Globally a novel, highly transmissible coronavirus—severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1, 2)—has infected more than 30 million people and has claimed almost 1 million lives, with the numbers still rising as of September 2020. Despite preventive measures, such as quarantines and lockdowns that help curb viral transmission, the virus rebounds after social restrictions are lifted. Safe and effective therapeutics and vaccines remain urgently needed.

Like other zoonotic coronaviruses, SARS-CoV-2 expresses a surface spike (S) glycoprotein, which consists of S1 and S2 subunits that form a homotrimeric viral spike to interact with host cells. The interaction is mediated by the S1 receptor binding domain (RBD), which binds the peptidase domain of human angiotensin-converting enzyme 2 (hACE2) as a host receptor (3). Structural studies have revealed different conformations of the spike (4, 5). In the prefusion stage, the RBD switches between a closed conformation and an open conformation for hACE2 interaction. In the postfusion stage, the S2 undergoes a substantial conformational change to trigger host membrane fusion (6). Investigations of sera from COVID-19 convalescent individuals have led to the identification of potent neutralizing antibodies (NAbS) that primarily target the RBD but also non-RBD epitopes (7–13). High-quality NAbS may overcome the risks of Fc-associated antibody-dependent enhancement and are promising therapeutic candidates (14, 15).

V_{H}H antibodies, or nanobodies (Nbs), are minimal, monomeric antigen binding domains derived from camelid single-chain antibodies (16). Unlike immunoglobulin G (IgG) antibodies, Nbs are small (~15 kDa), highly soluble and stable, readily bioengineered into bi- or multivalent forms, and amenable to low-cost, efficient microbial production. Owing to their robust physicochemical properties, Nbs can be administered by inhalation, which makes them appealing therapeutic agents for treatment of respiratory viruses (17, 18). Recently, several SARS-CoV-2 neutralizing Nbs have been identified through the screening of SARS-CoV or Middle East respiratory syndrome (MERS) cross-reacting Nb libraries or the use of synthetic Nb libraries for RBD binding. However, these synthetic Nbs generally neutralize the virus at or below microgram-per-milliliter concentrations (12, 19–22) and thus are hundreds of times less potent than the most effective NAbS, likely due to monovalency and/or lack of affinity maturation (23, 24). The development of highly potent anti–SARS-CoV-2 Nbs may provide a means for versatile, cost-effective prophylaxis, therapeutics, and point-of-care diagnosis.

To produce high-quality SARS-CoV-2 neutralizing Nbs, we immunized a llama with the recombinant RBD. Compared with the preimmunized serum sample, the postimmunized serum showed potent and specific serologic activities toward RBD binding with a titer of $1.75 \times 10^8$ (fig. S1A). The serum efficiently neutralized the pseudotyped SARS-CoV-2 at a half-maximal neutralization titer (NT_{50}) of $\sim 310,000$ (fig. SIB), orders of magnitude higher than that of the convalescent sera obtained from recovered COVID-19 patients (7, 8). To further characterize these activities, we separated the single-chain V_{H}H antibodies from the IgGs. We confirmed that the single-chain antibodies achieve specific, high-affinity binding to the RBD and possess subnanomolar half-maximal inhibitory concentration (IC_{50} = 509 pM) against the pseudotyped virus (fig. SIC).

We identified thousands of high-affinity V_{H}H Nbs from the RBD-immunized llama serum by using a robust proteomic strategy that we recently developed (25) (fig. S2A). This repertoire includes ~350 distinct CDR3s (CDRs, complementarity-determining regions). For *Escherichia coli* expression, we selected 109 highly diverse Nb sequences from the repertoire with distinct CDR3s to cover various biophysical, structural, and potentially different antiviral properties. Ninety-four Nbs were purified and tested for RBD binding by enzyme-linked immunosorbent assay (ELISA), from which we confirmed 71 RBD-specific binders (fig. S2, B and C, and tables S1 and S4). Of these RBD-specific binders, 49 Nbs presented high solubility and high affinity (ELISA IC_{50} below 30 nM; Fig. 1A) and were promising candidates for functional characterization. We used a SARS-CoV-2–green fluorescent protein (GFP) pseudovirus neutralization assay to screen and characterize the antiviral activities of these high-affinity Nbs. Of the tested Nbs, 94% neutralize the pseudotype virus below 3 μM (Fig. 1B), and 90% neutralize below 500 nM. Only 20 to 40% of the high-affinity RBD-specific monoclonal antibodies identified from patient sera have been reported to possess comparable potency (7, 8). More than three-quarters (76%) of the Nbs efficiently neutralized the pseudovirus below 50 nM, and 6% had neutralization activities below 0.5 nM. We selected the 18 most potent Nbs on the basis of the pseudovirus GFP reporter screen and measured their potency accurately using the pseudovirus-luciferase reporter assay. Finally, we used the plaque reduction neutralization test (PRNT) assay (26) to evaluate the potential of 14 Nbs to neutralize the SARS-CoV-2 Munich strain. All of the Nbs reached 100% neutralization and neutralized the virus in a dose-dependent manner. The IC_{50} values range from single-digit nanograms-per-milliliter amounts to below the nanograms-per-milliliter level. Of the most potent Nbs, three of them (89, 20, and 21) showed neutralization of 2.1 ng/ml (0.133 nM), 1.6 ng/ml (0.102 nM), and 0.7 ng/ml (0.045 nM), respectively, in the pseudovirus assay (Fig. 1C) and 0.154, 0.048, and 0.022 nM, respectively, in the SARS-CoV-2 assay (Fig. 1, D and E). Overall, there was an excellent

**Versatile and multivalent nanobodies efficiently neutralize SARS-CoV-2**

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Cost-effective, efficacious therapeutics are urgently needed to combat the COVID-19 pandemic. In this study, we used cameld immunization and proteomics to identify a large repertoire of highly potent neutralizing nanobodies (Nbs) to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein receptor binding domain (RBD). We discovered Nbs with picomolar to femtomolar affinities that inhibit viral infection at concentrations below the nanograms-per-milliliter level, and we determined a structure of one of the most potent Nbs in complex with the RBD. Structural proteomics and integrative modeling revealed multiple distinct and nonoverlapping epitopes and indicated an array of potential neutralization mechanisms. We bioengineered multivalent Nb constructs that achieved ultrahigh neutralization potency (half-maximal inhibitory concentration as low as 0.058 ng/ml) and may prevent mutational escape. These thermostable Nbs can be rapidly produced in bulk from microbes and resist lyophilization and aerosolization.
correlation between the two neutralization assays ($R^2 = 0.92$; fig. S3).

We measured the binding kinetics of Nbs 89, 20, and 21 by surface plasmon resonance (SPR) (fig. S4, A and B). Nbs 89 and 20 have affinities of 108 and 10.4 pM, respectively, and the most potent Nb21 did not show detectable dissociation from the RBD during 20 min of SPR analysis. The subpicomolar affinity of Nb21 potentially explains its unusual neutralization potency (Fig. 1F). From the Nb21 potentially explains its unusual neutralization potency (Fig. 1F). From the E. coli periplasmic preparations, we determined the thermostability of Nbs 89, 20, and 21 to be 65.9°, 71.8°, and 72.8°C, respectively (fig. S4C). Finally, we tested the on-shelf stability of Nb21, which remained soluble after ~6 weeks of storage at room temperature after purification. No multimeric forms or aggregations were detected by size exclusion chromatography (SEC) (fig. S4D). Together, these results suggest that these neutralizing Nbs have valuable physicochemical properties for advanced therapeutic applications.

We employed an integrative approach by using SEC, cross-linking and mass spectrometry, and structural modeling for epitope mapping (27–30). First, we performed SEC experiments to distinguish between Nbs that share the same RBD epitope with Nb21 and those that bind to nonoverlapping epitopes. According to SEC profiles, Nbs 9, 16, 17, 20, 64, 82, 89, 99, and 107 competed with Nb21 for RBD binding (Fig. 2A and fig. S5), which indicates that their epitopes overlap substantially. By contrast, higher-mass species (from early elution volumes) corresponding to the trimeric complexes composed of Nb21, RBD, and one of the Nbs (34, 36, 93, 105, and 95) were evident (Fig. 2B and fig. S6, A to H). Moreover, Nb105 competed with Nbs 34 and 95, which did not compete for RBD interaction, suggesting the presence of two distinct and nonoverlapping epitopes. Second, we used disuccinimidyl suberate (DSS) to cross-link Nb-RBD complexes, and we used mass spectrometry to identify, on average, four intermolecular cross-links for Nbs 20, 93, 34, 95, and 105. The cross-links were used to map the RBD epitopes derived from the SEC data (materials and methods). Our cross-linking models identified five epitopes (I, II, III, IV, and V) corresponding to Nbs 20, 93, 34, 95, and 105 (Fig. 2C). The models satisfied 90% of the cross-links with an average precision of 7.8 Å (Fig. 2D and table S2). Our analysis confirmed the presence of a dominant epitope I (e.g., epitopes of Nbs 20 and 21) overlapping with the hACE2 binding site. Epitope II also colocalized with the nonconserved hACE2 binding site. Both epitopes I and II Nbs can compete with hACE2 binding to the RBD at very low concentrations in vitro (fig. STA). Epitopes III to V colocalized with conserved sites (fig. S7, B and C). Notably, epitope I Nbs had significantly shorter CDR3s (four amino acids shorter; $P = 0.005$) than other epitope binders (fig. S6I). Despite this, most of the selected Nbs potently inhibited the virus with an IC$_{50}$ below 30 ng/ml (2 nM) (table S1).

To explore the molecular mechanisms that underlie the potent neutralization activities of epitope I Nbs, we determined a crystal structure of the RBD-Nb20 complex at a resolution of 3.3 Å by molecular replacement (materials and methods, table S3, and fig. S13). Most of the residues in the RBD (Asn334 to Gly526) and in the entire Nb20, particularly those at the protein interaction interface, are well resolved.
There are two nearly identical copies of RBD-Nb20 complexes in one asymmetric unit, with a root mean square deviation of 0.277 Å over 287 Cα atoms. In the structure, all three CDRs of Nb20 interact with the RBD by binding to its large extended external loop with two short β strands (Fig. 3A) (3f). Glu484 of the RBD forms hydrogen bonding and ionic interactions with the side chains of Arg31 (CDR1) and Tyr104 (CDR3) of Nb20, whereas Gln493 of the RBD forms hydrogen bonds with the main-chain carbonyl of Ala29 (CDR1) and the side chain of Arg97 (CDR3) of Nb20. These interactions constitute a major polar interaction network at the RBD and Nb20 interface. Arg31 of Nb20 also engages in a cation–π interaction with the side chain of Phe490 of the RBD (Fig. 3B). In addition, Met65 from the CDR2 of Nb20 packs against residues Leu452, Phe490, and Leu492 of the RBD. In our superimposed structure, Asn52 forms a new H bond with Asn450 of the RBD (fig. S9B). Although Asn55 does not engage in additional interactions with the RBD, it creates a salt bridge with the side chain of Arg31, which stabilizes the polar interaction network among Arg31 and Tyr104 of Nb21 and Gln493 of the RBD (fig. S9B). All of those likely contribute to a slower off-rate of Nb21 (Fig. 1F and fig. S4A) and stronger neutralization potency. Structural comparison of RBD-Nb20 or RBD-Nb21 and RBD-hACE2 [Protein Data Bank (PDB) ID 6LZG] (3f) clearly showed that the interfaces for Nb20 or Nb21 and hACE2 partially overlap (Fig. 3D and fig. S9C). Notably, the CDR1 and CDR3 of Nb20 or Nb21 would clash with the first helix of hACE2, the primary binding site for the RBD (fig. S9D).

To understand the antiviral efficacy of our Nbs, we superimposed RBD-Nb complexes on different spike conformations according to cryo–electron microscopy (cryo-EM) structures. We found that three copies of Nb20 or Nb21 can simultaneously bind all three RBDs in their “down” conformations (PDB ID 6VXX) (4) that correspond to the inactive spike (Fig. 4B). On the basis of our crystal structure, we further modeled the structure of the best neutralizer Nb21 with the RBD (materials and methods). Only four residues vary between Nbs 20 and 21 (fig. S9A), all of which are on CDRs. Two substitutions are at the RBD binding interface. Ser23 and Met65 in the CDR2 of Nb20 are replaced by two asparagine residues (Asn23 and Asn65) in Nb21. In our superimposed structure, Asn52 forms a new H bond of Nb20 or Nb21 and Gln493 of the RBD (fig. S9B). Although Asn55 does not engage in additional interactions with the RBD, it creates a salt bridge with the side chain of Arg31, which stabilizes the polar interaction network among Arg31 and Tyr104 of Nb21 and Gln493 of the RBD (fig. S9B). All of those likely contribute to a slower off-rate of Nb21 (Fig. 1F and fig. S4A) and stronger neutralization potency. Structural comparison of RBD-Nb20 or RBD-Nb21 and RBD-hACE2 [Protein Data Bank (PDB) ID 6LZG] (3f) clearly showed that the interfaces for Nb20 or Nb21 and hACE2 partially overlap (Fig. 3D and fig. S9C). Notably, the CDR1 and CDR3 of Nb20 or Nb21 would clash with the first helix of hACE2, the primary binding site for the RBD (fig. S9D).

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Epitope mapping enabled us to bioengineer homo- and heterodimeric and homotrimeric Nbs. Homodimers and -trimers based on Nb20 or Nb21 were designed to increase the antiviral activities through avidity binding to the trimeric spike. Heterodimers pairing Nb21 with Nbs that bind a different epitope were designed to prevent viral escape. The homodimers and -trimers used flexible linker sequences of 25 (GS) or 31 (EK) amino acids (materials and methods). The heterodimers used flexible linkers of 12 amino acids.

Through a pseudovirus luciferase assay, we found up to ~30-fold improvement for the homotrimeric constructs of Nb213 (IC50 = 1.3 pM) and Nb203 (IC50 = 4.1 pM) compared with the respective monomeric form (Figs. 1, C and E, and 5, A and C). Similar results were obtained from the SARS-CoV-2 PRNT (Fig. 5, B and C, and fig. S11A). The improvements are likely greater than indicated by these values, as the measured values may reflect the assay’s lower detection limits. For the heterodimeric constructs (i.e., Nb21-Nb34), we observed up to a fourfold increase of potency. The multivalent constructs retained similar physicochemical properties to those of the monomeric Nbs (including high solubility, yield, and thermostability) and remained intact (nonproteolyzed) under the neutralization assay condition (fig. Xiang et al., Science 370, 1479–1484 (2020)).
S10). They remained highly potent for pseudovirus neutralization after lyophilization and aerosolization (materials and methods and fig. S11, B to G), indicating the marked stability and potential flexibility of administration. Most of the RBD mutations observed in the Global Initiative on Sharing Avian Influenza Data (GISAID) (35) are very low in frequency (<0.0025), which may increase under NB selection. Therefore, a cocktail consisting of ultrapotent, multivalent constructs that simultaneously bind a variety of epitopes with potentially different neutralization mechanisms will likely efficiently block virus mutational escape (Fig. 5E and fig. S12) (9, 36–38).

In our study, in vivo antibody affinity maturation followed by advanced proteomics (25) enabled the rapid discovery of a diverse repertoire of high-affinity RBD Nb constructs. This is unusual for natural, single-domain antibody fragments. We demonstrated the simplicity and versatility of Nb bioengineering and the desirable physicochemical properties of the monomeric Nbs and their multivalent forms. To our knowledge, the multivalent constructs represent the most potent SARS-CoV-2 neutralizers to date. Flexible and efficient administration, such as inhalation, may further improve their antiviral efficacy while minimizing the dose, cost, and potential toxicity for clinical applications. The high sequence similarity between Nbs and human IgGs may restrain the immunogenicity (39). It is possible to fuse the antiviral Nbs with highly stable albumin-Nb constructs (40) to improve pharmacokinetics. These high-quality Nbs can also be applied as rapid and economic point-of-care diagnostics.

We envision that the Nb technology described here will contribute to curbing the current pandemic and possibly a future event.

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42. I. V. N. and W. P. D. analyzed the data. Y. S. supervised the work.
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of chemical cross-link and mass spectrometric analysis have been deposited into the MassIVE data repository with accession code MSV000086098. Plasmids are being deposited at Addgene and are available from Y.S. in the interim. This work is licensed under a Creative Commons Attribution 4.0 International (CC BY 4.0) license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. To view a copy of this license, visit https://creativecommons.org/licenses/by/4.0/. This license does not apply to figures/photos/artwork or other content included in the article that is credited to a third party; obtain authorization from the rights holder before using such material.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S13

Tables S1 to S4

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Versatile and multivalent nanobodies efficiently neutralize SARS-CoV-2
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Nanobodies that neutralize
Monoclonal antibodies that bind to the spike protein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) show therapeutic promise but must be produced in mammalian cells and need to be delivered intravenously. By contrast, single-domain antibodies called nanobodies can be produced in bacteria or yeast, and their stability may enable aerosol delivery. Two papers now report nanobodies that bind tightly to spike and efficiently neutralize SARS-CoV-2 in cells. Schoof et al. screened a yeast surface display of synthetic nanobodies and Xiang et al. screened anti-spike nanobodies produced by a llama. Both groups identified highly potent nanobodies that lock the spike protein in an inactive conformation. Multivalent constructs of selected nanobodies achieved even more potent neutralization.

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