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

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The 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. Cryogenic electron microscopy (cryo-EM) revealed that one nanobody, Nb6, binds Spike in a fully inactive conformation with its receptor binding domains (RBDs) 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 last two decades, three zoonotic  $\beta$ -coronaviruses have entered the human population, causing severe respiratory symptoms with high mortality (1–3). The COVID-19 pandemic is caused by 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 novel therapeutic and prophylactic approaches thus remains essential.

Coronavirus virions are bounded by a membrane that contains the homotrimeric transmembrane glycoprotein Spike responsible for virus entry into the host cell (8, 9). The

surface-exposed portion of Spike is composed of two domains, S<sub>1</sub> and S<sub>2</sub> (10). S<sub>1</sub> binds the host cell receptor angiotensin converting enzyme 2 (ACE2), while S<sub>2</sub> catalyzes fusion of the viral and host cell membranes (11–13). Contained within S<sub>1</sub> 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 S<sub>2</sub> 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  $>2 \times 10^9$  synthetic nanobody sequences for binders to the Spike ectodomain (17). We used a mutant form of SARS-CoV-2 Spike (Spike<sup>S2P</sup>) as the antigen (15). Spike<sup>S2P</sup> lacks one of the two proteolytic cleavage sites between the S<sub>1</sub> and S<sub>2</sub> domains and introduces two mutations and a trimerization domain to stabilize the pre-fusion conformation. We labeled Spike<sup>S2P</sup> 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 unique nanobodies that bound Spike<sup>S2P</sup> 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 Spike<sup>S2P</sup> and to RBD alone with a  $K_D$  of 210nM and 41nM, respectively (Fig. 1C and table S1). Class II, exemplified by nanobody Nb3, binds to Spike<sup>S2P</sup> ( $K_D=61$ 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 Spike<sup>S2P</sup> simultaneously (Fig. 1D).

Class I nanobodies show a consistently faster association rate constant ( $k_a$ ) for nanobody binding to the isolated RBD than to Spike<sup>S2P</sup> (table S1), which suggests that RBD accessibility influences the  $K_D$ . We next tested the efficacy of Class I and Class II nanobodies to inhibit binding of fluorescently labeled Spike<sup>S2P</sup> to ACE2-expressing HEK293 cells (Fig. 1E and table S1). Class I nanobodies Nb6 and Nb11 emerged as two of the most potent clones with  $IC_{50}$  values of 370 and 540nM, respectively. Class II nanobodies showed little to no activity in this assay. We prioritized two Class I nanobodies, Nb6 and Nb11, that combine potent Spike<sup>S2P</sup> binding with relatively small differences in  $k_a$  between binding to Spike<sup>S2P</sup> or RBD. For Class II nanobodies, we prioritized Nb3 because of its relative yield during purification (table S1).

To define the binding sites of Nb6 and Nb11, we determined their cryogenic electron microscopy (cryo-EM) structures bound to Spike<sup>S2P</sup> (Fig. 2, A and B; figs. S1 to S3; and table S2). Both nanobodies recognize RBD epitopes that overlap the ACE2 binding site (Fig. 2E). For Nb6 and Nb11, we resolved nanobody binding to both the open and closed

conformations of Spike<sup>S2P</sup>. We obtained a 3.0Å map of Nb6 bound to closed Spike<sup>S2P</sup>, which enabled modeling of the Nb6-Spike<sup>S2P</sup> complex (Fig. 2A), including the complementarity determining regions (CDRs). We also obtained lower resolution maps for Nb6 bound to open Spike<sup>S2P</sup> (3.8Å), and Nb11 bound to open and closed Spike<sup>S2P</sup> (4.2Å, and 3.7Å, respectively). For these lower resolution maps, we could define the nanobody's binding orientation but not accurately model the CDRs.

Nb6 bound to closed Spike<sup>S2P</sup> straddles the interface between two adjacent RBDs. The majority 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 pre-organizes 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 Spike<sup>S2P</sup> 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 52Å distance between adjacent Nb6 monomers bound to down-state RBDs in closed Spike<sup>S2P</sup> (fig. S4). These linkers are too short to span the 72Å 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 with longer linker length (fig. S4). By contrast, the minimum distance between adjacent Nb11 monomers bound to either open or closed Spike<sup>S2P</sup> is 68Å. We predicted that multivalent binding by Nb6 constructs would display significantly slowed dissociation rates due to enhanced avidity.

In SPR experiments, both bivalent Nb6 with a 15 amino acid linker (Nb6-bi) and trivalent Nb6 with two 20 amino acid linkers (Nb6-tri) dissociate from Spike<sup>S2P</sup> in a biphasic manner. The dissociation phase can be fitted to two components: a fast phase with kinetic rate constants  $k_{d1}$  of  $2.7 \times 10^{-2} \text{s}^{-1}$  for Nb6-bi and  $2.9 \times 10^{-2} \text{s}^{-1}$  for Nb6-tri, which are close to that observed for monovalent Nb6 ( $k_d=5.6 \times 10^{-2} \text{s}^{-1}$ ) and a slow phase that is dependent on avidity ( $k_{d2}=3.1 \times 10^{-4} \text{s}^{-1}$  for Nb6-bi and  $k_{d2}<1.0 \times 10^{-6} \text{s}^{-1}$  for Nb6-tri, respectively) (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 Spike<sup>S2P</sup> 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  $1 \times 10^{-6} \text{s}^{-1}$  and subpicomolar affinity. This measurement remains an upper boundary estimate because the measurement is limited by the intrinsic dissociation rate of Spike<sup>S2P</sup> from the SPR chip imposed by the chemistry used to immobilize Spike<sup>S2P</sup>. The true dissociation rate, therefore,

may be significantly lower.

Biphasic dissociation could be explained by a slow inter-conversion 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 re-equilibrate as the RBD flips into the down-state, eventually allowing Nb6-tri to trap all RBDs in closed Spike<sup>S2P</sup>. To test this directly, we varied the association time for Nb6-tri binding to Spike<sup>S2P</sup>. Indeed, we observed an exponential decrease in the percent fast-phase with a  $t_{1/2}$  of 65s (Fig. 3B), which, we surmise, reflects the timescale of conversion between the RBD up- and down-states in Spike<sup>S2P</sup>. 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<sup>S2P</sup> 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<sup>S2P</sup> (18). Two neighboring surface residues on the S<sub>1</sub> N-terminal domain of Spike (M177 and H207) were protected in the presence of 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 N-terminal domain of Spike with Fab fragments that are non-competitive with the host cell receptor (20, 21). Further SPR experiments demonstrated that Nb3 can bind Spike<sup>S2P</sup> simultaneously with monovalent ACE2 (fig. S6). We hypothesized that multivalent display of Nb3 on the surface of yeast may account for the partial decrease in Spike<sup>S2P</sup> binding observed in the presence of ACE2-Fc. Indeed, a trivalent construct of Nb3 with 15 amino acid linkers (Nb3-tri) inhibited Spike<sup>S2P</sup> binding to ACE2 cells with an  $IC_{50}$  of 41nM (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 pseudotyped lentivirus using a previously described assay (22). Nb6 and Nb11 inhibited pseudovirus infection with  $IC_{50}$  values of 2.0 $\mu$ M and 2.4 $\mu$ M, respectively. Nb3 inhibited pseudovirus infection with an  $IC_{50}$  of 3.9 $\mu$ M (Fig. 3C and table S1). Nb6-tri shows a 2000-fold enhancement of inhibitory activity, with an  $IC_{50}$  of 1.2nM, whereas trimerization of Nb11 and Nb3 resulted in more modest gains of 40- and 10-fold (51nM and 400nM), 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  $IC_{50}$  of 160pM (Fig. 3D). Nb3-tri neutralized SARS-CoV-2

with an average  $IC_{50}$  of 140nM (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 in CDR1 and P105Y in CDR3. We incorporated these mutations into Nb6 to generate matured Nb6 (mNb6), which binds with 500-fold increased affinity to Spike<sup>S2P</sup> (Fig. 4A). mNb6 inhibits both pseudovirus and live SARS-CoV-2 infection with low nanomolar potency, a ~200-fold improvement compared to Nb6 (Fig. 4B and table S1).

A 2.9Å cryo-EM structure shows that mNb6 binds to closed Spike<sup>S2P</sup> (Fig. 4C and fig. S7). mNb6 induces a slight rearrangement of the down-state RBDs as compared to Spike<sup>S2P</sup> bound to Nb6, inducing a 9° rotation of the RBD away from the central three-fold symmetry axis. This deviation likely arises from a different interaction between CDR3 and Spike<sup>S2P</sup>, which nudges the RBDs into a new resting position (Fig. 4D). While the I27Y substitution optimizes local contacts between CDR1 in its original binding site on the RBD, the P105Y substitution leads to a marked rearrangement of CDR3 in mNb6 (Fig. 4, E and F). This conformational change yields a different set of contacts between mNb6 CDR3 and the adjacent RBD. An X-ray crystal structure of mNb6 alone revealed dramatic conformational differences in CDR1 and CDR3 between free and Spike<sup>S2P</sup>-bound mNb6 (Fig. 4G and table S3). Although differences in loop conformation in the crystal structure may arise from crystal lattice contacts, they are suggestive of conformational heterogeneity for unbound mNb6 and induced-fit rearrangements upon binding to Spike<sup>S2P</sup>.

The binding orientation of mNb6 is similar to that of Nb6, suggesting that multivalent design would likewise enhance binding affinity. Unlike Nb6-tri, trivalent mNb6 with a 20 amino acid linker (mNb6-tri) bound to Spike<sup>S2P</sup> with no observable fast-phase dissociation and no measurable dissociation over ten minutes, yielding an upper bound for the dissociation rate constant  $k_d$  of  $1.0 \times 10^{-6} s^{-1}$  ( $t_{1/2} > 8$  days) and a  $K_D$  of <1pM (Fig. 4A). mNb6-tri displays further gains in potency in both pseudovirus and live SARS-CoV-2 infection assays with  $IC_{50}$  values of 120 pM (5.0ng/mL) and 54pM (2.3ng/mL), respectively (Fig. 4B and table S1). Given the sub-picomolar affinity observed by SPR, it is likely that these viral neutralization potencies reflect the lower limit of the assays. mNb6-tri is therefore an exceptionally potent SARS-CoV-2 neutralizing molecule.

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 chromatography and preserved high affinity binding to Spike<sup>S2P</sup> (Fig. 5, A and B, and fig. S9). Finally, mNb6-tri retains potent inhibition of pseudovirus and live SARS-CoV-2 infection after aerosolization, lyophilization, or heat treatment for 1 hour at 50°C (Fig. 5C and fig. S9).

Strategies to prevent SARS-CoV-2 entry into the host cell aim to block the ACE2-RBD interaction. Although high-affinity monoclonal antibodies are leading the way as potential therapeutics (20, 23–30), they are expensive to produce by mammalian cell expression and need to be intravenously administered by healthcare professionals (31). Large doses are needed for prophylactic use, as only a small fraction of systemic antibodies cross the epithelial cell layers lining the airways (32). By contrast, nanobodies can be inexpensively produced in bacteria or yeast. The inherent stability of nanobodies enables aerosolized delivery directly to the nasal and lung epithelia (33). Indeed, aerosol delivery of a trimeric nanobody targeting respiratory syncytial virus (ALX-0171) was recently demonstrated to be effective in substantially decreasing measurable viral load in hospitalized infants (34). Finally, potential immunogenicity of camelid-derived nanobodies can be mitigated by established humanization strategies (35).

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 RBDs yielded profound gains in potency. Furthermore, because RBDs must be in the up-state to engage with ACE2, conformational control of RBD accessibility serves as an added neutralization mechanism (30). Indeed, when mNb6-tri engages with Spike, it prevents ACE2 binding by both directly occluding the binding site and by locking the RBDs into an inactive conformation.

Our discovery of Class II neutralizing nanobodies demonstrates potentially novel mechanisms of disrupting Spike function. Pairing of Class I and Class II nanobodies in a prophylactic or therapeutic cocktail could provide both potent neutralization and prevention of escape variants (23). The combined stability, potency, and diverse epitope engagement of our anti-Spike nanobodies therefore provide a unique potential prophylactic and therapeutic strategy to limit the continued toll of the COVID-19 pandemic.

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and RBD-nanobody affinity determination, developed, performed and analyzed SPR binning, experiments, determined optimal freezing conditions for cryo-EM experiments, processed, refined and generated figures for Nb6, Nb11, and mNb6 EM datasets. R.A. Saunders expressed and purified ACE2 and nanobodies, developed and performed cell-based assays for inhibition of Spike<sup>S2P</sup> binding and pseudovirus assays for determining nanobody efficacy. S.Sangwan expressed and purified Spike<sup>S2P</sup>, RBD, ACE2-Fc, and nanobodies, processed cryo-EM data, optimized RBD-nanobody complexes for crystallography, grew crystals of mNb6, collected diffraction data, and refined the X-ray crystal structure of mNb6. V.Rezelj tested efficacy of nanobody constructs in live SARS-CoV-2 infection assays under the guidance of M.Vignuzzi. N.Hoppe purified nanobodies, developed, performed and analyzed SPR binning experiments, developed performed and analyzed variable Nb6-bi and Nb6-tri association experiments, and performed thermal melting stability assays for nanobody constructs. M.Boone developed approaches to express and purify nanobodies from *Pichia pastoris* and developed, performed, and analyzed approaches to quantify nanobody efficacy in live virus assays. C.B.Billesbølle expressed and purified Spike<sup>S2P</sup>, generated affinity maturation library for Nb6, performed yeast display selections to identify mNb6, and built the synthetic yeast nanobody library with J.Liang. I.Deshpande expressed and purified nanobody constructs. B.S.Zha performed live SARS-CoV-2 virus assays to test nanobody efficacy with guidance from O.Rosenberg. C.R.Simoneau and K.Leon performed live SARS-CoV-2 virus assays to test nanobody efficacy with guidance from M.Ott. K.M.White performed live SARS-CoV-2 virus assays to test nanobody efficacy with guidance from A.García-Sastre. A.W.Barile-Hill performed SPR experiments. A.A.Anand, N.Dobzinski, B.Barsi-Rhyne, and Y.Liu. assisted in cloning, expression, and purification of nanobody and pseudovirus constructs. V.Belyy performed single-molecule nanobody-Spike<sup>S2P</sup> interaction studies. S.Nock prepared media and coordinated lab usage during UCSF's partial shutdown. M.Zimanyi and S.Gupta performed radiolytic footprinting experiments with guidance from C.Y.Ralston and analyzed mass spectrometry data generated by D.L.Swaney. Several members of the QCRG Structural Biology Consortium played an exceptionally important role for this project. C.Azumaya and C.Puchades determined optimal freezing conditions for cryo-EM experiments, optimized data collection approaches, and collected cryo-EM datasets. A.F.Brilot, A.Rizo, A.M.Smith, F.Moss, D.Bulkley, T.Popsiech collected cryo-EM data on Spike<sup>S2P</sup>-nanobody complexes. S.Dickinson, H.C.Nguyen, C.M.Chio, U.S.Chio, M.Gupta, M.Jin, F.Li, Y.Liu, G.E.Merz, K.Zhang, M.Sun analyzed cryo-EM data from 15 Spike<sup>S2P</sup>-nanobody complex datasets. H.T.Kratochvil set up crystallization trials of various RBD-nanobody complexes, and crystallized, collected diffraction data for, and refined the mNb6 structure. M.C.Thompson collected, processed, and refined the mNb6 structure. R.Trenker, D.Diwanji, K.Schaefer expressed and purified Spike<sup>S2P</sup>, and S.Pourmal purified RBD. A.Manglik expressed and purified Spike<sup>S2P</sup>, labeled Spike<sup>S2P</sup> 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.Walter and A.Manglik. **Competing interests:** M.Schoof, B.Faust, R.A.Saunders, N.Hoppe, P.Walter, and A.Manglik are inventors on a provisional patent describing anti-Spike nanobodies described in this manuscript. P.Walter is a co-founder and consultant to Praxis Biotech LLC, with an equity interest in the company. The García-Sastre Laboratory has received research support from Pfizer, Senhwa Biosciences, Kenall Manufacturing, Light Sources and 7Hills Pharma. A.García-Sastre has consulting agreements for the following companies involving cash and/or stock: Vivaldi Biosciences, Contrafect, 7Hills Pharma, Avimex, Valneva, Accurius 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 code 7KKJ. Coordinates for Spike<sup>S2P</sup>:Nb6 and Spike<sup>S2P</sup>:mNb6 complexes have been deposited in the Protein Data Bank under accession codes 7KKK and 7KKL, respectively. Maps for Spike<sup>S2P</sup>:Nb6, Spike<sup>S2P</sup>:Nb11, and Spike<sup>S2P</sup>:mNb6 have been deposited in the Electron Microscopy Data Bank under accession codes EMD-22908 (Spike<sup>S2P</sup>-Nb6 Open), EMD-22907 (Spike<sup>S2P</sup>-Nb6 Closed), EMD-22911 (Spike<sup>S2P</sup>-Nb11 Open), EMD-22909 (Spike<sup>S2P</sup>-Nb11 Closed),

EMD-22910 (Spike<sup>S2P</sup>-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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Materials and Methods

Figs. S1 to S9

Tables S1 to S4

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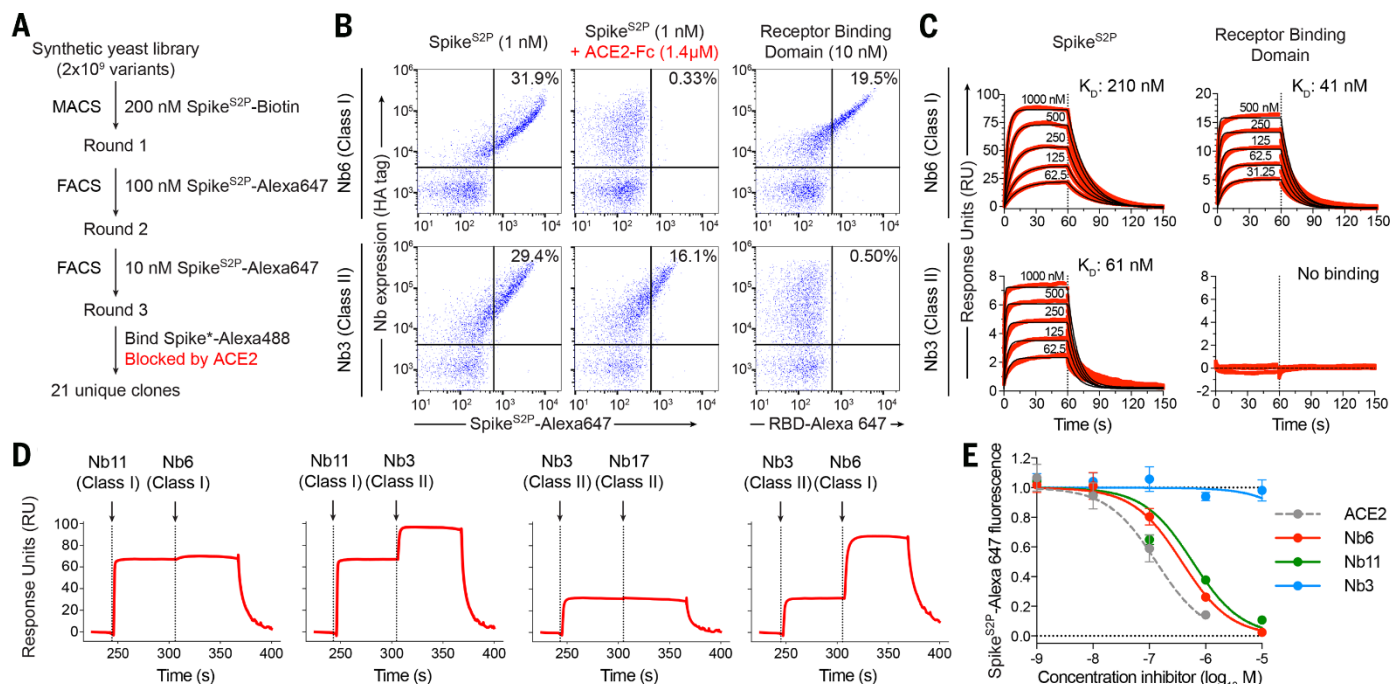
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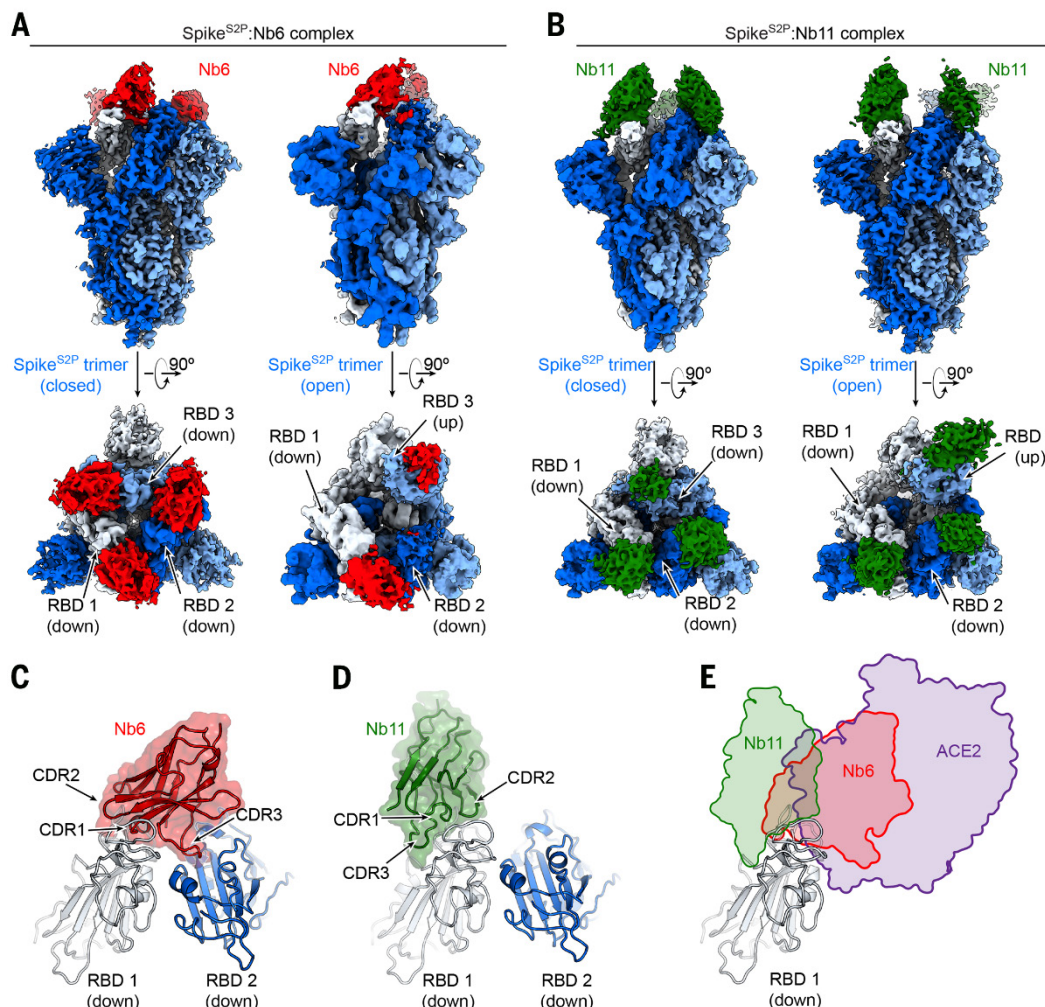
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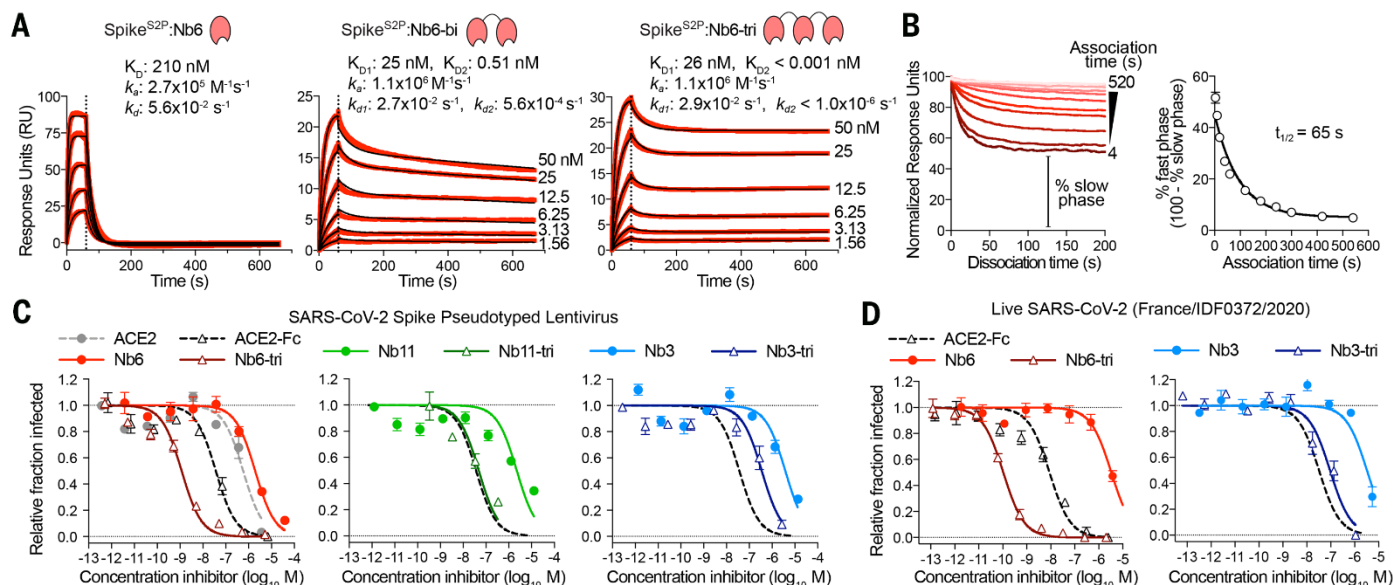


**Fig. 1. Discovery of two distinct classes of anti-Spike nanobodies.** (A) Selection strategy for identification of anti-Spike nanobodies that disrupt Spike-ACE2 interactions using magnetic bead selections (MACS) or fluorescence activated cell sorting (FACS). (B) Flow cytometry of yeast displaying Nb6 (a Class I nanobody) or Nb3 (a Class II nanobody). Nb6 binds Spike<sup>S2P</sup>-Alexa 647 and receptor binding domain (RBD-Alexa 647). Nb6 binding to Spike<sup>S2P</sup> is completely disrupted by an excess (1.4  $\mu$ M) of ACE2-Fc. Nb3 binds Spike<sup>S2P</sup>, but not the RBD. Nb3 binding to Spike<sup>S2P</sup> is partially decreased by ACE2-Fc. (C) SPR of Nb6 and Nb3 binding to either Spike<sup>S2P</sup> 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 Spike<sup>S2P</sup> show that Class I and Class II nanobodies can bind Spike<sup>S2P</sup> simultaneously. By contrast, two Class I nanobodies or Class II nanobodies do not bind simultaneously. (E) Nanobody inhibition of 1 nM Spike<sup>S2P</sup>-Alexa 647 binding to ACE2 expressing HEK293T cells.  $n = 3$  (ACE2, Nb3) or 5 (Nb6, Nb11) biological replicates. All error bars represent s.e.m.

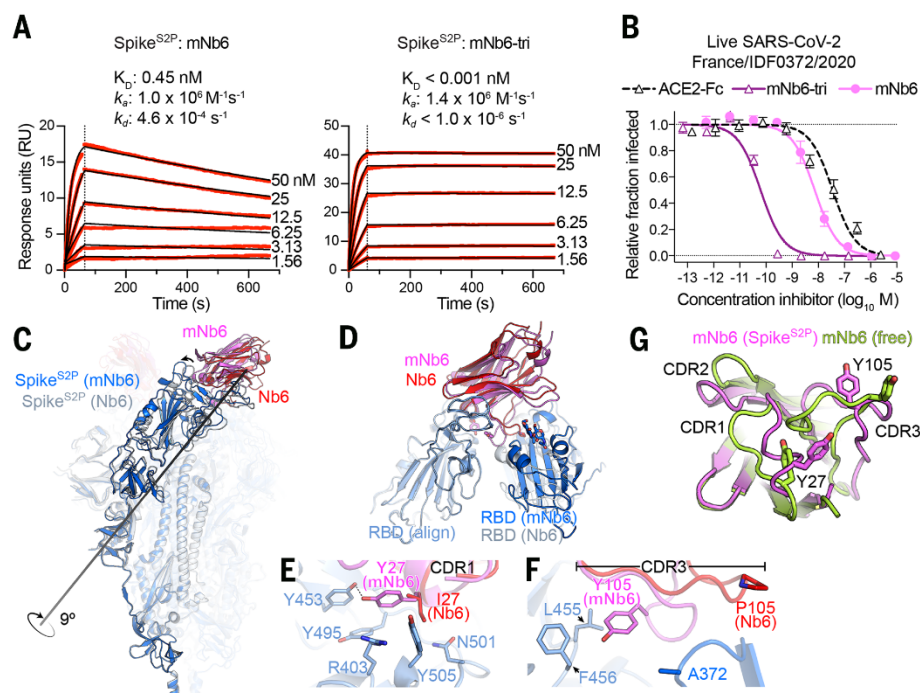




**Fig. 2. Cryo-EM structures of Nb6 and Nb11 bound to Spike.** (A) Cryo-EM maps of Spike<sup>S2P</sup>-Nb6 complex in either closed (left) or open (right) Spike<sup>S2P</sup> conformation. (B) Cryo-EM maps of Spike<sup>S2P</sup>-Nb11 complex in either closed (left) or open (right) Spike<sup>S2P</sup> conformation. The top views show receptor binding domain (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 Nb6 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.

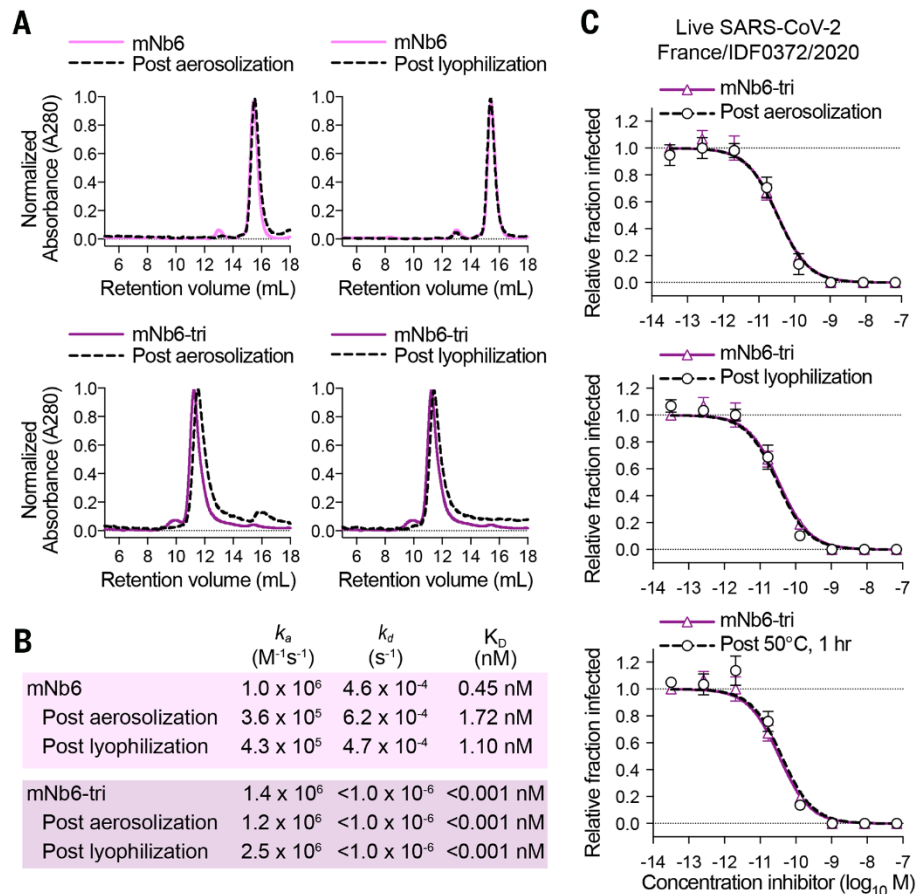


**Fig. 3. Multivalency improves nanobody affinity and inhibitory efficacy.** (A) SPR of Nb6 and multivalent variants. Red traces show raw data and black lines show global kinetic fit for Nb6 and independent fits for association and dissociation phases for Nb6-bi and Nb6-tri. (B) Dissociation phase SPR traces for Nb6-tri after variable association time ranging from 4 to 520 s. Curves were normalized to maximal signal at the beginning of the dissociation phase. Percent fast phase 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 replicate with  $n = 3$  (right panel) or 4 (left panel) technical replicates per concentration.  $n = 3$  biological replicates for all but Nb3 and Nb3-tri ( $n = 2$ ). All error bars represent s.e.m.



**Fig. 4. Affinity maturation of Nb6 yields a picomolar SARS-CoV-2 neutralizing molecule.** (A) SPR of mNb6 and mNb6-tri binding to immobilized Spike<sup>S2P</sup>. 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 s.e.m. (C) Comparison of closed Spike<sup>S2P</sup> bound to mNb6 and Nb6. Rotational axis for RBD movement is highlighted. (D) Comparison of receptor binding domain (RBD) engagement by Nb6 and mNb6. One RBD was used to align both structures (RBD align), demonstrating changes in Nb6 and mNb6 position 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 pi-pi and pi-cation interactions with the RBD. (F) CDR3 of Nb6 and mNb6 binding to the RBD demonstrating a large conformational rearrangement of the entire loop in mNb6. (G) Comparison of mNb6 complementarity determining regions in either the cryo-EM structure of the Spike<sup>S2P</sup>-mNb6 complex or an X-ray crystal structure of mNb6 alone.





**Fig. 5. mNb6 and mNb6-tri retain activity after aerosolization, lyophilization, and heat treatment.** (A) Size exclusion chromatography of nanobodies after lyophilization or aerosolization. (B) Summary table of SPR kinetics data and affinities for aerosolized or lyophilized mNb6 and mNb6-tri. (C) Inhibition of SARS-CoV-2 infection of VeroE6 cells by mNb6-tri after aerosolization, lyophilization, or heat treatment at 50°C for 1 hour. Representative biological replicate with  $n = 2$ . Technical replicates  $n = 3$  per concentration.

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

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