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Opinion

Slaying SARS-CoV-2 One (Single-domain) Antibody at a Time

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Camelid-derived and synthetic single-domain antibodies (sdAbs) are emerging as potent weapons against the novel coronavirus, SARS-CoV-2. sdAbs are small, compact, thermostable immunoglobulin elements capable of binding targets with subnanomolar affinities. By leveraging the power of phage- and yeast surface-display technologies, rare sdAbs can be isolated from highly diverse and complex antibody libraries. Once in hand, sdAbs can be engineered to improve binding affinity, avidity, target specificities, and biodistribution. In this Opinion piece we highlight a series of sophisticated studies describing the identification of ultrapotent sdAbs directed against the receptor-binding domain (RBD) of the SARS-CoV-2 Spike protein. We discuss the possible applications of these antibodies in the global fight against COVID-19.

The highly contagious novel coronavirus, SARS-CoV-2, continues to infect tens of millions worldwide, with the estimated number of deaths from COVID-19 fast approaching two million. SARS-CoV-2 is a spherical particle containing a 30 kb positive-sense RNA genome associated with a nucleocapsid core surrounded by an envelope [1]. The surface of SARS-CoV-2 is studded with ~40 copies of a trimeric club-shaped glycoprotein called Spike [2,3]. Spike's RBD promotes virus attachment to host cells via angiotensin-converting enzyme 2 (ACE2) [4,5]. From what is known about other coronaviruses, the SARS-CoV-2 Spike undergoes a conformation change following ACE2 binding that results in membrane fusion and delivery of the virus and its genomic contents into the cytoplasm of host cells, where replication ensues [6]. SARS-CoV-2 infection of the airways triggers an inflammatory cytokine storm that can progress to life-threatening acute respiratory distress syndrome (ARDS) [7,8].

The devastating impact of the SARS-CoV-2 pandemic has mobilized efforts around the globe to develop drugs, vaccines, and other intervention strategies to arrest the novel coronavirus in its tracks and stop the spread of COVID-19. Of particular interest is a series of recent reports describing camelid-derived and synthetic sdAbs with potent (nanomolar) and even ultrapotent (picomolar) SARS-CoV-2-neutralizing activities [9–13]. X-ray crystallography and cryogenic electron microscopy (cryo-EM) has revealed that the most potent sdAbs target a common region on the RBD that blocks ACE2 interactions, thereby preventing SARS-CoV-2 attachment to host cells. Here, we summarize the strategies used to isolate these unique sdAbs and speculate on how to best use them in the fight against SARS-CoV-2.

The Versatile World of sdAbs

While antibodies come in all shapes and sizes, the conventional arrangement in most mammals is typified by human IgG in which a heavy chain (H) and light chain (L) pair homodimerize to form a Y-shaped molecule (150 kDa) (Figure 1). The variable domains of the H (V_H) and L (V_L) chains of each arm of the 'Y' are in close spatial proximity to each other and form a single interface involved in target (antigen) recognition. The V_H and V_L elements each contain three hypervariable complementarity determining regions (CDRs) that, over the course of an immune response, may undergo

Highlights

sdAbs are small, thermostable immunoglobulin elements that were first discovered in camels.

Recent reports describe the isolation of sdAbs specific to the novel coronavirus, SARS-CoV-2, using phage- and yeast-display libraries.

sdAbs with SARS-CoV-2 neutralizing activity target the trimeric Spike protein and block entry of the virus into host cells.

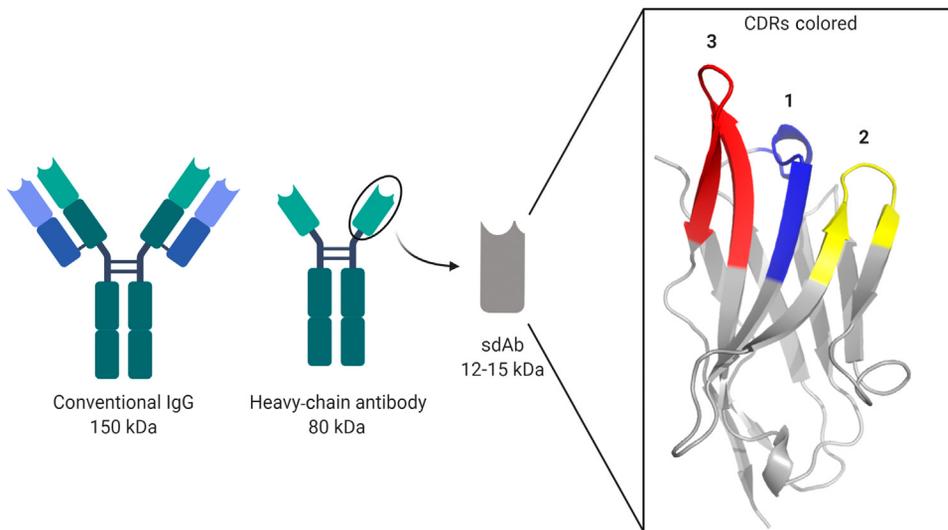
Ultrapotent sdAbs have broad applications as antivirals and diagnostics in the fight against COVID-19.

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Trends in Microbiology

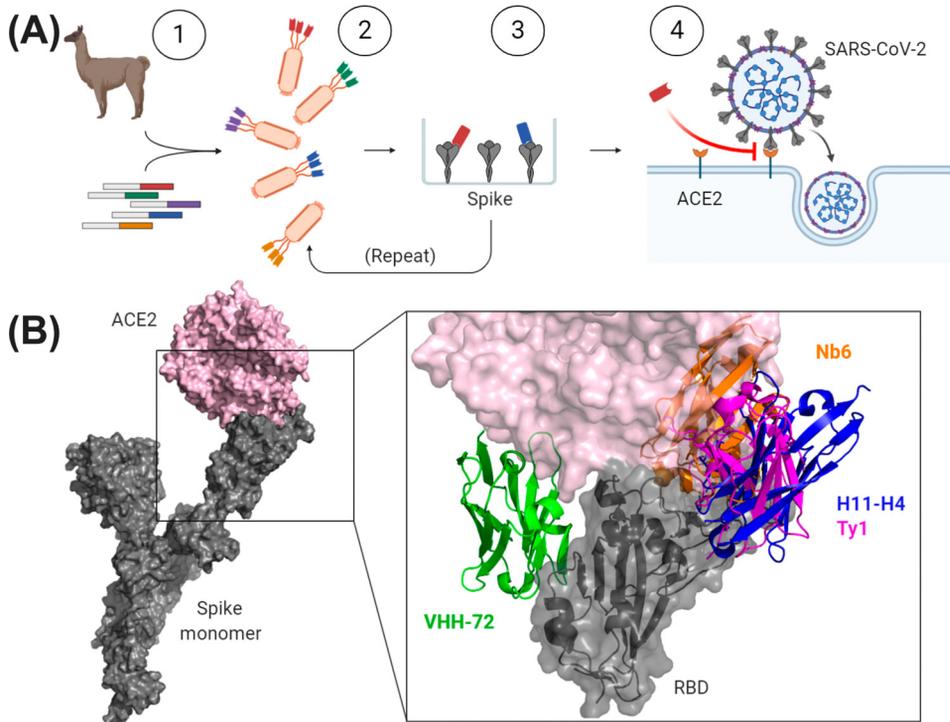
Figure 1. Structures of Conventional, Heavy-Chain-Only (HCAb), and Single-Domain Antibodies (sdAbs). Conventional IgG antibodies consist of H and L chain pairs that form Y-shaped structures. Camelids (e.g., alpacas, llamas) make a heavy-chain-only (HCAb) class of antibody. The variable elements (V_H) of HCAbs, when expressed as autonomous units, are known as single-domain antibodies (sdAbs) or nanobodies. The crystal structure of a representative sdAb (PDB ID 6OBC) is shown with the complementarity determining regions (CDRs) 1, 2, and 3 colored blue, yellow, and red, respectively. Image created in BioRender.

multiple rounds of affinity maturation to achieve a 'best fit' with a pathogen target [14]. While it is not uncommon that V_H elements dominate an antibody–antigen interaction, rarely does a V_H or V_L element on its own have sufficient affinity to engage with an antigen.

Heavy-chain-only antibodies (HCAbs) are different. First discovered in Arabian camels (*Camelus dromedarius*), HCAbs are now known to be present across the Camelidae family, including llamas and alpacas [15]. HCAbs consist of two heavy chains (homodimers) without light chain partners (Figure 1). Antigen recognition is confined to the terminal V_H domain or V_HH . Similar to the V_H domains of conventional immunoglobulins, HCAb V_HH domains or sdAbs consist of nine β -strands arranged in two β -sheets with CDR 1–3 elements located on one face. For reasons having to do with the absence of a light chain, V_HH s have a propensity for concave surfaces, including active sites and receptor-binding pockets [16,17]. Indeed, V_HH s are notorious for their ability to contact catalytic residues and mimic substrates. For a thorough discussion of the evolution, immunology, and structural biology of V_HH s, we refer the reader to comprehensive reviews on the subject [18].

What makes sdAbs so appealing is that they retain affinity and specificity for target antigens when expressed as recombinant fusion proteins on the surface of bacteriophage M13 ('phage display') or yeast ('yeast display') [19]. In the case of phage display, antibodies are isolated by subjecting the phage-display libraries to repeated rounds of affinity selection on antigen-coated tubes or magnetic beads (Figure 2). While specific methodologies are beyond the scope of this article, reiterative enrichment strategies enable the isolation of exceedingly rare sdAbs from large and diverse antibody libraries. Moreover, because the V_HH -coding DNA is packaged within the isolated phage particle or yeast cells, antibodies are readily cloned and expressed in *Escherichia coli*.

Single-domain antibody libraries come in three flavors: immune, naïve and synthetic (Figure 2). Immune libraries are generated from camelids that have been immunized (often repeatedly) with



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Figure 2. Discovery of Receptor-Binding Domain (RBD)-Specific Single-Domain Antibodies (sdAbs). A depiction of a workflow for isolating sdAbs against SARS-CoV-2 Spike and RBD. (A) (1) Isolate peripheral blood mononuclear cells from naïve or immune llamas or alpacas. (2) PCR-amplify and clone V_HH-encoding genes into M13-phage vector to create a library of high complexity. (3) Affinity-enrich for V_HHs that bind to Spike (or RBD). (4) Clone and express individual V_HHs and test for SARS-CoV-2-neutralizing activity. (B) Left, structure of SARS-CoV-2 Spike monomer bound to ACE2 (PDB ID: 6M0J). Right, SARS-CoV-2 RBD (6ZXN) superimposed with VHH-72 (6WAQ), H11-H4 (6ZH9), Ty1 (6ZXN), and Nb6 (7KKK). Image created in BioRender.

antigens of interest [18]. The primary advantage of such libraries is that they are enriched in antigen-specific sdAbs that arose as a result of natural, immune-mediated affinity-maturation, thereby greatly increasing the yield and diversity of high-affinity antibodies (low or subnanomolar) against desired targets. Indeed, two rounds of selection with such libraries can yield families of antibodies with extraordinary high binding affinities. However, immune libraries are not accessible to everyone as they require access to camelids, large amounts of antigen for immunizations, and the technical expertise necessary to assemble and validate a diverse and complex phage- or yeast-display library.

Naïve and synthetic libraries, on the other hand, are commercially available and theoretically ready at a moment's notice. Naïve libraries are derived from pre-existing B cell repertoires of one or more nonimmunized animals, while synthetic libraries are constructed *in vitro* using a combination of bioinformatics and structural biology information available in GenBank and the PDB [20]. In either case, once constructed, the power of phage- and yeast-display technologies enables the isolation of rare sdAbs against targets of interest. However, synthetic and naïve libraries generally yield sdAbs of lower affinities than immune libraries, thereby necessitating *in vitro* affinity maturation and further rounds of screening to obtain sdAbs with low or subnanomolar activities [11,13,21].

Discovery of sdAbs That Target the SARS-CoV-2 Spike Protein

Leveraging the strategies outlined in the previous text, there have been a flurry of studies in the past several months describing the discovery, structural analysis, and engineering of ultrapotent

sdAbs targeting the SARS-CoV-2 Spike protein [9–13]. In fact, according to the coronavirus antibody database (CoV-AbDab), there are now more than 300 SARS-CoV-2-specific sdAbs [22]. Here we summarize strategies used to discover some of the most potent RBD-specific sdAbs and speculate as to their potential to contribute to the fight against COVID-19.

The first sdAbs with SARS-CoV-2 neutralizing activity were reported within months of the appearance of the novel coronavirus onto the world's stage [12]. By chance, Wrapp and colleagues had generated a V_HH-phage display library from a llama immunized with the Spike proteins of two related human beta coronaviruses, MERS and SARS-CoV-1 [23]. The Spike proteins of SARS-CoV-1 and SARS-CoV-2 are ~75% similar to each other [24]. One sdAb, called VHH-72, was identified as targeting the RBD of SARS-CoV-1 Spike protein, but reacted with an epitope shared with SARS-CoV-2 RBD. The structure of VHH-72 bound to SARS-CoV-1 RBD revealed its binding site as being outside the ACE2 receptor binding motif (Figure 2). While VHH-72 by itself was not particularly potent, its SARS-CoV-2 neutralizing activity was enhanced when it was expressed as a bivalent human Fc IgG fusion protein (Table 1; Figure 3).

In a more concerted effort to target the SARS-CoV-2 RBD specifically, Hanke and colleagues immunized an alpaca with a cocktail of the S1 subunit of the Spike protein and isolated RBD (Table 1) [9]. As depicted in Figure 2, the investigators cloned V_HH-encoding genes from the alpaca into an M13 vector and subjected the resulting V_HH-phage display library to two rounds of affinity enrichment with immobilized RBD as 'bait'. Next-generation sequencing (NGS) revealed that the panning strategy resulted in a ~10 000-fold enrichment for RBD-specific antibodies. A single RBD-specific V_HH, named Ty1, was identified. Ty1 blocked RBD–ACE2 interactions and neutralized SARS-CoV-2 pseudoviruses (Table 1). The structure of Ty1 in complex with the SARS-CoV-2 trimeric Spike revealed Ty1's epitope as being within the region of RBD known as the receptor-binding motif (RBM), which interacts directly with ACE2 (Figure 2).

In a study in *Science*, Xiang and colleagues upped the ante by describing the isolation of ~350 unique sdAbs against SARS-CoV-2 RBD from a V_HH-phage display library derived from a single RBD-immunized llama [13]. The remarkable yield of unique sdAbs was achieved using an integrative proteomics pipeline. A series of down selection strategies eventually yielded 18 unique sdAbs with low nanomolar and even subnanomolar affinities for RBD and potent neutralizing activities against the SARS-CoV-2 Munich strain (Table 1). Through a series of competitive binding assays and cross-linking studies, the investigators identified five distinct epitopes on RBD with the ACE2 interface region being the most immunodominant. The investigators solved the crystal structure of a particularly potent sdAb, Nb20, bound to RBD (Table 1).

Table 1. RBD-Specific sdAbs against SARS-CoV-2

V _H H	Origin	'bait'	IC ₅₀ ^a	PDB	Refs
VHH-72	SARS S-immunized llama	S ^b	0.2 µg/ml	6WAQ	[12]
Ty1	RBD-immunized alpaca	RBD	0.77 µg/ml	6ZXX	[9]
H11–H4	Naïve llama, matured	RBD	4–6 nM	6ZH9	[10]
Nb20	RBD-immunized llama	RBD	0.048 nM	7JVB	[13]
mNb6	Synthetic, matured	S trimer	2.5 nM	7KKK	[11]

^aVHH-72 and Ty1 IC₅₀ values were generated using pseudoviruses, whereas H11–H4, Nb20, and mNb6 were derived using SARS-CoV-2 PRNT assay.

^bSpike (S) protein from SARS-CoV-1.

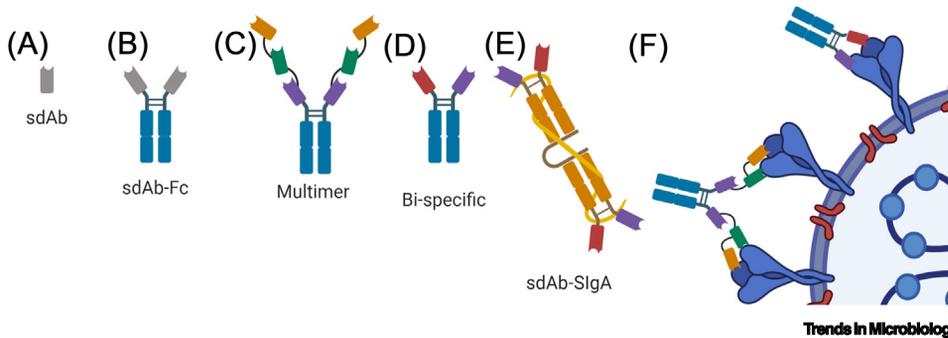


Figure 3. Engineering Single-Domain Antibodies (sdAbs) for Therapeutic Applications. (A,B) High-affinity monomeric sdAbs engineered onto IgG Fc regions allow enhanced avidity through bivalency, increased serum half-life, and potential Fc-mediated effector functions. (C) Multimerization of sdAbs through flexible linker regions can increase avidity through simultaneous binding of adjacent subunits. (D) Multimeric sdAbs including single bispecific sdAb–Fc molecules generated by knob-into-holes or lock-and-key techniques. (E) sdAbs similarly engineered onto IgA Fc scaffolds would allow for efficient antigen targeting to mucosal compartments, as well as potentially enhanced valency and antigen aggregation. (F) Multimeric sdAb constructs, targeting surface antigens of SARS-CoV-2, particularly the Spike RBD, may allow broad coverage of neutralizing epitopes on the virus, yielding ultra-potent antibody neutralization through multiple mechanisms. Image created in BioRender.

Synthetic and naïve phage- and yeast-display libraries have also yielded high-affinity sdAbs against SARS-CoV-2. In a report in *Nature Structural and Molecular Biology*, Huo and colleagues screened for RBD-specific sdAbs from a V_H H-phage display library derived from a naïve llama (Table 1). The screen yielded a single low-affinity RBD-specific V_H H, H11. In an effort to improve the binding characteristics of H11, the CDR3 element was subjected to random mutagenesis, cloned into a V_H H-phage display vector and re-panned on RBD. Ultimately, the screens gave rise to two derivatives, H11-D4 and H11-H4, that bound RBD with 20- to 50-fold improved affinity compared to H11. Both H11-D4 and H11-H4 blocked RBD interactions with ACE2 and neutralized SARS-CoV-2 *in vitro* (Table 1). The structures of the two V_H Hs in complex with RBD were solved and, remarkably, recognize virtually the same epitope on RBD as Ty1 (Figure 2).

Schoof and colleagues took a different tack and used a commercially available synthetic V_H H yeast-display library to fish for sdAbs capable of recognizing RBD with trimeric full-length Spike as bait [11]. In their article in *Science*, they describe a screen that involved a combination of magnetic bead-based enrichment and fluorescence-activated cell sorting, which ultimately uncovered 21 unique sdAbs. The sdAbs fell into two classes: class I bound RBD and competed with ACE2; class II recognized RBD, but outside the ACE2 interface. Class I sdAbs, notably Nb6, proved to be the most effective at neutralizing SARS-CoV-2 (Table 1). The cryo-EM structure of Nb6 in complex with trimeric Spike revealed that the antibody recognizes RBD in a 'closed' or down conformation (Figure 2). Moreover, Nb6 interacts simultaneously with two adjacent RBDs, effectively disabling two of the three RBD elements from being able to interact with ACE2. Using this information, the investigators engineered bivalent and trivalent Nb6 variants with ultrahigh binding affinities for Spike and potent SARS-COV-2 neutralizing activities. Introduction of point mutations in CDR1 and CDR3 improved Nb6's neutralizing activity of SARS-CoV-2 by 200-fold.

Advancing sdAbs from Bench to Bedside in the Battle against SARS-CoV-2

We now confront the question of how the armamentarium of newly discovered ultrapotent RBD-specific sdAbs can contribute to the battle against SARS-CoV-2. The immediate next step is to determine whether the newly identified sdAbs are able to neutralize SARS-CoV-2 in an animal

model of COVID-19 [25]. In this respect, two recent reports in *PNAS* and *Cell* provide a roadmap going forward [26,27]. In those reports, Li and colleagues isolated, by phage-display technologies, human V_H elements against RBD and tested them as recombinant IgGs in three different animal models: wild-type mice challenged intranasally with a mouse-adapted SARS-CoV-2 strain, human ACE2-transgenic mice challenged intranasally with SARS-CoV-2, and Syrian golden hamsters challenged intranasally with SARS-CoV-2. Antibodies were administered by injection before or after virus exposure and protection was assessed by viral load and lung histopathology at different time points after challenge. The investigators also performed dose–response studies and some limited pharmacokinetic analyses, thereby providing important benchmarks by which other sdAbs can now be evaluated.

If one or more of the camelid-derived ultrapotent RBD-specific sdAbs showcased in the previous text prove effective in animal models of COVID-19, then clinical studies may be warranted. There is also precedent in this realm. In 2019, the first single sdAb-based biologic, Caplacizumab, was approved by the FDA to treat a rare blood-clotting disorder [28]. Caplacizumab is a llama-derived sdAb that has been genetically engineered to resemble human-derived V_H elements ('humanized') [29]. Moreover, the humanized sdAb was dimerized (i.e., two identical sdAbs are joined via an alanine linker) to create a bivalent molecule, which is expressed in *E. coli* as a secreted protein [29,30]. Caplacizumab, administered intravenously, was evaluated in a nonhuman primate model before Phase I clinical trials [31]. It is now approved for use in the USA and abroad [28].

If a SARS-CoV-2 sdAb successfully makes it through preclinical and clinical testing regimens, the next consideration is whether it can compete with the MAb-based therapies from Eli Lilly [32] and Regeneron [33] that were recently granted emergency use authorization (EUA) by the United States Food and Drug Administration (FDA) for individuals with mild or moderate COVID-19. Both the Eli Lilly MAb (bamlanivimab) and the Regeneron MAb cocktail (casirivimab and imdevimab) are human IgGs that target the SARS-CoV-2 Spike RBD and block ACE2 interactions [32–34]. The binding affinities and SARS-CoV-2-neutralizing activities of the Regeneron MAbs have been reported and are similar to the sdAbs listed in Table 1 [34]. Therefore, to be competitive, an sdAb-based therapeutic would need to be of equal or greater *in vivo* potency than the existing Eli Lilly and Regeneron MAbs and have additional attributes to incentivize their use in the USA and abroad. At least in theory, sdAb-based therapeutics, if made in *E. coli* or yeast, would be considerably less expensive to manufacture than the existing human MAbs made in mammalian cells and priced in excess of US\$1000 per dose [28]. sdAbs also have the added benefit of stability at high temperatures (66–72°C) and being amenable to lyophilization without loss of biological activity, thereby reducing the need for refrigerated transport and storage [35].

Attacking SARS-CoV-2 on the Front Lines (of the Airways and Gut)

However, the future of sdAbs may not be so much going head-to-head with existing human MAb therapies for COVID-19, but, rather, venturing into territories where conventional MAbs cannot tread. Perhaps the most exciting prospect is direct delivery of sdAbs into the lungs as a strategy to intercept SARS-CoV-2 before it accesses ACE2 and takes up residence within airway epithelial cells [9–13]. As noted by others, the small size and intrinsic thermostability of sdAbs make them particularly attractive as candidates for aerosol or particle delivery [11,13]. Indeed, direct delivery or *in situ* expression of sdAbs within the context of respiratory infections is an area of active pursuit [36]. For example, a recently completed Phase 2b clinical trial evaluated a trivalent sdAb, called ALX-0171, as treatment for respiratory syncytial virus (RSV) infection [37]. Children admitted to hospital for acute severe RSV received two doses of ALX-0171 by nebulizer. Patients that received ALX-0171 cleared RSV, as detected in nasal swabs, faster than the placebo group,

demonstrating the ability of passively administered antibody to clear virus from the upper airways. Although the clinical course of RSV infection was not altered by ALX-0171 intervention, the study nonetheless represents a milestone in the application of sdAbs to severe respiratory virus infections.

Other delivery platforms and strategies should be considered when confronting SARS-CoV-2 (Figure 3). Ian Wilson's group at The Scripps Research Institute, for example, used structure-based design to engineer a multimeric, multiepitope targeted sdAb fusion protein (MD3606) against influenza virus [38]. MD3606 is truly an engineering feat in that it consists of three different sdAbs linked in tandem and dimerized by fusion with the Fc region of human IgG1. Mice that received MD3606 by injection or recombinant adeno-associated virus (AAV)-mediated gene delivery were protected from lethal-dose influenza A and B virus challenge.

sdAb applications do not need to be limited to the lung. SARS-CoV-2 is also found at high levels in the gastrointestinal tract, and shedding of virus in stools can occur in asymptomatic individuals [39,40]. IgA is the primary antibody type found in mucosal secretions, including saliva, the upper airways, and especially the gastrointestinal tract (Figure 3). In the context of the gut, it was recently shown that passively administered recombinant sdAb-IgA fusion proteins have biological activity. Specifically, an sdAb-IgA construct given to piglets in their feed was sufficient to protect them against an experimental enterotoxigenic *E. coli* infection [35,41]. The prospect of IgA-based sdAbs is particularly exciting considering that several reports indicated that human IgA is able to neutralize SARS-CoV-2 better than IgG [42,43].

Finally, with the advent of nucleic acid-based vaccines for SARS-CoV-2 now a reality, it is likely that mRNA-based antibody prophylactic or therapeutic strategies will soon follow with sdAbs leading the way. In fact, it has already been shown that a single injection of mRNA-lipid nanoparticles encoding engineered botulinum toxin sdAbs into mice resulted in serum antibody responses capable of affording immunity to a lethal toxin challenge [44]. We have reported that alpaca-derived sdAbs targeting the active site of ricin toxin were able to neutralize the toxin when expressed as intrabodies in the cytoplasm of Vero cells [17]. In fact, it is not farfetched to think that sdAbs might be identified that could function intracellularly to interfere with SARS-CoV-2 machinery involved in entry, replication, or host cell egress considering that a group from Wuhan University reported on this strategy for influenza virus more than decade ago [45]. For a more elaborate discussion on targeting virus machinery within infected cells, see De Vlieger *et al.* [46].

Concluding Remarks

With the COVID-19 pandemic showing no signs of abating, it is imperative that all avenues be pursued to arrest the spread of the novel coronavirus. If the results of the past several months are any indication, the prospect of sdAbs contributing to the fight against SARS-CoV-2 is high. Particularly intriguing is the prospect of delivering sdAbs directly into the lung as a means of slaying SARS-CoV-2 before it gains a foothold in host cells. In terms of basic research, the sdAbs described to date have identified critical contact points on RBD involved in ACE2 interaction and demonstrated that these epitopes constitute sites of vulnerability on the surface of SARS-CoV-2. Finally, although not discussed in detail, the ultrahigh-affinity SARS-CoV-2-specific sdAbs may have utility as diagnostic tools [47]. sdAbs can serve as cost-effective standards for ELISAs or virus-neutralizing assays. The thermostability of sdAbs makes them particularly attractive for point-of-care diagnostic applications. One could envision the use of high-affinity, high-specificity sdAbs in antigen-based detection platforms like SARS-CoV-2 lateral flow devices with saliva.

Outstanding Questions

How do ultrapotent sdAbs specific to the RBD of the SARS-CoV-2 Spike protein compare to existing monoclonal IgG antibodies at neutralizing the virus in an animal model of COVID-19?

Does delivery of RBD-specific sdAbs into the lungs by nebulization prevent or reduce the severity of SARS-CoV-2 infection?

Are there neutralizing epitopes on the SARS-CoV-2 Spike protein beyond those already identified on RBD?

Can SARS-CoV-2 neutralizing activities of sdAbs be further enhanced by structure-based design?

Acknowledgments

We thank Drs Charles Shoemaker (Tufts University), Michael Rudolph (New York Structural Biology Center) and Yang Wang (MassBiologics) for discussion and insights into V_H/sdAb applications. We also gratefully acknowledge support from the National Cancer Institute's Serological Sciences Network for COVID-19 (award U01CA260508). N.J.M. is supported in part by award U01 CA260508 from the National Cancer Institute (NCI), National Institutes of Health, USA. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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