

1 **D614G mutation of SARS-CoV-2 spike protein enhances viral infectivity**

2 ***Running Title*** : D614G mutant spike increases SARS-CoV-2 infectivity

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20

21 **Abstract**

22 Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory
23 syndrome coronavirus 2 (SARS-CoV-2). The spike (S) protein that mediates
24 SARS-CoV-2 entry into host cells is a major target for vaccines and therapeutics.
25 Thus, insights into its sequence variations are key to understanding the infection
26 and antigenicity of SARS-CoV-2. A dominant mutational variant at position 614 of
27 the S protein (aspartate to glycine, D614G mutation) was observed in the SARS-
28 CoV-2 genome sequence obtained from the Nextstrain database. Using a
29 pseudovirus-based assay, we identified that S-D614 and S-G614 protein
30 pseudotyped viruses share a common receptor, human angiotensin-converting
31 enzyme 2 (ACE2), which could be blocked by recombinant ACE2 with the fused Fc
32 region of human IgG1. However, S-D614 and S-G614 protein demonstrated
33 functional differences. First, S-G614 protein could be cleaved by serine protease
34 elastase-2 more efficiently. Second, S-G614 pseudovirus infected 293T-ACE2
35 cells significantly more efficiently than did the S-D614 pseudovirus, especially in
36 the presence of elastase-2. Third, an elastase inhibitor approved for clinical use
37 blocked elastase-enhanced S-G614 pseudovirus infection. Moreover, 93% (65/70)
38 convalescent sera from patients with COVID-19 could neutralize both S-D614 and
39 S-G614 pseudoviruses with comparable efficiencies, but about 7% (5/70)
40 convalescent sera showed reduced neutralizing activity against the S-G614
41 pseudovirus. These findings have important implications for SARS-CoV-2
42 transmission and immune interventions.

43 **Keywords:** antiviral therapeutics, coronavirus, COVID-19, D614G mutation,
44 infectivity, neutralizing antibodies, pseudovirus, SARS-CoV-2, spike protein

45

46 **Introduction**

47 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel
48 coronavirus reported in 2019 that caused the recent outbreak of coronavirus
49 disease 2019 (COVID-19)¹. By July 4, 2020, the World Health Organization (WHO)
50 reported that 10.92 million people worldwide had been infected with SARS-CoV-2,
51 and that 523 000 individuals had died of COVID-19. This pandemic has had a
52 significant adverse impact on international, social, and economic activities.

53 Coronaviruses are enveloped, positive-stranded RNA viruses that contain the
54 largest known RNA genomes to date. The RNA genome of SARS-CoV-2 has been
55 rapidly sequenced to facilitate diagnostic testing, molecular epidemiologic source
56 tracking, and development of vaccines and therapeutic strategies². The mutation
57 rate for RNA viruses is extremely high, which may contribute to their transmission
58 and virulence. The only significant variation in the SARS-CoV-2 spike (S) protein is
59 a non-synonymous D614G (aspartate (D) to glycine (G)) mutation³. Primary data
60 showed that S-G614 is a more pathogenic strain of SARS-CoV-2 with high
61 transmission efficiency³. However, whether the D614G mutation in the S protein
62 affects viral entry and infectivity in a cellular model is still unclear.

63

64 The S protein of coronavirus, the major determinant of host and tissue tropism, is a

65 major target for vaccines, neutralizing antibodies, and viral entry inhibitors^{4,5}.

66 Similar to SARS-CoV, the cellular receptor of SARS-CoV-2 is angiotensin-

67 converting enzyme 2 (ACE2); however, the SARS-CoV-2 S protein has a 10- to

68 20-fold higher affinity for ACE2 than the corresponding S protein of SARS-CoV^{6,7}.

69 Coronaviruses use two distinct pathways for cell entry: protease-mediated cell-

70 surface and endosomal pathways⁸. The S proteins of several coronaviruses are

71 cleaved by host proteases into S1 subunit for receptor binding and S2 subunit for

72 membrane fusion at the entry step of infection. Several cellular proteases including

73 furin, transmembrane protease serine 2 (TMPRSS2), and cathepsin (Cat) B/L are

74 critical for priming the SARS-CoV-2 S protein to enhance ACE2-mediated viral

75 entry⁴. Recently, Bhattacharyya et al. (2020) reported that a novel serine protease

76 (elastase-2) cleavage site was introduced into the S-G614 protein of SARS-CoV-

77 2⁹. However, it is unknown whether the S-G614 protein can be processed and

78 activated by elastase-2 in a cellular model. The S protein plays a key role in the

79 evolution of coronaviruses to evade the host immune system. It is still uncertain

80 whether the D614G mutation affects the antigenic properties of the S protein.

81 Meanwhile, whether elastase-2 inhibitors and convalescent serum samples of

82 COVID-19 can block the infection of the D614G variant of SARS-CoV-2 remains

83 unknown.

84

85 In this study, we analyzed the S gene sequences of SARS-CoV-2 submitted to the

86 Global Initiative on Sharing All Influenza Data (GISAID) database. We examined

87 the expression and cleavage of S-D614 and S-G614 protein in cell lines. Using a
88 luciferase (Luc)-expressing lentiviral pseudotype system, we established a
89 quantitative pseudovirus-based assay for the evaluation of SARS-CoV-2 cell entry
90 mediated by the viral S protein variants. We also compared the neutralizing
91 sensitivity of the S-D614 and S-G614 protein pseudoviruses to convalescent sera
92 from patients with COVID-19. Our study provides further insights into the
93 transmission and immune interventions of this newly emerged virus.

94

95 **Results**

96 **D614G mutation of SARS-CoV-2 S protein was globally distributed**

97 The S protein of SARS-CoV-2, which contains 1,273 amino acids, forms a trimeric
98 spike on the virion surface and plays an essential role in viral entry. We analyzed
99 the SARS-CoV-2 S protein amino acid sequence from viral genomic sequences in
100 the GISAID database. In line with prior reports, we found a globally distributed S
101 protein mutation, D614 to G614, which represented 64.6% of all the analyzed
102 sequences (Fig. 1 and Table 1). Among the top 10 most abundant non-
103 synonymous mutations observed in the S protein, the relative abundance of the
104 D614G mutant (the clade G) was the highest around the world, indicating that
105 G614 strain may be selectively advantageous. Since the S protein is critical to
106 coronavirus infection, we sought to explore the potential impact of the most
107 prevalent D614G mutation on the S protein structure, expression, and function.
108 Using the cryo-electron microscopy structure of S protein (PDB ID: 6ZGE)

109 determined by Wrobel et al.¹⁰, we analyzed the potential effects of the D614G
110 mutation. As shown in Fig. 1b, residue D614 is located at the C-terminal region of
111 the S1 domain, which directly associates with S2. On the one hand, residue D614
112 forms a hydrogen bond with backbone of G593 and a salt bridge with K854, and
113 then is capped by the folded 833-855 motif of chain B (Fig. 1c). D614G mutation
114 would abolish these interactions. This change may destabilize the locked
115 conformation to promote receptor binding domain (RBD) opening and potentially
116 increase virus-receptor binding and membrane fusion activities. On the other hand,
117 we observed that D614 is remarkably close to the N-linked glycosylation site N616
118 (Fig 1c). Thus, the D614G mutation may enhance the fitness of SARS-CoV-2 by
119 increasing S protein stability and participating in glycosylation. Further studies are
120 required to determine the structure of S-G614 protein.

121

122 **D614G mutation enhanced the cleavage of S protein variant by proteases**

123 As the D614G mutation is proximal to the S1 cleavage domain, we predicted
124 potential cleavage sites of proteases in S protein variants using PROSPER¹¹, and
125 identified a novel serine protease (elastase-2) cleavage site at residues 615-616
126 on the S1-S2 junction of the S-G614 protein (Fig. 2a, Supplementary information,
127 Table S2). To evaluate the expression and cleavage of SARS-CoV-2 S protein in a
128 human cell line, the codon-optimized S protein-expressing plasmids (pS-D614 and
129 pS-G614) were transfected into HEK 293T cells. The immunoblot analysis of
130 whole cell lysates revealed that both S-D614 and S-G614 proteins showed two

131 major protein bands (unprocessed S and cleaved S1 subunit), when allowed to
132 react with the monoclonal antibody targeting the RBD on the SARS-CoV-2 S
133 protein (Fig. 2b). However, the pS-G614-transfected cells showed a stronger S1
134 signal than pS-D614-transfected cells, indicating that the D614G mutation altered
135 the cleavability of the S protein by cellular proteases. Moreover, the elastase
136 inhibitor sivelestat sodium significantly decreased the S1 signal of S-D614 protein
137 (Fig. 2b). These data indicate that the D614G mutation of SARS-CoV-2 S
138 facilitates its cleavage by host serine protease elastase-2. The coronavirus S
139 protein must be cleaved by host proteases to enable membrane fusion, which is
140 critical for viral entry. Next, we sought to explore the impact of D614G mutation on
141 viral entry.

142

143 **Evaluating viral entry efficacies between S-D614 and S-G614 pseudotyped** 144 **lentiviral particles**

145 Lentiviral vectors can be pseudotyped with various heterologous viral
146 glycoproteins that modulate cellular tropism and entry properties¹². Due to the
147 highly pathogenic nature of SARS-CoV-2, infectious SARS-CoV-2 must be
148 handled in a biosafety level 3 (BSL-3) facility. We generated pseudotyped SARS-
149 CoV-2 based on the viral S protein using a lentiviral system, which introduced a
150 Luc (luciferase) reporter gene for quantification of SARS-CoV-2 S-mediated entry.
151 Thereafter, pNL4-3.Luc.R-E- was co-transfected with pS-D614 and pS-G614 to
152 package the SARS-CoV-2 S pseudotyped single-round Luc virus in HEK 293T

153 cells.

154 The titers of S-D614 and S-G614 protein pseudotyped viruses were determined by
155 reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)
156 expressed as the number of viral RNA genomes per mL, and then adjusted to the
157 same concentration (3.8×10^4 copies in 50 μ L) for the following experiments. The
158 virus infectivity was determined by a Luc assay expressed as relative
159 luminescence units (RLU). HEK 293T cells expressing human ACE2 (293T-ACE2)
160 were used to test the correlation between ACE2 expression and pseudoviral
161 susceptibility. The vesicular stomatitis virus G (VSV-G) pseudovirus was used as
162 control. As shown in Fig. 3a, both HEK 293T and 293T-ACE2 cells could be
163 effectively transduced by VSV-G pseudovirus. However, the entry of S-D614 and
164 S-G614 pseudoviruses is highly dependent on its cellular receptor ACE2
165 expression. The 293T-ACE2 cells showed an approximately 250-fold and 530-fold
166 increase in Luc activity when transduced by S-D614 and S-G614 pseudoviruses
167 compared to HEK 293T cells, respectively (Fig. 3a). We then detected the
168 inhibitory ability of ACE2-Ig, a fusion protein consisting of the extracellular domain
169 (Met 1-Ser 740) of human ACE2 linked to the Fc region of human IgG1 at the C-
170 terminus¹³. Both S-D614 and S-G614 pseudoviruses were potently inhibited by
171 ACE2-Ig, and the IC₅₀ (the concentration causing 50% inhibition of pseudoviral
172 infection) values were 0.13 and 0.15 μ g/mL, respectively (Fig. 3b). To further
173 compare the viral entry efficiency mediated by S variants, we detected the Luc
174 activity at different time points post-infection. With the G614 S variant, the increase

175 in viral transduction over the D614 variant was 2.2-fold at 48 h post-infection. The
176 highest transduction efficiency (approximately 2.5×10^4 RLU) was observed 72 h
177 post-infection with the S-G614 pseudovirus, which was approximately 2.4-fold
178 higher than that of the S-D614 pseudovirus (Fig. 3c). These data suggest that the
179 D614G mutation in S protein significantly promotes viral entry into ACE2-
180 expressing cells, and ACE2-Ig efficiently blocks both wild-type and mutant S
181 pseudotype virus infection.

182 We next explored the mechanism by which S-G614 increased pseudoviral
183 infectivity. The proteolytic activation of S protein is required for coronavirus
184 infectivity, and the protease-mediated cell-surface pathway is critical for SARS-
185 CoV-2 entry⁴. Since we observed S-G614 could be more efficiently cleaved by the
186 host protease when exogenously expressed in 293T cells, we assumed that host
187 proteases may be involved in the enhancement of S-G614 viral entry. As shown in
188 Fig. 3d, when 293T-ACE2 cells were treated with 100 $\mu\text{g}/\text{mL}$ elastase before virus
189 infection, the RLU value in S-G614 pseudovirus-infected cells (6.9×10^4) was
190 about 3.1 fold higher than that of the S-D614 (2.2×10^4), indicating that residue
191 G614 facilitates elastase-induced viral entry. In addition, the clinically proven
192 serine protease inhibitor sivelestat sodium, which is active against elastase, dose-
193 dependently blocked S-G614-driven entry into 293T-ACE2 in the presence of 100
194 $\mu\text{g}/\text{mL}$ elastase (Fig. 3e). These data indicated that the infectivity of S-G614
195 pseudovirus containing an additional elastase-2 cleavage site is enhanced by
196 exogenous elastase; therefore, S-G614 pseudovirus is more sensitive to sivelestat

197 sodium than the S-D614 pseudovirus.

198 We also tested two other protease inhibitors, camostat mesylate and E-64d, which
199 block host TMPRSS2 and CatB/L, respectively. In 293T-ACE2 cells lacking
200 TMPRSS2, the serine protease inhibitor camostat mesylate did not inhibit S-D614
201 or S-G614 pseudoviral infection (Fig. S1a), while the cysteine protease inhibitor E-
202 64d significantly blocked the entry of these two pseudoviruses, with IC₅₀ values of
203 0.37 μM and 0.24 μM for S-D614 and S-G614 pseudoviruses, respectively (Fig.
204 S1b). As expected, these protease inhibitors had no impact on VSV-G pseudovirus
205 infection. Together, these results suggest that S-mediated viral entry into 293T-
206 ACE2 cells deficient in TMPRSS2 is endosomal cysteine protease CatB/L-
207 dependent; therefore, S-D614 and S-G614 pseudoviruses showed similar
208 sensitivity to the CatB/L inhibitor E-64d in 293T-ACE2 cells.

209

210 **Neutralization effect of convalescent sera from patients with COVID-19** 211 **against S-D614 and S-G614 pseudoviruses**

212 Neutralizing antibodies are important for prevention of and possible recovery from
213 viral infections. However, as viruses mutate during replication and spread, host
214 neutralizing antibodies generated in the earlier phase of the infection may not be
215 as effective later on^{14,15}. To test whether D614G mutations could affect the
216 neutralization sensitivity of the virus, the neutralization activity of serum samples
217 from convalescent patients with COVID-19 against SARS-CoV-2 S-D614 and S-
218 G614 pseudoviruses were evaluated. To perform the neutralization assay, 50 μL

219 pseudovirus (3.8×10^4 copies) was incubated with serially diluted sera. As shown
220 in Fig. 4a, the inhibition rate of sera from convalescent COVID-19 patients was
221 analyzed at a single dilution of 1:1000, among the 70 tested sera, 65 of them
222 showed neutralizing activities against both S-D614 and S-G614 pseudoviruses
223 with comparable efficiencies. However, five sera samples (patients 1#, 7#, 40#,
224 42# and 52#) showed decreased inhibition rate against the S-G614 pseudovirus.
225 The serum from patient 1# failed to neutralize the S-G614 pseudovirus, even
226 though it neutralized about 30% of the S-D614 pseudovirus at a 1:1000 dilution.
227 Then, the inhibition curves and half-maximal inhibitory dose (ID_{50}) of sera samples
228 from five convalescent patients and one healthy donor were analyzed. Sera from
229 patients 17# and 39#, which were able to neutralize both pseudoviruses to similar
230 degrees, showed similar ID_{50} values (Fig. 4b). However, sera from patients 1#, 7#,
231 40#, 42# and 52# showed relative high neutralizing activity against the S-D614
232 pseudovirus with an ID_{50} ranging from 729 to 1524, but showed decreased
233 neutralizing activity against the S-G614 pseudovirus with an ID_{50} ranging from 216
234 to 367, indicating a 2.5- to 5.9-fold reduction in neutralizing titers (Fig. 4b). These
235 data indicate that most antisera (93%) from patients, likely infected with earlier
236 SARS-CoV-2 variant, can cross neutralize S-G614 variant, although D614G
237 mutation decreases the neutralization sensitivity to 7% convalescent sera.

238

239 **Discussion**

240 Pseudovirus-based assays have been widely used for the study of cellular tropism,

241 receptor recognition, viral inhibitors, and evaluation of neutralizing antibodies
242 without the requirement of BSL-3 laboratories. We constructed pseudotyped
243 SARS-CoV-2 based on the viral S protein using a lentiviral system, which
244 incorporated a Luc reporter gene for easy quantification of coronavirus S protein-
245 mediated entry. We investigated the major mutation in the S protein at position 614
246 and found that serine protease elastase-2 participates in the proteolytic activation
247 of the S-G614 protein, thereby enhancing viral entry into 293T-ACE2 cells (Fig. 5).
248 We demonstrated the potential role of the elastase-2 inhibitor sivelestat in blocking
249 S-G614 pseudovirus infection in the presence of elastase. Although S-D614 and
250 S-G614 variants could be similarly neutralized by most antisera, we found that the
251 S-G614 pseudovirus was more resistant to some neutralizing antisera than S-
252 D614 pseudovirus.

253

254 Several findings stand out in our study: First, we found that the entry efficiency of
255 S-G614 pseudotyped virus was about 2.4 times higher than that of the S-D614
256 pseudovirus when viral input doses were normalized, suggesting that D614G
257 mutation promotes the infectivity of SARS-CoV-2 and enhances viral
258 transmissibility. Since the pseudoviruses were employed in a single round of
259 infection assay, this seemingly small increase in entry activity could cause a large
260 difference in viral infectivity *in vivo*. Yao et al. (2020) reported that a patient-
261 derived viral isolate ZJU-1, which harbors the D614G mutation, has a viral load 19
262 times higher than isolate ZJU-8 (harboring the S-D614) when Vero-E6 cells were

263 infected¹⁶. However, the ZJU-1 isolate contained two other non-synonymous
264 mutations in open reading frame 1a (ORF1a) and envelope (E) gene. Our results
265 also elucidated the cause of increased entry efficiency due to the D614G mutation
266 in the S protein of SARS-CoV-2. The S-G614 protein contains a novel serine
267 protease cleavage site, so it could be cleaved by host elastase-2 more efficiently.
268 Previously studies on SARS-CoV demonstrated that the protease-mediated cell
269 surface entry facilitated a 100- to 1,000-fold increase in efficient infection
270 compared to the endosomal pathway in the absence of proteases¹⁷.

271

272 Second, we found that elastase-enhanced S-G614 pseudoviral infection could be
273 partially blocked by sivelestat sodium. Elastase-2, also known as neutrophil
274 elastase, plays an important role in degenerative and inflammatory diseases.
275 Sivelestat, a drug approved to treat acute respiratory distress syndrome (ARDS) in
276 Japan and South Korea, has a beneficial effect on the pulmonary function of
277 patients with ARDS and systemic inflammatory response syndrome¹⁸. About 10–
278 15% of patients with COVID-19 progress to ARDS¹⁹. Since sivelestat may not only
279 mitigate the damage of neutrophil elastase on lung connective tissue, but also limit
280 virus spread by inhibiting S protein processing, Mohamed et al. (2020) advocated
281 the use of sivelestat to alleviate neutrophil-induced damage in critically ill patients
282 with COVID-19²⁰. Our *in vitro* results also indicate that sivelestat sodium or similar
283 neutrophil elastase inhibitors might be an effective option for treatment of COVID-
284 19 caused by SARS-CoV-2 harboring the D614G mutation.

285

286 Third, we observed that the S-G614 pseudovirus was more resistant to
287 neutralization by convalescent sera from patients, likely infected in mid- to late-
288 January when wild-type (D614) virus was mainly circulating in China. Koyama et
289 al. (2020) reported that D614G is located in one of the predicted B-cell epitopes of
290 SARS-CoV-2 S protein, and this is a highly immunodominant region and may
291 affect the effectiveness of vaccine with wild-type S protein¹⁴. D614 is conserved in
292 the S protein of SARS-CoV in 2003. Previous studies of SARS-CoV suggested
293 that the peptide S₅₉₇₋₆₂₅ is a major immunodominant peptide in humans and elicits
294 a long-term B-cell memory response after natural infection with SARS-CoV²¹.
295 Regions between amino acids 614 and 621 of SARS-CoV-2 S protein were also
296 identified as a B-cell epitope by different methods, and the change in D614G may
297 affect the antigenicity of this region²². In our study, we observed that 7% (5/70)
298 convalescent sera showed markedly different neutralization activities between S-
299 G614 and S-G61 protein pseudotyped viruses, indicating that the D614G mutation
300 reduces the sensitivity to neutralizing antibodies from some patients infected with
301 earlier SARS-CoV-2 variant. Whether these patients were at high risk of reinfection
302 with the S-G614 variant should be explored in further studies. It will also be
303 important to determine the breadth of the neutralizing capacity of vaccine-induced
304 neutralizing antibodies.

305

306 Recently, several groups also reported that S-G614 enhances viral infectivity

307 based on pseudovirus assays²³⁻²⁶, but due to the small sample size, they found no
308 effect on the neutralization sensitivity of the virus^{23,24,26}. Given the evolving nature
309 of the SARS-CoV-2 RNA genome, antibody treatment and vaccine design require
310 further considerations to accommodate D614G and other mutations that may
311 affect the immunogenicity of the virus.

312

313 Our study had some limitations. First, 19 amino acids of S protein on the C-
314 terminal were not included in order to improve the packaging efficiency of the
315 SARS-CoV-2 S protein pseudotyped virus, and this pseudovirus only recapitulates
316 viral entry events. Therefore, additional assays with authentic SARS-CoV-2
317 viruses are required. Second, we only tested neutralizing antibodies against the S
318 protein. Previous studies on SARS-CoV indicated that only a small fraction of
319 memory B cells specific for SARS-CoV antigens are directed against neutralizing
320 epitopes present on the S protein²⁷. Third, in addition to D614G, further studies on
321 other mutations in the S protein are needed to evaluate their impact on SARS-
322 CoV-2 infectivity, pathogenicity, and immunogenicity. Further studies are needed
323 to determine the impact of these mutations on the severity of COVID-19.

324

325 In summary, we established a SARS-CoV-2 S protein-mediated pseudoviral entry
326 assay and explored the cellular entry of S-D614 and S-G614 pseudotyped viruses.
327 Our study provided evidence that the D614G mutation introduces an additional
328 elastase-2 cut site in the S protein, thereby promoting its cleavage and viral cell

329 entry, resulting in SARS-CoV-2 becoming more infectious. Importantly, the D614G
330 mutation reduced the sensitivity of the virus to serum neutralizing antibodies in 7%
331 convalescent patients with COVID-19. Our study will be helpful for understanding
332 SARS-CoV-2 transmission and for the design of vaccines and therapeutic
333 interventions against COVID-19.

334

335 **Materials and Methods**

336 **Plasmids.** The codon-optimized gene encoding SARS-CoV-2 S protein (GenBank:
337 QHD43416) with C-terminal 19-amino acid deletion was synthesized by Sino
338 Biological Inc (Beijing, China), and cloned into the *KpnI* and *XbaI* restriction sites
339 of pCMV3 vector (pCMV3-SARS-CoV-2-S-C19del, denoted as pS-D614). The
340 D614G mutant S-expressing plasmid (denoted as pS-G614) was constructed by
341 site-directed mutagenesis, with pS-D614 plasmid as a template. The HIV-1 NL4-3
342 Δ Env Vpr luciferase reporter vector (pNL4-3.Luc.R-E-) constructed by N. Landau²⁸
343 was provided by Prof. Cheguo Cai from Wuhan University (Wuhan, China). The
344 VSV-G-expressing plasmid pMD2.G was provided by Prof. Ding Xue from
345 Tsinghua University (Beijing, China). The expression plasmid for human ACE2 was
346 obtained from GeneCopoeia (Guangzhou, China).

347

348 **Cell lines.** HEK 293T cells were purchased from the American Type Culture
349 Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's
350 modified Eagle medium (DMEM; Hyclone, Waltham, MA, USA) supplemented with

351 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 mg/mL of
352 streptomycin, and 100 units/mL of penicillin at 37 °C in 5% CO₂. HEK 293T cells
353 transfected with human ACE2 (293T-ACE2) were cultured under the same
354 conditions with the addition of G418 (0.5 mg/mL) to the medium. Elastase (from
355 porcine pancreas) was obtained from MACKLIN Biochemical Co. (Shanghai,
356 China).

357

358 **Antibodies and inhibitors.** The anti-RBD monoclonal antibody against the SARS-
359 CoV-2 S protein was kindly provided by Prof. Aishun Jin from Chongqing Medical
360 University. Recombinant human ACE2 linked to the Fc domain of human IgG1
361 (ACE2-Ig) was purchased from Sino Biological Inc. Sivelestat sodium
362 (MedChemExpress, Monmouth Junction, NJ, USA) Camostat mesylate (Tokyo
363 Chemical Industry, Tokyo, Japan), and aloxistatin (E-64d; MedChemExpress) were
364 dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 50 mM.

365

366 **Sera samples.** A total of 70 convalescent sera samples from patients with COVID-
367 19 (at 2–4 weeks after symptom onset) were collected from three designated
368 hospitals in Chongqing from February 1 to February 10, 2020 (Supplementary
369 information, Table S1). All sera were tested positive using magnetic
370 chemiluminescence enzyme immunoassay (MCLIA) kits supplied by BioScience
371 Co. (Tianjin, China)²⁹. Patient sera were incubated at 56 °C for 30 min to inactivate
372 the complement prior to experiments.

373

374 **SARS-CoV-2 genome analysis**

375 The online Nextstrain analysis tool (<https://nextstrain.org/ncov>) was used to track
376 the D614G mutation in SARS-CoV-2 genomes. All the 2,834 genomes sampled
377 between Dec 20, 2019 and Jun 12, 2020 were visualized using the ‘rectangular’
378 layout. The mutations were labeled on branches.

379 All complete SARS-CoV-2 S gene sequences were downloaded from The National
380 Center for Biotechnology Information (NCBI) website

381 (<https://www.ncbi.nlm.nih.gov/sars-cov-2/>) on Jun 1, 2020. We obtained 4,701 S
382 coding sequences from NCBI, and after excluding partial and frameshift

383 sequences, 4,649 completed S sequences were used for further analysis. All S
384 nucleotide sequences were translated to amino acid sequences. The nucleotide

385 and amino acid sequences of S were aligned with multiple sequence alignment
386 software MULTiple Sequence Comparison by Log-Expectation (MUSCLE)

387 separately. The ‘Wuhan-Hu-1’ strain (NC_045512) was used to as the reference
388 sequence, and the mutations were extracted using private PERL scripts.

389

390 **Western blot analysis of SARS-CoV-2 S protein expression.** To analyze S

391 protein expression in cells, S-D614- and S-G614-expressing plasmids were

392 transfected into HEK 293T cells. Total protein was extracted from cells using radio

393 immunoprecipitation assay Lysis Buffer (CoWin Biosciences, Beijing, China)

394 containing 1 mM phenylmethylsulfonyl fluoride (Beyotime, Shanghai, China). Equal

395 amounts of protein samples were electrophoretically separated by 10% sodium
396 dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to
397 polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The
398 immunoblots were probed with the indicated antibodies. Protein bands were
399 visualized using SuperSignal West Pico Chemiluminescent Substrate kits (Bio-
400 Rad, Hercules, CA, USA) and quantified by densitometry using ImageJ software
401 (NCBI, Bethesda, MD, USA).

402

403 **Production and titration of SARS-CoV-2 S pseudoviruses.** SARS-CoV-2
404 pseudotyped viruses were produced as previously described with some
405 modifications³⁰. Briefly, 5×10^6 HEK 293T cells were co-transfected with 6 μ g each
406 of pNL4-3.Luc.R-E- and recombinant SARS-CoV-2 S plasmids (pS-D614 or pS-
407 G614) using Lipofectamine 3000 Transfection Reagent (Invitrogen) according to
408 the manufacturer's instructions. The S-D614 and S-G614 protein pseudotyped
409 viruses in supernatants were harvested 48 h after transfection, centrifuged, filtered
410 through a 0.45 μ m filter, and stored at -80°C . The pMD2.G was co-transfected
411 with the pNL4-3.Luc.R-E- plasmid to package the VSV-G pseudovirus.
412 The titers of the pseudoviruses were calculated by determining the number of viral
413 RNA genomes per mL of viral stock solution using RT-qPCR with primers and a
414 probe that target LTR³¹. Sense primer: 5'-TGTGTGCCCGTCTGTTGTGT-3', anti-
415 sense primer: 5'-GAGTCCTGCGTCGAGAGAGC-3', probe: 5'-FAM-
416 CAGTGGCGCCCGAACAGGGA-BHQ1-3'. Briefly, viral RNAs were extracted

417 using TRIzol (Invitrogen, Rockville, MD) and treated with RNase-free DNase
418 (Promega, Madison, WI, USA) and re-purified using mini columns. Then, the RNA
419 was amplified using the TaqMan One-Step RT-PCR Master Mix Reagents (Applied
420 Biosystems, Thermo Fisher). A known quantity of pNL4-3.Luc.R-E- vector was
421 used to generate standard curves. The S-D614 and S-G614 protein pseudotyped
422 viruses were adjusted to the same titer (copies/mL) for the following experiments.

423

424 **SARS-CoV-2 S-mediated pseudoviral entry assay.** To detect S variant-mediated
425 viral entry, 293T-ACE2 cells (2×10^4) grown on 96-well plates were infected with
426 the same amount of S-D614 or S-G614 pseudovirus (3.8×10^4 copies in 50 μ L).
427 The cells were transferred to fresh DMEM medium 8 h post-infection, and RLU
428 was measured 24-72 h post-infection using Luciferase Assay Reagent (Promega,
429 Madison, WI, USA) according to the manufacturer's protocol³².

430

431 **Neutralization and inhibition assays.** The 293T-ACE2 cells (2×10^4 cells/well)
432 were seeded on 96-well plates. For the neutralization assay, 50 μ L pseudoviruses,
433 equivalent to 3.8×10^4 vector genomes, were incubated with serial dilutions of
434 sera samples from patients and normal human serum as a negative control for 1 h
435 at 37 °C, then added to the 293T-ACE2 cells (with three replicates for each
436 dilution). For the inhibition assay, the cells were pretreated with elastase for 5 min
437 and then infected with pseudotyped viruses in the presence of various
438 concentrations of sivelestat sodium. After incubation for 12 h, the medium was

439 replaced with fresh cell culture medium. Luciferase activity was measured 72 h
440 after infection and the percentage of neutralization was calculated using GraphPad
441 Prism 6.0 software (GraphPad Software, San Diego, CA, USA). Percentage of
442 RLU reduction (inhibition rate) was calculated as: $1 - (\text{RLU of sample sera-control}$
443 $\text{wells}) / (\text{RLU from mock control sera-control wells}) \times 100\%$. The titers of
444 neutralizing antibodies were calculated as 50% inhibitory dose (ID₅₀).

445

446 **Statistical analyses.** Statistical analyses of the data were performed using
447 GraphPad Prism version 6.0 software. Quantitative data in histograms are shown
448 as means \pm SD. Statistical significance was determined using ANOVA for multiple
449 comparisons. Student's *t*-tests were applied to compare the two groups.
450 Differences with *P* values < 0.05 were deemed statistically significant.

451

452 **Ethical approval.** The study was approved by the Ethics Commission of
453 Chongqing Medical University (ref. no. 2020003). Written informed consent was
454 waived by the Ethics Commission of the designated hospital for emerging
455 infectious diseases.

456

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469

470 **Author contributions**

471 A-L.H., N.T., and K.W. conceived the project and supervised the study. J.H., C-
472 L.H., Q-Z.G. and G-J.Z. performed most experiments. J.H. and K.W. performed
473 serum neutralization assay. H-J.D. performed SARS-CoV-2 genome analysis. L-Y.
474 H. performed structural analysis of S protein. Q-X.L., J.C. and X-X. C. collected the
475 serum samples. J.H. and Q-Z.G. contributed to the statistical analysis. K.W. and
476 N.T. wrote the manuscript. All the authors analyzed the final data, reviewed and
477 approved the final version.

478

479 **Competing interests**

480 The authors declare no competing interests.

481

482

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- 563
- 564

565 **Table 1. Mutations in spike protein of SARS-CoV-2.**

Mutations in Spike Protein	No. of mutation	No. of wildtype	Total No. of sequences	Mutation (%)
D614G	2995	1637	4632	64.659
A829T	37	4602	4639	0.798
L5F	33	4614	4647	0.710
H146Y	26	4594	4620	0.563
P1263L	15	4631	4646	0.323
V483A	12	4387	4399	0.273
S939F	12	4626	4638	0.259
R78M	10	4623	4633	0.216
E583D	9	4639	4648	0.194
A845S	9	4632	4641	0.194

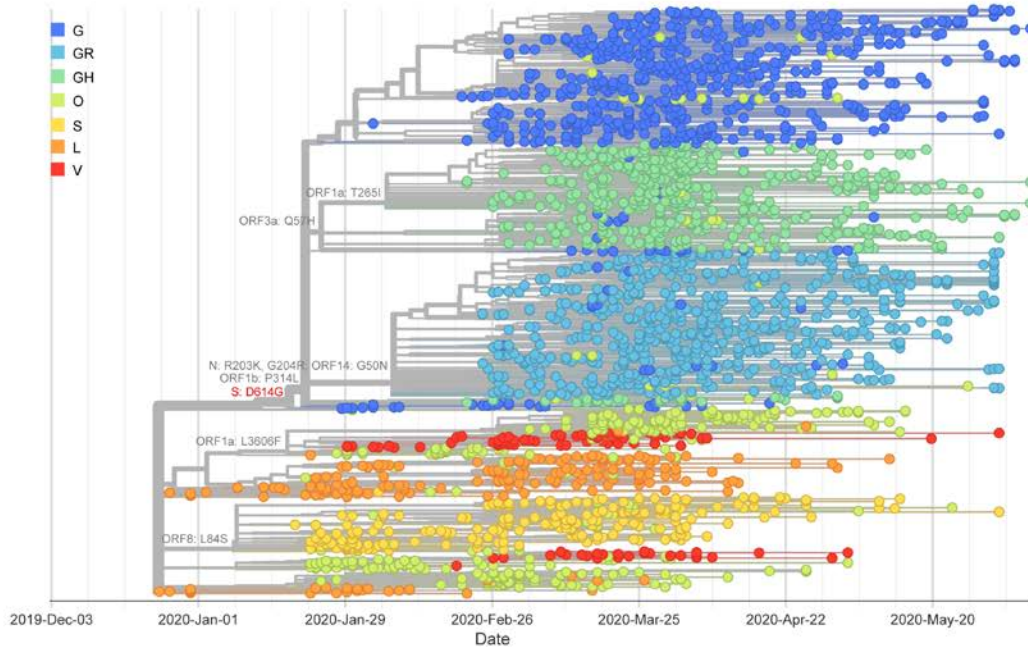
566 Top 10 abundant non-synonymous mutations observed in S protein of SARS-CoV-

567 2.

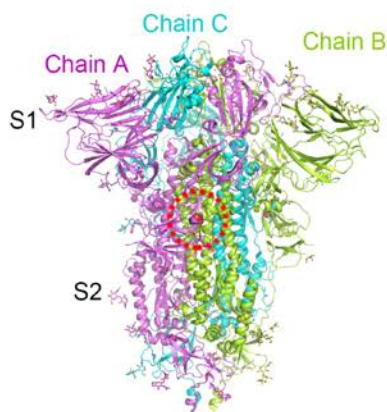
568 **Figures and Figure legends:**

Figure 1

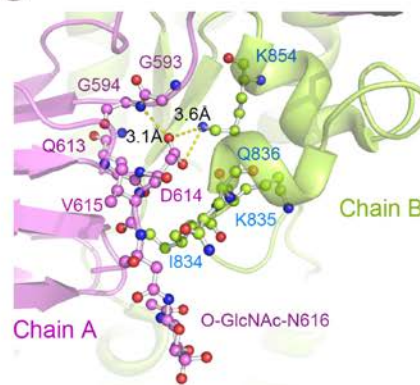
a



b



c



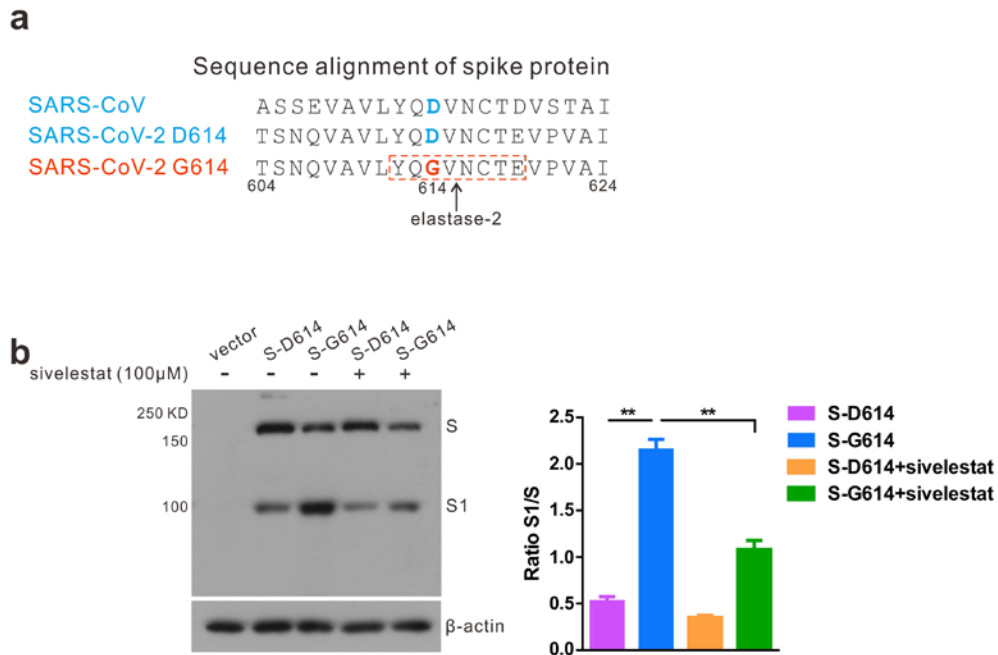
569

570 **Fig. 1. Phylogenetic timetree of SARS-CoV-2 and structural features of its**

571 **spike (S) protein. a** Phylogenetic analysis of SARS-CoV-2 sequences from

572 GISAID database. Prevalence of S-D614G genomes over time produced by the
573 Nextstrain analysis tool using GISAID dataset ($n = 2,834$ genomes samples from
574 January 2020 to May 2020). **b** Cartoon representation of the trimeric SARS-CoV-2
575 spike structure (PDB ID: 6ZGE). Three spike chains are colored in violet, lemon,
576 and aquamarine, respectively. D614 is located at the C-terminal of the S1 subunit
577 and is shown as violet spheres in the red dashed circle. **c** D614 involves the
578 interactions between two S chains. Residues are highlighted by violet or lemon
579 spheres and sticks. Yellow dashed lines indicate hydrogen bonds or salt bridges.
580 PyMOL software (Schrödinger, LLC) was used to generate all rendered structural
581 images.

Figure 2



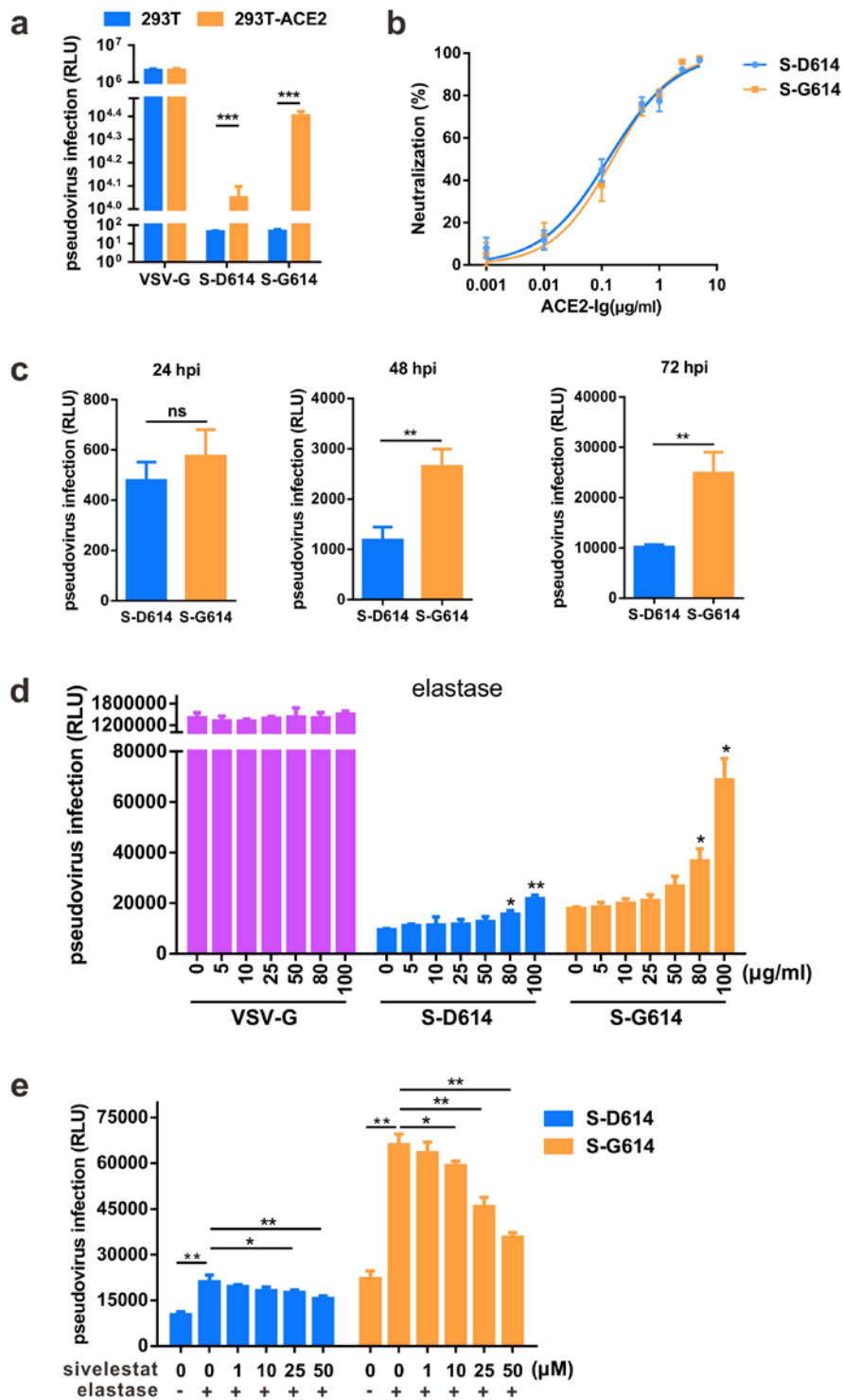
582

583 **Fig. 2 Detection of S-D614 and S-G614 protein expression and cleavage. a** An

584 additional serine protease (elastase-2) cleavage site in S1-S2 junction was

585 identified in S-614G protein of SARS-CoV-2. **b** Detection of S protein expression
586 in HEK 293T cells by Western blot using the anti-RBD (receptor-binding domain)
587 monoclonal antibody. Cells were transfected with pS-D614 or pS-G614 plasmids
588 or with an empty vector and incubated with or without sivelestat sodium. To
589 compare the S1 and S ratio, integrated density of S1/S was quantitatively
590 analyzed using ImageJ software. $n = 3$, \pm SD. $**P < 0.01$.

Figure 3



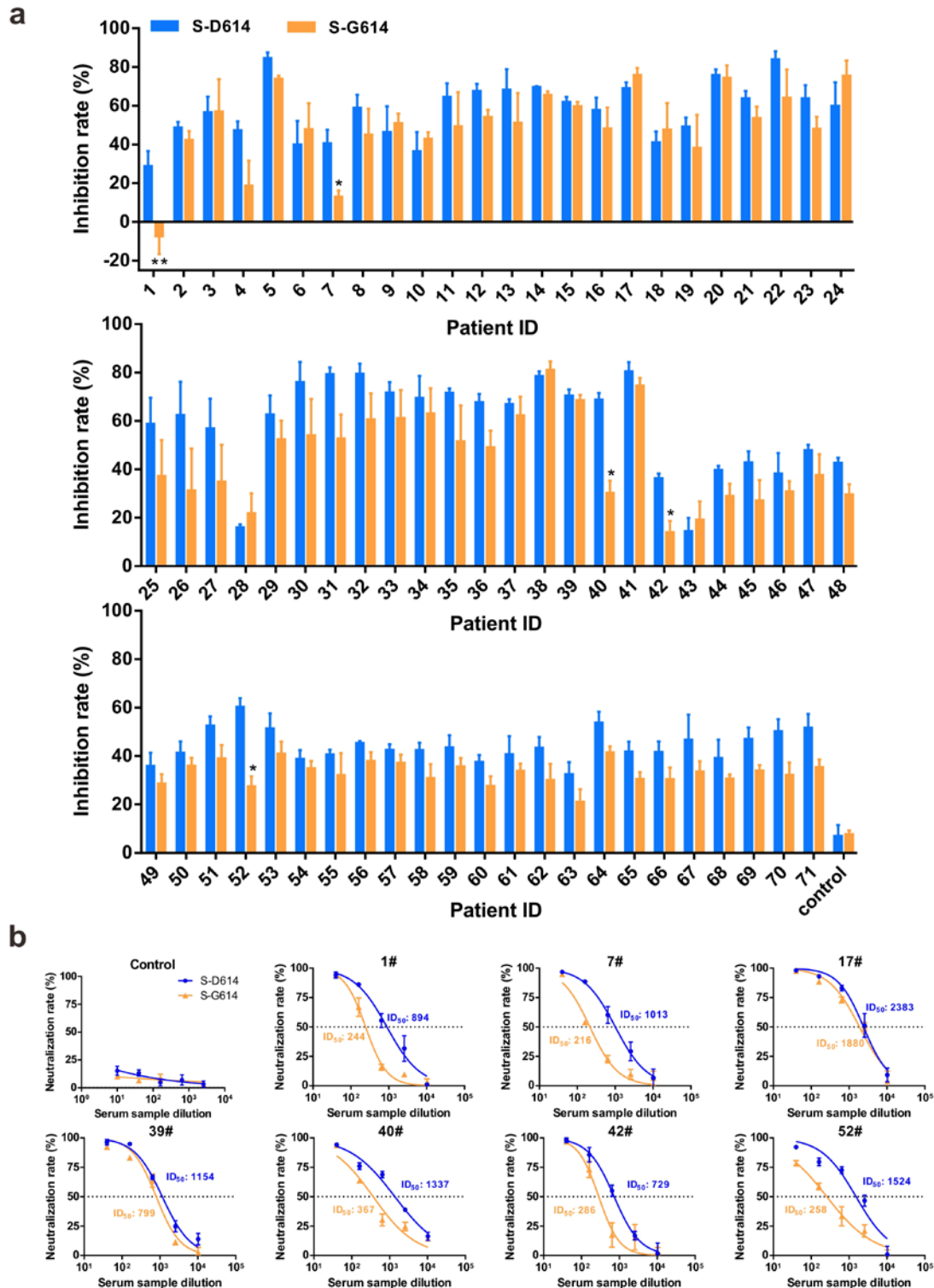
591

592 **Fig. 3 The S-G614 protein pseudotyped virus showed increased infectivity. a**

593 HEK 293T and 293T-ACE2 (human angiotensin-converting enzyme 2) cells were

594 infected with lentiviruses pseudotyped with VSV-G and SARS-CoV-2 S protein
595 variants. Virus titers were quantified by RT-qPCR and adjusted to 3.8×10^4 copies
596 in 50 μ L to normalize input virus doses. The relative luminescence units (RLU)
597 detected 72 h post-infection (hpi). **b** Inhibition of pseudoviral entry by ACE2-Ig.
598 Pseudoviruses were pre-incubated with ACE2-Ig and added to 293T-ACE2 cells,
599 then RLU was measured at 72 hpi. **c** Viral entry efficiency mediated by S variants.
600 The RLU was measured at 24-72 hpi. **d-e** D614G mutation facilitates elastase-2
601 induced pseudoviral entry. 293T-ACE2 cells were treated with elastase for 5 min
602 and then infected with pseudotyped viruses containing the S-D614 or S-G614
603 mutant in the presence of various concentrations of sivelestat sodium. RLU was
604 measured at 72 hpi. n = 3, \pm SD. * P < 0.05, ** P < 0.01. ns, not significant.

Figure 4



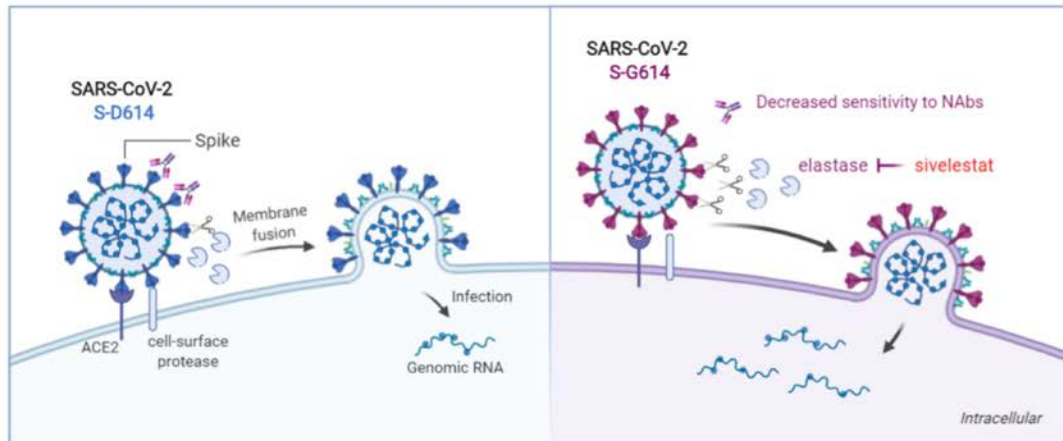
605

606 **Fig. 4 Detection of neutralizing antibodies in convalescent sera against spike**

607 **(S)-D614 and S-G614 protein pseudotyped viruses. a The inhibition rate of**

608 convalescent sera from 70 patients with COVID-19 against S-D614 and S-G614
609 pseudoviruses. A serum sample from healthy individual was tested as a negative
610 control. Convalescent sera were collected 2–4 weeks after symptom onset from
611 confirmed case patients (1#–70#). Sera was analyzed at a single dilution of
612 1:1000. RLU was measured at 72 hpi. We then compared the RLU of serum
613 neutralized sample to the control and calculated the inhibition rate. n = 3, \pm SD. **b**
614 The inhibition curves for serum samples from seven convalescent patients and a
615 healthy donor. The initial dilution was 1:40, followed by 4-fold serial dilution.
616 Neutralization titers were calculated as 50% inhibitory dose (ID₅₀), expressed as
617 the serum dilution at which RLUs were reduced by 50% compared with virus
618 control wells after subtraction of background RLU in cell control wells.
619

Figure 5



620

621 **Fig. 5 Proposed working model of this study.** Protease-mediated cell-surface

622 pathway is critical for SARS-CoV-2 entry and infection. D614G mutation introduces

623 an additional elastase-2 cut site in the spike protein of SARS-CoV-2, thereby
624 promoting its cleavage and enhancing viral entry into host cells. The elastase-2
625 inhibitor sivelestat partially blocks S-G614 pseudovirus infection in the presence of
626 elastase. Both S-D614 and S-G614 variants could be similarly neutralized by most
627 antisera, however, S-G614 mutant is more resistant to neutralizing antibodies
628 (NAbs) from some patients infected with earlier SARS-CoV-2 variant.