

1 **Title: Multivalency transforms SARS-CoV-2 antibodies into broad and ultrapotent**  
2 **neutralizers**

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43 **Abstract**

44 The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes  
45 Coronavirus Disease 2019 (COVID-19), has caused a global pandemic. Antibodies are powerful  
46 biotherapeutics to fight viral infections; however, discovery of the most potent and broadly acting  
47 clones can be lengthy. Here, we used the human apoferritin protomer as a modular subunit to drive  
48 oligomerization of antibody fragments and transform antibodies targeting SARS-CoV-2 into  
49 exceptionally potent neutralizers. Using this platform, half-maximal inhibitory concentration  
50 ( $IC_{50}$ ) values as low as  $9 \times 10^{-14}$  M were achieved as a result of up to 10,000-fold potency  
51 enhancements. Combination of three different antibody specificities and the fragment  
52 crystallizable (Fc) domain on a single multivalent molecule conferred the ability to overcome viral  
53 sequence variability together with outstanding potency and Ig-like *in vivo* bioavailability. This  
54 MULTi-specific, multi-Affinity antiBODY (Multabody; or MB) platform contributes a new class  
55 of medical countermeasures against COVID-19 and an efficient approach to rapidly deploy potent  
56 and broadly-acting therapeutics against infectious diseases of global health importance.

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58 **One Sentence Summary:** multimerization platform transforms antibodies emerging from  
59 discovery screens into potent neutralizers that can overcome SARS-CoV-2 sequence diversity.

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## 64 Introduction

65 The continuous threat to public health from respiratory viruses such as the novel SARS-CoV-2  
66 underscores the urgent need to rapidly develop and deploy prophylactic and therapeutic  
67 interventions to combat pandemics. Monoclonal antibodies (mAbs) have been used effectively for  
68 the treatment of infectious diseases as exemplified by palivizumab for the prevention of respiratory  
69 syncytial virus in high-risk infants<sup>1</sup> or Zmapp, mAb114 and REGN-EB3 for the treatment of  
70 Ebola<sup>2</sup>. Consequently, mAbs targeting the Spike (S) protein of SARS-CoV-2 have been a focus  
71 for the development of medical countermeasures against COVID-19. To date, several antibodies  
72 targeting the S protein have been identified<sup>3-19</sup> and several are under clinical evaluation<sup>20,21</sup>.  
73 Receptor binding domain (RBD)-directed mAbs that interfere with binding to angiotensin  
74 converting enzyme 2 (ACE2), the receptor for cell entry<sup>22</sup>, are usually associated with high  
75 neutralization potencies<sup>11,12,14</sup>.  
76 mAbs can be isolated by B-cell sorting from infected donors, immunized animals or by identifying  
77 binders in pre-assembled libraries. Despite these methodologies being robust and reliable for the  
78 discovery of virus-specific mAbs, identification of the best antibody clone can be a lengthy  
79 process. For example, RNA viruses have a high mutation rate. Indeed, 198 naturally occurring  
80 mutations in the S protein of SARS-CoV-2 have been already registered in the GISAID database<sup>23</sup>.  
81 Identification of broadly neutralizing mAbs (bnAbs) that target the most conserved epitopes to  
82 overcome viral escape mutations is therefore critical. This approach has been employed to discover  
83 bnAbs against human immune deficiency virus 1 (HIV-1)<sup>24</sup>, Influenza<sup>25</sup>, and Ebola<sup>26</sup>. However,  
84 their identification required extensive sampling and high-throughput sequencing and consequently  
85 several years of research. Hence, there is an unmet need for the development of a platform that

86 bridges antibody discovery and the rapid identification and deployment of highly potent and  
87 broadly neutralizing mAbs.

88 The potency of an antibody is greatly affected by its ability to simultaneously interact multiple  
89 times with its epitope<sup>27-29</sup>. This enhanced apparent affinity, known as avidity, has been previously  
90 reported to increase the neutralization potency of nanobodies<sup>30,31</sup> and IgGs over Fabs<sup>9,16,18</sup> against  
91 SARS-CoV-2. In order to rapidly propel mAbs that emerge during initial screening efforts into  
92 potent neutralizers against SARS-CoV-2, we have developed an antibody-scaffold technology,  
93 using the human apoferritin protomer as a modular subunit, to multimerize antibody fragments.  
94 We demonstrate the ability of this technology to combine one to three different Fab specificities  
95 together with Fc on the same molecule. The resulting MB molecules can increase mAb potency  
96 by up to four orders of magnitude and resist sequence variability in the spike protein. Therefore,  
97 the MB offers a versatile “plug-and-play” platform to enhance anti-viral characteristics of  
98 prophylactic/therapeutic molecules in the fight against the SARS-CoV-2 pandemic, and to be  
99 quickly deployed in the setting of emerging pandemics.

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## 101 **Results**

### 102 **Avidity enhances neutralization potency**

103 We used the self-assembly of the light chain of human apoferritin to multimerize antigen binding  
104 moieties targeting the SARS-CoV-2 S glycoprotein. Apoferritin protomers self-assemble into an  
105 octahedrally symmetric structure with an ~6 nm hydrodynamic radius ( $R_h$ ) composed of 24  
106 identical polypeptides<sup>32</sup>. The N terminus of each apoferritin subunit points outwards of the  
107 spherical nanocage and is therefore accessible for the genetic fusion of proteins of interest. Upon

108 folding, apoferritin protomers act as building blocks that drive the multimerization of the 24  
109 proteins fused to their N termini (**Fig. 1a**).

110 First, we investigated the impact of multivalency on the ability of the single chain variable domain  
111 VHH-72 to block viral infection. VHH-72 has been previously described to neutralize SARS-CoV-  
112 2 when fused to a Fc domain, but not in its monovalent format<sup>30</sup>. The light chain of human  
113 apoferritin displaying 24 copies of VHH-72 assembled into monodisperse, well-formed spherical  
114 particles (**Fig. 1b-c**) and showed an enhanced binding avidity to the S protein (**Fig. 1d**) in  
115 comparison to bivalent VHH-72-Fc. Strikingly, display of VHH-72 on the light chain of human  
116 apoferritin achieved a 1000-fold increase in neutralization potency against SARS-CoV-2  
117 pseudovirus (PsV) compared to the conventional Fc fusion (**Fig. 1e**).

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### 119 **Multabodies have IgG-like properties**

120 The Fc confers *in vivo* half-life and effector functions to IgGs through interaction with neonatal  
121 Fc receptor (FcRn) and Fc gamma receptors (FcγR), respectively. To confer these IgG-like  
122 properties to the MB, we next sought to incorporate both Fabs and Fc domains. Because a Fab is  
123 a hetero-dimer consisting of a light and a heavy chain, and the Fc is a homodimer, we created  
124 single-chain Fab (scFab) and Fc (scFc) polypeptide constructs to allow for their direct fusion to  
125 the N terminus of the apoferritin protomer. As a first step, we generated a species-matched  
126 surrogate molecule that consists of mouse light chain apoferritin fusions to a mouse scFab and a  
127 mouse scFc (IgG2a subtype), or a modified mouse scFc (LALAP mutation) to assess their,  
128 biodistribution, immunogenicity and pharmacokinetics *in vivo* (**Fig. S1**). As expected, binding  
129 affinity of the WT MB to mouse Fc receptors was enhanced in comparison to the parental IgG and

130 the LALAP mutation reduced binding affinity to Fc $\gamma$ R1 (**Fig. S1a**) Subcutaneous administration  
131 of these MBs in C57BL/6 or BALB/c mice was well tolerated with no decrease in body weight or  
132 visible adverse events. *In vivo* bioavailability and biodistribution of the MB were similar to the  
133 parental IgG (**Fig. S1b and c**). Confirming the role of the Fc in mediating *in vivo* bioavailability,  
134 serum half-life could be extended by mutations in the Fc (**Fig. S1b**). Presumably because all  
135 sequences derived from the host, the MB did not induce an anti-drug antibody response in mice  
136 (**Fig. S1d**).

137 In view of these results, we aimed to generate MBs derived from the previously reported IgG  
138 BD23<sup>5</sup> and 4A8<sup>6</sup> that target the SARS-CoV-2 spike RBD and N-terminal domain (NTD),  
139 respectively. Addition of scFc into the MB reduces the number of scFabs that can be  
140 multimerized. In order to endow the MB platform with Fc without compromising Fab avidity and  
141 hence neutralization potency, we engineered the apoferritin protomer to accommodate more than  
142 24 components per particle. Based on its four-helical bundle fold, the human apoferritin protomer  
143 was split into two halves: the two N-terminal  $\alpha$  helices (N-Ferritin) and the two C-terminal  $\alpha$   
144 helices (C-Ferritin). In this configuration, the scFc fragment of human IgG1 and the scFab of anti-  
145 SARS-CoV-2 IgGs were genetically fused at the N terminus of each apoferritin half, respectively.  
146 Split apoferritin complementation led to hetero-dimerization of the two halves and in turn, full  
147 apoferritin self-assembly displaying more than 24 copies of scFc and scFab on the nanocage  
148 periphery (**Fig. 2a**). Conveniently, this design allows for the straightforward purification of the  
149 MB using Protein A akin to IgG purification.

150 This split MB design forms 16 nm hydrodynamic radius ( $R_h$ ) spherical particles with an  
151 uninterrupted ring of density and regularly spaced protruding scFabs and scFc (**Fig. 2b and 2c**).  
152 Hence, the MB is on the lower size range of natural IgMs<sup>33</sup> but packs more weight on a similar

153 size to achieve multi-valency and multi-specificity. Binding kinetics experiments demonstrated  
154 that avidity of the MB for the Spike was preserved upon addition of Fc fragments (**Fig. 2d**).  
155 Binding to human Fc $\gamma$ RI and FcRn at endosomal pH confirmed that scFc was properly folded in  
156 the split MB design and that LALAP and I253A mutations lowered binding affinities to Fc $\gamma$ RI and  
157 FcRn, respectively (**Fig. 2e**). SARS-CoV-2 PsV neutralization assays with the split design MBs  
158 showed that enhanced binding affinity for the S protein translates into an improved neutralization  
159 potency in comparison to their IgG counterparts, with a ~1600-fold and ~2500-fold increase for  
160 BD23 and A48, respectively (**Fig. 2f**). Combined, this data supported the further exploration of  
161 the MB as an IgG-like platform that confers extraordinary multivalency.

162

### 163 **From antibody discovery to ultrapotent neutralizers**

164 We next assessed the ability of the MB platform to transform mAb binders identified from initial  
165 phage display screens into potent neutralizers against SARS-CoV-2 (**Fig. 3a**). Following standard  
166 biopanning protocols, 20 human mAb binders with moderate affinities that range from  $10^{-6}$  to  $10^{-8}$   
167 M against the RBD of SARS-CoV-2 were selected (**Table S1**). These mAbs were produced as  
168 full-length IgGs and MBs and their capacity to block viral infection was compared in a  
169 neutralization assay against SARS-CoV-2 PsV (**Fig. 3b** and **Fig. S2a**). Notably, MBs preserved  
170 the thermostability of their parental IgGs (**Fig. S2b-c**) and significantly enhanced the potency of  
171 18 out of 20 (90%) IgGs by up to four orders of magnitude. The largest increment was observed  
172 for mAb 298 which went from an IC<sub>50</sub> of ~30  $\mu$ g/mL as an IgG to 0.0001  $\mu$ g/mL as a MB.  
173 Strikingly, 11 mAbs were converted from non-neutralizing IgGs to neutralizing MBs in the tested  
174 concentration ranges. Seven MBs displayed exceptional potency with IC<sub>50</sub> values between 0.2-2  
175 ng/mL against SARS-CoV-2 PsV using two different target cells (293T-ACE2 and HeLa-ACE2

176 cells) (**Fig. 3b** and **Fig. S3a**). The enhanced neutralization potency of the MB was further  
177 confirmed with authentic SARS-CoV-2 virus for the mAbs with the highest potency (**Fig. 3c** and  
178 **Fig. S3b**). Retrospectively, all IgGs and MBs were tested for their ability to bind to the S protein  
179 and the RBD of SARS-CoV-2 (**Fig. S4**). Increased avidity resulted in higher apparent binding  
180 affinities with no detectable off-rates against the S protein, most likely due to inter-spike  
181 crosslinking that translates into high neutralization potency (**Fig. 3b-d** and **Fig. S4**). Overall, the  
182 data shows that the MB platform is compatible with rapid delivery of ultrapotent IgG-like  
183 molecules.

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### 185 **Epitope mapping**

186 Based on their neutralization potency, seven mAbs were selected for further characterization: 298  
187 (IGHV1-46/IGKV4-1), 82 (IGHV1-46/IGKV1-39), 46 (IGHV3-23/IGKV1-39), 324 (IGHV1-  
188 69/IGKV1-39), 236 (IGHV1-69/IGKV2-28), 52 (IGHV1-69/IGKV1-39) and 80 (IGHV1-  
189 69/IGKV4-1) (**Fig. 3b** and **Table S1**). Epitope binning experiments showed that these mAbs target  
190 two main sites on the RBD, with one of these bins overlapping with the ACE2 binding site (**Fig.**  
191 **4a** and **Fig. S5**). Cryo-EM structures of Fab-SARS-CoV-2 S complexes at a global resolution of  
192  $\sim 6\text{-}7$  Å confirmed that mAbs 324, 298, 80 bind overlapping epitopes (**Fig. 4b**, **Fig. S6a-c** and  
193 **Table S2**). To gain insight into the binding of mAbs targeting the second bin, we obtained the  
194 cryo-EM structure of Fab 46 in complex with the RBD at a global resolution of 4.0 Å (**Fig. 4c**,  
195 **Fig. S6d** and **Table S2**), and the crystal structure of Fabs 298 and 52 as a ternary complex with  
196 the RBD at 2.95 Å resolution (**Fig. 4d**, **Fig. S7** and **Table S3**).

197 The crystal structure shows that mAb 298 binds almost exclusively to the ACE2 receptor binding  
198 motif (RBM) of the RBD (residues 438-506). In fact, out of 16 RBD residues involved in binding

199 mAb 298, 12 are also involved in ACE2-RBD binding (**Fig. S7a-c** and **Table S4 and S5**). The  
200 RBM is stabilized by 11 hydrogen bonds from heavy and light chain residues of mAb 298. In  
201 addition, RBM Phe486 is contacted by 11 mAb 298 residues burying approximately 170 Å<sup>2</sup> (24%  
202 of the total buried surface area on RBD) and hence is central to the antibody-antigen interaction  
203 (**Fig. S7a** and **Table S5**).

204 Detailed analysis of the RBD-52 Fab interface reveals that the epitope of mAb 52 is shifted towards  
205 the core of the RBD encompassing 20 residues of the RBM and 7 residues in the core domain (**Fig.**  
206 **4c**, **Fig. S7b** and **Table S6** and **S7**). In agreement with the competition data, antibody 52 and  
207 antibody 46 share a similar binding site, although they approach the RBD with slightly different  
208 angles (**Fig. 4c-d** and **Fig. S7d**). Inspection of previously reported structures of RBD-antibody  
209 complexes reveal that antibodies 46 and 52 target a site of vulnerability on the SARS-CoV-2 spike  
210 that has not been described previously (**Fig. 4e**). The epitope targeted by these antibodies is  
211 partially occluded by the NTD in the S “closed” conformation, suggesting that the mechanism of  
212 action for this new class of antibodies could involve Spike destabilization. Together, these data  
213 demonstrate that the enhanced potency observed for the MB platform is associated with mAbs that  
214 target both overlapping and distinct epitope bins.

215

### 216 **Multabodies overcome Spike sequence variability**

217 To explore whether MBs could resist viral escape, we tested the effect of four naturally occurring  
218 RBD mutations (L452R, A475V, N439K and V483A)<sup>34</sup> (**Fig. 5a**) on the binding and neutralization  
219 of the seven human mAbs of highest potency (**Fig. 5b-c**). In addition, the impact of mutating  
220 Asn234, a glycosylation site, to Gln was also assessed because the absence of glycosylation at this



221 site has been previously reported to decrease sensitivity to neutralizing antibodies targeting the  
222 RBD<sup>34</sup>. The more infectious PsV variant D614G<sup>35</sup> was also included in the panel. Mutation L452R  
223 significantly decreased binding and potency of antibodies 52 and 46, while antibody 298 was  
224 sensitive to mutation A475V (**Fig. 5b-c**). Deletion of the N-linked glycan at position Asn234  
225 increased viral resistance to the majority of the antibodies, especially mAbs 46, 80 and 324,  
226 emphasizing the important role of glycans in viral antigenicity (**Fig. 5c**). Strikingly, the following  
227 mAbs were minimally impacted in their exceptional neutralization potency by any S mutation  
228 when present in the MB format: 298, 80, 324, and 236 (**Fig. 5d**). Mutation L452R decreased the  
229 sensitivity of the 46-MB and 52-MB but in contrast to their parental IgGs, they remained  
230 neutralizing against this PsV variant (**Fig. 5d**). The more infectious SARS-CoV-2 PsV variant  
231 D614G was neutralized with similar potency as the WT PsV for both IgGs and MBs (**Fig. 5c** and  
232 **Fig. S8a**).

233 MB cocktails consisting of three monospecific MB resulted in pan-neutralization across all PsV  
234 variants without a significant loss in potency and hence achieved a 100-1000-fold higher potency  
235 compared to the corresponding IgG cocktails (**Fig. 5e** and **Fig. S8c-d**). In order to achieve breadth  
236 within a single molecule, we next generated tri-specific MBs by combining multimerization  
237 subunits displaying three different Fabs in the same MB assembly (**Fig. S8b**). Notably, the  
238 resulting tri-specific MBs exhibited pan-neutralization while preserving the exceptional  
239 neutralization potency of the monospecific versions (**Fig. 5e** and **Fig. S8c-d**). The most notable  
240 increase in potency was for the 298-324-46 combination, where the IgG cocktail neutralized WT  
241 SARS-CoV-2 PsV with an  $\sim 0.5$   $\mu\text{g}/\text{mL}$  ( $3.3 \times 10^{-9}$  M)  $\text{IC}_{50}$ , while the tri-specific MB achieved  
242 broad neutralization with an exceptional  $\text{IC}_{50}$  of  $\sim 0.0005$   $\mu\text{g}/\text{mL}$  ( $2.2 \times 10^{-13}$  M). The potency

243 enhancement of MB cocktails and tri-specific MBs in comparison to their corresponding IgG tri-  
244 specific cocktails was also observed with live replicating SARS-CoV-2 virus (**Fig. S8e**).

245

## 246 **Discussion**

247 We have developed an antibody-multimerization platform to increase avidity of mAbs targeting  
248 SARS-CoV-2. The seven most potent MBs have IC<sub>50</sub> values of 0.2 to 2 ng/mL ( $9 \times 10^{-14}$  to  $9 \times 10^{-13}$   
249 M) against SARS-CoV-2 PsVs and therefore are, to our knowledge, within the most potent  
250 antibody-like molecules reported to date against SARS-CoV-2.

251 The MB platform was designed to include key favorable attributes from a developability  
252 perspective. First, the ability to augment antibody potency is independent of antibody sequence,  
253 format or epitope targeted. The modularity and flexibility of the platform was exemplified by  
254 enhancing the potency of a VHH and multiple Fabs that target non-overlapping regions on two  
255 SARS-CoV-2 S sub-domains (RBD and NTD). Second, in contrast to other approaches to enhance  
256 avidity such as tandem fusions of single chain variable fragments<sup>36,37</sup>, which suffer from low  
257 stability, MBs self-assemble into highly stable particles with aggregation temperatures values  
258 similar to those of their parental IgGs. Third, alternative multimerization strategies like  
259 streptavidin<sup>38</sup>, verotoxin B subunit scaffolds<sup>39</sup> or viral-like nanoparticles<sup>40</sup> face immunogenicity  
260 challenges and/or poor bioavailability because of the absence of an Fc fragment and therefore the  
261 inability to undergo FcRn-mediated recycling. The light chain of apoferritin is fully human,  
262 biologically inactive, has been engineered to include Fc domains, and despite multimerization of  
263 >24 Fab/Fc fragments, has a hydrodynamic radius similar to an IgM. As such, a surrogate mouse  
264 MB did not elicit anti-drug antibodies in mice, was detectable in the sera for over a week, and has  
265 similar biodistribution as its parental IgG.

266 Different increases in neutralization potency were observed for different mAb sequences tested on  
267 the MB against SARS-CoV-2. This suggests that the ability of the MB to enhance potency may  
268 depend on epitope location on the Spike or the geometry of how the Fabs engage the antigen to  
269 achieve neutralization. The fact that 2 out of 20 SARS-CoV-2 RBD binders were not rescued by  
270 the MB platform also suggests limitations based on mAb sequences and binding properties.  
271 Nevertheless, the capacity of the MB to transform avidity into neutralization potency across a  
272 range of epitope specificities on the SARS-CoV-2 Spike highlights the potential for using this  
273 technology broadly. It will be interesting to explore the potency-enhancement capacity of the MB  
274 platform against viruses with low surface spike density like HIV<sup>41</sup>, or against other targets like the  
275 tumor necrosis factor receptor superfamily, where bivalency of conventional antibodies limits their  
276 efficient activation<sup>42</sup>.

277 Virus escape can arise in response to selective pressure from treatments or during natural selection.  
278 A conventional approach to combat escape mutants is the use of antibody cocktails targeting  
279 different epitopes. MBs showed a lower susceptibility to S mutations in comparison to their  
280 parental IgGs, presumably because the loss in affinity was compensated by enhanced binding  
281 avidity. Hence, when used in cocktails, the MB overcame viral sequence variability with  
282 exceptional potency. In addition, the split MB design allows combination of multiple antibody  
283 specificities within a single multimerized molecule resulting in similar potency and breadth as the  
284 MB cocktails. To our knowledge, these MBs represent the first tri-specifics described against  
285 SARS-CoV-2. Multi-specificity within the same particle could offer additional advantages such as  
286 intra-S avidity and synergy for the right combination of mAbs, setting the stage for further  
287 investigation of different combinations of mAb specificities on the MB.

288 Overall, the MB platform provides a tool to surpass antibody affinity limits and generate broad  
289 and potent neutralizing molecules without the need for extensive antibody discovery or  
290 engineering efforts. This platform will accelerate the timeline from discovery to development of  
291 antibodies as medical countermeasures against COVID-19 and in future pandemics.

292

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401

402



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443 maps have been deposited at the Electron Microscopy Data Band (EMDB) with accession codes  
444 EMD-22738, EMD-22739, EMD-22740, EMD-22741 (Table S2). The crystal structure of 298-52-  
445 RBD complex (Table S3) is available from the Protein Data Bank under accession code PDB:  
446 7K9Z. Sequences of the monoclonal antibodies are available in Table S1. Other data are available  
447 from the corresponding author upon reasonable request.  
448

449 **SUPPLEMENTARY MATERIALS**

450 Materials and Methods

451 Figs S1 to S8

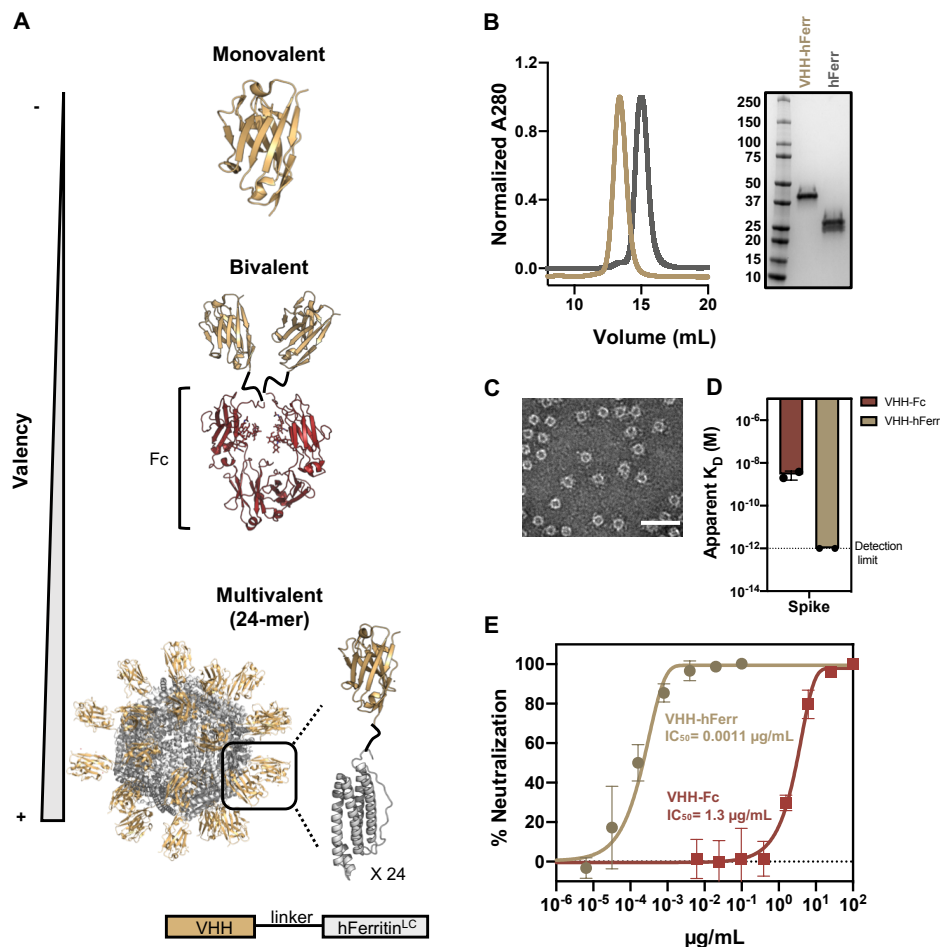
452 Tables S1 to S7

453 References (46-49)

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455

456 **Figures**



457

458 **Fig. 1. Avidity enhances binding and neutralization of VHH against SARS-CoV-2.** (A)

459 Schematic representation of a monomeric VHH domain and its multimerization using a

460 conventional Fc scaffold or human apoferritin. (B) Size exclusion chromatography and SDS-

461 PAGE of apoferritin alone (*gray*) and VHH-72 apoferritin particles (*gold*). (C) Negative stain

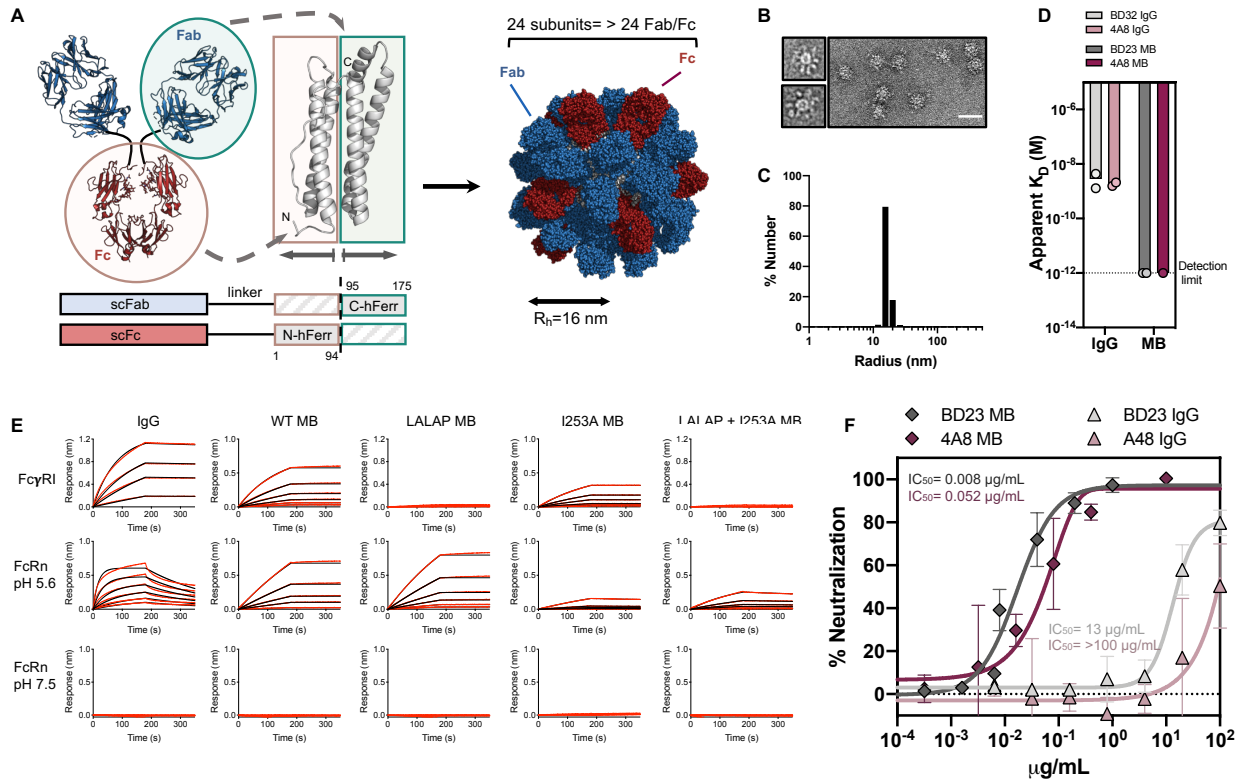
462 electron microscopy of VHH-72 apoferritin particles. (Scale bar 50 nm). (D) Comparison of the

463 binding avidity (apparent  $K_D$ ) of VHH-72 to SARS-CoV-2 S protein when displayed in a bivalent

464 (red) or 24-mer (gold) format. Apparent  $K_D$  higher than  $10^{-12}$  M (dash line) is beyond the

465 instrument detection limit. (E) Neutralization potency against SARS-CoV-2 PsV (color coding is

466 as in d). One representative out of two experiments with similar results is shown.



467

468 **Fig. 2. Protein engineering to multimerize IgG-like particles against SARS-CoV-2. (A)**

469 Schematic representation of the human apoferritin split design. (B) Negative stain electron

470 micrograph of the MB. (Scale bar 50 nm). (C) Hydrodynamic radius ( $R_h$ ) of the MB. (D) Avidity

471 effect on the binding (apparent  $K_D$ ) of A48 (purple) and BD23 (gray) to the SARS-CoV-2 Spike.

472 (E) Kinetic curves of BD23 IgG and MB with different Fc sequence variants binding to FcγRI (top

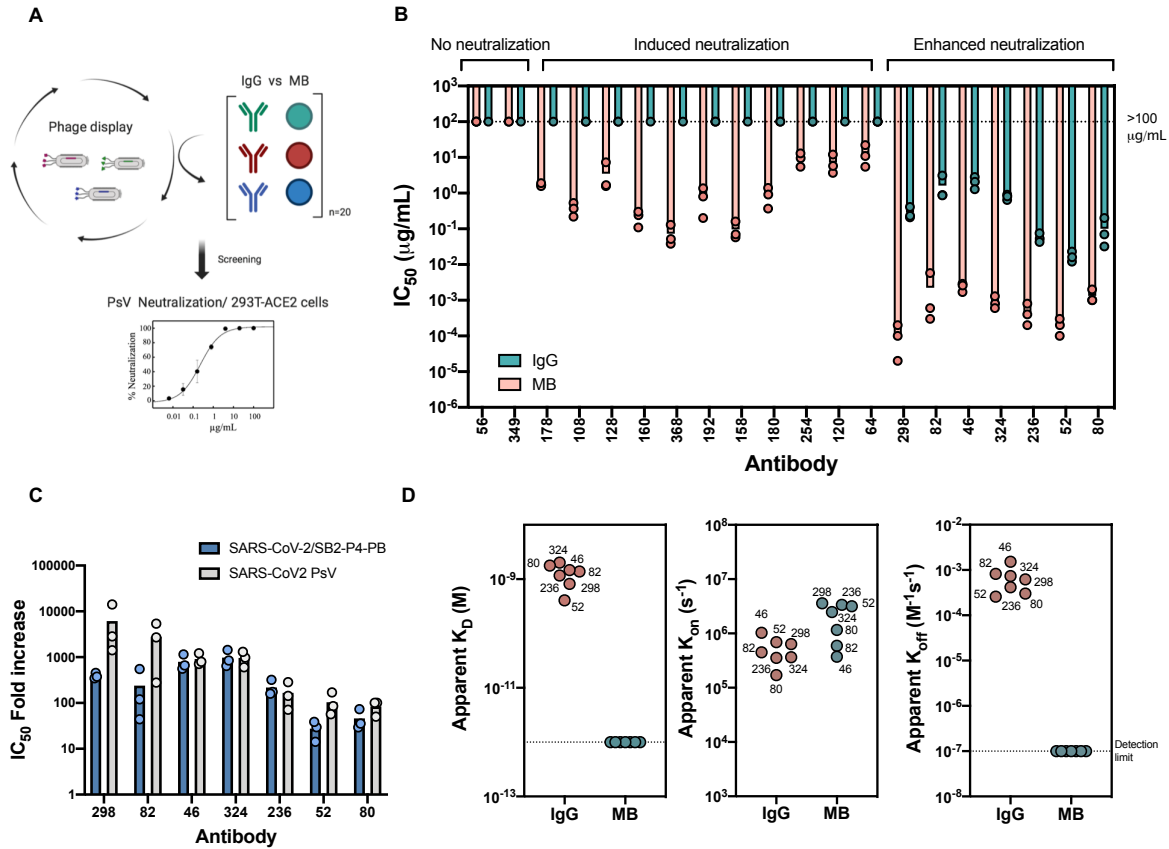
473 row), FcRn at endosomal pH (middle row) and FcRn at physiological pH (bottom row). Black

474 lines represent raw data whereas red lines represent global fits. (F) Neutralization of SARS-CoV-

475 2 PsV by A48 and BD23 IgGs and MBs. The mean values  $\pm$  SD for two technical replicates for a

476 representative experiment is shown.

477



478

479 **Fig. 3. The Multibody enhances the potency of human mAbs from phage display. (A)** Work

480 flow for the identification of potent anti-SARS-CoV-2 neutralizers using the MB technology. **(B)**

481 Comparison of neutralization potency between IgGs and MBs that display the same human Fab

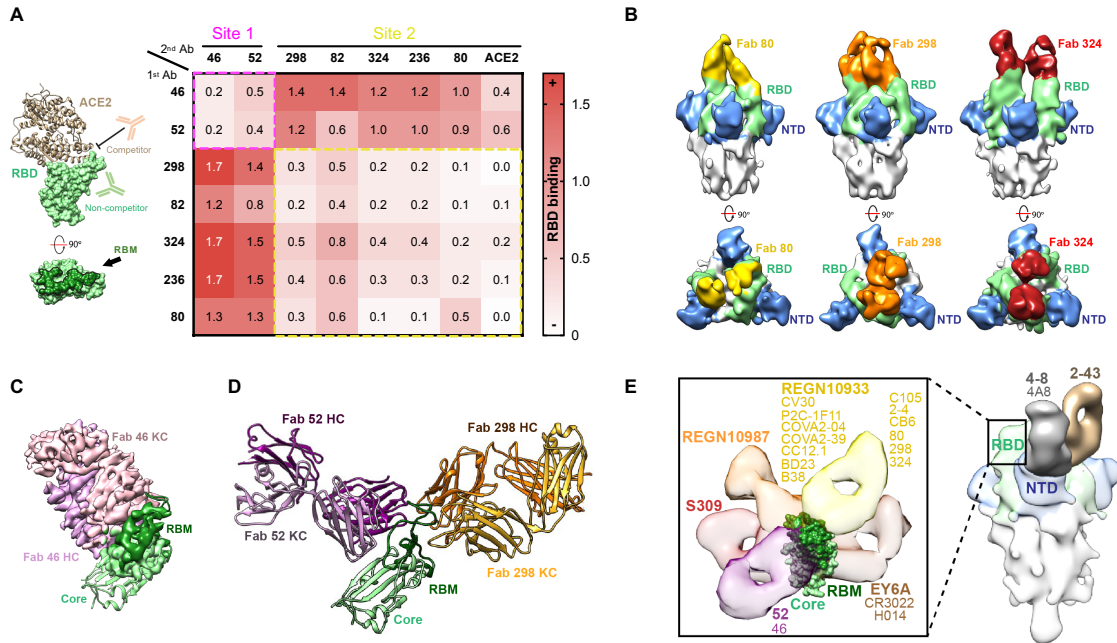
482 sequences derived from phage display. **(C)**  $\text{IC}_{50}$  values fold increase upon multimerization. **(D)**

483 Apparent affinity ( $K_D$ ), association ( $k_{on}$ ) and dissociation ( $k_{off}$ ) rates of the most potent neutralizing

484 MBs compared to their IgG counterparts for binding the SARS-CoV-2 S protein. Three biological

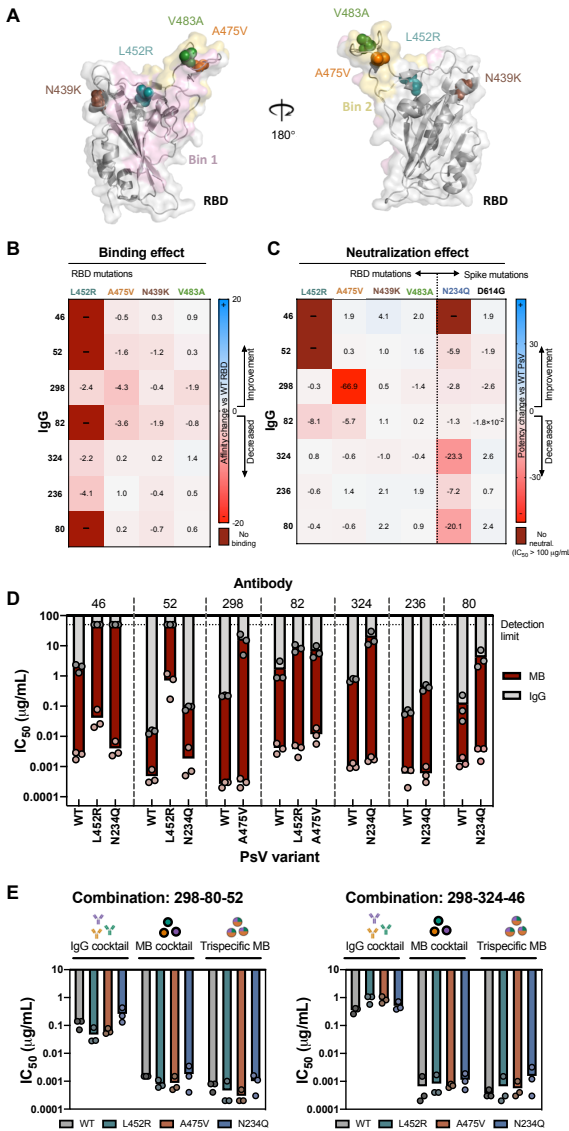
485 replicates and their mean are shown for  $\text{IC}_{50}$  values in (b) and (c).

486



487

488 **Fig. 4. Epitope delineation of the most potent mAb specificities.** (A) Surface and cartoon  
 489 representation of RBD (*light green* for the core and *dark green* for RBM) and ACE2<sup>46</sup> (*light*  
 490 *brown*) binding. Heat map showing binding competition experiments. Epitope bins are highlighted  
 491 by dashed-line boxes. (B) 15.0 Å filtered cryo-EM reconstruction of the Spike (*grey*) in complex  
 492 with Fab 80 (*yellow*), 298 (*orange*) and 324 (*red*). The RBD and NTD are shown in *green* and  
 493 *blue*, respectively. (C) Cryo-EM reconstruction of Fab 46 (*pink*) and RBD (*green*) complex. A  
 494 RBD cartoon<sup>46</sup> is fitted into the partial density observed for the RBD. (D) Crystal structure of the  
 495 ternary complex formed by Fab 52 (*purple*), Fab 298 (*orange*) and RBD (*green*). (E) Composite  
 496 image depicting antibodies targeting SARS-CoV-2 S with available PDB or EMD  
 497 entries<sup>3,4,6,8,10,16,17,47,48</sup>. Inset: close up view of antibodies targeting different antigenic sites on the  
 498 RBD. The mAb with the lowest reported IC<sub>50</sub> value against SARS-CoV-2 PsV was selected as a  
 499 representative antibody of the bin (color coding as in b).



500

501 **Fig. 5. Multabodies overcome SARS-CoV-2 sequence diversity.** (A) Cartoon representation of  
 502 the RBD showing four naturally occurring mutations as spheres. The epitopes of mAbs 52 (*light*  
 503 *pink*) and 298 (*yellow*) are shown as representative epitopes of each bin. (B) Affinity and (C) IC<sub>50</sub>  
 504 fold-change comparison between WT and mutated RBD and PsV, respectively. (D) Neutralization  
 505 potency of IgG (*grey bars*) vs MB (*dark red bars*) against SARS-CoV-2 PsV variants in  
 506 comparison to WT PsV. (E) Neutralization potency comparison of two IgG cocktails (three IgGs),  
 507 monospecific MB cocktails (three MBs) and tri-specific MBs against WT SARS-CoV-2 PsV and



508 variants. mAbs sensitive to one or more PsV variants were selected to generate the cocktails and  
509 the tri-specific MBs. The mean of three biological replicates are shown in (b), (c), (d) and (e).

510

## Supplementary Materials for

### **Multivalency transforms SARS-CoV-2 antibodies into broad and ultrapotent neutralizers**

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Materials and Methods  
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## **Materials and Methods**

### **Protein expression and purification**

Genes encoding VHH-human apoferritin fusion, Fc fusions, Fabs, IgG, and RBD mutants were synthesized and cloned by GeneArt (Life Technologies) into the pcDNA3.4 expression vector. All constructs were expressed transiently in HEK293F cells (Thermo Fisher Scientific) at a density of  $0.8 \times 10^6$  cells/mL with 50  $\mu$ g of DNA per 200 mL of cells using FectoPRO (Polyplus Transfections) in a 1:1 ratio unless specified otherwise. After 6-7 days of incubation at 125 rpm oscillation at 37° C, 8% CO<sub>2</sub>, and 70% humidity in a Multitron Pro shaker (Infors HT), cell suspensions were harvested by centrifugation at 5000  $\times$ g for 15 min and supernatants were filtered through a 0.22  $\mu$ m Steritop filter (EMD Millipore). Fabs and IgGs were transiently expressed by co-transfecting 90  $\mu$ g of the LC and the HC in a 1:2 ratio and purified using KappaSelect affinity column (GE Healthcare) and HiTrap Protein A HP column (GE Healthcare), respectively with 100 mM glycine pH 2.2 as the elution buffer. Eluted fractions were immediately neutralized with 1 M Tris-HCl, pH 9.0 and further purified using a Superdex 200 Increase size exclusion column (GE Healthcare). Fc fusions of ACE2 and VHH-72 were purified the same way as IgGs. The VHH-72 apoferritin fusion was purified by hydrophobic interaction chromatography using a HiTrap Phenyl HP column and the eluted fraction was loaded onto a Superose 6 10/300 GL size exclusion column (GE Healthcare) in 20 mM sodium phosphate pH 8.0, 150 mM NaCl. The prefusion S ectodomain (BEI NR52394), wild type RBD (BEI NR52309) and mutant RBDs were purified using a HisTrap Ni-NTA column (GE Healthcare) followed by Superose 6 and Superdex 200 Increase size exclusion columns (GE Healthcare), respectively in 20 mM phosphate pH 8.0, 150 mM NaCl buffer.

### **Design, expression and purification of split Multabodies**

Genes encoding scFab and scFc fragments linked to half apoferritin were generated by deletion of residues 1 to 95 (C-Ferritin) and 95 to 175 (N-Ferritin) of the light chain of human apoferritin using the KOD-Plus mutagenesis kit (Toyobo, Osaka, Japan). Transient transfection of the split Multabodies in HEK 293F cells were obtained by mixing 66 µg of the plasmids scFab-human apoferritin: scFc-human N-Ferritin: scFab-C-Ferritin in a 2:1:1 ratio. In the case of multispecific Multabodies, a 4:2:1:1 ratio of scFab1-human apoferritin: scFc-human N-Ferritin: scFab2-C-Ferritin: scFab3-C-Ferritin was used. The DNA mixture was filtered and incubated at RT with 66 µl of FectoPRO before adding to the cell culture. Split Multabodies were purified by affinity chromatography using a HiTrap Protein A HP column (GE Healthcare) with 20 mM Tris pH 8.0, 3 M MgCl<sub>2</sub> and 10% glycerol elution buffer. Fractions containing the protein were concentrated and further purified by gel filtration on a Superose 6 10/300 GL column (GE Healthcare).

### **Negative-stain electron microscopy**

3 µL of Multabody at a concentration approximately of 0.02 mg/mL was placed on the surface of a carbon-coated copper grid that had previously been glow-discharged in air for 15 sec, allowed to adsorb for 30 s, and stained with 3 µL of 2% uranyl formate. Excess stain was removed immediately from the grid using Whatman No. 1 filter paper and an additional 3 µL of 2% uranyl formate was added for 20 s. Grids were imaged with a FEI Tecnai T20 electron microscope operating at 200 kV and equipped with an Orius charge-coupled device (CCD) camera (Gatan Inc).

### **Biolayer interferometry**

Direct binding kinetics measurements were conducted using an Octet RED96 BLI system (Sartorius ForteBio) in PBS pH 7.4, 0.01% BSA and 0.002% Tween at 25° C. His-tagged RBD or SARS-CoV-2 Spike protein was loaded onto Ni-NTA (NTA) biosensors (Sartorius ForteBio) to

reach a BLI signal response of 0.8 nm. Association rates were measured by transferring the loaded biosensors to wells containing a 2-fold dilution series from 250 to 16 nM (Fabs), 125 to 4 nM (IgG), and 16 to 0.5 nM (MB). Dissociation rates were measured by dipping the biosensors into buffer-containing wells. The duration of each of these two steps was 180 s. Fc characterization in the split Multabody design was assessed by measuring binding to hFcγRI and hFcRn. To probe the theoretical capacity of the Multabodies to undergo endosomal recycling, binding to the hFcRn β2-microglobulin complex was measured at physiological (7.5) and endosomal (5.6) pH. Competition assays were performed in a two-step binding process. Ni-NTA biosensors preloaded with His-tagged RBD were first dipped into wells containing the primary antibody at 50 μg/mL for 180 s. After a 30 s baseline period, the sensors were dipped into wells containing the second antibody at 50 μg/ml for an additional 300 s.

### **Dynamic light scattering**

The hydrodynamic radius (Rh) of the Multabody was determined by dynamic light scattering (DLS) using a DynaPro Plate Reader III (Wyatt Technology). 20 μL of the Multabody at a concentration of 1 mg/mL was added to a 384-well black, clear bottom plate (Corning) and measured at a fixed temperature of 25° C with a duration of 5 s per read. Particle size determination and polydispersity were obtained from the accumulation of 5 reads using the Dynamics software (Wyatt Technology).

### **Aggregation temperature measurements**

Aggregation temperature ( $T_{agg}$ ) of the Multabodies and parental IgGs were determined using a UNit instrument (Unchained Labs). Samples were concentrated to 1.0 mg/mL and subjected to a thermal ramp from 25 to 95° C with 1° C increments.  $T_{agg}$  was determined as the temperature at which 50% increase in the static light scattering at a 266 nm wavelength relative to baseline was

observed (i.e. the maximum value of the differential curve). The average and the standard error of two independent measurements were calculated using the UNit analysis software.

### **Pharmacokinetics and immunogenicity studies**

A surrogate Multabody composed of the scFab and scFc fragments of mouse HD37 (anti-hCD19) IgG2a fused to the N-terminus of the light chain of mouse apoferritin (mFerritin) was used for the study. HD37 scFab-mFerritin: Fc-mFerritin: mFerritin in a 2:1:1 ratio was transfected and purified following the procedure described above. L234A, L235A and P329G mutations were introduced in the mouse IgG2a Fc-construct to silence effector functions of the Multabody<sup>43</sup>. *In vivo* studies were performed using 12-week-old male C57BL/6 mice purchased from Charles River (Strain code: 027), housed in individually-vented cages. All procedures were approved by the Local Animal Care Committee at the University of Toronto Scarborough. A single injection of approximately 5 mg/kg of Multabodies or control samples (HD37 IgG -IgG1 and IgG2a subtypes) and *Helicobacter pylori* ferritin (hpFerritin)-PfCSP malaria peptide in 200  $\mu$ L of PBS (pH 7.5) were subcutaneously injected. Blood samples were collected at multiple time points and serum samples were assessed for levels of circulating antibodies and anti-drug antibodies (ADA) by ELISA. Briefly, 96-well Pierce Nickel Coated Plates (Thermo Fisher) were coated with 50  $\mu$ L at 0.5  $\mu$ g/ml of the His<sub>6x</sub>-tagged antigen hCD19 to determine circulating HD37-specific concentrations using reagent-specific standard curves for IgGs and Multabodies. For ADA determination, Nunc MaxiSorp plates (Biolegend) were coated with a 12-mer HD37 scFab-mFerritin or with the hpFerritin-PfCSP malaria peptide. HRP-ProteinA (Invitrogen) was used as a secondary molecule and the chemiluminescence signal was quantified using a Synergy Neo2 Multi-Mode Assay Microplate Reader (Biotek Instruments).

### **Biodistribution studies**

8-week-old BALB/c mice were purchased from The Jackson Laboratory and housed in individually-vented caging. All procedures were approved by the Local Animal Care Committee at the University of Toronto. The HD37 IgG2a Multibody or HD37 IgG2a control were fluorescently conjugated with Alexa-647 using an Alexa Fluor™ 647 Antibody Labeling kit (Invitrogen) as per manufacturer instructions. PerkinElmer IVIS Spectrum (PerkinElmer) was used to conduct non-invasive biodistribution experiments. BALB/c mice were injected subcutaneously into the loose skin over the shoulders with approximately 5 mg/kg of the MB or control samples in 200 µL of PBS (pH 7.5) and imaged at time 0, 1h, 6h, 24h, 2d, 3d, 4d, 8d, 11d following injection. Prior to imaging, mice were placed in an anesthesia induction chamber containing a mixture of isoflurane and oxygen for 1 min. Anesthetized mice were then placed in the prone position at the center of a built-in heated docking system within the IVIS imaging system (maintained at 37°C and supplied with a mixture of isoflurane and oxygen). For whole body 2D imaging, mice were imaged for 1-2 s (excitation 640 nm, emission 680 nm) inside the imaging system. Data were analyzed and reconstructed with the IVIS software (Living Image Software for IVIS) using 640 nm/680 nm laser.

### **Panning of Phage libraries against the RBD of SARS-CoV-2**

143 healthy human subjects were used for the assembly of a SuperHuman 2.0 library. CDR-H3 diversity was sourced by PCR from naive CD27-IgM<sup>+</sup> B-cells while other CDR diversity was sourced from CD27<sup>+</sup>IgG<sup>+</sup> B cells from the therapeutic frameworks IGHV1-46, IGHV1-69, IGHV3-15, IGHV3-23, IGKV1-39, IGKV2-28, IGKV3-15 and IGKV4-1 by using PCR overlap extension. The light and heavy chains were transformed to exceed 1e<sup>8</sup> and 7.6e<sup>10</sup> transformants for each chain framework, respectively. Illumina MiSeq replicate rarefaction analysis was used to assure a recovery of over 98% unique clones. In addition, Protein L and Protein A were used (10

min incubation at 70° C) to select thermostable and well-expressing light chain diversity. The EXPi-293 mammalian expression system was used for expression of the receptor Binding domain (RBD)-Fc-Avi tag construct of the SARS-CoV-2. This protein was subsequently purified by protein G Dynabeads, biotinylated and quality-controlled for biotinylation and binding to ACE2 recombinant protein (Sino Biologics Inc). The SuperHuman 2.0 Phage library ( $5 \times 10^{12}$ ) was heated for 10 min at 72° C and de-selected against Protein G Dynabeads™ (Invitrogen), M-280 Streptavidin Dynabeads™ (Invitrogen), Histone from Calf Thymus (Sigma), Human IgG (Sigma) and ssDNA-Biotin NNK from Integrated DNA Technologies and DNA-Biotin NNK from Integrated DNA Technologies. Next, the library was panned against the RBD-captured by M-280 Streptavidin Dynabeads™ using an automated protocol on Kingfisher FLEX (ThermoFisher). Selected phages were acid eluted from the beads and neutralized using Tris-HCl pH 7.9 (Teknova). ER2738 cells were infected with the neutralized phage pools at  $OD_{600}=0.5$  at a 1:10 ratio and after 40 min incubation at 37° C and 100 rpm, the phage pools were centrifuged and incubated on agar with antibiotic selection overnight at 30° C. The rescued phages were precipitated by PEG and subjected to three additional rounds of soluble-phase automated panning. PBST/1% BSA buffer and/or PBS/1% BSA was used in the de-selection, washes and selection rounds.

### **Screening of anti-SARS-CoV-2 scFvs in bacterial periplasmic extracts with SARS-CoV-2 RBD**

Anti-SARS-CoV-2 RBD scFvs selected from phage display were expressed and screened using high-throughput surface plasmon resonance (SPR) on Carterra LSA Array SPR instrument (Carterra) equipped with HC200M sensor chip (Carterra) at 25° C. A V5 epitope tag was added to the scFv to enable capture via immobilized anti-V5 antibody (Abcam, Cambridge, MA) that was pre-immobilized on the chip surface by standard amine-coupling. Briefly: the chip surface was



first activated by 10 min injection of a 1:1:1 (v/v/v) mixture of 0.4 M 1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide hydrochloride (EDC), 0.1 M N-hydroxysulfosuccinimide (sNHS) and 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) pH 5.5. Then, 50 µg/ml of anti-V5 tag antibody prepared in 10 mM sodium acetate pH 4.3 was coupled for 14 min and the excess reactive esters were blocked with 1 M ethanolamine HCl pH 8.5 during a 10 min injection. For screening, a 384-ligand array comprising of crude bacterial periplasmic extracts (PPE) containing the scFvs (1 spot per scFv) was prepared. Each extract was prepared at a 2-fold dilution in running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.01% (v/v) Tween-20 (HBSTE)) and printed on the anti-V5 surface for 15 min. SARS-CoV-2 RBD Avi Tev His tagged was then prepared at 0, 3.7, 11.1, 33.3, 100, 37, and 300 nM in 10 mM HEPES pH 7.4, 150 mM NaCl, 0.01% (v/v) Tween-20 (HBST) supplemented with 0.5 mg/ml BSA and injected as analyte for 5 min with a 15 min dissociation time. Samples were injected in ascending concentration without any regeneration step. Binding data from the local reference spots was used to subtracted signal from the active spots and the nearest buffer blank analyte responses were subtracted to double-reference the data. The double-referenced data were fitted to a simple 1:1 Langmuir binding model in Carterra's Kinetic Inspection Tool (version Oct. 2019). 20 medium-affinity binders from phage display screening were selected for the present study.

### **Virus production and pseudovirus neutralization assays**

SARS-CoV-2 pseudotyped viruses (PsV) were generated using an HIV-based lentiviral system as previously described<sup>44</sup> with few modifications. Briefly, 293T cells were co-transfected with a lentiviral backbone encoding the luciferase reporter gene (BEI NR52516), a plasmid expressing the Spike (BEI NR52310) and plasmids encoding the HIV structural and regulatory proteins Tat (BEI NR52518), Gag-pol (BEI NR52517) and Rev (BEI NR52519). 24 h post transfection at 37°

C, 5 mM sodium butyrate was added to the media and the cells were incubated for an additional 24-30 h at 30° C. SARS-CoV-2 Spike mutant D614G was kindly provided by D.R. Burton (The Scripps Research Institute) and the rest of the PsV mutants were generated using the KOD-Plus mutagenesis kit (Toyobo, Osaka, Japan). Neutralization was determined in a single-cycle neutralization assay using 293T-ACE2 cells (BEI NR52511) and HeLa-ACE2 cells (kindly provided by D.R. Burton; The Scripps Research Institute). PsV neutralization was monitored by adding Britelite plus reagent (PerkinElmer) to the cells and measuring luminescence in relative light units (RLUs) using a Synergy Neo2 Multi-Mode Assay Microplate Reader (Biotek Instruments). IC<sub>50</sub> fold increase was calculated as: IgG<sub>IC50</sub> (μg/mL) / MB<sub>IC50</sub> (μg/mL). Two to three biological replicates with two technical replicates each were performed.

#### **Authentic virus neutralization assays**

VeroE6 cells were seeded in a 96F plate at a concentration of 30,000/well in DMEM supplemented with 100U Penicillin, 100U Streptomycin and 10% FBS. Cells were allowed to adhere to the plate and rest overnight. After 24 h, 5-fold serial dilutions of the IgG and MB samples were prepared in DMEM supplemented with 100U Penicillin and 100U Streptomycin in a 96R plate in quadruplicates (25 uL/well). 25 uL of SARS-CoV-2/SB2-P4-PB<sup>45</sup> Clone 1 was added to each well at 100TCID<sub>50</sub>/well and incubated for 1 h at 37 °C with shaking every 15 min. After co-culturing, the media from the VeroE6 plate was removed, and 50 uL antibody-virus sample was used to inoculate VeroE6 cells in quadruplicates for 1 h at 37 °C, 5% CO<sub>2</sub>, shaking every 15 min. After 1 h inoculation, the inoculum was removed and 200 uL of fresh DMEM supplemented with 100U Penicillin, 100U Streptomycin and 2% FBS was added to each well. The plates were further incubated for 5 days. The cytopathic effect (CPE) was monitored and PRISM was used to calculate IC<sub>50</sub> values. Three biological replicates with four technical replicates each were performed.

### **Cross-linking of Spike protein with Fabs 80, 298 and 324**

100 µg of Spike trimer was mixed with 2x molar excess of Fab 80, 298 or 324 in 20 mM HEPES pH 7.0, 150 mM NaCl. Proteins were crosslinked by addition of 0.075% (v/v) glutaraldehyde (Sigma Aldrich) and incubated at room temperature for 120 min. Complexes were purified via size exclusion chromatography (Superose6 Increase 10/300 GL, GE Healthcare), concentrated to 0.5 mg/mL and directly used for cryo-EM grid preparation.

### **Cross-linking of Fab 46-RBD complex**

100 µg of Fab 46 was mixed with 2x molar excess of RBD in 20 mM HEPES pH 7.0, 150 mM NaCl. The complex was crosslinked by addition of 0.05% (v/v) glutaraldehyde (Sigma Aldrich) and incubated at room temperature for 45 min. The cross-linked complex was purified via size exclusion chromatography (Superdex 200 Increase 10/300 GL, GE Healthcare), concentrated to 2.0 mg/ml and directly used for cryo-EM grid preparation.

### **Cryo-EM data collection and image processing**

3 µl of sample was deposited on holey gold grids prepared in-house<sup>51</sup>, which were glow-discharged in air for 15 s with a PELCO easiGlow (Ted Pella) before use. Sample was blotted for 6 s with a modified FEI Mark III Vitrobot (maintained at 4° C and 100% humidity) using an offset of -5, and subsequently plunge-frozen in a mixture of liquid ethane and propane. Data was acquired at 300 kV with a Thermo Fisher Scientific Titan Krios G3 electron microscope and prototype Falcon 4 camera operating in electron counting mode at 250 frames/s. Movies were collected for 9.6 s with 29 exposure fractions, a camera exposure rate of ~5 e<sup>-</sup>/pix/s, and total specimen exposure of ~44 e<sup>-</sup>/Å<sup>2</sup>. No objective aperture was used. The pixel size was calibrated at 1.03 Å/pixel from a gold diffraction standard. The microscope was automated with the *EPU* software package and data collection was monitored with *cryoSPARC Live*<sup>52</sup>.

To overcome preferred orientation encountered with some of the samples, tilted data collection was employed<sup>53</sup>. For the Spike-Fab 80 complex, 820 0° tilted movies and 2790 40° tilted movies were collected. For the Spike-Fab 298 complex, 4259 0° tilted movies and 3513 40° tilted movies were collected. For the Spike-Fab 324 complex, 1098 0° tilted movies and 3380 40° tilted movies were collected. For the RBD-Fab 46 complex, 4722 0° tilted movies were collected. For 0° tilted movies, cryoSPARC patch motion correction was performed. For 40° tilted movies, *Relion* MotionCorr<sup>54,55</sup> was used. Micrographs were then imported into *cryoSPARC* and patch CTF estimation was performed. Templates generated from 2D classification during the *cryoSPARC Live* session were used for template selection of particles. 2D classification was used to remove junk particle images, resulting in a dataset of 80,951 particle images for the Spike-Fab 80 complex, 203,138 particle images for the Spike-Fab 298 complex, 64,365 particle images for the Spike-Fab 324 complex, and 2,143,629 particle images for the RBD-Fab 46 complex. Multiple rounds of multi-class *ab initio* refinement were used to clean-up the particle image stacks, and homogeneous refinement was used to obtain consensus structures. For tilted particles, particle polishing was done within *Relion* at this stage and re-imported back into *cryoSPARC*. For the Spike-Fab complexes, extensive flexibility was observed. 3D variability analysis was performed<sup>56</sup> and together with heterogeneous refinement used to classify out the different states present. Non-uniform refinement was then performed on the final set of particle images<sup>57</sup>. For the RBD-Fab 46 complex, *cryoSPARC ab initio* refinement with three classes was used iteratively to clean up the particle image stack. Thereafter, the particle image stack with refined Euler angles was brought into *cisTEM* for reconstruction<sup>58</sup> to produce a 4.0 Å resolution map. Transfer of data between *Relion* and *cryoSPARC* was done with *pyem*<sup>59</sup>.

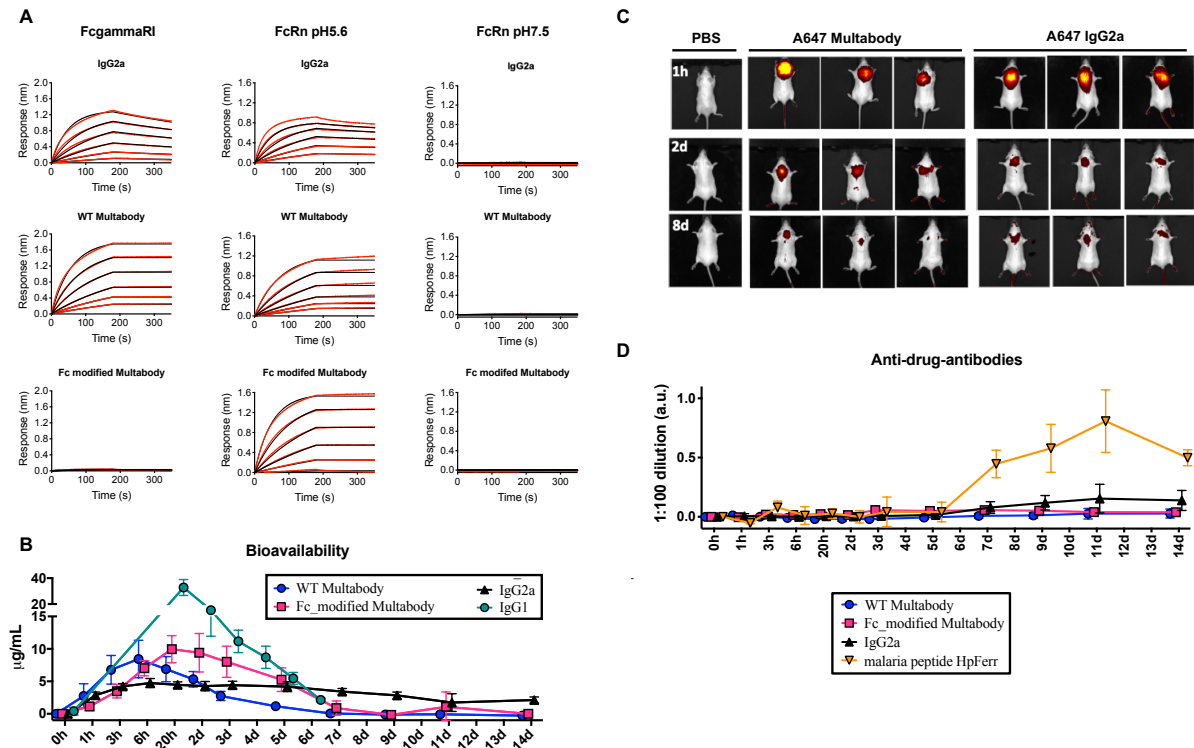
### **Crystallization and structure determination**

A ternary complex of 52 Fab-298 Fab-RBD was obtained by mixing 200  $\mu\text{g}$  of RBD with 2X molar excess of each Fab in 20 mM TRIS pH 8.0, 150 mM NaCl and subsequently purified via size exclusion chromatography (Superdex 200 Increase 10/300 GL, GE Healthcare). Fractions containing the complex were concentrated to 7.3 mg/ml and mixed in a 1:1 ratio with 20% (w/v) 2-propanol, 20% (w/v) PEG 4000, 0.1 M sodium citrate pH 5.6. Crystals appeared after  $\sim$ 1 day and were cryoprotected in 10% (v/v) ethylene glycol before being flash-frozen in liquid nitrogen. Data were collected on the 23-ID-D beamline at the Argonne National Laboratory Advanced Photon Source. The dataset was processed using XDS and XPREP<sup>46</sup>. Phases were determined by molecular replacement using Phaser<sup>47</sup> with CNTO88 Fab as a model for 52 Fab (PDB ID: 4DN3), 20358 Fab as a model for 298 Fab (PDB ID: 5CZX), and PDB ID: 6XDG as a search model for the RBD. Refinement of the structure was performed using phenix.refine<sup>48</sup> and iterations of manual building and refinement in Coot<sup>49</sup>. Access to all software was supported through SBGrid<sup>50</sup>.

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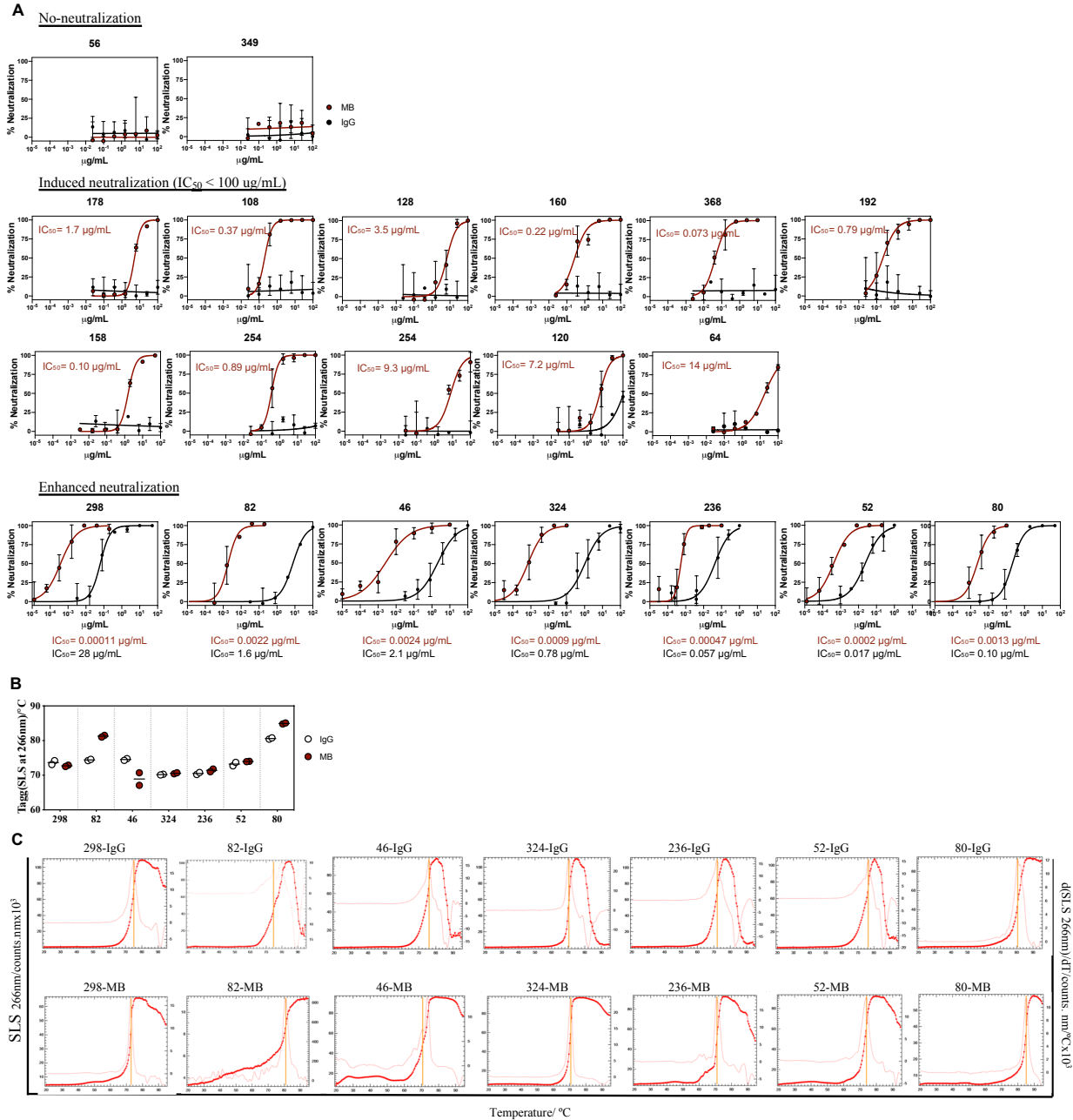
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**Fig. S1.**



**Bioavailability, biodistribution and immunogenicity of a surrogate Multobody in mice. (A)** Binding kinetics of WT and Fc-modified (LALAP mutation) MB to mouse Fc $\gamma$ RI (left) and mouse FcRn at endosomal (middle) and physiological (right) pH in comparison to the parental IgG. 2-fold dilution series from 100 to 3 nM (IgG), and 10 to 0.3 nM (MB) were used. Black lines represent raw data whereas red lines represent global fits. **(B)** Five male C57BL/6 mice per group were used to assess the serum concentration of a surrogate mouse MB, a Fc-modified MB (LALAP mutation) and parental mouse IgGs (IgG1 and IgG2a subtype) after subcutaneous administration of 5 mg/kg. **(C)** MB and IgG2a samples were labeled with Alexa-647 for visualization of their biodistribution post subcutaneous injection into three BALB/c mice/group via live non-invasive 2D whole body imaging. **(D)** Five male C57BL/6 mice per group were used to assess any anti-drug antibody response induced by the mouse surrogate Multobody in comparison to parental IgG and a species-mismatched malaria PfCSP peptide fused to *Helicobacter pylori* ferritin (HpFerr).

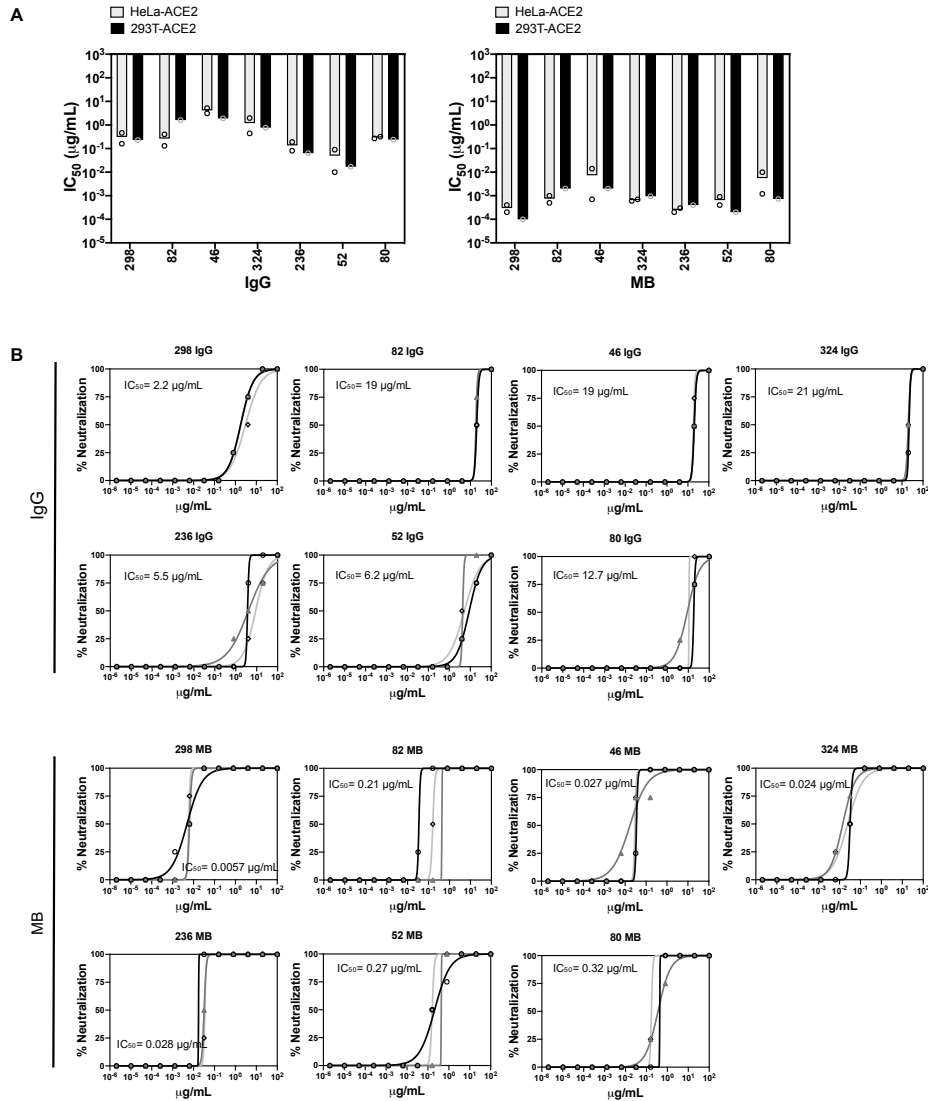
**Fig. S2.**



**Neutralization and thermostability of SARS-CoV-2 RBD-targeting Multibodies and their parental IgGs.** (A) Representative neutralization titration curves of 20 antibodies against SARS-CoV-2 PsV when displayed as IgGs (black) and MBs (dark red). The mean  $IC_{50}$  value of three biological replicates, each with two technical replicates are displayed for comparison. (B) Comparison of the aggregation temperature ( $T_{agg}$ ) of the seven most potent IgGs (white) and their respective MBs (dark red). (C) Static light scattering (SLS) at 266 nm *versus* temperature plots (dark red) from (b).  $T_{agg}$  values are calculated from the maximum of the differential curves (light red) and indicated with yellow lines.

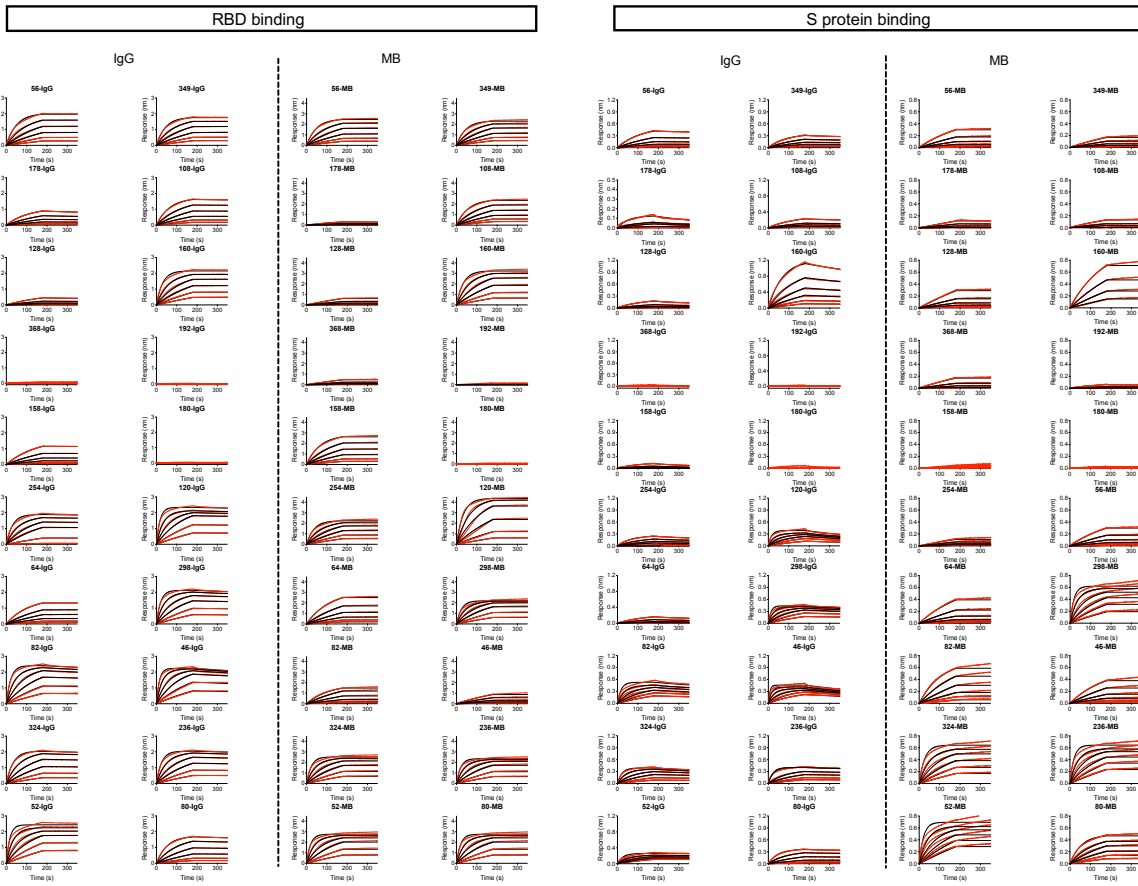


**Fig. S3.**



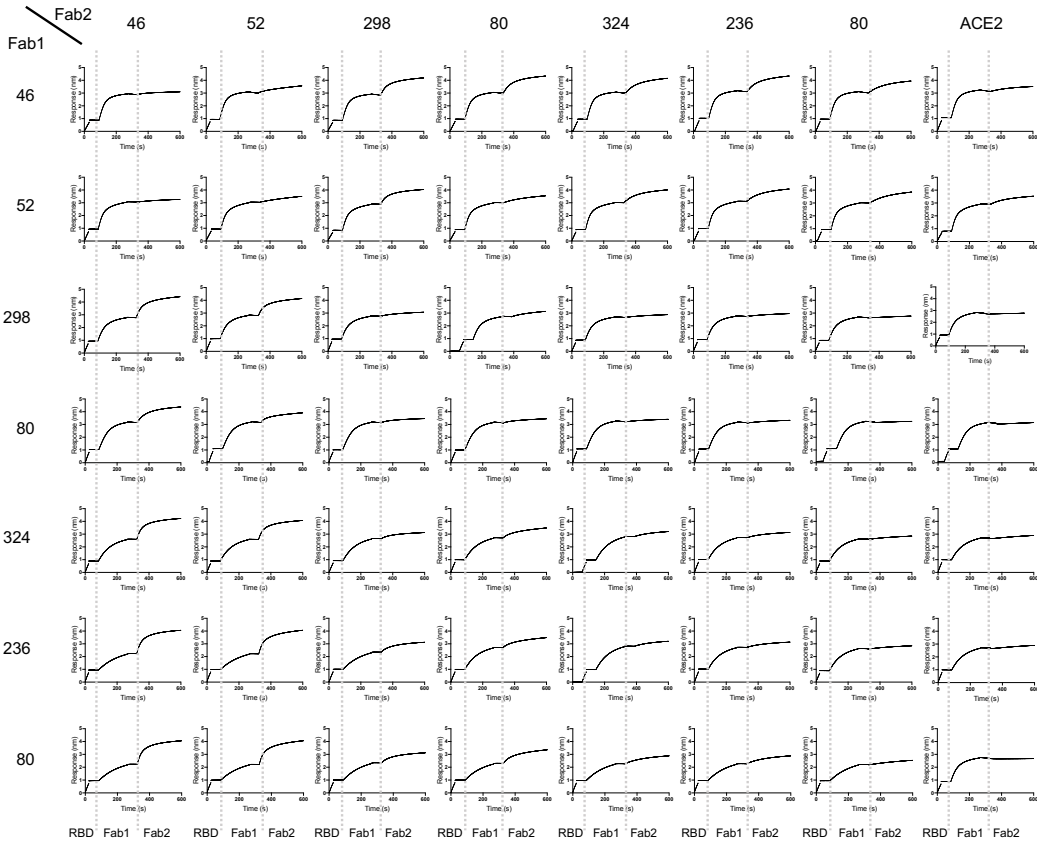
**Neutralization profiles of selected IgGs and MBs in different assays.** (A) Similar neutralization profiles of IgGs (left) vs MBs (right) against pseudotyped SARS-CoV-2 PsV targeting 293T-ACE2 (black) and HeLa-ACE2 (gray) target cells. The mean IC<sub>50</sub> value and individual IC<sub>50</sub> values of three and two biological replicates are shown for 293T-ACE2 and HeLa-ACE2 cells, respectively. (B) Neutralization titration curves of three biological replicates (different shades of gray) against the authentic SARS-CoV-2/SB2-P4-PB strain<sup>45</sup>. The mean IC<sub>50</sub> is indicated. The less sensitive neutralization phenotype observed against authentic virus in comparison to PsV is in agreement with previous reports<sup>5,13,14,17</sup>. However, other studies have observed similar values<sup>3,11,12,16</sup> between the two assays. This discrepancy makes cross-comparison of antibody potencies against live replicating virus difficult and is likely due to differences in the length of time of the neutralization experiment. Short incubation times will minimize the number of replications that the virus can undergo, resembling the one replication cycle of the PsV assays.

**Fig. S4.**



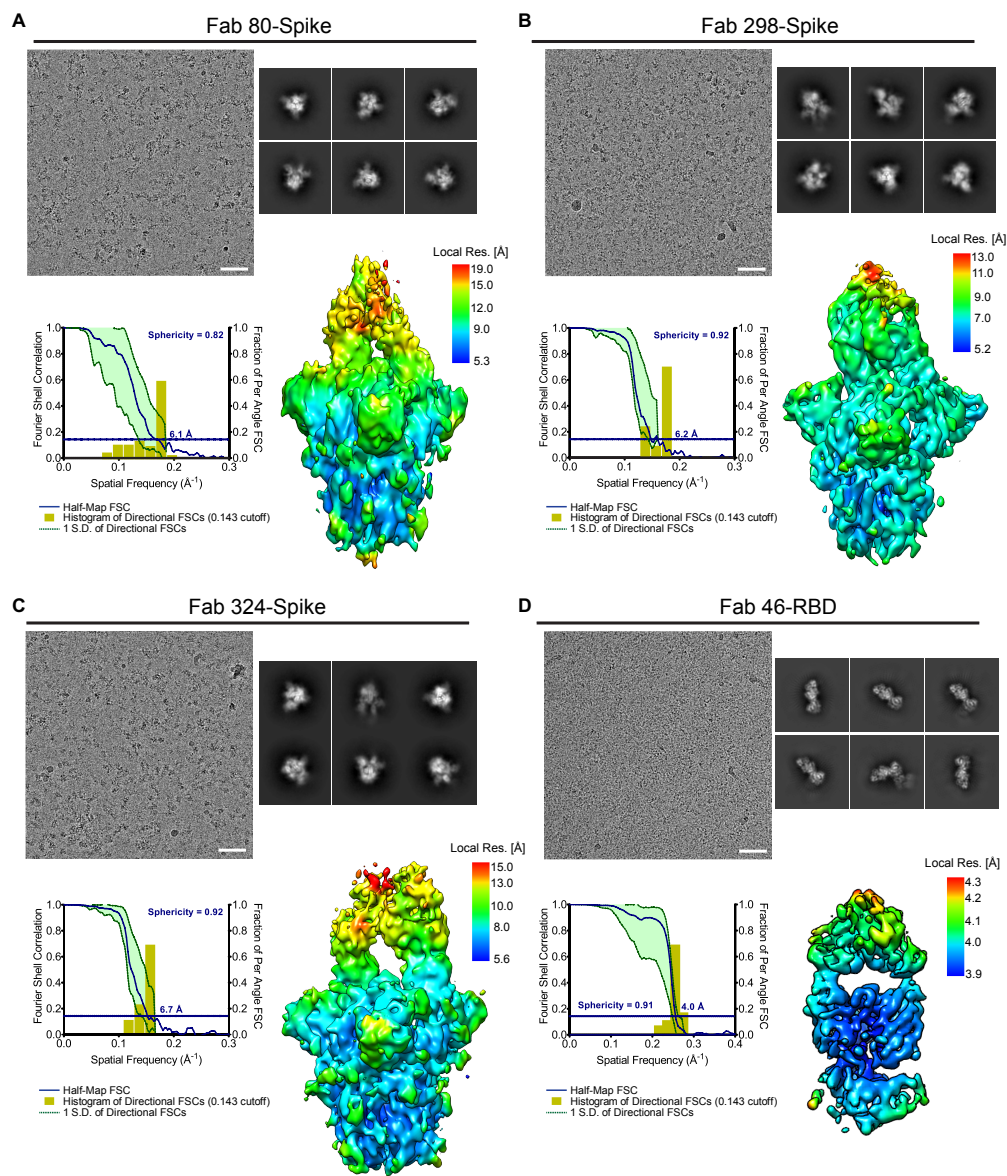
**Binding profiles of IgGs and MBs.** BLI response curves of IgG and MBs binding to RBD (left) and S protein (right) of SARS-CoV-2 immobilized onto Ni-NTA biosensors. 2-fold dilution series from 125 to 4 nM (IgG), and 16 to 0.5 nM (MB) were used. Black lines represent raw data whereas red lines represent global fits.

Fig. S5.



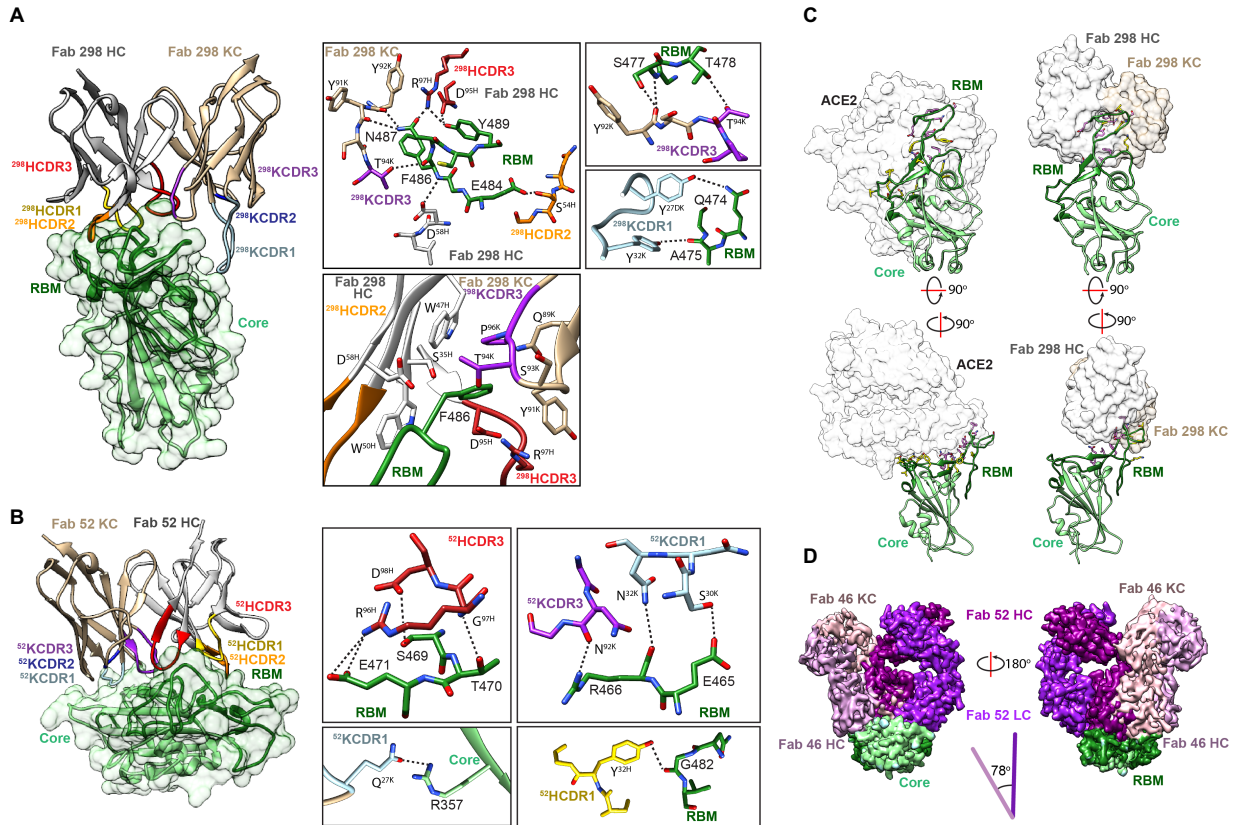
**Epitope binning.** mAb binding competition experiments to His-tagged RBD as measured by biolayer interferometry (BLI). 50  $\mu\text{g}/\text{ml}$  of mAb 1 was incubated for 3 min followed by incubation with 50  $\mu\text{g}/\text{ml}$  of mAb 2 for 5 min.

**Fig. S6.**



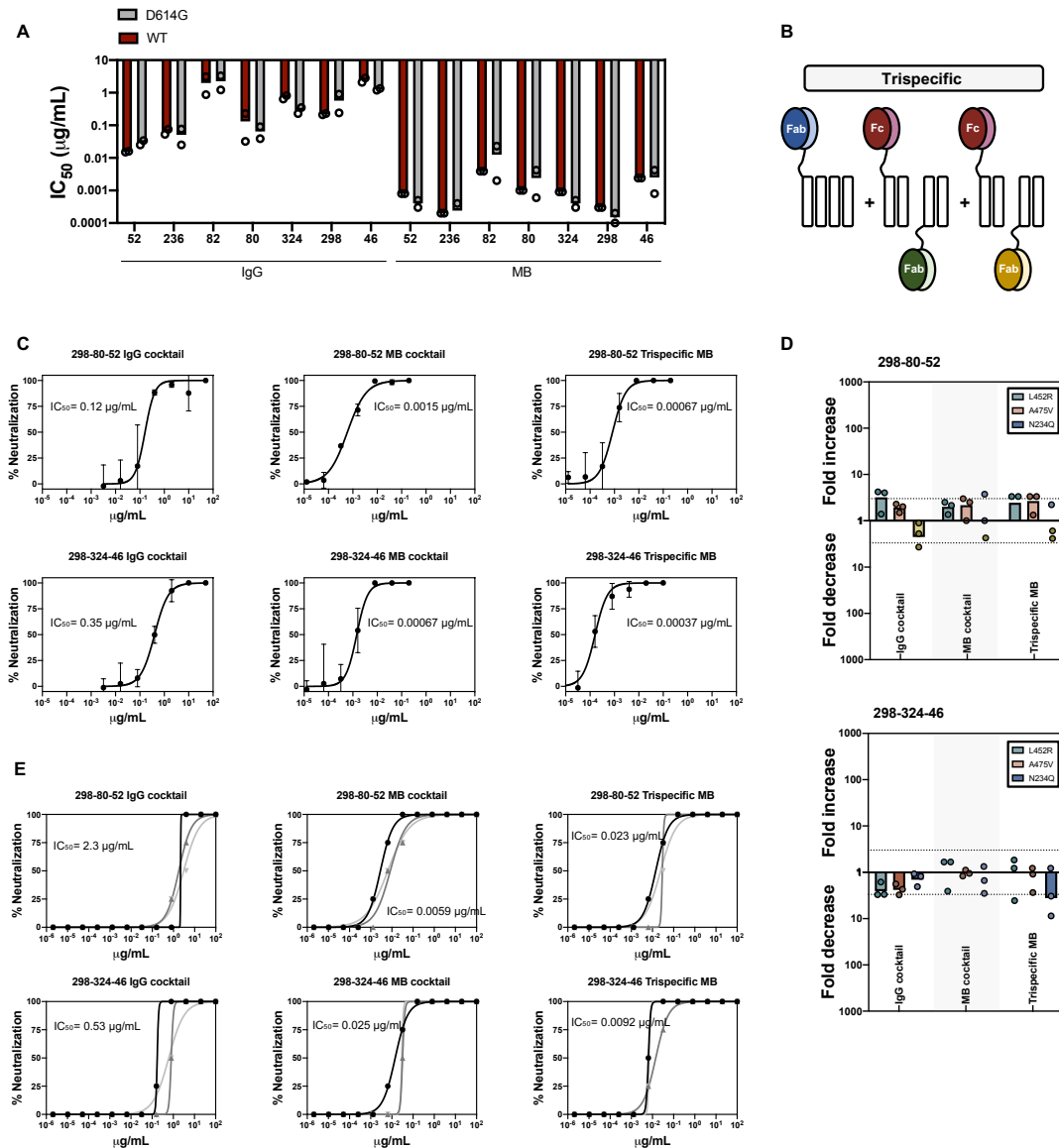
**Cryo-EM analysis of the Fab-Spike and Fab-RBD complexes.** Representative cryo-EM micrograph (Scale bar 50 nm, top left), selected 2D class averages (top right), Fourier shell correlation curve from the final 3D non-uniform refinement (bottom left) and local resolution (Å) plotted on the surface of the cryo-EM map (bottom right) are shown for the Fab 80-Spike complex (A), the Fab 298-Spike complex (B), the Fab 324-Spike complex (C), and the Fab 46-RBD complex (D).

**Fig. S7.**



**Binding interfaces of mAbs 52 and 298 and the RBD.** Interaction of mAbs 298 (A) and 52 (B) with RBD (light and dark green for the core and RBM regions, respectively) is mediated by complementarity determining regions (CDR) heavy (H) 1 (yellow), H2 (orange), H3 (red), kappa light (K) 1 (light blue) and K3 (purple) (left panels). Critical binding residues are shown in sticks (right panels). H-bonds and salt bridges are depicted as black dashed lines. L and H chains of Fabs are shown in tan and white, respectively. (C) Bottom and side views of ACE2 (left) and Fab 298 (right) bound to RBD. RBD side-chains that are part of the binding interface of the ACE2-RBD and Fab 298-RBD complexes are depicted in pink, while RBD side-chains unique to a given interface are shown in yellow. Surfaces of ACE2, variable regions of Fab 298 HC and Fab 298 KC are shown in white, grey and tan, respectively. The RBD is colored as in (a). (D) Superposition of Fabs 46 (light pink) and 52 (dark pink) when bound to the RBD (green) reveals a distinct angle of approach for the two mAbs.

**Fig. S8.**



**The MB platform potently overcomes SARS-CoV-2 sequence variability.** (A) Comparison of the neutralization potency of selected IgGs and MBs against WT PsV (dark red) and the more infectious D614G PsV (grey). (B) Schematic representation of a tri-specific MB generated by combination of three Fab specificities and the Fc fragment using the MB split design. (C) Cocktails and tri-specific MBs that combine the specificities of mAbs 298, 80 and 52, or 298, 324 and 46 were generated and tested against WT PsV. (D) Neutralization potency change of cocktails and tri-specific MBs against pseudotyped SARS-CoV-2 variants in comparison to WT PsV. PsV variants that were sensitive to individual antibodies within the cocktails were selected. The area within the dotted lines represent a 3-fold change in  $IC_{50}$  value. This threshold was established as the cutoff to establish increased sensitivity (up bars) and increased resistance (down bars). (E) Neutralization titration curves showing three biological replicates of cocktails and tri-specific MBs against the authentic SARS-CoV-2/SB2-P4-PB strain<sup>45</sup>. Mean  $IC_{50}$  values of three biological replicates are shown.

**Table S2. Cryo-EM data collection and image processing**

	<b>Fab 80-Spike</b>	<b>Fab 298-Spike</b>	<b>Fab 324-Spike</b>	<b>Fab 46-RBD</b>
<b>EMDB ID</b>	EMD-22739	EMD-22740	EMD-22741	EMD-22738
<b>Data Collection</b>				
<b>Electron microscope</b>	Titan Krios G3	Titan Krios G3	Titan Krios G3	Titan Krios G3
<b>Camera</b>	Falcon 4EC	Falcon 4EC	Falcon 4EC	Falcon 4EC
<b>Voltage (kV)</b>	300	300	300	300
<b>Nominal magnification</b>	75,000	75,000	75,000	75,000
<b>Calibrated physical pixel size (Å)</b>	1.03	1.03	1.03	1.03
<b>Total exposure (e-/Å<sup>2</sup>)</b>	44	44	44	44
<b>Number of frames</b>	29	29	29	29
<b>Image Processing</b>				
<b>Motion correction software</b>	<i>cryoSPARC v2, Relion MotionCorr</i>	<i>cryoSPARC v2, Relion MotionCorr</i>	<i>cryoSPARC v2, Relion MotionCorr</i>	<i>cryoSPARCv2</i>
<b>CTF estimation software</b>	<i>cryoSPARCv2</i>	<i>cryoSPARCv2</i>	<i>cryoSPARCv2</i>	<i>cryoSPARCv2</i>
<b>Particle selection software</b>	<i>cryoSPARCv2</i>	<i>cryoSPARCv2</i>	<i>cryoSPARCv2</i>	<i>cryoSPARCv2</i>
<b>3D map classification and refinement software</b>	<i>cryoSPARCv2</i>	<i>cryoSPARCv2</i>	<i>cryoSPARCv2</i>	<i>cisTEM</i>
<b>Micrographs used (total)</b>	3610	7772	4478	4722
<b>0° tilt</b>	820	4259	1098	4722
<b>40° tilt</b>	2790	3513	3380	0
<b>Global resolution (Å)</b>	6.2	6.2	6	4
<b>Particles in final maps</b>	7,525	26,972	18,595	32,283



**Table S3. X-ray crystallography data collection and refinement statistics**

	<b>Fab 52- Fab 298- SARS-CoV-2 RBD</b>
<b>PDB ID</b>	7K9Z
<b>Data Collection</b>	
Wavelength (Å)	1.03317
Space group	P 3 <sub>2</sub> 2 1
Cell dimensions	
a,b,c (Å)	87.6, 87.6, 325.1
$\alpha, \beta, \gamma$ (°)	90.0, 90.0, 120.0
Resolution (Å)	39.66-2.95 (3.05-2.95)
No. Molecules in ASU	1
No. Total observations	496,550 (43,958)
No unique observations	31,545 (3,060)
Multiplicity	15.7 (14.3)
R <sub>merge</sub> (%)	16.8 (74.2)
R <sub>pim</sub> (%)	4.3 (20.1)
<I/ $\sigma$ I>	12.3 (1.4)
CC <sub>1/2</sub>	99.8 (86.3)
Completeness (%)	99.9 (99.9)
<b>Refinement</b>	
Non-hydrogen atoms	8061
Macromolecule	8047
Glycan	14
R <sub>work</sub> /R <sub>free</sub>	0.259/0.286
Rms deviations	
Bond lengths (Å)	0.002
Bond angles (°)	0.53
Ramachandran plot	
Favored regions (%)	95.6
Allowed regions (%)	4.1
Outliers (%)	0.3
Rotamer Outliers (%)	2.6
B-factors (Å <sup>2</sup> )	
Wilson B-factor	78.6
Average B-factors	103.9
Average macromolecule	103.9
Average glycan	114.3



**Table S4. RBD residues contacting Fab 298 identified by PISA (Krissinel and Henrick, 2007).**

RBD	Residue	BSA (Å <sup>2</sup> )	Interaction	Fab 298 (H-HC, K-KC)
453	Tyr	2	vdW	H-Thr31, H-Ile100
455	Leu	20	vdW	H-Thr31, H-Ile100
456	Phe	30	vdW	H-Thr31, H-Tyr32
458	Lys	1	vdW	K-Ser27F
474	Gln	12	vdW	K-Tyr27D
	Gln <sup>NE2</sup>		HB	K-Tyr27D <sup>OH</sup>
475	Ala	45	vdW	K-Tyr27D, H-Tyr32, K-Tyr32, H-Arg97
	Ala <sup>O</sup>		HB	K-Tyr32 <sup>OH</sup>
476	Gly	23	vdW	K-Tyr27D, K-Tyr32, K-Tyr91, K-Tyr92, K-Ser93, H-Arg97
477	Ser	75	vdW	K-Tyr27D, K-Tyr92, K-Ser93, K-Thr94
	Ser <sup>N</sup>		HB	K-Tyr92 <sup>O</sup>
	Ser <sup>OG</sup>		HB	K-Tyr92 <sup>O</sup>
478	Thr	41	vdW	K-Tyr27D, K-Tyr92, K-Ser93, K-Thr94
	Thr <sup>OG1</sup>		HB	K-Thr94 <sup>OG1</sup>
484	Glu	74	vdW	H-Trp50, H-Ser52, H-Ser54, H-Gly55, H-Gly56, H-Thr57, H-Asp58
	Glu <sup>OE2</sup>		HB	H-Ser54 <sup>OG</sup>
485	Gly	28	vdW	H-Trp50, H-Thr57, H-Asp58
486	Phe	169	vdW	K-Gln89, K-Tyr91, K-Ser93, K-Thr94, K-Pro96, H-Ser35, H-Trp47, H-Trp50, H-Asp58, H-Asp95, H-Arg97
	Phe <sup>N</sup>		HB	H-Asp58 <sup>OD2</sup>
	Phe <sup>O</sup>		HB	K-Thr94 <sup>OG1</sup>
487	Asn	41	vdW	K-Tyr32, K-Tyr91, K-Ser93, K-Thr94, H-Asp95, H-Arg97
	Asn <sup>OD1</sup>		HB	H-Arg97 <sup>NH2</sup>
	Asn <sup>ND2</sup>		HB	K-Tyr91 <sup>O</sup> , K-Tyr92 <sup>O</sup>
488	Cys	1	vdW	H-Trp50
489	Tyr	84	vdW	H-Ser30, H-Thr31, H-Tyr32, H-Trp50, H-Asp95, H-Arg97,
	Tyr <sup>OH</sup>		HB	H-Arg97 <sup>NH2</sup> , H-Asp95 <sup>OD2</sup>
493	Gln	45	vdW	H-Ser30, H-Thr31, H-Ile100
<b>Total BSA (Å<sup>2</sup>):</b>		691		

vdW: van der Waals interaction (5.0 Å cut-off)

HB: hydrogen bond (3.8 Å cut-off)

SB: salt bridge (4.0 Å cut-off)

**Table S5. Fab 298 residues contacting RBD identified by PISA (Krissinel and Henrick, 2007).**

<b>Fab 298</b>	<b>Residue</b>	<b>Chain</b>	<b>BSA (Å<sup>2</sup>)</b>	<b>Interaction</b>	<b>RBD</b>
<b>30</b>	Ser	H	9	vdW	Tyr489, Gln493
<b>31</b>	Thr	H	68	vdW	Tyr453, Leu455, Phe456, Tyr489, Gln493
<b>32</b>	Tyr	H	57	vdW	Phe456, Ala475, Tyr489
<b>35</b>	Ser	H	8	vdW	Phe486
<b>47</b>	Trp	H	23	vdW	Phe486
<b>50</b>	Trp	H	79	vdW	Glu484, Gly485, Phe486, Cys488, Tyr489
<b>52</b>	Ser	H	8	vdW	Glu484
<b>54</b>	Ser	H	17	vdW	Glu484
	Ser <sup>OG</sup>			HB	Glu484 <sup>OE2</sup>
<b>55</b>	Gly	H	7	vdW	Glu484
<b>56</b>	Gly	H	7	vdW	Glu484
<b>57</b>	Thr	H	10	vdW	Glu484, Gly485
<b>58</b>	Asp	H	28	vdW	Glu484, Gly485, Phe486
	Asp <sup>OD2</sup>			HB	Phe486 <sup>N</sup>
<b>95</b>	Asp	H	15	vdW	Phe486, Asn487, Tyr489
	Asp <sup>OD2</sup>			HB	Tyr489 <sup>OH</sup>
<b>97</b>	Arg	H	61	vdW	Asn487, Tyr489
	Arg <sup>NH2</sup>			HB	Asn487 <sup>OD1</sup> , Tyr489 <sup>OH</sup>
<b>100</b>	Ile	H	7	vdW	Tyr453, Leu455, Gln493
<b>27D</b>	Tyr	K	59	vdW	Gln474, Ala475, Gly476, Ser477, Thr478
	Tyr <sup>OH</sup>			HB	Gln474 <sup>NE2</sup>
<b>27F</b>	Ser	K	1	vdW	Lys458
<b>32</b>	Tyr	K	28	vdW	Ala475, Gly476, Asn487
	Tyr <sup>OH</sup>			HB	Ala475 <sup>O</sup>
<b>89</b>	Gln	K	1	vdW	Phe486
<b>91</b>	Tyr	K	30	vdW	Gly476, Phe486, Asn487
	Tyr <sup>O</sup>			HB	Asn487 <sup>ND2</sup>
<b>92</b>	Tyr	K	40	vdW	Gly476, Ser477, Thr478
	Tyr <sup>O</sup>			HB	Asn487 <sup>ND2</sup>
	Tyr <sup>O</sup>			HB	Ser477 <sup>N</sup> , Ser477 <sup>OG</sup>
<b>93</b>	Ser	K	26	vdW	Gly476, Ser477, Thr478, Phe486, Ser487
<b>94</b>	Thr	K	57	vdW	Ser477, Thr478, Phe486, Asn487
	Thr <sup>OG1</sup>			HB	Thr478 <sup>OG1</sup> , Phe486 <sup>O</sup>
<b>96</b>	Pro	K	23	vdW	Phe486

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**Total BSA ( $\text{\AA}^2$ ):** 669

vdW: van der Waals interaction (5.0  $\text{\AA}$  cut-off)

HB: hydrogen bond (3.8  $\text{\AA}$  cut-off)

SB: salt bridge (4.0  $\text{\AA}$  cut-off)

**Table S6. RBD residues contacting Fab 52 identified by PISA (Krissinel and Henrick, 2007).**

RBD	Residue	BSA (Å <sup>2</sup> )	Interaction	Fab 52 (H-HC, K-KC)
346	Arg	45	vdW	H-Gln64
351	Tyr	29	vdW	K-Phe94, H-Ile52, H-Thr56, H-Asn58
352	Ala	19	vdW	K-Gly93, K-Phe94
354	Asn	6	vdW	K-Phe94
355	Arg	36	vdW	K-Ser0, K-Gln27
	Arg <sup>O</sup>		HB	K-Ser0 <sup>OG</sup>
356	Lys	13	vdW	K-Ser0
357	Arg	66	vdW	K-Ser0, K-Gln27
	Arg <sup>N</sup>		HB	K-Ser0 <sup>OG</sup>
	Arg <sup>NH2</sup>		HB	K-Gln27 <sup>OE1</sup>
449	Tyr	13	vdW	H-Phe554, H-Thr56
450	Asn	38	vdW	H-Phe554, H-Thr56
452	Leu	47	vdW	H-Ile52, H-Phe554, H-Gly55, H-Thr56
462	Lys	1	vdW	K-Ser30
465	Glu	28	vdW	K-Ser30, K-Asn31, K-Asn32, K-Asn92
	Glu <sup>OE2</sup>		HB	K-Ser30 <sup>OG</sup>
466	Arg	70	vdW	K-Asn32, K-Asn92, K-93Gly, K-Phe94
	Arg <sup>O</sup>		HB	K-Asn32 <sup>ND2</sup>
	Arg <sup>NH1</sup>		HB	K-Asn92 <sup>O</sup>
467	Asp	12	vdW	K-Asn32, K-Asn92, H-Asp98
468	Ile	102	vdW	K-Asn32, K-Gly91, K-Asn92, K-93Gly, K-Phe94, K-Leu96, H-Arg96, H-Gly97, H-Asp98
469	Ser	37	vdW	K-Asn32, H-Arg96, H-Gly97, H-Asp98
	Ser <sup>OG</sup>		HB	H-Asp98 <sup>OD1</sup>
470	Thr	67	vdW	H-Ser31, H-Tyr32, H-Gly33, H-Ile52, H-Asp95, H-Arg96, H-Gly97, H-Asp98
	Thr <sup>OG1</sup>		HB	H-Gly97 <sup>N</sup>
471	Glu	39	vdW	H-Tyr32, H-Arg96, H-Gly97, H-Asp98
	Glu <sup>OE1</sup>		SB	H-Arg96 <sup>NH2</sup>
	Glu <sup>OE2</sup>		SB	H-Arg96 <sup>NH2</sup>
472	Ile	7	vdW	H-Ser31, H-Tyr32, H-Arg96
481	Asn	1	vdW	H-Tyr32
482	Gly	42	vdW	H-Ser31, H-Tyr32
	Gly <sup>O</sup>		HB	H-Tyr32 <sup>OH</sup>
483	Val	12	vdW	H-Thr28, H-Ser31, H-Tyr32
484	Glu	53	vdW	H-Thr28, H-Phe29, H-Thr30, H-Ser31, H-Met54
490	Phe	87	vdW	H-Thr30, H-Ser31, H-Tyr32, H-Gly33, H-Ile52, H-Met54, H-Phe554
492	Leu	13	vdW	H-Ile52, H-Met54, H-Phe554
493	Gln	8	vdW	H-Phe554

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<b>494</b>	Ser	17	vdW	H-Phe554
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**Total BSA (Å<sup>2</sup>):** 904

vdW: van der Waals interaction (5.0 Å cut-off)

HB: hydrogen bond (3.8 Å cut-off)

SB: salt bridge (4.0 Å cut-off)

**Table S7. Fab 52 residues contacting RBD identified by PISA (Krissinel and Henrick, 2007).**

Fab 52	Residue	Chain	BSA (Å <sup>2</sup> )	Interaction	RBD
28	Thr	H	24	vdW	Val483, Glu484
30	Thr	H	3	vdW	Glu484, Phe490
31	Ser	H	66	vdW	Glu471, Gly482, Val483, Glu484, Phe490
32	Tyr	H	51	vdW	Thr470, Glu471, Ile472, Asn481, Gly482, Val483, Phe490
	Tyr <sup>OH</sup>	H		HB	Gly482 <sup>O</sup>
33	Gly	H	7	vdW	Thr470, Phe490
52	Ile	H	62	vdW	Tyr351, Leu452, Thr470, Phe490, Leu492
53	Met	H	38	vdW	Glu484, Phe490, Leu492
54	Phe	H	110	vdW	Tyr449, Asn450, Leu452, Phe490, Leu492, Gln493, Ser494
55	Gly	H	4	vdW	Leu452
56	Thr	H	57	vdW	Tyr351, Tyr449, Asn450, Leu452
58	Asn	H	10	vdW	Tyr351
64	Gln	H	43	vdW	Arg346
95	Asp	H	2	vdW	Thr470
96	Arg	H	64	vdW	Ile468, Ser469, Thr470, Glu471, Ile472
	Arg <sup>NH2</sup>	H		SB	Glu471 <sup>OE1</sup> , Glu471 <sup>OE2</sup>
97	Gly	H	64	vdW	Ile468, Ser469, Thr470, Glu471
	Gly <sup>N</sup>			HB	Thr470 <sup>OG1</sup>
98	Asp	H	28	vdW	Asp467, Ile468, Ser469, Thr470, Glu471
	Asp <sup>OD1</sup>			HB	Ser469 <sup>OG</sup>
0	Ser	K	77	vdW	Arg355, Lys356, Arg357
	Ser <sup>OG</sup>			HB	Arg355 <sup>O</sup> , Arg357 <sup>N</sup>
27	Gln	K	60	vdW	Arg355, Arg357
	Gln <sup>OE1</sup>			HB	Arg357 <sup>NH2</sup>
30	Ser	K	28	vdW	Lys462, Glu465
	Ser <sup>OG</sup>			HB	Glu465 <sup>OE2</sup>
32	Asn	K	19	vdW	Glu465, Arg466, Asp467, Ile468, Ser469
	Asn <sup>ND2</sup>			HB	Arg466 <sup>O</sup>
91	Gly	K	18	vdW	Ile468
92	Asn	K	56	vdW	Glu465, Arg466, Asp467, Ile468
	Asn <sup>O</sup>			HB	Arg466 <sup>NH1</sup>
93	Gly	K	22	vdW	Ala352, Arg466, Ile468
94	Phe	K	56	vdW	Tyr351, Ala352, Asn354, Arg466, Ile468

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<b>96</b>	Leu	K	5	vdW	Ile468
<b>Total BSA (Å<sup>2</sup>):</b>			974		

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vdW: van der Waals interaction (5.0 Å cut-off)

HB: hydrogen bond (3.8 Å cut-off)

SB: salt bridge (4.0 Å cut-off)