

1 **Broadly neutralizing and protective nanobodies against diverse sarbecoviruses**

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35 **Abstract**

36 As SARS-CoV-2 Omicron and other variants of concern continue spreading around the world,
37 development of antibodies and vaccines to confer broad and protective activity is a global
38 priority. Here, we report on the identification of a special group of nanobodies from immunized
39 alpaca with exceptional breadth and potency against diverse sarbecoviruses including SARS-
40 CoV-1, Omicron BA.1, and BA.2. Crystal structure analysis of one representative nanobody,
41 3-2A2-4, revealed a highly conserved epitope between the cryptic and the outer face of the
42 receptor binding domain (RBD). The epitope is readily accessible regardless of RBD in “up”
43 or “down” conformation and distinctive from the receptor ACE2 binding site. Passive delivery
44 of 3-2A2-4 protected K18-hACE2 mice from infection of authentic SARS-CoV-2 Delta and
45 Omicron. This group of nanobodies and the epitope identified should provide invaluable
46 reference for the development of next generation antibody therapies and vaccines against wide
47 varieties of SARS-CoV-2 infection and beyond.

48 **Keywords:** SARS-CoV-2, nanobody, cross-reactive, sarbecoviruses, conserved epitope,
49 animal model.

50

51 **INTRODUCTION**

52 As the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to rage
53 around the world, we have witnessed the rapid emergence and turnover of multiple variants of
54 concerns (VOCs) such as Alpha (B.1.1.7) initially identified in the United Kingdom; Beta
55 (B.1.351) in South Africa; Gamma (P.1) in Brazil; Delta (B.1.617.2) in India; and Omicron
56 (BA.1 and BA.2) in Botswana and South Africa ([https://www.who.int/en/activities/tracking-](https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/)
57 [SARS-CoV-2-variants/](https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/)). These VOCs are not only associated with steeply increased new
58 infections among unvaccinated but also break-through infections among the vaccinated
59 individuals¹⁻⁴. Increasing evidence suggests that substantial changes in their antigenic

60 properties have facilitated these VOCs to escape from serum neutralization of convalescent
61 and vaccinated individuals⁵⁻⁹. As a result, efficacies of all vaccine modalities as well as many
62 therapeutic antibodies approved for emergency use authorization (EUA) have been severely
63 compromised, particularly toward Omicron, followed by Beta, Delta, Gamma, and Alpha^{6,8,10-}
64 ¹⁴. Among these VOCs, Omicron is perhaps the most insidious as it generally causes milder
65 symptoms among healthy and vaccinated individuals but displays exceptionally high viral load
66 in the upper respiratory tract and extremely efficient in transmission¹⁵⁻¹⁷. As quiet as it seems,
67 Omicron has been actively replacing Delta and other local variants to become the most
68 dominant variant in many parts of the world¹⁸. Development of broader and more effective
69 therapies and vaccines against Omicron and future variants has therefore become an urgent and
70 global priority.

71 One striking aspect of Omicron is the largest number of mutations found in the spike (S)
72 protein among the VOCs identified thus far (<https://www.gisaid.org>). It is currently unknown
73 how Omicron accumulated such high number of mutations in such a short period, although
74 some speculated that it could be derived from immune-compromised individuals or straight
75 across from other animal species. BA.1 and BA.2 are the two major subvariants of Omicron
76 that are rapidly spreading and accounting for majority of the new and break-through infections
77 worldwide. The S protein of SARS-CoV-2 is the major target for neutralizing antibodies and
78 has been engineered and used in many of the vaccine modalities¹⁹⁻²¹. In the Omicron S protein,
79 there are approximately 35 substitutions compared to the prototype strain found in Wuhan,
80 China. Of which, at least 15 are located in the receptor-binding domain (RBD) and at least 8
81 in the N-terminal domain (NTD), although the exact number of substitutions vary among
82 various subvariant within Omicron (<https://www.gisaid.org>). Recently, several elegant studies
83 have pinpointed a few key substitutions in the S protein that are responsible for neutralization
84 escape, and many of which are shared among the VOCs. For example, the N501Y substitution

85 previously shown to enhance binding affinity to the receptor angiotensin-converting enzyme 2
86 (ACE2) is found in Alpha, Beta, Gamma, and Omicron ²². Delta and other variants such as
87 Epsilon, Lambda, and Kappa have substitutions L452R or L452Q within the RBD that also
88 facilitate virus escape ^{23,24}. Beta, Gamma, and Omicron each have three substitution sites in
89 common within the RBD, namely K417N/T, E484K/A, and N501Y, which resulted in marked
90 reduction or complete loss of neutralizing activities of many therapeutic antibodies and
91 immune serum from vaccinated individuals ^{5,6,25,26}. In the NTD, Alpha, Beta, Gamma, and
92 Omicron share deletions/insertions and substitutions within or near the “NTD supersite” that
93 largely consisted of the N1 (residues 14–26), N3 (residues 141–156), and N5 (residues 246–
94 260) loops ²⁷⁻³⁰. While these findings have clearly identified several critical substitutions that
95 confer viral escape from antibody neutralization, they also point to the critical substitutions
96 that must overcome in order to develop broad and protective antibody therapies and vaccines
97 against various VOCs including the most divergent Omicron BA.1 and BA.2.

98 We previously reported hundreds of spike-specific monoclonal antibodies (mAbs) from
99 SARS-CoV-2 convalescent individuals ³¹. While these mAbs are being screened for broad and
100 protective activity against Omicron and other VOCs, we decided to expand our search through
101 immunizing alpaca, as this animal species like those in the family of *Camelidae* has a small
102 and robust nanobody structure, allowing deeper and broader recognition of target antigens
103 thereby to improve the likelihood of success in finding the ideal type of antibodies mentioned
104 above. Structurally, nanobody is unique and composed of a single heavy chain with one
105 variable domain (VHH). The smaller size (<15 kDa) and longer VHH domain facilitate their
106 reach to targets that are otherwise inaccessible to conventional human antibodies ³². Their
107 special properties in specificity, stability, thermotolerance, low immunogenicity, and ease in
108 production in yeast and other cost-effective systems have made the nanobodies the ideal
109 candidate for therapeutic and prophylactic interventions against SARS-CoV-2 infection ³³⁻⁴⁶.

110 Through screening nanobody library from an immunized alpaca, we have identified a special
111 group of nanobodies with exceptional breadth and potency against diverse sarbecoviruses
112 including Omicron BA.1 and BA.2, SARS-CoV-1, as well as the key representatives from bats
113 and pangolins coronaviruses. The IC₅₀ reached as low as 0.042 µg/mL or 0.550 nM against
114 WT D614G and remained relatively stable across the entire panel of the tested viruses.
115 Although neutralization assays differ, this suggests they are amongst the most broad and potent
116 nanobodies described to date. Passive delivery of one of the representative nanobodies, 3-2A2-
117 4, protected K18-hACE2 mice from infection of authentic SARS-CoV-2 Delta and Omicron.
118 Structural analysis revealed 3-2A2-4 targeted a highly conserved epitope between the cryptic
119 and outer face of RBD, distinctive from the ACE2 binding site. This epitope is readily
120 accessible regardless of RBD in “up” or “down” conformations. These results clearly indicate
121 that we have identified a broadly neutralizing and protective nanobody that recognized a highly
122 conserved epitope among a diverse panel of sarbecoviruses. The nanobody and the epitope
123 identified should provide invaluable reference for the development of next generation antibody
124 therapies and vaccines against wide varieties of SARS-CoV-2 infection and beyond.

125

126 **RESULTS**

127 **Cross-neutralizing SARS-CoV-2 and SARS-CoV-1 nanobodies.** We started with selection
128 for cross-neutralizing SARS-CoV-2 and SARS-CoV-1 nanobodies, as these nanobodies would
129 be expected to have higher probability to cross-react with other members of sarbecoviruses. To
130 this end, we screened a yeast display VHH library constructed from the PBMCs of a serially
131 immunized alpaca with RBD and spike of prototype SARS-CoV-2 (see Materials and Methods).
132 Through iterative process of FACS-sorting, enriching, and finally expressing in the
133 recombinant form with human IgG1 Fc fragment, we identified a total of 593 nanobodies
134 capable of binding to the recombinant spike trimer of prototype SARS-CoV-2. Of which, 124

135 showed neutralizing activity to prototype SARS-CoV-2 pseudovirus with IC₅₀ ranging from
136 0.003 to 5.399 µg/mL or 0.039 to 71.039 nM (Figure 1). Among these, 91 demonstrated cross-
137 neutralizing activity to SARS-CoV-1, and all but two (No. 43 and 55) strongly bound to RBD
138 (Figure. 1). Phylogenetically, these cross-neutralizing nanobodies were segregated into four
139 major clusters (**a**, **b**, **c**, and **d**) (Figure 1, red in the left panel). Clusters **a** and **d** nanobodies had
140 equivalent average IC₅₀ to SARS-CoV-2 and SARS-CoV-1 (0.077 vs. 0.056 µg/mL or 1.013
141 vs. 0.737 nM) while clusters **b** and **c**, however, demonstrated stronger activity to SARS-CoV-
142 1 than to SARS-CoV-2 (0.036 vs. 0.499 µg/mL or 0.474 vs 6.566 nM). This suggests that the
143 epitopes recognized by clusters **b** and **c** nanobodies could be more exposed and/or easily
144 accessible on the SARS-CoV-1 spike.

145 Genetic analysis of the 91 cross-neutralizing nanobodies identified preferred usage of
146 several germline V-gene and J-gene, particularly for IGHV3S65 (39.6%), IGHV3-3 (37.4%),
147 IGHV3S53 (13.2%), and IGHJ4 (100%) (Figure S1a). Similar pattern of preference was also
148 noticed among the total of 124 isolated nanobodies (Figure S1b), and the 237 published
149 nanobodies in the CoV-AbDab database except for IGHV3S65 (Figure S1c). Such convergence
150 on germline gene usage may suggest that the combinations of the gene segments encode
151 nanobodies with unique structural and biochemical properties rendering them naturally
152 complementary in shape and strong in binding to the spike surface of SARS-CoV-2 and SARS-
153 CoV-1. Furthermore, the 91 cross-neutralizing nanobodies as well as the isolated 124
154 nanobodies were all dominated by 19-residue long CDR3 (Figure S1d and S1e) while those in
155 the CoV-AbDab by 17- or longer segments (Figure S1f). However, the CDR3 length per se
156 was unlikely a prerequisite for neutralization breadth across SARS-CoV-2 and SARS-CoV-1.
157 Nanobodies in clusters **a** and **d** had an average CDR3 length of 10 and 19 residues, respectively,
158 but demonstrated equivalent cross-neutralizing activity against SARS-CoV-2 and SARS-CoV-
159 1 (Figure 1). Interestingly, sequence logo plots identified 7 polar residues (GSYYYCS) in the

160 19-residue CDR3 that were rather prevalent among the 91 cross-neutralizing, the 124 isolated
161 nanobodies and the 237 published nanobodies in the CoV-AbDab database, although no
162 obvious patterns were found in 17- or 18-residue CDR3 sequences (Figure S1g, S1h, and S1i).

163

164 **Broadly neutralizing sarbecoviruses nanobodies.** To identify nanobodies that have broader
165 activity beyond prototype SARS-CoV-2 and SARS-CoV-1, we selected 32 cross-neutralizing
166 nanobodies based on their representation in neutralizing potency and locations on the
167 phylogenetic tree. Six SARS-CoV-2-specific neutralizing nanobodies were also selected and
168 used as controls. These selected nanobodies are indicated by the red triangles in Figure 1. We
169 first classified the 32 cross-neutralizing nanobodies into 3 groups based on their degree of
170 competition with ACE2 and VHH72, a previously published nanobody with known epitope on
171 the cryptic face of RBD that only became accessible when RBD in the “up” standing
172 conformation³⁵. As shown in Figure 2a, the Group 1 (G1) nanobodies strongly competed with
173 both ACE2 and VHH72 for binding to RBD, suggesting they also bound to the similar cryptic
174 epitopes on RBD from the angles that restricted ACE2 binding. The Group 2 (G2) nanobodies
175 moderately competed with ACE2 but strongly with VHH72, indicating similar cryptic epitopes
176 like those in G1 but less interfering with ACE2 binding. The Group 3 (G3) nanobodies,
177 however, moderately competed with ACE2 or VHH72, and their epitopes and binding poses
178 must deviate away from that of ACE2 and VHH72. The Group 4 (G4) control nanobodies
179 included the six SARS-CoV-2 only neutralizing nanobodies, and showed varying competition
180 activity with ACE2 and the weakest with VHH72 among all nanobodies studied here (Figure
181 2a). We next studied the neutralizing breadth of 38 nanobodies against a panel of 18
182 sarbecoviruses that dependent on ACE2 for entry, including 13 major SARS-CoV-2 variants,
183 SARS-CoV-1, and 4 representatives from bats and pangolins. These nanobodies demonstrated
184 diverse and distinctive neutralizing patterns, corresponding well to the 4 groups defined by the

185 competition assays (Figure 2a, Figures S2 and S3). The G1 nanobodies showed broad
186 neutralizing activity against all sarbecoviruses tested with average IC₅₀ ranging from 0.085
187 µg/mL or 1.114 nM against WT D614G. The IC₅₀s to Omicron BA.1 and BA.2 dropped below
188 the detection limit (BDL in dark red) against Omicron BA.1 and BA.2 while remained
189 relatively unchanged to other SARS-CoV-2 variants (in white), relative to WT D614G (Figure
190 2a, and Figure S2). Interestingly, many nanobodies showed improved neutralizing activity to
191 SARS-CoV-1, pangolin coronavirus GD, and bat coronavirus RaTG13 (in blue) while varied
192 considerably to pangolin coronavirus GX and bat coronavirus WIV16 (mixed with red and blue)
193 (Figure 2a, and Figure S3). The G2 nanobodies demonstrated relatively weaker neutralizing
194 activity against WT D614G than those in the G1 with average IC₅₀ ranging from 0.204 µg/mL
195 or 2.690 nM against WT D614G. However, they appeared to be less affected by BA.1. Nb70
196 could also neutralize BA.2 although with marked reduced activity. Subtle differences may exist
197 in the epitope specificity and binding pose between the G1 and G2 nanobodies. Like those in
198 the G1, the G2 nanobodies also demonstrated improved neutralizing activity to SARS-CoV-1,
199 and those representative coronaviruses from bats and pangolins (Figure 2a). The G3
200 nanobodies exhibited the broadest neutralizing activity against all sarbecoviruses tested with
201 average IC₅₀ ranging from 0.040 µg/mL or 0.530 nM against WT D614G, although partial
202 reduction was found to pangolin coronavirus GX. The G3 nanobodies therefore may recognize
203 conserved epitopes on RBD distinctive from the ACE2 and VHH72 binding sites. The G4
204 nanobodies, despite being the strongest against WT D614G with average IC₅₀ ranging from
205 0.014 µg/mL or 0.184 nM against WT D614G, had the poorest breadth against the 18
206 sarbecoviruses tested. Severe reduction or complete loss of neutralizing activities were found
207 in many nanobodies with only exception to SARS-CoV-2 variants (Alpha, Epsilon, and A23.1)
208 and pangolin coronavirus GD. As only minor variation existed among these viruses in the
209 receptor-binding motif (RBM) (<https://www.gisaid.org>), it was likely that the G4 nanobodies

210 targeted sites on RBD that either overlapped with or proximate to RBM, consistent with their
211 noticeable competition with ACE2 for binding to RBD (Figure 2a).

212 We then selected one nanobody each from G1 (1-2C7), G2 (Nb70), and G3 (3-2A2-4) to
213 test their neutralizing activity against authentic SARS-CoV-2 such as the wildtype (WT),
214 Alpha, Beta, Delta and Omicron variants, using focus reduction neutralization test (FRNT).
215 Consistent with their respective activities to pseudoviruses, 3-2A2-4 was the most broad and
216 potent among the three nanobodies with 0.102 $\mu\text{g}/\text{mL}$ against WT, 0.115 $\mu\text{g}/\text{mL}$ against Alpha,
217 0.098 $\mu\text{g}/\text{mL}$ against Beta, 0.130 $\mu\text{g}/\text{mL}$ against Delta, and 0.360 $\mu\text{g}/\text{mL}$ against Omicron BA.1
218 (Figure 2b). Nb70 had a relatively moderate IC₅₀ values with 1.337 $\mu\text{g}/\text{mL}$ against WT, 1.242
219 $\mu\text{g}/\text{mL}$ against Alpha, 1.635 $\mu\text{g}/\text{mL}$ against Beta, 1.210 $\mu\text{g}/\text{mL}$ against Delta, and 1.381 $\mu\text{g}/\text{mL}$
220 against Omicron BA.1. However, 1-2C7 had IC₅₀ values of 0.234, 0.270, 0.134, and 0.163
221 $\mu\text{g}/\text{mL}$ against WT, Alpha, Beta, and Delta, respectively, but failed to neutralize BA.1 at the
222 highest concentration (Figure 1b).

223

224 **Structural definition of three nanobodies epitopes.** We determined crystal structures of 1-
225 2C7, Nb70, and 3-2A2-4 bound to various recombinant RBDs (Figure 3). Of which, 1-2C7
226 bound to the SARS-CoV-2 Beta RBD (SARS-CoV-2 SA-RBD) was resolved at 1.8 Å
227 resolution (Figure 3a and Table S1) whereas 3-2A2-4 bound to the SARS-CoV-2 wild type
228 RBD (SARS-CoV-2 WT-RBD) at 2.4 Å resolution (Figure 3d and Table S1). The crystal
229 structure of Nb70 was determined in two forms. One was the ternary complex of Nb70 and
230 human antibody P2C-1F11 simultaneously bound to the SARS-CoV-2 WT-RBD at 2.4 Å
231 resolution (Figure 3b and Table S1). The other was the binary complex of Nb70 bound to the
232 SARS-CoV-1 wild type RBD (SARS CoV-1 WT-RBD) at 2.4 Å resolution (Figure 3c and
233 Table S1).

234 The crystal structures showed that both 1-2C7 and Nb70 bound to the cryptic face of
235 SARS-CoV-2 RBD, in complete agreement with the competition data shown in Figure 2a.
236 Their epitopes substantially overlapped with the cryptic epitopes recognized by VHH72 and
237 the typical Class 4 antibody CR3022 (Figures 3e, 3f, and 3g). Seven residues in the 1-2C7
238 epitope (Y369, N370, S375, T376, F377, K378 and P384) and nine residues in the Nb70
239 epitope (Y369, F377, K378, C379, Y380, G381, V382, S383 and P384) were also part of the
240 CR3022 epitope⁴⁷. However, 1-2C7 epitope deviated upward from the CR3022 epitope and
241 therefore expected to create steric hindrance to ACE2 (Figure 3e). Nb70 epitope, on the other
242 hand, was largely confined to the lower part of RBD cryptic face and further away from the
243 ACE2 binding site (Figure 3f). In addition, the Nb70 epitopes were highly conserved between
244 SARS-CoV-2 and SARS-CoV-1 (Figure 3f and 3g). A total of 17 residues were shared between
245 the two respective epitopes, namely Y369, F374, F377, K378, C379, Y380, G381, V382, S383,
246 R408, A411, P412, G413, Q414, D427, D428, and F429 on the SARS-CoV-2 RBD and Y356,
247 F361, F364, K365, C366, Y367, G368, V369, S370, R395, A398, P399, G400, Q401, D414,
248 D415, and F416 on the SARS-CoV-1 RBD (Figure 3f and 3g). These residues interacted with
249 R31, E52, N101, Y103 and D115 of Nb70 through hydrogen bonds, salt-bridges, and van der
250 Waals contacts (Figure 3i and 3j, Table S2). For instance, D115 formed hydrogen bonds and
251 salt bridges with K378 and Y380 at the Nb70-SARS-CoV-2 interface and with K365, Y367,
252 R395 and Q401 at the Nb70-SARS-CoV-1 interface (Figure 3i and 3j, Table S2). Such
253 conserved epitopes provided structural basis for the cross-neutralizing activity of Nb70
254 between SARS-CoV-2 and SARS-CoV-1. On the other hand, 3-2A2-4 bound to the bottom
255 part of RBD core subdomain between the cryptic and the outer face, distinctive from that of 1-
256 2C7 and Nb70 (Figure 3h). Five residues (T333, N334, L335, G339 and N343) within the 3-
257 2A2-4 epitope overlapped with that of the Class 3 antibody S309 (Figure 3h). Such binding

258 pose was not expected to have steric clash with either ACE2 or VHH72, compatible with the
259 competition results (Figure 2a).

260 The binding of 1-2C7, Nb70 and 3-2A2-4 to the trimeric spike is expected to be influenced
261 by the degree of exposure and accessibility of their epitopes when RBD fluctuates between the
262 “up” and “down” conformation. By superimposing the RBD-nanobody crystal structures onto
263 the spike cryo-EM structures (Figure S4), we found that 1-2C7 and Nb70 could bind to the
264 spike trimer only when two or three RBDs in the “up” state where sufficient space became
265 available for accessing the cryptic domain on the inner surface of RBD. By contrast, 3-2A2-4
266 was able to bind to the spike trimer regardless of RBD in either the “up” or “down”
267 conformation, suggesting its epitope was constantly exposed and readily accessible by the
268 nanobody.

269

270 **3-2A2-4 nanobody maintains neutralizing activity to Omicron BA.1 and BA.2**
271 **subvariants.** We next studied which mutations found in the Omicron variant potentially
272 responsible for the varying impact on neutralizing activity of 1-2C7, Nb70, and 3-2A2-4. Based
273 on the structural information on the epitopes, we focused on six single (G339D, S371L, S371F,
274 S373P, S375F, and T376A) and one triple (S371L/S373P/S375F) substitutions found in
275 Omicron variant that were either within or in close proximity to the respective epitopes (Figure
276 4a-4d). Pseudoviruses bearing these single and triple mutations were constructed and tested
277 against a serial dilution of 1-2C7, Nb70, and 3-2A2-4 (Figure 4e-4g). As single S375F
278 substitution failed to mediate detectable infection despite multiple attempts, this particular
279 mutant was removed from the subsequent studies. Among the viable mutants tested, 1-2C7 was
280 mostly affected by single S371F substitution, followed by S371L, S373P, and then G339D.
281 The triple mutations S371L/S373P/S375F resulted in complete loss of activity to the degree
282 that was compatible to Omicron subvariants BA. 1 and BA. 2 (Figure 4e). Nb70, however, was

283 only mildly affected by Omicron BA.1 and moderately by BA.2 (Figure 4f). Single S371
284 substitution led to marked reduction (S371L) or completed loss (S371F) of Nb70 activity, but
285 the remaining single (G339D and S373P) or triple (S371L/S373P/S375F) substitutions only
286 had moderate or no effect at all (Figure 4f). It was possible that the constellation of
287 S371L/S373P/S375F substitutions somehow restored the epitope structure disrupted by the
288 single S371L substitution, allowing Nb70 to resume binding and exerting neutralizing activity
289 to Omicron BA.1. However, S371F was responsible for dramatic reduction against Omicron
290 BA. 2 (Figure 4c). By contrast, 3-2A2-4 was the most resilient to Omicron variant among the
291 three nanobodies tested (Figure 4g). 3-2A2-4 remained similar neutralizing activity to BA.1,
292 BA. 2 and WT D614G, with IC₅₀ of 0.032 µg/mL, 0.047 µg/mL, and 0.043 µg/mL respectively.
293 Single substitutions such as G339D, S371L, or S373P had only moderate effect while the triple
294 S371L/S373P/S375F substitutions, like that occurred to Nb70, restored neutralizing activity
295 indistinguishable to that of WT D614G (Figure 4g). Lastly, sequence alignment of RBD
296 sequences from the multiple sarbecoviruses revealed that epitope residues of 3-2A2-4 and
297 Nb70 were more conserved than that of 1-2C7 (data not shown), providing molecular basis for
298 the broad and potent neutralizing activity of 3-2A2-4 against all sarbecoviruses tested including
299 Omicron BA.1 and BA.2.

300

301 **3-2A2-4 protects K18-hACE2 mice from infection with authentic SARS-CoV-2 Omicron**
302 **and Delta.** We next studied the protective potential of 3-2A2-4 against infection of authentic
303 Omicron and Delta variants in a K18-hACE2 mouse model of SARS-CoV-2 infection, as
304 previously described (Figure. 5a)⁴⁸. Specifically, the mice were intraperitoneally administered
305 with 3-2A2-4 at a dose of 10 mg/kg body weight 24 hours prior to intranasal challenge with
306 1.7×10^3 plaque-forming units (PFU) of authentic SARS-CoV-2 Omicron or 10^3 PFU of SARS-
307 CoV-2 Delta. The animals were then monitored daily throughout the following 14 days for

308 their body weight and survival. Half of the animals were euthanized on day 3 post inoculation
309 to obtain lung tissues for viral load and histopathological analysis.

310 In SARS-CoV-2 Omicron challenged groups, one out of the six untreated animals
311 succumbed to disease on day 11 post infection whereas all 3-2A2-4 treated mice remained
312 healthy and survived infection (Figure 5b). The changes in body weight followed the similar
313 trend of survival, with moderate loss in untreated compared to relative stability in 3-2A2-4
314 treated animals, although animals in both groups experienced minor body weight loss during
315 the first 6 days after challenge (Figure 5c). No detectable levels of live viruses were found in
316 the lungs of 3-2A2-4-treated mice on day 3 post challenge while that in untreated mice reached
317 an average as high as 796.7 PFU/tissue (Figure. 5d). Immunohistochemistry analysis revealed
318 that the lung tissue of 3-2A2-4-treated mice remained intact and no viral antigen positive cells
319 were detected (Figure. 5h). By contrast, the lung sections of untreated mice presented moderate
320 damage and inflammation with marked infiltration of inflammatory cells. Infected cells were
321 readily detectable using anti-N protein specific antibody (Figure. 5h).

322 In SARS-CoV-2 Delta challenged groups, untreated animals exhibited faster disease
323 progression and greater severity compared to those in the Omicron challenged group,
324 indicating Delta was more pathogenic than Omicron in this model of SARS-CoV-2 infection,
325 in complete agreement with those recently reported^{15,16}. All untreated animals succumbed to
326 diseases by day 6 after challenge, and associated with severe body weight loss (Figure 5e and
327 5f). By contrast, 3-2A2-4-treated group remained fully protected and maintained stable body
328 weights, except for one mouse had to be euthanized on day 6 after infection due to the
329 requirement of experimental protocol when body weight fell below 75% of baseline (Figure 5e
330 and 5f). No detectable levels of viruses in the lungs were found in 3-2A2-4-treated mice while
331 that in untreated mice reached as high as 8.1×10^4 PFU/tissue on average (Figure 5g). The lung
332 sections from untreated animals showed severe lung damage and inflammation with marked

333 infiltration of inflammatory cells. A large number of viral antigen positive pneumocytes were
334 clearly visible (Figure 5i). By contrast, in 3-2A2-4-treated mice, lung tissue remained
335 relatively intact and well-defined. Collectively, these results indicated that the broad and potent
336 neutralizing nanobody 3-2A2-4 conferred strong protection in vivo against challenge of
337 authentic SARS-CoV-2 Omicron and Delta.

338

339 **DISCUSSION**

340 The rapid emergence and spread of antigenically distinct SARS-CoV-2 variants such as
341 Omicron BA.1 and BA.2 have resulted in the substantial reduction and loss of activity of many
342 therapeutic antibodies and vaccines^{8,9,11-13}. Given the unusual huge number of mutations found
343 in the spike of Omicron BA.1 and BA.2, one of the urgent questions need to be addressed is
344 whether conserved and protective epitopes still exist on the spike trimer that can be targeted
345 for the development of broad and potent antibody therapies and vaccines. Here, we report on
346 the isolation and characterization of a unique group of nanobodies from immunized alpaca.
347 The most outstanding feature of these nanobodies, exemplified by 3-2A2-4, is their exceptional
348 breadth and potency against a highly diverse panel of sarbecoviruses including Omicron BA.1
349 and BA.2, SARS-CoV-1, and the key representatives from bats and pangolins coronaviruses.
350 The IC₅₀ reached as low as 0.042 µg/mL or 0.550 nM against WT D614G and remained
351 relatively stable across the entire panel of the tested viruses. Although neutralization assays
352 differ, this suggests they are amongst the broadest and most potent nanobodies described to
353 date³³⁻⁴⁶. Passive delivery of 3-2A2-4 protected K18-hACE2 mice from infection of two
354 widely spreading SARS-CoV-2 variants Delta and Omicron. Crystal structure analysis together
355 with modeling in the context of spike trimer revealed 3-2A2-4 recognized a highly conserved
356 epitope between the cryptic and outer face of RBD, distinctive from the ACE2 binding site,
357 and readily accessible in both “up” or “down” conformations. Such unique binding pose and

358 specificity provide structural basis for 3-2A2-4 and the other members of the G3 nanobodies
359 in withstanding the mutant residues commonly found in the major variants that compromised
360 many therapeutic antibodies approved for EUA ^{8,12}. These results clearly indicated that we have
361 identified a broad and protective nanobody that recognized a highly conserved epitope among
362 a diverse panel of sarbecoviruses including Omicron BA.1 and BA.2. The nanobody and the
363 epitope it recognized may serve as an important reference for the development of next
364 generation antibody therapies and vaccines against wide varieties of SARS-CoV-2 infection
365 and beyond.

366 Owing to their nanoscale and extended CDR3, nanobodies are expected to penetrate
367 deeper into the spike trimer and access to hidden epitopes that are less frequently exposed
368 and/or unreachable by conventional antibodies. This is particularly true for the cross-
369 neutralizing SARS-CoV-2 and SARS-CoV-1 nanobodies characterized here. Virtually all
370 nanobodies in the G1 and G2 were able to compete with a published nanobody VHH72 known
371 to recognize the hidden epitope accessible only when RBD is in the up conformation ³⁵. By
372 contrast, the nanobodies in the G3 bound to the epitopes that are readily accessible regardless
373 of up or down conformations of RBD. Interestingly, by screening and characterizing hundreds
374 of monoclonal antibodies from convalescent or vaccinated individuals, a small but convincing
375 number of cross-neutralizing antibodies with similar epitope specificity have also been found
376 ^{39,49-60}. The epitopes recognized by the G1, G2, and G3 nanobodies are therefore not only the
377 viable targets in alpaca but also in humans. In particular, the G1 and G2 nanobodies would fall
378 into the inner face antibodies exemplified by CR3022, H014, S2X259, COVA1-16, CV2-75,
379 and C1c-A3 whereas the G3 nanobodies with the escarpment face antibody 47D11 (Figure S5)
380 ⁶¹. Additional cross-neutralizing SARS-CoV-2 and SARS-CoV-1 antibodies targeting to other
381 faces of RBD have also been identified such as those to the cliff face (S2H97 and 6D60), top
382 face (S2X146), and outer face (S309 and BG10-19) (Figure S5). Recently, a human antibody

383 with broad reactivity to human beta-coronaviruses has been isolated that targets the conserved
384 S2 stem-helix, raising the possibility of development of pan- β -CoV therapies and vaccines ⁶².
385 More importantly, these cross-neutralizing and pan- β -CoV antibodies can be substantially
386 boosted by mRNA vaccines, particularly in individuals with pre-existing SARS-CoV-1 or
387 SARS-CoV-2 infection ⁶³⁻⁶⁵. Comprehensive characterization of these antibodies will provide
388 us with deeper insights into their ontogeny and potential ways of inducing broader and more
389 effective protection against the circulating and future variants.

390 Taken together, identification of 3-2A2-4 in this work and broadly neutralizing human
391 antibodies elsewhere has revealed the existence of highly conserved and vulnerable sites on
392 the RBD and spike trimer that could potentially be explored to trigger broader and more
393 protective immune responses. This would require preferential exposure of these conserved
394 regions while minimizing the receptor-binding motif that was predominantly recognized
395 during natural infection and vaccination. While to achieve this goal would be expected a huge
396 challenge, recent advances in structure-based vaccine design through understanding of antigen
397 and antibody interaction will undoubtedly provide new conceptual and technological toolbox
398 for this highly anticipated outcome. Identification of 3-2A2-4, as well as other broadly
399 neutralizing antibodies, represent the first but a crucial step for us to achieve the ultimate goal
400 of developing universal vaccine against all SARS-CoV-2 variants including Omicron BA.1
401 and BA. 2, and beyond.

402 Given the nature of immune response in alpaca is likely different from that in human, the
403 antibody responses and the epitopes recognized by the nanobodies may not be exactly
404 reflective of that in humans. Furthermore, our nanobody protection experiments were
405 performed exclusively in K18-hACE2 mice, which are inherently different in responses to
406 infection of different SARS-CoV-2 variants. Omicron resulted in relatively milder disease and
407 lower viral replication in lungs than Delta. The protection efficacies against the two variants

408 should therefore not be compared. Future studies in NHP and humans would be highly
409 desirable to verify and validate the protection results.

410

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427

428 **Author contributions**

429 L.Z., X.W. and J.C. conceived and designed the study. M.L., Y.R., Z.A. and B.C. performed
430 most of the experiments with assistance from Y.L., Q.L. J.H., Y.Y., Y.W., J.C., S.S. J.G., X.S.
431 and Q.Z. B.C. immunized the alpaca and constructed the yeast library. M.L., Y.L, Q.L, J.H.,
432 and Y.Y. isolated nanobodies and performed all evaluations. Y.R., J.C. and J.G. solved and

433 analyzed crystal structure of nanobody and RBD complexes. Z.A., Y.W. and J.C. performed
434 the nanobody protection experiment in K18-hACE2 mice. Z.Y. conducted the sequence
435 analysis. L.C. conducted the live SARS-CoV-2 neutralization assay. R.W. constructed the
436 pseudoviruses of SARS-CoV-2 and variants. M.L. and Y.R. had full access to data in the study,
437 generated figures and tables, and take responsibility for the integrity and accuracy of the data
438 presentation. L.Z., X.W, M.L. and Y.R. wrote the manuscript. All authors reviewed and
439 approved the final version of the manuscript.

440

441 **Declaration of interests**

442 B.C. is employee of NB BIOLAB Co., Ltd. The remaining authors declare that the research
443 was conducted in the absence of any commercial or financial relationships that could be
444 construed as a potential conflict of interest.

445

446 **FIGURE TITLES AND LEGENDS**

447 **Figure 1. Phylogenetic, neutralizing, binding, and genetic properties of isolated**
448 **nanobodies against SARS-CoV-2 and SARS-CoV-1.** Phylogenetic analysis of 124
449 neutralizing nanobodies isolated from the VHH library of an immunized alpaca. Cross-
450 neutralizing nanobodies against SARS-CoV-2 and SARS-CoV-1 are primarily clustered in
451 four groups (a, b, c, and d) and highlighted by the red branches. Neutralizing activities of each
452 nanobody to pseudoviruses carrying the spike of either prototype SARS-CoV-2 or SARS-CoV-
453 1 are shown by half-maximal inhibitory concentration IC₅₀ (μg/mL). The thirty-eight
454 representative nanobodies selected for further evaluation are indicated by red triangle in Nb ID
455 column. Results were calculated from three independent experiments and each performed in
456 technical duplicates. The binding activity of each nanobody to prototype spike trimer, RBD,
457 NTD, and S2 regions are indicated by the color scheme, with red for high binding, blue for
458 medium binding, and grey for low binding. The genetic features such as germline variable gene
459 segment (V), diversity gene segment (D), and junction gene (J) as well as CDR3 length of each
460 nanobody are indicated with various colors. See also Figure S1.

461

462 **Figure 2. Classification and neutralizing activity of isolated nanobodies against SARS-**
463 **CoV-2 variants and hACE2-dependent sarbecoviruses. (a)** Classification of nanobodies
464 into four major groups based on their degrees of competition with ACE2 and control VHH72
465 nanobody with known epitope specificity, measured by surface plasmon resonance (SPR).
466 "++++" indicates >90% competition; "++" 30-60%, and "+" 10-30%. Neutralizing activities of
467 nanobodies against WT D614G were presented by the actual values of 50% inhibitory
468 concentration (IC₅₀) while that against SARS-CoV-2 variants and hACE2-dependent
469 sarbecoviruses were in fold-changes relative to that of WT D614G. IC₅₀ values highlighted in
470 salmon indicate < 0.02 μg/mL; in yellow 0.02-0.1 μg/mL, and in green >0.1 μg/mL. “-”

471 indicates increased resistance and “+” indicates increased sensitivity to nanobody
472 neutralization. The fold-changes highlighted in red indicate that resistance increased at least 3-
473 fold; in blue, sensitivity increased at least threefold; and in white, resistance or sensitivity
474 increased less than 3-fold. BDL indicates that nanobodies at their highest concentration (13.33
475 µg/mL) failed to reach 50% neutralization. Results were calculated from three independent
476 experiments and each performed in technical duplicates. **(b)** Neutralizing activity of
477 representative nanobodies from G1, G2, and G3 against authentic SARS-CoV-2 of wildtype
478 (WT) and various VOCs such as Alpha, Beta, Delta and Omicron. Error bars indicate standard
479 error of mean between technical replicates. See also Figure S2 and S3.

480

481 **Figure 3. Crystal structures and epitope of three representative nanobodies bound to**
482 **SARS-CoV-2 RBD or SARS-CoV-1 RBD. (a, d)** Crystal structures of 1-2C7 and 3-2A2-4
483 bound to SARS-CoV-2 RBD. **(b)** Crystal structure of Nb70 and 1F11 bound to SARS-CoV-2
484 RBD. SARS-CoV-2 RBD is colored in cyan whereas 1-2C7 in orange, Nb70 in green, and 3-
485 2A2-4 in purple. **(c)** Crystal structure of Nb70 bound to SARS-CoV-1 RBD. Nb70 is shown in
486 green while the SARS-CoV-1 RBD in blue. **(e, h)** The epitope of 1-2C7 (orange) or of 3-2A2-
487 4 (purple) is respectively depicted on the surface of SARS-CoV-2 RBD. **(f)** The epitope of
488 Nb70 (green) depicted on the surface of SARS-CoV-2 RBD or **(g)** on the prototype SARS-
489 CoV-1 RBD. The same residues between SARS-CoV-2 and SARS-CoV-1 epitopes were
490 underlined. The epitope of CR3022 highlighted in red (PDB: 6YM0 on SARS-CoV-2 RBD
491 and PDB: 7JN5 on SARS-CoV-1 RBD) is superposed onto that of **(e)** 1-2C7, **(f)** Nb70, as well
492 as **(g)** Nb70 together with VHH72 epitope in white (PDB: 6WAQ). The epitope of S309 in
493 yellow (PDB: 7R6W) is superimposed onto **(h)** that of 3-2A2-4. **(i, j)** Conserved interactions
494 between Nb70 and SARS-CoV-2 RBD or SARS-CoV-1 RBD. See also Figure S4.

495

496 **Figure 4. Impact of Omicron mutations on neutralizing activity of the three**
497 **representative nanobodies. (a)** Binding modes of Nb70 (green), 1-2C7 (orange) and 3-2A2-
498 4 (purple) to prototype SARS-CoV-2 RBD with the relevant major mutations found in Omicron
499 highlighted in red. **(b, c, d)** The footprints of 1-2C7 (orange), Nb70 (green), and 3-2A2-4
500 (purple) on the surface of prototype SARS-CoV-2 RBD (cyan), relative to relevant major
501 mutations found in Omicron highlighted in red. **(e, f, g)** Neutralizing activity of 1-2C7, Nb70,
502 and 3-2A2-4 against pseudoviruses bearing the indicated mutations found in Omicron. Data
503 are presented as the means \pm SEM from three independent experiments.

504

505 **Figure 5. Efficacy of 3-2A2-4 prophylaxis against the authentic SARS-CoV-2 Omicron**
506 **and Delta in K18-hACE-2 mice. (a)** Experimental schedule for nanobody prophylaxis. Eight-
507 week-old K18-hACE2 transgenic female mice were administered with 10 mg/kg body weight
508 of 3-2A2-4 intraperitoneally or untreated 1 day prior to challenge with 1.7×10^3 plaque-forming
509 units (PFU) infectious SARS-CoV-2 Omicron or 10^3 PFU Delta via the intranasal route. The
510 survival percentage **(b and e)** and body weight **(c and f)** were recorded daily after infection
511 until the occurrence of death or until the end of experiment. The viral load in the lung tissue **(d**
512 **and g)** was tested by plaque assays in the tissue homogenates at 3 days post inoculation. Data
513 are presented as the means \pm SEM. Analysis of Mann-Whitney test was used. ****P<0.01. (h**
514 **and i)** HE and IHC staining of lung tissue from 3-2A2-4-treated or untreated mice at 3 days
515 post inoculation. VL, vascular lumen; BL, bronchiolar lumen. Scale bars, 50 μ m. Each image
516 is representative of each group.

517

518 **METHOD DETAILS**

519 **Cell lines.** HEK293T cells (ATCC, CRL-3216) and HeLa cells expressing hACE2 were kindly
520 provided by Dr. Qiang Ding at Tsinghua University. Both of these cell lines were maintained
521 at 37°C in 5% CO₂ in Dulbecco's minimal essential medium (DMEM) containing 10% (v/v)
522 heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin–streptomycin. FreeStyle
523 293F cells (Thermo Fisher Scientific, R79007) were maintained at 37°C in 5% CO₂ in SMM
524 293-TII expression medium (Sino Biological, M293TII). Sf9 cells (ATCC) were maintained at
525 27°C in Sf-900 II SFM medium. Hi5 cells (ATCC) were maintained at 27°C in SIM HF
526 medium.

527

528 **Expression and purification of recombinant proteins.** The genes encoding the ectodomain
529 of S trimer and S2 trimer of prototype SARS-CoV-2 Wuhan-Hu-1 strain (GenBank:
530 MN908947.3) were constructed as previously reported (Ruoke Wang, Immunity 2021). Both
531 S trimer (residues M1-Q1208) and S2 trimer (residues S686-Q1208) contained proline
532 substitutes at residue 986 and 987, a foldon trimerization motif, and a strep tag at C-terminal.
533 Additional “GSAS” substitution at furin cleavage site (residues 682-685) was also introduced
534 into the S trimer construct to improve overall stability. Both S trimer and S2 trimer were
535 expressed in the FreeStyle 293F cells and purified by Strep-Tactin Sepharose (IBA
536 Lifesciences) followed by gel filtration chromatography (GE Healthcare). The S trimer of
537 SARS-CoV-1, RBD of SARS-CoV-1 and SARS-CoV-2 prototype, and the N-terminal
538 peptidase domain of human ACE2 were expressed using the Bac-to-Bac Baculovirus
539 Expression System (Invitrogen, Carlsbad, CA, USA) and purified by Strep-Tactin Sepharose
540 (IBA Lifesciences) followed by gel filtration chromatography (GE Healthcare), as previously
541 reported⁶⁶. Briefly, the RBD of SARS-CoV-2 wildtype (residues Arg319–Lys529) with an N-
542 terminal gp67 signal peptide for secretion and a C-terminal 6×His tag for purification was

543 expressed using Hi5 cells and purified by Ni-NTA resin followed by gel filtration
544 chromatography (GE Healthcare) in HBS buffer (10 mM HEPES, pH 7.2, 150 mM NaCl). The
545 RBD of SARS-CoV-1 (residues Arg306–Leu515) and N-terminal peptidase domain of human
546 ACE2 (residues S19–D615) were expressed and purified by the same protocol as used for the
547 RBD of SARS-CoV-2 wildtype (see above). The SARS-CoV-2 NTD protein was purchased
548 from SinoBiological (40591-V49H).

549

550 **Immunization of alpaca, construction of yeast display VHH library, and isolation of VHH**
551 **yeasts specific for SARS-CoV-2 and SARS-CoV-1 spikes.** The animal experiment protocol
552 involving immunization, collection of blood samples, and construction of VHH library was
553 approved by IACUC at NBbiolab, Inc. in Chengdu, China. The immunization procedure
554 involved three-time subcutaneous injections of 200 µg recombinant RBD of SARS-CoV-2 in
555 Freund adjuvant, one-time subcutaneous injection of 10¹¹ viral particle AdC68-19S vaccine
556 expressing the wildtype S trimer⁶⁷, and two-time subcutaneous injections of 200 µg
557 recombinant SARS-CoV-2 S-2P protein in Freund adjuvant. Seven days after the last
558 immunization, blood samples were collected to isolate peripheral blood lymphocytes and
559 plasma. Total RNA was extracted from the peripheral blood lymphocytes and used as the
560 template for the first strand cDNA synthesis, using oligo dT as a primer. VHH sequences were
561 amplified by PCR, cloned into a yeast surface display vector pYD1, and introduced into the
562 electrocompetent EBY100 cells. The yeast library was first grown in SDCAA media at 30 °C
563 for 48 h. At the exponential growth phase, the yeast library was transferred to SGCAA media
564 for induction of VHH expression at 20 °C for 36 h. The yeast clones displaying VHHs specific
565 to SARS-CoV-2 or SARS-CoV-1 spikes were enriched by one round of MACS biopanning
566 followed by additional round of FACS biopanning. Specifically, induced yeast library was
567 collected and incubated with 100 nM SARS-CoV-2 S-2P protein or SARS-CoV-1 S protein on

568 ice for 30 min. The yeast library was washed with cold PBS+1%FBS for three times and
569 incubated with streptavidin microbeads on ice for 10 min. The mixture was passed through LS
570 column and the S protein-positive yeasts were harvested for further culture and induction of
571 VHH expression. Once again, induced yeasts were incubated with 100 nM SARS-CoV-2 S-2P
572 protein or SARS-CoV-1 S protein on ice for 30 min. After extensive wash with cold
573 PBS+1%FBS, the yeast clones were incubated with HA-Tag (6E2) mouse monoclonal
574 antibody conjugated with Alexa Fluor® 488 (1:100 dilution) and eBioscience™ streptavidin
575 conjugated with PE Conjugate (1:200 dilution) on ice for 30 min. The yeast clones were washed
576 three times with cold PBS+1%FBS before analyzed by FACS using Aria II (BD Biosciences).
577 Positive yeast clones were sorted and used for nanobody cloning.

578

579 **Molecular cloning and expression of VHH.** The sequences encoding various VHHs were
580 amplified from the sorted yeast clones and inserted into two different expression vectors
581 depending on the subsequent study objectives. One was to express VHH in conjunction with
582 human IgG1 Fc fragment to evaluate binding and neutralizing activity of VHHs. The other was
583 to express VHH with a 6xHis tag for crystal structural analysis. For the former, VHH genes
584 were cloned into the multiple cloning sites of pMD18T containing the upstream CMV promoter,
585 the secretory signal sequence from the mouse Ig heavy chain, and the downstream human IgG1
586 Fc gene fragment and SV40 poly (A) signal sequence. For the latter, selected VHH genes were
587 cloned into pVRC8400 vector with a 6xHis tag. Expression and production of nanobodies were
588 conducted by transfecting the expression vectors into the HEK293F cells using
589 polyethyleneimine (PEI) (Polysciences). After approximately 96 h, nanobodies containing
590 human IgG1 Fc in the culture supernatant were captured by AmMag Protein A Magnetic Beads
591 (Genscript L00695) and eluted by Glycine pH 3.0. Nanobodies with 6xHis tag were captured
592 by Ni-NTA Agaose (QIAGEN) and eluted by 500 nM Imidazole. All nanobodies were purified

593 by gel-filtration chromatography with Superdex 200 High-Performance column (GE
594 Healthcare). The exact concentration was determined by nanodrop 2000 Spectrophotometer
595 (Thermo Scientific).

596

597 **Production of SARS-CoV-2 and sarbecovirus pseudoviruses.** Pseudoviruses carrying the
598 full-length spike envelope of SARS-CoV-2 and sarbecoviruses were generated as previously
599 reported ⁶. Specifically, human immunodeficiency virus backbones expressing firefly
600 luciferase (pNL4-3-R-E-luciferase) and pcDNA3.1 vector encoding either SARS-CoV-2 or
601 sarbecovirus spike proteins were co-transfected into the HEK-293T cells (ATCC). Forty-eight
602 hours later, pseudoviruses in the viral supernatant were collected, centrifuged to remove cell
603 lysis, and stored at -80°C until use. The wildtype pseudovirus used throughout the analysis was
604 the prototype strain (GenBank: MN908947.3) with a D614G mutation (WT D614G). The
605 Alpha variant (Pango lineage B.1.1.7, GISAID: EPI_ISL_601443) included a total of 9
606 reported mutations in the spike protein (69-70del, 144del, N501Y, A570D, D614G, P681H,
607 T716I, S982A and D1118H). The Beta variant (Pango lineage B.1.351, GISAID:
608 EPI_ISL_700450) included 10 identified mutations in the spike such as L18F, D80A, D215G,
609 242-244del, S305T, K417N, E484K, N501Y, D614G and A701V. The Gamma variant (Pango
610 lineage P.1, GISAID: EPI_ISL_792681) had 12 reported mutations in the spike including L18F,
611 T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I and V1176F.
612 The Delta variant (Pango lineage B.1.617.2, GISAID: EPI_ISL_1534938) included 10 reported
613 mutations in the spike such as T19R, G142D, 156-157del, R158G, A222V, L452R, T478K,
614 D614G, P681R, D950N. The Omicron BA.1 variant (Pango lineage BA.1, GISAID:
615 EPI_ISL_6752027) was constructed with 34 mutations in the spike such as A67V, 69-70del,
616 T95I, G142D, 143-145del, 211del, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N,
617 N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K,

618 D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, and L981F. The
619 Omicron BA.2 variant (Pango lineage BA.2, GISAID: EPI_ISL_8515362) was constructed
620 with 29 mutations in the spike such as T19I, 24-26del, A27S, G142D, V213G, G339D, S371F,
621 S373P, S375F, T376A, D405N, R408S, K417N, N440K, S477N, T478K, E484A, Q493R,
622 Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, N969K and
623 Q954H. The Kappa variant (Pango lineage B.1.617.1, GISAID: EPI_ISL_1384866) was
624 constructed with 8 mutations in the spike such as T95I, G142D, E154L, L452R, E484Q,
625 D614G, P681R and N1071H. The Eta variant (Pango lineage B.1.525, GISAID: EPI_ISL_
626 2885901) included 8 mutations in the spike such as Q52R, A67V, 69-70del, Y144del, E484K,
627 D614G, Q677H and F888L. The Iota variant (Pango lineage B.1.526, GISAID: EPI_ISL_
628 2922249) was constructed with 6 mutations in the spike such as L5F, T95I, D253G, E484K,
629 D614G and A701V. The Lambda variant (Pango lineage C.37, GISAID: EPI_ISL_2930541)
630 was constructed with 8 mutations in the spike such as G75V, T76I, R246N, 247-253del, L452Q,
631 F490S, D614G and T859N. The Epsilon variant (Pango lineage B.1.429, GISAID: EPI_ISL_
632 2922315) was constructed with 4 mutations in the spike such as S13I, W152C, L452R and
633 D614G. The variant with Pango lineage A23.1, GISAID: EPI_ISL_2690464) was constructed
634 with 4 mutations in the spike such as F157L, V367F, Q613H and P681R. The G339D, S371L,
635 S371F, S373P, T376A, R408S and S371L+D373P+S375F were constructed with a D614G
636 mutation based on the WT strain. For sarbecoviruses, the cDNAs encoding the SARS-CoV-1
637 S glycoprotein (NCBI Accession NP_828851.1), Pangolin CoV GD spike (GenBank:
638 QLR06867.1), Pangolin CoV GX spike (GenBank: QIA48614.1), Bat SARS-like coronavirus
639 WIV16 (GenBank: ALK02457.1), Bat SARS-like RaTG13 spike (GenBank: QHR63300.2),
640 Bat SARS-like ZC45 (GenBank: AVP78031.1), Bat SARS-like ZXC21 (GenBank:
641 AVP78042.1) and Bat SARS-like coronavirus RsSHC014 (GenBank: AGZ48806.1) were

642 synthesized with codons optimized for protein expression (Genwiz Inc., China) and verified
643 by sequencing.

644

645 **Neutralization activity of nanobodies against pseudoviruses and live SARS-CoV-2.**

646 Neutralization activity of nanobodies were determined using SARS-CoV-2 pseudovirus and
647 authentic live virus as previously reported³¹. Nanobodies were 3-fold serially diluted in 96-
648 well cell culture plates, mixed with SARS-CoV-2 pseudovirus, and incubated at 37°C for 1 h.
649 HeLa-ACE2 cells were then added to the mixture of nanobody-pseudovirus, incubated at 37°C
650 for additional 48 h, and lysed for measuring luciferase-activity. The IC50 values were
651 calculated based on the reduction of 50% relative light units (Bright-Glo Luciferase Assay
652 Vector System, Promega, USA) compared to the virus-only control, using Prism 8.0 (GraphPad
653 Software Inc., USA).

654 For the authentic live virus assay, we used the focus reduction neutralization test (FRNT)
655 performed in a certified BSL3 facility at Shenzhen Third People's Hospital, China. Briefly,
656 serial dilutions of nanobodies were mixed with different authentic SARS-CoV-2 and incubated
657 for 1 h at 37°C. The mixtures were then transferred to 96-well plates seeded with Vero E6 cells
658 and incubated for 1 h at 37°C. After changing the medium, the plates were incubated at 37°C
659 for an additional 24 h. The cells were then fixed, permeabilized, and incubated with cross-
660 reactive rabbit anti-SARS-CoV-N IgG (Sino Biological, Inc., China) for 1 h at room
661 temperature before adding an HRP-conjugated goat anti-rabbit IgG antibody (Jackson
662 ImmunoResearch, USA). The reactions were developed using KPL TrueBlue peroxidase
663 substrate (Seracare Life Sciences Inc., USA). The number of SARS-CoV-2 foci was quantified
664 using an EliSpot reader (Cellular Technology Ltd. USA). The authentic SARS-CoV-2 used in
665 the assay including the WT, Alpha, Beta, and Delta were isolated locally. Their whole genome
666 sequences have been deposited into China National Center for Bioinformation, with accession

667 numbers GWHXXX00000000 for WT strain, GWHBFWX01000000 for Alpha strain,
668 GWHBDSE01000000 for Beta strain, and GWHBFWZ01000000 for Delta strain, which are
669 publicly accessible at <https://ngdc.cncb.ac.cn/gwh>.

670

671 **Phylogenetic tree and genetic analysis of nanobodies.** Neighbor-joining phylogenetic trees
672 were generated using MEGA version 10.1.8 with 1000 bootstrap replicates ⁶⁸. The IMGT/V-
673 QUEST program (http://www.imgt.org/IMGT_vquest/vquest) was used to analyze the
674 germline gene and the loop lengths of complementarity determining region 3 (CDR3) of each
675 nanobody. Chord diagrams showing the germline gene usages and V/J gene pairing were
676 analyzed and presented by the R package circlize version 0.4.13 ⁶⁹. The width of linking arc is
677 proportional to the number of nanobodies identified. Sequence logo were plotted using Python
678 package Logomaker ⁷⁰.

679

680 **Nanobody binding kinetics and competition with ACE2 measured by SPR.** The binding
681 kinetics of nanobodies to SARS-CoV-2 RBD were analyzed using SPR (Biacore 8K; GE
682 Healthcare). The nanobodies were captured by the ProA sensor chip and serial dilutions of
683 SARS-CoV-2 RBD were then flowed through at a rate of 30 $\mu\text{L}/\text{min}$ in PBS buffer (with 0.05%
684 Tween20) for 120s with a dissociation time 3600s. The resulting data were fitted to a 1:1
685 binding model using the Biacore 8K Evaluation software (GE Healthcare). To determine the
686 levels of nanobody competition with the human ACE2 and VHH72 of known epitope
687 specificity, the prototype SARS-CoV-2 RBD was immobilized to a CM5 sensor chip via the
688 amine group for a final RU around 250. In the first round, nanobodies (1 μM) or VHH72 (1
689 μM) were injected onto the chip for 120 s to reach the steady state for binding. In the second
690 round, nanobodies (1 μM) or VHH72 (1 μM) were injected onto the chip for 120 s followed by
691 injection of ACE2 (2 μM) or nanobodies (1 μM) for additional 120 s. In the third round, running

692 buffer was injected for 120 s followed by injection of ACE2 (2 μ M) or nanobodies (1 μ M) for
693 another 120 s. The sensorgrams of the three rounds were aligned from 120 to 240 s in Biacore
694 8K Evaluation software (GE Healthcare). The blocking efficacy was determined by a
695 comparison of the response units with and without prior antibody injection.

696

697 **Crystallization and data collection.** To obtain the complex of RBD bound to nanobodies,
698 RBD was incubated with each nanobody for 1 h on ice in HBS buffer. The mixture was then
699 purified using gel filtration chromatography. Fractions containing the complex were pooled
700 and concentrated to 10 mg ml⁻¹. Crystals were successfully grown at room temperature in
701 sitting drops, over wells containing 0.2 M Ammonium sulfate, 0.1 M Bis-Tris pH 4.4, 21% w/v
702 Polyethylene glycol 3,350 for Nb70-SARS-CoV-1 RBD, 0.15M DL-Malic acid pH 7.0, 20%
703 w/v Polyethylene glycol 3,350 for Nb70-1F11 fab-SARS-CoV-2 RBD, 0.2 M Ammonium
704 sulfate, 0.1M Bis Tris pH 5.5, 25% w/v Polyethylene glycol 3,350 for 1-2C7-SA RBD, 0.2 M
705 Ammonium formate, pH 6.6, 20% w/v Polyethylene glycol 3,350 for 3-2A2-4-SARS-CoV-2
706 RBD. Crystals were collected, soaked briefly in 0.2 M Ammonium sulfate, 0.1 M Bis-Tris pH
707 4.4, 21% w/v Polyethylene glycol 3,350 and 20% glycerol for Nb70-SARS-CoV-1 RBD, 0.15
708 M DL-Malic acid pH 7.0, 20% w/v Polyethylene glycol 3,350 and 20% glycerol for Nb70-
709 1F11 fab-SARS-CoV-2 RBD, 0.2 M Ammonium sulfate, 0.1 M Bis-Tris pH 5.5, 25% w/v
710 Polyethylene glycol 3,350 and 20% glycerol for 1-2C7-SA RBD, 0.2 M Ammonium formate,
711 pH 6.6, 20% w/v Polyethylene glycol 3,350 and 20% glycerol for 3-2A2-4-SARS-CoV-2 RBD
712 and were subsequently flash-frozen in liquid nitrogen. Diffraction data were collected at 100
713 K and a wavelength of 0.987 Å at the BL18U1 beam line for Nb70-SARS-CoV-1 RBD, Nb70-
714 1F11 fab-SARS-CoV-2 RBD and 1-2C7-SA RBD, 100 K and a wavelength of 1.07180 Å at
715 the BL02U1 beam line for 3-2A2-4-SARS-CoV-2 RBD of the Shanghai Synchrotron Research

716 Facility. Diffraction data were processed using the HKL3000 software (PMID:16855301) and
717 the data-processing statistics are listed in Table S1.

718

719 **Data availability.** The coordinates and structure factors for the 1-2C7-SARS-CoV-2 SA-RBD,
720 Nb70-SARS-CoV-2 WT-RBD-P2C-1F11, Nb70-SARS-CoV-1 WT-RBD, 3-2A2-4-SARS-
721 CoV-2 WT-RBD complex were deposited in Protein Data Bank with accession code 7X2M,
722 7X2K, 7X2J, 7X2L.

723 **Structure determination and refinement.** The structure was determined using the molecular
724 replacement method with PHASER in the CCP4 suite (PMID: 19461840). Density map
725 improvement by updating and refinement of the atoms was performed with ARP/wARP26
726 (PMID: 18094467). Subsequent model building and refinement were performed using COOT
727 (PMID: 15572765) and PHENIX (PMID: 12393927), respectively. Final Ramachandran
728 statistics: 96.54% favored, 3.46% allowed and 0.00% outliers for the final Nb70-SARS-CoV-
729 1 RBD structure, 97.28% favored, 2.59% allowed and 0.14% outliers for the final Nb70-1F11
730 fab-SARS-CoV-2 RBD structure, 97.15% favored, 2.85% allowed and 0.00% outliers for the
731 final 1-2C7-SA RBD structure and 93.89% favored, 5.79% allowed and 0.32% outliers for the
732 final 3-2A2-4-SARS-CoV-2 RBD structure. The structure refinement statistics are listed in
733 Table S1. All structure figures were generated with ChimeraX and Pymol (PMID: 28158668).

734 **Nanobody protection in K18-hACE2 mice** Animal experiments were conducted in a
735 Biosafety Level 3 (BSL-3) facility in accordance with the National University of Singapore
736 (NUS) Institutional Animal Care and Use Committee (IACUC) (protocol no. R20-0504), and
737 the NUS Institutional Biosafety Committee (IBC) and NUS Medicine BSL-3 Biosafety
738 Committee (BBC) approved SOPs. Eight-week-old female K18-hACE2 transgenic mice
739 (InVivos Ptd Ltd, Lim Chu Kang, Singapore) were used for this study. The mice were housed

740 and acclimatized in an ABSL-3 facility for 72 h prior to the start of the experiment. K18-
741 hACE2 transgenic mice were subjected to pretreatment of nanobody 3-2A2-4 (10 mg/kg)
742 delivered through intraperitoneal injection a day prior to infection. The viral challenge was
743 conducted through intranasal delivery in 25 μ l of either 1.7×10^3 PFU of the infectious SARS-
744 CoV-2 Omicron or 10^3 PFU of Delta variant. Baseline body weights were measured prior to
745 infection and monitored daily by two personnel post-infection for the duration of the
746 experiment. Mice were euthanized when their body weight fell below 75% of their baseline
747 body weight. To assess the viral load, mice from each experimental group were sacrificed 3
748 days post inoculation, with lung tissues harvested. Each organ was halved for the plaque assay
749 and histology analysis, respectively. Tissues were homogenized with 0.5 mL DMEM
750 supplemented with antibiotic and antimycotic (Gibco, Waltham, MA, USA) and titrated in
751 Vero E6 cells using plaque assays. For virus titer determination, supernatants from
752 homogenized tissues were diluted 10-fold serially in DMEM supplemented with antibiotic and
753 antimycotic. Of each serial diluted supernatant, 250 μ l was added to Vero E6 cells into 12-well
754 plates. After 1 h of incubation for virus adsorption, the inoculum was removed and washed
755 once with PBS. About 1.2% microcrystalline cellulose (MCC)-DMEM supplemented with
756 antibiotic and antimycotic overlay media was added to each well and incubated at 37°C, 5%
757 CO₂ for 72 h for plaque formation. The cells were then fixed in 10% formalin overnight and
758 counterstained with crystal violet. The number of plaques was determined and the virus titers
759 of individual samples were expressed in logarithm of PFU per organ. For histopathological
760 analyses, lung lobes were fixed in 3.7% formaldehyde solution prior to removal from BSL-3
761 containment. The tissues were routinely processed, embedded in paraffin blocks (Leica
762 Surgipath Paraplast), sectioned at 4- μ m thickness, and stained with H&E (Thermo Scientific)
763 following standard histological procedures. For immunohistochemistry, sections were
764 deparaffinized and rehydrated, followed by heat-mediated antigen retrieval, quenching of

765 endogenous peroxidases and protein blocking. Sections were then covered with rabbit anti-
766 SARS-CoV-2 N protein monoclonal antibody (Abcam; 1:1000) for 1 h at room temperature.
767 Subsequently, sections were incubated with rabbit-specific HRP polymer (secondary antibody),
768 visualized using chromogenic substrate DAB solution (Abcam), and counterstained with
769 hematoxylin.

770

771 **Statistical analysis.** The technical and independent experiment replicates were indicated in the
772 figure legends. Half-maximal inhibitory concentration (IC₅₀) of nanobodies was calculated by
773 the equation of four-parameter dose inhibition response using Graphpad Prism 8.0. The fold
774 change of the variants relative to D614G in neutralization were calculated by simple division
775 of respective IC₅₀ values. In animal experiments, a two-tailed unpaired Mann-Whitney test
776 was used to assess statistical significance. Statistical calculations were performed in GraphPad
777 Prism 8.0. Differences with p-values less than 0.05 were considered to be statistically
778 significant (**p < 0.01).

779

780

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960

Figure 2

a

Group	Nb ID	Nb Competition		WT D614G IC50(µg/mL)	WT D614G IC50(nM)	SARS-CoV-2 Variants														Other Sarbecoviruses			
		ACE2	VHH72			Fold changes in IC50 relative to WT D614G																	
						Alpha B.1.1.7	Beta B.1.351	Gamma P.1	Delta B.1.617.2	Omicron BA-1	Omicron BA.2	Kappa B.1.617.1	Eta B.1.525	Iota B.1.526	Lambda C.37	Epsilon B.1.429	A23.1 Uganda	SARS-CoV-1	Pangolin CoV GD				
G1	1-1H3	++++	++++	0.066	0.867	+3.0	+1.7	+5.1	+2.7	BDL	BDL	+1.3	+1.5	+1.3	+1.9	-1.5	+1.7	+6.4	+16.1	+1.3	+2.1	+24.6	
	1-2A1	++++	++++	0.054	0.715	+1.3	+3.3	+2.4	+1.1	BDL	BDL	+1.2	+1.3	+1.5	-1.0	-2.2	-1.1	+12.8	+8.5	+1.9	+1.6	+9.1	
	1-1F11	++++	++++	0.041	0.533	+1.2	+2.3	+1.9	-1.7	BDL	BDL	-1.1	+1.2	-1.1	-1.4	-1.4	-2.1	+2.9	+4.7	-47.4	-5.1	+7.3	
	2-E5	++++	++++	0.058	0.767	+1.2	+1.7	+2.4	-1.3	BDL	BDL	+1.4	+1.5	+1.1	+1.0	-1.3	-1.5	+1.5	+7.3	-3.5	+5.3	+10.7	
	2-1G11-5	++++	++++	0.049	0.843	+1.3	+2.6	+2.1	-1.8	BDL	BDL	+1.0	+1.2	+1.1	+1.1	-1.5	-1.0	-1.4	+6.0	+3.8	+3.0	+11.1	
	1-1B10	++++	++++	0.089	1.175	+2.3	+3.5	+3.2	+1.8	BDL	BDL	+2.0	+2.0	+1.9	+1.5	-2.9	+1.2	+17.2	+9.0	-5.0	+3.6	+4.8	
	1-B1	++++	++++	0.045	0.593	+1.0	+1.6	+2.1	-1.7	BDL	BDL	+1.2	-1.0	+1.1	-1.1	-1.4	-1.5	+1.8	+6.0	-19.4	+1.0	+6.7	
	1-1E4	++++	++++	0.052	0.679	+1.2	+2.0	+1.6	-1.4	BDL	BDL	-1.1	-1.0	+1.1	-1.5	-1.2	-1.7	+6.2	+6.1	-2.2	+2.0	+4.5	
	1-1E10	++++	++++	0.038	0.504	+1.1	+1.5	+1.2	-1.9	BDL	BDL	-1.5	-1.2	-1.8	-1.8	+1.1	-3.1	+1.1	+4.9	-147.4	-2.4	+5.8	
	1-2B1	++++	++++	0.062	0.817	+1.5	+2.1	+1.7	-1.3	BDL	BDL	+1.1	+1.3	+1.2	-1.5	-1.3	-1.7	+11.3	+6.4	+1.5	+1.5	+10.1	
	1-2C7	++++	++++	0.056	0.735	+2.0	+2.4	+2.4	+1.1	BDL	BDL	+1.1	+1.3	+1.1	-1.3	-1.9	-1.4	+8.8	+6.4	-6.5	+3.2	+6.1	
	1-1G2	++++	++++	0.073	0.954	+2.0	+3.4	+3.7	+1.0	BDL	BDL	+1.4	+1.5	+2.0	+1.2	-1.6	+2.0	+8.7	+7.8	-4.6	+4.4	+12.2	
	1-2D8	++++	++++	0.044	0.580	+1.1	+2.4	+1.9	-1.2	BDL	BDL	+1.1	+1.7	+1.3	+1.1	-1.4	-1.1	+19.1	+8.1	-8.0	+5.9	+8.7	
	1-2F2	++++	++++	0.045	0.597	+1.5	+2.4	+3.6	-1.3	BDL	BDL	-1.3	+1.1	+1.1	-1.1	-1.1	+1.2	+6.1	+8.2	-108.3	+2.7	+5.0	
	1-2B3	++++	++++	0.077	1.010	+1.4	+1.6	+2.3	-1.1	BDL	BDL	-1.1	+1.8	+1.2	-1.1	-1.6	-1.6	+12.8	+6.8	+3.4	+3.6	+7.6	
	1-1C7-4	++++	++++	0.070	0.927	+1.2	+1.8	+2.7	-1.1	BDL	BDL	+1.2	+1.3	+1.7	-1.2	-1.4	-1.5	+11.6	+7.2	+1.5	+1.5	+14.7	
	1-G1	++++	++++	0.079	1.041	+1.4	+2.0	+1.6	-1.6	BDL	BDL	-1.1	+1.0	-1.2	-1.6	-1.4	-2.0	+13.3	+6.1	+5.0	+2.7	+5.5	
	4-1H1	++++	++++	0.583	7.666	+5.7	+4.9	+7.3	+4.8	BDL	BDL	+5.9	+5.0	+4.6	+3.3	-11.3	+3.9	+5.0	+26.3	+14.6	+23.6	+28.9	
	4-1E1-4	++++	++++	0.201	2.645	+1.9	+2.0	+2.7	+1.6	BDL	BDL	+2.1	+2.1	+2.1	-1.2	-4.1	+1.4	+1.5	+6.9	+4.8	+3.9	+7.5	
	4-F10-1	++++	++++	0.029	0.379	+1.9	+2.7	+3.5	-2.2	BDL	BDL	-1.2	-1.0	+1.1	-1.3	-1.2	-1.4	+2.3	+6.3	+3.2	+2.0	+10.1	
2-2H5-1	++++	++++	0.073	0.954	+1.4	+3.7	+3.5	-1.1	BDL	BDL	+1.6	+1.9	+1.4	+1.4	-1.7	-1.8	+4.1	+13.7	+2.4	+6.5	+18.2		
1-2F8	++++	++++	0.057	0.753	+1.3	+1.5	+4.2	+1.6	BDL	BDL	+1.7	+1.3	+1.3	-1.3	-1.9	-9.4	-2.4	+3.9	BDL	-11.6	+9.3		
1-2D3-3	++++	++++	0.071	0.928	+1.2	+1.4	+3.8	+1.3	BDL	BDL	+1.8	+1.6	+1.8	+1.2	-2.3	-16.0	-3.3	+2.9	-104.2	-12.3	+9.5		
2-2F1	++++	++++	0.042	0.554	-1.4	+1.3	+1.4	-2.4	BDL	BDL	-1.6	-1.6	-1.7	-2.4	+1.7	-4.7	-1.1	+2.0	-1.4	-1.7	+3.6		
1-1C2	++++	++++	0.062	0.821	+1.9	+2.9	+2.7	+1.1	BDL	BDL	+1.3	+1.4	+1.4	+1.4	-7.4	-2.5	+1.1	+10.8	+9.9	+4.5	+3.7	+10.7	
G2	4-2E10	++	++++	0.067	0.878	+1.2	+1.5	+1.3	-1.1	-3.4	BDL	+1.3	+1.1	-1.1	-1.2	-2.0	-1.5	-1.2	+5.4	+4.4	+8.3	+6.4	
	Nb70	+	++++	0.263	3.462	+1.1	+1.1	+1.5	-1.4	-1.6	-13.8	+1.2	-1.3	-1.3	-1.4	-1.5	+1.2	+10.1	+12.0	+3.3	+4.9	+15.5	
	Nb3	++	++++	0.231	3.037	+1.4	+1.3	+2.2	-1.1	-13.3	BDL	+1.1	-1.3	-1.1	-1.1	-1.6	+1.3	+13.1	+14.7	+8.9	+10.2	+22.6	
G3	3-2A2-4	++	++	0.042	0.550	+1.8	+1.0	+2.1	+2.0	+1.3	-1.1	+1.6	+1.4	+1.2	-1.3	-1.1	-1.0	+2.7	+1.2	-12.0	-1.2	+1.1	
	4-1A10-1	++	++	0.037	0.481	+1.8	+1.1	+1.2	+1.3	+3.8	-1.1	-1.0	-1.1	-1.4	-1.2	-1.1	-1.0	+1.6	+2.4	-8.6	-1.0	+1.2	
	4-1A10-4	++	++	0.043	0.559	+1.6	+1.6	-1.2	+1.3	-1.1	-3.5	+1.8	+1.5	+1.4	-2.1	-1.3	-1.4	+1.1	+1.0	-8.2	-2.3	+1.0	
G4	1-E1-1	++++	+	0.023	0.297	+2.2	-5.2	-6.0	-1.0	BDL	-157.1	+2.8	-1.2	-1.1	+3.1	-2.2	+1.0	BDL	+3.1	BDL	BDL	BDL	
	1-C2	++++	+	0.005	0.068	-1.2	BDL	-2001.0	+1.2	-86.1	BDL	-461.9	BDL	BDL	-55.1	+3.4	+1.1	BDL	+1.7	BDL	BDL	-548.4	
	1-C3-4	++	+	0.019	0.254	+15.5	BDL	-758.9	+1.7	BDL	BDL	BDL	BDL	BDL	-183.6	-1.3	+6.3	BDL	+4.2	BDL	BDL	-125.8	
	1-2D3-1	++++	+	0.010	0.131	+2.3	BDL	BDL	-9.2	BDL	BDL	-27.9	BDL	BDL	-29.9	+10.2	+1.0	BDL	+1.0	-793.0	BDL	-421.6	
	3-2D7-2	++++	+	0.013	0.176	-3.1	BDL	-705.6	-28.5	BDL	BDL	-4.0	BDL	BDL	BDL	+119.5	+1.2	BDL	+2.3	BDL	-534.4	-297.7	
1-1G9	++++	+	0.014	0.179	+7.0	-126.4	-109.0	+2.2	BDL	BDL	+2.8	-7.3	-9.5	+4.4	-7.8	+1.0	BDL	+3.0	BDL	BDL	-100.7		

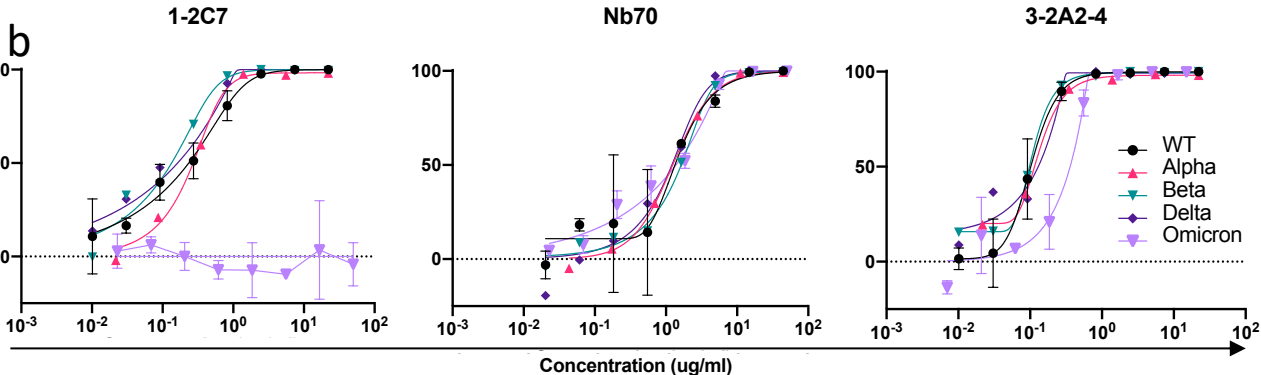


Figure 3

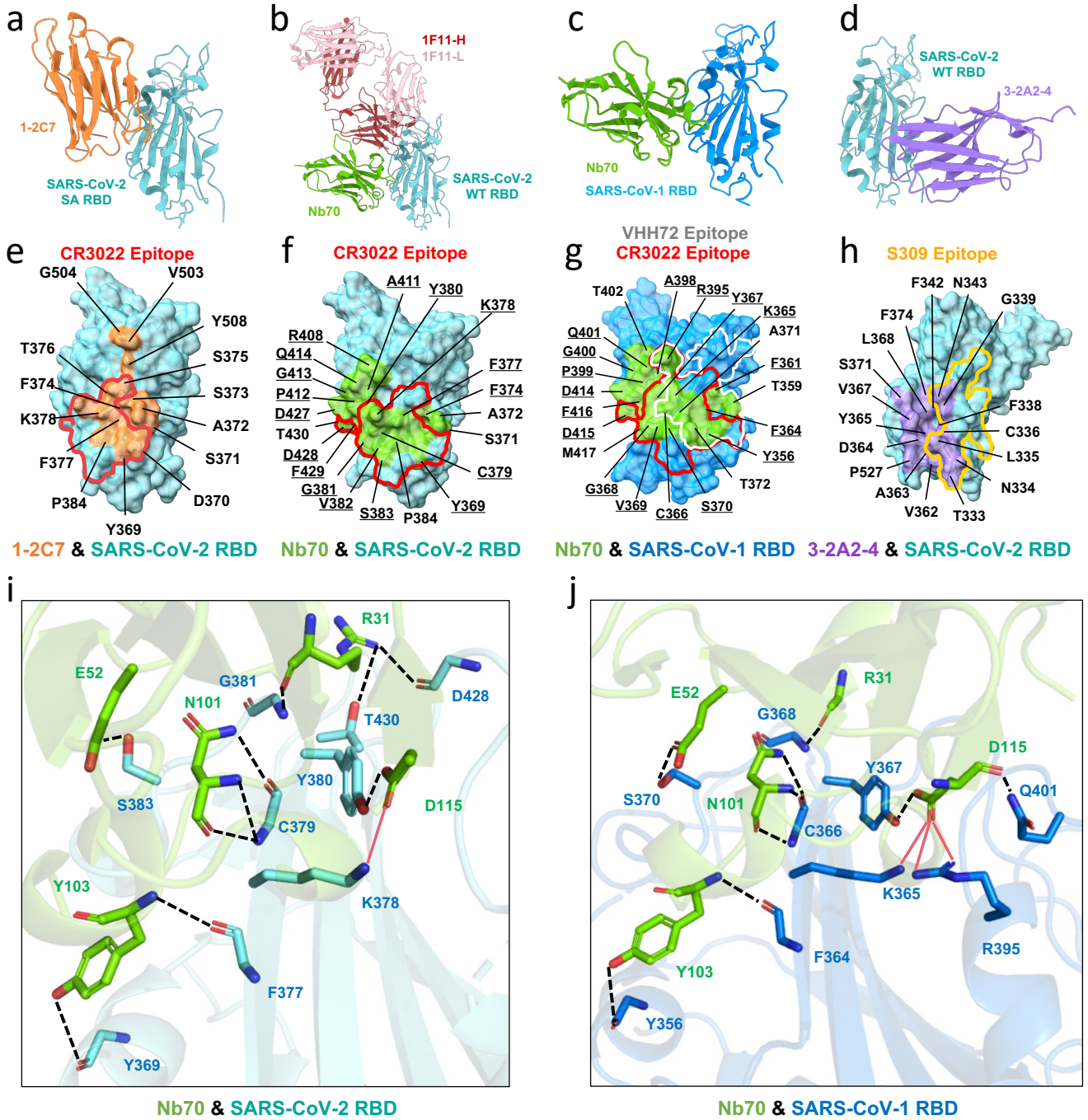


Figure 4

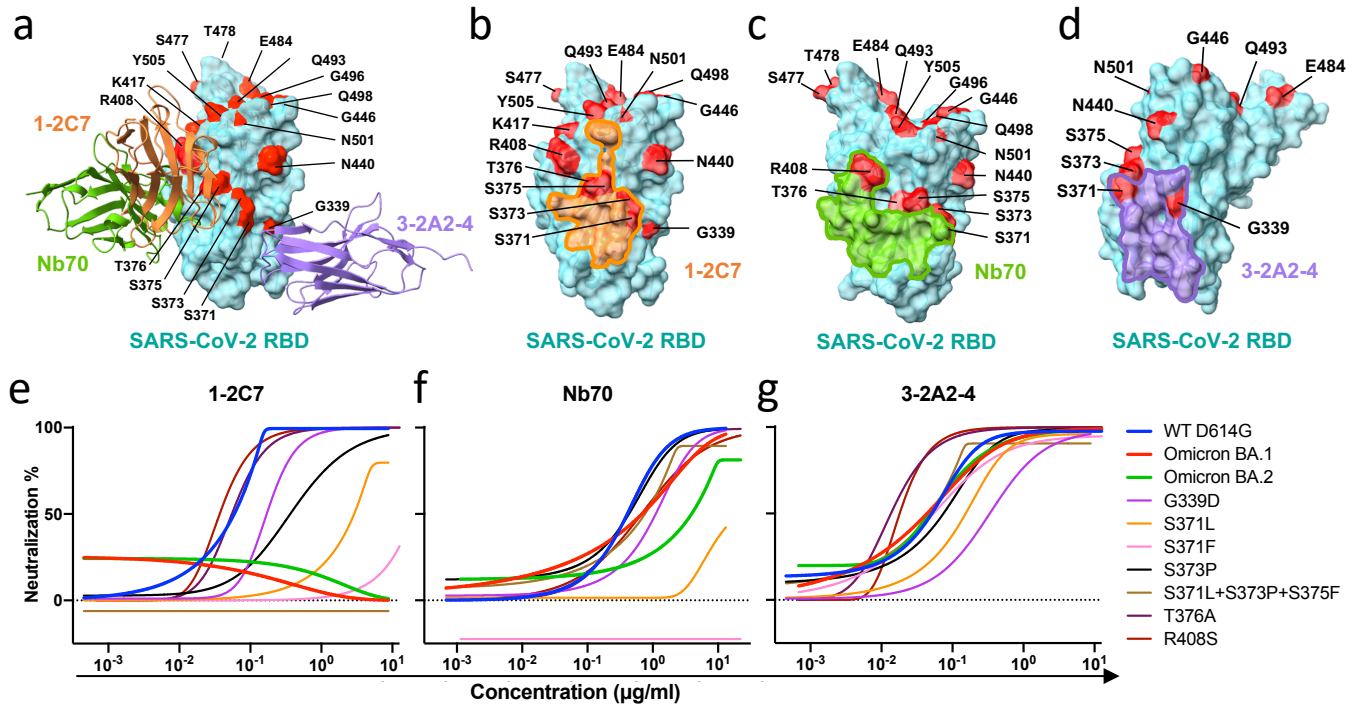
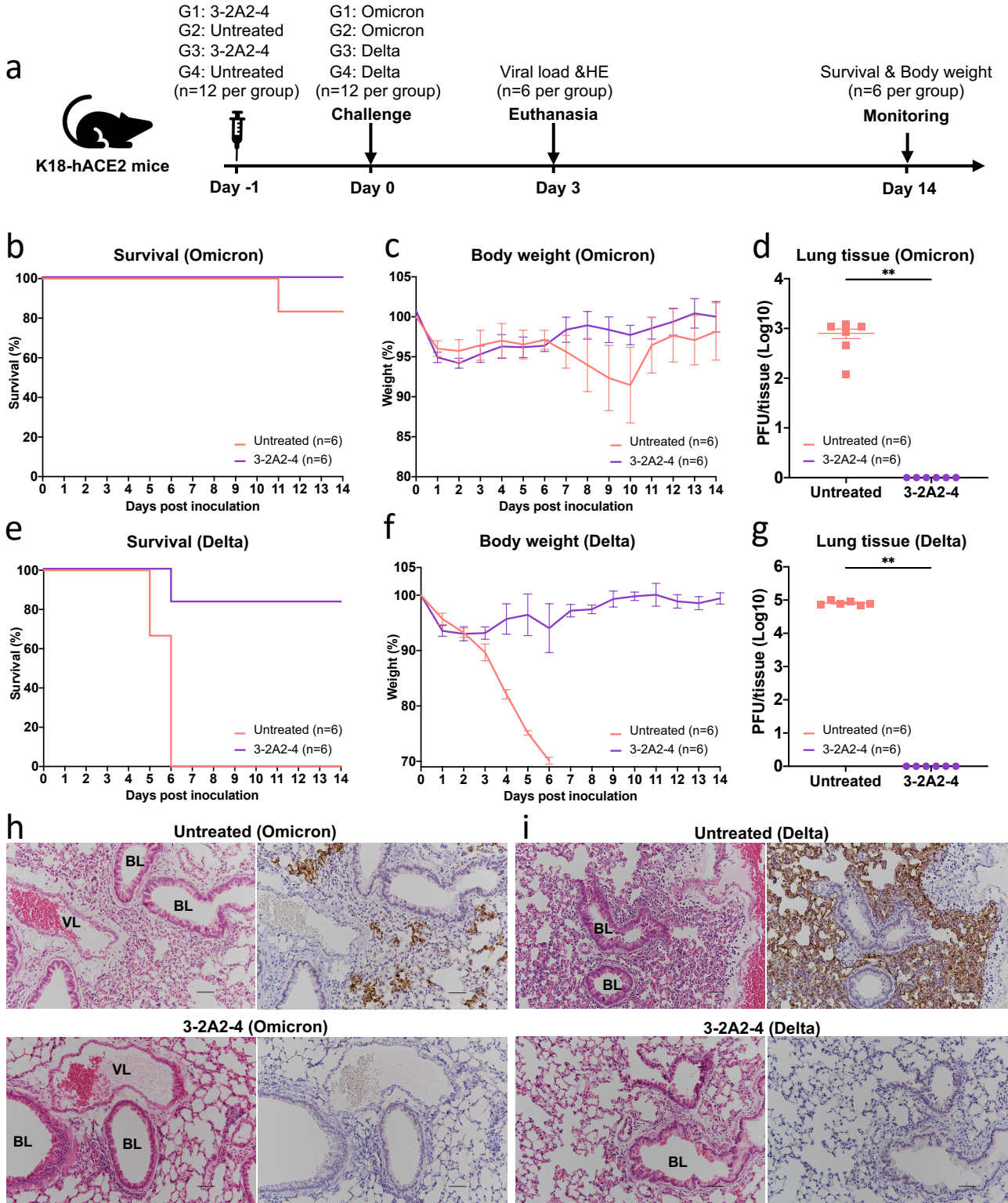


Figure 5



SUPPLEMENTAL INFORMATION

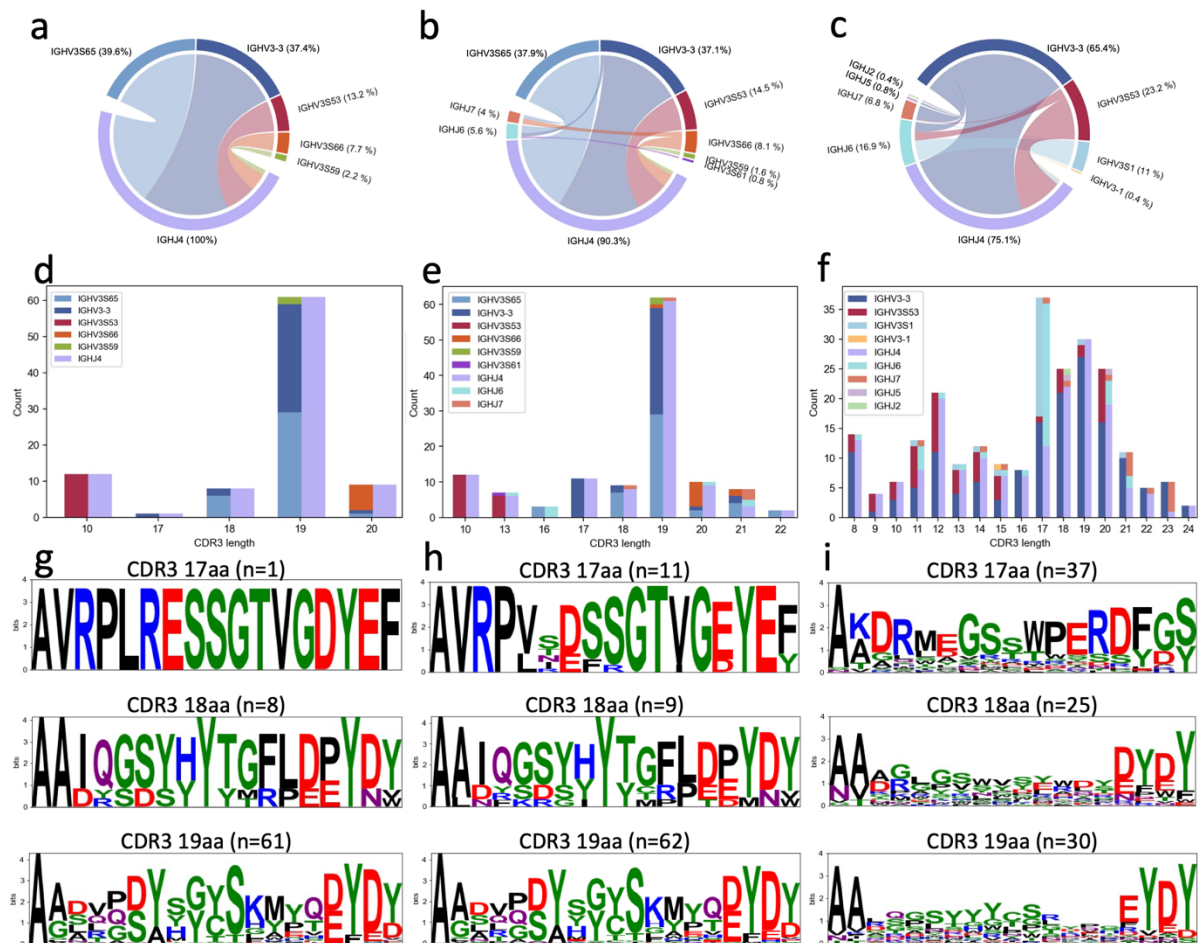


Figure S1, related to Figure 1. Genetic characterization and comparison between isolated and published nanobodies against SARS-CoV-2. (a, b, c) Chord diagrams comparing the V and J gene segments usage and pairing among the 91 cross-neutralizing, total 124 isolated, and published 237 nanobodies in the CoV-AbDab database. Each V and J segments are colored and indicated around the peripheral circle together with their percentage among the total number of nanobodies analyzed. V/J pairs are linked by colored arcs, and the size of which is proportional to the total number of nanobodies analyzed. **(d, e, f)** The bar plot showing the distribution and proportion of various CDR3 length among the 91 cross-neutralizing, total 124 isolated and 237 published nanobodies. The specific V and J gene usage associated with each CDR3 length are colored and shown. **(g, h, i)** Comparison of CDR3 logo sequence among the 91 cross-neutralizing, total 124 isolated, and 237 published nanobodies, analyzed separately for 17-residue (top), 18-residue (middle), and 19-residue (bottom) CDR3. The number of sequences analyzed for each CDR3 group are indicated.

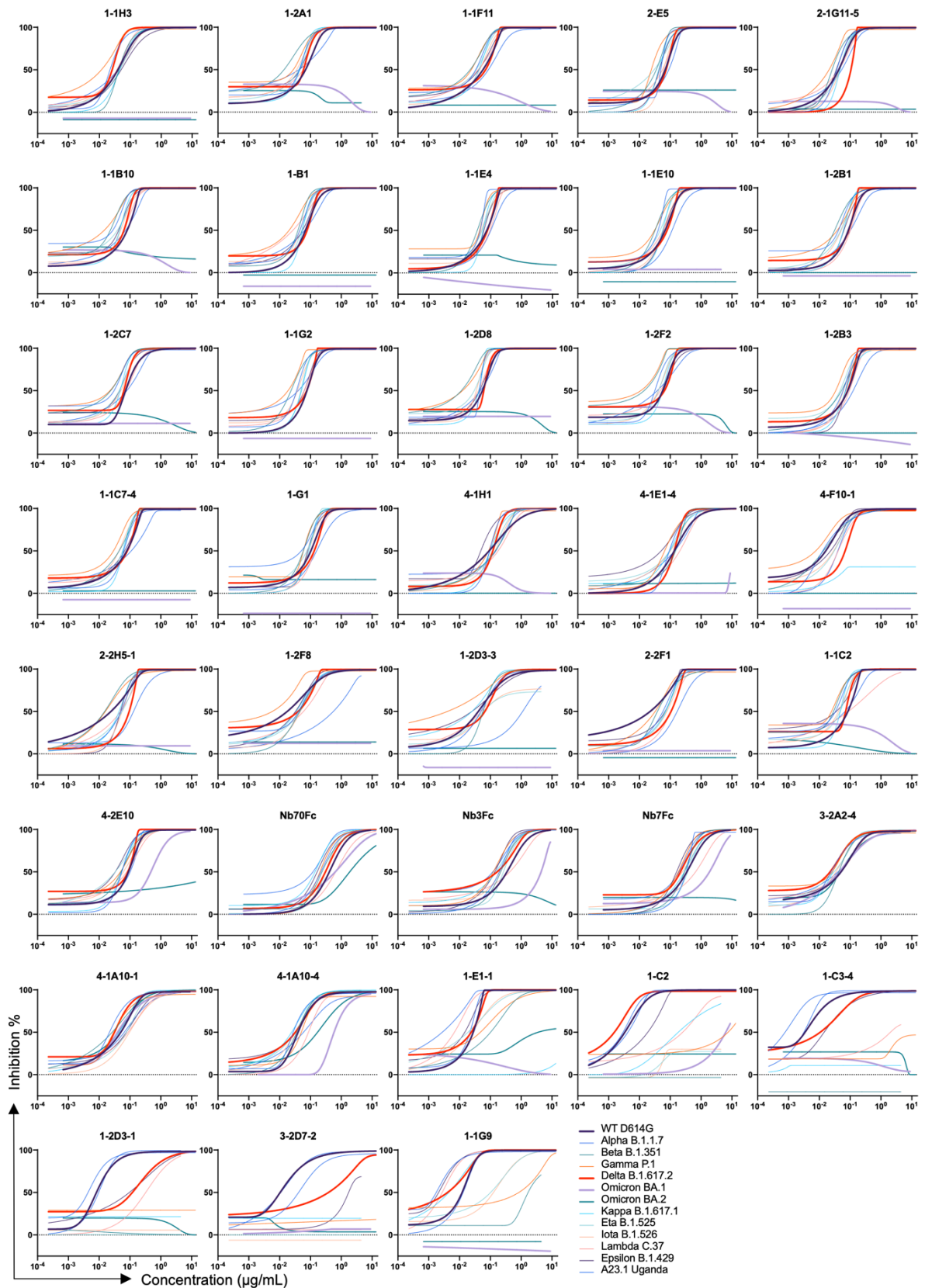


Figure S2, related to Figure 2. Neutralizing activity of isolated nanobodies against SARS-CoV-2 variants. Serial dilutions of each nanobody were evaluated against pseudoviruses carrying spike protein of prototype and variants of SARS-CoV-2. Neutralizing activity was defined as the percent reduction in luciferase activities compared to no antibody controls. Results presented are representatives of three independent experiments.

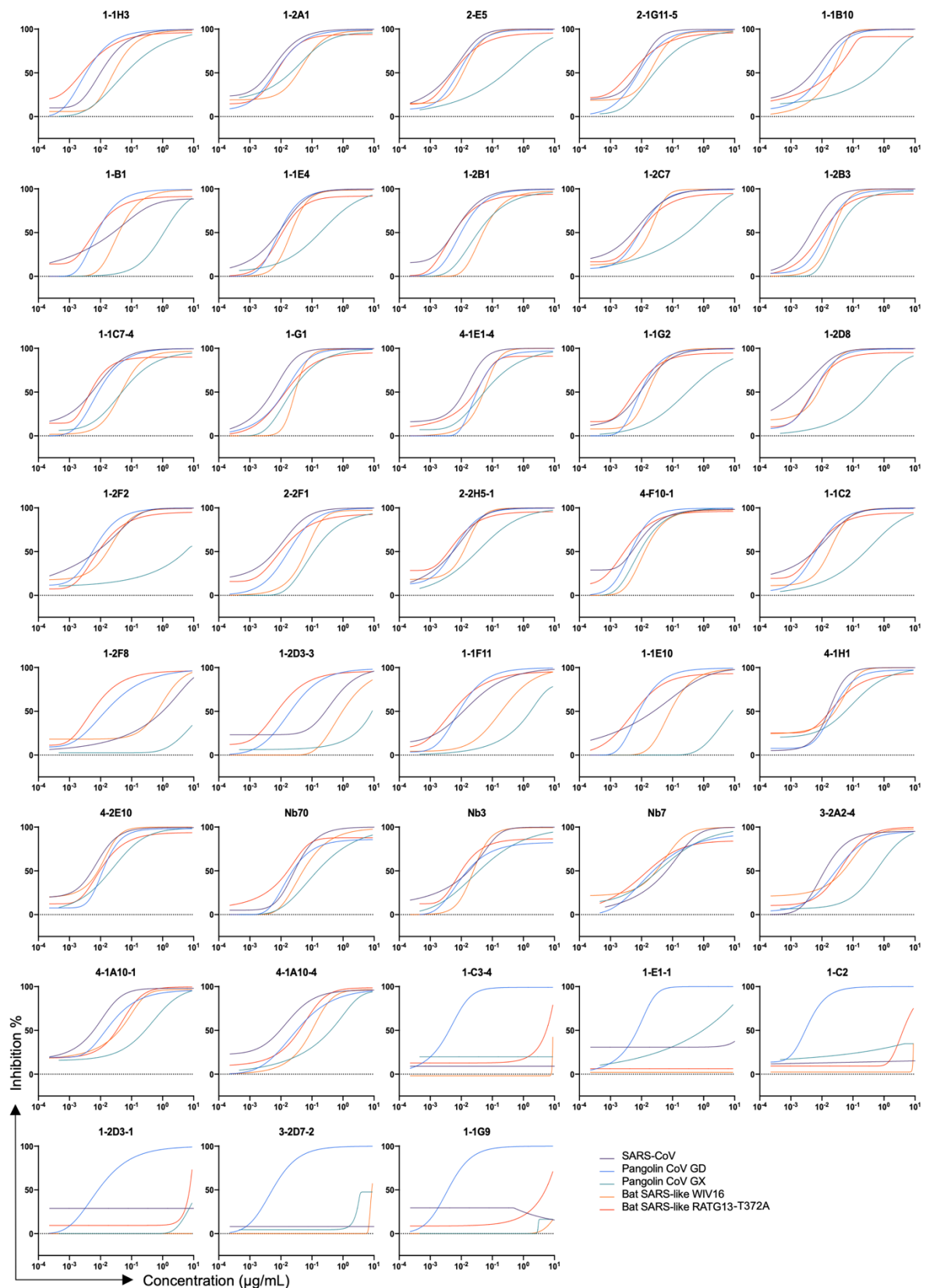


Figure S3, related to Figure 2. Neutralizing activity of isolated nanobodies against hACE2-dependent sarbecoviruses. Serial dilutions of each nanobody were tested against pseudoviruses carrying spike protein of various sarbecoviruses. Neutralizing activity was defined as the percent reduction in luciferase activities compared to no antibody controls. Results presented are representatives of three independent experiments.

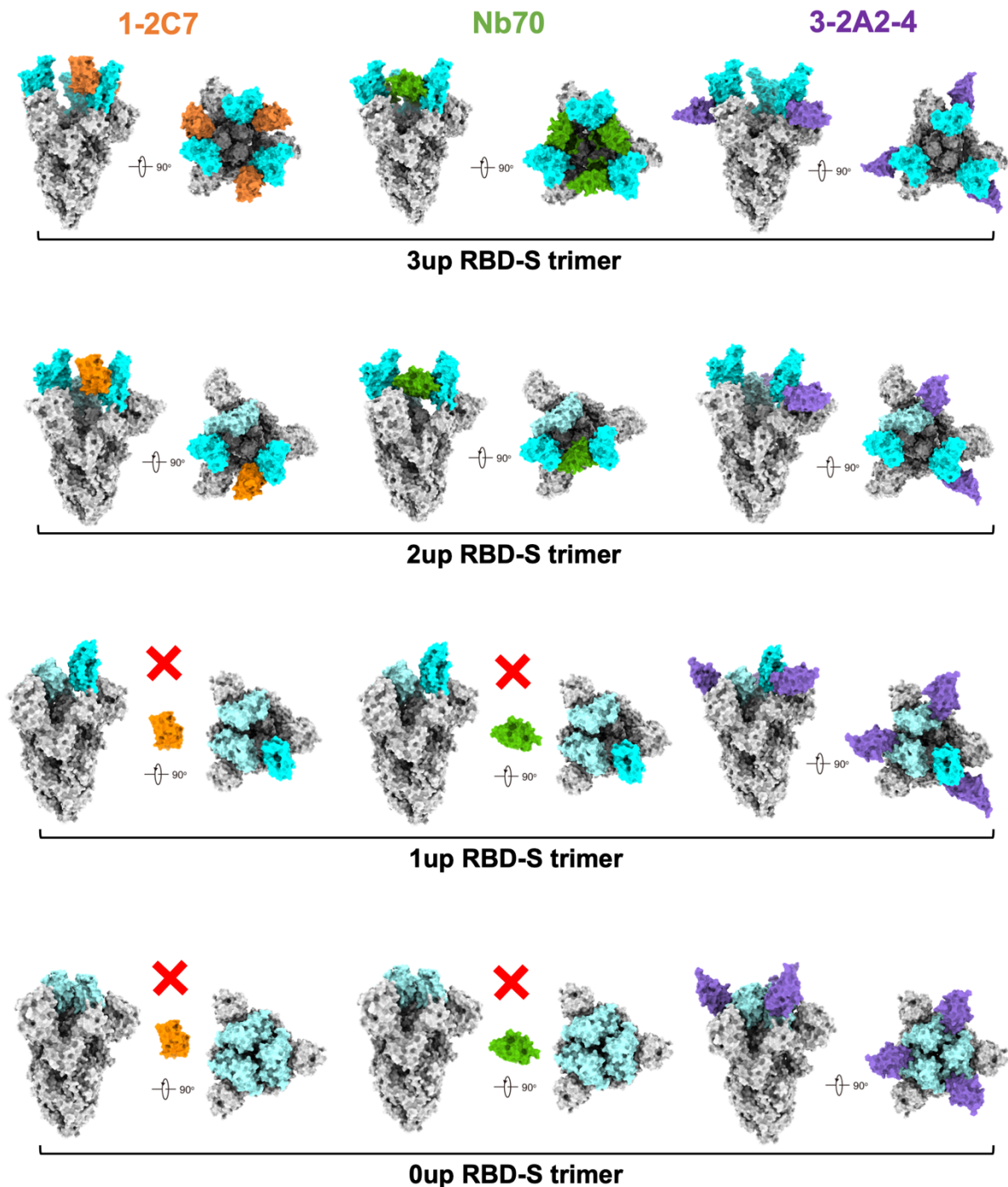


Figure S4, related to Figure 3. Proposed binding models of the three representative nanobodies to various RBD conformations in the context of prototype SARS-CoV-2 spike trimer. Crystal structures of 1-2C7, Nb70, and 3-2A2-4 bound to RBD are aligned to SARS-CoV-2 spike trimer in four different conformations: 1) 3up RBD-S trimer (PDB: 7KMS); 2) 2up RBD-S trimer (PDB: 7A93); 3) 1up RBD-S trimer (PDB: 6VYB); and 4) 0 up RBD-S trimer (PDB: 6VXX). The spike trimers are shown as a molecular surface, with up RBD colored in cyan, down RBD in light blue, NTD and S2 in grey. 1-2C7, Nb70, and 3-2A2-4 are colored in orange, green, and purple, respectively.

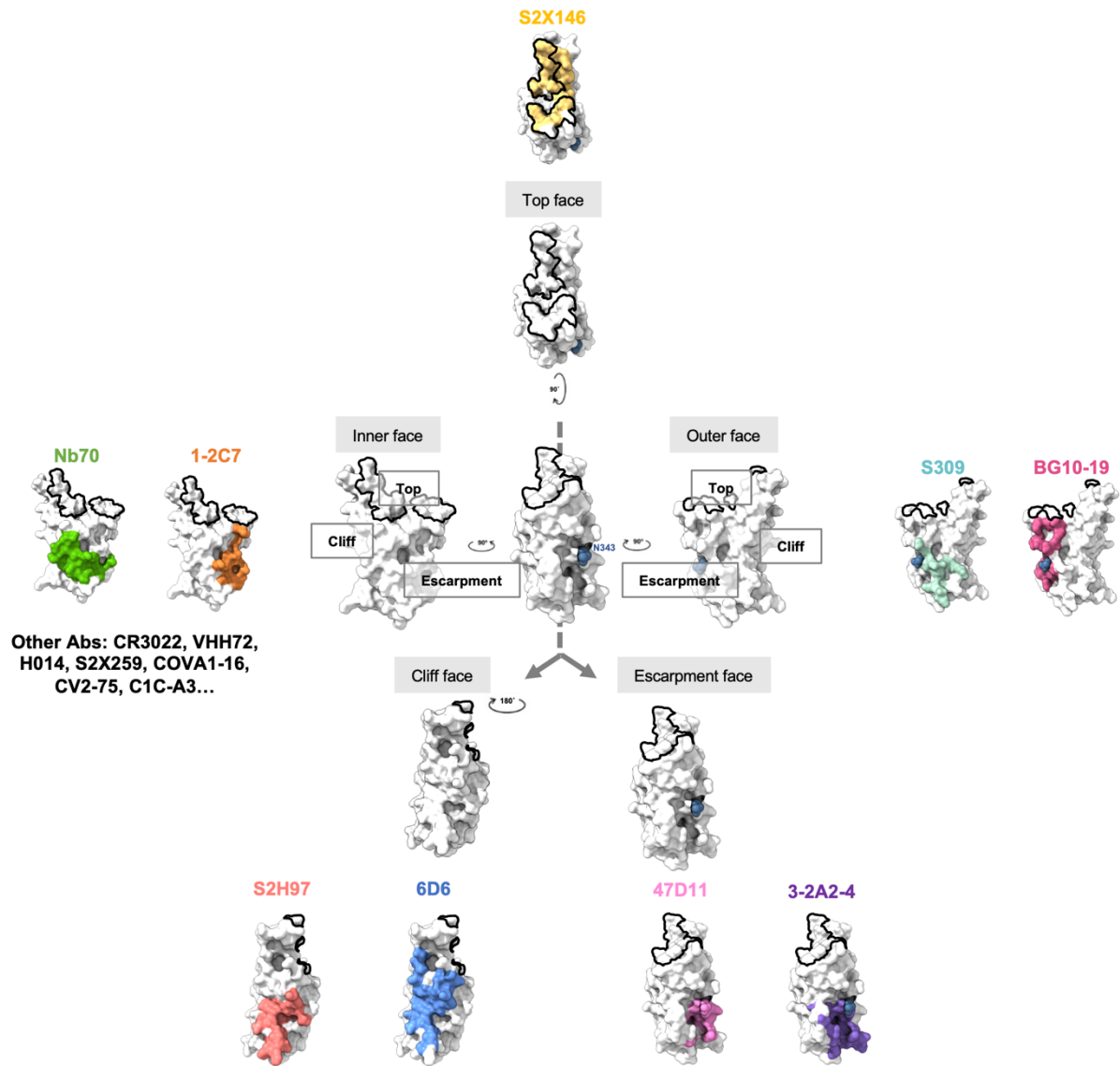


Figure S5. Structural illustration of SARS-CoV-1 and SARS-CoV-2 cross-neutralizing epitopes recognized by the three representative nanobodies and various published antibodies. Cross-neutralizing epitopes on the top face of RBD is recognized by S2X146, inner face by Nb70 and 1-2C7, outer face by S309 and BG10-19, cliff face by S2H97 and 6D6, and escarpment face by 47D11 and 3-2A2-4. The glycosylation site at position 343 (N343), conserved across the sarbecovirus subgenus, is colored in dark blue. The ACE2-binding site is outlined in black.

Table S1, related to Figure 3. Data collection and refinement statistics

	Nb70-SARS-CoV-1 RBD	Nb70-1F11 Fab- SARS-CoV- 2 WT RBD	1-2C7-SARS-CoV-2 SA RBD	3-2A2-4-SARS-CoV-2 WT RBD
Data collection				
Space group	P3 ₁ 21	P2 ₁ 2 ₁ 2 ₁	I23	P4 ₁ 2 ₁ 2
Cell dimensions				
a, b, c (Å)	108.413 108.413 94.119	94.629 95.47 104.139	143.619 143.619 143.619	89.168 89.168 129.154
α , β , γ (°)	90, 90, 120	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	50-2.4(2.486-2.4)	50-2.4(2.486-2.4)	50-1.8(1.864-1.8)	50-2.402 (2.488-2.402)
<i>R</i> _{merge}	0.135 (2.269)	0.213 (1.249)	0.172 (2.643)	0.152 (2.555)
<i>I</i> / <i>σ</i>	25.45 (1.10)	17.18 (1.74)	33.27 (1.57)	23.43 (1.54)
Completeness (%)	99.77 (99.84)	99.54 (99.89)	99.93 (100)	97.37 (83.09)
Redundancy	17.9 (14.3)	12.2 (8.5)	38.8(31.2)	22.1 (20.9)
Refinement				
Resolution (Å)	33.24-2.4	45.62-2.4	25.39-1.8	28.33-2.402
No. reflections	25337 (2493)	37381 (3676)	45590 (4537)	20419 (1695)
<i>R</i> _{work} / <i>R</i> _{free}	19.98/23.78	19.53/24.36	17.99/20.97	22.96/28.74
No. atoms				
Protein	375	745	323	315
Ligand/ion	28	14	39	49
Water	51	216	225	30
B-factors				
Protein	64.96	43.35	44.17	69.22
Ligand/ion	103.73	52.62	67.20	69.84
Water	61.38	62.66	46.31	77.73
R.m.s. deviations				

Bond lengths(Å)	0.008	0.008	0.007	0.010
Bond angles (°)	1.01	0.99	0.87	1.25
Ramachandran				
Favored (%)	96.54	97.28	97.15	93.89
Allowed (%)	3.46	2.59	2.85	5.79
Outliers (%)	0.00	0.14	0.00	0.32

Table S2, related to Figure 3. Contact residues of the Nbs-RBD interfaces

Nb70	SARS-CoV-1 RBD	Nb70	SARS-CoV-2 RBD	1-2C7	SARS-CoV-2 RBD	3-2A2-4	SARS-CoV-2 RBD
Q1	G400	Q1	G413	Q1	L378	S29	D364
	T402	T28	D427	L31	Y369		V367
T28	D414		D428		P384	L31	L335
	D415	R31	Y380	R57	A372		V362
R31	Y367		G381	R100	T376		A363
	G368		D428		F377		D364
	D415		F429		L378		P527
	F416		T430	P101	Y369	V33	L335
	M417	Y32	Y380		F374	L53	T333
Y32	Y367		P412		S375		N334
	P399	E52	S383		F377		L335
E52	S370	W53	G381	S102	Y369		V362
W53	V369		V382		S371	D54	T333
	G368		S383		A372	E98	L335
	S370	G100	C379		S373	N99	V367
G100	C366		Y380		F374	G100	A363
	Y367	N101	L378		F377		D364
N101	L365		C379	A103	Y369		V367
	C366		G381		N370	G101	L335
	G368		V382		S371		C336
	V369		P384	H104	N370		F338
	A371	Q102	F377		S371		G339
Q102	F364		L378		A372	F102	F338
	L365		P384	Y105	S371		G339
	A371	Y103	Y369		A372		F342
Y103	Y356		F377		F374		N343
	F364		P384		S375		D364
	A371	Y104	S371	Y109	V503		Y365

	T372		A372	T111	V503		L368
Y104	T359		F374		G504	F103	F342
	F361	Y114	R408	Q112	S375		N343
N112	R395	D115	L378		Y508		V367
E113	R395		Y380	D114	L378		S371
Y114	R395		R408				F374
D115	L365		A411			Y104	G339
	Y367		Q414				N343
	R395	F116	P412				
	A398		G413				
	Q401		Q414				
F116	P399	W117	R408				
	G400						
	Q401						
