CORONAVIRUS

An ultrapotent synthetic nanobody neutralizes SARS-CoV-2 by stabilizing inactive Spike

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which enables aerosol-mediated delivery of this potent neutralizer directly to the airway epithelia.

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binds Spike in a fully inactive conformation with its receptor binding domains locked into their inaccessible down state, incapable of binding ACE2. Affinity maturation and structure-guided design of multivalency yielded a trivalent nanobody, mNb6-tri, with femtomolar affinity for Spike and picomolar neutralization of SARS-CoV-2 infection. mNb6-tri retains function after aerosolization, lyophilization, and heat treatment, which enables aerosol-mediated delivery of this potent neutralizer directly to the airway epithelia.

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus enters host cells via an interaction between its Spike protein and the host cell receptor angiotensin-converting enzyme 2 (ACE2). By screening a yeast surface-displayed library of synthetic nanobody sequences, we developed nanobodies that disrupt the interaction between Spike and ACE2. Cryo–electron microscopy (cryo-EM) revealed that one nanobody, Nb6, binds Spike in a fully inactive conformation with its receptor binding domains locked into their inaccessible down state, incapable of binding ACE2. Affinity maturation and structure-guided design of multivalency yielded a trivalent nanobody, mNb6-tri, with femtomolar affinity for Spike and picomolar neutralization of SARS-CoV-2 infection. mNb6-tri retains function after aerosolization, lyophilization, and heat treatment, which enables aerosol-mediated delivery of this potent neutralizer directly to the airway epithelia.

over the past two decades, three zoonotic β-coronaviruses have entered the human population, causing severe respiratory symptoms with high mortality (1–3). The COVID-19 pandemic is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the most readily transmissible of these three coronaviruses (4–7). No preventive treatment has been approved for any coronavirus to date, and the timeline for an effective and broadly available vaccine for SARS-CoV-2 remains uncertain. The development of new therapeutic and prophylactic approaches thus remains essential.

Coronavirus virions are bounded by a membrane that contains the homotrimeric transmembrane glycoprotein Spike, which is responsible for virus entry into the host cell (8, 9). The surface-exposed portion of Spike is composed of two domains, S1 and S2 (10). S1 binds the host cell receptor angiotensin-converting enzyme 2 (ACE2), whereas S2 catalyzes fusion of the viral and host cell membranes (11–13). Contained within S1 is the receptor binding domain (RBD), which directly binds to ACE2, and the N-terminal domain (NTD). The RBD is attached to the body of Spike by a flexible region and can exist in an inaccessible down state or an accessible up state (14, 15). Binding to ACE2 requires the RBD to occupy the up state and enables cleavage by host proteases, triggering a conformational change in S2 required for viral entry (16). In SARS-CoV-2 virions, Spike exchanges between an active, open conformation with at least one RBD in the up state and an inactive, closed conformation with all RBDs in the down state (8, 9).

We isolated single-domain antibodies (nanobodies) that neutralize SARS-CoV-2 by screening a yeast surface-displayed library of synthetic nanobody sequences for binders to the Spike ectodomain (17). We used a mutant form of SARS-CoV-2 Spike (Spike32P) as the antigen (15). Spike32P lacks one of the two proteolytic cleavage sites between the S1 and S2 domains and introduces two mutations and a trimerization domain to stabilize the prefusion conformation. We labeled Spike32P with biotin or with fluorescent dyes and selected nanobody-displaying yeast over multiple rounds, first by magnetic bead binding and then by fluorescence-activated cell sorting (Fig. 1A).

Three rounds of selection yielded 21 distinct nanobodies that bound Spike32P and showed decreased binding in the presence of a dimeric construct of the ACE2 extracellular domain (ACE2-Fc). These nanobodies fall into two classes. Class I binds the RBD and competes directly with ACE2-Fc (Fig. 1B). A prototypical example of this class is nanobody Nb6, which binds to Spike32P and to RBD alone with a dissociation constant (Kd) of 210 and 41 nM, respectively (Fig. 1C and table S1). Class II, exemplified by nanobody Nb3, binds to Spike32P (Kd = 61 nM) but displays no binding to RBD alone (Fig. 1C and table S1). In the presence of excess ACE2-Fc, binding of Nb6 and other class I nanobodies is blocked entirely, whereas binding of Nb3 and other class II nanobodies is moderately decreased (Fig. 1B). These results suggest that class I nanobodies target the RBD to block ACE2 binding, whereas class II nanobodies target other epitopes. Indeed, surface plasmon resonance (SPR) experiments demonstrate that class I and class II nanobodies can bind Spike32P simultaneously (Fig. 1D).

Class I nanobodies show a consistently faster association rate constant (kₐ) for nanobody binding to the isolated RBD than to Spike32P (table S1), which suggests that RBD accessibility influences the kₐ. We next tested the efficacy of class I and class II nanobodies to inhibit binding of fluorescently labeled Spike32P to ACE2-expressing human embryonic kidney (HEK) 293 cells (Fig. 1E and table S1). Class I nanobodies Nb6 and Nb11 emerged as two of the most potent clones, with half-maximal inhibitory concentration (IC₅₀) values of 370 and 540 nM, respectively. Class II nanobodies showed little to no activity in this assay. We prioritized two class I nanobodies, Nb6 and Nb11, that combine potent Spike32P binding with relatively small differences in kₐ between binding to Spike32P or RBD. For class II nanobodies, we prioritized Nb3 because of its relative yield during purification (table S1).

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To define the binding sites of Nb6 and Nb11, we determined their cryo-electron microscopy (cryo-EM) structures bound to SpikeS2P (Fig. 2, A and B; figs. S1 to S3, and table S2). Both nanobodies recognize RBD epitopes that overlap the ACE2 binding site (Fig. 2C). For Nb6 and Nb11, we resolved nanobody binding to both the open and closed conformations of SpikeS2P. We obtained a 3.0-Å map of Nb6 bound to closed SpikeS2P, which enabled modeling of the Nb6-SpikeS2P complex (Fig. 2A), including the complementarity-determining regions (CDRs). We also obtained lower-resolution maps for Nb6 bound to open SpikeS2P (3.8 Å), and Nb11 bound to open and closed SpikeS2P (4.2 and 3.7 Å, respectively). For these lower-resolution maps, we could define the nanobody’s binding orientation but not accurately model the CDers.

Nb6 bound to closed SpikeS2P straddles the interface between two adjacent RBDs. Most of the contacting surfaces are contributed by CDR1 and CDR2 of Nb6 (Fig. 2C). CDR3 contacts the adjacent RBD positioned counterclockwise when viewed from the top (Fig. 2C). The binding of one Nb6 therefore stabilizes two adjacent RBDs in the down state and likely preorganizes the binding site for a second and third Nb6 molecule to stabilize the closed Spike conformation. By contrast, Nb11 bound to down-state RBDs only contacts a single RBD (Fig. 2D).

The structure of Nb6 bound to closed SpikeS2P enabled us to engineer bivalent and trivalent nanobodies predicted to lock all RBDs in the down state. We inserted flexible Gly-Ser linkers of either 15 or 20 amino acids to span the 52-Å distance between adjacent Nb6 monomers bound to down-state RBDs in closed SpikeS2P (fig. S4). These linkers are too short to span the 72-Å distance between Nb6 molecules bound to open Spike. Moreover, steric clashes would prevent binding of three RBDs in open Spike with a single up-state RBD even in the absence of linker length (fig. S4). By contrast, the minimum distance between adjacent Nb11 monomers bound to either open or closed SpikeS2P is 68 Å. We predicted that multivalent binding by Nb6 constructs would display substantially slowed dissociation rates owing to enhanced avidity.

In SPR experiments, both bivalent Nb6 with a 15-mer amino acid linker (Nb6-bi) and trivalent Nb6 with two 20-mer amino acid linkers (Nb6-tri) dissociate from SpikeS2P in a biphasic manner. The dissociation phase can be fitted to two components: a fast phase with kinetic rate constants $k_{d1}$ of $2.7 \times 10^{-2}$ s$^{-1}$ for Nb6-bi and $2.9 \times 10^{-2}$ s$^{-1}$ for Nb6-tri, which are close to that observed for monovalent Nb6 ($k_d = 5.6 \times 10^{-2}$ s$^{-1}$), and a slow phase that is dependent on avidity ($k_{d2} = 3.1 \times 10^{-4}$ s$^{-1}$ for Nb6-bi and $k_{d2} < 1.0 \times 10^{-4}$ s$^{-1}$ for Nb6-tri) (Fig. 3A). The relatively similar $k_d$ for the fast phase suggests that a fraction of the observed binding for the multivalent constructs is nanobody binding to a single SpikeS2P RBD. By contrast, the slow dissociation phase of Nb6-bi and Nb6-tri indicates engagement of two or three RBDs. We observed no dissociation for the slow phase of Nb6-tri over 10 min, indicating an upper boundary for $k_{d2}$ of $1 \times 10^{-4}$ s$^{-1}$ and subpicomolar affinity. This measurement remains an upper boundary estimate because the measurement is limited by the intrinsic dissociation rate of SpikeS2P from the SPR chip imposed by the chemistry used to immobilize SpikeS2P. The true dissociation rate, therefore, may be considerably lower.

Biphasic dissociation could be explained by a slow interconversion between up- and down-state RBDs, with conversion to the more stable down state required for multivalent binding: A single domain of Nb6-tri engaged with an up-state RBD would dissociate rapidly. The system would then reequilibrate as the RBD flips into the down state, eventually allowing Nb6-tri to trap all RBDs in closed SpikeS2P. To test this directly, we varied the association time for Nb6-tri binding to SpikeS2P. Indeed,

**Fig. 1. Discovery of two distinct classes of anti-Spike nanobodies.**

(A) Selection strategy for identification of anti-Spike nanobodies that disrupt Spike-ACE2 interactions using magnetic bead selections (MACS) or fluorescence-activated cell sorting (FACS). By contrast, Nb11 (a class II nanobody) binds to SpikeS2P but not the RBD. Nb3 binding to SpikeS2P is partially decreased by ACE2-Fc. (C) SPR of Nb6 and Nb3 binding to either SpikeS2P or RBD. Red traces are raw data, and global kinetic fits are shown in black. Nb3 shows no binding to RBD. (D) SPR experiments with immobilized SpikeS2P show that class I and class II nanobodies can bind SpikeS2P simultaneously. By contrast, two class I nanobodies or class II nanobodies do not bind simultaneously. (E) Nanobody inhibition of 1 nM SpikeS2P-Alexa 647 binding to ACE2-expressing HEK293T cells. n = 3 (ACE2, Nb3) or n = 5 (Nb6, Nb11) biological replicates. All error bars represent SEM.
we observed an exponential decrease in the percentage of fast-phase dissociation with a half-life ($t_{1/2}$) of 65 s (Fig. 3B), which, we surmise, reflects the time scale of conversion between the RBD up and down states in Spike$^{3D}$. Taken together, dimerization and trimerization of Nb6 afforded 750-fold and >200,000-fold gains in $K_D$, respectively.

Unable to determine the binding site of Nb3 by cryo-EM, we turned to radiolytic hydroxyl radical footprinting. We exposed apo- or Nb3-bound Spike$^{3D}$ to synchrotron x-ray radiation to label solvent-exposed amino acids with hydroxyl radicals, which we subsequently quantified by mass spectrometry of protease-digested Spike$^{3D}$ (18). Two neighboring surface residues on the $S_1$ NTD of Spike (Met$^{177}$ and His$^{207}$) were protected in the presence of Nb6.

**Fig. 2. Cryo-EM structures of Nb6 and Nb11 bound to Spike.**

(A) Cryo-EM maps of the Spike$^{3D}$-Nb6 complex in either closed (left) or open (right) Spike$^{3D}$ conformation. (B) Cryo-EM maps of the Spike$^{3D}$-Nb11 complex in either closed (left) or open (right) Spike$^{3D}$ conformation. The top views show RBD up or down states. (C) Nb6 straddles the interface of two down-state RBDs, with CDR3 reaching over to an adjacent RBD. (D) Nb11 binds a single RBD in the down state (displayed) or similarly in the up state. No cross-RBD contacts are made by Nb11 in either RBD up or down state. (E) Comparison of RBD epitopes engaged by ACE2 (purple), Nb6 (red), or Nb11 (green). Both Nb11 and Nb6 directly compete with ACE2 binding.

**Fig. 3. Multivalency improves nanobody affinity and inhibitory efficacy.** (A) SPR of Nb6 and multivalent variants. Red traces show raw data, and black lines show global kinetic fit for Nb6 and independent fits for association and dissociation phases for Nb6-bi and Nb6-tri. (B) Dissociation phase SPR traces for Nb6-tri after variable association times ranging from 4 to 520 s. Curves were normalized to maximal signal at the beginning of the dissociation phase. Percent fast-phase dissociation is plotted as a function of association time (right) with a single exponential fit. $n = 3$ independent biological replicates. (C) Inhibition of pseudotyped lentivirus infection of ACE2-expressing HEK293T cells. $n = 3$ biological replicates for all but Nb11-tri ($n = 2$). (D) Inhibition of live SARS-CoV-2 virus. Representative biological replicates with $n = 3$ (right) or $n = 4$ (left) technical replicates per concentration. $n = 3$ biological replicates for all but Nb3 and Nb3-tri ($n = 2$). All error bars represent SEM.
Nb3 at a level consistent with prior observations of antibody-antigen interactions by hydroxyl radical footprinting (fig. S5) (19). Previously discovered coronavirus neutralizing antibodies bind an epitope within the NTD of Spike with Fab fragments that are noncompetitive with the host cell receptor (20, 21). Further SPR experiments demonstrated that Nb3 can bind SpikeS2P simultaneously with monovalent ACE2 (fig. S6). We hypothesized that the multivalent display of Nb3 on the surface of yeast may account for the partial decrease in SpikeS2P binding observed in the presence of ACE2-Fc. Indeed, a trivalent construct of Nb3 with 15-ami-no acid linkers (Nb3-tri) inhibited SpikeS2P binding to ACE2 cells with an IC50 of 41 nM (fig. S6). How Nb3-tri disrupts Spike-ACE2 interactions remains unclear.

We next tested the neutralization activity of monovalent and trivalent versions of our top class I (Nb6 and Nb11) and class II (Nb3) nanobodies against SARS-CoV-2 pseudovirus, using a previously described assay (22). Nb6 and Nb11 inhibited pseudovirus infection with IC50 values of 2.0 and 2.4 μM, respectively. Nb3 inhibited pseudovirus infection with an IC50 of 3.9 μM (Fig. 3C and table S1). Nb6-tri shows a 2000-fold enhancement of inhibitory activity, with an IC50 of 1.2 nM, whereas trimerization of Nb11 and Nb3 resulted in more modest gains of 40- and 10-fold (51 and 400 nM), respectively (Fig. 3C). We confirmed these neutralization activities with a viral plaque assay using live SARS-CoV-2 virus infection of VeroE6 cells. Here, Nb6-tri proved exceptionally potent, neutralizing SARS-CoV-2 with an average IC50 of 160 pM (Fig. 3D). Nb3-tri neutralized SARS-CoV-2 with an average IC50 of 140 nM (Fig. 3D).

We further optimized the potency of Nb6 by selecting a saturation mutagenesis library targeting all three CDRs. Two rounds of selection identified high-affinity clones with two penetrant mutations: I27Y (Ile27→Tyr) in CDR1 and P105Y (Pro105→Tyr) in CDR3. We incorporated these mutations into Nb6 to generate matured Nb6 (mNb6), which binds with 500-fold increased affinity to SpikeS2Pbinding to immobilized SpikeS2P. Red traces show raw data, and black lines show global kinetic fit. No dissociation was observed for mNb6-tri over 10 min. (B) mNb6 and mNb6-tri inhibit SARS-CoV-2 infection of VeroE6 cells in a plaque assay. Representative biological replicate with n = 4 technical replicates per concentration. n = 3 biological replicates for all samples. All error bars represent SEM. (C) Comparison of closed SpikeS2P bound to Nb6 and Nb6-tri. Rotational axis for RBD movement is highlighted. (D) Comparison of RBD engagement by Nb6 and mNb6-tri. One RBD was used to align both structures (RBD align), demonstrating changes in Nb6 and mNb6-tri and the adjacent RBD. (E) CDR1 of Nb6 and mNb6 binding to the RBD. As compared to I27 in Nb6; Y27 of mNb6 hydrogen bonds to Y453 and optimizes π-π and π-cation interactions with the RBD. N, Asp; R, Arg. (F) CDR3 of Nb6 and mNb6 binding to the RBD demonstrating a large conformational rearrangement of the entire loop in mNb6. A, Ala; L, Leu; F, Phe. (G) Comparison of mNb6 and Nb6-tri with mNb6-tri with mNb6-tri (fig. S8). However, the potency for mNb6 viral neutralization was unchanged with increasing concentrations of Nb3-tri, suggesting minimal synergy between these two nanobodies.

We next tested whether viral neutralization by the class I nanobody mNb6 is potentially synergistic with the class II nanobody Nb3-tri. In pseudovirus neutralization assays, we observed an additive effect when combining Nb3-tri with mNb6 (fig. S8). However, the potency for mNb6 viral neutralization was unchanged with increasing concentrations of Nb3-tri, suggesting minimal synergy between these two nanobodies.

We next tested Nb6 and its derivatives for stability. Circular dichroism revealed melting temperatures of 66.9°, 62.0°, 67.6°, and 61.4°C for Nb6, Nb6-tri, mNb6, and mNb6-tri, respectively (fig. S9). Moreover, mNb6 and mNb6-tri were stable to lyophilization and to aerosolization, showing no aggregation by size exclusion...
Nanobody multimerization has been shown to improve target affinity by avidity (33, 36). In the case of Nb6 and mNb6, structure-guided design of a multimeric construct that simultaneously engages all three RBBDs yielded profound gains in potency. Furthermore, because RBBDs must be in the up state to engage with ACE2, conformational control of RBBD accessibility serves as an added neutralization mechanism (30). Indeed, when mNb6-tri engages with Spike, it prevents ACE2 binding both by directly occluding the binding site and by locking the RBBDs into an inactive conformation.

Our discovery of class II neutralizing nanobodies demonstrates potentially new mechanisms of disrupting Spike function. The pairing of class I and class II nanobodies in a prophylactic or therapeutic cocktail could provide both potent neutralization and prevention of escape variants (29). The combined stability, potency, and diverse epitope engagement of our anti-Spike nanobodies therefore provide a distinctive potential prophylactic and therapeutic strategy to limit the continued toll of the COVID-19 pandemic.

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EM datasets. R.A.S. expressed and purified ACE2 and nanobodies and determined optimal freezing conditions for cryo-EM experiments; and developed, performed, and analyzed SPR experiments for SpikeS2P and RBD-nanobody affinity determination; developed, performed, and analyzed SPR binning and experiments; determined optimal freezing conditions for cryo-EM experiments; and performed thermal melting stability assays with guidance from M.O. K.M.W. performed live SARS-CoV-2 virus assays to test nanobody efficacy with guidance from C.Y.R. and analyzed mass spectrometry data generated by D.L.S. Several members of the QCRG Structural Biology Consortium played an exceptionally important role for this project: C.M.A. and C.P. determined optimal freezing conditions for cryo-EM experiments, optimized data collection approaches, and collected cryo-EM datasets. A.F.B., A.N.R., A.M.S., F.M., D.B., and T.P. collected cryo-EM data on SpikeS2P–nanobody complexes. S.D., H.C.N., C.M.C., U.S.C., M.G., M.J., F.L., Y.A., G.E.M., K.Z., and M.S. analyzed cryo-EM data from 15 SpikeS2P–nanobody complex datasets. H.T.K. set up crystallization trials of various RBD-nanobody complexes and crystallized, collected diffraction data for, and refined the mNb6 structure. M.C.T. collected, processed, and refined the mNb6 structure. R.T., D.D., and K.S. expressed and purified SpikeS2P, and S.P. purified RBD. A.M. expressed and purified SpikeS2P, labeled SpikeS2P for biochemical studies, designed selection strategies for nanobody discovery, cloned nanobodies for expression, designed affinity maturation libraries and performed selections, analyzed SPR data, and performed nanobody stability studies. The overall project was supervised by P.W. and A.M.

Competing interests: M.S., B.F., R.A.S., N.H., P.W., and A.M. are inventors on a provisional patent describing the anti-Spike nanobodies described in this manuscript. P.W. is a cofounder and consultant to Praxis Biotech LLC with an equity interest in the company. The Garcia-Sastre Laboratory has received research support from Pfizer, Senhwa Biosciences, Kenall Manufacturing, Light Sources, and 7Hills Pharma. A.G.-S. has consulting agreements for the following companies involving cash and/or stock: Vivaldi Biosciences, Contrafect, 7Hills Pharma, Avimex, Valneva, Accuris, and EsperovaX.

Data and materials availability: All data generated or analyzed during this study are included in this published article and its supplementary materials. Crystallographic coordinates and structure factors for mNb6 have been deposited in the Protein Data Bank under accession codes 7KKJ. Coordinates for SpikeS2P–mNb6 and SpikeS2P–mNb6 complexes have been deposited in the Protein Data Bank under accession codes 7KKJ and 7KKL, respectively. Maps for SpikeS2P–mNb6, SpikeS2P–Nb11, and SpikeS2P–mNb6 have been deposited in the Electron Microscopy Data Bank under accession codes EMD-22908 (SpikeS2P–mNb6 Open), EMD-22907 (SpikeS2P–mNb6 Closed), EMD-22911 (SpikeS2P–Nb11 Open), EMD-22909 (SpikeS2P–Nb11 Closed), and EMD-22910 (SpikeS2P–mNb6 Closed). The yeast-displayed library used to generate nanobodies in this study and the plasmids for nanobody constructs used in this study are available under a material transfer agreement with the University of California, San Francisco. 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.

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science.sciencemag.org/content/370/6523/1473/suppl/DC1 Materials and Methods
Figs. S1 to S9
Tables S1 to S5
QCRG Structural Biology Consortium Author List
References (37–63)
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An ultrapotent synthetic nanobody neutralizes SARS-CoV-2 by stabilizing inactive Spike


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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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