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Versatile and multivalent nanobodies efficiently neutralize SARS-CoV-2

Yufei Xiang¹, Sham Nambulli^{2,3*}, Zhengyun Xiao^{1*}, Heng Liu^{4*}, Zhe Sang^{1,5}, W. Paul Duprex^{2,3},
Dina Schneidman-Duhovny^{6†}, Cheng Zhang^{4†}, Yi Shi^{1,5†}

¹Department of Cell Biology, University of Pittsburgh, Pittsburgh, PA, USA. ²Center for Vaccine Research, University of Pittsburgh, Pittsburgh, PA, USA. ³Department of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA, USA. ⁴Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA, USA. ⁵Pitt/CMU Program for Computational Biology, Pittsburgh, PA, USA. ⁶School of Computer Science and Engineering, Institute of Life Sciences, The Hebrew University of Jerusalem, Israel.

*These authors contributed equally to this work.

†Corresponding author. Email: dina.schneidman@mail.huji.ac.il (D.S.-D.); chengzh@pitt.edu (C.Z.); yi.shi@pitt.edu (Y.S.)

Cost-effective, efficacious therapeutics are urgently needed against the COVID-19 pandemic. Here, we used camelid immunization and proteomics to identify a large repertoire of highly potent neutralizing nanobodies (Nbs) to the SARS-CoV-2 spike (S) protein receptor-binding domain (RBD). We discovered Nbs with picomolar to femtomolar affinities that inhibit viral infection at sub-ng/ml concentration and determined a structure of one of the most potent in complex with RBD. Structural proteomics and integrative modeling revealed multiple distinct and non-overlapping epitopes and indicated an array of potential neutralization mechanisms. We constructed multivalent Nb constructs that achieved ultrahigh neutralization potency (IC50s 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.

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 1,000,000 lives, with the numbers still rising as of September 2020. Despite preventive measures, such as quarantines and lock-downs that help curb viral transmission, the virus rebounds after lifting social restrictions. Safe and effective therapeutics and vaccines remain in dire need.

Like other zoonotic coronaviruses, SARS-CoV-2 expresses a surface spike (S) glycoprotein, which consists of S1 and S2 subunits forming 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 (PD) of angiotensin-converting enzyme-2 (hACE2) as a host receptor (3). Structural studies have revealed different conformations of the spike (4, 5). In the pre-fusion stage, the RBD switches between a closed conformation, and an open conformation for hACE2 interaction. In the post-fusion stage, the S2 undergoes a dramatic conformational change to trigger host membrane fusion (6). Investigations into COVID-19 convalescence individuals' sera have led to the identification of potent neutralizing antibodies (NAbs) primarily targeting the RBD but also non-RBD epitopes (7–13). High-quality NAbs may overcome the risks of Fc-associated antibody-dependent enhancement (ADE) and are promising therapeutic candidates (14, 15).

V_HH antibodies or nanobodies (Nbs) are minimal, monomeric antigen-binding domains derived from camelid single-chain antibodies (16). Unlike IgG antibodies, Nbs are small (~15 kDa), highly soluble and stable, readily bioengineered into bi/multivalent forms, and are amenable to low-cost, efficient microbial production. Due to their robust physicochemical properties, Nbs can be administered by inhalation, making their use against the respiratory viruses very appealing (17, 18). Recently, several SARS-CoV-2 neutralizing Nbs have been identified, by screening SARS-CoV or Middle East respiratory syndrome (MERS) cross-reacting Nbs or using synthetic Nb libraries for RBD binding. However, these synthetic Nbs generally neutralize the virus at µg to sub-µg/ml concentration (12, 19–22), which are hundreds of times less potent than the best NAbs, likely due to monovalency and lack of affinity maturation (23, 24). The development of highly potent anti-SARS-CoV-2 Nbs may provide a novel means for versatile, cost-effective therapeutics and point-of-care diagnosis.

To produce high-quality SARS-CoV-2 neutralizing Nbs, we immunized a llama with the recombinant RBD. Compared to the pre-bleed, the post-immunized serum showed potent and specific serologic activities toward RBD binding with a titer of 1.75×10^6 (fig. S1A). The serum efficiently neutralized the pseudotyped SARS-CoV-2 at the half-maximal neutralization titer (NT50) of ~310,000 (fig. S1B), orders of magnitude

higher than the convalescent sera obtained from recovered COVID-19 patients (7, 8). To further characterize these activities, we separated the single-chain V_HH antibodies from the IgGs. We confirmed that the single-chain antibodies achieve specific, high-affinity binding to the RBD and possess sub-nM half-maximal inhibitory concentration (IC₅₀ = 509 pM) against the pseudotyped virus (fig. S1C).

We identified thousands of high-affinity V_HH Nbs from the RBD-immunized llama serum using a robust proteomic strategy that we have recently developed (25) (fig. S2A). This repertoire includes ~350 unique CDR3s (complementarity-determining regions). For *E. coli* expression, we selected 109 highly diverse Nb sequences from the repertoire with unique CDR3s to cover various biophysical, structural, and potentially different antiviral properties. 94 Nbs were purified and tested for RBD binding by 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 IC₅₀ below 30 nM, Fig. 1A), and were promising candidates for functional characterizations. We used a SARS-CoV-2-GFP pseudovirus neutralization assay to screen and characterize the antiviral activities of these high-affinity Nbs. 94% of the tested Nbs neutralize the pseudotype virus below 3 μ M (Fig. 1B), with 90% of them below 500 nM. Only 20-40% of high-affinity RBD-specific mAbs identified from patient sera have been reported to possess comparable potency (7, 8). Over 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 most potent 18 based on the pseudovirus GFP reporter screen and measured their potency accurately using the pseudovirus-luciferase reporter assay. Finally, we tested the potential of 14 to neutralize the SARS-CoV-2 Munich strain using the PRNT50 assay (26). All the Nbs reached 100% neutralization and neutralized the virus in a dose-dependent manner. The IC₅₀s span from single-digit ng/ml to sub-ng/ml, of the 3 most potent Nbs 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 nM, 0.048 nM, and 0.022 nM, in the SARS-CoV-2 assay (Fig. 1, D and E). Overall, there was an excellent 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 an affinity of 108 pM and 10.4 pM, and the most potent Nb21 did not show detectable dissociation from the RBD during 20 min SPR analysis. The sub-picomolar affinity of Nb21 potentially explains its unusual neutralization potency (Fig. 1F). We determined the thermostability of Nbs 89, 20, and 21 from the *E. coli* periplasmic preparations 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 excellent physicochemical properties for advanced therapeutic applications.

We employed an integrative approach by SEC, cross-linking/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 as Nb21 and those that bind to non-overlapping epitopes. Nbs 9, 16, 17, 20, 64, 82, 89, 99, and 107 competed with Nb21 for RBD binding based on SEC profiles (Fig. 2A and fig. S5), indicating that their epitopes significantly overlap. In 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 Nb34 and Nb95, which did not compete for RBD interaction, suggesting the presence of two distinct and non-overlapping epitopes. Second, we cross-linked Nb-RBD complexes by DSS (disuccinimidyl suberate) and identified on average, four intermolecular cross-links by MS for Nbs 20, 93, 34, 95, and 105. The cross-links were used to map the RBD epitopes derived from the SEC data (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 co-localized with the non-conserved hACE2 binding site. Both epitopes I and II Nbs can compete with hACE2 binding to RBD at very low concentrations in vitro (fig. S7A). Epitopes III-V co-localized with conserved sites (fig. S7, B and C). Interestingly, epitope I Nbs had significantly shorter CDR3 (four amino acids shorter, $p = 0.005$) than other epitope binders (fig. S6I). Despite this, the vast majority of the selected Nbs potently inhibited the virus with an IC₅₀ 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 (Methods, table S3, and fig. S13). Most of the residues in RBD (N334-G526) and the entire Nb20, particularly those at the protein interaction interface, are well resolved. There are two copies of RBD-Nb20 complexes in one asymmetric unit, which are almost identical with an RMSD of 0.277 Å over 287 Ca 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) (31). E484 of RBD forms hydrogen bonding and ionic interactions with the side chains of R31 (CDR1) and Y104 (CDR3) of Nb20, while Q493 of RBD forms hydrogen bonds with the main chain carbonyl of A29 (CDR1) and the side chain of R97 (CDR3) of Nb20. These interactions constitute a major polar interaction network at the RBD and Nb20 interface. R31 of Nb20 also engages in a cation- π interaction with the side chain of F490 of the RBD (Fig. 3B). In addition, M55 from the CDR2 of Nb20 packs against residues L452, F490, and L492 of RBD to form hydrophobic interactions at the interface. Another small patch of hydrophobic interactions is formed among residues V483 of RBD and F45 and L59 from the framework β -sheet of Nb20 (Fig. 3C).

The binding mode of Nb20 to RBD is distinct from other reported neutralizing Nbs, which generally recognize similar epitopes in the RBD external loop region (32–34) (fig. S8). The extensive hydrophobic and polar interactions (Fig. 3, B and C) between RBD and Nb20 stem from the remarkable shape complementarity (Fig. 3D) between the CDRs and the external RBD loop, leading to ultrahigh-affinity (~ 10 pM). We further modeled the structure of the best neutralizer Nb21 with RBD based on our crystal structure (Methods). Only four residues vary between Nb20 and Nb21 (fig. S9A), all of which are on CDRs. Two substitutions are at the RBD binding interface. S52 and M55 in the CDR2 of Nb20 are replaced by two asparagine residues N52 and N55 in Nb21. In our superimposed structure, N52 forms a new H-bond with N450 of RBD (fig. S9B). While N55 does not engage in additional interactions with RBD, it creates a salt bridge with the side chain of R31, which stabilizes the polar interaction network among R31 and Y104 of Nb21 and Q484 of 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-Nb20/21 and RBD-hACE2 (PDB 6LZG) (31) clearly showed that the interfaces for Nb20/21 and hACE2 partially overlap (Fig. 3D and fig. S9C). Notably, the CDR1 and CDR3 of Nb20/21 would clash with the first helix of hACE2, the primary binding site for RBD (fig. S9D).

To understand the antiviral efficacy of our Nbs, we superimposed RBD-Nb complexes to different spike conformations based on cryoEM structures. We found that three copies of Nb20/21 can simultaneously bind all three RBDs in their “down” conformations (PDB 6VXX) (4) that correspond to the inactive spike (Fig. 4B). Our analysis indicates a potential mechanism by which Nbs 20 and 21 (Epitope I) lock RBDs in their down conformation with ultrahigh affinity. Combined with the steric interference with hACE2 binding in the RBD open conformation (Fig. 4A), these mechanisms may explain the exceptional neutralization potencies of Epitope I Nbs.

Other epitope-binders do not fit into this inactive conformation without steric clashes and appear to use different neutralization strategies (Fig. 4C). For example, Epitope II:

Nb 93 co-localizes with the hACE2 binding site and can bind the spike in the one RBD “up” conformation (Fig. 4D, PDB 6VSB) (3). It may neutralize the virus by blocking the hACE2 binding site. Epitope III and IV Nbs can only bind when two or three RBDs are in their “up” conformations (PDB 6XCN) (24) where the epitopes are exposed. In the all RBDs “up” conformation, three copies of Nbs can directly interact with the trimeric spike. Interestingly, through RBD binding, Epitope III: Nb34 can be accommodated on top of the trimer to lock the helices of S2 in the prefusion stage, preventing their large conformational changes for membrane fusion (Fig. 4E). When superimposed onto the all “up” conformation, Epitope IV: Nb95 is proximal to the rigid NTD of the trimer, presumably restricting the flexibility of the spike domains (Fig. 4F).

Epitope mapping enabled us to bioengineer homo- and hetero-dimeric and homo-trimeric Nbs. Homodimers/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/trimers used flexible linker sequences of 25 (GS) or 31 (EK) amino acids (Methods). The heterodimers used flexible linkers of 12 amino acids.

We found up to ~ 30 fold improvement for the homotrimeric constructs of Nb21₃ (IC₅₀ = 1.3 pM) and Nb20₃ (IC₅₀ = 4.1 pM) compared to the respective monomeric form by the pseudovirus luciferase assay (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 these values indicate, as the measured values may reflect the assay’s lower detection limits. For the heterodimeric constructs, up to a 4-fold increase of potency (i.e., Nb21-Nb34) was observed. The multivalent constructs retained similar physicochemical properties to the monomeric Nbs, including high solubility, yield, thermostability, and remained intact (non-proteolyzed) under the neutralization assay condition (fig. S10). They remained highly potent for pseudovirus neutralization after lyophilization and aerosolization (Methods and fig. S11, B to G), indicating the outstanding stability and potential flexibility of administration. The majority of the RBD mutations observed in GISAID (35) are very low in frequency (<0.0025) which may increase under Nb selection. Therefore, a cocktail consisting of ultrapotent, multivalent constructs that bind simultaneously a variety of epitopes with potentially different neutralization mechanisms will likely efficiently block virus mutational escape (Fig. 5E and fig. S12) (9, 36–38).

Here, in vivo antibody affinity maturation followed by advanced proteomics (25) enabled the rapid discovery of a diverse repertoire of high-affinity RBD Nbs, including an ultrapotent neutralizer with sub-picomolar affinity, which is

unprecedented for natural, single-domain antibodies. We demonstrated the simplicity and versatility of Nb bioengineering and the outstanding 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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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S13

Tables S1 to S4

References (41–60)

MDAR Reproducibility Checklist

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Fig. 1. Production and characterizations of high-affinity RBD Nbs for SARS-CoV-2 neutralization. (A) The binding affinities of 71 Nbs toward RBD by ELISA. The pie chart shows the number of Nbs according to affinity and solubility. (B) Screening of 49 high-affinity Nbs with high-expression level by SARS-CoV-2-GFP pseudovirus neutralization assay. $n = 1$ for Nbs with neutralization potency $IC_{50} \leq 50$ nM, $n = 2$ for Nbs with neutralization potency $IC_{50} > 50$ nM. (C) The neutralization potency of 18 highly potent Nbs was calculated based on the pseudotyped SARS-CoV-2 neutralization assay (luciferase). Purple, red, and yellow lines denote Nbs 20, 21, and 89 with $IC_{50} < 0.2$ nM. Two different purifications of the pseudovirus were used. The average neutralization percentage was shown for each data point ($n = 5$ for Nbs 20, 21; $n = 2$ for all other Nbs). (D) The neutralization potency of 14 neutralizing Nbs by SARS-CoV-2 plaque reduction neutralization test (PRNT). The average neutralization percentage was shown for each data point ($n = 4$ for Nbs 20, 21, and 89; $n = 2$ for other Nbs). (E) A table summary of pseudovirus and SARS-CoV-2 neutralization potencies of 18 Nbs. N/A: not tested. (F) The SPR binding kinetics measurement of Nb21.

Fig. 2. Nb epitope mapping by integrative structural proteomics. (A) A summary of Nb epitopes based on size exclusion chromatography (SEC) analysis. Light salmon color: Nbs that bind the same RBD epitope. Sea green: Nbs of different epitopes. (B) A representation of SEC profiling of RBD, RBD-Nb21 complex, and RBD-Nb21-Nb105 complex. The y-axis represents UV 280 nm absorbance units (mAu). (C) A cartoon model showing the localization of five Nbs that bind different epitopes: Nb20 (medium purple), Nb34 (light sea green), Nb93 (salmon), Nb105 (pale goldenrod), and Nb95 (light pink) in complex with the RBD (gray). The Blue and red lines represent DSS cross-links shorter or longer than 28 Å, respectively. (D) Top 10 scoring cross-linking based models for each Nb (cartoons) on top of the RBD surface.

Fig. 3. Crystal structure analysis of an ultrahigh affinity Nb in complex with the RBD. (A) Cartoon presentation of Nb20 in complex with the RBD. CDR1, 2, and 3 are in red, green, and orange, respectively. (B) Zoomed-in view of an extensive polar interaction network that centers on R35 of Nb20. (C) Zoomed-in view of hydrophobic interactions. (D) Surface presentation of the Nb20-RBD and hACE2-RBD complex (PDB: 6M0J).

Fig. 4. Potential mechanisms of SARS-CoV-2 neutralization by Nbs. (A) hACE2 (blue) binding to spike trimer conformation (wheat, beige, and gray colors) with one RBD up (PDBs 6VSB, 6LZG). (B) Nb20 (Epitope I, medium purple) partially overlaps with the hACE2 binding site and can bind the closed spike conformation with all RBDs down (PDB 6VXX). (C) A summary of spike conformations accessible (+) to the Nbs of different epitopes. (D) Nb93 (Epitope II, salmon) partially overlaps with the hACE2 binding site and can bind to spike conformations with at least one RBD up (PDB 6VSB). (E and F) Nb34 (Epitope III, light sea blue) and Nb95 (Epitope IV, light pink) do not overlap with the hACE2 binding site and bind to spike conformations with at least two open RBDs (PDB 6XC�).

Fig. 5. Development of multivalent Nb cocktails for highly efficient SARS-CoV-2 neutralization. (A) Pseudotyped SARS-CoV-2 neutralization assay of multivalent Nbs. The average neutralization percentage of each data point was shown ($n = 2$). ANTE-CoV2-Nab20T_{GS}/E_K: homo-trimeric Nb20 with the GS/E_K linker; ANTE-CoV2-Nab21T_{GS}/E_K: homo-trimeric Nb21 with the GS/E_K linker. (B) SARS-CoV-2 PRNT of monomeric and trimeric forms of Nbs 20 and 21. The average neutralization percentage of each data point was shown ($n = 2$ for the trimers, $n = 4$ for the monomers). (C) A summary table of the neutralization potency measurements of the multivalent Nbs. N/A: not tested. (D) Mapping mutations to localization of Nb epitopes on the RBD. The x-axis corresponds to the RBD residue numbers (333 to 533). Rows in different colors represent different epitope residues. Epitope I: 351, 449-450, 452-453, 455-456, 470, 472, 483-486, 488-496; Epitope II: 403, 405-406, 408,409, 413-417, 419-421, 424, 427, 455-461, 473-478, 487, 489, 505; Epitope III: 53, 355, 379-383, 392-393, 396,412-413, 424-431, 460-466, 514-520; Epitope IV: 333-349, 351-359, 361, 394, 396-399, 464-466, 468, 510-511, 516; Epitope V: 353, 355-383, 387, 392-394, 396, 420, 426-431, 457,459-468, 514, 520.