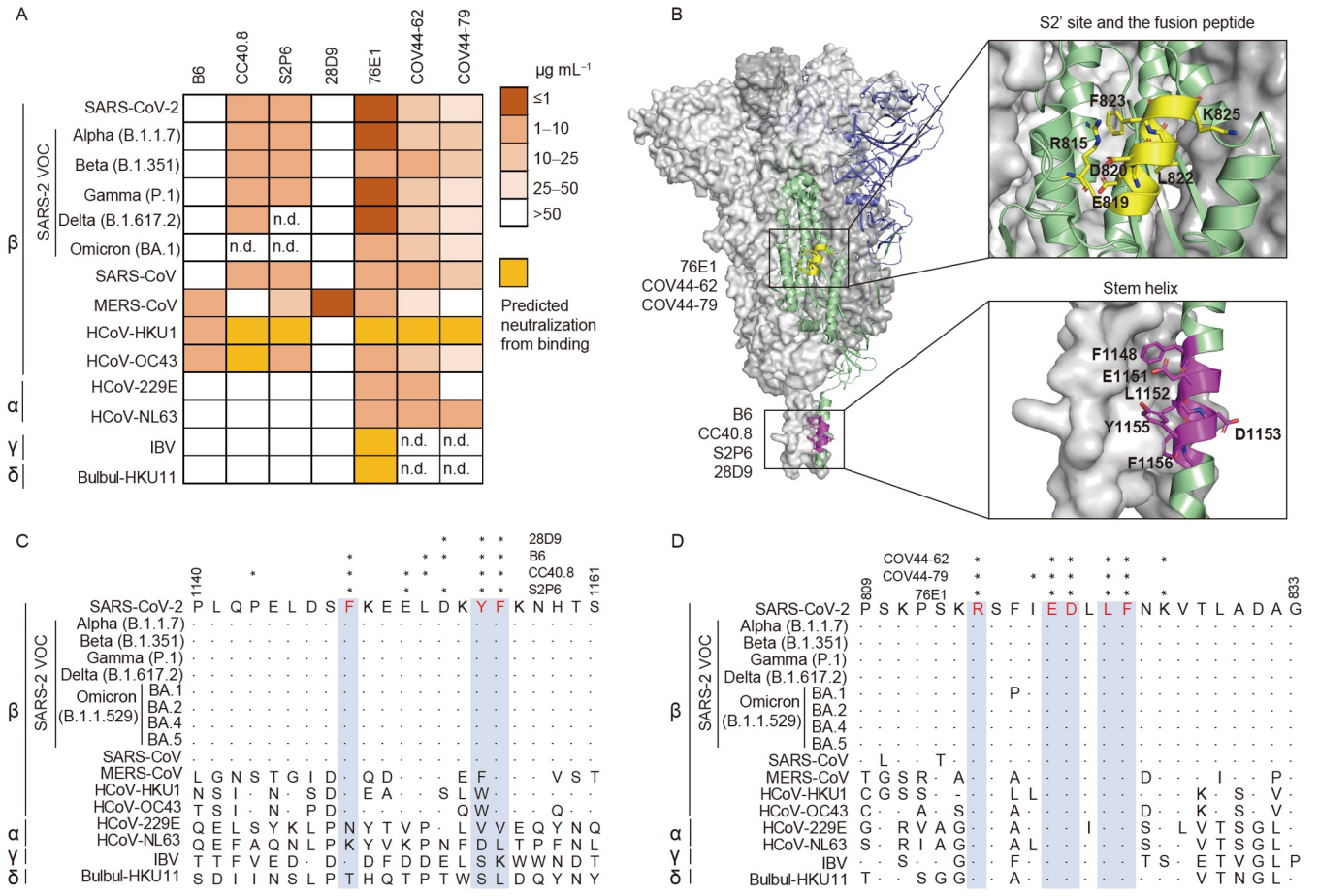

Figure 6 Epitope specificity and breadth of CoV S2-targeting NAbs. A, Neutralizing breadth of CoV S2-targeting NAbs derived from published data (see Table 1 for references). The colour gradient used in the table indicates the neutralizing potency of each antibody against different CoVs. n,d, not determined in the published papers. B, Epitope display of S2-targeting NAbs. Two conserved epitopes were discovered: (i) the S2′ site and fusion peptide for 76E1, COV44-62 and COV44-79 (yellow); and (ii) the stem helix region for B6, 28D9, CC40.8 and S2P6 (magenta). Two protomers of the prefusion S conformation (PDB ID: 6XR8) are shown as a map in light grey and dark grey, and the other protomer is shown as a cartoon with S1 in purple and S2 in green. The side chains of the critical epitope residues are shown. Sequence alignment of the stem helix (peptide$_{1140\text{-}1161}$, SARS-CoV-2 numbering) (C) and S2′ site and the fusion peptide (peptide$_{809\text{-}833}$, SARS-CoV-2 numbering) (D) from SARS-CoV-2 VOCs and viral isolates represent a diverse group of CoVs across four genera. The critical epitope residues for each antibody are indicated with "*". Common critical epitope residues are shown in red. "." indicates an amino acid identical to SARS-CoV-2.

tional cross-reactivity with alpha-, gamma- and deltacoronaviruses because the stem helix of betacoronaviruses varies significantly from those of other genera (Sauer et al., 2021; Tortorici et al., 2021; Zhou et al., 2022) (Figure 6A, C).

The ideal concept of broad spectrum is the capability of an antibody to neutralize all four CoV genera. Recently, our lab reported an antibody (76E1) with unprecedented breadth against various CoVs, including all four CoV genera and SARS-CoV-2 VOCs, by targeting the highly conserved S2′ cleavage site and fusion peptide within the peptide $_{809}$PSKPSKRSFIEDLLFNKVTLADAGF$_{833}$ (Figure 6A, B, D, and Table 2). A similar epitope and neutralizing breadth have been reported for other antibodies, such as COV44-62 and COV44-79 (Dacon et al., 2022). In general, the higher conservation of this epitope determines the outstanding broad range of reactivity of these mAbs. Interestingly, the 76E1 epitope is partially buried in the structure of the SARS-CoV-2 S trimer in the prefusion state but is exposed when the S protein binds to ACE2. This observation suggests that 76E1 binds to the epitope at an intermediate state of the S trimer during the transition from the prefusion to the intermediate state, thereby blocking membrane fusion and viral entry. Consistently, 76E1 acts synergistically with ACE2 or some ACE2 mimicry RBD antibodies to prevent SARS-CoV-2 infection, suggesting two possible mechanisms: synergy by which 76E1 binds to its epitope in an S protein conformation that must be primed by receptor binding; and synergy between the receptor blocking process and membrane fusion process. Similar to stem helix-targeting mAbs, 76E1 and COV44-62 and COV44-79 exhibit moderate neutralizing activities.

In summary, the two epitopes that these bNAbs bind are promising sites for developing pan-coronavirus vaccines and antivirals. However, four challenges should be considered in the development of vaccines and antibodies when targeting the stem helix, the S2′ site and the fusion peptide. First, not all peptide$_{809\text{-}833}$ binding antibodies possess neutralizing activities. In fact, a nonneutralizing property of these antibodies is likely because they do not target the S2′ site and inhibit S2′ cleavage, indicating that the S2′ site is not sufficiently immunogenic to induce a strong antibody response. A critical problem is how to rationally design antigens to induce mAbs specifically targeting the S2′ site. Second, the neutralizing potency of these S2-targeting antibodies is relatively lower than that of some best-in-class RBD antibodies, such as LY-CoV1404 (Westendorf et al., 2022) and BD-55-5514 (Cao et al., 2022b). This phenomenon is consistent with influenza virus, for which haemagglutinin (HA) stem antibodies are usually less potent than HA head antibodies (Kallewaard et al., 2016). A higher antibody dosage might be needed when treating patients in clinical trials, which would increase manufacturing costs.

Third, the lack of a standardized correlation between protection in patients and *in vitro* antibody neutralizing activity hinders the value of bNAbs with regard to clinical outcomes. As RBD antibodies and S2 antibodies use different mechanisms to combat CoVs and the Fc effective functions of different mAbs, cell-based *in vitro* neutralizing assays (neutralizing potency in cells) may not fully represent protective efficacy in patients. No studies have been published describing the *in vivo* protective efficacy between RBD antibodies and S2 antibodies concurrently, and a parallel comparison should be performed to quantitatively determine the protective effects of RBD antibodies and S2 antibodies as prophylactics and treatments *in vivo*. Fourth, S2-specific NAbs are present at low titres in convalescent or vaccinee plasma and at low frequencies in their memory B-cell repertoire, which might be attributed to the key residues involved in the antibody-antigen interactions that are partially buried in the S trimer (Tortorici et al., 2021; Zhou et al., 2022). The moderate neutralization potency of these S2 antibodies may result from limited epitope exposure and the

Table 2 Antibodies targeting the S2 subdomain with panβ-coronavirus or pancoronavirus neutralizing activity[a)]

Breath	Name	Target	Epitope	Neutralizing mechanism	VH germline	VL germline	Source	Platform	Fc	PDB ID	Reference
Panβ-coronavirus	B6	S2	Stem helix	Fusion inhibition	IGHV1-19	IGKV8-27	Mice	Hybridoma	WT	7M5E, 7M51, 7M52, 7M53, 7M55	(Sauer et al., 2021)
	CC40.8	S2	Stem helix	Fusion inhibition	IGHV3-23	IGLV3-10	COVID-19 convalescent patients	Single B-cell	WT	7SJS	(Zhou et al., 2022)
	S2P6	S2	Stem helix	Fusion inhibition	IGHV1-46	IGKV3-20	COVID-19 convalescent patients	Single B cells	WT	7RNJ	(Tortorici et al., 2021)
	28D9	S2	Stem helix	Fusion inhibition	n	n	Humanized mice	MERS-CoV S, hybridoma	WT	n	(Wang et al., 2021a)
Pancoronavirus	76E1	S2	S2' site and FP	Fusion inhibition	IGHV3-43	IGLV2-8	COVID-19 convalescent patients	Single B-cell cloning	WT	7X9E	(Sun et al., 2022b)
	COV44-62	S2	S2' site and FP	Fusion inhibition	IGHV1-2	IGLV2-8	COVID-19 convalescent patients	Single B cells	n	8D36	(Dacon et al., 2022)
	COV44-79	S2	S2' site and FP	Fusion inhibition	IGHV3-30	IGKV1-12	COVID-19 convalescent patients	Single B cells	WT	8DAO	(Dacon et al., 2022)

a) Note: Due to space limitations, some research papers are not cited.

lower immunogenicity of these epitopes. Vaccine design based on S2 should consider these points.

Combinatorial use of antibodies

Different SARS-CoV-2 variant strains have evaded host immunity and caused global COVID-19 waves since the first outbreak in late 2019 (Starr et al., 2020; Zhou et al., 2020). Regarding antibody therapies, combinatorial use of antibodies (antibody cocktail or bispecific antibody) targeting distinct epitopes of SARS-CoV-2 is a promising approach to overcome antibody evasion of SARS-CoV-2 (Baum et al., 2020; Shiakolas et al., 2022; Wang et al., 2021b; Yao et al., 2021). Some antibody cocktails have been approved for clinical use to cover a larger range of epitopes of SARS-CoV-2, thus providing broader protection with high potency (Weinreich et al., 2021).

AZD7442 is a cocktail of two fully human SARS-CoV-2 neutralizing mAbs, AZD8895 (Tixagevimab) and AZD1061 (Cilgavimab), which are derived from B cells of convalescent patients previously infected with SARS-CoV-2. AZD8895 and AZD1061 simultaneously bind to distinct, nonoverlapping epitopes of the SARS-CoV-2 RBD to potently neutralize the virus (Levin et al., 2022; Loo et al., 2022; Zost et al., 2020a; Zost et al., 2020b). AZD7442 has been shown to neutralize SARS-CoV-2 and its VOCs *in vitro* and exerts prophylactic and therapeutic effects on nonhuman primates (Levin et al., 2022; Loo et al., 2022). For Omicron variant strains, despite losses in neutralization potency in cell culture, AZD7442 treatments reduced BA.1, BA.1.1, and BA.2 lung infection in susceptible mice that express human ACE2 (K18-hACE2) in prophylactic and therapeutic settings (Case et al., 2022). The neutralization potency of another antibody cocktail REGN-COV-2 (Ronapreve) which is a cocktail of REGN10933 (Casirivimab) and REGN10987 (Imdevimab) is not substantially affected by RBD mutations in early variants, with a broader spectrum of neutralization than the mono-use of these antibodies (Baum et al., 2020; Hansen et al., 2020). However, the new versions of Omicron have evolved large amount of immune evasion mutations within the S protein, especially in the RBD, and striking loss of neutralizing activity against Omicron variants has been proven for REGN-COV-2 (Cao et al., 2022a; Tada et al., 2022).

Antibody cocktails with more than two antibodies with clear structural information and rational combinations have been reported. Sun et al. reported the formulation of a cocktail containing three or four NAbs, including two humanized NAbs, H014 and HB27, and one fully human NAb named P17, targeting the RBD and conferring effective protection against SARS-CoV-2 in animal models. Notably, H014 exhibited cross-neutralization activity against SARS-CoV and SARS-CoV-2, while others were SARS-CoV-2 specific. In addition, the NTD-binding fully human antibody FC05 might also be included in the cocktail, which would significantly enhance the neutralizing potency by promoting the full occupation of RBD and NTD (Sun et al., 2021). Many newly developed antibody cocktails with broader neutralizing spectra, including different versions of Omicron, have been reported to combat the problem of Omicron escape. Wang et al. identified three potent bNAbs against SARS-CoV-2 VOCs, including Omicron, with structural insights revealing how the three antibodies were resistant to most of the RBD mutations in VOCs and showed that these antibodies exhibit synergic neutralization and protect against the Omicron variant *in vitro* and *in vivo*. Importantly, the antibody cocktail XMA01/XMA04 also showed potent *in vivo* neutralizing activity against the Beta strain, which was reported to escape the LY-CoV555 (Bamlanivimab)/LY-CoV016 (Etesevimab) cocktail (Tada et al., 2022; Wang et al., 2022b). Nikitin et al. developed an antibody cocktail IMM-BCP-01 that consists of three patient-derived bNAbs targeting nonoverlapping epitopes of the S protein. Two antibodies, IMM20184 and IMM20190, block ACE2 binding, while the other antibody, IMM20253, alters the conformation of the S trimer, promoting the release of S monomers. The antibody cocktail decreased Omicron SARS-CoV-2 infection in the lungs of Syrian golden hamsters *in vivo*, and potently neutralized multiple SARS-CoV-2 VOCs, including Delta, Omicron BA.1 and BA.2 (Nikitin et al., 2022).

The construction of different NAbs into one molecule to generate bispecific antibodies (bsAbs) or multispecific antibodies is a promising strategy for combating the rapidly evolving virus. The bsAbs design could reduce the complexity of antibody cocktails through simultaneous targeting 2 or more distinct epitopes in a single entity. Furthermore, the bsAbs engineering provides the opportunity to tailor multifunctional molecules to match the proposed mechanism of action (Nyakatura et al., 2017). Inspired by the review reported by Aran F. Labrijn et al., obligate bsAbs are antibodies in which physical linkage of binding domains creates a novel functionality, that is, a function that cannot be accomplished by an antibody mixture (Labrijn et al., 2019). The discovery of bNAbs against CoVs and elucidation of the underlying mechanism may facilitate the design of bsAbs under temporal obligate or spatial obligate conditions with therapeutic advantages over antibody mixtures. Li et al. reported the development and evaluation of an engineered bsAb human mAb against SARS-CoV-2. They explored two bsAb formats, dual variable domain immunoglobulin (Ig) and IgG-single-chain variable fragment (IgG-ScFv). The IgG-ScFv named bsAb15 showed better *in vitro* and *in vivo* performance than both dual variable domain Ig and the cocktail strategy, which is a successful example of novel

functionality achived by rational design (Crowe, 2022; Li et al., 2022c). In addition, Li et al. reported the engineered, bispecific, single-domain antibody bn03 that binds simultaneously and synergistically to one RBD of the S trimer and inhalation of bn03 effectively treated SARS-CoV-2 infection in hACE2 mice. The bsAb targets conserved epitopes outside of the RBM of the SARS-CoV-2 S RBD and broadly neutralizes SARS-CoV-2 variants (Li et al., 2022a).

The bNAbs discussed in this review that retain activity against Omicron variants or CoVs other than SARS-CoV-2 provide solutions for the current Omicron problem and a potential new CoV pandemic in the future. Furthermore, antibody cocktails or bsAbs consisting of bNAbs targeting nonoverlapping epitopes might provide better protection against newly emerged SARS-CoV-2 variants or even a new CoV. For example, the S2-targeting bNAb 76E1 is synergistic with RBD-targeting ACE2 or ACE2 mimicry antibodies, and potential value for use of the combination has been noted (Sun et al., 2022b).

Clinical use of SARS-CoV-2 antibodies

The use of SARS-CoV-2 NAbs as prophylactics or as a therapy for infection has been investigated in several clinical trials, which facilitated the approval or emergency use authorization of the antibody therapies, including Amubarvimab/Romlusevimab combination (BRII-196/BRII-198), Sotrovimab (derived from S309), Bamlanivimab (LY-CoV555) and Etesevimab (LY-CoV016) cocktail, REGN-COV2 (Casirivimab/Imdevimab), AZD7442 (Evesheld, Tixagevimab/Cilgavimab) and Bebtelovimab (LY-CoV-1404), although some of them have not been recommended due to the variant problem (Hoy, 2022; Takashita et al., 2022b).

The phase 3 clinical trial for COVID-19 prevention with the long-acting antibody cocktail AZD7442 involving revealed that intramuscular injection of a single dose of AZD7442 in adults (⩾18 years of age) who had an increased risk of an inadequate response to vaccination against COVID-19, an increased risk of exposure to SARS-CoV-2, or both provided a relative risk reduction of 82.8% at a median of 6 months (Levin et al., 2022). AZD7442 has been approved for clinical use in the UK and the USA (Kmietowicz, 2021). The treatment might provide protection against COVID-19 for the group of 500,000 immunocompromised people in the UK and more individuals in other countries, including those with blood cancers, those taking immunosuppressive drugs after an organ transplant, or those with conditions such as multiple sclerosis and rheumatoid arthritis (Wise, 2022).

For the case of Sotrovimab, the phase 3 multinational, randomized, double-blind, placebo-controlled trial designed to examine the efficacy and safety of the antibody in treating high-risk outpatients with mild-to-moderate COVID-19 and symptom onset within the previous 5 days, 291 people who received a single 1-hour intravenous infusion of sotrovimab (500 mg) were significantly less likely to be hospitalized (for >24 h) for any cause or die through day 29 (Gupta et al., 2021).

According to the data from HHS (U.S. Department of Health & Human Services), from November 2020 to August 28, 2022, about 3,948,374 courses of courses of antibodies have been administered in the US including 459,572 courses of Evusheld (AZD7442), 497,253 courses of Bebtelovimab, 2,078,587 courses of REGN-COV2, 610,753 courses of Bamlanivimab/Etesevimab and 302,209 courses of Sotrovimab (https://aspr.hhs.gov/COVID-19/Therapeutics/orders/Pages/default.aspx). However, on January 24, 2022, FDA limited the use of Bamlanivimab/Etesevimab and REGEN-COV to treat COVID-19 due to the Omicron variants which escaped the certain nAbs (https://www.fda.gov/).

Perspectives and conclusions

A series of studies revealed that the protective serum NAb level against SARS-CoV-2 wanes quickly after vaccination or natural infection (Levin et al., 2021; Pérez-Alós et al., 2022), suggesting that humoral immunity to CoVs is rather short-lived compared to some other viruses, such as measles virus, for which life-long antibody immunity is observed. Considering the continuous mutation of SARS-CoV-2, the vaccines and antibody therapies developed based on the original strains have largely been weakened. Currently, Bebtelovimab (LY-CoV1404) is the only neutralizing mAb therapy recommended by the NIH for nonhospitalized adults with COVID-19 due to its activity against a broad range of SARS-CoV-2 variants, including the Omicron variant and its BA.1, BA.1.1, BA.2 BA.2.12.1, BA.3, BA.4 and BA.5 subvariants (https://www.covid19treatmentguidelines.nih.gov).

In the Omicron wave, the mortality of elderly people with certain underlying medical conditions who did not complete the full course of vaccination is much higher than that of the other groups of people (Lu et al., 2022). Severe acute hepatitis in children has also been reported recently (Mücke and Zeuzem, 2022; Zeng and Huang, 2022). For cases in Israel, 11 of 12 patients had COVID-19 in recent months, and most of them were too young to be eligible for vaccination, suggesting a relationship between children with severe acute hepatitis and SARS-CoV-2 infection. Young children who have not been included in the COVID-19 vaccination program are at high risk of SARS-CoV-2 infection. Passive immunity acquired by bNAbs against SARS-CoV-2 variants and other human CoVs may provide immediate prophylactic

and therapeutic protection against SARS-CoV-2 variants and other human CoVs (Estcourt, 2021; Keller and Stiehm, 2000). Once the safety and effectiveness of bNAbs have been proven in clinical trials, these groups of people can be prophylactically protected by long-half-life antibody therapeutics during the pandemic.

Great achievements have been made in the field of small molecules against SARS-CoV-2. The FAD has approved or issued the Emergency Use Authorization (EUA) for the antiviral drugs Veklury (remdesivir), Nirmatrelvir with ritonavir (Paxlovid) and Molnupiravir (Lagevrio) as well as the immune modulator Olumiant (baricitinib) for the treatment of COVID-19. These drugs have been proven to be broadly effective and easy to administer with cost advantages. Antibody therapeutics are relatively expensive and have a short treatment window period; thus, their competitiveness is becoming weaker than that of small-molecule drugs. However, no evidence is available that small-molecule drugs prevent SARS-CoV-2 infection, while antibody therapies have been approved for the prophylaxis of infection in high-risk groups of people (Levin et al., 2022). Effective antibodies that provide immediate protection with relatively few side effects, together with small molecules that are convenient to use in the community and vaccines that aim to achieve herd immunity, might be used to build a 3-D protection network for people of all age groups to fight against SARS-CoV-2 variants.

The NAbs targeting the RBD show very high neutralizing potency *in vitro* and thus they represent the majority of approved antibody therapies for COVID-19. However, variants with escape mutations in the RBD circulate worldwide and cause many waves of SARS-CoV-2 infection, despite the wide use of vaccines. SARS-CoV-2 variants have been shown to escape RBD-directed antibody therapies, such as LY-CoV016, LY-CoV555, casirivimab and tixagevimab, with Beta, Gamma and Omicron variants showing a greater degree of escape than other VOCs, which limits the clinical use of these antibodies (Takashita et al., 2022a). Some newly identified antibodies such as LY-CoV-1404 and BD55-5514 retained the potency against major variants identified to date; however, RBD mutants K444Q and V445A reduced the binding and neutralizing activity of LY-CoV-1404, which revealed the risk of immune escape of these antibodies to emerging SARS-CoV-2 variants (Westendorf et al., 2022). As discussed in this review, although S2-targeting antibodies show a broader neutralizing spectrum and are more resistant to RBD mutations, their *in vitro* neutralizing activity is much lower than that of RBD antibodies. A head-to-head comparison of *in vivo* efficacy should be performed to determine the protection provided by S2- and RBD- antibodies.

Regarding vaccines, considering that some SARS-CoV-2 variants can substantially evade the immune response, especially Omicron and its descendent lineages, the WHO Technical Advisory Group on COVID-19 Vaccine Composition asserted on 11 January 2022 the opinion that current COVID-19 vaccines may need to be updated to ensure continued effectiveness against Omicron and future variants (https://news.un.org/en/story/2022/01/1109562). Major vaccine producers around the world are racing to develop upgraded vaccines against Omicron. However, the Omicron variants BA.2.12.1, BA.4 and BA.5 have been observed to evolve mutations to specifically evade the humoral immunity elicited by BA.1 infection (Cao et al., 2022b; Yamasoba et al., 2022), indicating that vaccines based on early Omicron strains may fall behind the evolution of the virus. Most of the bNAbs against SARS-CoV-2 variants, sarbecoviruses or human CoVs were isolated from convalescent patients or vaccinees, which provides good clues that the idea of a broad spectrum or universal vaccine might be successful if a new vaccine design method is used to induce epitope-specific bNAbs (Correia et al., 2014). The highly conserved epitopes of bNAbs against SARS-CoV-2 variants targeting the RBD have been well characterized, and are basically conformational (Figure 4C, D). S2-targeting antibodies with a broader neutralizing spectrum have also been studied and basically target linear epitopes (Figure 6B, C). The epitope information defined by elegant structural analysis and the mechanism underlying broad neutralization have shed light on the design of universal vaccines against SARS-CoV-2 and other human CoVs. The concept of epitope-focused vaccine design might be applied to develop universal vaccines for CoVs, which require structure-based antigen design, extensive *in vitro* and *in vivo* evaluations and exploratory clinical trials to select the best formulation and delivery system (Rappuoli et al., 2016). Although effective universal vaccines against CoVs have not yet been reported, breakthroughs in this field are expected in the near future.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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