

1 **An emerging SARS-CoV-2 mutant evading cellular immunity and increasing**
2 **viral infectivity**

3
4 Chihiro Motozono¹, Mako Toyoda¹, Jiri Zahradnik², Terumasa Ikeda³, Akatsuki
5 Saito^{4,5,6}, Toong Seng Tan¹, Isaac Ngare¹, Hesham Nasser³, Izumi Kimura⁷, Keiya
6 Uriu⁷, Yusuke Kosugi⁷, Shiho Torii^{8,9,10}, Akiko Yonekawa¹¹, Nobuyuki Shimono¹¹,
7 Yoji Nagasaki¹², Rumi Minami¹³, Takashi Toya¹⁴, Noritaka Sekiya^{15,16}, Takasuke
8 Fukuhara¹⁷, Yoshiharu Matsuura^{8,9,10}, Gideon Schreiber², The Genotype to
9 Phenotype Japan (G2P-Japan) consortium, So Nakagawa^{18,19*}, Takamasa Ueno^{1*},
10 Kei Sato^{7,19,20,21*}

11
12 ¹ Division of Infection and immunity, Joint Research Center for Human Retrovirus
13 infection, Kumamoto University, Kumamoto 8600811, Japan

14 ²Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100,
15 Israel

16 ³ Division of Molecular Virology and Genetics, Joint Research Center for Human
17 Retrovirus infection, Kumamoto University, Kumamoto 8600811, Japan

18 ⁴ Department of Veterinary Science, Faculty of Agriculture, University of Miyazaki,
19 Miyazaki 8892192, Japan

20 ⁵ Center for Animal Disease Control, University of Miyazaki, Miyazaki 8892192,
21 Japan

22 ⁶ Graduate School of Medicine and Veterinary Medicine, University of Miyazaki,
23 Miyazaki 8892192, Japan

24 ⁷ Division of Systems Virology, Department of Infectious Disease Control,
25 International Research Center for Infectious Diseases, The Institute of Medical
26 Science, The University of Tokyo, Tokyo 1088639, Japan

27 ⁸ Department of Molecular Virology, Research Institute for Microbial Diseases,
28 Osaka University, Osaka 5650871, Japan

29 ⁹Division of Microbiology and Immunology, Center for Infectious Diseases Education
30 and Research, Osaka University, Osaka 5650871, Japan

31 ¹⁰ Laboratory of Virus Control, Research Institute for Microbial Diseases, Osaka
32 University, Osaka 5650871, Japan

33 ¹¹ Department of Medicine and Biosystemic Science, Graduate School of Medical
34 Sciences, Kyushu University, Fukuoka 8128582, Japan

35 ¹² Division of Infectious Diseases, Clinical Research Institute, National
36 Hospitalization Organization, Kyushu Medical Center, Fukuoka 8108563, Japan

37 ¹³ Internal Medicine, Clinical Research Institute, National Hospital Organization,
38 Kyushu Medical Center, Fukuoka 8108563, Japan

39 ¹⁴ Hematology Division, Tokyo Metropolitan Cancer and Infectious Diseases Center
40 Komagome Hospital, Tokyo 1138677, Japan.

41 ¹⁵ Department of Infection Prevention and Control, Tokyo Metropolitan Cancer and
42 Infectious Diseases Center Komagome Hospital, Tokyo 1138677, Japan.

43 ¹⁶ Department of Clinical Laboratory, Tokyo Metropolitan Cancer and Infectious
44 Diseases Center Komagome Hospital, Tokyo 1138677, Japan.

45 ¹⁷ Department of Microbiology and Immunology, Graduate School of Medicine,
46 Hokkaido University, Hokkaido 0608638, Japan

47 ¹⁸ Department of Molecular Life Science, Tokai University School of Medicine,
48 Kanagawa 2591193, Japan

49 ¹⁹ CREST, Japan Science and Technology Agency, Saitama 3220012, Japan

50 ²⁰ Twitter: @SystemsVirology

51 ²¹ Lead Contact

52 *Correspondences:

53 so@tokai.ac.jp (S.N.)

54 uenotaka@kumamoto-u.ac.jp (T.U.)

55 KeiSato@g.ecc.u-tokyo.ac.jp (K.S.)

56

57 **Conflict of interest:** The authors declare that no competing interests exist.

58 **Short title:** SARS-CoV-2 variants escape from cellular immunity (50/50 characters)

59 **Keywords:** SARS-CoV-2; COVID-19; cellular immunity; spike protein; receptor
60 binding motif; mink; zoonosis; naturally occurring variants; B.1.427/429; B.1.298;
61 L452R; Y453F

62

63

64 **Highlights** (85 characters including spaces)

65 ● L452R (in B.1.427/429) and Y453F (in B.1.298) variants in S RBM have
66 emerged

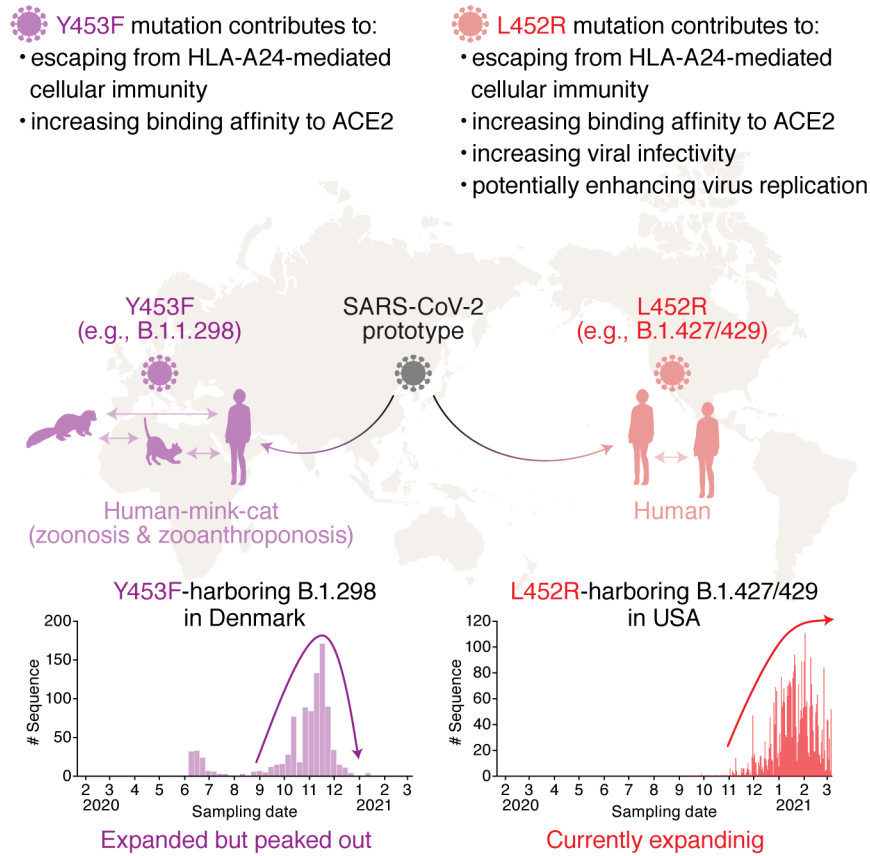
67 ● L452R and Y453F mutants escape from HLA-24-restricted cellular immunity

68 ● L452R increases viral infectivity and potentially promotes viral replication

69 ● Epidemic of L452R-harboring B.1.427/429 variants has been expanding in USA

70

71 Graphical Abstract



72

73 **Summary** (144/150 words)

74 During the current SARS-CoV-2 pandemic that is devastating the modern societies
75 worldwide, many variants that naturally acquire multiple mutations have emerged.
76 Emerging mutations can affect viral properties such as infectivity and immune
77 resistance. Although the sensitivity of naturally occurring SARS-CoV-2 variants to
78 humoral immunity has recently been investigated, that to human leukocyte antigen
79 (HLA)-restricted cellular immunity remains unaddressed. Here we demonstrate that
80 two recently emerging mutants in the receptor binding domain of the SARS-CoV-2
81 spike protein, L452R (in B.1.427/429) and Y453F (in B.1.298), can escape from the
82 HLA-24-restricted cellular immunity. These mutations reinforce the affinity to viral
83 receptor ACE2, and notably, the L452R mutation increases protein stability, viral
84 infectivity, and potentially promotes viral replication. Our data suggest that the HLA-
85 restricted cellular immunity potentially affects the evolution of viral phenotypes, and
86 the escape from cellular immunity can be a further threat of the SARS-CoV-2
87 pandemic.

88 **Introduction**

89 Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe
90 acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Since an unusual
91 outbreak in Wuhan, Hubei province, China, in December 2019 ([Wu et al., 2020](#); [Zhou
92 et al., 2020](#)), SARS-CoV-2 has rapidly spread all over the world, and as of March
93 2021, SARS-CoV-2 is an ongoing pandemic: more than one hundred million cases
94 of infections have been reported worldwide, and more than two million people died
95 of COVID-19 ([WHO, 2020a](#)).

96 During the current pandemic, a variety of SARS-CoV-2 mutants have
97 emerged and some of them have dominantly spread [reviewed in ([Plante et al.,
98 2021](#))]. A well-studied SARS-CoV-2 mutant harbors D614G substitution in the spike
99 (S) protein. Recent studies have revealed that the D614G mutation increases the
100 SARS-CoV-2 binding affinity to ACE2, the SARS-CoV-2 receptor ([Ozono et al.,
101 2021](#); [Yurkovetskiy et al., 2020](#); [Zhou et al., 2021](#)), infectivity ([Ozono et al., 2021](#);
102 [Yurkovetskiy et al., 2020](#); [Zhou et al., 2021](#)), fitness ([Hou et al., 2020](#); [Plante et al.,
103 2020](#); [Zhou et al., 2021](#)), and transmissibility in the human population ([Volz et al.,
104 2021](#)). However, there is no evidence suggesting that the D614G variant is
105 associated with viral pathogenicity and lethality ([Hou et al., 2020](#); [Korber et al., 2020](#);
106 [Plante et al., 2020](#)). Additionally, since the fall of 2020, new SARS-CoV-2 variants
107 such as B.1.1.7 (also known as a variant of concern 202012/01 or 20I/501Y.V1),
108 B.1.351 (also known as 20H/501Y.V2), and P.1 (also known as 501Y.V3) lineages
109 emerged in the UK, South Africa, and Brazil, respectively, and have rapidly spread
110 worldwide ([CDC, 2020](#)). At the end of 2020, another lineage, B.1.427/429 (also
111 known as CAL.20C), has been predominant particularly in California state, the USA
112 ([Deng et al., 2021](#); [Zhang et al., 2021](#)). Moreover, cross-species viral infection can
113 accelerate the emergence of diversified viruses [reviewed in ([Banerjee et al., 2021](#);
114 [Parrish et al., 2008](#))]. In the case of SARS-CoV-2, a variety of mammals such as
115 nonhuman primates ([Chandrashekar et al., 2020](#); [Munster et al., 2020](#); [Yu et al.,
116 2020](#)) and carnivores ([Halfmann et al., 2020](#); [Kim et al., 2020](#); [Shi et al., 2020](#)) are
117 prone to its infection ([Damas et al., 2020](#); [Martinez-Hernandez et al., 2020](#); [OIE,
118 2021](#)). Strikingly, the emergence of a SARS-CoV-2 variant, B.1.298, has likely to be
119 associated with the outbreak in farmed minks in Denmark ([Koopmans, 2021](#); [WHO,
120 2020b](#)), and phylogenetic analysis has provided evidence of mink-to-human
121 transmission of SARS-CoV-2 within Danish mink farms ([Oude Munnink et al., 2021](#)).
122 Because newly emerging variants can potentially change viral infectivity,
123 transmissibility and pathogenicity, deep monitoring of the SARS-CoV-2 strains
124 circulating globally and locally and evaluating the effects of mutations detected on
125 virological characteristics are urgent and crucial.

126 The emergence of mutated viruses is mainly due to error-prone viral
127 replication, and the spread of emerged variants is attributed to the escape from
128 immune selective pressures [reviewed in (Duffy et al., 2008)]. In fact, several SARS-
129 CoV-2 mutants can be resistant to the neutralization mediated by the antibodies from
130 COVID-19 patients (Baum et al., 2020; Chen et al., 2021; Liu et al., 2021c; McCarthy
131 et al., 2021; Weisblum et al., 2020) as well as those from vaccinated individuals (Liu
132 et al., 2021b). Although the B.1.1.7 variant is sensitive to convalescent and
133 vaccinated sera (Collier et al., 2021; Garcia-Beltran et al., 2021; Shen et al., 2021;
134 Supasa et al., 2021; Wang et al., 2021), the B.1.351 and P.1 variants are relatively
135 resistant to anti-SARS-CoV-2 humoral immunity (Garcia-Beltran et al., 2021;
136 Hoffmann et al., 2021a; Wang et al., 2021).

137 In addition to the humoral immunity mediated by neutralizing antibodies,
138 another protection system against pathogens is the cellular immunity mediated by
139 cytotoxic T lymphocytes (CTLs) [reviewed in (Fryer et al., 2012; Leslie et al., 2004)].
140 CTLs recognize the nonself epitopes that are presented on virus-infected cells via
141 human leukocyte antigen (HLA) class I molecules, and therefore, the CTL-mediated
142 antiviral immunity is HLA-restricted [reviewed in (La Gruta et al., 2018)]. Recent
143 studies have reported the HLA-restricted SARS-CoV-2-derived epitopes that can be
144 recognized by human CTLs (Kared et al., 2021; Kiyotani et al., 2020; Nelde et al.,
145 2021; Schulien et al., 2021; Wilson et al., 2021). More importantly, Bert et al. have
146 recently reported that the functionality of virus-specific cellular immunity is inversely
147 correlated to the COVID-19 severity (Le Bert et al., 2021). Therefore, it is
148 conceivable to assume that the HLA-restricted CTLs play crucial roles in controlling
149 SARS-CoV-2 infection and COVID-19 disorders. However, comparing to humoral
150 immune responses, it remains unclear whether the SARS-CoV-2 variants can
151 potentially escape from cellular immunity.

152 In this study, we investigate the possibility for the emergence of the SARS-
153 CoV-2 mutants that can escape from the HLA-restricted cellular immunity. We
154 demonstrate that at least two naturally occurring substitutions in the receptor binding
155 motif (RBM; residues 438-506) of the SARS-CoV-2 S protein, L452R and Y453F,
156 which were identified in the two major variants, B.1.427/429 (L452R) and B.1.1.298
157 (Y453F), can be resistant to the cellular immunity in the context of HLA-A*24:02, an
158 allele of HLA-I. More intriguingly, the L452R and Y453F mutants increase the binding
159 affinity to ACE2, and the experiments using pseudoviruses show that the L452R
160 substitution increases viral infectivity. Furthermore, we artificially generate the
161 SARS-CoV-2 harboring these point mutations by reverse genetics and demonstrate
162 that the L452R mutants enhance viral replication capacity.

163 Results

164 Evasion from the HLA-A24-restricted CTL responses by acquiring mutations 165 in the RBM of SARS-CoV-2 S protein

166 We set out to address the possibility of the emergence of the naturally occurring
167 mutants that can potentially confer the resistance to antigen recognition by HLA-
168 restricted cellular immunity. A bioinformatic study has suggested that the 9-mer
169 peptide in the RBM, NYNYLYRLF (we designate this peptide "NF9"), which spans
170 448-456 in the S protein, can be the potential epitope presented by HLA-A24
171 (Kiyotani et al., 2020), an HLA-I allele widely distributed all over the world and
172 particularly predominant in East and Southeast Asian area (**Table S1**). Additionally,
173 three immunological analyses using COVID-19 convalescents have shown that the
174 NF9 peptide is an immunodominant epitope presented by HLA-A*24:02 (Gao et al.,
175 2021; Hu et al., 2020; Kared et al., 2021). To verify these observations, we obtained
176 the peripheral blood mononuclear cells (PBMCs) from nine COVID-19
177 convalescents with HLA-A*24:02 and stimulated these cells with the NF9 peptide.
178 As shown in **Figure 1A**, a fraction of CD8⁺ T cells upregulated two activation markers,
179 CD25 and CD137, in response to the stimulation with NF9. In the nine samples of
180 COVID-19 convalescents with HLA-A*24:02, the percentage of the CD25⁺CD137⁺
181 cells in the presence of the NF9 peptide (5.3% in median) was significantly higher
182 than that in the absence of the NF9 peptide (0.49% in median) (**Figure 1B**; $P=0.016$
183 by Wilcoxon signed-rank test). Additionally, the stimulation with the NF9 peptide did
184 not upregulate CD25 and CD137 in the CD8⁺ T cells of three seronegative samples
185 with HLA-A*24:02 and the percentage of the CD25⁺CD137⁺ cells in seronegative
186 samples (0.93% in median) was significantly lower than that in COVID-19
187 convalescent samples (**Figure 1B**; $P=0.011$ by Mann-Whitney U test). Consistent
188 with previous reports (Gao et al., 2021; Hu et al., 2020; Kared et al., 2021; Kiyotani
189 et al., 2020), our data suggest that the NF9 peptide is an immunodominant HLA-
190 A*24:02-restricted epitope recognized by the CD8⁺ T cells of COVID-19
191 convalescents in our cohort.

192 We next assessed the profile of cytokine production by the NF9 stimulation.
193 As shown in **Figure 1C**, the stimulation with the NF9 peptide induced the production
194 of IFN- γ , TNF- α and IL-2 in the CD8⁺ T cells of a COVID-19 convalescent. The
195 analysis using six COVID-19 convalescent samples showed that CD8⁺ T cells
196 produce multiple cytokines in response to the NF9 stimulation (**Figure 1D**),
197 demonstrating the multifunctional nature of the NF9-specific CD8⁺ T cells of COVID-
198 19 convalescents. Moreover, the cytotoxic potential of the NF9-specific CD8⁺ T cells
199 was assessed by staining with surface CD107a, a degranulation marker (**Figure 1E**).
200 As shown in **Figure 1F**, the percentage of CD107a⁺ cells in the CD8⁺ T cells with

201 the NF9 peptide (12.9% in median) was significantly higher than that without the NF9
202 peptide (0.83% in median) (; $P=0.031$ by Wilcoxon signed-rank test), suggesting the
203 cytotoxic potential of the NF9-specific CD8⁺ T cells.

204 To assess the presence of naturally occurring variants harboring mutations
205 in this region (residues 448-456 in the S protein), we analyzed the diversity of SARS-
206 CoV-2 during the current pandemic. We downloaded 750,243 viral genome
207 sequences from the global initiative on sharing all influenza data (GISAID) database
208 (<https://www.gisaid.org>; as of March 15, 2021). The L452R substitution was most
209 frequent among the sequences analyzed (5,677 sequence), and 1,380 sequences
210 reported contained the Y453F substitution (**Table 1**). Notably, the B.1.427/429 and
211 B.1.1.298 lineages ([CDC, 2020](https://www.cdc.gov/media/releases/2020/s111920-covid-19-variants.html)) in the PANGO lineages ([https://cov-
212 lineages.org/index.html](https://cov-lineages.org/index.html)) mainly harbor the L452R and Y453F mutations,
213 respectively (**Figure 1G and Table S2**).

214 To address the possibility that the naturally occurring mutations in the NF9
215 region, L452R and Y453F, evade the NF9-specific CD8⁺ T cells of HLA-A24-positive
216 COVID-19 convalescents, two NF9 derivatives containing either L452R or Y453F
217 substitution (NF9-L452R and NF9-Y453F) were prepared and used for the
218 stimulation experiments. As shown in **Figure S1**, parental NF9 induced IFN- γ
219 expression in a dose-dependent manner. In contrast, the induction level of IFN- γ
220 expression by the NF9-Y453F derivative was significantly lower than that by parental
221 NF9, and more intriguingly, the NF9-L452R derivative did not induce IFN- γ
222 expression even at the highest concentration tested (10 nM) (**Figure S1**). In the five
223 HLA-A24-positive COVID-19 convalescent samples, parental NF9 peptide
224 significantly induced IFN- γ expression, while the NF9-L452R and NF9-Y453F
225 derivatives did not (**Figures 1H and 1I**). Altogether, these results suggest that the
226 NF9 peptide, which is derived from the RBM of SARS-CoV-2 S protein, is an
227 immunodominant epitope of HLA-A24, and two naturally occurring mutants, L452R
228 and Y453F, evade the HLA-A24-restricted cellular immunity.

229

230 **Augmentation of the binding affinity to ACE2 by the L452 and Y453 mutations**

231 We next addressed whether the mutations of interest affect the efficacy of virus
232 infection. Structural analyses have shown that the Y453 and N501 residues in the
233 RBM are located on the interface between the SARS-CoV-2 RBM and human ACE2
234 and directly contribute to the binding to human ACE2, while the L452 residue is not
235 on the RBM-ACE2 interface ([Lan et al., 2020](#); [Wang et al., 2020](#); [Zhao et al., 2020](#))
236 (**Figure 2A**). To directly assess the effect of these mutations in the RBM on the
237 binding affinity to ACE2, we prepared the yeasts expressing parental SARS-CoV-2
238 receptor binding domain RBD (residues 336-528) and its derivatives (L452R, Y453F

239 and N501Y) and performed *in vitro* binding assay using the yeast surface display of
240 the RBD and soluble ACE2 protein. Consistent with recent studies including ours
241 ([Supasa et al., 2021](#); [Zahradník et al., 2021b](#)), the N501Y mutation, which is a
242 common mutation in the B.1.1.7, B.1.351 and P.1 variants [reviewed in ([Plante et al.,](#)
243 [2021](#))] as well as the Y453F mutation ([Bayarri-Olmos et al., 2021](#); [Zahradník et al.,](#)
244 [2021b](#)) significantly increased the binding affinity to human ACE2 (**Figures 2B and**
245 **2C**; RBD parental $K_D = 2.05 \pm 0.26$ nM; RBD N501Y $K_D = 0.59 \pm 0.03$ nM; and RBD
246 Y453F $K_D = 0.51 \pm 0.06$ nM). We also found that the L452R mutant significantly
247 increased the binding affinity to human ACE2 (**Figures 2B and 2C**; RBD L452R K_D
248 $= 1.20 \pm 0.06$ nM). Intriguingly, the L452R mutations increased the surface
249 expression, which reflects protein stability ([Traxlmayr and Obinger, 2012](#)), while the
250 Y453F and N501Y mutations decreased (**Figure 2D**).

251

252 **Increase of pseudovirus infectivity by the L452R mutation**

253 To directly analyze the effect of the mutations of interest on viral infectivity, we
254 prepared the HIV-1-based reporter virus pseudotyped with the SARS-CoV-2 S
255 protein and its mutants and the 293 cells transiently expressing human ACE2 and
256 TMPRSS2. As shown in **Figure 2E**, although the N501Y mutation faintly affected
257 viral infectivity in this assay, the L452R mutations significantly increased viral
258 infectivity compared to parental S protein. In contrast to the yeast display assay
259 (**Figures 2B and 2C**), the infectivity of the Y453F mutant was significantly lower than
260 that of parental S protein (**Figure 2E**). Altogether, these findings suggest that the
261 L452R substitution increases the binding affinity of the SARS-CoV-2 RBD to human
262 ACE2, protein stability, and viral infectivity. Although the L452 residue is not directly
263 located at the binding interface (**Figure 2A**), structural analysis and *in silico*
264 mutagenesis suggested that the L452R substitution can cause a gain of
265 electrostatics complementarity ([Selzer et al., 2000](#)) (**Figure 2F**). Because the
266 residue 452 is located close proximity to the negatively charged patch of ACE2
267 residues (E35, E37, D38), the increase of viral infectivity by the L452R substitution
268 can be attributed to the increase in the electrostatic interaction with ACE2.

269

270 **Promotion of SARS-CoV-2 replication in cell cultures by the L452 mutation**

271 To investigate the effect of the mutations in the RBM on viral replication, we artificially
272 generated the recombinant SARS-CoV-2 viruses that harbor the mutations of
273 interest as well as parental recombinant virus by a reverse genetics system ([Torii et](#)
274 [al., 2021](#)). The nucleotide similarity of SARS-CoV-2 strain WK-521 (GISAID ID:
275 EPI_ISL_408667) ([Matsuyama et al., 2020](#)), the backbone of the artificially
276 generated recombinant SARS-CoV-2, to strain Wuhan-Hu-1 (GenBank:

277 NC_045512.2) (Wu et al., 2020) is 99.91% (27 nucleotides difference) and the
278 sequences encoding the S protein between these two strains are identical, indicating
279 that the strain WK-521 is a SARS-CoV-2 prototype. We verified the insertions of the
280 targeted mutations in the generated viruses by direct sequencing (**Figure 3A**) and
281 performed virus replication assay using these recombinant viruses. As shown in
282 **Figure 3B**, we revealed that the growth of the L452R mutant in VeroE6/TMPRSS2
283 cells was significantly higher than that of parental virus. Together with the findings in
284 the binding assay (**Figures 2B-2D**) and the assay using pseudoviruses (**Figure 2E**),
285 our results suggest that the L452R mutation potentially increase viral replication.

286

287 **Dynamics of the spread of the RBM mutants during the current pandemic**

288 We finally assessed the epidemic dynamics of the naturally occurring variants
289 containing the substitutions in L452 and Y453. As shown in **Figure 4A and Table**
290 **S3**, the L452R mutants were mainly found (3,967 sequences) in the B.1.427/B.1.429
291 lineage that forms a single clade (Deng et al., 2021). Although the L452R mutant
292 was first detected the B.1.39 lineage in Denmark on March 17, 2020 (GISAID ID:
293 EPI_ISL_429311) (**Table 1**), this variant did not spread. The oldest sequence that
294 contains the L452R mutation in the B.1.427/B.1.429 lineage was isolated in Quintana
295 Roo state, Mexico, on July 6, 2020 (GISAID ID: EPI_ISL_942929) (**Table 1**), and the
296 L452R-harboring mutants have been first detected in California state, the USA, on
297 September 28, 2020 (GISAID ID: EPI_ISL_730092 and EPI_ISL_730345) (**Figure**
298 **4B**). The B.1.427/B.1.429 lineage harboring the L452R mutation has started
299 expanding in California state, the USA, at the beginning of November, 2020 (**Figure**
300 **4B, top**). In 2021, this lineage has expanded throughout the USA, and currently, is
301 one of the most predominant lineages in the country (**Figure 4B, bottom and Table**
302 **S4**).

303 For the Y453F mutation, 1,274 out of the 1,380 mutated sequences belong
304 to the B.1.1.298 lineage, which has been exclusively detected in Denmark (**Table**
305 **S3**). The oldest sequence that contains the Y453F mutation in the B.1.1.298 lineage
306 was isolated from a human in Denmark on April 20, 2020 (GISAID ID:
307 EPI_ISL_714253) (**Figure 4C**). Intriguingly, the B.1.1.298 variants containing either
308 Y453 or F453 are detected not only in humans but also in minks (**Figure 4D**).

309 The phylogenetic analysis of the whole genome sequences of the B.1.1.298 lineage
310 SARS-CoV-2 suggested multiple SARS-CoV-2 transmissions between humans to
311 minks (**Figure S2**). Additionally, the three sequences that contain the Y453F
312 mutation were isolated from cats in Denmark: the two sequences out of them
313 (GISAID ID: EPI_ISL_683164 and EPI_ISL_683166) made a single clade, while the
314 other one (GISAID ID: EPI_ISL_683165) had a distinct origin (**Figure S2**). These

315 results suggest that this SARS-CoV-2 variant has transmitted from humans to cats
316 multiple times and some of them may spread among Danish cat population. However,
317 the epidemic of a fraction of the B.1.1.298 lineage containing the Y453F mutation in
318 Denmark peaked during October to November, 2020, and subsequently, gradually
319 reduced (**Figure 4D**). The variant containing the Y453F mutation was last collected
320 in Denmark on January 18, 2021 (GISAID ID: EPI_ISL_925998) and it has not been
321 reported worldwide since then (**Figure 4D**).

322 **Discussion**

323 In the present study, we demonstrated that at least two naturally occurring mutations
324 in the SARS-CoV-2 RBM, L452R and Y453F, escape from an HLA-restricted cellular
325 immunity and further reinforce the affinity to viral receptor ACE2. We further
326 demonstrate that the L452R mutation increase the stability of S protein, viral
327 infectivity and thereby enhances viral replication. Our data suggests that the L452R
328 mutant escapes from the HLA-A24-restricted cellular immunity and further
329 strengthens its infectivity.

330 Lines of recent studies have shown the emergence of the SARS-CoV-2
331 variants that evade the anti-SARS-CoV-2 neutralizing humoral immunity (Baum et
332 al., 2020; Chen et al., 2021; Garcia-Beltran et al., 2021; Hoffmann et al., 2021a; Liu
333 et al., 2021b; Liu et al., 2021c; McCarthy et al., 2021; Wang et al., 2021; Weisblum
334 et al., 2020) and have alerted the risk of the spread of immune escape variants. In
335 addition to humoral immunity, Zuo et al. have recently reported that functional SARS-
336 CoV-2-specific cellular immune responses are retained at least 6 months following
337 infection (Zuo et al., 2021). Le Bert et al. have also shown that functional cellular
338 immune responses can contribute to controlling the disease progression of COVID-
339 19 (Le Bert et al., 2021). These observations suggest the importance of cellular
340 immunity eliciting efficient antiviral effects. However, the possibility of the emergence
341 of SARS-CoV-2 variants that can evade cellular immunity has not been addressed
342 yet. Here we demonstrated the L452R and Y453F mutations can contribute to
343 escaping from an HLA-restricted cellular immunity. In addition to our findings, recent
344 papers have documented that the L452R (Deng et al., 2021; Li et al., 2020) and
345 Y453F substitutions (Baum et al., 2020; Hoffmann et al., 2021b) are potentially
346 resistant to neutralization antibodies, suggesting that these mutants can evade both
347 humoral and an HLA-restricted cellular immunity. Furthermore, we demonstrated
348 that the L452R mutation significantly improve the viral replication capacity by
349 increasing the binding affinity to human ACE2 and protein stability. Altogether, our
350 findings suggest that the emergence of the variants that can escape from the HLA-
351 restricted cellular immunity and further enhance viral replication capacity is another
352 potential risk for deteriorating the COVID-19 pandemic situation.

353 As suggested in previous reports (Koopmans, 2021; Oude Munnink et al.,
354 2021; WHO, 2020b), our data showed that the B.1.1.298 variant possessing the
355 Y453F substitution is closely associated with the outbreak in minks in Denmark.
356 Although it remains unclear whether the emergence of the Y453F mutant potentially
357 associates with the evasion from the acquired immunity in mink, here we showed
358 that this mutation can be resistant to the HLA-A24-restricted human cellular immunity.
359 Because the Y453F mutation did not increase the infection efficacy using mink ACE2,

360 our results suggest that the emergence of this mutant is not due to improving viral
361 fitness to mink. Nevertheless, the host range of SARS-CoV-2, in terms of the use of
362 ACE2 molecule for infection receptor, is broad in a variety of mammals (Liu et al.,
363 2021a; OIE, 2021). More importantly, although murine ACE2 cannot be used for the
364 infection of prototype SARS-CoV-2, recent studies have revealed that some SARS-
365 CoV-2 variants including the B.1.351 and P.1 variants gained the ability to use
366 murine ACE2 for infection and expanded their host range to mice (Li et al., 2021;
367 Montagutelli et al., 2021). In addition to the evasion from human acquired immunity,
368 zoonotic and zoonoanthroposis SARS-CoV-2 transmissions can contribute to the
369 accumulation of mutations in the spreading viruses and further impact viral
370 phenotypes including infectivity, replication efficacy, pathogenicity, transmissibility
371 and even host range. Therefore, the surveillance on the emergence of novel variants
372 even in nonhuman mammals and assessing their potentials to adapt to use
373 nonhuman ACE2 for infection receptor will be critical.

374 In contrast to the B.1.1.298 variant, the B.1.427/429 variant that harbors
375 L452R substitution seem to emerge during the spread in human population,
376 particularly in the California state in the USA, one of the hot spots of the SARS-CoV-
377 2 outbreak in the USA [<https://coronavirus.jhu.edu> (as of April 2, 2021)]. Because
378 the L452R mutation reinforces the binding affinity to human ACE2 and further
379 enhances viral replication capacity, this variant might have emerged to improve viral
380 fitness in humans. Another possibility is that the L452R mutant has emerged to
381 evade the HLA-A24-restricted cellular immunity: HLA-A24 is relatively predominant
382 in East Asian individuals (Gonzalez-Galarza et al., 2020), and the proportion of Asian
383 American in California is highest in the USA (CDC, 2019). For instance, the
384 proportion of the HLA-A24-positive individuals is ~20% in San Diego (Moore et al.,
385 2018), a city of California, where more than 270,000 SARS-CoV-2 infection cases
386 reported so far [<https://coronavirus.jhu.edu> (as of April 2, 2021)]. Therefore, it might
387 be conceivable to assume that the emergence of the L452R mutant (or the
388 B.1.427/429 lineage) was driven by the HLA-A24-mediated cellular immunity.

389 In addition to the escape from antiviral acquired immunity, recent studies
390 have shown that the emerging variants during the current pandemic, particularly the
391 B.1.1.7 variant, can even increase viral pathogenicity and the mortality of COVID-19
392 (Challen et al., 2021; Davies et al., 2021; Grint et al., 2021). Importantly, the HLA-
393 A24 individuals are relatively highly frequent in East and Southeast Asian countries,
394 such as Japan (Japanese, allele frequency=0.364, n=1,550) and Malaysia
395 (Malaysian, allele frequency=0.361, n=1,974) (Gonzalez-Galarza et al., 2020)
396 (Table S1), where both the confirmed cases and the mortality of COVID-19 are
397 relatively lower than European countries and the USA so far

398 [<https://coronavirus.jhu.edu> (as of April 2, 2021)]. A limitation of this study is that the
399 effects of the L452 and Y453 substitutions on viral pathogenicity, mortality and
400 transmissibility remain unaddressed. To fully characterize the virological features of
401 these mutants, further investigations using animal models and epidemiological data
402 will be required. Nevertheless, here we showed direct evidence suggesting that the
403 mutations in the RBM including L452R (in the B.1.427/429 lineage) and Y453F (in
404 the B1.1.298 lineage) potentially escape from the HLA-A24-restricted cellular
405 immunity, and further, the L452R mutant increase its replication capacity. Therefore,
406 these variants, particularly those possessing the L452R mutations, such as the
407 B.1.427/429 lineage, can be the potential threat for these countries and regions with
408 predominant HLA-A24 individuals, and deep surveillance and tracing the epidemic
409 of these variants will be urgently required.

410 **STAR★METHODS**

411 ● KEY RESOURCES TABLE

412 ● RESOURCE AVAILABILITY

413 ○ Lead Contact

414 ○ Materials Availability

415 ○ Data and Code Availability

416 ● EXPERIMENTAL MODEL AND SUBJECT DETAILS

417 ○ Ethics Statement

418 ○ Cell Culture

419 ● METHOD DETAILS

420 ○ Viral Genomes and Phylogenetic Analyses

421 ○ Activation Induced Marker Assay

422 ○ Analysis of Multifunctionality and Cytotoxic Potential of CD8⁺ T cells

423 ○ Plasmid Construction

424 ○ Preparation of Soluble Human ACE2

425 ○ Preparation of the Yeast-Based SARS-CoV-2 RBD Expression System

426 ○ Analysis of the Binding Affinity of the SARS-CoV-2 S RBD Variants to

427 Human ACE2 by Yeast Surface Display

428 ○ Pseudovirus Assay

429 ○ Lentiviral Transduction

430 ○ Protein Structure

431 ○ SARS-CoV-2 Reverse Genetics

432 ○ Plaque Assay

433 ○ SARS-CoV-2 Infection

434 ○ Real-time RT-PCR

435 ● QUANTIFICATION AND STATISTICAL ANALYSIS

436

437 **Supplemental Information**

438 Supplemental Information includes 2 figures and 6 tables and can be found with this

439 article online at <http://...>

440 **Author Contributions**

441 C.M., M.T., J.Z., T.I., A.S., T.S.T., I.N., H.N., I.K., K.U., and K.S. performed the
442 experiments.

443 S.T., T.F., G.S., and Y.M. prepared experimental materials.

444 J.Z. and Y.K. performed structural analysis.

445 S.N. performed molecular phylogenetic analysis.

446 A.Y., N.Shimoto, Y.N., R.M., T.T., and N.Sekiya performed clinical analysis and
447 collected clinical samples.

448 C.M., M.T., J.Z., T.I., A.S., S.N., T.U. and K.S. designed the experiments and
449 interpreted the results.

450 K.S. wrote the original manuscript.

451 C.M., J.Z., T.I., A.S., S.N, and T.U. modified the manuscript.

452 All authors reviewed and proofread the manuscript.

453 The Genotype to Phenotype Japan (G2P-Japan) consortium contributed to the
454 project administration.

455

456 **Consortia**

457 The Genotype to Phenotype Japan (G2P-Japan) consortium: Mai Fujimi, Hirotake
458 Furihata, Haruyo Hasebe, Kazuko Kitazato, Naoko Misawa, Mikari Motomura, Akiko
459 Oide, Sachiko Sakata, Ryo Shimizu, Mai Suganami, Miyoko Takahashi, Jiaqi Wu,
460 Miyabishara Yokoyama, and Yuan Yue

461

462 **Acknowledgments**

463 We would like to thank all members belonging to The Genotype to Phenotype Japan
464 (G2P-Japan) consortium. We thank Drs. Sho Fujiwara, Kazuaki Fukushima, Masaru
465 Tanaka and Akifumi Imamura (Tokyo Metropolitan Cancer and Infectious Diseases
466 Center Komagome Hospital, Japan) for supporting the collection of COVID-19
467 convalescent samples, Dr. Mizuki Kitamatsu and Mr. Yoshiki Aritsu (Kindai
468 University, Japan) for supporting the preparation of synthetic peptides, Drs. Hiroyuki
469 Kishi and Hiroshi Hamana (University of Toyama, Japan) for helpful suggestion, Dr.
470 Kenzo Tokunaga (National Institute of Infectious Diseases, Japan) for providing pC-
471 SARS2-S, Dr. Shuetsu Fukushi (National Institute of Infectious Diseases, Japan) for
472 providing pTarget-human ACE2, and Dr. Masafumi Takiguchi (Kumamoto
473 University, Japan) for providing C1R-A2402 cells. The super-computing resource
474 was provided by Human Genome Center at The University of Tokyo and the NIG
475 supercomputer at ROIS National Institute of Genetics.

476 This study was supported in part by AMED Research Program on Emerging
477 and Re-emerging Infectious Diseases 20fk0108163 (to A.S.), 20fk0108146 (to K.S.),

478 19fk0108171 (to S.N. and K.S.), 20fk0108270 (to K.S.) and 20fk0108413 (to T.I.,
479 S.N. and K.S.); AMED Research Program on HIV/AIDS 20fk0410019 (to T.U. and
480 K.S.), 20fk0410014 (to K.S.) and 21fk0410039 (to K.S.); AMED Japan Program for
481 Infectious Diseases Research and Infrastructure 20wm0325009 (to A.S.); JST J-
482 RAPID JPMJJR2007 (to K.S.); JST SICORP (e-ASIA) JPMJSC20U1 (to K.S.); JST
483 CREST JPMJCR20H6 (to S.N) and JPMJCR20H4 (to K.S); JSPS KAKENHI Grant-
484 in-Aid for Scientific Research B 18H02662 (to K.S.); JSPS KAKENHI Grant-in-Aid
485 for Scientific Research on Innovative Areas 16H06429 (to S.N. and K.S.), 16K21723
486 (to S.N. and K.S.), 17H05823 (to S.N.), 17H05813 (to K.S.), 19H04843 (to S.N.) and
487 19H04826 (to K.S.); JSPS Fund for the Promotion of Joint International Research
488 (Fostering Joint International Research) 18KK0447 (to K.S.); JSPS Core-to-Core
489 Program JPJSCCB20190009 (to T.U.); JSPS Research Fellow DC1 19J20488 (to
490 I.K.); JSPS Leading Initiative for Excellent Young Researchers (LEADER) (to T.I.);
491 ONO Medical Research Foundation (to K.S.); Ichiro Kanehara Foundation (to K.S.);
492 Lotte Foundation (to K.S.); Mochida Memorial Foundation for Medical and
493 Pharmaceutical Research (to K.S.); Daiichi Sankyo Foundation of Life Science (to
494 K.S.); Sumitomo Foundation (to K.S.); Uehara Foundation (to K.S.); Takeda Science
495 Foundation (to C.M., T.I. and K.S.); The Tokyo Biochemical Research Foundation
496 (to K.S.); Mitsubishi Foundation (to T.I.); Shin-Nihon Foundation of Advanced
497 Medical Research (to T.I.); An intramural grant from Kumamoto University COVID-
498 19 Research Projects (AMABIE) (to C.M., T.I. and T.U.); Kumamoto University
499 International Collaborative Research Grants (to T.U.);
500 Intercontinental Research and Educational Platform Aiming for Eradication of
501 HIV/AIDS (to T.I. and T.U.); and 2020 Tokai University School of Medicine Research
502 Aid (to S.N.). T.S.T and I.N. are the recipients of the doctoral course scholarship
503 from Japanese Government.

504 **References**

- 505 Anderson, B.D., Ikeda, T., Moghadasi, S.A., Martin, A.S., Brown, W.L., and Harris,
506 R.S. (2018). Natural APOBEC3C variants can elicit differential HIV-1 restriction
507 activity. *Retrovirology* 15, 78.
- 508 Banerjee, A., Mossman, K., and Baker, M.L. (2021). Zoonanthropotic potential of
509 SARS-CoV-2 and implications of reintroduction into human populations. *Cell Host*
510 *Microbe* 29, 160-164.
- 511 Baum, A., Fulton, B.O., Wloga, E., Copin, R., Pascal, K.E., Russo, V., Giordano, S.,
512 Lanza, K., Negron, N., Ni, M., *et al.* (2020). Antibody cocktail to SARS-CoV-2 spike
513 protein prevents rapid mutational escape seen with individual antibodies. *Science*
514 369, 1014-1018.
- 515 Bayarri-Olmos, R., Rosbjerg, A., Johnsen, L.B., Helgstrand, C., Bak-Thomsen, T.,
516 Garred, P., and Skjoedt, M.-O. (2021). The SARS-CoV-2 Y453F mink variant
517 displays a striking increase in ACE-2 affinity but does not challenge antibody
518 neutralization. *BioRxiv*, 428834.
- 519 Capella-Gutierrez, S., Silla-Martinez, J.M., and Gabaldon, T. (2009). trimAl: a tool
520 for automated alignment trimming in large-scale phylogenetic analyses.
521 *Bioinformatics* 25, 1972-1973.
- 522 CDC (2019). "Birth: final data for 2018".
523 https://www.cdc.gov/nchs/data/nvsr/nvsr68/nvsr68_13-508.pdf.
- 524 CDC (2020). "Emerging SARS-CoV-2 variants (updated January 28, 2021)".
525 [https://www.cdc.gov/coronavirus/2019-ncov/more/science-and-research/scientific-](https://www.cdc.gov/coronavirus/2019-ncov/more/science-and-research/scientific-brief-emerging-variants.html)
526 [brief-emerging-variants.html](https://www.cdc.gov/coronavirus/2019-ncov/more/science-and-research/scientific-brief-emerging-variants.html).
- 527 Challen, R., Brooks-Pollock, E., Read, J.M., Dyson, L., Tsaneva-Atanasova, K., and
528 Danon, L. (2021). Risk of mortality in patients infected with SARS-CoV-2 variant of
529 concern 202012/1: matched cohort study. *BMJ* 372, n579.
- 530 Chandrashekar, A., Liu, J., Martinot, A.J., McMahan, K., Mercado, N.B., Peter, L.,
531 Tostanoski, L.H., Yu, J., Maliga, Z., Nekorchuk, M., *et al.* (2020). SARS-CoV-2
532 infection protects against rechallenge in rhesus macaques. *Science* 369, 812-817.
- 533 Chen, R.E., Zhang, X., Case, J.B., Winkler, E.S., Liu, Y., VanBlargan, L.A., Liu, J.,
534 Errico, J.M., Xie, X., Suryadevara, N., *et al.* (2021). Resistance of SARS-CoV-2
535 variants to neutralization by monoclonal and serum-derived polyclonal antibodies.
536 *Nat Med*.
- 537 Collier, D.A., De Marco, A., Ferreira, I., Meng, B., Datir, R., Walls, A.C., Kemp, S.S.,
538 Bassi, J., Pinto, D., Fregni, C.S., *et al.* (2021). Sensitivity of SARS-CoV-2 B.1.1.7 to
539 mRNA vaccine-elicited antibodies. *Nature*.
- 540 Damas, J., Hughes, G.M., Keough, K.C., Painter, C.A., Persky, N.S., Corbo, M.,
541 Hiller, M., Koepfli, K.P., Pfenning, A.R., Zhao, H., *et al.* (2020). Broad host range of

542 SARS-CoV-2 predicted by comparative and structural analysis of ACE2 in
543 vertebrates. *Proc Natl Acad Sci U S A* 117, 22311-22322.

544 Darriba, D., Posada, D., Kozlov, A.M., Stamatakis, A., Morel, B., and Flouri, T. (2020).
545 ModelTest-NG: A New and Scalable Tool for the Selection of DNA and Protein
546 Evolutionary Models. *Mol Biol Evol* 37, 291-294.

547 Davies, N.G., Jarvis, C.I., Group, C.C.-W., Edmunds, W.J., Jewell, N.P., Diaz-Ordaz,
548 K., and Keogh, R.H. (2021). Increased mortality in community-tested cases of
549 SARS-CoV-2 lineage B.1.1.7. *Nature*.

550 Deng, X., Garcia-Knight, M.A., Khalid, M.M., Servellita, V., Wang, C., Morris, M.K.,
551 Sotomayor-González, A., Glasner, D.R., Reyes, K.R., Gliwa, A.S., *et al.* (2021).
552 Transmission, infectivity, and antibody neutralization of an emerging SARS-CoV-2
553 variant in California carrying a L452R spike protein mutation. *MedRxiv*, 21252647.

554 Duffy, S., Shackelton, L.A., and Holmes, E.C. (2008). Rates of evolutionary change
555 in viruses: patterns and determinants. *Nat Rev Genet* 9, 267-276.

556 Fryer, H.R., Frater, J., Duda, A., Palmer, D., Phillips, R.E., and McLean, A.R. (2012).
557 Cytotoxic T-lymphocyte escape mutations identified by HLA association favor those
558 which escape and revert rapidly. *J Virol* 86, 8568-8580.

559 Fukushi, S., Mizutani, T., Sakai, K., Saijo, M., Taguchi, F., Yokoyama, M., Kurane,
560 I., and Morikawa, S. (2007). Amino acid substitutions in the s2 region enhance
561 severe acute respiratory syndrome coronavirus infectivity in rat angiotensin-
562 converting enzyme 2-expressing cells. *J Virol* 81, 10831-10834.

563 Gao, A., Chen, Z., Amitai, A., Doelger, J., Mallajosyula, V., Sundquist, E., Segal,
564 F.P., Carrington, M., Davis, M.M., Streeck, H., *et al.* (2021). Learning from HIV-1 to
565 predict the immunogenicity of T cell epitopes in SARS-COV-2. *iScience*, 102311.

566 Garcia-Beltran, W.F., Lam, E.C., St Denis, K., Nitido, A.D., Garcia, Z.H., Hauser,
567 B.M., Feldman, J., Pavlovic, M.N., Gregory, D.J., Poznansky, M.C., *et al.* (2021).
568 Multiple SARS-CoV-2 variants escape neutralization by vaccine-induced humoral
569 immunity. *Cell*.

570 Gonzalez-Galarza, F.F., McCabe, A., Santos, E., Jones, J., Takeshita, L., Ortega-
571 Rivera, N.D., Cid-Pavon, G.M.D., Ramsbottom, K., Ghattaoraya, G., Alfievic, A., *et*
572 *al.* (2020). Allele frequency net database (AFND) 2020 update: gold-standard data
573 classification, open access genotype data and new query tools. *Nucleic Acids Res*
574 48, D783-D788.

575 Grint, D.J., Wing, K., Williamson, E., McDonald, H.I., Bhaskaran, K., Evans, D.,
576 Evans, S.J., Walker, A.J., Hickman, G., Nightingale, E., *et al.* (2021). Case fatality
577 risk of the SARS-CoV-2 variant of concern B.1.1.7 in England. *MedRxiv*, 21252528.

578 Halfmann, P.J., Hatta, M., Chiba, S., Maemura, T., Fan, S., Takeda, M., Kinoshita,
579 N., Hattori, S.I., Sakai-Tagawa, Y., Iwatsuki-Horimoto, K., *et al.* (2020). Transmission

580 of SARS-CoV-2 in Domestic Cats. *N Engl J Med* 383, 592-594.

581 Hoffmann, M., Arora, P., Groß, R., Seidel, A., Hörnich, B.F., Hahn, A.S., Krüger, N.,
582 Graichen, L., Hofmann-Winkler, H., Kempf, A., *et al.* (2021a). SARS-CoV-2 variants
583 B.1.351 and P.1 escape from neutralizing antibodies. *Cell Pre-proof*.

584 Hoffmann, M., Zhang, L., Krüger, N., Graichen, L., Kleine-Weber, H., Hofmann-
585 Winkler, H., Kempf, A., Nessler, S., Riggert, J., Winkler, M.S., *et al.* (2021b). SARS-
586 CoV-2 mutations acquired in mink reduce antibody-mediated neutralization. *BioRxiv*,
587 430998.

588 Hou, Y.J., Chiba, S., Halfmann, P., Ehre, C., Kuroda, M., Dinnon, K.H., 3rd, Leist,
589 S.R., Schafer, A., Nakajima, N., Takahashi, K., *et al.* (2020). SARS-CoV-2 D614G
590 variant exhibits efficient replication *ex vivo* and transmission *in vivo*. *Science* 370,
591 1464-1468.

592 Hu, C., Shen, M., Han, X., Chen, Q., Li, L., Chen, S., Zhang, J., Gao, F., Wang, W.,
593 Wang, Y., *et al.* (2020). Identification of cross-reactive CD8⁺ T cell receptors with
594 high functional avidity to a SARS-CoV-2 immunodominant epitope and its natural
595 mutant variants. *BioRxiv*, 364729.

596 Ikeda, T., Molan, A.M., Jarvis, M.C., Carpenter, M.A., Salamango, D.J., Brown, W.L.,
597 and Harris, R.S. (2019). HIV-1 restriction by endogenous APOBEC3G in the myeloid
598 cell line THP-1. *J Gen Virol* 100, 1140-1152.

599 Karaki, S., Kariyone, A., Kato, N., Kano, K., Iwakura, Y., and Takiguchi, M. (1993).
600 HLA-B51 transgenic mice as recipients for production of polymorphic HLA-A, B-
601 specific antibodies. *Immunogenetics* 37, 139-142.

602 Kared, H., Redd, A.D., Bloch, E.M., Bonny, T.S., Sumatoh, H., Kairi, F., Carbajo, D.,
603 Abel, B., Newell, E.W., Bettinotti, M.P., *et al.* (2021). SARS-CoV-2-specific CD8⁺ T
604 cell responses in convalescent COVID-19 individuals. *J Clin Invest* 131.

605 Katoh, K., and Standley, D.M. (2013). MAFFT multiple sequence alignment software
606 version 7: improvements in performance and usability. *Mol Biol Evol* 30, 772-780.

607 Kim, Y.I., Kim, S.G., Kim, S.M., Kim, E.H., Park, S.J., Yu, K.M., Chang, J.H., Kim,
608 E.J., Lee, S., Casel, M.A.B., *et al.* (2020). Infection and Rapid Transmission of
609 SARS-CoV-2 in Ferrets. *Cell Host Microbe* 27, 704-709 e702.

610 Kiyotani, K., Toyoshima, Y., Nemoto, K., and Nakamura, Y. (2020). Bioinformatic
611 prediction of potential T cell epitopes for SARS-Cov-2. *J Hum Genet* 65, 569-575.

612 Koopmans, M. (2021). SARS-CoV-2 and the human-animal interface: outbreaks on
613 mink farms. *Lancet Infect Dis* 21, 18-19.

614 Korber, B., Fischer, W.M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W.,
615 Hengartner, N., Giorgi, E.E., Bhattacharya, T., Foley, B., *et al.* (2020). Tracking
616 Changes in SARS-CoV-2 Spike: Evidence that D614G Increases Infectivity of the
617 COVID-19 Virus. *Cell* 182, 812-827.

- 618 Kozlov, A.M., Darriba, D., Flouri, T., Morel, B., and Stamatakis, A. (2019). RAXML-
619 NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic
620 inference. *Bioinformatics* 35, 4453-4455.
- 621 La Gruta, N.L., Gras, S., Daley, S.R., Thomas, P.G., and Rossjohn, J. (2018).
622 Understanding the drivers of MHC restriction of T cell receptors. *Nat Rev Immunol*
623 18, 467-478.
- 624 Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., Zhang, Q., Shi, X., Wang, Q.,
625 Zhang, L., *et al.* (2020). Structure of the SARS-CoV-2 spike receptor-binding domain
626 bound to the ACE2 receptor. *Nature* 581, 215-220.
- 627 Le Bert, N., Clapham, H.E., Tan, A.T., Chia, W.N., Tham, C.Y.L., Lim, J.M.,
628 Kunasegaran, K., Tan, L.W.L., Dutertre, C.A., Shankar, N., *et al.* (2021). Highly
629 functional virus-specific cellular immune response in asymptomatic SARS-CoV-2
630 infection. *J Exp Med* 218.
- 631 Leslie, A.J., Pfafferott, K.J., Chetty, P., Draenert, R., Addo, M.M., Feeney, M., Tang,
632 Y., Holmes, E.C., Allen, T., Prado, J.G., *et al.* (2004). HIV evolution: CTL escape
633 mutation and reversion after transmission. *Nat Med* 10, 282-289.
- 634 Li, Q., Nie, J., Wu, J., Zhang, L., Ding, R., Wang, H., Zhang, Y., Li, T., Liu, S., Zhang,
635 M., *et al.* (2021). SARS-CoV-2 501Y.V2 variants lack higher infectivity but do have
636 immune escape. *Cell* 184, 1-10 (pre-proof).
- 637 Li, Q., Wu, J., Nie, J., Zhang, L., Hao, H., Liu, S., Zhao, C., Zhang, Q., Liu, H., Nie,
638 L., *et al.* (2020). The impact of mutations in SARS-CoV-2 spike on viral infectivity
639 and antigenicity. *Cell* 182, 1284-1294 e1289.
- 640 Liu, Y., Hu, G., Wang, Y., Ren, W., Zhao, X., Ji, F., Zhu, Y., Feng, F., Gong, M., Ju,
641 X., *et al.* (2021a). Functional and genetic analysis of viral receptor ACE2 orthologs
642 reveals a broad potential host range of SARS-CoV-2. *Proc Natl Acad Sci U S A* 118.
- 643 Liu, Y., Liu, J., Xia, H., Zhang, X., Fontes-Garfias, C.R., Swanson, K.A., Cai, H.,
644 Sarkar, R., Chen, W., Cutler, M., *et al.* (2021b). Neutralizing Activity of BNT162b2-
645 Elicited Serum. *N Engl J Med*.
- 646 Liu, Z., VanBlargan, L.A., Bloyet, L.M., Rothlauf, P.W., Chen, R.E., Stumpf, S., Zhao,
647 H., Errico, J.M., Theel, E.S., Liebeskind, M.J., *et al.* (2021c). Identification of SARS-
648 CoV-2 spike mutations that attenuate monoclonal and serum antibody neutralization.
649 *Cell Host Microbe* 29, 477-488 e474.
- 650 Martinez-Hernandez, F., Isaak-Delgado, A.B., Alfonso-Toledo, J.A., Munoz-Garcia,
651 C.I., Villalobos, G., Arechiga-Ceballos, N., and Rendon-Franco, E. (2020).
652 Assessing the SARS-CoV-2 threat to wildlife: Potential risk to a broad range of
653 mammals. *Perspect Ecol Conserv* 18, 223-234.
- 654 Matsuyama, S., Nao, N., Shirato, K., Kawase, M., Saito, S., Takayama, I., Nagata,
655 N., Sekizuka, T., Katoh, H., Kato, F., *et al.* (2020). Enhanced isolation of SARS-CoV-

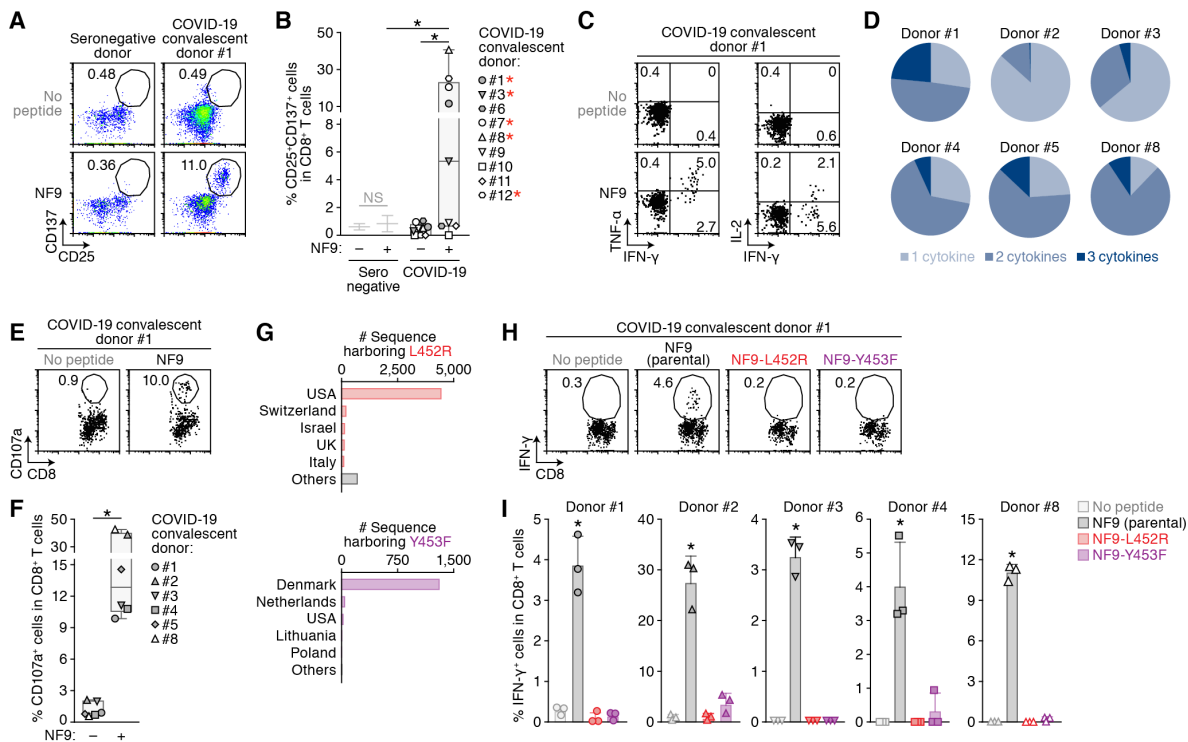
656 2 by TMPRSS2-expressing cells. *Proc Natl Acad Sci U S A* 117, 7001-7003.
657 McCarthy, K.R., Rennick, L.J., Nambulli, S., Robinson-McCarthy, L.R., Bain, W.G.,
658 Haidar, G., and Duprex, W.P. (2021). Recurrent deletions in the SARS-CoV-2 spike
659 glycoprotein drive antibody escape. *Science* 371, 1139-1142.
660 Montagutelli, X., Prot, M., Levillayer, L., Salazar, E.B., Jouvion, G., Conquet, L.,
661 Donati, F., Albert, M., Gambaro, F., Behillil, S., *et al.* (2021). The B1.351 and P.1
662 variants extend SARS-CoV-2 host range to mice. *BioRxiv*, 436013.
663 Moore, E., Grifoni, A., Weiskopf, D., Schulten, V., Arlehamn, C.S.L., Angelo, M.,
664 Pham, J., Leary, S., Sidney, J., Broide, D., *et al.* (2018). Sequence-based HLA-A, B,
665 C, DP, DQ, and DR typing of 496 adults from San Diego, California, USA. *Hum*
666 *Immunol* 79, 821-822.
667 Munster, V.J., Feldmann, F., Williamson, B.N., van Doremalen, N., Perez-Perez, L.,
668 Schulz, J., Meade-White, K., Okumura, A., Callison, J., Brumbaugh, B., *et al.* (2020).
669 Respiratory disease in rhesus macaques inoculated with SARS-CoV-2. *Nature* 585,
670 268-272.
671 Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M.,
672 and Trono, D. (1996). In vivo gene delivery and stable transduction of nondividing
673 cells by a lentiviral vector. *Science* 272, 263-267.
674 Nelde, A., Bilich, T., Heitmann, J.S., Maringer, Y., Salih, H.R., Roerden, M., Lubke,
675 M., Bauer, J., Rieth, J., Wacker, M., *et al.* (2021). SARS-CoV-2-derived peptides
676 define heterologous and COVID-19-induced T cell recognition. *Nat Immunol* 22, 74-
677 85.
678 Niwa, H., Yamamura, K., and Miyazaki, J. (1991). Efficient selection for high-
679 expression transfectants with a novel eukaryotic vector. *Gene* 108, 193-199.
680 OIE (2021). "Infection with SARS-CoV-2 in animals (January 2021)".
681 https://www.oie.int/fileadmin/Home/MM/EN_Factsheet_SARS-CoV-2.pdf.
682 Oude Munnink, B.B., Sikkema, R.S., Nieuwenhuijse, D.F., Molenaar, R.J., Munger,
683 E., Molenkamp, R., van der Spek, A., Tolsma, P., Rietveld, A., Brouwer, M., *et al.*
684 (2021). Transmission of SARS-CoV-2 on mink farms between humans and mink and
685 back to humans. *Science* 371, 172-177.
686 Ozono, S., Zhang, Y., Ode, H., Sano, K., Tan, T.S., Imai, K., Miyoshi, K., Kishigami,
687 S., Ueno, T., Iwatani, Y., *et al.* (2021). SARS-CoV-2 D614G spike mutation increases
688 entry efficiency with enhanced ACE2-binding affinity. *Nat Commun* 12, 848.
689 Ozono, S., Zhang, Y., Tobiume, M., Kishigami, S., and Tokunaga, K. (2020). Super-
690 rapid quantitation of the production of HIV-1 harboring a luminescent peptide tag. *J*
691 *Biol Chem* 295, 13023-13030.
692 Parrish, C.R., Holmes, E.C., Morens, D.M., Park, E.C., Burke, D.S., Calisher, C.H.,
693 Laughlin, C.A., Saif, L.J., and Daszak, P. (2008). Cross-species virus transmission

694 and the emergence of new epidemic diseases. *Microbiol Mol Biol Rev* 72, 457-470.
695 Peleg, Y., and Unger, T. (2014). Application of the Restriction-Free (RF) cloning for
696 multicomponents assembly. *Methods Mol Biol* 1116, 73-87.
697 Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng,
698 E.C., and Ferrin, T.E. (2004). UCSF Chimera—A visualization system for exploratory
699 research and analysis. *J Comp Chem* 25, 1605-1612.
700 Plante, J.A., Liu, Y., Liu, J., Xia, H., Johnson, B.A., Lokugamage, K.G., Zhang, X.,
701 Muruato, A.E., Zou, J., Fontes-Garfias, C.R., *et al.* (2020). Spike mutation D614G
702 alters SARS-CoV-2 fitness. *Nature*.
703 Plante, J.A., Mitchell, B.M., Plante, K.S., Debbink, K., Weaver, S.C., and Menachery,
704 V.D. (2021). The Variant Gambit: COVID's Next Move. *Cell Pre-proof*.
705 Rambaut, A., Holmes, E.C., O'Toole, A., Hill, V., McCrone, J.T., Ruis, C., du Plessis,
706 L., and Pybus, O.G. (2020). A dynamic nomenclature proposal for SARS-CoV-2
707 lineages to assist genomic epidemiology. *Nat Microbiol* 5, 1403-1407.
708 Rausch, T., Fritz, M.H., Untergasser, A., and Benes, V. (2020). Tracy: basecalling,
709 alignment, assembly and deconvolution of sanger chromatogram trace files. *BMC*
710 *Genomics* 21, 230.
711 Schulien, I., Kemming, J., Oberhardt, V., Wild, K., Seidel, L.M., Killmer, S., Sagar,
712 Daul, F., Salvat Lago, M., Decker, A., *et al.* (2021). Characterization of pre-existing
713 and induced SARS-CoV-2-specific CD8(+) T cells. *Nat Med* 27, 78-85.
714 Selzer, T., Albeck, S., and Schreiber, G. (2000). Rational design of faster associating
715 and tighter binding protein complexes. *Nat Struct Biol* 7, 537-541.
716 Shema Mugisha, C., Vuong, H.R., Puray-Chavez, M., Bailey, A.L., Fox, J.M., Chen,
717 R.E., Wessel, A.W., Scott, J.M., Harastani, H.H., Boon, A.C.M., *et al.* (2020). A
718 Simplified Quantitative Real-Time PCR Assay for Monitoring SARS-CoV-2 Growth
719 in Cell Culture. *mSphere* 5.
720 Shen, X., Tang, H., McDanal, C., Wagh, K., Fischer, W., Theiler, J., Yoon, H., Li, D.,
721 Haynes, B.F., Sanders, K.O., *et al.* (2021). SARS-CoV-2 variant B.1.1.7 is
722 susceptible to neutralizing antibodies elicited by ancestral spike vaccines. *Cell Host*
723 *Microbe*.
724 Shi, J., Wen, Z., Zhong, G., Yang, H., Wang, C., Huang, B., Liu, R., He, X., Shuai,
725 L., Sun, Z., *et al.* (2020). Susceptibility of ferrets, cats, dogs, and other domesticated
726 animals to SARS-coronavirus 2. *Science* 368, 1016-1020.
727 Supasa, P., Zhou, D., Dejnirattisai, W., Liu, C., Mentzer, A.J., Ginn, H.M., Zhao, Y.,
728 Duyvesteyn, H.M.E., Nutalai, R., Tuekprakhon, A., *et al.* (2021). Reduced
729 neutralization of SARS-CoV-2 B.1.1.7 variant by convalescent and vaccine sera. *Cell*
730 184, 1-11 (pre-proof).
731 Torii, S., Ono, C., Suzuki, R., Morioka, Y., Anzai, I., Fauzyah, Y., Maeda, Y., Kamitani,

- 732 W., Fukuhara, T., and Matsuura, Y. (2021). Establishment of a reverse genetics
733 system for SARS-CoV-2 using circular polymerase extension reaction. *Cell Rep Pre-*
734 *proof*.
- 735 Traxlmayr, M.W., and Obinger, C. (2012). Directed evolution of proteins for
736 increased stability and expression using yeast display. *Arch Biochem Biophys* 526,
737 174-180.
- 738 Volz, E., Hill, V., McCrone, J.T., Price, A., Jorgensen, D., O'Toole, A., Southgate, J.,
739 Johnson, R., Jackson, B., Nascimento, F.F., *et al.* (2021). Evaluating the Effects of
740 SARS-CoV-2 Spike Mutation D614G on Transmissibility and Pathogenicity. *Cell* 184,
741 64-75 e11.
- 742 Wang, P., Nair, M.S., Liu, L., Iketani, S., Luo, Y., Guo, Y., Wang, M., Yu, J., Zhang,
743 B., Kwong, P.D., *et al.* (2021). Antibody Resistance of SARS-CoV-2 Variants B.1.351
744 and B.1.1.7. *Nature*.
- 745 Wang, Q., Zhang, Y., Wu, L., Niu, S., Song, C., Zhang, Z., Lu, G., Qiao, C., Hu, Y.,
746 Yuen, K.Y., *et al.* (2020). Structural and Functional Basis of SARS-CoV-2 Entry by
747 Using Human ACE2. *Cell* 181, 894-904 e899.
- 748 Weisblum, Y., Schmidt, F., Zhang, F., DaSilva, J., Poston, D., Lorenzi, J.C.,
749 Muecksch, F., Rutkowska, M., Hoffmann, H.H., Michailidis, E., *et al.* (2020). Escape
750 from neutralizing antibodies by SARS-CoV-2 spike protein variants. *Elife* 9.
- 751 WHO (2020a). "Coronavirus disease 2019".
752 <https://www.who.int/emergencies/diseases/novel-coronavirus-2019>.
- 753 WHO (2020b). "SARS-CoV-2 mink-associated variant strain – Denmark (November
754 6, 2020)". [https://www.who.int/csr/don/06-november-2020-mink-associated-sars-](https://www.who.int/csr/don/06-november-2020-mink-associated-sars-cov2-denmark/en/)
755 [cov2-denmark/en/](https://www.who.int/csr/don/06-november-2020-mink-associated-sars-cov2-denmark/en/).
- 756 Wilson, E.A., Hirneise, G., Singharoy, A., and Anderson, K.S. (2021). Total predicted
757 MHC-I epitope load is inversely associated with population mortality from SARS-
758 CoV-2. *Cell Rep Med* 2, 100221.
- 759 Wolf, M., Kuball, J., Ho, W.Y., Nguyen, H., Manley, T.J., Bleakley, M., and
760 Greenberg, P.D. (2007). Activation-induced expression of CD137 permits detection,
761 isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen
762 without requiring knowledge of epitope specificities. *Blood* 110, 201-210.
- 763 Wu, F., Zhao, S., Yu, B., Chen, Y.M., Wang, W., Song, Z.G., Hu, Y., Tao, Z.W., Tian,
764 J.H., Pei, Y.Y., *et al.* (2020). A new coronavirus associated with human respiratory
765 disease in China. *Nature* 579, 265-269.
- 766 Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y., and Zhou, Q. (2020). Structural basis for
767 the recognition of SARS-CoV-2 by full-length human ACE2. *Science* 367, 1444-1448.
- 768 Yu, J., Tostanoski, L.H., Peter, L., Mercado, N.B., McMahan, K., Mahrokhian, S.H.,
769 Nkolola, J.P., Liu, J., Li, Z., Chandrashekar, A., *et al.* (2020). DNA vaccine protection

770 against SARS-CoV-2 in rhesus macaques. *Science* 369, 806-811.
771 Yurkovetskiy, L., Wang, X., Pascal, K.E., Tomkins-Tinch, C., Nyalile, T.P., Wang, Y.,
772 Baum, A., Diehl, W.E., Dauphin, A., Carbone, C., *et al.* (2020). Structural and
773 Functional Analysis of the D614G SARS-CoV-2 Spike Protein Variant. *Cell* 183, 739-
774 751 e738.
775 Zahradník, J., Dey, D., Marciano, S., and Schreiber, G. (2021a). An enhanced yeast
776 display platform demonstrates the binding plasticity under various selection
777 pressures. *BioRxiv*, 423176.
778 Zahradník, J., Marciano, S., Shemesh, M., Zoler, E., Chiaravalli, J., Meyer, B.,
779 Rudich, Y., Dym, O., Elad, N., and Schreiber, G. (2021b). SARS-CoV-2 RBD in vitro
780 evolution follows contagious mutation spread
781 yet generates an able infection inhibitor. *BioRxiv*, 425392.
782 Zhang, W., Davis, B.D., Chen, S.S., Sincuir Martinez, J.M., Plummer, J.T., and Vail,
783 E. (2021). Emergence of a novel SARS-CoV-2 variant in Southern California. *JAMA*,
784 e211612.
785 Zhao, X., Chen, D., Szabla, R., Zheng, M., Li, G., Du, P., Zheng, S., Li, X., Song, C.,
786 Li, R., *et al.* (2020). Broad and Differential Animal Angiotensin-Converting Enzyme
787 2 Receptor Usage by SARS-CoV-2. *J Virol* 94.
788 Zhou, B., Thi Nhu Thao, T., Hoffmann, D., Taddeo, A., Ebert, N., Labroussaa, F.,
789 Pohlmann, A., King, J., Steiner, S., Kelly, J.N., *et al.* (2021). SARS-CoV-2 spike
790 D614G change enhances replication and transmission. *Nature*.
791 Zhou, P., Yang, X.L., Wang, X.G., Hu, B., Zhang, L., Zhang, W., Si, H.R., Zhu, Y.,
792 Li, B., Huang, C.L., *et al.* (2020). A pneumonia outbreak associated with a new
793 coronavirus of probable bat origin. *Nature* 579, 270-273.
794 Zuo, J., Dowell, A.C., Pearce, H., Verma, K., Long, H.M., Begum, J., Aiano, F., Amin-
795 Chowdhury, Z., Hallis, B., Stapley, L., *et al.* (2021). Robust SARS-CoV-2-specific T
796 cell immunity is maintained at 6 months following primary infection. *Nat Immunol*.
797

798 **Figures & figure legends**
799



800

801 **Figure 1. Escape of the two naturally occurring SARS-CoV-2 mutations from**
802 **the S RBM-specific CD8⁺ T cells.**

803 (A and B) Detection of the HLA-A24-restricted NF9-specific CTLs. The HLA-
804 A*24:02-positive CTL lines of 3 seronegative donors and 9 COVID-19 convalescents
805 were stimulated with or without 1 μM NF9 peptide (NYNYLYRLF, residues 448-456
806 of the SARS-CoV-2 S protein). Representative FACS plots showing the surface
807 expression of CD25 and CD137 in the CD8⁺ T cell subset (i.e., CD3⁺CD8⁺ cells) of
808 a seronegative donor (left) and a COVID-19 convalescent donor #1 (right) (A) and
809 the median of the percentage of CD25⁺CD137⁺ cells in CD8⁺ T cells (B) are shown.
810 In B, the COVID-19 convalescent samples >3 SD of the median of NF9-stimulated
811 seronegative samples are indicated with red asterisks.

812 (C and D) Multifunctionality of the HLA-A24-restricted NF9-specific CTLs. The HLA-
813 A*24:02-positive CTL lines of 6 COVID-19 convalescents were stimulated with or
814 without 10 nM NF9 peptide. Representative FACS plots showing the intracellular
815 expression of IFN-γ, TNF-α and IL-2 in the CD8⁺ T cell subset of a COVID-19
816 convalescent (donor #1) (C) and the pie charts showing the proportion of cytokine
817 positive cells in each convalescent sample (D) are shown.

818 (E and F) Potential killing activity of the HLA-A24-restricted NF9-specific CTLs. The
819 HLA-A*24:02-positive CTL lines of 6 COVID-19 convalescents were stimulated with

820 the C1R-A2402 cells pulsed with or without 10 nM NF9 peptide. Representative
821 FACS plots showing the surface expression of CD107a in the CD8⁺ T cell subset of
822 a COVID-19 convalescent (donor #1) (**E**) and the median of the percentage of
823 CD107a⁺ cells in CD8⁺ T cells (**F**) are shown.

824 (**G**) Distribution of the L452R and Y453F mutants during the current pandemic. The
825 top 5 countries where the variants harboring the L452R (top) and Y453F (bottom)
826 mutations are shown. The raw data are summarized in **Table S2**.

827 (**H and I**) Mutations escaped from the HLA-A24-restricted NF9-specific CTLs. The
828 HLA-A*24:02-positive CTL lines of 5 COVID-19 convalescents were stimulated with
829 1 nM NF9 peptide or its derivatives: NF9-L452R (NYNYRYR_LF) and NF9-Y453F
830 (NYNYLFR_LF). Representative FACS plots showing the intracellular expression of
831 IFN-γ in the CD8⁺ T cell subset of a COVID-19 convalescent (donor #1) (**H**) and the
832 mean of the percentage of IFN-γ⁺ cells in CD8⁺ T cells (**I**) are shown.

833 In **A, E and H**, the numbers in the FACS plot represent the percentage of gated cells
834 in CD8⁺ T cells. In **C**, the number represents the percentage of the cells in each
835 quadrant.

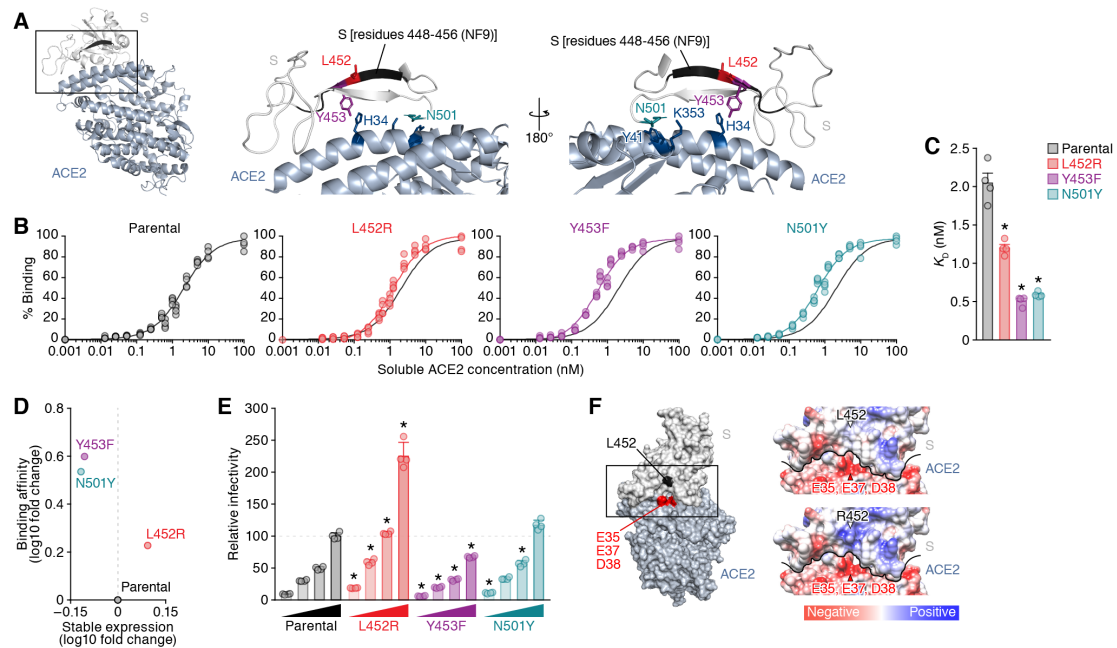
836 In **B**, a statistically significant difference (*, $P < 0.05$) between SARS-CoV-2
837 seronegative and COVID-19 convalescent samples is determined by Mann-Whitney
838 U test, and a statistically significant difference (*, $P < 0.05$) between with and without
839 NF9 peptide in COVID-19 convalescent samples is determined by Wilcoxon signed-
840 rank test.

841 In **F**, each symbol of the COVID-19 convalescent data represents the mean of
842 technical triplicate. Statistically significant differences (*, $P < 0.05$) between with and
843 without NF9 peptide in COVID-19 convalescent samples are determined by
844 Wilcoxon signed-rank test.

845 In **I**, the assay was performed in triplicate, and the means are shown with SD.
846 Statistically significant differences (*, $P < 0.05$) versus "no are determined by ANOVA
847 with multiple comparisons by Bonferroni correction.

848 See also **Figure S1** and **Tables S1 and S2**.

849



850

851 **Figure 2. Increase of the binding affinity to ACE2 and viral infectivity by the**
 852 **L452 mutation.**

853 **(A)** Location of the NF9 peptide (residues 448-456) in the cocrystal structure of the
 854 SARS-CoV-2 S and human ACE2 proteins (PDB: 6M17) (Yan et al., 2020). An
 855 overview (left), the enlarged view of the boxed area in the left panel (middle) and the
 856 view of the middle panel rotated 180° on the y-axis (right) are shown. The residues
 857 448-456 of SARS-CoV-2 S (corresponding to the NF9 peptide) are shown in black.
 858 **(B-D)** Binding affinity of SARS-CoV-2 S RBD to ACE2 by yeast surface display. The
 859 percentage of the binding of the SARS-CoV-2 S RBD expressing on yeast to soluble
 860 ACE2 **(B)** and the K_D values **(C)** are shown. **(D)** The level of stable expression of the
 861 SARS-CoV-2 RBD on yeast (x-axis) and the binding affinity to ACE2 (y-axis)
 862 compared to parental RBD. In **B**, the fitting curve of parental RBD is shown in all
 863 panels as black lines.

864 **(E)** Pseudovirus assay. The HIV-based reporter virus pseudotyped with the parental
 865 SARS-CoV-2 S or its derivatives (L452R, Y453F and N501Y) were inoculated into
 866 the 293 cells transiently expressing human ACE2 and TMPRSS2 at 4 different doses
 867 (1, 3, 5 and 10 ng p24 antigens). The percentages of the infectivity compared to the
 868 virus pseudotyped with parental S (10 ng p24 antigen) are shown.

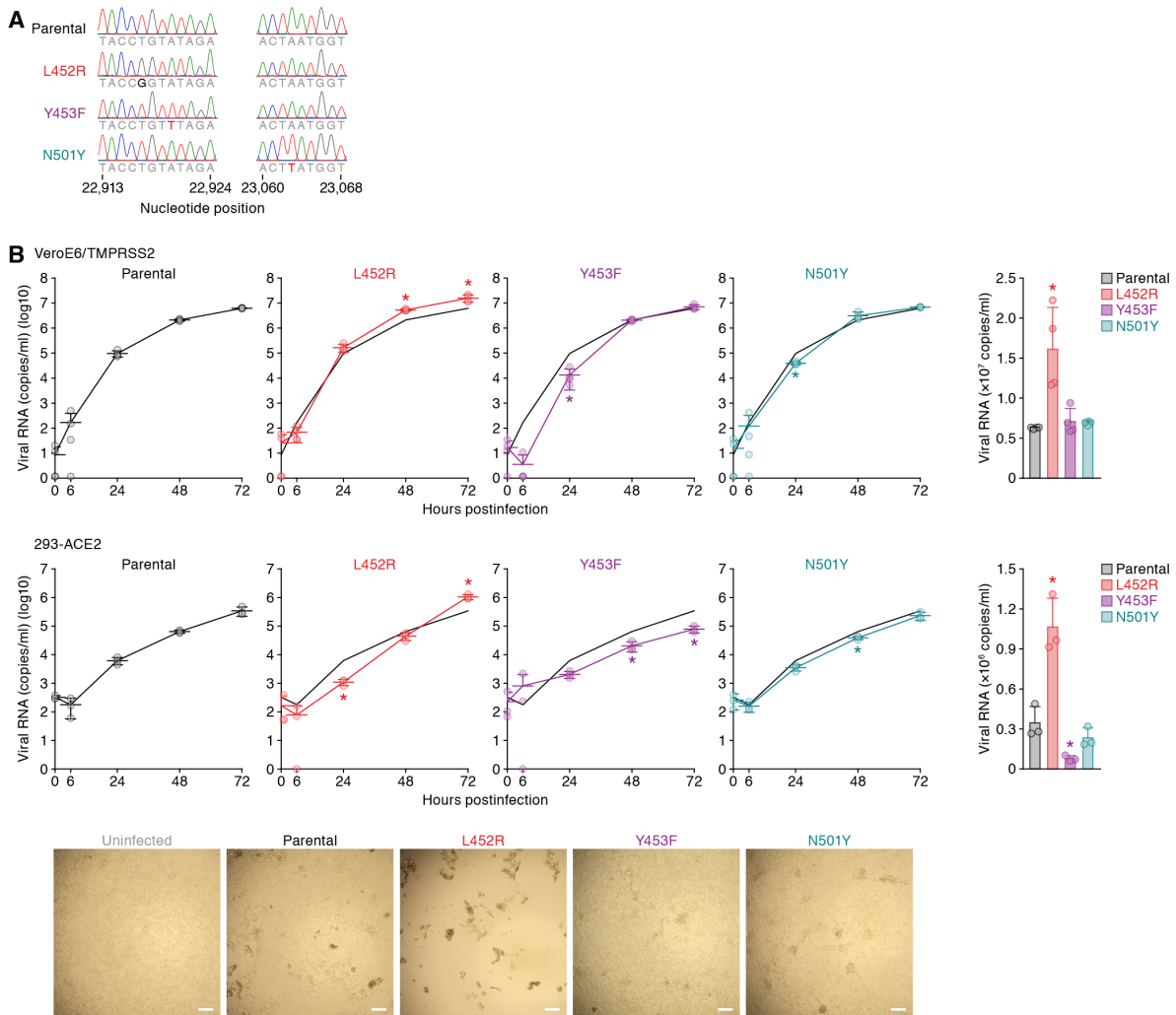
869 **(F)** Gain of electrostatic complementarity by the L452R substitution. (Left) The
 870 surface structure of the SARS-CoV-2 S and ACE2 (PDB: 6M17) (Yan et al., 2020).
 871 The residue 452 of the SARS-CoV-2 S and the negatively charged patch on ACE2
 872 (residues E35, E37 and D38) are indicated by black and red. The boxed area is

873 enlarged in the upper right panel. (Right) Coulombic surface coloring at the
874 structures of the SARS-CoV-2 S and ACE2 (PDB: 6M17) (Yan et al., 2020) (top) and
875 a model of the L452R substitution (bottom). The black line indicates the border
876 between SARS-CoV-2 S and ACE2.

877 In **B and E**, these assays were performed in quadruplicate.

878 In **C**, statistically significant differences (*, $P < 0.05$) versus parental S are determined
879 by Mann-Whitney U test.

880 In **E**, statistically significant differences (*, $P < 0.05$) versus parental S at the same
881 dose are determined by ANOVA with multiple comparisons by Bonferroni correction.
882



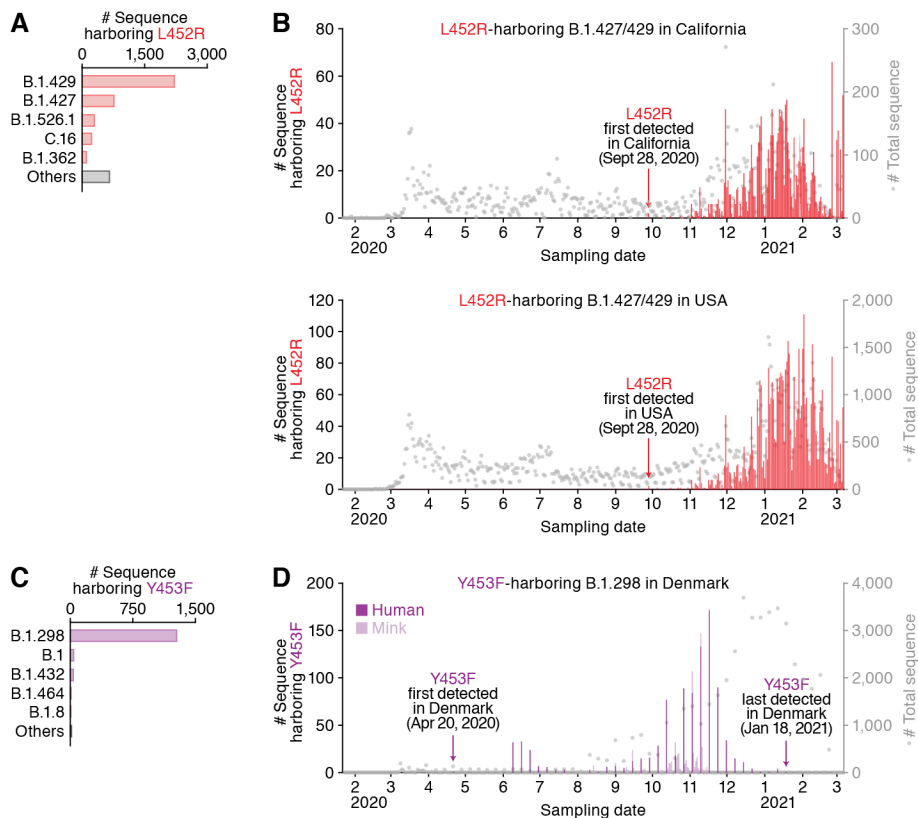
883

884 **Figure 3. Enhancement of viral replication by the L452 mutation.**

885 (A) Chromatograms of the mutated regions of the SARS-CoV-2 viruses artificially
 886 generated by reverse genetics. The chromatograms of the nucleotide positions
 887 22,913-22,924 (left) and 23,060-23,068 (right) of parental SARS-CoV-2, the L452R,
 888 Y453F and N501Y mutants are shown.

889 (B) Growth kinetics of parental SARS-CoV-2 and the SARS-CoV-2 mutants.
 890 Parental SARS-CoV-2, the L452R, Y453F and N501Y mutants (100 pfu) were
 891 inoculated into VeroE6/TMPRSS2 cells (top) and 293-ACE2 cells (middle) and the
 892 copy number of viral RNA in the culture supernatant was quantified by real-time RT-
 893 PCR. (Left) The growth curve of the viruses inoculated. The result of parental virus
 894 is shown in all panels as a black line. (Right) The amount of viral RNA in the culture
 895 supernatant at 72 h postinfection. The assays were performed in quadruplicate
 896 (VeroE6/TMPRSS2 cells) or triplicate (293-ACE2 cells), and statistically significant
 897 differences (*, $P < 0.05$) versus parental S are determined by Student's *t* test. (Bottom)

898 Representative figures of the blight fields of 293-ACE2 cells infected with the viruses
899 indicated at 72 h post infection are also shown. Bars, 200 μm .
900



901

902 **Figure 4. Epidemic dynamics of the B.1.298 and B.1.427/429 lineages during**
 903 **the current pandemic.**

904 The PANGO lineages harboring the L452R (**A and B**) and Y453F (**C and D**) and
 905 their epidemic dynamics are summarized.

906 (**A and C**) Distribution of the L452R and Y453F mutants during the current pandemic.
 907 The top 5 PANGO lineages (<https://cov-lineages.org/index.html>) that harbor the
 908 L452R (**A**) and Y453F (**C**) mutations are shown. The raw data are summarized in
 909 **Table S3.**

910 (**B and D**) Epidemic dynamics of the L452R-harboring B.1.427/429 lineage in
 911 California state, the USA (**B, top**) and the USA (**B, bottom**) and the Y453F-harboring
 912 B.1.298 lineage in Denmark (**D**). The numbers of the sequences harboring mutation
 913 per day (left y-axis, bars) and the numbers of total sequences per day (right y-axis,
 914 dots), from January 22, 2020 to March 6, 2021, are summarized. Note that a L452R
 915 variant isolated from gorilla and three Y453F variants isolated from cats are not
 916 included.

917 See also **Figure S2** and **Tables S3** and **S4**.

Table 1. Naturally occurring mutations in the residues 448-456 of the SARS-CoV-2 S protein

Mutation	# Sequence	Date, first detected	Country, first detected	Dominant PANGO lineage	Date, first detected in the dominant lineage	Country, first detected in the dominant lineage	Nonhuman hosts detected ^a
L452R	5,677	March 17, 2020	Denmark	B.1.427/429	July 6, 2020	Mexico	Gorilla (1)
Y453F	1,380	April 20, 2020	Denmark	B.1.298	April 20, 2020	Denmark	Mink (339) and cat (3)
N450K	181	March 27, 2020	UK	B.1.214.2	December 5, 2020	UK	-
L452M	140	April 13, 2020	Japan	B.1.22/36	July 2, 2020	Netherlands	Mink (32)
L452Q	111	March 6, 2020	Spain	B.1.1.374	October 23, 2020	Belarus	-

918 ^a The number in parenthesis indicates the number of sequence reported.

- 919 **Table S1.** Distribution of HLA-A24 allele in each population, related to Figure 1
920
- 921 **Table S2.** Countries where the naturally occurring mutations in the residues 448-
922 456 of the SARS-CoV-2 S protein were isolated, related to Figure 1
923
- 924 **Table S3.** The PANGO lineages harboring the L452R and Y453F mutations, related
925 to Figure 4
926
- 927 **Table S4.** The PANGO lineages dominantly expanding in the USA, related to Figure
928 4
929
- 930 **Table S5.** HLA-A*24:02-positive COVID-19 convalescent samples used in this study,
931 related to Figure 1
932
- 933 **Table S6.** The SARS-CoV-2 genomic region encoded in each template and the
934 primers used for the preparation of each fragment for CPER, related to Figure 3

935 **STAR★METHODS**

936

937 **KEY RESOURCES TABLE**

938 **RESOURCE AVAILABILITY**

939 **Lead Contact**

940 Further information and requests for resources and reagents should be directed to
941 and will be fulfilled by the Lead Contact, Kei Sato (KeiSato@g.ecc.u-tokyo.ac.jp).

942

943 **Materials Availability**

944 All unique reagents generated in this study are listed in the Key Resources Table
945 and available from the Lead Contact with a completed Materials Transfer Agreement.

946

947 **Data and Code Availability**

948 Additional Supplemental Items are available from Mendeley Data at <http://...>

949

950 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

951 **Ethics Statement**

952 All protocols involving the human subjects recruiting at Kyushu University Hospital,
953 Japan, National Hospital Organization Kyushu Medical Center, Japan, and Tokyo
954 Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Japan,
955 were reviewed and approved by the Ethics Committee for Epidemiological and
956 General Research at the Faculty of Life Science, Kumamoto University (approval
957 numbers 2066 and 461). All human subjects provided written informed consent.

958

959 **Cell Culture**

960 Human PBMCs were obtained from a total of 15 subjects harboring HLA-A*24:02
961 including 12 COVID-19 convalescents and 3 seronegatives (**Table S5**). The PBMCs
962 were purified by a density gradient centrifugation using Ficoll-Paque Plus (GE
963 Healthcare Life Sciences, cat# 17-1440-03) and stored in liquid nitrogen until further
964 use. The C1R cells expressing HLA-A*2402 (C1R-A2402) ([Karaki et al., 1993](#)) were
965 maintained in RPMI1640 medium (Thermo Fisher Scientific, cat# 11875101)
966 containing 10% fetal calf serum (FCS) and 1% antibiotics (penicillin and
967 streptomycin; PS).

968 HEK293 cells (a human embryonic kidney cell line; ATCC CRL-1573) and HEK293T
969 cells (a human embryonic kidney cell line; ATCC CRL-3216) were maintained in

970 Dulbecco's modified Eagle's medium (high glucose) (Wako, cat# 044-29765)
971 containing 10% FCS and 1% PS.
972 Vero cells [an African green monkey (*Chlorocebus sabaeus*) kidney cell line;
973 JCRB0111] were maintained in Eagle's minimum essential medium (Wako, cat#
974 051-07615) containing 10% FCS and 1% PS.
975 VeroE6/TMPRSS2 cells [an African green monkey (*Chlorocebus sabaeus*) kidney
976 cell line; JCRB1819] ([Matsuyama et al., 2020](#)) were maintained in Dulbecco's
977 modified Eagle's medium (low glucose) (Wako, cat# 041-29775) containing 10%
978 FCS, G418 (1 mg/ml; Nacalai Tesque, cat# G8168-10ML) and 1% PS.
979 HEK293-C34 cells, the *IFNAR1* KO HEK293 cells expressing human ACE2 and
980 TMPRSS2 by doxycycline treatment ([Torii et al., 2021](#)), were maintained in
981 Dulbecco's modified Eagle's medium (high glucose) (Sigma-Aldrich, cat# R8758-
982 500ML) containing 10% FCS, Blasticidin (10 µg/ml; Invivogen, cat# ant-bl-1) and 1%
983 PS.
984 Expi293F cells (Thermo Fisher Scientific, cat# A14527) were maintained in Expi293
985 expression medium (Thermo Fisher Scientific, cat# A1435101).

986

987 **METHOD DETAILS**

988 **Viral Genomes and Phylogenetic Analyses**

989 All viral genome sequences and annotation information used in this study were
990 downloaded from GISAID (<https://www.gisaid.org>) as of March 15, 2021 (750,243
991 sequences). We used the viral nucleotide sequences that do not contain any
992 undetermined nucleotides in the region coding S protein for the analysis (581,367
993 sequences). The SARS-CoV-2 variants containing the L452R or Y453F mutation
994 were sorted from the verified 581,367 sequences (**Tables S2 and S3**). To infer the
995 phylogeny of B.1.1.298 lineage (**Figure S2**), we collected the 657 sequences
996 belonging to the B.1.1.298 lineage that do not contain any undetermined nucleotides.
997 We aligned whole genome sequences by using FFT-NS-2 program in an MAFFT
998 suite v7.467 ([Katoh and Standley, 2013](#)) and removed gapped regions using trimAl
999 v1.4.rev22 with a gappyout option ([Capella-Gutierrez et al., 2009](#)). We selected
1000 GTR+I as the best-fit nucleotide substitution model using ModelTest-NG v0.1.5
1001 ([Darriba et al., 2020](#)). Using the model, we generated a maximum-likelihood based
1002 phylogenetic tree using RAXML-NG v1.0.0 ([Kozlov et al., 2019](#)) with a bootstrap test
1003 (n=100).

1004

1005 **Activation Induced Marker Assay**

1006 The expansion of antigen-specific human CD8⁺ T cells and the analysis of the
1007 surface expression levels of activation markers, CD25 and CD137, were performed

1008 as previously described (Wolfl et al., 2007). Briefly, human PBMCs were pulsed with
1009 1 µg/ml of the SARS-CoV-2 PepTivator peptide pools (“S overlap peptides”) (Miltenyi
1010 Biotec, cat# 130-126-700) and maintained in RPMI 1640 medium (Thermo Fisher
1011 Scientific, cat# 11875101) containing 10% FCS and 30 U/ml recombinant human IL-
1012 2 (Peprotec, cat# 200-02) for 10-14 days. The *in vitro* expanded CD8⁺ T cells (i.e.,
1013 the CTL lines) were stimulated with or without the NF9 peptide (NYNYLYRLF,
1014 residues 448-456 of the SARS-CoV-2 S protein; synthesized by Scrum Inc.). After
1015 the incubation at 37°C for 1 h, the cells were washed and the surface proteins (CD3,
1016 CD8, CD14, CD19, CD25 and CD137) were stained with the antibodies listed in **Key**
1017 **Resources Table**. The dead cells were stained with 7-aminoactinomycin D
1018 (Biolegend, cat# 420404). After the incubation for 20 min on ice, the cells were fixed
1019 with 1% paraformaldehyde (Nacalai Tesque, cat# 09154-85) and the levels of protein
1020 surface expression were analyzed by flow cytometry using a FACS Canto II (BD
1021 Biosciences). The data obtained by flow cytometry were analyzed by FlowJo
1022 software (Tree Star).

1023

1024 **Analysis of Multifunctionality and Cytotoxic Potential of CD8⁺ T cells**

1025 C1R-A2402 cells were pulsed with or without the NF9 peptide or its derivatives [the
1026 NF9-L452R peptide (NYNYRYRLF, L5R in NF9) and the NF9-Y453F peptide
1027 (NYNYLERLF, Y6F in NF9); synthesized by Scrum Inc.] at concentrations from 0.1
1028 to 10 nM at 37°C for 1 h. The cells were washed twice with PBS, mixed with the CTL
1029 lines generated from COVID-19 convalescents (see above) and incubated with
1030 RPMI 1640 medium (Thermo Fisher Scientific, cat# 11875101) containing 10% FCS,
1031 5 µg/ml brefeldin A (Sigma-Aldrich, cat# B7651), 2 µM monensin (Biolegend, cat#
1032 420701) and BV421-anti-CD107a antibody (Biolegend, cat# 420404) in a 96-well U
1033 plate at 37°C for 5 h. Then, the cells were washed and the surface proteins (CD3,
1034 CD8, CD14 and CD19) were stained with the antibodies listed in **Key Resources**
1035 **Table**. The dead cells were stained with 7-aminoactinomycin D (Biolegend, cat#
1036 420404). After the incubation at 37°C for 30 min, the cells were fixed and
1037 permeabilized with Cytofix/Cytoperm Fixation/Permeabilization solution kit (BD
1038 Biosciences, cat# 554714) and the intracellular proteins (IFN-γ, TNF-α and IL-2)
1039 were stained with the antibodies listed in **Key Resources Table**. After the incubation
1040 at room temperature for 30 min, the cells were washed and the levels of protein
1041 expression were analyzed by flow cytometry using a FACS Canto II (BD
1042 Biosciences). The data obtained by flow cytometry were analyzed by FlowJo
1043 software (Tree Star).

1044

1045 **Plasmid Construction**

1046 The plasmids expressing the SARS-CoV-2 S protein (pCAGGS-SARS2-S) and its
1047 mutants (pCAGGS-S-L452R, pCAGGS-SARS2-S-Y453F and pCAGGS-SARS2-S-
1048 N501Y) were generated by site-directed mutagenesis PCR using pC-SARS2-S
1049 (kindly provided by Kenzo Tokunaga) ([Ozono et al., 2021](#)) as the template and the
1050 following primers: S forward, 5'-TTG GGTACC ATG TTT GTG TTC CTG GTG CTG-
1051 3'; S reverse, 5'-GTG GCGGCCGC TCT AGA TTC AGG TGT AGT GCA GTT T-3';
1052 S Y453F forward, 5'-GTG GGA GGC AAC TAC AAC TAC CTC TTC AGA-3'; and S
1053 L452R/Y453F reverse, 5'-GTT GTA GTT GCC TCC CAC CTT-3'; S N501Y forward,
1054 5'-TCC TAT GGC TTC CAA CCA ACC TAT GGA-3'; and S N501Y reverse, 5'-TGG
1055 TTG GAA GCC ATA GGA TTG-3'. The resultant PCR fragment was digested with
1056 KpnI and NotI and inserted into the KpnI-NotI site of pCAGGS vector ([Niwa et al.,
1057 1991](#)).

1058 To construct the expression plasmid for human ACE2 (GenBank:
1059 NM_021804.3) (pLV-EF1a-human ACE2-IRES-Puro), the MluI-SmaI fragment
1060 pTarget-human ACE2 (kindly provided by Shuetsu Fukushi) ([Fukushi et al., 2007](#))
1061 was inserted into the MluI-HpaI site of pLV-EF1a-IRES-Puro (Addgene #85132).
1062 Nucleotide sequences were determined by a DNA sequencing service (Fasmac),
1063 and the sequence data were analyzed by Sequencher v5.1 software (Gene Codes
1064 Corporation).

1065

1066 **Preparation of Soluble Human ACE2**

1067 To prepare soluble human ACE2, the expression plasmid for the extracellular
1068 domain of human ACE2 (residues 18-740) based on pHL-sec (Addgene, cat# 99845)
1069 ([Zahradník et al., 2021a](#)) was transfected into Expi293F cells using ExpiFectamine
1070 293 transfection kit (Thermo Fisher Scientific, cat# A14525) according to the
1071 manufacturer's protocol. Three days posttransfection, the culture medium was
1072 harvested, centrifuged, and filtrated through a 0.45- μ m pore size filter (Thermo
1073 Fisher Scientific, cat# 09-740-114). The filtered medium was applied on a 5-ml of
1074 HisTrap Fast Flow column (Cytiva, cat# 17-5255-01) equilibrated by phosphate
1075 buffered saline (PBS) using ÄKTA pure chromatography system (Cytiva). The
1076 column was washed with PBS and the pure human ACE2 protein (residues 18-740)
1077 was eluted using the PBS supplemented with 300 mM imidazole (pH 7.4). Using
1078 Ultracel-3 regenerated cellulose membrane (Merck, cat# UFC900324), the buffer
1079 was exchanged to PBS and the purified protein was concentrated. The purity of
1080 prepared protein was analyzed by a Tycho NT.6 system (NanoTemper).

1081

1082 **Preparation of the Yeast-Based SARS-CoV-2 RBD Expression System**

1083 A labeling-free yeast surface display plasmid pJYDC1 (Addgene, cat# 162458)
1084 ([Zahradník et al., 2021b](#)) encoding the SARS-CoV-2 S RBD (residues 336-528)
1085 (pJYDC1-RBD) ([Zahradník et al., 2021a](#)) was modified by the restriction enzyme-
1086 free cloning procedure ([Peleg and Unger, 2014](#)). To prepare the plasmids with the
1087 mutated RBD, megaprimers were amplified by PCR using KAPA HiFi HotStart
1088 ReadyMix kit (Roche, cat# KK2601) and the following primers: RBD L452R forward:
1089 5'-GGA CAG CAA GGT GGG AGG CAA CTA CAA CTA CAG ATA CAG ACT GTT
1090 CAG GAA GAG CAA C-3'; RBD Y453F reverse: 5'-CTC AAA TGG TTT CAG GTT
1091 GCT CTT CCT GAA CAG TCT GAA GAG GTA GTT GTA GTT GCC TCC C-3';
1092 RBD N501Y reverse: 5'-GTA TGG TTG GTA GCC CAC TCC ATA GGT TGG TTG
1093 GAA GCC ATA GGA TTG-3'; pCT_seq Reverse: 5'-CAT GGG AAA ACA TGT TGT
1094 TTA CGG AG-3'; and pCTCON_seq Forward: 5'-GCA GCC CCA TAA ACA CAC
1095 AGT AT-3', according to the manufacturer's protocol. The PCR products were
1096 integrated into pJYDC1 by integration PCR as previously described ([Peleg and
1097 Unger, 2014](#)).

1098

1099 **Analysis of the Binding Affinity of the SARS-CoV-2 S RBD Variants to Human** 1100 **ACE2 by Yeast Surface Display**

1101 The pJYDC1-based yeast display plasmids expressing SARS-CoV-2 RBD and its
1102 mutants were transformed into yeast (*Saccharomyces cerevisiae*; strain EBY100,
1103 ATCC MYA-4941) and selected by the growth on the SD-W plates ([Peleg and Unger,
1104 2014](#)). Single colonies were grown in 1-ml liquid SD-CAA medium ([Zahradník et al.,
1105 2021a](#)) overnight at 30°C (220 rpm) and used to inoculate the expression cultures in
1106 the 1/9 medium ([Zahradník et al., 2021a](#)) with 1 nM bilirubin (Sigma-Aldrich, cat#
1107 14370-1G). The cells were washed with PBS-B buffer [PBS supplemented with
1108 bovine serum albumin (1 g/l)] and aliquoted in analysis solutions. The analysis
1109 solutions consist of the PBS-B buffer with the 14 different concentrations (covering
1110 the range from 100 nM to 1 pM) of the human ACE2 protein (residues 18-740) that
1111 is labeled with CF640R succinimidyl ester (Biotium, cat# 92108). The volume of the
1112 analysis solution was adjusted (1-100 ml) in order to reduce the effect of ligand
1113 depletion ([Zahradník et al., 2021b](#)). The yeasts expressing the SARS-CoV-2 S RBDs
1114 were incubated with the analysis solution overnight to allow for equilibrium.
1115 Subsequently, the yeasts were washed with the PBS-B buffer, passed through a 40-
1116 µm cell strainer (SPL Life Sciences, cat# 93040), and the binding affinity to the
1117 CF640R-labeled human ACE2 protein (residues 18-740) was analyzed using an
1118 Accuri C6 flow cytometer (BD Biosciences). The fluorescent signal was processed
1119 as previously described ([Zahradník et al., 2021b](#)) and the standard non-cooperative

1120 Hill equation was fitted by nonlinear least-squares regression using Python v3.7
1121 (<https://www.python.org>).

1122

1123 **Pseudovirus Assay**

1124 To prepare the pseudoviruses, the lentivirus (HIV)-based, luciferase-expressing
1125 reporter viruses that are pseudotyped with the SARS-CoV-2 S protein and its
1126 derivatives, HEK293T cells (1×10^6 cells) were cotransfected with 1 μ g of psPAX2-
1127 IN/HiBiT ([Ozono et al., 2020](#)), and 1 μ g of pWPI-Luc2 ([Ozono et al., 2020](#)), and 500
1128 ng of the plasmids expressing parental S or its derivatives (L452R, Y453F or N501Y)
1129 using Lipofectamine 3000 (Thermo Fisher Scientific, cat# L3000015) according to
1130 the manufacturer's protocol. Two days posttransfection, the culture supernatants
1131 were harvested, centrifuged, and treated with 37.5 U/ml DNase I (Roche, cat#
1132 Sigma-Aldrich, cat# 11284932001) at 37°C for 30 min. The amount of the
1133 pseudoviruses prepared was quantified by HiBiT assay and the measured value was
1134 normalized to the level of HIV p24 antigen as previously described ([Ozono et al.,](#)
1135 [2021](#); [Ozono et al., 2020](#)). The pseudoviruses prepared were stored at -80°C until
1136 use.

1137 To prepare the target cells for pseudovirus infection, HEK293T cells ($1 \times$
1138 10^6 cells) were cotransfected with 250 ng of pC-TMPRSS2 ([Ozono et al., 2021](#)) and
1139 500 ng of pC-ACE2 (a human ACE2 expression plasmid) ([Ozono et al., 2021](#)) using
1140 Lipofectamine 2000 (Thermo Fisher Scientific, cat# 11668019) according to the
1141 manufacturer's protocol. Two days posttransfection, the transfected cells (22,000
1142 cells/100 μ l) were seeded into 96-well plates and infected with 100 μ l of the
1143 pseudoviruses prepared at 4 different doses (1, 3, 5 and 10 ng of p24 antigen). Two
1144 days postinfection, the infected cells were lysed with One-Glo luciferase assay
1145 system (Promega, cat# E6130), and the luminescent signal was measured by using
1146 a CentroXS3 plate reader (Berthold Technologies).

1147

1148 **Lentiviral Transduction**

1149 Lentiviral transduction was performed as described previously ([Anderson et al.,](#)
1150 [2018](#); [Ikeda et al., 2019](#)). Briefly, the VSV-G-pseudotyped lenvirus vector expressing
1151 human ACE2 was generated by transfecting 2.5 μ g of pLV-EF1a-human ACE2-
1152 IRES-Puro plasmid with 1.67 μ g of p Δ -NRF (expressing HIV-1 *gag*, *pol*, *rev*, and *tat*
1153 genes) ([Naldini et al., 1996](#)) and 0.83 μ g of pVSV-G (expressing VSV-G; Addgene,
1154 cat#138479) into 293T cells (3×10^6 cells) using TransIT-LT1 (Takara, cat#
1155 MIR2300) according to the manufacturer's protocol. Two days posttransfection, the
1156 culture supernatants were harvested, centrifuged, and the supernatants were filtered
1157 with 0.45 μ m pore size filter (Millipore, cat# SLGVR33RB) and collected as the

1158 lentiviral vector. The lentivirus vectors were concentrated by centrifugation (at 22,000
1159 × g for 2 h at 4°C) and the concentrated lentiviral vectors were inoculated into the
1160 target cells and incubated at 37°C. Two days posttransduction, the transduced cells
1161 were placed under the drug selection using the culture medium containing 1 µg/ml
1162 puromycin (Invivogen, cat# ant-pr-1). The puromycin-selected cells with relatively
1163 higher ACE2 expression were sorted by a FACS Aria II (BD Biosciences) and
1164 expanded. After the expansion, the expression level of surface ACE2 was verified
1165 by a FACS Canto II (BD Biosciences). For the staining of surface ACE2, a goat anti-
1166 ACE2 polyclonal antibody (R&D systems, cat# AF933) and an APC-conjugated
1167 donkey anti-goat IgG (R&D systems, cat# F0108) were used. A normal goat IgG
1168 (R&D systems, cat# AB-108-C) was used as the negative control of this assay.

1169

1170 **Protein Structure**

1171 The 3D visualization of the SARS-CoV-2 S and human ACE2 proteins (**Figures 2A**
1172 **and 2F**) was generated using PyMOL v2.3 (<https://pymol.org/2/>) with the cocrystal
1173 structure of the SARS-CoV-2 S and human ACE2 proteins (PDB: 6M17) ([Yan et al.,](#)
1174 [2020](#)). The L452R substitution (**Figure 2F**) was prepared using UCSF Chimera
1175 v1.13 ([Pettersen et al., 2004](#)).

1176

1177 **SARS-CoV-2 Reverse Genetics**

1178 Recombinant SARS-CoV-2 was generated by circular polymerase extension
1179 reaction (CPER) as previously described ([Torii et al., 2021](#)). In brief, the 9 DNA
1180 fragments encoding the partial genome of SARS-CoV-2 (strain WK-521; GISAID ID:
1181 EPI_ISL_408667) ([Matsuyama et al., 2020](#)) were prepared by PCR using
1182 PrimeSTAR GXL DNA polymerase (Takara, cat# R050A). Additionally, a linker
1183 fragment encoding hepatitis delta virus ribozyme (HDVr), bovine growth hormone
1184 (BGH) polyA signal and cytomegalovirus (CMV) promoter was prepared by PCR.
1185 The corresponding SARS-CoV-2 genomic region and the templates and the primers
1186 of this PCR are summarized in **Table S6**. The obtained 10 DNA fragments were
1187 mixed and used for the CPER ([Torii et al., 2021](#)).

1188

1189 To produce the recombinant SARS-CoV-2, the CPER products were
1190 transfected into HEK293-C34 cells using TransIT-LT1 (Takara, cat# MIR2300)
1191 according to the manufacturer's protocol. One day posttransfection, the culture
1192 medium was replaced with Dulbecco's modified Eagle's medium (high glucose)
1193 (Sigma-Aldrich, cat# R8758-500ML) containing 2% FCS, 1% PS and doxycycline (1
1194 µg/ml; Takara, cat# 1311N). Six days posttransfection, the culture medium was
1195 harvested, centrifuged, and the supernatants were collected as the seed virus. To

1196 was treated with 2 μ l TURBO DNase (Thermo Fisher Scientific, cat# AM2238) and
1197 incubated at 37 °C for 1 h. The complete removal of the CPER products (i.e., SARS-
1198 CoV-2-related DNA) from the seed virus was verified by PCR. To prepare the
1199 working virus of the recombinant SARS-CoV-2 for the virological experiments
1200 (**Figure 3**), 100 μ l of the seed virus was inoculated into VeroE6/TMPRSS2 cells
1201 (5,000,000 cells in T-75 flask). One hour after infection, the culture medium was
1202 replaced with Dulbecco's modified Eagle's medium (low glucose) (Wako, cat# 041-
1203 29775) containing 2% FCS and 1% PS. Two days postinfection, the culture medium
1204 was harvested, centrifuged, and the supernatants were collected as the working
1205 virus.

1206 To generate the recombinant SARS-CoV-2 mutants, mutations were
1207 inserted into the fragment 8 (**Table S6**) using GENEART Site-Directed mutagenesis
1208 system (Thermo Fisher Scientific, cat# A13312) and the following primers: Fragment
1209 8_S L452R forward, 5'-CTA AGG TTG GTG GTA ATT ATA ATT ACC GGT ATA
1210 GAT TGT TTA GGA AGT CTA ATC-3'; Fragment 8_S L452R reverse, 5'-GAT TAG
1211 ACT TCC TAA ACA ATC TAT ACC GGT AAT TAT AAT TAC CAC CAA CCT TAG-
1212 3'; Fragment 8_S Y453F forward, 5'-GGT TGG TGG TAA TTA TAA TTA CCT GTT
1213 TAG ATT GTT TAG GAA GTC TAA TCT C-3'; Fragment 8_S Y453F reverse, 5'-
1214 GAG ATT AGA CTT CCT AAA CAA TCT AAA CAG GTA ATT ATA ATT ACC ACC
1215 AAC C-3'; Fragment 8_S N501Y forward, 5'-CAA TCA TAT GGT TTC CAA CCC
1216 ACT TAT GGT GTT GGT TAC CAA CCA TAC AG-3'; and Fragment 8_S N501Y
1217 reverse, 5'-CTG TAT GGT TGG TAA CCA ACA CCA TAA GTG GGT TGG AAA
1218 CCA TAT GAT TG-3', according to the manufacturer's protocol. Nucleotide
1219 sequences were determined by a DNA sequencing service (Fasmac), and the
1220 sequence data were analyzed by Sequencher version 5.1 software (Gene Codes
1221 Corporation). The CPER for the preparation of SARS-CoV-2 mutants was performed
1222 using the mutated fragment 8 instead of the parental fragment 8. Subsequent
1223 experimental procedures correspond to the procedure for parental SARS-CoV-2
1224 preparation (described above). To verify the inserted mutation in the working viruses,
1225 viral RNA was extracted using QIAamp viral RNA mini kit (Qiagen, cat# 52906). Viral
1226 RNA was reversed transcribed using SuperScript III reverse transcriptase (Thermo
1227 Fisher Scientific, cat# 18080085) according to manufacturers' protocols. The DNA
1228 fragments including the mutations inserted were obtained by RT-PCR using
1229 PrimeSTAR GXL DNA polymerase (Takara, cat# R050A) and the following primers:
1230 WK-521 22607-22630 forward: 5'-GCA TCT GTT TAT GCT TGG AAC AGG-3'; and
1231 WK-521 23342-23367 reverse: 5'-CCT GGT GTT ATA ACA CTG ACA CCA CC-3'.
1232 Nucleotide sequences were determined as described above, and the sequence

1233 chromatograms (**Figure 3A**) were visualized using a web application Tracy
1234 (<https://www.gear-genomics.com/teal/>) (Rausch et al., 2020).

1235

1236 **Plaque Assay**

1237 One day prior to infection, 200,000 Vero cells were seeded into the 12-well plate.
1238 The virus was diluted with serum-free virus dilution buffer [1 × minimum essential
1239 medium (Temin's modification) (Thermo Fisher Scientific, cat# 11935046) with 20
1240 mM Hepes, non-essential amino acid (Thermo Fisher Scientific, cat# 11140-050) and
1241 antibiotics]. After removing the culture media, Vero cells were infected with 500 µl of
1242 the diluted virus at 37 °C. Two hours postinfection, 1 ml of mounting solution [1 ×
1243 minimum essential medium containing 3% FCS and 1.5% carboxymethyl cellulose
1244 (Sigma, cat# C9481-500G)] was overlaid and incubated at 37 °C. Three days
1245 postinfection, the culture media were removed, and the cells were washed with PBS
1246 three times and fixed with 10% formaldehyde (Nacalai Tesque, cat# 37152-51) or
1247 4% paraformaldehyde (Nacalai Tesque, cat# 09154-85). The fixed cells were
1248 washed with city water, dried up, and stained with staining solution [2% crystal violet
1249 (Nacalai Tesque, cat# 09804-52) or 0.1% methylene blue (Nacalai Tesque, cat#
1250 22412-14) in water]. The stained cells were washed with city water, dried up, and
1251 the number of plaques was counted to calculate plaque forming unit (pfu).

1252

1253 **SARS-CoV-2 Infection**

1254 One day prior to infection, 10,000 cells of VeroE6/TMPRSS2 and 293-ACE2 cells
1255 were seeded into the 96-well plate. Recombinant SARS-CoV-2 (100 pfu) was
1256 inoculated and incubated at 37 °C for 1 h. The infected cells were washed and
1257 replaced with 180 µl of culture media. The culture supernatant (10 µl) was harvested
1258 at 0, 6, 24, 48 and 72 hours postinfection and used for real-time PCR to quantify the
1259 copy number of viral RNA.

1260

1261 **Real-time RT-PCR**

1262 The amount of viral RNA in the culture supernatant was quantified by real-time RT-
1263 PCR as previously described (Shema Mugisha et al., 2020) with some modifications.
1264 In brief, 5 µl of culture supernatants was mixed with 5 µl of 2 × RNA lysis buffer [2%
1265 Triton X-100, 50 mM KCl, 100 mM Tris-HCl (pH 7.4), 40% glycerol, 0.8 U/µl
1266 recombinant RNase inhibitor (Takara, cat# 2313B)] and incubated at room
1267 temperature for 10 min. RNase-free water (90 µl) was added and the diluted sample
1268 (2.5 µl) was used as the template of real-time RT-PCR. Real-time RT-PCR was
1269 performed according to the manufacturer's protocol using the One Step TB Green
1270 PrimeScript PLUS RT-PCR kit (Takara, cat# RR096A) and the following primers:

1271 Forward *N*, 5'-AGC CTC TTC TCG TTC CTC ATC AC-3'; and Reverse *N*, 5'-CCG
1272 CCA TTG CCA GCC ATT C-3'. The copy number of viral RNA was standardized by
1273 SARS-CoV-2 direct detection RT-qPCR kit (Takara, cat# RC300A). The fluorescent
1274 signal was acquired on a QuantStudio 3 Real-Time PCR systems (Thermo Fisher
1275 Scientific), a CFX Connect Real-Time PCR Detection system (Bio-Rad) or a 7500
1276 Real Time PCR System (Applied Biosystems) was used.

1277

1278 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1279 Data analyses were performed using Prism 7 (GraphPad Software). Unless
1280 otherwise stated, the data are presented as means with SD. Statistically significant
1281 differences were determined as described in the figure legend. Statistical details can
1282 be found directly in the corresponding figure legends.

1283 **KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FITC-conjugated anti-human CD3 antibody	Biolegend	Cat# 300440; RRID: AB_2562046
BV510-conjugated anti-human CD3 antibody	Biolegend	Cat# 317331; RRID: AB_2561376
APC-Cy7-conjugated anti-human CD8 antibody	Biolegend	Cat# 301016; RRID: AB_314134
PerCP-Cy5.5-conjugated anti-human CD14 antibody	Biolegend	Cat# 325622; RRID: AB_893250
PerCP-Cy5.5-conjugated anti-human CD19 antibody	Biolegend	Cat# 302230; RRID: AB_2073119
PE-Cy7-conjugated anti-human CD25 antibody	Biolegend	Cat# 356107; RRID: AB_2561974
APC-conjugated anti-human CD137 antibody	Biolegend	Cat# 309809; RRID: AB_830671
BV421-conjugated anti-human CD107a antibody	Biolegend	Cat# 328625; RRID: AB_10899581
PE-conjugated anti-human IFN- γ antibody	BD Biosciences	Cat# 554552; RRID: AB_395474
PE-Cy7-conjugated anti-human TNF- α antibody	Biolegend	Cat# 502930; RRID: AB_2204079
APC-conjugated anti-human IL-2 antibody	Biolegend	Cat# 500310; RRID: AB_315097
Anti-ACE2 antibody	R&D systems	Cat# AF933; RRID: RRID: AB_355722
APC-conjugated anti-goat IgG	R&D systems	Cat# F0108; RRID: AB_573124
Goat normal IgG	R&D systems	Cat# AB-108-C; RRID: AB_354267
Bacterial and Virus Strains		
SARS-CoV-2 (strain WK-521)	(Matsuyama et al., 2020)	GISAID ID: EPI_ISL_408667
Biological Samples		
Human PBMCs	This study	N/A

Chemicals, Peptides, and Recombinant Proteins		
Ficoll-Paque Plus	GE Healthcare Life Sciences	Cat# 17-1440-03
RPMI1640 medium	Thermo Fisher Scientific	Cat# 11875101
Fetal calf serum (FCS)	Sigma-Aldrich	Cat# 172012-500ML
Penicillin streptomycin (PS)	Sigma-Aldrich	Cat# P4333-100ML
Dulbecco's modified Eagle's medium (high glucose)	Wako	Cat# 044-29765
Eagle's minimum essential medium (with L-glutamine and phenol red)	Wako	Cat# 051-07615
Dulbecco's modified Eagle's medium (low glucose)	Wako	Cat# 041-29775
G418	Nacalai Tesque	Cat# G8168-10ML
Dulbecco's modified Eagle's medium (high glucose)	Sigma-Aldrich	Cat# R8758-500ML
Blasticidin	Invivogen	Cat# ant-bl-1
Expi293 expression medium	Thermo Fisher Scientific	Cat# A1435101
SARS-CoV-2 PepTivator peptide pools	Miltenyi Biotec	Cat# 130-126-700
Recombinant human IL-2	Peptotec	Cat# 200-02
NF9 peptide (NYYLYRLF, residues 448-456 of the SARS-CoV-2 S protein)	This study	N/A
L452R peptide (NYYRYRLF, L5R in NF9)	This study	N/A
Y453F peptide (NYYLFRFLF, Y6F in NF9)	This study	N/A
7-aminoactinomycin D	Biolegend	Cat# 420404
Paraformaldehyde	Nacalai Tesque	Cat# 09154-85
Brefeldin A	Sigma-Aldrich	Cat# B7651
Monensin	Biolegend	Cat# 420701
PrimeSTAR GXL DNA polymerase	Takara	Cat# R050A
KpnI	New England Biolab	Cat# R0142S
NotI	New England Biolab	Cat# R1089S
MluI-HF	New England Biolab	Cat# R3198L
SmaI	New England Biolab	Cat# R0141L
HpaI	New England Biolab	Cat# R0105L
BamHI-HF	New England Biolab	Cat# R3136L
Bilirubin	Sigma-Aldrich	Cat# 14370-1G
CF640R succinimidyl ester	Biotium	Cat# 92108

Lipofectamine 3000	Thermo Fisher Scientific	Cat# L3000015
Lipofectamine 2000	Thermo Fisher Scientific	Cat# 11668019
DNase I	Sigma-Aldrich	Cat# 11284932001
TransIT-LT1	Takara	Cat# MIR2300
Puromycin	Invivogen	Cat# ant-pr-1
Doxycycline	Takara	Cat# 1311N
TURBO DNase	Thermo Fisher Scientific	Cat# AM2238
Minimum essential medium (2×), no phenol red	Thermo Fisher Scientific	Cat# 11935046
Non-essential amino acid	Thermo Fisher Scientific	Cat# 11140-050
Carboxymethyl cellulose	Sigma-Aldrich	Cat# C9481-500G
Formaldehyde	Nacalai Tesque	Cat# 37152-51
Crystal violet	Nacalai Tesque	Cat# 09804-52
Methylene blue	Nacalai Tesque	Cat# 22412-14
Recombinant RNase inhibitor	Takara	Cat# 2313B
Critical Commercial Assays		
Cytofix/Cytoperm Fixation/Permeabilization solution kit	BD Biosciences	Cat# 554714
QIAamp RNA blood mini kit	Qiagen	Cat# 52304
SuperScript III reverse transcriptase	Thermo Fisher Scientific	Cat# 18080085
ExpiFectamine 293 transfection kit	Thermo Fisher Scientific	Cat# A14525
KAPA HiFi HotStart ReadyMix kit	Roche	Cat# KK2601
One-Glo luciferase assay system	Promega	Cat# E6130
GENEART Site-Directed mutagenesis system	Thermo Fisher Scientific	Cat# A13312
QIAamp viral RNA mini kit	Qiagen	Cat# 52906
One Step TB Green PrimeScript PLUS RT-PCR kit	Takara	Cat# RR096A
SARS-CoV-2 direct detection RT-qPCR kit	Takara	Cat# RC300A
Deposited Data		

Experimental Models: Cell Lines		
Human: C1R-A2402 cells	(Karaki et al., 1993)	N/A
Human: HEK293 cells	ATCC	CRL-1573
Human: HEK293T cells	ATCC	CRL-3216
African green monkey (<i>Chlorocebus sabaeus</i>): Vero cells	JCRB	JCRB0111
African green monkey (<i>Chlorocebus sabaeus</i>): VeroE6/TMPRSS2 cells	JCRB	JCRB1819
Human: 293-C34 cells	(Torii et al., 2021)	N/A
Human: Expi293F cells	Thermo Fisher Scientific	Cat# A14527
Yeast (<i>Saccharomyces cerevisiae</i>): strain EBY100	ATCC	MYA-4941
Experimental Models: Organisms/Strains		
Oligonucleotides		
Forward primer for the preparation of S expression plasmid (pCAGGS-SARS2-S): TTG GGTACC ATG TTT GTG TTC CTG GTG CTG	This study	N/A
Reverse primer for the preparation of S expression plasmid (pCAGGS-SARS2-S): GTG GCGGCCGC TCT AGA TTC AGG TGT AGT GCA GTT T	This study	N/A
Forward primer for the preparation of S L452R expression plasmid (pCAGGS-SARS2-S-L452R): GTG GGA GGC AAC TAC AAC TAC CGT TAC	This study	N/A
Forward primer for the preparation of S Y453F expression plasmid (pCAGGS-SARS2-S-Y453F): GTG GGA GGC AAC TAC AAC TAC CTC TTC AGA	This study	N/A
Reverse primer for the preparation of S L452M/L452R/L453F expression plasmid: GTT GTA GTT GCC TCC CAC CTT	This study	N/A
Forward primer for the preparation of S N501Y expression plasmid (pCAGGS-SARS2-S-N501Y): TCC TAT GGC TTC CAA CCA ACC TAT GGA	This study	N/A
Reverse primer for the preparation of S N501Y expression plasmid (pCAGGS-SARS2-S-N501Y): TGG TTG GAA GCC ATA GGA TTG	This study	N/A

Primer for the preparation of RBD L452R expression plasmid (RBD L452R): GGA CAG CAA GGT GGG AGG CAA CTA CAA CTA CAG ATA CAG ACT GTT CAG GAA GAG CAA C	This study	N/A
Primer for the preparation of RBD Y453F expression plasmid (RBD Y453F): CTC AAA TGG TTT CAG GTT GCT CTT CCT GAA CAG TCT GAA GAG GTA GTT GTA GTT GCC TCC C	This study	N/A
Primer for the preparation of RBD N501Y expression plasmid (RBD N501Y): GTA TGG TTG GTA GCC CAC TCC ATA GGT TGG TTG GAA GCC ATA GGA TTG	This study	N/A
Primer for the preparation of the RBD expression plasmid CAT GGG AAA ACA TGT TGT TTA CGG AG	This study	N/A
Primer for the preparation of the RBD expression plasmid preparation: GCA GCC CCA TAA ACA CAC AGT AT	This study	N/A
Forward primer for the preparation of Fragment 8, S L452R: CTA AGG TTG GTG GTA ATT ATA ATT ACC GGT ATA GAT TGT TTA GGA AGT CTA ATC	This study	N/A
Reverse primer for the preparation of Fragment 8, S L452R: GAT TAG ACT TCC TAA ACA ATC TAT ACC GGT AAT TAT AAT TAC CAC CAA CCT TAG	This study	N/A
Forward primer for the preparation of Fragment 8, S Y453F: GGT TGG TGG TAA TTA TAA TTA CCT GTT TAG ATT GTT TAG GAA GTC TAA TCT C	This study	N/A
Reverse primer for the preparation of Fragment 8, S Y453F: GAG ATT AGA CTT CCT AAA CAA TCT AAA CAG GTA ATT ATA ATT ACC ACC AAC C	This study	N/A
Forward primer for the preparation of Fragment 8, S N501Y: CAA TCA TAT GGT TTC CAA CCC ACT TAT GGT GTT GGT TAC CAA CCA TAC AG	This study	N/A
Reverse primer for the preparation of Fragment 8, S N501Y: CTG TAT GGT TGG TAA CCA ACA CCA TAA GTG GGT TGG AAA CCA TAT GAT TG	This study	N/A
SARS-CoV-2 S forward primer for the mutation verification (WK-521, 22607-22630 forward): GCA TCT GTT TAT GCT TGG AAC AGG	(Torii et al., 2021)	N/A

SARS-CoV-2 S reverse primer for the mutation verification (WK-521, 23342-23367 reverse): CCT GGT GTT ATA ACA CTG ACA CCA CC	(Torii et al., 2021)	N/A
SARS-CoV-2 N forward primer for real-time RT-PCR: AGC CTC TTC TCG TTC CTC ATC AC	This study	N/A
SARS-CoV-2 N reverse primer for real-time RT-PCR: CCG CCA TTG CCA GCC ATT C	This study	N/A
Recombinant DNA		
Plasmid: pC-SARS2-S	(Ozono et al., 2021)	N/A
Plasmid: pCAGGS	(Niwa et al., 1991)	N/A
Plasmid: pCAGGS-SARS2-S	This study	N/A
Plasmid: pCAGGS-SARS2-S-L452R	This study	N/A
Plasmid: pCAGGS-SARS2-S-Y453F	This study	N/A
Plasmid: pCAGGS-SARS2-S-N501Y	This study	N/A
Plasmid: pTargeT-human ACE2	(Fukushi et al., 2007)	N/A
Plasmid: pLV-EF1a-IRES-Puro	Addgene	Cat# #85132
Plasmid: pLV-EF1a-human ACE2-IRES-Puro	This study	N/A
Plasmid: pHL-sec	Addgene	Cat# 99845
Plasmid: pHL-sec-human ACE2 (residues 18-740)	(Zahradnik et al., 2021a)	N/A
Plasmid: pJYDC1	Addgene	Cat# 162458
Plasmid: pJYDC1-SARS-CoV-2 S RBD (residues 336-528)	(Zahradnik et al., 2021a)	N/A
Plasmid: pJYDC1-SARS-CoV-2 S RBD L452R	This study	N/A
Plasmid: pJYDC1-SARS-CoV-2 S RBD Y453F	This study	N/A
Plasmid: pJYDC1-SARS-CoV-2 S RBD N501Y	This study	N/A
Plasmid: psPAX2-IN/HiBiT	(Ozono et al., 2020)	N/A
Plasmid: pWPI-Luc2	(Ozono et al., 2020)	N/A
Plasmid: pC-TMPRSS2	(Ozono et al., 2021)	N/A
Plasmid: pC-ACE2	(Ozono et al., 2021)	N/A
Plasmid: pΔ-NRF	(Naldini et al., 1996)	N/A
Plasmid: pVSV-G	Addgene	Cat# 138479
Plasmid: template plasmids for the preparation of the partial SARS-CoV-2 genome fragments for CPER, see Table S6	(Torii et al., 2021)	N/A
Software and Algorithms		

MAFFT suite (v7.467)	(Kato and Standley, 2013)	https://mafft.cbrc.jp/alignment/software/
ModelTest-NG (v0.1.5)	(Darriba et al., 2020)	https://github.com/ddarriba/modeltest
RAxML-NG (v1.0.0)	(Kozlov et al., 2019)	https://github.com/amkozlov/raxml-ng
FigTree (v1.4.4)	Andrew Rambaut	http://tree.bio.ed.ac.uk/software/figtree
trimAl (v1.4.rev22)	(Capella-Gutierrez et al., 2009)	http://trimal.cgenomics.org
Python (v3.7)	Python Software Foundation	https://www.python.org
Sequencher (v5.1)	Gene Codes Corporation	N/A
PyMOL (v2.3.0)	Schrödinger	https://pymol.org/2/
UCSF Chimera (v1.13)	(Pettersen et al., 2004)	N/A
Prism	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Tracy	(Rausch et al., 2020)	https://www.gear-genomics.com/teal/
Other		
GISAID	Freunde von GISAID e.V.	https://www.gisaid.org
Pangolin	(Rambaut et al., 2020)	https://covid19-lineages.org/pangolin.html
0.45-µm pore size filter	Thermo Fisher Scientific	Cat# 09-740-114
40-µm cell strainer	SPL Life Sciences	Cat# 93040
HisTrap Fast Flow column	Cytiva	Cat# 17-5255-01
Ultracel-3 regenerated cellulose membrane	Merck	Cat# UFC900324
0.45-µm pore size filter	Merck	Cat# SLGVR33RB