Figure S1: ELISA curves of Nbs for RBD mutant binding (related to Figure 1).
Figure S2: Structure representations of SARS-CoV-2 spike trimer glycoprotein and mutations for two prevalent circulating strains (related to Figures 1 and 5).
Figure S3: Pseudovirus assay results for individual Nbs (related to Figure 1).
Figure S4

A

~900k particles were auto-picked from 2574 micrographs

B

C

D

E

F

~135k particles

3D classification

C1 symmetry imposed

3D refinement

Focused refinement

Resolution Å

Fourier Shell Correlation

RBD

Nb21

1-up-2-down RBDs

2-up-1-down RBDs

Up RBD

Down RBD

Up RBD

Down RBD

1-up-2-down RBDs

2-up-1-down RBDs

CDR2

N55

M55

N52

S52

T470

Y449

V24

A27

V32

CDR3

Y106

Q1

CDR1

T105

I105

L77

A27

T105

I105

L77

A27

T105

I105

L77

A27

T105

I105

L77

A27
Figure S4. Cryo-EM Structure determination of S with Nb21, local refinement of RBD with Nb21 and comparison of Nb21 with Nb20 (related to Figure 2).

(A) Cryo-EM Data Processing workflow showing the strategies and particle cohort sizes used to generate the maps discussed in this work. ~900K particles were picked based on the 2D class averages of S with Nb21 for 3D classification. Two major classes with the largest proportions were further refined. One class refined to 3.6 Å corresponds to S with 1-up-2-down RBDs and the other class refined to 3.9 Å corresponds to S with 2-up-1-down RBDs. S is colored in dark gray. Nb21 is colored in blue.

(B) Fourier Shell Correlation and local resolution estimations for S and NB21 complexes. The red line represents FSC = 0.143.

(C) Focused Refinement of one down RBD with Nb21.

(D-F) Structural comparison of RBD with Nb21 and with Nb20. Nb21 is colored blue while Nb20 is colored yellow. RBD is colored dark gray and cyan in the structures with Nb21 and Nb20, respectively. Nb21 differs from Nb20 by four residues (all on CDRs). Its RBD binding is very similar to that of Nb20. The two structures can be well aligned with a root mean square deviation (RMSD) of 1.8 Å (all atoms) (Figure S4D). Here, S52 and M55 on CDR2 in Nb20, are replaced by N52 and N55 in Nb21, which form additional polar interactions with the RBD (Figure S4E). A27 (CDR1) and L105 (CDR3) of Nb20 are replaced by L27 and T105 in Nb21 (Figure S4F). While the two residues do not bind RBD directly, the side chain of L27 is buried inside Nb21 to form additional hydrophobic interactions with V24, V32, and I77. The small short side chain of T105 allows the neighboring residue Y106 to point towards the first N-terminal residue Q1 to form a hydrogen bond. This interaction, which is missing in the structure of Nb20:RBD as it is impeded by the presence of the large side chain of I105 in the analogous position of Nb20 I105. These additional interactions may help stabilize CDR1 and CDR3 loops to strengthen Nb21:RBD interactions.
Figure S5. Assessment of the RBD:Nb21 interactions using both computational binding energy calculation and experimental mutagenesis (related to Figure 2).

(A) Decomposition of relative binding free energy contribution from individual residues of RBD (top, gray) and Nb21 (bottom, blue) for these more than -1 kcal/mol.

(B) ELISA assay showing Nb21 point mutant R31D fails to bind RBD.
Figure S6

(A) ~880K particles were picked based on the 2D class averages of S with Nb95 for 3D classification. Two major classes with the largest proportions were further refined. One class refined to 3.8 Å corresponds to S with 2-up-1-down RBDs and the other class refined to 3.7 Å corresponds to S with 3-up RBDs. S is colored in dark gray. Nb95 is colored in teal. FSC estimations for these two complexes are shown at the lower-left corner. The red line represents FSC=0.143.

(B) Local resolution distribution for the two S and Nb95 complexes.

(C) Focused Refinement of one down RBD with NB95. The down RBD showed better density compared to up RBDs.

Figure S6. Cryo-EM Structure determination of S with Nb95 and focused refinement (related to Figure 3).

(A) ~880K particles were picked based on the 2D class averages of S with Nb95 for 3D classification. Two major classes with the largest proportions were further refined. One class refined to 3.8 Å corresponds to S with 2-up-1-down RBDs and the other class refined to 3.7 Å corresponds to S with 3-up RBDs. S is colored in dark gray. Nb95 is colored in teal. FSC estimations for these two complexes are shown at the lower-left corner. The red line represents FSC=0.143.

(B) Local resolution distribution for the two S and Nb95 complexes.

(C) Focused Refinement of one down RBD with NB95. The down RBD showed better density compared to up RBDs.
Figure S7

(A) ~756k particles were auto-picked from 2405 micrographs. 182332 particles were selected after 2D classification. Two major classes were observed with clear features of 3-up RBDs and 2-up-1-down RBDs. We focused on the 2-up-1-down class for 3D refinement and obtained a structure with a global resolution of 3.5 Å corresponding to 0.143FSC shown at the lower-left panel.

(B) Local resolution distribution for the S and Nb34 complex. Focused Refinement of one down RBD with NB34. The down RBD showed better density compared to up RBDs.

Figure S7. Cryo-EM Structure determination of S with Nb34 and focused refinement (related to Figure 3).

(A) ~756K particles were picked based on the 2D class averages of S with Nb34 for 3D classification. Two major classes were observed with clear features of 3-up RBDs and 2-up-1-down RBDs. We focused on the 2-up-1-down class for 3D refinement and obtained a structure with a global resolution of 3.5 Å corresponding to 0.143FSC shown at the lower-left panel.

(B) Local resolution distribution for the S and Nb34 complex.

(C) Focused Refinement of one down RBD with NB34. The down RBD showed better density compared to up RBDs.
Figure S8

(A) Representative micrograph and 2D class averages of Nb105:S complex.
(B) Gold-standard Fourier shell correlation (FSC) and Euler angular distribution.
(C) Representative micrograph and 2D class averages of Nb105:RBD:Nb21 complex.
(D) Gold-standard Fourier shell correlation (FSC) and Euler angular distribution.
(E) Local resolution estimation for Nb105:RBD:Nb21 complex.
(F) Rigid docking of Nb105:RBD complex to the interface of the dimeric S. The interface highlighted with the green line is between the Nb framework and RBS.

Figure S8. Cryo-EM analysis of Nb105:S and Nb105:RBD: Nb21 complexes (related to Figure 3).

(A) Representative micrograph and 2D class averages of Nb105:S complex.
(B) Gold-standard Fourier shell correlation (FSC) and Euler angular distribution.
(C) Representative micrograph and 2D class averages of Nb105:RBD:Nb21 complex.
(D) Gold-standard Fourier shell correlation (FSC) and Euler angular distribution.
(E) Local resolution estimation for Nb105:RBD:Nb21 complex.
(F) Rigid docking of Nb105:RBD complex to the interface of the dimeric S. The interface highlighted with the green line is between the Nb framework and RBS.
Figure S9. Cryo-EM analysis of Nb17:S and Nb17:RBD:Nb105 complexes (related to Figure 4).
(A) Representative micrograph and 2D class averages of Nb17:S complex.
(B) Gold-standard Fourier shell correlation (FSC) and Euler angular distribution.
(C) Local resolution estimation for Nb17:S complex.
(D) Focused classification of the flexible region in Nb17:S complex. The density of Nb17 in class 1 (cyan) is smeared due to motion along the y-direction, class 2 (magenta) has well resolved RBD, Nb17, and NTD density, and both densities of RBD and Nb17 is lost due to motion along the x-direction.
(E) Representative micrograph and 2D class averages of Nb17:RBD: Nb105 sample.
(F) Local resolution estimation for Nb105:RBD: Nb21 sample.
(G) Interface residues of Nb17:RBD complex.
(H) Alignment of Nb17:RBD to Nb21:RBD showing the large overlap between Nb17 CDR3 with Nb21 CDR2 and partially Nb21 CDR1.
Figure S10. EM Analysis of Nb36 with S and RBD (related to Figure 4).
(A) Representative negative stain EM micrographs of spike protein in the presence of an increased concentration of Nb36. An example of an intact trimeric spike particle is highlighted by a blue arrow, and an example of a disrupted spike particle is highlighted by a red arrow.

(B) Thermal melting profile of S protein in the presence of an increased concentration of Nb36.

(C) Representative micrograph and 2D class averages of Nb36:RBD: Nb21 complex.

(D) Gold-standard Fourier shell correlation (FSC) and Euler angular distribution.

(E) Local resolution estimation for Nb36:RBD: Nb21 complex.

Figure S11

Figure S11. Analysis of the interactions between the RBD residues of I468 and T470 and highly potent neutralizing Nbs from class III (related to Figure 4).
Figure S12. Comparison of neutralizing Nbs and mAbs for RBD binding (related to Figure 7).

(A) The heatmap shows the binding difference between Nbs and Fabs in terms of paratope residue utility despite overall similar epitope regions.

(B) Heatmaps show the difference in preference of epitope-paratope residues between Nbs and Fabs. The comparisons were made separately for RBS binders and non-RBS binders. Nbs with at least 30% overlapping residues with ACE2 binding sites were considered RBS binders.

(C) Illustrations of dominated electrostatic interactions formed between arginine from Nb CDRs and RBD residues. RBD was colored in dark gray, Nbs were colored in khaki, E484 (RBD) was colored in red, F490 (RBD) was colored in teal and R (Nb CDRs) was colored in blue.
Figure S13. Analysis of interactions of E484 (RBD) with neutralizing Nbs and mAbs (related to Figure 7).

Superposition of Fab-RBD structures showing E484 (RBD) forms hydrogen and/or hydrophobic interactions with the respective residues of Fabs. The side chains of residues tyrosine, serine, threonine and arginine in close contact E484 are shown in stick representation. RBD: dark gray, Fab VH: light blue, Fab LH: light green, and residue E484 (RBD): red.
Tables S1-4:

Table S1. Summary of all RBD mutants, related to Figure 1.

Table S2. Statistics for 3D reconstruction and model refinement for Nb:S complexes, related to Figures 2, 3 and 4.

Table S3. Statistics for 3D reconstruction and model refinement for 2Nbs:RBD complexes, related to Figures 3 and 4.

Table S4. Summary of structural comparisons between mAbs and Nbs, related to Figure 6 and 7.