Broad neutralization of SARS-CoV-2 variants by an inhalable bispecific single-domain antibody

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24

25 **Abstract**

26 The effectiveness of SARS-CoV-2 vaccines and therapeutic antibodies has been
 27 limited by the continuous emergence of viral variants, and by the restricted diffusion
 28 of antibodies from circulation into the sites of respiratory virus infection. Here, we
 29 report the identification of two highly conserved regions on Omicron variant RBD
 30 recognized by broadly neutralizing antibodies. Based on this finding, we generated a
 31 bispecific single-domain antibody that was able to simultaneously and synergistically
 32 bind these two regions on a single Omicron variant RBD as revealed by Cryo-EM
 33 structures. This inhalable antibody exhibited exquisite neutralization breadth and
 34 therapeutic efficacy in mouse models of SARS-CoV-2 infections. The structures also
 35 deciphered an uncommon cryptic epitope within the spike trimeric interface that may
 36 have implications for the design of broadly protective SARS-CoV-2 vaccines and
 37 therapeutics.

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47 Since the emergence of the original strain in late 2019, five SARS-CoV-2 variants,
 48 namely Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and the
 49 newly identified Omicron (B.1.1.529) have been defined as variants of concern
 50 (VOCs) by the World Health Organization, inducing waves of prevalence. Notably,
 51 the Omicron variant bears over 30 mutations in the viral spike protein with 15
 52 mutations in the receptor-binding domain (RBD) (**fig. S1A**), significantly higher than
 53 other VOCs that only possess no more than three RBD mutations. Remarkable
 54 resistance of the Omicron variant against the neutralization by antibodies and serum
 55 have been reported, challenging the protective efficacy of the vaccines and therapeutic
 56 antibodies(1-4).

57 To assess the extent of immune escape of the SARS-CoV-2 Omicron variant, we
 58 first mapped its mutations on the RBD, which is the major target for neutralizing
 59 antibodies, and compared these regions with the binding epitopes of several reported
 60 antibodies (**fig. S1B**). According to their spatial location on spike, the RBD-targeting
 61 antibodies can be grouped into three types: those bind to the angiotensin converting
 62 enzyme 2 (ACE2) receptor-binding motif (RBM), the cryptic epitopes hidden or
 63 partially hidden inside the trimeric interface, and the lateral surface epitopes outside
 64 the trimeric interface, respectively (**Fig. 1A**). It is notable that the majority of RBD
 65 mutations (9 out of 15) found in Omicron were located in the RBM and the other 6
 66 mutations resided in the ridge side of the RBD core, leaving the cryptic site hidden
 67 inside the spike trimer and a part of lateral surface essentially unchanged. In
 68 alignment with this, more mutations were involved in the epitopes of apex

69 RBM-binding antibodies than the other two clusters (**Fig. 1B**).

70 To confirm this finding, we measured the binding and neutralization titer of
 71 plasma collected from 7 vaccine recipients who received three doses of inactivated
 72 SARS-CoV-2 vaccine. The average median binding titer (ED_{50}) of serum declined by
 73 12.8-fold for Omicron RBD, while the neutralizing potency (average median
 74 neutralizing titer, ID_{50}) decreased by 5.1-fold against Omicron pseudovirus (**Fig. 1C**
 75 **and figs. S2, A and B**). Interestingly, we found that some vaccinees had high binding
 76 and neutralizing antibody titers against the wild-type (WT) SARS-CoV-2, but
 77 relatively low neutralization titers against the Omicron variant. To understand the
 78 underlying mechanism, we performed epitope binning by saturating the RBM of
 79 SARS-CoV-2 RBD with ACE2, and then loaded plasma to allow the binding of
 80 non-RBM antibodies (**Fig. 1D and figs. S2, C and D**). The Spearman's rank relation
 81 test was applied to measure the potential relationship between ID_{50} with binding of
 82 RBM or non-RBM antibodies. This analysis revealed a high correlation of the
 83 percentage of non-RBM antibodies in plasma and the Omicron neutralizing potency,
 84 implying the functional importance of non-RBM antibodies. Consistently, we found
 85 that CB6(5) and P2B-2F6(6), monoclonal antibodies that bind to RBM and potently
 86 block ACE2 from binding to the SARS-CoV-2 RBD, both lost completely the binding
 87 ability to Omicron RBD and neutralizing potency against Omicron pseudovirus (**Fig.**
 88 **1E and fig. S3**). In contrast, the potency of some antibodies recognizing the lateral
 89 surface epitopes, exemplified by S309(7) and n3113v, a mutant of previously reported
 90 human single-domain antibody n3113(8, 9), was less affected by Omicron mutations

(**Fig. 1E and fig. S3**). Similarly, the antibody CR3022 targets highly conserved cryptic epitopes inside the trimeric interface(*10, 11*), and none of the mutations found in Omicron were involved in CR3022 interaction (**Fig. 1B**). As a consequence, the binding of CR3022 to Omicron RBD was only partially diminished (**Fig. 1E and fig. S3**). Such an indiscriminant influence by Omicron on antibody potency was also found in n3130v, a variant of human single-domain antibody n3130(8) which may have epitopes partially overlapped with CR3022 as revealed by its competition with CR3022 for the binding to SARS-CoV-2 RBD. Taken together, our results suggest that the ACE2-competing antibodies, which constitute the majority of RBD-specific antibodies elicited by natural infection or vaccination(*12*), may play a pivotal role in the immune escape of the SARS-CoV-2 Omicron variant. Furthermore, two conserved RBD regions targeted by broadly neutralizing antibodies were identified, including a part of lateral surface (exemplified by n3113v epitope), and the cryptic site inside the trimeric interface (exemplified by n3130v epitope).

To further ensure neutralizing breadth, we connected two single-domain antibodies (n3113v and n3130v) that recognize conserved but distinct RBD regions using a flexible glycine-serine linker. The resulting bispecific antibody, designated as bn03, exhibits superior biophysical properties, and exists in pure monomeric form with a molecular mass of 27 kDa as demonstrated by size exclusion chromatography (**Fig. 2A**). The synergistic effect of the two arms of bn03 can be demonstrated by the fact that it exhibited more potent neutralizing efficacy against SARS-CoV-2 than the cocktail of n3113v and n3130v (**Fig. 2B**), and that bn03 was still able to bind RBD

113 that has already been saturated with either n3113v or n3130v (**fig. S4**). Furthermore,
 114 bn03 could broadly neutralize WT and all the five VOCs, and also strongly bind all
 115 the RBDs (**Figs. 2, C and D**). To further investigate the neutralization mechanism of
 116 bn03, we determined the cryo-EM structure of the prefusion stabilized SARS-CoV-2
 117 Omicron S ectodomain trimer complexed with bn03 (Omicron S-bn03). During data
 118 processing, three conformational states were observed and we determined three
 119 structures to the resolutions of 3.3 Å, 2.9 Å and 3.0 Å, respectively (**figs. S5 and S6**).

120 In S-bn03 structure of State 1 (~15% particles), two RBDs are in the down
 121 position at a slightly different angle, allowing only one down RBD binds a
 122 single-domain antibody n3113v. The other RBD is in the “up” conformation, and
 123 binds n3113v and n3130v simultaneously, each targeting a unique epitope. In S-bn03
 124 “State 3” structure (~30% particles), only one RBD is in the down position which
 125 binds a n3113v. The other two RBDs are in the up position, both interacting with
 126 n3113v and n3130v. Majority of particles are at the State 2 conformation (~55%
 127 particles), which includes one n3113v-bound down RBD, one n3113v-n3130v-bound
 128 up RBD and one n3113v-bound RBD in the half-up state, presenting an intermediate
 129 state that the RBD domain is flipping from down to the up state (**Fig. 2E**). Since most
 130 apo-state spike trimers exit in the conformation with one RBD up, the result suggested
 131 that bn03 tends to induce the 2-up conformation.

132 Both n3113v and n3130v target epitopes outside the RBM region (**Fig. 3A**). The
 133 n3113v targets an epitope that is exposed in both up and down state RBD. The
 134 binding of n3113v with S-RBD buries 973 Å² surface area. A total of 30 residues from

135 RBD are involved in the interaction. The interactions of n3113v is mainly mediated
 136 by hydrogen bonds between the complementarity determining regions (CDRs) and
 137 RBD. Residues R346, T345, N354, Y351 and T470 from CRDs participate in the
 138 interaction with RBD, forming 8 pairs of hydrogen bonds and 1 pair of salt bridge.
 139 The R346 is engaged in 2 hydrogen bonds (with W99 and W108) and a salt bridge
 140 (with D106) interactions. Other than CDR, the framework region 44-48 also forms
 141 tight interactions with RBD by forming hydrogen bonds and hydrophobic interactions.
 142 The n3130v targets an epitope in between the cryptic side and the outside, which is
 143 only accessible when the RBD is in the up conformation. Totally 20 residues from
 144 RBD are involved in the interactions, burying 842 Å² surface area. All three CDRs are
 145 involved in the binding by forming hydrogen bonds and hydrophobic interactions.
 146 CDR3 formed 5 hydrogen bonds with the loop region of RBD (515-519). H519 is
 147 clapped by F107 and Y105 by π - π stacking. F102 also forms π - π stacking with F429.
 148 Besides, the residues D30 on CDR1 and Y59 on CDR2 are also involved in the
 149 interaction by forming hydrogen bonds with E465, R157 and N394 respectively.

150 Totally 49 out of 50 residues involved in n3113v and n3130v interactions remain
 151 unchanged between WT RBD and Omicron RBD (**Fig. 3B**). K493 is the only residue
 152 that was mutated from Q. K493 is involved in a π -cation interaction with Y58 from
 153 n3113v. The mutation from Q and K did not affect the interaction, thus not affecting
 154 the binding affinity of n3113v on RBD. The well conserved epitopes explain the
 155 broad neutralization activity of bn03.

156 It has been reported that the camelid-derived nanobodies can be delivered via

157 inhalation for the treatment of pulmonary disease due to their favorable properties of
158 small size, high stability and solubility(13, 14). Because of the similarities in
159 structures and biophysical properties between nanobodies and human single-domain
160 antibodies, here we explored the potential of delivering human single-domain
161 antibodies via inhalation route. For this purpose, we employed a previously described
162 high-pressure microsyringe for the intratracheally administration of antibodies to the
163 lung of mice as a fine aerosolized spray(15). This device enables quantitative and
164 uniformly intratracheal aerosol dosing of therapeutics to mice(16). Besides, the
165 n3113v was conjugated with DyLight 800 to enable the *in vivo* imaging of the
166 single-domain antibody. To assess the tissue distribution, Balb/c mice were treated
167 with n3113v at a dose of 12 mg/mL through inhalation (INH) or intraperitoneal
168 injection (IP), respectively (**Fig. 4A**). Bio-imaging of the mice at different time points
169 indicated that the inhalation administration effectively delivered the single-domain
170 antibodies to chest cavity, while the antibodies retained essentially almost in abdomen
171 in the IP group (**Fig. 4B**). We also dissect the mice and measured the fluorescence in
172 different organs at 4 and 6 hours post antibody administration (**Fig. 4C**). Most of the
173 antibodies were detected in lung in the INH group. In contrast, the antibodies
174 concentrated mainly in liver and kidney but very few in lung in the IP group.
175 Therefore, we further quantified the antibody concentrations in lung and plasma, and
176 confirmed that a dramatically larger number of antibodies could be delivered to lung
177 via the route of inhalation than IP administration (**Fig. 4D**). Next, we used the Next
178 Generation Impactor (NGI), a high-performance cascade impactor with seven stages,

179 to investigate the aerosol performances of antibodies by a vibrating mesh nebulizer
180 (**Fig. 4E**). The human single-domain antibodies displayed well drug deposition on
181 stage 2 through stage 6, representing the potential for aerosol deposition within the
182 respiratory tract. In contrast, most of the IgG antibodies remained in the device and
183 barely distributed to the impactor stages under the same conditions. These data
184 confirmed inhalation as a favorable route for delivery of human single-domain
185 antibodies.

186 Next, we evaluated the therapeutic efficacy of inhaled human single-domain
187 antibodies in human ACE2 (hACE2)-transgenic mouse models infected with the
188 authentic SARS-CoV-2 virus. Post intranasal injection of SARS-CoV-2, mice were
189 treated daily with a dose of n3113v by INH or IP. The viral copies in lung were
190 slightly reduced in the IP group, which may due to the short half-time of
191 single-domain antibody in the circulation, but significantly reduced by inhalation of
192 n3113v (around 1,000-fold, **Fig. 4F**). We further evaluated the *in vivo* efficacy of
193 bn03, which also displayed well inhalability on NGI (**fig. S7A**), and unchanged
194 binding efficacy before or after inhalation by the high-pressure microsprayer (**fig.**
195 **S7B**). The single-dose pharmacokinetics study in mice demonstrated the much higher
196 concentrations of bn03 in lung than in blood circulation via inhalation (**fig. S7C**). Two
197 hACE2-transgenic mouse models of mild and severe symptoms were used for the
198 evaluation, and bn03 exhibited robust therapeutic effect in both models by reducing
199 the viral RNA copies (**Figs 4, G and I**) and alleviating the lung injury through inhaled
200 administration (**Figs 4, H and J**). Co-culture of the lung homogenates of mice from

201 the severe symptom group indicated that inhalation of bn03 almost eliminated entirely
202 the live virus in lung (**Fig. 4K**), reflecting the efficiency of the bispecific antibody.

203 The continuous emergence of new SARS-CoV-2 variants highlights the urgent
204 need to develop broad-spectrum vaccines and antibodies. Our study identified two
205 highly conserved regions on RBD that can be recognized by broadly neutralizing
206 antibodies, and showed the superiority of the novel cryptic epitope revealed by
207 n3130v in evading viral escape. Moreover, our study also demonstrates that human
208 single-domain antibodies can be efficiently delivered to lung and effectively treat
209 pulmonary disease via inhalation route. They are derived from fully human sequences,
210 and thus may have higher safety profile and clinic efficacy compared to
211 camelid-derived nanobodies. Importantly, the bispecific single-domain antibody bn03
212 was able to simultaneously and synergistically bind two distinct epitopes on a single
213 RBD of the SARS-CoV-2 Omicron variant. Its small molecular size (27 kDa) not only
214 confers inhalable properties, but also enables the deep penetration of bn03 into
215 trimeric interface of the spike protein to target the highly conserved cryptic epitopes.
216 Because of its unique epitope profile, we propose that such antibody may prove to
217 possess exceptional neutralization breadth against current and future SARS-CoV-2
218 variants.

219

220 **Figure legend**

221 **Fig. 1. Non-RBM antibodies confer resistance against the Omicron variant. (A)**

222 Epitope clustering of RBD-targeting antibodies on spike and RBD. The Omicron S

223 was shown as surface with RBD colored in grey. The mutations were highlighted in
224 red. The three classes of epitopes were circled in S and highlighted in RBD as blue,
225 green and yellow, respectively. **(B)** The amino-acid mutations in RBD region of
226 Omicron variant were shown as colored boxes. The key mutations found in binding
227 sites of three classes of antibody, such as RBM-binding site, lateral site and cryptic
228 site, were displayed. **(C)** Binding and neutralization of vaccinee plasma against WT
229 and Omicron variant. Values denoted the geometric mean titer. **(D)** Illustration of
230 RBD mAbs, RBM mAbs and non-RBM mAbs in plasma, as measured by BLI (left).
231 Correlation coefficient between plasma neutralization ID₅₀ against the Omicron
232 variant with RBM mAbs or non-RBM mAbs (right), analyzed by Spearman's rank
233 correlation test. **(E)** The binding affinity and neutralization of six antibodies. Values
234 indicated the fold change relative to WT.

235 **Fig. 2. The design of broadly neutralizing bispecific single-domain antibody bn03**
236 **and Omicron S-bn03 complex structure.** **(A)** Size exclusion (SEC) chromatography
237 profile of the bispecific antibody bn03. **(B)** Neutralization of SARS-CoV-2
238 pseudoviruses by a panel of single-domain antibodies, including n3113v, n3130v,
239 combination of n3113v and n3130v, and bn03. Three independent experiments were
240 performed. **(C)** Neutralizing potency of bn03 against WT and several variants of
241 concern pseudoviruses including the Alpha, Beta, Gamma, Delta, and Omicron. **(D)**
242 Binding affinity of bn03 to WT and five VOCs, as measured by BLI. **(E)** Cryo-EM
243 structures of Omicron spike in complex with bispecific antibody bn03. Two
244 perpendicular views of Omicron spike-bn03 are deciphered as surface, with n3113v in

245 magenta, n3130v in blue, and the trimeric spike in cyan, pink and yellow. Bispecific
246 antibody bn03 bound to Omicron S trimers in 3 states.

247 **Fig. 3. Mutations on Omicron RBD are unlikely to affect epitopes of bn03. (A)**

248 Close-up view of the interactions between bn03 and Omicron RBD. The Omicron
249 RBD is displayed in yellow surface. N3113v and n3130v are shown as cartoon
250 colored in magenta and blue, respectively. The interaction of n3113v **(B)** and n3130v
251 **(C)** with Omicron RBD. The residues involved in interactions are represented as
252 sticks. Polar interactions are indicated as dotted lines. **(D)** The epitopes of n3113v and
253 n3130v on Omicron RBD. Omicron RBD is shown as yellow surface. Four degrees of
254 surface representation of Omicron RBD with epitopes of n3113v and n3130v
255 highlighted in magenta and blue, respectively are shown. The mutations in Omicron
256 are colored in red.

257 **Fig. 4. Bn03 potently inhibit SARS-CoV-2 infection by inhalation. (A)** Schematic

258 diagram of n3113v biodistribution in mice by inhalation and intraperitoneal injection.
259 **(B)** Bio-imaging of mice body at different time-points (left) and fluorescence intensity
260 ratio of thoracic cavity against abdomen (right). **(C)** Organ imaging (left) and
261 quantification of fluorescence signal (right). Lu: lung, K: kidney, H: heart, S: spleen,
262 Li: liver. **(D)** Concentration of n3113v in lung (left) and the ratio of n3113v in lung
263 relative to plasma. **(E)** The aerosol performances of n3113v. The cutoff diameter (μm)
264 of particles was 0.98 (Moc), 1.36 (Stage 7), 2.08 (Stage 6), 3.3 (Stage 5), 5.39 (Stage
265 4), 8.61 (Stage 3), 14.1 (Stage 2), above 14.1 (Stage 1). The Device represents
266 antibody failed to become aerosolized. **(F)** Experimental design of therapeutic

267 evaluation of n3113v by inhalation and intraperitoneal injection and lung viral load in
268 authentic SARS-CoV-2 infected hACE2-transgenic mice. **(G-J)** Therapeutic efficacy
269 of bn03 in hACE2-transgenic mice with mild (G) or severe symptom (I) by inhalation
270 (n=3-5). Lung viral load (G, I) and lung histology (H, J) in infected mice with
271 indicated treatments. **(K)** Determination of FFU with serum in 1:10 dilutions.
272 Ordinary one-way ANOVA was used in the statistical analysis. *** $p < 0.001$,
273 ** $p < 0.01$, * $p < 0.05$, ns means no significance.

274

275 **Data availability statements**

276 The cryo-EM map and the coordinates of SARS-CoV-2 Omicron S complexed with
277 Bn03 have been deposited to the Electron Microscopy Data Bank (EMDB) and
278 Protein Data Bank (PDB) with accession numbers EMD-32501 and PDB 7WHJ
279 (State 1, two down and 1up RBDs), EMD-7WHI and PDB 32500 (State2, 1up, 1down,
280 and 1 half-up RBDs), EMD-32503 and PDB 7WHK (State3, 2 up and 1 down RBDs)
281 and EMD-32502 (NTD-RBD-Bn03 local refinement).

282

283 **Ethics statements**

284 All the procedures related to animal handling, care, and the treatment were performed
285 and approved by the Ethics Committee of the School of Basic Medical Sciences at
286 Fudan University in accordance with the recommendations in the Guide for the Care
287 and Use of Laboratory Animals of Fudan University. The authentic SARS-CoV-2
288 infection and sample collection were performed in BSL-3 lab of Fudan University.

289

290 **Acknowledgements**

291 We are grateful to Qi Shao and Zhixin Jing from Sinepharm for the support in NGI
292 experiments, and Q. Wang, Y. Zhong and other staff from Core Facility of
293 Microbiology and Parasitology, Shanghai Medical College, for help with experiments.

294 This work was supported by grants from the National Key R&D Program of China
295 (2019YFA0904400), National Natural Science Foundation of China (82041003,
296 81822027, 32070938), Chinese Academy of Medical Sciences (2019PT350002),
297 Shanghai Municipal Health Commission (GWV-10.2-XD01, GWV-10.2-YQ06) and
298 Science and Technology Commission of Shanghai Municipality (20411950402,
299 20XD1401200, 18DZ2210200, 20DZ2254600, 20DZ2261200).

300

301 **Conflict of interests**

302 C.L., Y.W. and T.Y. are listed as inventors on two patent applications related to this
303 work.

304

305 **Author contributions**

306 T.Y., Y.W., L.S. and C.T. initiated, planned and supervised the project. C.L. performed
307 most of the experiments with assistance from K.H., Y.K., Y.Z., Y.X., Q.D., L.L., and
308 S.J., W.Z., X.Z., M.Z., Z.C. and L.S. collected Cryo-EM data and solved the
309 structures. Y.Z. and X.G. performed animal studies in BSL-3 lab. W.S. performed
310 pseudovirus neutralization assay. The manuscript was written by T.Y., Z.Y. and Y.W.
311 and reviewed, commented and approved by all the authors.

312

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349

Figure 1

Figure 2

Figure 3

Figure 4

Supplementary Figure 1

A

B

Supplementary Figure 2

A

B

C

D

	Vaccinee 1	Vaccinee 2	Vaccinee 3	Vaccinee 4	Vaccinee 5	Vaccinee 6	Vaccinee 7
WT binding titer (ED ₅₀)	274.30	222.30	2455.00	433.70	1889.00	696.00	332.60
Omicron binding titer (ED ₅₀)	26.19	50.50	23.18	50.30	220.00	78.76	43.70
WT neutralizing titer (ID ₅₀)	284.70	112.50	1049.00	53.72	1587.00	251.70	35.35
Omicron neutralizing titer (ID ₅₀)	0.04	9.28	66.82	113.00	439.94	30.84	0.27
RBD mAbs	0.36	0.36	1.33	0.44	1.00	0.42	0.13
RBM mAbs	0.35	0.29	1.01	0.19	0.76	0.33	0.12
Non-RBM mAbs	0.01	0.07	0.32	0.25	0.24	0.09	0.01

Supplementary Figure 3

A

B

C

Supplementary Figure 4

Supplementary Figure 5

Supplementary Figure 6

Supplementary Figure 7