Effect of SARS-CoV-2 spike mutations on animal ACE2 usage and in vitro neutralization sensitivity

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Abstract

The emergence of SARS-CoV-2 variants poses greater challenges to the control of COVID-19 pandemic. Here, we parallelly investigated three important characteristics of seven SARS-CoV-2 variants, including two mink-associated variants, the B.1.617.1 variant, and the four WHO-designated variants of concerns (B.1.1.7, B.1.351, P.1, and B.1.617.2). We first investigated the ability of these variants to bind and use animal ACE2 orthologs as entry receptor. We found that, in contrast to a prototype variant, the B.1.1.7, B.1.351, and P.1 variants had significantly enhanced affinities to cattle, pig, and mouse ACE2 proteins, suggesting increased susceptibility of these species to these SARS-CoV-2 variants. We then evaluated in vitro neutralization sensitivity of these variants to four monoclonal antibodies in clinical use. We observed that all the variants were partially or completely resistant against at least one of the four tested antibodies, with B.1.351 and P.1 showing significant resistance to three of them. As ACE2-Ig is a broad-spectrum anti-SARS-CoV-2 drug candidate, we then evaluated in vitro neutralization sensitivity of these variants to eight ACE2-Ig constructs previously described in three different studies. All the SARS-CoV-2 variants were efficiently neutralized by these ACE2-Ig constructs. Interestingly, compared to the prototype variant, most tested variants including the variants of concern B.1.1.7, B.1.351, P.1, and B.1.617.2 showed significantly increased (up to ~15-fold) neutralization sensitivity to ACE2-Ig constructs that are not heavily mutated in the spike-binding interface of the soluble ACE2 domain, suggesting that SARS-CoV-2 evolves toward better utilizing ACE2, and that ACE2-Ig is an attractive drug candidate for coping with SARS-CoV-2 mutations.

Keywords： SARS-CoV-2; spike mutation; variant of concern; receptor; ACE2; host range; neutralizing antibody; ACE2-Ig

Introduction

As of June 23rd 2021, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiological agent of the ongoing coronavirus disease 2019 (COVID-19) pandemic, has already caused about 178 million confirmed infections and over 3.8 million documented deaths over the world, according to World Health Organization (WHO) online updates. The pandemic has triggered unprecedentedly extensive worldwide efforts to develop countermeasures against COVID-19, and a number of encouraging progresses have been achieved in developing prophylactic vaccines and antibody therapeutics[1-12]. So far, there are more than six prophylactic COVID-19 vaccines that have been authorized by different countries for emergency use. These include two mRNA vaccines (Pfizer-BioNTech, US; Moderna, US)[1,2], two inactivated vaccines (Sinopharm, China; Sinovac, China)[3-5], and two adenoviral vectored vaccines (Sputnik V, Russia; AstraZeneca-Oxford, UK)[6,7]. Over 2 billion doses of these vaccines have been administered worldwide. There are also some convalescent patient-derived antibodies that have been authorized for emergency use by the US FDA, such as Regeneron's antibody cocktail consisting of casirivimab (REGN10933) and imdevimab (REGN10987)[8,9], and Eli Lilly's antibody cocktail consisting of etesevimab (LY-CoV016) and bamlanivimab (LY-CoV555)[10-12]. So far, all the SARS-CoV-2 vaccines and antibody therapeutics in clinical use were developed on the basis of the prototype SARS-CoV-2 strain.

SARS-CoV-2 is a betacoronavirus that has broad host ranges[13-16]. Receptor usage is a critical determinant for the host range, as well as an effective neutralization target, of coronaviruses [16,17]. SARS-CoV-2 utilizes ACE2 as a key cellular receptor to infect cells[13,18,19]. Antibodies targeting the interactions between ACE2 and SARS-CoV-2 spike receptor-binding domain (RBD) efficiently neutralize SARS-CoV-2 infection and reduced viral load in animal models and COVID-19 patients[8-12]. As human ACE2 residues on the spike RBD-binding interface are highly conserved across a number of mammalian-ACE2 orthologs, mutations within the spike

67 RBD region might easily alter cross-species receptor usage by SARS-CoV-2, as well as
68 sensitivity to SARS-CoV-2 neutralization agents. Indeed, we recently found that SARS-CoV-
69 2 can use human ACE2 and a wide range of animal-ACE2 orthologs, but not mouse ACE2, for
70 cell entry [14]. But a single amino-acid change within the spike receptor-binding domain (RBD;
71 Q498H, Q498Y, or N501Y) could be sufficient to confer SARS-CoV-2 the ability to utilize
72 mouse ACE2[ref.20-23].

73 SARS-CoV-2 is a single-stranded RNA virus with moderate mutation and recombination
74 frequencies[24,25]. A number of spontaneous and selection-pressure-driven mutations of SARS-
75 CoV-2 genome have been identified in viral variants that emerged during the course of the
76 pandemic[13,26-35]. With more and more SARS-CoV-2 variants being identified to carry diverse
77 spike mutations within the RBD region, it's possible that some of the spike mutations might
78 alter the host range of the virus, or compromise the efficacy of vaccines or neutralizing
79 antibodies developed on the basis of the prototype SARS-CoV-2 strain. In this study, we
80 investigated cross-species receptor usage of multiple circulating SARS-CoV-2 variants that
81 emerged during the pandemic, as well as sensitivity of these variants to four neutralizing
82 antibodies in clinical use and a broad-spectrum anti-SARS-CoV-2 drug candidate.

83 Results

84 Spike variants and related mutations investigated in this study

85 We first cloned twelve spike genes of nine SARS-CoV-2 natural variants. They include a spike
86 gene of an early isolate WHU01[ref.36], representing the prototype SARS-CoV-2 variant, a spike
87 gene of the well-studied D614G variant[35], two spike genes of a mink-associated variant that
88 carries a Y453F signature mutation in the RBD region[31,32,38], a spike gene of the B.1.617.1
89 variant, and seven spike genes of the four WHO-designated variants of concern (VOCs),
90 B.1.1.7, B.1.351, P.1, and B.1.617.2[ref.27-30] (**Figure 1A**). The VOC B.1.1.7, also called

501Y.V1, was first identified in the UK in late summer to early autumn 2020, then spread rapidly across the UK, and now has been identified in at least 114 countries[39-41]. B.1.1.7 has an N501Y signature mutation in the RBD region, and carries seven additional mutations in the remaining region of the spike protein[27,28]. Two spike sequences, one that only carries the N501Y mutation and the other that carries all the spike mutations, were used in the following studies (**Figure 1A**). The VOC B.1.351, also called 501Y.V2, was first detected in South Africa from samples collected in early August, then rapidly became dominant locally, and now has been identified in at least 68 countries. B.1.351 variant has three signature amino-acid substitutions (K417N-E484K-N501Y) in the RBD region and a number of additional mutations in the remaining regions of the spike protein[29]. Similarly, two spike sequences, one that only carries the K417N-E484K-N501Y mutations and the other that carries all the spike mutations, were used in the following studies (**Figure 1A**). The VOC P.1, also called 501Y.V3, emerged in Brazil around mid-November 2020, and now has rapidly spread to at least 37 countries. P.1 variant has three signature substitutions (K417T-E484K-N501Y) in the RBD region, similar to the RBD mutations of the B.1.351 variant. It also carries seven additional lineage-defining amino-acid substitutions in the remaining regions of the spike protein[30]. Again, two spike sequences, one that only carries the K417T-E484K-N501Y mutations and the other that carries all the spike mutations, were used in the following studies (**Figure 1A**). The VOC B.1.617.2, emerged in India around September 2020, and now has rapidly spread to over 75 countries. B.1.617.2 variant has two signature substitutions (L452R-T478K) in the RBD region. It also carries six additional lineage-defining amino-acid substitutions in the remaining regions of the spike protein. A spike sequence that carries all the B.1.617.2-signature mutations was used in the following studies (**Figure 1A**). All the residues associated with the above mentioned RBD mutations are indicated in the structure of human ACE2 in complex with the RBD of a prototype SARS-CoV-2 variant (**Figure 1B**).

116 **Pseudoviruses of the B.1.1.7, B.1.351, and P.1 variants displayed patterns of animal-**
117 **ACE2 tropism distinct from the patterns of other variants**

118 To evaluate ACE2 ortholog-mediated viral entry of the above-mentioned SARS-CoV-2
119 variants, we produced luciferase reporter retroviruses pseudotyped with one of these different
120 spike variants. These reporter pseudoviruses were used to infect 293T cells expressing each of
121 eight ACE2 orthologs (**Figure 2 and Table 1**), including the human ACE2 and ACE2
122 orthologs of cattle (*Bos taurus*), pig (*Sus scrofa domesticus*), cat (*Felis catus*), rabbit
123 (*Oryctolagus cuniculus*), bat (*Rhinolophus sinicus* isolate Rs-3357), rat (*Rattus norvegicus*),
124 and mouse (*Mus musculus*). Parallel infection experiments using 293T cells transfected with
125 an empty vector plasmid were included as controls. Consistent with our previous report[14], the
126 early isolate WHU01 efficiently infected 293T cells expressing most of the tested ACE2
127 orthologs except for that of rat and mouse (**Figure 2A**). While a similar pattern was also
128 observed with the variants D614G, and Y453F (**Figure 2B and C**), distinct patterns were
129 observed with the remaining variants (**Figure 2D-L**). Significant changes were observed with
130 the VOCs B.1.1.7, B.1.351, and P.1, all of which showed ability to use ACE2 orthologs of rat
131 and mouse for entry (**Figure 2E-J**). In addition, the variants B.1.351 and P.1 showed complete
132 loss of the ability to use bat ACE2 for entry (**Figure 2G-J**). These data suggest that the
133 mutations acquired by different variants might have changed SARS-CoV-2 RBD affinity to
134 different ACE2 orthologs.

135 **Y453F-RBD and the B.1.617.2-RBD have enhanced affinity to human ACE2**

136 We then sought to quantitatively study the ability of the variants to utilize various animal
137 orthologs of ACE2. The extracellular domains of the eight ACE2 orthologs and the RBD
138 domains of seven spike variants, including WHU01, Y453F, B.1.1.7, B.1.351, P.1, B.1.617.1,
139 and B.1.617.2, were expressed in 293F cells as immunoglobulin Fc fusion proteins. Purified
140 ACE2 and RBD recombinant proteins were then used as immobilized receptors and analytes,

141 respectively, in a bio-layer interferometry (BLI)-based assay to measure the interaction kinetics
142 of fifty-six RBD-ACE2 pairs (**Figure 3 and Table 2**). When the RBD of early SARS-CoV-2
143 isolate WHU01 was tested against different ACE2 proteins, strong interactions were observed
144 for human, cattle, pig, cat, and rabbit ACE2 proteins, with human ACE2 showing the highest
145 affinity to this RBD. No any binding signal was detected for mouse ACE2 (**Figure 3H)**. We
146 then compared the kinetics data of each RBD variants to the data of the WHU01 variant. We
147 observed that the Y453F mutation increased RBD affinity to human ACE2 by ~4.7-fold
148 (**Figure 3A and Table 2**), consistent with a very recent report[42]. In addition, the B.1.617.2
149 RBD but not the B.1.617.1 RBD was also found with >2-fold higher affinity to human ACE2
150 (**Figure 3A and Table 2**), consistent with the fast spreading of this variant across the world.

151 **N501Y mutation enhances RBD affinity to most of the tested ACE2 proteins, especially**
152 **cattle and pig ACE2 proteins**

153 In the case of the B.1.1.7 RBD which has a N501Y signature mutation, we observed 2.9-fold
154 increase in the RBD affinity to human ACE2 (**Figure 3A and Table 2**). This is consistent with
155 the binding data from other reports and the increased infectivity of the B.1.1.7 variant[39-41,43-45].
156 Structural studies show that the Tyr501 residue of the B.1.1.7 RBD inserts into a cavity at the
157 binding interface and forms a perpendicular π–π stacking interaction with the Tyr41residue of
158 ACE2[ref.45]. Perhaps because of a same mechanism, the B.1.1.7 RBD also displayed
159 significantly increased affinity to all the other tested ACE2 proteins, except for bat ACE2 that
160 has a His41 rather than a Tyr41 (**Figure 3B-H and Table 2**). More pronounced affinity
161 increase was observed with cattle ACE2 (5.0-fold) and pig ACE2 (6.1-fold), making the
162 affinities of cattle and pig ACE2 proteins to B.1.1.7 RBD slightly higher than that of human
163 ACE2 to WHU01 RBD (**Figure 3B and C, and Table 2**). Only very weak binding signals
164 were detected for mouse ACE2, though the B.1.1.7 pseudovirus efficiently utilized mouse

165 ACE2 for entry (**Figure 2E, F, and 3H**), reflecting the sensitivity difference of the two assays
166 for discriminating weak and strong interactions.

167 **The RBDs of B.1.351 and P.1 have increased affinities to cattle, pig, and mouse ACE2**
168 **proteins**

169 In the case of the RBDs of B.1.351 and P.1 which share the E484K-N501Y signature mutations,
170 almost identical patterns were observed (**Figure 3**). Compared to WHU01 RBD, B.1.351 and
171 P.1 RBDs respectively also showed 2.9- and 2.7-fold higher affinities to cattle ACE2, and 3.4-
172 and 2.9-fold higher affinities to pig ACE2 protein, possibly because of the shared N501Y
173 mutation in these two variants (**Figure 3B and C, and Table 2**). On the other hand, consistent
174 with the pseudovirus infection data (**Figure 2G-J**), the B.1.351 and P.1 RBDs showed a
175 complete loss of interaction with bat ACE2, and a clear gain of interaction with mouse ACE2
176 (**Figure 3F and H**). Additional pseudovirus infection experiments showed that every single
177 RBD mutation (K417N, K417T, E484K, or N501Y) of the B.1.351 and P.1 variants contribute
178 to the utilization of mouse ACE2 as a functional receptor, with the N501Y mutation showed
179 the most prominent effect (**Figure 4**). A Y41A single mutation in mouse ACE2 significantly
180 impaired infectivity of all the single-spike-mutation pseudoviruses, but not the triple-spike-
181 mutation pseudoviruses, while Y41A-H353A double mutation in mouse ACE2 significantly
182 impaired infectivity of all the pseudoviruses, but much less pronounced effect was observed
183 with the triple-spike-mutation pseudoviruses (**Figure 4**). These data suggest that the B.1.351
184 and P.1 RBDs gain binding to mouse ACE2 through the mutated RBD residues forming
185 multiple new interactions with ACE2.

186 **Spike mutations cause escape from potent neutralizing antibodies in clinical use**

187 We then performed pseudovirus neutralization assays to evaluate sensitivity of these diverse
188 SARS-CoV-2 variants to four therapeutic antibodies in clinical use (etesevimab/LY-CoV016,

189 bamlanivimab/LY-CoV555, casirivimab/REGN10933, and imdevimab/REGN10987)[8-12].
190 While both the early strain WHU01 and the D614G variant were highly sensitive to all the four
191 antibodies, all the RBD-mutated variants showed partial or complete resistance to at least one
192 antibody (**Figure 5**). Specifically, both of the variants carrying a Y453F mutation showed
193 strong resistance to REGN10933 (**Figure 5C and D**). The VOC B1.1.7 showed partial
194 resistance to LY-CoV016 (**Figure 5E and F**). It is of note that the VOCs B.1.351 and P.1
195 showed strong resistance to REGN10933, and complete resistance to both LY-CoV016 and
196 LY-CoV555, the two components of an antibody-cocktail therapy authorized for emergency
197 use by the U.S. FDA[10-12] (**Figure 5G-J**). The B.1.617.1 variant showed complete resistance to
198 LY-CoV555 and partial resistance to REGN10933 (**Figure 5K)**. Although the VOC B.1.617.2
199 also showed strong resistance to LY-CoV555, it's sensitivity to LY-CoV016 was found
200 significantly increased (**Figure 5L**). Consistent with these neutralization data, analyzing
201 structural data of these antibodies in complex with the original RBD revealed contact of LY-
202 CoV016 with RBD residues Lys417, LY-CoV555 with RBD residues Glu484 and Phe486,
203 REGN10933 with RBD residues Lys417, Tyr453, Glu484, and Phe486, and REGN10987 with
204 RBD residues Asn439 and Gln498 (**Figure 5M-P**).

205 **The impact of RBD mutations on antibody affinity**

206 We then performed BLI assays to quantitatively measure the kinetics of antibody-RBD
207 interactions (**Figure 6 and Table 3**). The binding data are fully consistent with the pseudovirus
208 neutralization data. Specifically, the mink-associated Y453F mutation decreased RBD affinity
209 to REGN10933 (**Figure 6C**). The B.1.1.7-signature mutation N501Y decreased RBD binding
210 to LY-CoV016 (**Figure 6A**). The B.1.351 RBD showed significantly decreased binding to
211 REGN10933, and complete loss of interaction with LY-CoV016 and LY-CoV555 (**Figure 6A-**
212 **C**). The P.1 RBD showed significantly decreased binding to REGN10933, weakly-detectable
213 interaction with LY-CoV016, and complete loss of interaction with LY-CoV555 (**Figure 6A-**

214 **C**). The B.1.617.1 RBD showed complete loss of interaction with LY-CoV555 (**Figure 6B**).
215 Consistent with the neutralization data, the B.1.617.2 RBD showed significantly decreased
216 affinity to LY-Cov555, but increased affinity to REGN10933 (**Figure 5L, 6B, and 6C**). These
217 data demonstrate that SARS-CoV-2 variants could easily develop resistance to neutralization
218 antibodies or even antibody cocktails in clinical use, highlighting the necessity of developing
219 broad-spectrum anti-coronavirus agents.

220 **Spike mutations change sensitivity of SARS-CoV-2 variants to different ACE2-Ig**
221 **constructs**

222 ACE2-Ig is a recombinant Fc fusion protein of soluble human ACE2. Recently we developed
223 a panel of ACE2-Ig constructs that potently neutralize SARS-CoV-2 early isolate and three
224 related but distinct coronaviruses, demonstrating that ACE2-Ig is a promising broadly anti-
225 coronavirus drug candidate [14]. Here we evaluated *in vitro* neutralization sensitivity of the
226 twelve SARS-CoV-2 variants to eight representative ACE2-Ig constructs previously described
227 in three different studies, including three constructs from our recent study (ACE2-Ig-v1,
228 ACE2-Ig-v1.1, ACE2-Ig-v3)[14], one construct from Chan *et al* (ACE2-Ig-Chan-v2.4)[46], and
229 four constructs from Glasgow *et al* (ACE2-Ig-Glasgow-293, ACE2-Ig-Glasgow-310, ACE2-
230 Ig-Glasgow-311, ACE2-Ig-Glasgow-313)[47]. Because we previously found that the CLD
231 domain of human ACE2 has an ~20-fold contribution to ACE2-Ig's neutralization activity
232 against SARS-CoV-2 pseudoviruses[14], we used CLD-containing soluble ACE2 domains for all
233 the constructs tested in this study. We got three interesting findings here (**Figure 7**). First, all
234 the twelve SARS-CoV-2 variants were potently neutralized by all the eight ACE2-Ig constructs,
235 demonstrating that ACE2-Ig is a broad-spectrum anti-SARS-CoV-2 agent. Second, the mink-
236 associated variant Y453F-F486L-N501T showed partial resistance to ACE2-Ig-Glasgow-310
237 and ACE2-Ig-Glasgow-313, but clearly increased sensitivity to ACE2-Ig-v1, ACE2-Ig-v1.1
238 and ACE2-Ig-v3, the constructs that are not heavily mutated in the spike-binding interface of

239 the soluble ACE2 domain (**Figure 7A-C**, **F**, **H**). These data suggest that extensively mutating
240 the ACE2 residues near the RBD-binding interface should be avoided. It might result in
241 compromised neutralization breadth, not to mention the risk of eliciting anti-drug antibody
242 (ADA) immune response. Third, compared to the early isolate WHU01, most circulating
243 variants, including the four VOCs B.1.1.7, B.1.351, P.1, and B.1.617.2 showed significantly
244 increased (up to ~15-fold) neutralization sensitivity to ACE2-Ig-v1, ACE2-Ig-v1.1 and ACE2-
245 Ig-v3, the constructs that carry a wild-type or a D30E-mutated ACE2 domain (**Figure 7A-C**).

246 Discussion

247 In this study, we parallelly investigated multiple circulating SARS-CoV-2 variants, including
248 two mink-associated variants, the B.1.258 and B.1.617 variants, and the VOCs B.1.1.7, B.1.351,
249 and P.1. As receptor usage is a critical determinant for the host range of coronaviruses, we
250 first investigated the ability of these variants to utilize animal orthologs of ACE2 as an entry
251 receptor. We found that, in contrast to an early isolate WHU01, the VOCs B.1.351, and P.1
252 were able to bind and use mouse ACE2 for entry, suggesting that these variants may have
253 gained ability to infect mouse (**Figure 2G-J and 3H**). Consistent with our finding, during the
254 preparation of this manuscript, an *in vivo* study reported that the VOCs B.1.351 and P.1 were
255 able to infect mouse and replicate to high titers in the lungs[48]. Mice are vaccine-inaccessible
256 rodent species that have large population size and could access the territories of both humans
257 and domestic animals. The fact that the B.1.351 and P.1 variants have gained ability to infect
258 mice raises the possibility of wild rodents becoming a second SARS-CoV-2 reservoir, adding
259 one more concerning factor to these variants.

260 In addition, we found that multiple variants, especially the VOCs B.1.1.7, B.1.351, and P.1,
261 have significantly increased affinity to cattle and pig ACE2 proteins (**Figure 3 and Table 1**).
262 Multiple previous studies have shown that cattle and pigs are not or only weakly susceptible to

263 experimental infection with SARS-CoV-2[15,49-52]. Consistently, here we found that both cattle
264 and pig ACE2 proteins have significantly lower affinities than that of human ACE2 to the RBD
265 of an early isolate WHU01. However, in the case of the RBDs of the three VOCs, binding
266 affinities of cattle and pig ACE2 proteins to these RBDs are comparable to that of human ACE2
267 to WHU01 RBD (**Table 2**). Cattle and pig ACE2 affinities to B.1.1.7 RBD are even slightly
268 higher than that of human ACE2 to WHU01 RBD. It has been recently reported that the RBDs
269 in the B.1.1.7 and B.1.351 spike proteins are more accessible than that of the early isolate[43,44].
270 This could further enable these variants to efficiently use cattle and pig ACE2 proteins. These
271 data altogether suggest that cattle and pigs might now become susceptible to SARS-CoV-2.
272 Cattle and pigs are extremely important livestock animals which serve as two major sources of
273 meat for humans. It might be necessary to perform *in vivo* studies to re-evaluate the
274 susceptibility of these species to SARS-CoV-2 variants, especially the widely circulating
275 VOCs. On the other hand, the SARS-CoV-2 infected population is already in a huge size, with
276 over 160 million confirmed infections worldwide. As of 29 March 2021, the VOC B.1.1.7
277 comprises roughly 95% of new SARS-CoV-2 infections in England. It has now been identified
278 in at least 114 countries and exhibits a similar transmission increase (59 to 74%) in Denmark,
279 Switzerland, and the United States[39-41]. So far, the VOCs B.1.351 and P.1 have also been
280 identified in at least 68 and 37 countries, respectively. The enormous number of SARS-CoV-
281 2 infection cases and high prevalence of these VOCs worldwide make it more and more
282 necessary to closely monitor cattle and pigs for SARS-CoV-2 natural infections.

283 Our data about neutralization sensitivity of the SARS-CoV-2 variants to four monoclonal
284 antibodies are consistent with multiple other studies[53-58]. Although, compared to the early
285 isolate WHU01, all the tested variants carrying mutations in the RBD region showed partial or
286 fully escape from at least one and up to three of the four tested antibodies (**Figure 5 and 6**),
287 none of the variants escaped ACE2-Ig (**Figure 7**), a broad-spectrum anti-SARS-CoV-2 drug

288 candidate. More importantly, we found that most of the tested SARS-CoV-2 variants had
289 increased (up to ~15-fold) sensitivity to ACE2-Ig constructs that carry a wild-type or a single-
290 point-mutated ACE2 domain (**Figure 7A-B**). This might be explained by increased affinity of
291 SARS-CoV-2 variant to human ACE2 or increased accessibility of the RBDs of SARS-CoV-2
292 variants. Indeed, we observed a 4.7-fold, 2.9-fold, 2.0-fold and 1.6-fold increase in the affinity
293 of human ACE2 to the RBDs of the mink-associated Y453F variant, the B.1.1.7 variant, the
294 B.1.351 variant, and the P.1 variant, respectively (**Table 2**). On the other hand, recent structural
295 studies have shown that spike trimers of multiple SARS-CoV-2 variants, including a mink-
296 associate variant and the VOCs B.1.1.7, B.1.1.28, and B1.351, have significantly higher
297 propensity to adopt 'RBD-up' or open state than the D614G variant does[43,44]. These data
298 suggest that SARS-CoV-2 was evolving toward better utilization of ACE2 as a receptor, either
299 through increasing RBD affinity to ACE2, or through better exposure of its RBDs, or through
300 both. It further indicates that ACE2 is still likely an essential receptor for SARS-CoV-2. ACE2
301 also serves as a cellular receptor for a number of other coronaviruses, including SARS-CoV,
302 HCoV-NL63, Pangolin-CoV-2020, Bat-CoV RaTG13, and some other SARS-like CoVs found
303 in bats[14,16,59]. ACE2-Ig is therefore a promising broadly anti-coronavirus drug candidate that
304 might be used to treat and prevent infection of these diverse coronaviruses and their variants.
305 It might also be a good alternative anti-SARS-CoV-2 agent for the populations who are not
306 responsive to, or don't have access to any prophylactic vaccines.

307 There are a couple of limitations of the current study. SARS-CoV-2 spike-pseudotyped
308 reporter viruses, instead of live viruses, were used to study virus-ACE2 interaction and
309 neutralization sensitivity of multiple circulating SARS-CoV-2 variants. It is difficult to obtain
310 or access live SARS-CoV-2 variants, and even more difficult to simultaneously obtain multiple
311 VOC live viruses and the mink-associated variant. Utilization of pseudotyped reporter virus
312 to study virus-receptor interaction and neutralization is a well-established and widely used

313 method in the field for studying various enveloped viruses, such as HIV[60,61], influenza virus[62-
314 64], and coronaviruses including SARS-CoV-2[ref.14,34,47,64-68]. Findings about virus-receptor
315 interaction and neutralization based on pseudovirus systems normally correlate well with that
316 based on live viruses. In addition, we applied quantitative binding kinetics assays to validate
317 all the key findings based on pseudovirus infection assays. This largely compensate the
318 limitation of using pseudoviruses. On the other hand, ACE2-Ig is a promising broadly anti-
319 SARS-CoV-2 drug candidate. More studies under *in vivo* conditions will be necessary to
320 validate its potential for clinical applications. We have recently obtained a preliminary result
321 showing that a single-dose injection or intranasal administration of an ACE2-Ig construct could
322 significantly lower the viral load in the lungs of a COVID-19 mouse model (unpublished data).
323 More mouse studies to test and optimize the protein's *in vivo* pharmacokinetic properties and
324 anti-SARS-CoV-2 efficacy are ongoing.

325 Materials and Methods

326 **Cells.** 293T cells and HeLa cells were kindly provided by Stem Cell Bank, Chinese Academy
327 of Sciences, confirmed mycoplasma-free by the provider, and maintained in Dulbecco's
328 Modified Eagle Medium (DMEM, Life Technologies) at 37 °C in a 5% CO_2-humidified
329 incubator. Growth medium was supplemented with 2 mM Glutamax-I (Gibco, Cat. No.
330 35050061), 100 μM non-essential amino acids (Gibco, Cat. No. 11140050), 100 U/mL
331 penicillin and 100 μg/mL streptomycin (Gibco, Cat. No. 15140122), and 10% heat-inactivated
332 FBS (Gibco, Cat. No. 10099141C). HeLa-based stable cells expressing human ACE2 were
333 maintained under the same culture condition as HeLa, except that 3 μg/mL of puromycin was
334 added to the growth medium. 293F cells for recombinant protein production were generously
335 provided by Dr. Yu J. Cao (School of Chemical Biology and Biotechnology, Peking University

336 Shenzhen Graduate School) and maintained in SMM 293-TII serum-free medium (Sino
337 Biological, Cat. No. M293TII) at 37 °C, 8% CO_2, in a shaker incubator at 125 rpm.

338 **Plasmids.** DNA fragment encoding spike protein of SARS-CoV-2 WHU01 (GenBank:
339 MN988668.1) was synthesized by the Beijing Genomic Institute (BGI, China) and then cloned
340 into pCAGGS plasmid between EcoRI and XhoI restriction sites. Plasmids encoding SARS-
341 CoV-2 spike variants were generated according to the in-fusion cloning protocol. To facilitate
342 SARS-CoV-2 pseudovirus production, spike sequences for WHU01 and all the variants
343 investigated in this study all contain a furin-cleavage site mutation (ΔPRRA) or a GSAS
344 substitution of the PRRA furin-cleavage site. We had shown in our previous study that the
345 ΔPRRA mutation does not affect SARS-CoV-2 cross-species receptor usage or neutralization
346 sensitivity[14]. The retroviral reporter plasmids encoding a Gaussia luciferase reporter gene were
347 constructed by cloning the reporter genes into the pQCXIP plasmid (Clontech). DNA
348 fragments encoding C-terminally S-tagged ACE2 orthologs were synthesized in pUC57
349 backbone plasmid by Sangon Biotech (Shanghai, China). These fragments were then cloned
350 into pQCXIP plasmid (Clontech) between SbfI and NotI restriction sites. Plasmids encoding
351 recombinant RBD and soluble ACE2 variants were generated by cloning each of the gene
352 fragments into a pCAGGS-based mouse-IgG2a or human IgG1 Fc fusion protein expression
353 plasmid between NotI and BspEI sites. DNA fragments encoding heavy and light chains of
354 anti-SARS-CoV-2 antibodies were synthesized by Sangon Biotech (Shanghai, China) and then
355 cloned into a pCAGGS plasmid. Four antibodies (LY-CoV016, LY-CoV-555, REGN10933,
356 and REGN10987)[ref.8-12] that constitute two antibody cocktails authorized by the U.S. FDA for
357 emergency use were included in this study.

358 **Western Blot to detect S-tagged ACE2 (ACE2-S-tag) expression in 293T cells.** 293T cells
359 at 30% density in each well of 96 well plates were reverse transfected with 0.60 μg of plasmid
360 in complex with 0.15 μL of lipofectamine 2000 (Life Technologies, Cat. No. 11668019).

361 Twenty-four hours after transfection, cells in each well were lysed with 40 μL lysis buffer and
362 5 μL of the lysate was used for western blot. ACE2-S-tag expression was detected by using a
363 mouse anti-S-tag monoclonal antibody 6.2 (Invitrogen, Cat. No. MA1-981), and an HRP-
364 conjugated goat anti-mouse IgG Fc secondary antibody (Invitrogen, Cat. No. 31437). Beta-
365 actin was used as an internal control.

366 **Production of reporter retroviruses pseudotyped with SARS-CoV-2 spike variants.** MLV
367 retroviral vector-based SARS-CoV-2 spike pseudotypes were produced according to our
368 previous study[14], with minor changes. In brief, 293T cells were seeded at 30% density in 150
369 mm dish at 12-15 hours before transfection. Cells were then transfected with 67.5 μg of
370 polyethylenimine (PEI) Max 40,000 (Polysciences, Inc, Cat. No. 24765-1) in complex with
371 3.15 μg of plasmid encoding a spike variant, 15.75 μg of plasmid encoding murine leukemia
372 virus (MLV) Gag and Pol proteins, and 15.75 μg of a pQCXIP-based luciferase reporter
373 plasmid. Eight hours after transfection, cell culture medium was refreshed and changed to
374 growth medium containing 2% FBS (Gibco, Cat. No. 10099141C) and 25 mM HEPES (Gibco,
375 Cat. No. 15630080). Cell culture supernatants were collected at 36-48 hours post transfection,
376 spun down at 3000×g for 10 min, and filtered through 0.45 μm filter units to remove cell debris.
377 SARS-CoV-2 spike-pseudotyped viruses were then concentrated 10 times at 2000×g using 100
378 kDa cut-off Amicon Ultra-15 Centrifugal Filter Units (Millipore. Cat. No. UFC910024).

379 **Pseudovirus Titration.** Pseudovirus titer were determined by $TCID_{50}$ followed a previous
380 protocol[69]. Viruses were diluted 100 times as a working solution and then serially diluted in a
381 ½ log10 manner. Human ACE2 expressed HeLa cells were infected with those diluted viruses
382 in 96-well plates. Culture supernatants were refreshed every 12 hours and loaded to a Gaussia
383 luciferase assay at 48 hours post infection. $TCID_{50}$ of each pseudovirus was calculated by the
384 Reed-Muench method.

385 **Western Blot to detect spike protein (C9-tag) and MLV P30 protein in SARS-CoV-2**
386 **pseudovirus-containing supernatant.** Pseudovirus-containing supernatants (8000 TCID50
387 of each pseudovirus) were mixed with SDS-PAGE loading buffer and boiled at 95°C for 10min.
388 Spike protein (C9-tag) on the surface of pseudovirus was detected using a mouse anti-C9-tag
389 monoclonal antibody 1D4 (Invitrogen, Cat. No. MA1-722), and an HRP-conjugated goat anti-
390 mouse IgG Fc secondary antibody (Invitrogen, Cat. No. 31437). MLV P30 protein within the
391 pseudovirus capsid was detected using a rabbit anti-MLV-P30 polyclonal antibody (Origene,
392 Cat. No. AP33447PU-N) and an HRP-conjugated goat anti-rabbit IgG Fc secondary antibody
393 (Invitrogen, Cat. No. 31463).

394 **SARS-CoV-2 pseudovirus infection of 293T cells expressing ACE2 orthologs.** Pseudovirus
395 infection assay was performed according to our previous study[14]. In brief, 293T cells at 30%
396 density in each well of gelatin (Millipore, ES-006-B) pre-coated 96-well plates were reverse
397 transfected with 0.15 μL of lipofectamine 2000 (Life Technologies, Cat. No. 11668019) in
398 complex with 60 ng of a vector control plasmid or a plasmid encoding ACE2 orthologs or
399 ACE2 mutants. Twenty-four hours later, cells in each well were infected with 2000 $TCID_{50}$
400 SARS-CoV-2 pseudovirus in 100 μL of culture medium containing 2% FBS (Gibco, Cat. No.
401 10099141C). Culture medium was refreshed every 12 hours. Cell culture supernatants were
402 collected and subjected to a Gaussia luciferase assay at 48 hours post infection.

403 **Gaussia luciferase luminescence flash assay.** To measure Gaussia luciferase expression, 20
404 μL of cell culture supernatant of each sample and 100 μL of assay buffer containing 4 μM
405 coelenterazine native (Biosynth Carbosynth, Cat. No. C-7001) were added to one well of a 96-
406 well black opaque assay plate (Corning, Cat. No. 3915), and measured with Centro LB 960
407 microplate luminometer (Berthold Technologies) for 0.1 second/well.

408 **Production and Purification of ACE2-Ig protein and SARS-CoV-2 antibodies.** 293F cells
409 at the density of 6×10^5 cells/mL were seeded into 100 mL SMM 293-TII serum-free medium

410 (Sino Biological, Cat. No. M293TII) one day before transfection. Cells were then transfected
411 with 100 μg plasmid in complex with 250 μg PEI MAX 4000 (Polysciences, Inc, Cat. No.
412 24765-1). Cell culture supernatants were collected at 48 to 72 hours post transfection. Human
413 IgG1 Fc-containing proteins were purified using Protein A Sepharose CL-4B (GE Healthcare,
414 Cat. No. 17-0780-01), eluted with 0.1 M citric acid at pH 4.5 and neutralized with 1 M Tris-
415 HCl at pH 9.0. Buffers were then exchanged to PBS and proteins were concentrated by 30 kDa
416 cut-off Amicon Ultra-15 Centrifugal Filter Units (Millipore, Cat. No. UFC903096).

417 **SARS-CoV-2 pseudovirus neutralization assay.** Pseudovirus neutralization experiments
418 were performed following our previous study[14]. In brief, SARS-CoV-2 spike variant-
419 pseudotyped luciferase reporter viruses were pre-diluted in DMEM (2% FBS, heat-inactivated)
420 containing titrated amounts of an ACE2-Ig construct or an anti-SARS-CoV-2 antibody. Virus-
421 inhibitor mixtures were incubated at 37 °C for 30min, then added to HeLa-hACE2 cells in 96-
422 well plates and incubated overnight at 37 °C. Virus-inhibitor-containing supernatant was then
423 removed and changed with 150 μL of fresh DMEM (2% FBS) and incubated at 37 °C. Cell
424 culture supernatants were collected for Gaussia luciferase assay at 48 h post infection.

425 **Biolayer interferometry (BLI) assay.** The BLI assays were performed on a Fortebio Octet
426 RED384 instrument, with the temperature and shaking speed at 30 °C and 1000 rpm
427 respectively. ACE2-hFc constructs were diluted to 5 μg/mL in 1× assay buffer containing 150
428 mM NaCl, 0.1% Tween-20, 10mM HEPES and 0.1%BSA (pH 7.4), and used as ligands for
429 the assays. RBD-mFc constructs were serially diluted to 100nM, 50nM, 25 nM, 12.5 nM, and
430 6.25 nM in the 1× assay buffer. Each experiment group started with a 10 min warm-up for
431 pre-hydration of AHC biosensors, followed by cycles of baseline (60 s), loading (60 s),
432 baseline2 (60s), association (100 s), dissociation (600 s) and regeneration plus neutralization
433 (30 s). A 1:1 Langmuir binding model was applied for data processing. All fitted diagrams

434 (global fit) display the entire association window and the first 200 s (or 100 s only for house
435 mouse and Chinese rufous horseshoe bat ACE2-related assays) of dissociation phase.

436 **Data collection and analysis.** All the experiments were independently performed for two or
437 three times by two different people. Image Lab Software (Bio-Rad) was used to collect SDS-
438 PAGE and Western-Blot image data. MikroWin 2000 Software (Berthold Technologies) was
439 used to collect luciferase assay data. The BLI sensorgrams were recorded by Octet Data
440 Acquisition 12.0 software and were fitted using Octet Data Analysis HT 12.0 software.
441 GraphPad Prism 6.0 software was used for figure preparation and statistical analyses.

442 **Data availability.** The study did not generate unique datasets or code. Our research resources,
443 including methods, plasmids, and protocols, are available upon reasonable request to qualified
444 academic investigators for noncommercial research purposes. All reagents developed in this
445 study, such as vector plasmids, as well as detailed methods, will be made available upon written
446 request.

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459 Conflict of interests

460 Shenzhen Bay Laboratory has filed a PCT patent application for multiple ACE2-Ig constructs.

461 Author contributions

462 G.Z. conceived and designed this study. W.Y., D.M., H.W., X.T., C.D., H.P., and Y.L.
463 generated experimental materials. W.Y., D.M., and Y.L. performed all experiments. W.Y.,
464 D.M., Y.L., and G.Z. analyzed all data. C.L., H.L., M.F., and J.Z. contributed key resources.
465 G.Z. and H.W. wrote the manuscript.

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624 ## Figure Legends

625 **Figure 1. SARS-CoV-2 spike variants and related mutations investigated in the following**
626 **studies.** (**A**) Twelve SARS-CoV-2 spike genes investigated in this study are illustrated. The
627 receptor binding domain (RBD) is in red and the receptor binding motif (RBM) is in yellow.
628 Spike mutations associated with each variant are indicated. (**B**) Interactions between SARS-
629 CoV-2 RBD (red) and ACE2 (blue) involve a large number of contact residues (PDB accession
630 no. 6M0J). The so-called receptor binding motif (RBM) within the RBD is indicated in yellow.
631 The ACE2 residues in less than 4 Å from RBD atoms are shown. All SARS-CoV-2 variant-
632 associated RBD mutations investigated in this study are shown and labelled.

633 **Figure 2. Animal-ACE2 tropism of SARS-CoV-2-variant pseudoviruses. (A-L)** 293T cells
634 expressing one of the eight indicated ACE2 orthologs were infected with each of twelve
635 pseudoviruses corresponding to the twelve SARS-CoV-2 spike variants shown in Figure 1.
636 ACE2-mediated pseudovirus entry was measured by a luciferase reporter expression at 48
637 hours post infection. Data shown are representative of three independent experiments
638 performed by two different people with similar results, and data points represent mean ± s.d.
639 of three biological replicates. (**M**) MLV retroviral vector-based pseudoviruses of the indicated
640 SARS-CoV-2 variants were validated for the presence of spike protein (C9-tag) and MLV-Gag
641 p30 protein. (**N**) 293T cells transfected with the indicated ACE2 genes (S-tagged) were
642 collected at 48 hours post transfection. Expression levels of the indicated ACE2 proteins were
643 detected using Western Blot.

644 **Figure 3. Interaction kinetics of fifty-six RBD-ACE2 pairs.** (**A-H**) A bio-layer
645 interferometry (BLI)-based assay was used to measure the kinetics of RBD-ACE2 interactions.
646 Recombinant RBD proteins of seven SARS-CoV-2 variants, including WHU01, B.1.298,
647 B.1.1.7, B.1.351, P.1, B.1.617.1, and B.1.617.2 were sequentially used as analytes to measure

648 their interactions with recombinant ACE2 proteins of eight species, including human (A), cattle
649 (B), pig (C), cat (D), rabbit (E), bat (F), rat (G), mouse (H). The black lines are the binding
650 sensorgrams for each analyte at 100 nM, 50 nM, 25 nM, 12.5 nM, or 6.25 nM. The red lines
651 show fits of the data to a 1:1 Langmuir binding model (global fit). Full kinetics data, including
652 the on-rate (k_a, $M^{-1}s^{-1}$), off-rate (k_{dis}, s^{-1}), and affinity (K_D, nM) for all the fifty-six RBD-ACE2
653 interaction pairs, are provided in Table 2.

654 **Figure 4. The effects of K417N, K417T, E484K, N501Y mutations on the ability of SARS-**
655 **CoV-2 pseudovirus to utilize mouse ACE2. (A-G)** SARS-CoV-2 pseudoviruses carrying the
656 indicated spike mutations were used to infect 293T cells expressing human ACE2 or a mouse
657 ACE2 mutant. 293T cells transfected with an empty vector were used as a control. ACE2-
658 mediated pseudovirus entry was measured by a luciferase reporter expression at 48 hours post
659 infection. Luminescence values were divided by the values observed with human ACE2 to
660 calculate Relative Infection (%) values. (**H**) Expression levels of the ACE2 constructs used in the
661 above experiments were detected using Western blotting. Data shown in A-G are representative of
662 two independent experiments performed by two different people with similar results, and data
663 points represent mean ± s.d. of three biological replicates.

664 **Figure 5. Neutralization sensitivity of SARS-CoV-2 variants to four monoclonal**
665 **antibodies in clinical use.** (**A-L**) Twelve SARS-CoV-2 variant pseudoviruses were tested for
666 their neutralization sensitivity to four monoclonal antibodies (LY-CoV016/etesevimab, LY-
667 CoV555/bamlanivimab, REGN10933/casirivimab, REGN10987/imdevimab) that constitute
668 two antibody cocktails authorized by the U.S. FDA for emergency use. HeLa-hACE2 cells, a
669 stable cell line that overexpresses human ACE2, were infected with each of the twelve
670 pseudoviruses in the presence of the indicated neutralizing antibodies at the indicated
671 concentrations. Viral entry was measured by luciferase reporter expression at 48 hours post

672 infection. Luminescence values observed at each concentration were divided by the values
673 observed at concentration zero to calculate percentage-of-infection (Infection%) values. Data
674 shown are representative of three independent experiments performed by two different people
675 with similar results, and data points represent mean ± s.d. of three biological replicates. (**M-P**)
676 Structures of SARS-CoV-2 RBD in complex with LY-CoV016 (M; PDB accession no. 7C01),
677 LY-CoV555 (N; PDB accession no. 7MKG), REGN10933 (O; PDB accession no. 6XDG), or
678 REGN10987 (P; PDB accession no. 6XDG) are analyzed. The RBD and RBM are shown in
679 red and yellow, respectively. Antibody heavy chain and light chain are shown in blue and
680 green, respectively. Antibody residues in less than 4 Å from RBD atoms and RBD residues
681 associated with SARS-CoV-2 mutations are shown as sticks. SARS-CoV-2 mutation-
682 associated RBD residues that have direct contact with antibody residues or reside within 5 Å
683 from antibody atoms are shown as spheres and labelled.

684 **Figure 6. Interaction kinetics of twenty-eight RBD-antibody pairs.** (**A-D**) The BLI-based
685 assay was used to measure the kinetics of RBD-antibody interactions. Recombinant RBD
686 proteins of seven SARS-CoV-2 spike variants, including WHU01, Y453F, B.1.1.7, B.1.351,
687 P.1, B.1.617.1, and B.1.617.2 were sequentially used as analytes to measure their interactions
688 with four monoclonal antibodies, including LY-CoV016 (A), LY-CoV555 (B), REGN10933
689 (C), and REGN10987 (D). The black lines are the binding sensorgrams for each analyte at 100
690 nM, 50 nM, 25 nM, 12.5 nM, or 6.25 nM. The red lines show fits of the data to a 1:1 Langmuir
691 binding model (global fit). The affinity (K_D, nM) for each interaction is indicated in the insets
692 of the figures. Full kinetics data, including the on-rate (k_a, $M^{-1}s^{-1}$), off-rate (k_{dis}, s^{-1}), and
693 affinity (K_D, nM) for each interaction, are provided in Table 3.

694 **Figure 7. Neutralization sensitivity of SARS-CoV-2 variants to eight ACE2-Ig constructs.**
695 (**A-H**) Eight recombinant ACE2-Ig protein constructs, including three from our recent study

696 (ACE2-Ig-v1, ACE2-Ig-v1.1, ACE2-Ig-v3; A-C) [ref.14], one from Chan *et al* (ACE2-Ig-Chan-
697 v2.4; D) [ref.46]; and four from Glasgow *et al* (ACE2-Ig-Glasgow-293, ACE2-Ig-Glasgow-310,
698 ACE2-Ig-Glasgow-311, ACE2-Ig-Glasgow-313; E-H) [ref.47], were tested for their neutralization
699 potencies against the twelve SARS-CoV-2 variant pseudoviruses. HeLa-hACE2 cells were
700 infected with each of the twelve pseudoviruses in the presence of the indicated ACE2-Ig
701 proteins at the indicated concentrations. Viral entry was measured by luciferase reporter
702 expression at 48 hours post infection. Luminescence values observed at each concentration
703 were divided by the values observed at concentration zero to calculate percentage-of-infection
704 (Infection%) values. Data shown are representative of three independent experiments
705 performed by two different people with similar results, and data points represent mean ± s.d.
706 of three biological replicates.

Tables 1-3

Table 1. Species and accession numbers of the amino-acid sequences of the eight ACE2 orthologs investigated in this study.

No.	Species name used in the text	Binomial name	NCBI Reference Sequence ID or *Genebank ID*
1	Human	*Homo sapiens*	NM_021804.3
2	Cattle	*Bos Taurus*	NM_001024502.4
3	Pig	*Sus scrofa domesticus*	XM_021079374.1
4	Cat	*Felis catus*	NM_001039456.1
5	Rabbit	*Oryctolagus cuniculus*	XM_002719845.3
6	Bat	*Rhinolophus sinicus* (isolate Rs-3357)	*KC881004.1*
7	Rat	*Rattus norvegicus*	NM_001012006.1
8	Mouse	*Mus musculus*	NM_027286.4

Table 2. Interaction kinetics data, including the BLI Response (nm) at 100 nM analyte, on-rate (k_a, $M^{-1}s^{-1}$), off-rate (k_{dis}, s^{-1}), and affinity (K_D, nM), of the fifty-six ACE2-RBD pairs

Loading Sample (ACE2-hFc)	Analytes (RBD-mFc)	Response (nm)	k_a (x10^5 M-1 s-1)	k_{dis} (x10^{-4} s-1)	K_D (nM)
Human ACE2	WHU01	0.25	6.28±0.02	4.48±0.05	0.71±0.01
Human ACE2	Y453F	0.23	4.56±0.01	0.69±0.04	0.15±0.01
Human ACE2	B.1.1.7	0.25	5.79±0.02	1.42±0.05	0.24±0.01
Human ACE2	B.1.351	0.24	5.17±0.03	1.82±0.09	0.35±0.02
Human ACE2	P.1	0.21	9.98±0.06	4.36±0.09	0.44±0.01
Human ACE2	B.1.617.1	0.29	7.78±0.03	3.13±0.06	0.40±0.01
Human ACE2	B.1.617.2	0.24	7.50±0.03	2.24±0.06	0.30±0.01
Cattle ACE2	WHU01	0.28	6.93±0.04	18.23±0.10	2.63±0.02
Cattle ACE2	Y453F	0.27	5.53±0.02	4.29±0.06	0.78±0.01
Cattle ACE2	B.1.1.7	0.27	6.06±0.02	3.20±0.06	0.53±0.01
Cattle ACE2	B.1.351	0.26	7.73±0.03	6.91±0.07	0.89±0.01
Cattle ACE2	P.1	0.22	8.66±0.09	8.51±0.17	0.98±0.02
Cattle ACE2	B.1.617.1	0.23	10.12±0.06	12.54±0.10	1.24±0.01
Cattle ACE2	B.1.617.2	0.22	8.56±0.06	9.45±0.10	1.10±0.01
Pig ACE2	WHU01	0.28	8.17±0.06	29.82±0.13	3.65±0.03
Pig ACE2	Y453F	0.25	5.58±0.03	7.33±0.07	1.31±0.01
Pig ACE2	B.1.1.7	0.28	5.87±0.02	3.53±0.06	0.60±0.01
Pig ACE2	B.1.351	0.24	5.47±0.03	5.85±0.07	1.07±0.01
Pig ACE2	P.1	0.21	7.42±0.08	9.30±0.17	1.25±0.03
Pig ACE2	B.1.617.1	0.21	11.74±0.09	19.31±0.12	1.65±0.02
Pig ACE2	B.1.617.2	0.21	10.86±0.08	19.78±0.13	1.82±0.02
Cat ACE2	WHU01	0.30	7.72±0.06	14.01±0.13	1.81±0.02
Cat ACE2	Y453F	0.27	6.72±0.03	2.08±0.07	0.31±0.01
Cat ACE2	B.1.1.7	0.28	7.69±0.05	5.69±0.10	0.74±0.01
Cat ACE2	B.1.351	0.26	7.49±0.06	14.67±0.13	1.96±0.02
Cat ACE2	P.1	0.20	8.98±0.06	16.74±0.12	1.87±0.02
Cat ACE2	B.1.617.1	0.28	7.93±0.04	7.29±0.09	0.92±0.01
Cat ACE2	B.1.617.2	0.24	7.62±0.04	7.33±0.09	0.96±0.01

Table 2-continued. Interaction kinetics data, including the BLI Response (nm) at 100 nM analyte, on-rate (k_a, $M^{-1}s^{-1}$), off-rate (k_{dis}, s^{-1}), and affinity (K_D, nM), of the fifty-six ACE2-RBD pairs

Loading Sample (ACE2-hFc)	Analytes (RBD-mFc)	Response (nm)	k_a (x10^5 M-1 s-1)	k_{dis} (x10^{-4} s-1)	K_D (nM)
Rabbit ACE2	WHU01	0.23	8.14±0.05	7.88±0.09	0.97±0.01
Rabbit ACE2	Y453F	0.22	7.48±0.04	5.48±0.09	0.73±0.01
Rabbit ACE2	B.1.1.7	0.24	8.81±0.04	2.87±0.07	0.33±0.01
Rabbit ACE2	B.1.351	0.23	6.65±0.03	5.86±0.08	0.88±0.01
Rabbit ACE2	P.1	0.23	7.83±0.04	5.48±0.08	0.70±0.01
Rabbit ACE2	B.1.617.1	0.24	11.17±0.06	5.37±0.08	0.48±0.01
Rabbit ACE2	B.1.617.2	0.23	9.30±0.05	5.23±0.09	0.56±0.01
Bat ACE2	WHU01	0.14	8.89±0.13	129.7±0.79	14.59±0.23
Bat ACE2	Y453F	0.10	12.4±0.23	108.7±0.91	8.76±0.18
Bat ACE2	B.1.1.7	0.10	9.48±0.17	55.63±0.75	5.87±0.13
Bat ACE2	B.1.351	0.00	N.D.	N.D.	N.D.
Bat ACE2	P.1	-0.02	N.D.	N.D.	N.D.
Bat ACE2	B.1.617.1	0.05	16.43±0.40	84.95±1.04	5.17±0.14
Bat ACE2	B.1.617.2	0.10	9.62±0.14	125.90±0.78	13.09±0.20
Rat ACE2	WHU01	0.07	9.83±0.07	27.10±0.13	2.76±0.02
Rat ACE2	Y453F	0.07	7.15±0.05	9.54±0.10	1.34±0.02
Rat ACE2	B.1.1.7	0.12	12.80±0.11	14.03±0.13	1.10±0.01
Rat ACE2	B.1.351	0.10	13.42±0.14	19.70±0.17	1.47±0.02
Rat ACE2	P.1	0.13	9.95±0.06	10.55±0.10	1.06±0.01
Rat ACE2	B.1.617.1	0.07	12.97±0.11	14.59±0.13	1.13±0.01
Rat ACE2	B.1.617.2	0.06	14.10±0.12	15.77±0.14	1.12±0.01
Mouse ACE2	WHU01	0.00	N.D.	N.D.	N.D.
Mouse ACE2	Y453F	0.01	N.D.	N.D.	N.D.
Mouse ACE2	B.1.1.7	0.02	2.77±0.08	62.14±0.94	22.45±0.76
Mouse ACE2	B.1.351	0.21	14.96±0.19	48.93±0.50	3.27±0.05
Mouse ACE2	P.1	0.19	20.26±0.29	47.43±0.52	2.34±0.04
Mouse ACE2	B.1.617.1	0.01	N.D. (0.00)	N.D. (0.00)	N.D. (0.00)
Mouse ACE2	B.1.617.2	-0.01	N.D. (0.00)	N.D. (0.00)	N.D. (0.00)

Table 3. Interaction kinetics data, including the BLI Response (nm) at 100 nM analyte, on-rate (k_a, $M^{-1}s^{-1}$), off-rate (k_{dis}, s^{-1}), and affinity (K_D, nM), of the twenty-eight mAb-RBD pairs

Loading Sample (Antibody)	Analytes (RBD-mFc)	Response (nm)	k_a (x10^5 M-1 s-1)	k_{dis} (x10^{-4} s-1)	K_D (nM)
LY-CoV016	WHU01	0.57	5.98±0.01	2.10±0.04	0.35±0.01
LY-CoV016	Y453F	0.51	5.51±0.02	3.62±0.05	0.66±0.01
LY-CoV016	B.1.1.7	0.52	6.31±0.03	9.25±0.08	1.47±0.02
LY-CoV016	B.1.351	0.03	N.D.	N.D.	N.D.
LY-CoV016	P.1	0.16	5.19±0.05	15.37±0.16	2.96±0.04
LY-CoV016	B.1.617.1	0.52	6.13±0.02	3.33±0.04	0.54±0.01
LY-CoV016	B.1.617.2	0.46	5.95±0.01	2.50±0.04	0.42±0.01
LY-CoV555	WHU01	0.50	6.30±0.01	0.62±0.03	0.10±0.00
LY-CoV555	Y453F	0.45	5.53±0.01	1.06±0.03	0.19±0.01
LY-CoV555	B.1.1.7	0.49	5.12±0.01	0.06±0.03	0.01±0.01
LY-CoV555	B.1.351	0.00	N.D.	N.D.	N.D.
LY-CoV555	P.1	0.02	N.D.	N.D.	N.D.
LY-CoV555	B.1.617.1	0.02	N.D.	N.D.	N.D.
LY-CoV555	B.1.617.2	0.33	5.09±0.04	54.35±0.18	10.68±0.09
REGN10933	WHU01	0.63	7.26±0.03	1.27±0.06	0.18±0.01
REGN10933	Y453F	0.50	9.92±0.09	30.82±0.17	3.11±0.03
REGN10933	B.1.1.7	0.62	7.81±0.02	1.52±0.05	0.19±0.01
REGN10933	B.1.351	0.50	9.03±0.07	22.4±0.14	2.48±0.03
REGN10933	P.1	0.40	8.47±0.06	18.52±0.12	2.19±0.02
REGN10933	B.1.617.1	0.51	9.07±0.03	3.45±0.06	0.38±0.01
REGN10933	B.1.617.2	0.52	8.51±0.03	0.14±0.05	0.02±0.01
REGN10987	WHU01	0.66	6.09±0.02	1.10±0.04	0.18±0.01
REGN10987	Y453F	0.62	5.49±0.01	0.90±0.03	0.16±0.01
REGN10987	B.1.1.7	0.66	6.12±0.01	0.60±0.04	0.10±0.01
REGN10987	B.1.351	0.64	6.42±0.02	0.90±0.04	0.14±0.01
REGN10987	P.1	0.57	5.90±0.02	1.09±0.05	0.18±0.01
REGN10987	B.1.617.1	0.67	6.41±0.02	0.80±0.04	0.13±0.01
REGN10987	B.1.617.2	0.62	5.96±0.02	1.51±0.04	0.25±0.01

Figures 1-7

Figure 1

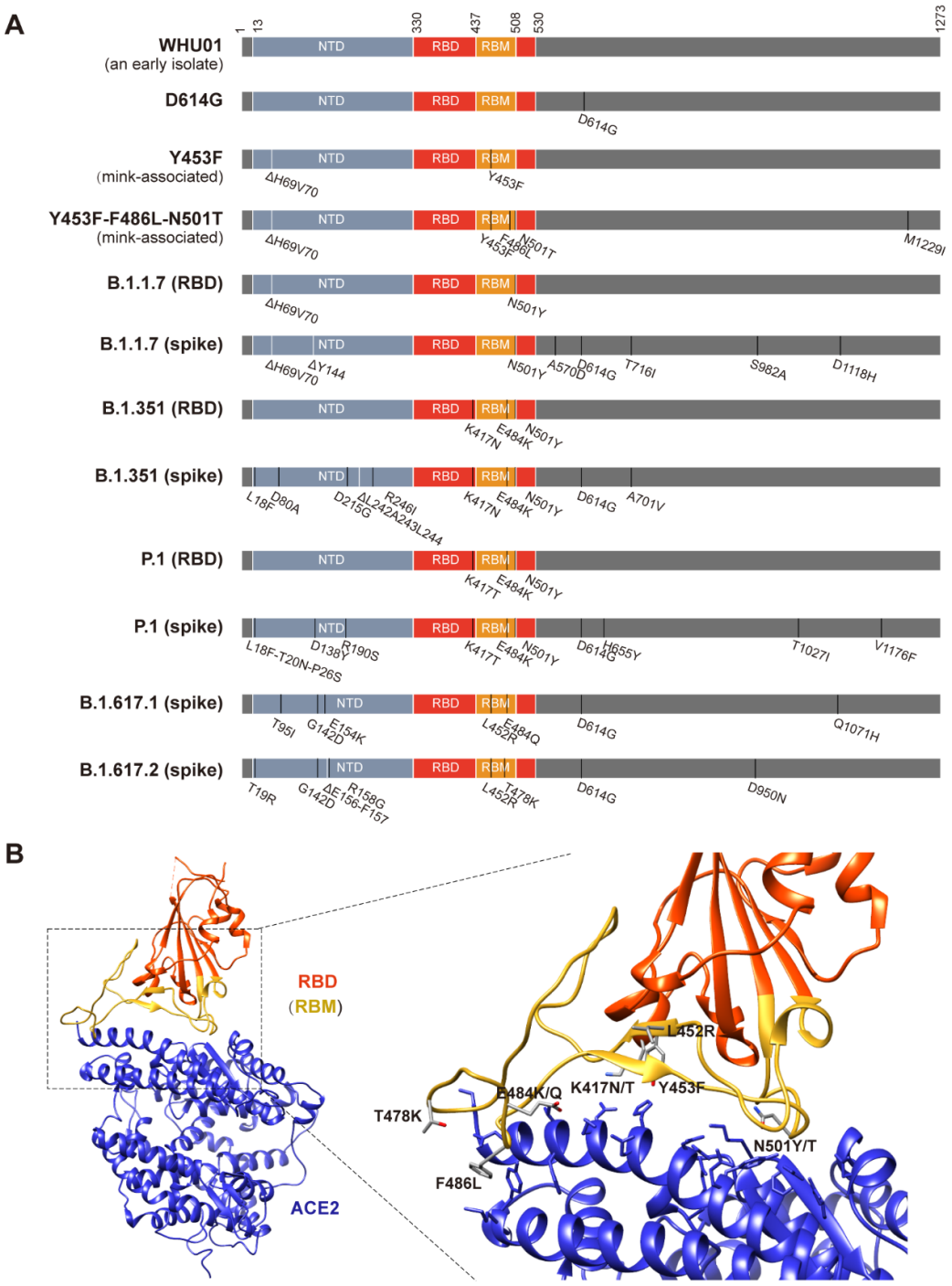


Figure 2

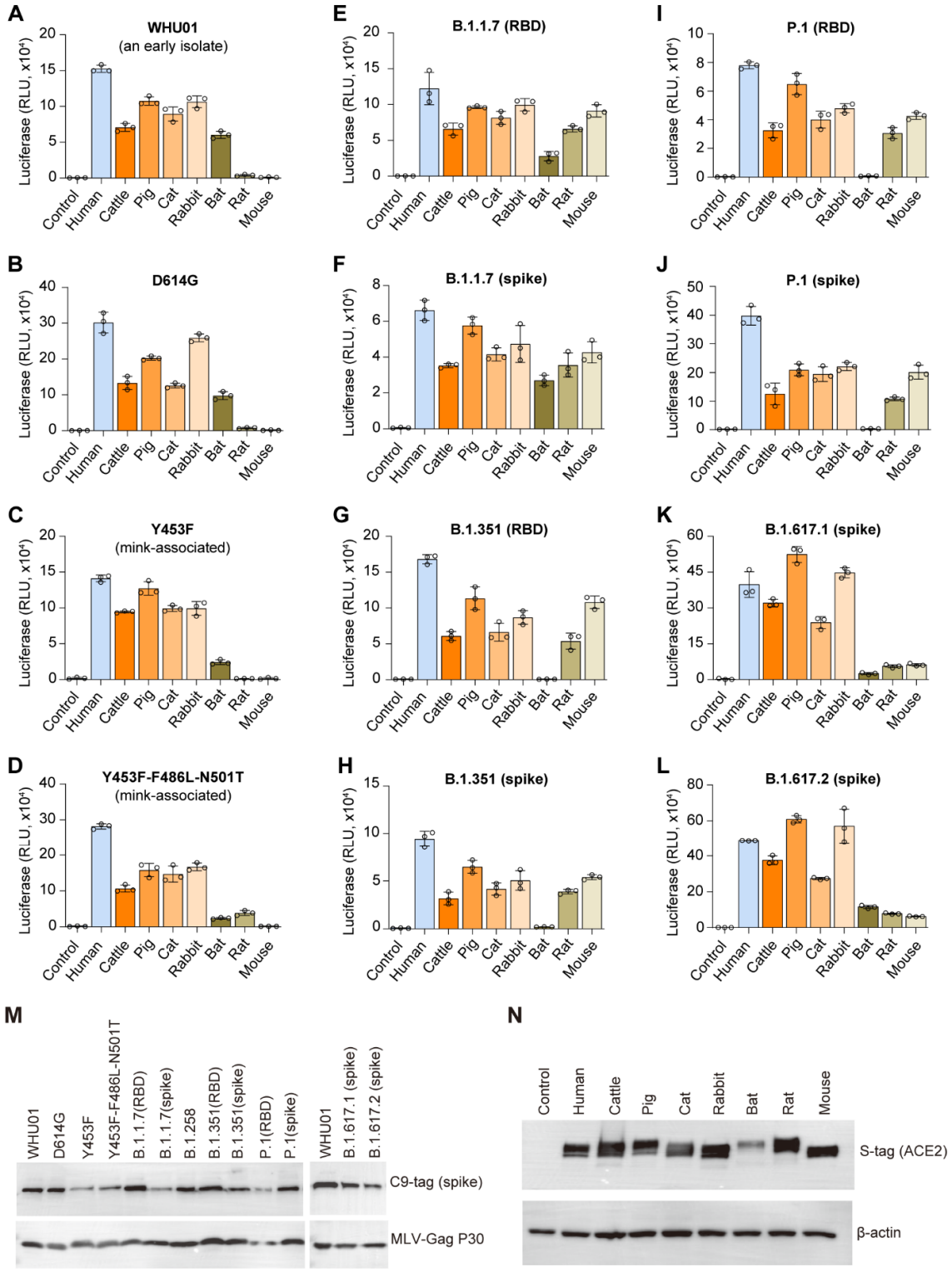

A
WHU01
(an early isolate)
B
D614G
C
Y453F
(mink-associated)
D
Y453F-F486L-N501T
(mink-associated)
E
B.1.1.7 (RBD)
F
B.1.1.7 (spike)
G
B.1.351 (RBD)
H
B.1.351 (spike)
I
P.1 (RBD)
J
P.1 (spike)
K
B.1.617.1 (spike)
L
B.1.617.2 (spike)
Luciferase (RLU, x10⁴)
Control
Human
Cattle
Pig
Cat
Rabbit
Bat
Rat
Mouse
M
WHU01
D614G
Y453F
Y453F-F486L-N501T
B.1.1.7(RBD)
B.1.1.7(spike)
B.1.258
B.1.351(RBD)
B.1.351(spike)
P.1(RBD)
P.1(spike)
WHU01
B.1.617.1 (spike)
B.1.617.2 (spike)
C9-tag (spike)
MLV-Gag P30
N
Control
Human
Cattle
Pig
Cat
Rabbit
Bat
Rat
Mouse
S-tag (ACE2)
β-actin

Figure 3

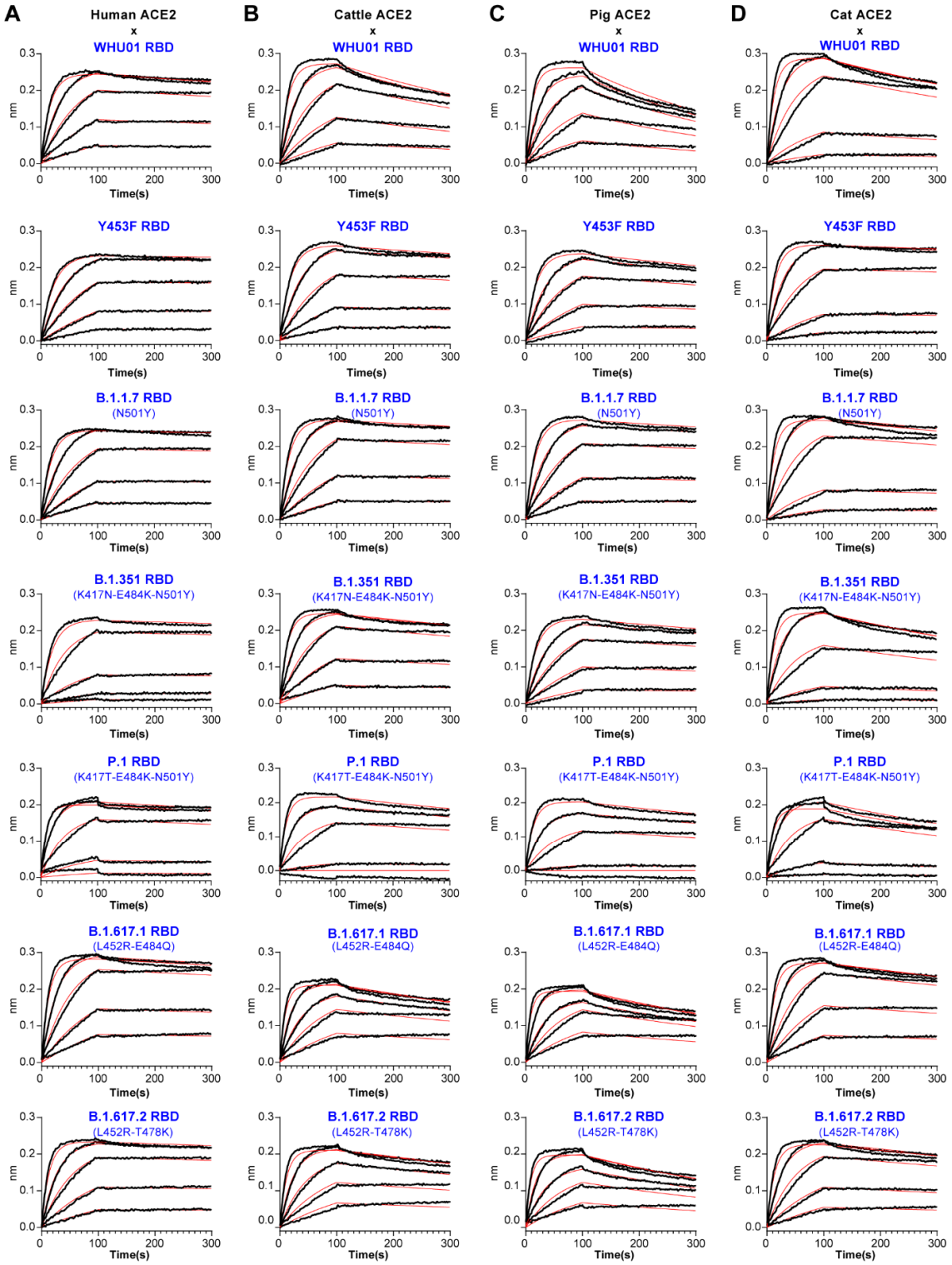

A
Human ACE2
x
B
Cattle ACE2
x
C
Pig ACE2
x
D
Cat ACE2
x
WHU01 RBD
Y453F RBD
B.1.1.7 RBD
(N501Y)
B.1.351 RBD
(K417N-E484K-N501Y)
P.1 RBD
(K417T-E484K-N501Y)
B.1.617.1 RBD
(L452R-E484Q)
B.1.617.2 RBD
(L452R-T478K)
nm
Time(s)

Figure 3-continued

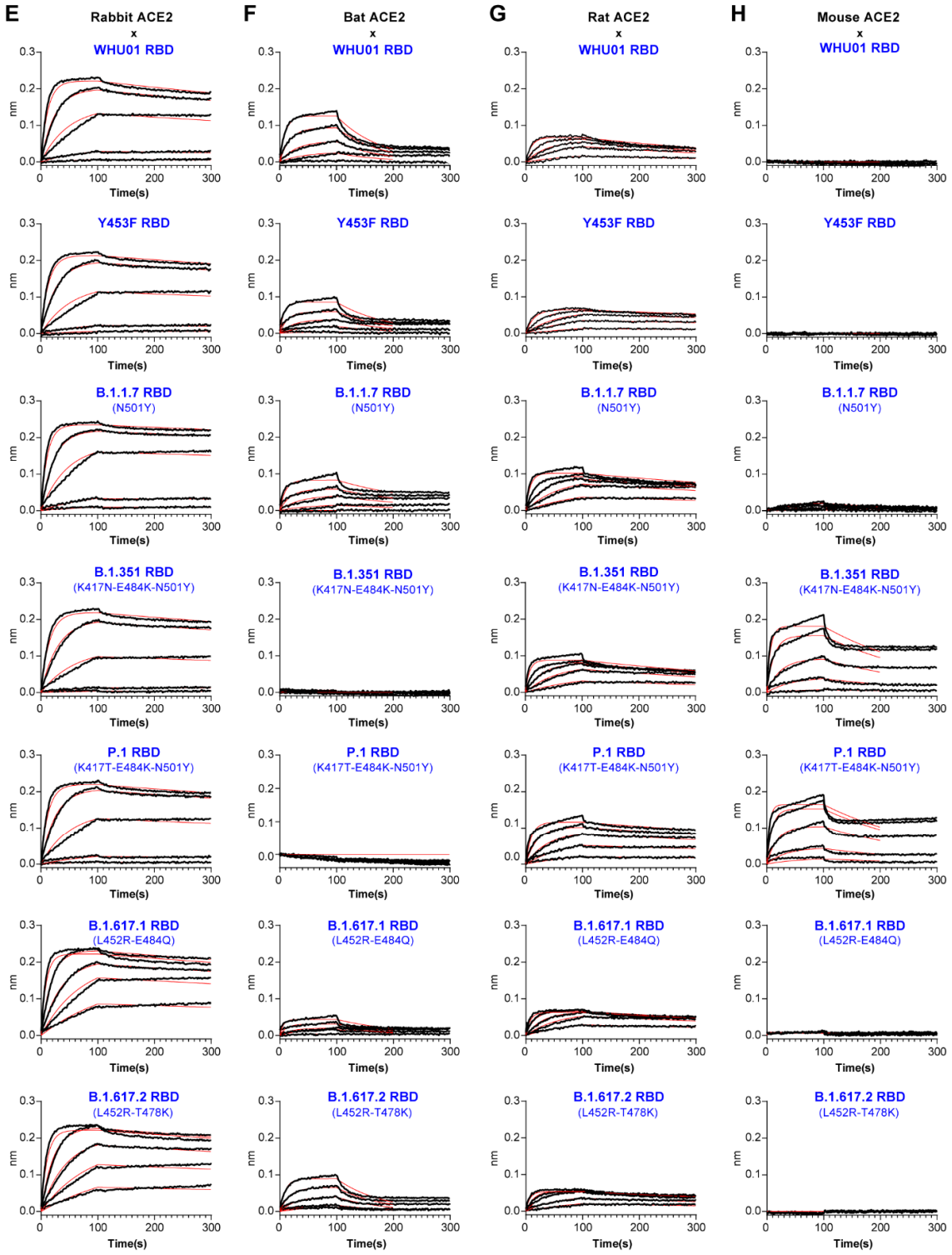


Figure 4

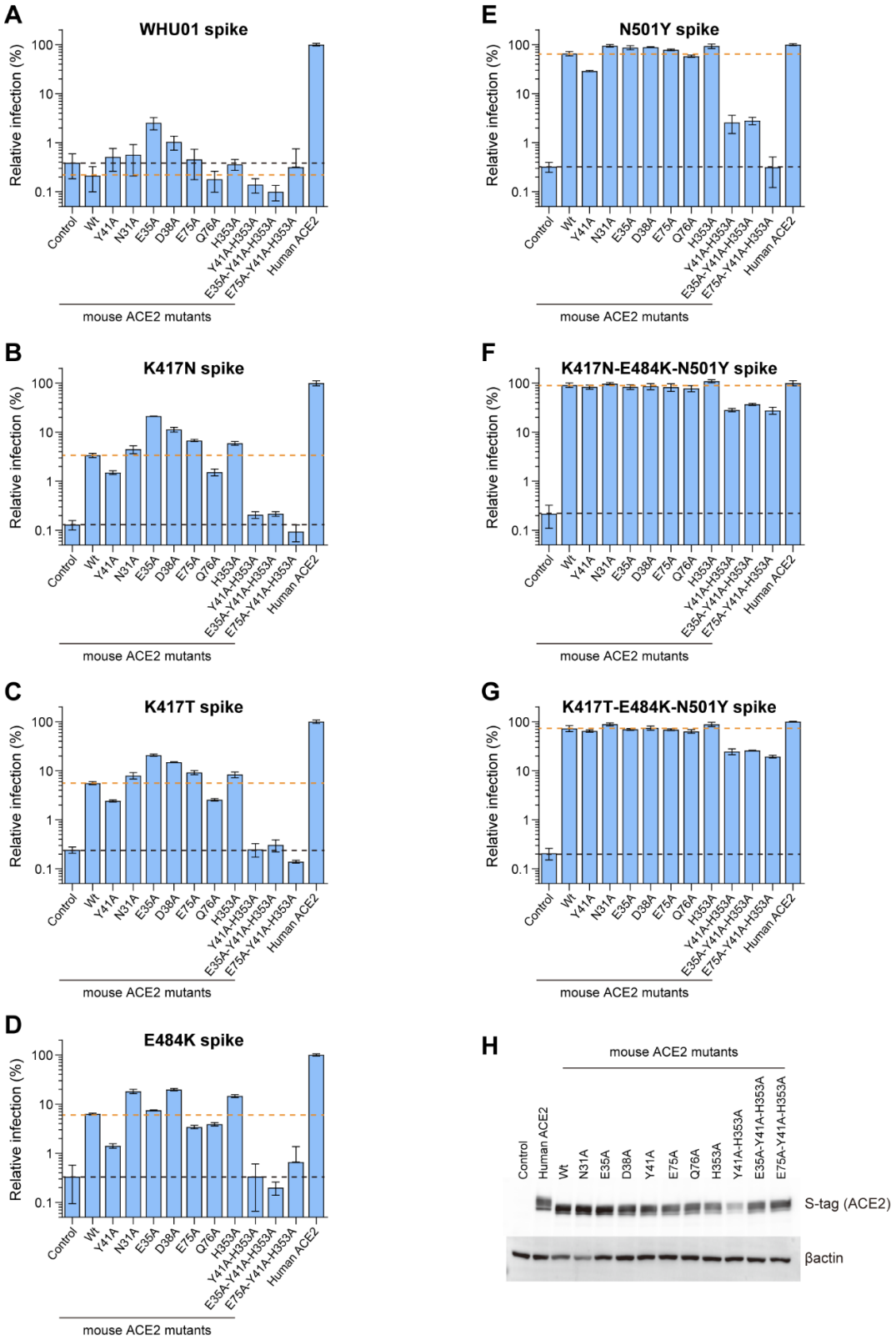

A
WHU01 spike
B
K417N spike
C
K417T spike
D
E484K spike
E
N501Y spike
F
K417N-E484K-N501Y spike
G
K417T-E484K-N501Y spike
Relative infection (%)
100
10
1
0.1
Control
Wt
Y41A
N31A
E35A
D38A
E75A
Q76A
H353A
Y41A-H353A
E35A-Y41A-H353A
E75A-Y41A-H353A
Human ACE2
mouse ACE2 mutants
H
mouse ACE2 mutants
Control
Human ACE2
Wt
N31A
E35A
D38A
Y41A
E75A
Q76A
H353A
Y41A-H353A
E35A-Y41A-H353A
E75A-Y41A-H353A
S-tag (ACE2)
βactin

Figure 5

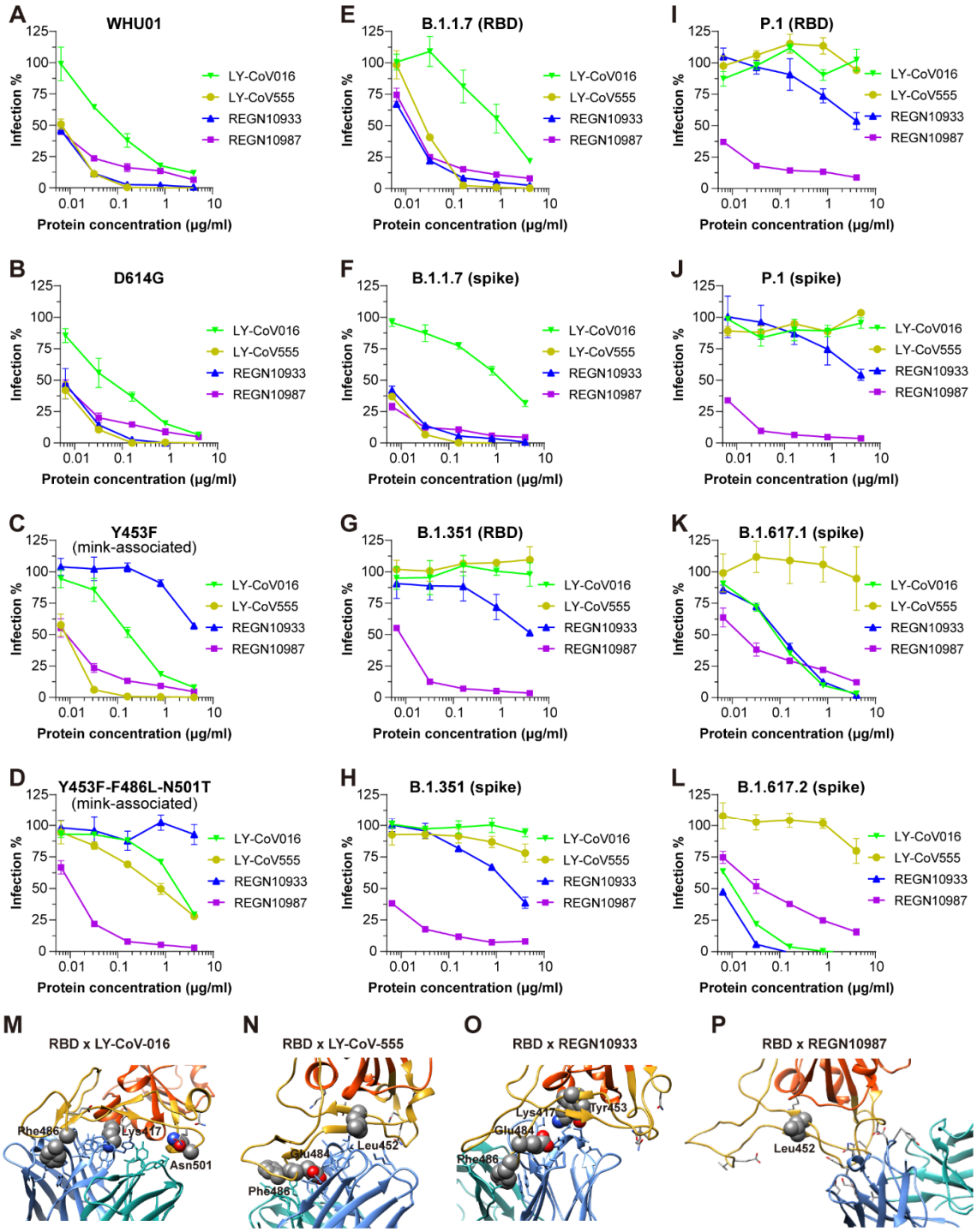

A
WHU01
B
D614G
C
Y453F
(mink-associated)
D
Y453F-F486L-N501T
(mink-associated)
E
B.1.1.7 (RBD)
F
B.1.1.7 (spike)
G
B.1.351 (RBD)
H
B.1.351 (spike)
I
P.1 (RBD)
J
P.1 (spike)
K
B.1.617.1 (spike)
L
B.1.617.2 (spike)
Infection %
Protein concentration (μg/ml)
LY-CoV016
LY-CoV555
REGN10933
REGN10987
M
RBD x LY-CoV-016
Phe486
Lys417
Asn501
N
RBD x LY-CoV-555
Glu484
Leu452
Phe486
O
RBD x REGN10933
Lys417
Tyr453
Glu484
Phe486
P
RBD x REGN10987
Leu452

Figure 6

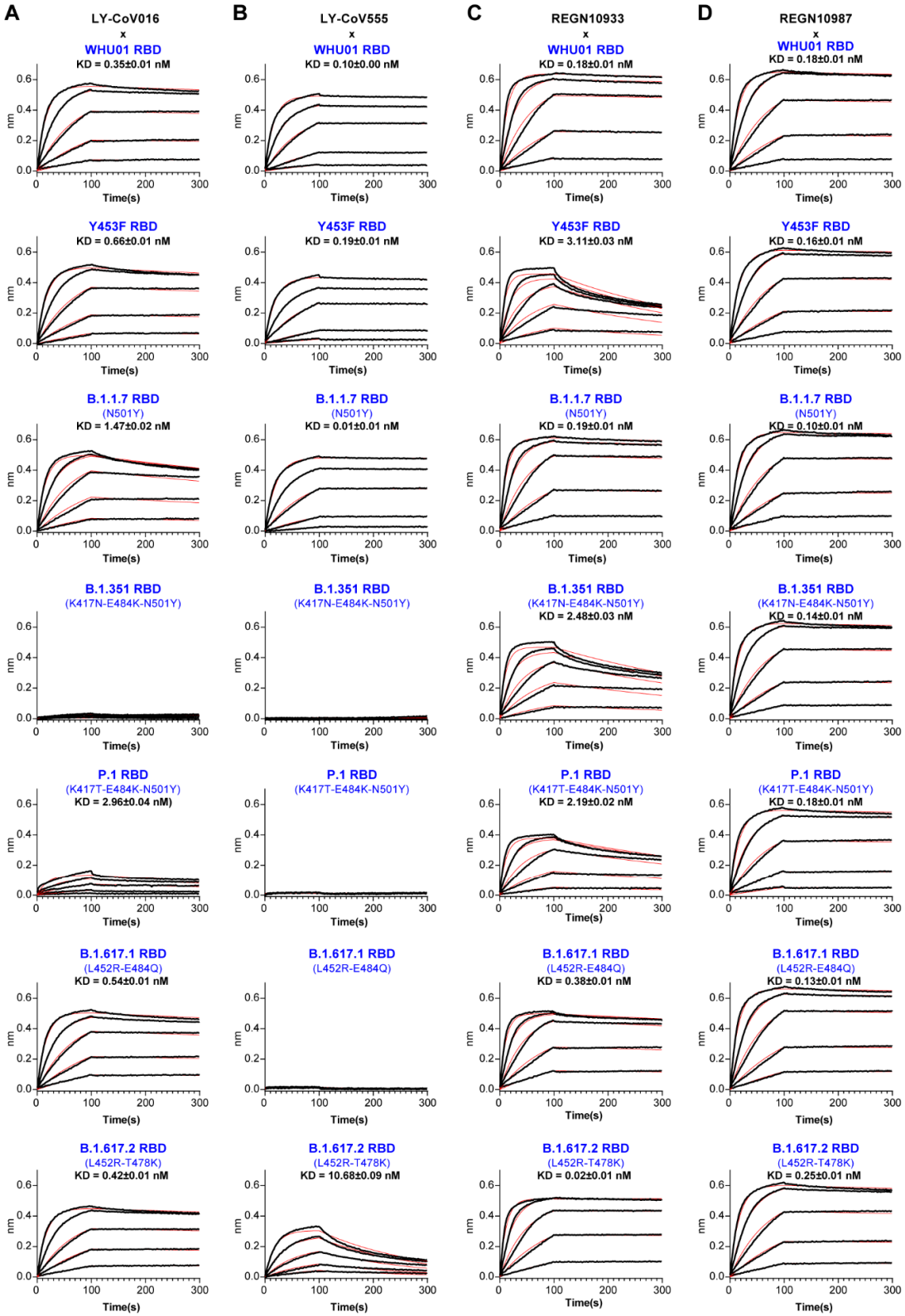
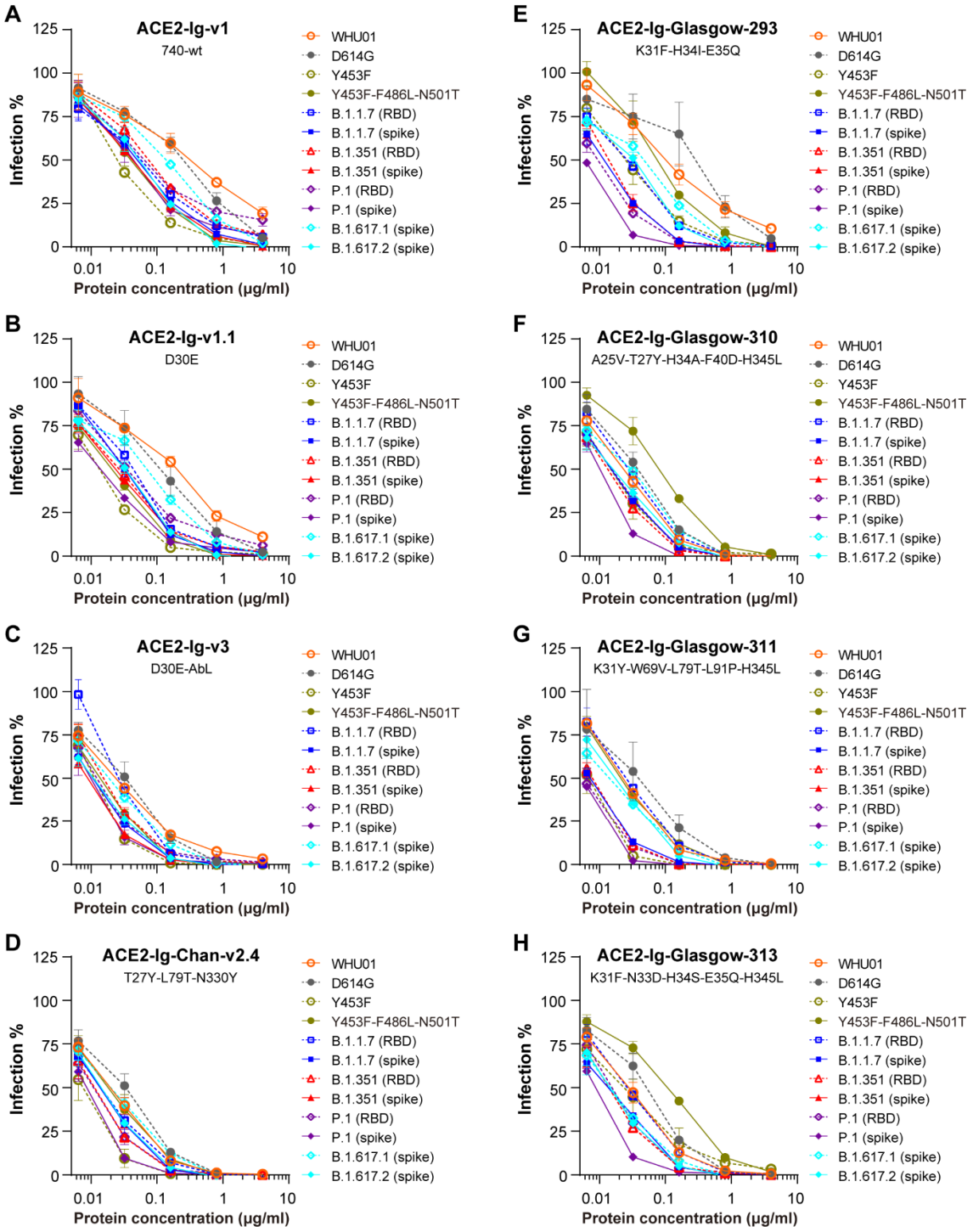

A
LY-CoV016
x
WHU01 RBD
KD = 0.35±0.01 nM
Y453F RBD
KD = 0.66±0.01 nM
B.1.1.7 RBD
(N501Y)
KD = 1.47±0.02 nM
B.1.351 RBD
(K417N-E484K-N501Y)
P.1 RBD
(K417T-E484K-N501Y)
KD = 2.96±0.04 nM)
B.1.617.1 RBD
(L452R-E484Q)
KD = 0.54±0.01 nM
B.1.617.2 RBD
(L452R-T478K)
KD = 0.42±0.01 nM
B
LY-CoV555
x
WHU01 RBD
KD = 0.10±0.00 nM
Y453F RBD
KD = 0.19±0.01 nM
B.1.1.7 RBD
(N501Y)
KD = 0.01±0.01 nM
B.1.351 RBD
(K417N-E484K-N501Y)
P.1 RBD
(K417T-E484K-N501Y)
B.1.617.1 RBD
(L452R-E484Q)
B.1.617.2 RBD
(L452R-T478K)
KD = 10.68±0.09 nM
C
REGN10933
x
WHU01 RBD
KD = 0.18±0.01 nM
Y453F RBD
KD = 3.11±0.03 nM
B.1.1.7 RBD
(N501Y)
KD = 0.19±0.01 nM
B.1.351 RBD
(K417N-E484K-N501Y)
KD = 2.48±0.03 nM
P.1 RBD
(K417T-E484K-N501Y)
KD = 2.19±0.02 nM
B.1.617.1 RBD
(L452R-E484Q)
KD = 0.38±0.01 nM
B.1.617.2 RBD
(L452R-T478K)
KD = 0.02±0.01 nM
D
REGN10987
x
WHU01 RBD
KD = 0.18±0.01 nM
Y453F RBD
KD = 0.16±0.01 nM
B.1.1.7 RBD
(N501Y)
KD = 0.10±0.01 nM
B.1.351 RBD
(K417N-E484K-N501Y)
KD = 0.14±0.01 nM
P.1 RBD
(K417T-E484K-N501Y)
KD = 0.18±0.01 nM
B.1.617.1 RBD
(L452R-E484Q)
KD = 0.13±0.01 nM
B.1.617.2 RBD
(L452R-T478K)
KD = 0.25±0.01 nM
nm
Time(s)

Figure 7


A
ACE2-Ig-v1
740-wt
B
ACE2-Ig-v1.1
D30E
C
ACE2-Ig-v3
D30E-AbL
D
ACE2-Ig-Chan-v2.4
T27Y-L79T-N330Y
E
ACE2-Ig-Glasgow-293
K31F-H34I-E35Q
F
ACE2-Ig-Glasgow-310
A25V-T27Y-H34A-F40D-H345L
G
ACE2-Ig-Glasgow-311
K31Y-W69V-L79T-L91P-H345L
H
ACE2-Ig-Glasgow-313
K31F-N33D-H34S-E35Q-H345L
Infection %
Protein concentration (µg/ml)
0
25
50
75
100
125
0.01
0.1
1
10
WHU01
D614G
Y453F
Y453F-F486L-N501T
B.1.1.7 (RBD)
B.1.1.7 (spike)
B.1.351 (RBD)
B.1.351 (spike)
P.1 (RBD)
P.1 (spike)
B.1.617.1 (spike)
B.1.617.2 (spike)