

Virological characteristics of the SARS-CoV-2 Omicron BA.2.75

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- 80 **Conflict of interest:** Yuki Yamamoto and Tetsuharu Nagamoto are founders
81 and shareholders of HiLung, Inc. Yuki Yamamoto is a co-inventor of patents
82 (PCT/JP2016/057254; "Method for inducing differentiation of alveolar epithelial

83 cells", PCT/JP2016/059786, "Method of producing airway epithelial cells"). The
84 other authors declare that no competing interests exist.

85

86 **Short title:** Characteristics of SARS-CoV-2 BA.2.75 (37/50 characters)

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88 immune resistance; antiviral drug resistance; pathogenicity

89 **Abstract** (150/150 words)

90 SARS-CoV-2 Omicron BA.2.75 emerged in May 2022. BA.2.75 is a BA.2
 91 descendant but is phylogenetically different from BA.5, the currently
 92 predominant BA.2 descendant. Here, we showed that the effective reproduction
 93 number of BA.2.75 is greater than that of BA.5. While the sensitivity of BA.2.75
 94 to vaccination- and BA.1/2 breakthrough infection-induced humoral immunity
 95 was comparable to that of BA.2, the immunogenicity of BA.2.75 was different
 96 from that of BA.2 and BA.5. Three clinically-available antiviral drugs were
 97 effective against BA.2.75. BA.2.75 spike exhibited a profound higher affinity to
 98 human ACE2 than BA.2 and BA.5 spikes. The fusogenicity, growth efficiency in
 99 human alveolar epithelial cells, and intrinsic pathogenicity in hamsters of
 100 BA.2.75 were comparable to those of BA.5 but were greater than those of BA.2.
 101 Our multiscale investigations suggest that BA.2.75 acquired virological
 102 properties independently of BA.5, and the potential risk of BA.2.75 to global
 103 health is greater than that of BA.5.

104 Introduction

105 By the end of 2021, five SARS-CoV-2 variants-of-concern (VOCs) were
 106 classified by the WHO (WHO, 2022). These are the Alpha [also known as
 107 lineage B.1.1.7 based on the PANGO classification (<https://cov-lineages.org>);
 108 clade 20I based on the Nextstrain classification (<https://nextstrain.org>)], Beta
 109 (lineage B.1.351; clade 20H), Gamma (lineage P.1; clade 20J), Delta (lineages
 110 B.1.617.2 and AY; clades 21I and 21J), and Omicron (lineages B.1.1.529 and
 111 BA; clade 21K) variants. Since these five VOCs are phylogenetically unrelated to
 112 each other, SARS-CoV-2 evolution until the end of 2021 was posed by the
 113 antigenic shift. At the beginning of 2022, Omicron BA.1 variant (clade 21K)
 114 outcompeted the other variants and spread globally. Thereafter, BA.2 (clade
 115 21L) and BA.4/5 (clades 22A and 22B) continuously emerged from South Africa,
 116 while BA.2.12.1 (clade 22C) emerged in the USA. As of the beginning of August
 117 2022, Omicron BA.5 (clade 22B) is the most predominant SARS-CoV-2 variant
 118 in the world. In contrast to the five VOCs detected in 2021, the Omicron
 119 subvariants are phylogenetically related. Therefore, the evolution of
 120 SARS-CoV-2 Omicron subvariants since the end of 2021 is posed by the
 121 antigenic drift.

122 Newly emerging SARS-CoV-2 variants need to be carefully and rapidly
 123 assessed for a potential increase in their growth efficiency in the human
 124 population [i.e., relative effective reproduction number (R_e)], their evasion from
 125 antiviral immunity, and their pathogenicity. Resistance to antiviral humoral
 126 immunity can be mainly determined by substitutions in the spike (S) protein. For
 127 instance, Omicron BA.1 (Cao et al., 2021; Cele et al., 2021; Dejnirattisai et al.,
 128 2022; Garcia-Beltran et al., 2021; Liu et al., 2021; Meng et al., 2022; Planas et
 129 al., 2021; Takashita et al., 2022a; VanBlargan et al., 2022) , BA.2 (Bruel et al.,
 130 2022; Takashita et al., 2022b; Yamasoba et al., 2022c), and BA.5 (Arora et al.,
 131 2022; Cao et al., 2022; Gruell et al., 2022; Hachmann et al., 2022; Khan et al.,
 132 2022; Kimura et al., 2022c; Lyke et al., 2022; Qu et al., 2022; Tuekprakhon et al.,
 133 2022; Wang et al., 2022; Yamasoba et al., 2022c) exhibit profound resistance to
 134 neutralizing antibodies induced by vaccination, natural SARS-CoV-2 infection,
 135 and therapeutic monoclonal antibodies. Particularly, newly spreading
 136 SARS-CoV-2 variants tend to be resistant to the humoral immunity induced by
 137 the infection with prior variant; for instance, BA.2 is resistant to BA.1
 138 breakthrough infection sera (Qu et al., 2022; Tuekprakhon et al., 2022;
 139 Yamasoba et al., 2022b), and BA.5 is resistant to BA.2 breakthrough infection
 140 sera (Hachmann et al., 2022; Kimura et al., 2022c; Wang et al., 2022). Therefore,
 141 acquiring immune resistance to previously dominant variant is a key factor in
 142 outcompeting previous variants, thereby obtaining relatively increased R_e
 143 compared to the previously dominant variant. Viral pathogenicity is also closely
 144 associated with the phenotype of viral S protein. Particularly, we have proposed

145 that the fusogenicity of viral S protein in *in vitro* cell cultures is associated with
146 viral pathogenicity *in vivo* (Kimura *et al.*, 2022c; Saito *et al.*, 2022; Suzuki *et al.*,
147 2022; Yamasoba *et al.*, 2022b).

148 As mentioned above, major SARS-CoV-2 phenotypes can be defined
149 by the function of the viral S protein. SARS-CoV-2 S protein bears two major
150 domains, receptor binding domain (RBD) and N-terminal domain (NTD)
151 [reviewed in (Harvey *et al.*, 2021; Mittal *et al.*, 2022)]. RBD is crucial for the
152 binding to human angiotensin-converting enzyme 2 (ACE2) receptor for the cell
153 attachment and entry, and therefore, this domain has been considered a major
154 target for neutralizing antibodies to block viral infection [reviewed in (Barnes *et al.*, 2020; Harvey *et al.*, 2021; Jackson *et al.*, 2022)]. On the other hand, NTD is
155 an immunodominant domain that can be recognized by antibodies, and some
156 antibodies targeting NTD potentially neutralize viral infection (Cerutti *et al.*, 2021;
157 Chi *et al.*, 2020; Liu *et al.*, 2020; Lok, 2021; McCallum *et al.*, 2021; Suryadevara
158 *et al.*, 2021; Voss *et al.*, 2021), despite our limited understanding of its virological
160 function.

161 The Omicron BA.2.75 variant, a new BA.2 subvariant, was first
162 detected in India in May 2022 (WHO, 2022). Because an early preliminary
163 investigation suggested the potential increase in the relative R_e value of BA.2.75
164 compared to BA.5 and the original BA.2 (GitHub, 2022), BA.2.75 has been
165 flagged as the most concerning variant that can potentially outcompete BA.5 and
166 be the next predominant variant in the future. In fact, on July 19, 2022, the WHO
167 classified this variant as a VOC lineage under monitoring (VOC-LUM) together
168 with the other BA.2 subvariants, including BA.5, which bear the substitution at
169 the L452 residue in their S proteins (WHO, 2022). On July 23, 2022, Nextstrain
170 (<https://nextstrain.org>) classified BA.2.75 as a new clade, 22D. Compared to the
171 BA.2 S, BA.4/5 bears four mutations in its S protein (Kimura *et al.*, 2022c;
172 Yamasoba *et al.*, 2022b). On the other hand, the majority of BA.2.75 S bears
173 nine mutations: K147E, W152R, F157L, I210V, and G257S substitutions are
174 located in the NTD, while D339H, G446S, N460K, and R493Q substitutions are
175 located in the RBD. The mutation number in the BA.2.75 S is larger than that in
176 the BA.4/5 S, and notably, some of the substitutions detected in the BA.2.75 S
177 show the signs of convergent evolution (Zahradnik *et al.*, 2022). These notions
178 raise the possibility that the phenotype of BA.2.75 S is critically different from
179 previous BA.2 subvariants. In fact, we have recently revealed that the S protein
180 of BA.2.75 exhibits different sensitivity towards several therapeutic monoclonal
181 antibodies from those of BA.2 and BA.5 (Yamasoba *et al.*, 2022a). However, the
182 virological phenotype of BA.2.75, including its R_e , potential evasion from antiviral
183 humoral immunity, sensitivity to currently recommended antiviral small
184 compounds, virological properties of its S protein, and intrinsic pathogenicity

185 remains unclear. Here, we elucidate the features of newly emerging
186 SARS-CoV-2 Omicron BA.2.75 subvariant.

187 Results

188 Epidemics of BA.2.75 in India

189 As of the beginning of August 2022, the Omicron BA.5 variant is predominant in
190 the world and is outcompeting the BA.2 variant. However, a novel BA.2
191 subvariant, BA.2.75, emerged and rapidly spread in India since May 2022.
192 Although BA.2.75 and BA.5 (and BA.4) belong to the BA.2 subvariant clade,
193 BA.2.75 is phylogenetically distinct from the BA.4/5 clade (**Figure 1A**).
194 Compared to BA.2, BA.2.75 harbors 14 amino acid substitutions, including nine
195 substitutions in the S protein (**Figures 1B and S1A**). Of these, only one
196 revertant mutation (S:R493Q) is shared with BA.5. In India, BA.5 and BA.2.75
197 spread in different regions each other: BA.5 spreads in the south part including
198 Tamil Nadu and Telangana states, while BA.2.75 spreads the other parts
199 including Himachal Pradesh, Odisha, Haryana, Rajasthan, and Maharashtra
200 states (**Figures 1C and 1D**). To compare the relative R_e between BA.5 and
201 BA.2.75 in India with adjusting the regional differences, we constructed a
202 Bayesian hierarchical model that can estimate both state-specific R_e values and
203 the value averaged in India (**Figures 1E and S1B and Table S1**). The R_e value
204 of BA.5 is 1.19-fold higher than that of BA.2 [95% credible interval (CI):
205 1.14–1.24] on average in India (**Figure 1E**). This value is comparative to the
206 relative R_e value of BA.5 in South Africa (1.21) estimated in our recent study
207 (Kimura *et al.*, 2022c). Of note, the R_e value of BA.2.75 is 1.34-fold higher than
208 that of BA.2 (95% CI: 1.29–1.38), and the R_e value of BA.2.75 is 1.13-fold higher
209 than that of BA.5 (95% CI: 1.06–1.20) (**Figures 1E and S1C**). Furthermore, in
210 the Indian states analyzed, where both BA.5 and BA.2.75 are dominant, such as
211 Telangana and Tamil Nadu (for BA. 5-dominant states) and Odisha, Haryana,
212 Rajasthan, and Maharashtra (for BA.2.75-dominant states), the R_e value of
213 BA.2.75 was greater than that of BA.5 (**Figures S1B and S1C**). Together, our
214 data suggest that BA.2.75 bears the potential to spread more rapidly than BA.5
215 and will be predominant in some regions including India in the near future.

217 Sensitivity of BA.2.75 to antiviral humoral immunity and antiviral drugs

218 Recent studies, including ours, showed that newly emerging Omicron
219 subvariants such as BA.5 exhibit higher resistance to the humoral immunity
220 induced by vaccination and natural infections with prior SARS-CoV-2 variants
221 including BA.1 and BA.2 (Hachmann *et al.*, 2022; Kimura *et al.*, 2022c; Wang *et al.*, 2022). Additionally, we have recently demonstrated that BA.2.75 is more
222 resistant to a therapeutic monoclonal antibody, bebtelovimab, compared to BA.2
223 and BA.5 (Yamasoba *et al.*, 2022a). To investigate the sensitivity of BA.2.75 to
224 antiviral humoral immunity, we prepared pseudoviruses bearing the S proteins of
225 D614G-bearing ancestral B.1.1, BA.2, BA.5 and BA.2.75. Human sera were
226 collected from vaccinated and infected individuals (listed in **Table S2**). The
227

228 2-dose vaccine sera were ineffective against all Omicron subvariants tested,
229 including BA.2.75 (**Figure 2A**). Although BA.5 was significantly more resistant to
230 3-dose vaccine sera than BA.2, which is consistent with previous studies
231 (Hachmann *et al.*, 2022; Kimura *et al.*, 2022c; Wang *et al.*, 2022), the sensitivity
232 of BA.2.75 to these sera was comparable to that of BA.2 (**Figures 2B and 2C**).
233 We then assessed the sensitivity of BA.2.75 to the convalescent sera from
234 individuals who were infected with BA.1 and BA.2 after 2-dose or 3-dose
235 vaccination (i.e., breakthrough infection). Similar to the previous reports
236 including ours (Hachmann *et al.*, 2022; Kimura *et al.*, 2022c; Wang *et al.*, 2022),
237 BA.5 exhibited significant resistance to breakthrough infection sera compared to
238 BA.2, while the sensitivity of BA.2.75 to these sera was comparable to that of
239 BA.2 (**Figures 2D and 2E**). These results suggest that BA.2.75 is not resistant
240 to the humoral immunity induced by vaccination and the infection with prior
241 Omicron subvariants including BA.1 and BA.2. Since the Delta variant emerged
242 and caused a huge surge of infection in India in the middle of 2021 (Mlcochova
243 *et al.*, 2021), it is hypothesized that BA.2.75 evades the immunity induced by
244 Delta. To address this possibility, we used Delta infection sera. However, the
245 sensitivity of all Omicron subvariants tested, including BA.2.75, to Delta infection
246 sera was similar (**Figure 2F**), implying that previous Delta infection is not
247 associated with the emergence of BA.2.75 in India.

248 To further address the difference in immunogenicity among Omicron
249 subvariants, we used the sera obtained from infected hamsters at 16 days
250 postinfection (d.p.i., i.e., after recovery) (Kimura *et al.*, 2022c; Suzuki *et al.*,
251 2022; Yamasoba *et al.*, 2022b). While BA.1 infection hamster sera were
252 ineffective against BA.2, BA.5 and BA.2.75 (**Figure 2G**), both BA.5 (17-fold,
253 $P=0.031$ by Wilcoxon signed-rank test) and BA.2.75 (23-fold, $P=0.031$ by
254 Wilcoxon signed-rank test) exhibited significant resistance to BA.2 infection
255 hamster sera than BA.2 (**Figure 2H**). These results suggest that the
256 immunogenicity of BA.5 and BA.2.75 is different from BA.2. Notably, BA.2
257 (5.1-fold, $P=0.031$ by Wilcoxon signed-rank test) and BA.2.75 (12-fold, $P=0.031$
258 by Wilcoxon signed-rank test) exhibited significant resistance to BA.5 infection
259 hamster sera (**Figure 2I**). These results suggest that the immunogenicity of BA.5
260 and BA.2.75 is also different. To identify the substitutions responsible for the
261 different immunogenicity of BA.2.75 S from BA.2 S and BA.5 S, we prepared the
262 BA.2 S-based derivatives that bear respective BA.2.75 substitutions. The
263 neutralization assay using BA.2-infected hamster sera showed that the G446S
264 and R493Q substitutions contribute to the resistance of BA.2.75 to BA.2-induced
265 immunity (**Figure 2H**). Because the R493Q substitution is shared with BA.5
266 (**Figures 1B and S1A**), it can be suggested that this substitution contributes to
267 the resistance of BA.5 to BA.2-induced immunity (**Figure 2H**). In the case of
268 BA.5-infected hamster sera, multiple substitutions, including the K147E, W152R,

269 F157L, I210V, G446S and N460K, associated with the resistance of BA.2.75 to
270 BA.5-induced immunity (**Figure 2I**).

271 To evaluate the sensitivity of BA.2.75 to three antiviral drugs,
272 Remdesivir, EIDD-1931 (an active metabolite of Molnupiravir) and Nirmatrelvir
273 (also known as PF-07321332), we used a clinical isolate of BA.2.75 (strain
274 TY41-716; GISAID ID: EPI_ISL_13969765). As controls, we also used clinical
275 isolates of B.1.1 (strain TKYE610670; GISAID ID: EPI_ISL_479681) (Suzuki *et al.*,
276 2022), BA.2 (strain TY40-385; GISAID ID: EPI_ISL_9595859) (Kimura *et al.*,
277 2022c), BA.5 (strain TKYS14631; GISAID ID: EPI_ISL_12812500) (Tamura *et al.*,
278 2022). These viruses were inoculated into human airway organoids (AO), a
279 physiologically relevant model (Sano *et al.*, 2022), and treated with three
280 antiviral drugs. As shown in **Table 1** and **Figure S2A**, Remdesivir had a stronger
281 antiviral effect ($EC_{50}=0.63 \mu M$) against B.2.75 than other variants, B.1.1, BA.2
282 and BA.5. EIDD-1931 inhibited BA.2 and BA.2.75 ($EC_{50}=0.02 \mu M$ and $0.08 \mu M$,
283 respectively) more potently than B.1.1 and BA.5 ($EC_{50}=0.24 \mu M$ and $0.21 \mu M$,
284 respectively). For Nirmatrelvir, no differences in antiviral efficacy were observed
285 between four variants ($EC_{50}=0.84 \mu M$, $0.85 \mu M$, $0.63 \mu M$ and $0.81 \mu M$ for B.1.1,
286 BA.2, BA.5 and BA.2.75, respectively). Altogether, it is suggested that all three
287 drugs exhibit antiviral effects against BA.2.75, and particularly, EIDD-1931 is
288 effective against BA.2.75.

289

290 **Virological characteristics of BA.2.75 S *in vitro***

291 To investigate the virological properties of BA.2.75 S, we measured the
292 pseudovirus infectivity. As shown in **Figure 3A**, the pseudovirus infectivity of
293 BA.2.75 was significantly (12.5-fold) higher than that of BA.2. To assess the
294 association of TMPRSS2 usage with the increased pseudovirus infectivity of
295 BA.2.75, we used both HEK293-ACE2/TMPRSS2 cells and HEK293-ACE2 cells,
296 on which endogenous surface TMPRSS2 is undetectable (Yamasoba *et al.*,
297 2022b), as target cells. Consistent with our recent study (Kimura *et al.*, 2022c),
298 the fold increase in pseudovirus infectivity of BA.5 caused by TMPRSS2
299 expression on the target cells was not observed (**Figure S3A**). Similarly, the
300 infectivity of BA.2.75 pseudovirus was not increased by TMPRSS2 expression
301 (**Figure S3A**), suggesting that TMPRSS2 is not associated with an increase in
302 pseudovirus infectivity of BA.2.75. To determine the substitutions that are
303 responsible for the increased pseudovirus infectivity of BA.2.75, we used a
304 series of BA.2 derivatives that bears the BA.2.75-specific substitutions. Three
305 substitutions in the NTD, K147E, F157L, and I210V, and two substitutions in the
306 RBD, N460K and R493Q, significantly increased infectivity (**Figure 3A**). Notably,
307 the N460K substitution increased infectivity by 44-fold (**Figure 3A**). On the other
308 hand, a substitution in the NTD, W152R, significantly (8.9-fold) decreased
309 infectivity (**Figure 3A**). The BA.2 derivative bearing the three substitutions in the

310 NTD in close proximity to each other, K147E, W152R and F157L, exhibited
311 comparable infectivity to BA.2 (**Figure 3A**).

312 To decipher the binding properties of BA.2.75 S RBD to human ACE2
313 and the role of each substitution, we measured the ACE2 binding affinity of the S
314 RBDs of BA.2.75 as well as those of BA.2 derivatives bearing D339H, G446S,
315 N460K and R493Q substitutions by an enhanced surface display system
316 (Zahradnik et al., 2021a). Intriguingly, the BA.2.75 S RBD showed a strongly
317 tight binding with 146 ± 6 pM affinity (**Figure 3B**). Out of the four BA.2-based
318 derivatives, only the BA.2 N460K substitution exhibited a significantly increased
319 binding affinity than BA.2 (**Figure 3B**). Consistent with the results of pseudovirus
320 assay (**Figure 3A**), these observations suggest that the N460K substitution is
321 critical to characterize the virological phenotype of BA.2.75 S. To reveal the
322 structural effect of the N460K substitution, we generated a structural model of
323 BA.2.75 S RBD using AlphaFold2 (Mirdita et al., 2022). Calculating the
324 electrostatic potential of this model in comparison with the S RBDs of B.1.1 and
325 BA.2 showed that K460 of BA.2.75 S RBD is positively charged (**Figure 3C**),
326 and the K460 is complementary to the negative charged binding site on human
327 ACE2 (**Figure 3D**). These structural observations suggest that N460K
328 substitution contributes to increased electrostatic complementary binding
329 between the BA.2.75 S RBD and human ACE2.

330 Although the N460K substitution significantly increased binding affinity
331 (**Figure 3B**), the binding affinity of the BA.2 N460K was still 5-fold lower than
332 that of BA.2.75 (**Figure 3B**). Therefore, the extraordinary tight binding of
333 BA.2.75 cannot be explained by the N460K alone, and it is hypothesized that the
334 additional substitutions conferred negative effects in the BA.2 background. In
335 particular, the D339H substitution requires two nucleotide changes in the codon
336 to occur. Such changes are still relatively rare in the evolution of SARS-CoV-2,
337 reinforcing the importance and corresponding fitness advantage. To analyze the
338 potential impact of this substitution, we additionally prepared the BA.2.75 H339D
339 derivative and measured its affinity. The K_D value of this mutant was significantly
340 (3-fold) lower than that of the parental BA.2.75 (**Figure 3B**). The structural model
341 computed by AlphaFold2 (Mirdita et al., 2022) suggested that the loss of
342 ion-dipole interaction between the D339 and the N343 allowed for the N343 side
343 chain repositioning (**Figure S3B**). These data suggest that the D339H
344 substitution potentially influences the position of the linoleic acid binding loop
345 between residues 367–378 (Toelzer et al., 2020) and thereby increases binding
346 affinity to ACE2.

347 To further reveal the virological property of BA.2.75 S, we performed a
348 cell-based fusion assay (Kimura et al., 2022b; Kimura et al., 2022c; Motozono et
349 al., 2021; Saito et al., 2022; Suzuki et al., 2022; Yamasoba et al., 2022b) using
350 Calu-3 cells as target cells. Flow cytometry analysis showed that the surface

expression level of BA.2.75 is comparable to that of BA.2 (**Figure 3E**). Consistent with our recent study (Kimura *et al.*, 2022c), the fusogenicity of BA.5 was significantly higher than that of BA.2, and notably, the BA.2.75 S was also significantly more fusogenic than the BA.2 S (**Figure 3F**). Altogether, these results suggest that BA.2.75 S exhibits higher binding affinity to human ACE2 and higher fusogenicity.

Virological characteristics of BA.2.75 clinical isolate *in vitro*

To evaluate the growth capacity of BA.2.75, a clinical isolate of BA.2.75 (strain TY41-716; GISAID ID: EPI_ISL_13969765) was inoculated in a variety of *in vitro* cell culture systems. As controls, we also used clinical isolates of B.1.1 (strain TKYE610670; GISAID ID: EPI_ISL_479681) (Suzuki *et al.*, 2022), Delta (B.1.617.2, strain TKYTK1734; GISAID ID: EPI_ISL_2378732) (Saito *et al.*, 2022), BA.2 (strain TY40-385; GISAID ID: EPI_ISL_9595859) (Kimura *et al.*, 2022c) and BA.5 (strain TKYS14631; GISAID ID: EPI_ISL_12812500) (Tamura *et al.*, 2022). The growth efficacy of B.1.1 and Delta was significantly higher than that of BA.2 in Vero cells (**Figure 4A**), VeroE6/TMPRSS2 cells (**Figure 4B**), HEK293-ACE2/TMPRSS2 cells (**Figure 4C**), AO-derived air-liquid interface (AO-ALI) model (**Figure 4D**), human iPSC cell (iPSC)-derived airway epithelial cells (**Figure 4E**) and lung epithelial cells (**Figure 4F**). BA.5 replicated more efficiently than BA.2 with statistically significant differences in the five cell culture systems except AO-ALI (**Figures 4A–4F**). The growth efficacy of BA.2.75 was significantly higher than that of BA.2 in Vero cells (**Figure 4A**), VeroE6/TMPRSS2 cells (**Figure 4B**), HEK293-ACE2/TMPRSS2 cells (**Figure 4C**), and iPSC-derived lung epithelial cells (**Figure 4F**), while the growth efficacy of BA.2.75 and BA.2 were comparable in the two airway epithelial cell systems (**Figures 4D and 4E**).

To evaluate the effect of BA.2.75 on the airway epithelial and endothelial barriers, airway-on-a-chips (**Figure S3C**) were used. By measuring the amount of virus that invades from the top channel (airway channel; **Figure 4G**) to the bottom channel (blood vessel channel; **Figure 4H**), the ability of viruses to disrupt the airway epithelial and endothelial barriers can be evaluated. Notably, the amount of virus that invades to the blood vessel channel of BA.2.75-, BA.5- and B.1.1-infected airway-on-chips was significantly higher than that of BA.2-infected one (**Figure 4I**). These results suggest that BA.2.75 exhibits more severe airway epithelial and endothelial barrier disruption than BA.2.

To further address the fusogenic capacity of BA.2.75, we performed plaque assay using VeroE6/TMPRSS2 cells. Consistent with our previous studies using a Delta isolate (Saito *et al.*, 2022) as well as the recombinant SARS-CoV-2 bearing the B.1.1 S (Yamasoba *et al.*, 2022a), BA.2 S (Yamasoba

et al., 2022a), and BA.5 S (Kimura *et al.*, 2022c), the plaques formed by the infections of clinical isolates of B.1.1, Delta and BA.5 were significantly bigger than those formed by the infection of BA.2 (**Figure 4J**). Notably, BA.2.75 infection also showed significantly bigger plaques than BA.2 infection (**Figure 4J**). Together with the results of cell-based fusion assay (**Figure 3F**) and airway-on-a-chip infection experiments (**Figures 4G–4I**), these observations suggest that BA.2.75 is more fusogenic than BA.2, and the fusogenicity of BA.2.75 is comparable to that of BA.5.

Virological characteristics of BA.2.75 *in vivo*

As we proposed in our prior studies (Kimura *et al.*, 2022c; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Yamasoba *et al.*, 2022b), the fusogenicity of the S proteins of SARS-CoV-2 variants is closely associated with the intrinsic pathogenicity in an experimental hamster model. Here we revealed that both BA.5 and BA.2.75 are more fusogenic than BA.2 in the *in vitro* cell culture systems (**Figures 3 and 4**). Given that the recombinant SARS-CoV-2 bearing the BA.5 S (Kimura *et al.*, 2022c) as well as a clinical isolate of BA.5 (Tamura *et al.*, 2022) exhibited relatively higher pathogenicity than BA.2 in hamsters, it is hypothesized that BA.2.75 is also intrinsically more pathogenic than BA.2. To address this possibility, we intranasally inoculated a BA.2.75 isolate into hamsters. As controls, we also used clinical isolates of Delta, BA.2 and BA.5. While we followed our established experimental protocol (Kimura *et al.*, 2022c; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Yamasoba *et al.*, 2022b), the viral titers of clinical isolates of Omicron subvariants were relatively low. Therefore, we set out to conduct animal experiments in this study with relatively lower titer inoculum (1,000 TCID₅₀ per hamster) than our previous studies (10,000 TCID₅₀ per hamster) (Kimura *et al.*, 2022c; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Yamasoba *et al.*, 2022b). Nevertheless, consistent with our previous study (Saito *et al.*, 2022), the Delta infection exhibited the most severe weight changes among the five groups (**Figure 5A**). While the body weight of BA.2-infected hamsters was similar to that of uninfected hamsters, those of BA.5- and BA.2.75-infected hamsters were significantly lower than that of uninfected hamsters (**Figure 5A**).

We then quantitatively analyzed the pulmonary function of infected hamsters as reflected by three parameters, enhanced pause (Penh), the ratio of time to peak expiratory flow relative to the total expiratory time (Rpef), and breath per minute (BPM), which are surrogate markers for bronchoconstriction or airway obstruction. Subcutaneous oxygen saturation (SpO₂) was also routinely measured. Although the SpO₂ values were comparable among the five groups, Delta infection resulted in significant differences in the other three respiratory parameters compared to BA.2 (**Figure 5A**), suggesting that Delta is more pathogenic than BA.2. There were no differences in the values of Penh,

433 Rpef and BPM between BA.5 and BA.2, and the values of Penh and Rpef of
434 BA.2.75-infected hamsters were comparable to those of BA.2 (**Figure 5A**).
435 However, the BPM value of BA.2.75 was significantly lower than that of BA.2
436 (**Figure 5A**), suggesting that BA.2.75 is slightly more pathogenic than BA.2.

437 To address the viral spread in infected hamsters, we routinely
438 measured the viral RNA load in the oral swab. Although the viral RNA loads of
439 the hamsters infected with Delta, BA.2 and BA.5 were comparable, the viral load
440 in the swabs of BA.2.75-infected hamsters was relatively highly maintained by 7
441 d.p.i. and was significantly higher than that of BA.2-infected hamsters (**Figure**
442 **5B**). To address the possibility that BA.2.75 more efficiently spread in the
443 respiratory tissues, we collected the lungs of infected hamsters at 2 and 5 d.p.i.,
444 and the collected tissues were separated into the hilum and periphery regions.
445 Although the viral RNA loads in both the hilum and periphery of four infection
446 groups were comparable at 2 d.p.i. (**Figure 5C, top**), those of the hamsters
447 infected with Delta, BA.5 and BA.2.75 were significantly higher than those
448 infected with BA.2 at 5 d.p.i. (**Figure 5C, bottom**).

449 To further address the virus spread in the respiratory tissues,
450 immunohistochemical (IHC) analysis targeting viral nucleocapsid (N) protein was
451 conducted. Similar to our previous studies (Kimura *et al.*, 2022c; Suzuki *et al.*,
452 2022; Yamasoba *et al.*, 2022b), epithelial cells in the upper tracheae of infected
453 hamsters were sporadically positive for viral N protein at 2 d.p.i., but there were
454 no significant differences among four viruses including BA.2.75 (**Figure S4A**). In
455 the alveolar space around the bronchi/bronchioles at 2 d.p.i., the N-positive cells
456 were detected in Delta-infected hamsters. On the other hand, the N proteins
457 strongly remained in the lobar bronchi in BA.5- and BA.2.75-infected hamsters
458 (**Figures 5D, top, and S4B**). While few N-positive cells were detected in the
459 alveolar space of BA.2- and BA.5-infected hamsters, it was notable that the N
460 positivity spread into the alveolar space in BA.2.75-infected hamsters (**Figures**
461 **5D, top, and S4B**). The quantification of the N-positive area in total of four lung
462 lobes at 2 d.p.i. (**Figure S4B**) showed that the N-positive areas of Delta- and
463 BA.2.75-infected hamsters were significantly greater than that of BA.2-infected
464 hamsters (**Figure 5E, top**). At 5 d.p.i., although the N-positive cells were hardly
465 detected in the lungs infected with BA.2, a few N-positive cells were detected in
466 the peripheral alveolar space in Delta, BA.5, BA.2.75 (**Figures 5D, bottom, and**
467 **S4C**). The quantification of the N-positive area in the four lung lobes at 5 d.p.i.
468 (**Figure S4C**) further showed that the N-positive areas of Delta- and BA.5- and
469 BA.2.75-infected hamsters were significantly greater than that of BA.2-infected
470 hamsters (**Figure 5E, bottom**). These data suggest that BA.2 targets only a
471 portion of bronchial/bronchiolar epithelium and was less efficiently transmitted to
472 the neighboring epithelial cells. On the other hand, BA.5 and BA.2.75 infections
473 seemed to persist in the bronchial/bronchiolar epithelium, and particularly,

BA.2.75 invaded the alveolar space more efficiently than BA.5 at the early stage of infection. Altogether, the IHC data suggest that among Omicron subvariants, BA.2.75 more efficiently spread into the alveolar space than BA.2 and BA.5, with persistent infection in the bronchi/bronchioles.

Pathogenicity of BA.2.75

To investigate the intrinsic pathogenicity of BA.2.75, the formalin-fixed right lungs of infected hamsters at 2 and 5 d.p.i. were analyzed by carefully identifying the four lobules and main bronchus and lobar bronchi sectioning each lobe along with the bronchial branches. Histopathological scoring was performed according to the criteria described in our previous studies (Kimura *et al.*, 2022c; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Yamasoba *et al.*, 2022b): (i) bronchitis/bronchiolitis (an inflammatory indicator at early stage of infection), (ii) hemorrhage/congestion, (iii) alveolar damage with epithelial apoptosis and macrophage infiltration, (iv) emergence of type II pneumocytes, and (v) hyperplasia of type II pneumocytes were evaluated by certified pathologists and the degree of these pathological findings were arbitrarily scored using four-tiered system as 0 (negative), 1 (weak), 2 (moderate), and 3 (severe). Consistent with our previous studies (Saito *et al.*, 2022; Suzuki *et al.*, 2022), all five parameters as well as the total score of Delta-infected hamsters were significantly higher than those of BA.2-infected hamsters (**Figures 5F and 5G**), suggesting that Delta is more pathogenic than BA.2. When we compare the histopathological scores of Omicron subvariants, the scores indicating hemorrhage or congestion and total histology scores of BA.5 and BA.2.75 were significantly greater than those of BA.2 (**Figures 5F and 5G**). Similar to our recent studies (Kimura *et al.*, 2022c; Tamura *et al.*, 2022), BA.5 is intrinsically more pathogenic than BA.2, and notably, our results suggest that BA.2.75 exhibits more significant inflammation than BA.2. To clarify the area of pneumonia, the inflammatory area, which is mainly composed of the type II pneumocytes with some inflammatory cell types, such as neutrophils, lymphocytes, and macrophages, is termed the area of type II pneumocytes and was morphometrically analyzed (**Figure S4D**). As summarized in **Figure 5H**, at 5 d.p.i., the percentages of the area of type II pneumocytes of Delta, BA.5 and BA.2.75 were significantly higher than that of BA.2. Altogether, these findings suggest that BA.2.75 infection intrinsically induces greater inflammation and exhibits higher pathogenicity than BA.2.

509 Discussion

510 Here, we characterized the virological property of the Omicron BA.2.75 variant,
511 such as the growth rate in the human population, resistance to antiviral humoral
512 immunity and antiviral drugs, functions of S protein *in vitro*, and intrinsic
513 pathogenicity.

514 In terms of the emergence geography and phylogeny, BA.5 and
515 BA.2.75 emerged independently. Nevertheless, the results of cell-based fusion
516 assay, airway-on-a-chip assay and plaque assay suggested that both BA.5 and
517 BA.2.75 acquired higher fusogenicity after the divergence from BA.2. Our data
518 including a recent study (Kimura *et al.*, 2022c) suggest that the critical
519 substitution responsible for the higher fusogenicity of BA.5 and BA.2.75 S
520 proteins are different: the L452R substitution for BA.5 S, and the D339H/N460K
521 substitution for BA.2.75 S.

522 The higher fusogenicity attributed by the increased binding affinity of
523 the L452R-bearing S RBD to human ACE2 was reported in previous studies
524 focusing on the S proteins of previous SARS-CoV-2 variants including Epsilon
525 (Motozono *et al.*, 2021), Delta (Saito *et al.*, 2022) and Omicron BA.5 (Kimura *et al.*, 2022c) variants. The prominently increased ACE2 binding affinity caused by
526 the N460K substitution was also reported in our previous study (Zahradnik *et al.*,
527 2021b). We also demonstrated that the D339H, which is unique in the BA.2.75 S,
528 contributes to increased ACE2 binding affinity. Our data suggest that the N460K
529 and D339H substitutions cooperatively determine the higher fusogenicity of
530 BA.2.75 S. In our previous studies focusing on Delta (Saito *et al.*, 2022),
531 Omicron BA.1 (Suzuki *et al.*, 2022), BA.2 (Yamasoba *et al.*, 2022b) and BA.5
532 (Kimura *et al.*, 2022c), we proposed a close association between the S-mediated
533 fusogenicity *in vitro* and the pathogenicity in a hamster model. Consistent with
534 our hypothesis, here we demonstrated that, compared to BA.2, BA.2.75 exhibits
535 higher fusogenicity *in vitro* and efficient viral spread in the lungs of infected
536 hamsters, which leads to enhanced inflammation in the lung and higher
537 pathogenicity *in vivo*. Moreover, *in vitro* experiments using a variety of cell
538 culture systems showed that BA.2.75 replicates more efficiently than BA.2 in
539 alveolar epithelial cells but not in airway epithelial cells. Altogether, our results
540 suggest that BA.2.75 exhibits higher fusogenicity and pathogenicity via evolution
541 of its S protein independently of BA.5.

542 Consistent with our previous study (Yamasoba *et al.*, 2022b),
543 neutralization experiments showed that BA.5 was significantly more resistant to
544 the humoral immunity induced by vaccination and breakthrough infections of
545 prior Omicron subvariants. On the other hand, the sensitivity of BA.2.75 to these
546 antisera was comparable to BA.2. More importantly, BA.2.75 was highly
547 resistant to the BA.5-induced immunity. These results suggest that, although
548 both BA.2.75 and BA.5 are descendants of BA.2, their immunogenicity is
549

different from each other. Furthermore, compared to BA.2, the sensitivity of BA.2.75 and BA.5 to therapeutic monoclonal antibodies was also different (Yamasoba *et al.*, 2022a). The G446S was also closely associated with the resistance of BA.2.75 to the antiviral effects of BA.2- and BA.5-infected hamster sera. Because the G446S significantly decreases ACE2 affinity of S RBD, this substitution was acquired to evade antiviral immunity, and the other substitutions in RBD, particularly N460K, contributed to compensate for the decreased ACE2 binding affinity by G446S.

Another remarkable substitution pattern in the BA.2.75 S is the multiple substitutions in the S NTD. Particularly, three out of the five substitutions in the NTD (K147E, W152R and F157L) are located in a well-studied region, the NTD supersite. Previous studies showed that the mutations in the NTD supersite are responsible for the resistance to antiviral monoclonal antibodies (Cerutti *et al.*, 2021; Chi *et al.*, 2020; Liu *et al.*, 2020; Lok, 2021; McCallum *et al.*, 2021; Suryadevara *et al.*, 2021; Voss *et al.*, 2021). In fact, our results suggested that these three substitutions in the NTD supersite are closely associated with the evasion from BA.5-induced humoral immunity, in addition to the G446S in RBD. In fact, the W152 has been shown as a mutational hot spot of SARS-CoV-2 (Kubik *et al.*, 2021). Therefore, BA.2.75 might mutate this specific residue to evade neutralization by sera of convalescent or vaccinated individuals.

According to the “COVID-19 Treatment Guidelines” issued by NIH (NIH, 2022), the use of Paxlovid (Ritonavir and Nirmatrelvir), Remdesivir and Molnupiravir (a prodrug of EIDD-1931) is highly recommended as treatment of patients who do not require hospitalization or oxygen supplement. Because the evolution of SARS-CoV-2 is unpredictable, timely and accurate testing of the efficacy of currently available antiviral drugs is indispensable to treat patients infected with a new variant. Our results using physiologically relevant human AO demonstrated that BA.2.75 retained the sensitivity to major small-molecule anti-SARS-CoV-2 drugs including Remdesivir, EIDD-1931 and Nirmatrelvir. Interestingly, BA.2.75 was more sensitive to Remdesivir than other stains, and a similar tendency was observed with EIDD-1931. In terms of drug testing, previous studies addressed the antiviral activity of these drugs against BA.2 and BA.5 using immortalized cell lines such as VeroE6/TMPRSS2 cells (Takashita *et al.*, 2022c), Caco-2-F03 cells (Bojkova *et al.*, 2022) and Calu-3 cells (Carlin *et al.*, 2022) but the effects of these antiviral drugs were different each other, and these results were also different from ours (**Table 1**). In addition, a previous study demonstrated that VeroE6 cells have a low capacity to metabolize Remdesivir, leading to a weak antiviral activity (Pruijssers *et al.*, 2020). These results suggest that the experimental system significantly affects the outcome of antiviral drug efficacy, raising the importance to evaluate the efficacy of antiviral drugs using

590 physiologically relevant systems, such as organoids and organ-on-a-chip
591 systems.

592 Our investigation using the viral genome surveillance data reported
593 from India suggested that BA.2.75 bears the potential to outcompete BA.2 as
594 well as BA.5, the most predominant variant in the world as of August 2022.
595 Following the worldwide spread of BA.5, it is probable that the number of
596 individuals infected with BA.5 will increase. Together with our findings showing
597 the higher resistance of BA.2.75 to the BA.5-induced immunity, there appears to
598 be sufficient plausibility that BA.2.75 evades the BA.5-induced immunity, and
599 this property will confer this variant to more efficient spread in the countries
600 where BA.5 has been widely spreading, such as Australia and Japan.
601 Additionally, here we showed that the intrinsic pathogenicity of BA.2.75 in
602 hamsters is comparable to BA.5 and higher than that of BA.2. Since a recent
603 study showed that the hospitalization risk of BA.5 was significantly higher than
604 that of BA.2 in the once-boosted vaccinated population (Kislaya et al., 2022), it is
605 not unreasonable to infer that the intrinsic pathogenicity in infected hamsters
606 reflects to the severity and outcome in infected humans to a meaningful extent.

607 In summary, our multiscale investigations revealed the growth rate in
608 the human population, fusogenicity and intrinsic pathogenicity of BA.2.75 are
609 greater than BA.2. These features of BA.2.75 suggests the potential risk of this
610 variant to global health. Since BA.2.75 shows significantly higher R_e than BA.2
611 and BA.5 in India, this variant will probably transmit to and initiate outcompeting
612 BA.2 and BA.5 in some countries other than India in the near future. To assess
613 the potential risk of BA.2.75 to global health, this variant should be under
614 monitoring carefully and continuously through worldwide cooperation of in-depth
615 viral genomic surveillance.

616 **STAR[®]METHODS**

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654 **Supplemental Information**

655 Additional Supplemental Items are available upon request.

656

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687 **Conflict of interest**

688 The authors declare that no competing interests exist.

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1066 **Table 1. Effects of three antiviral drugs against BA.2.75 in AO**

	EC ₅₀ (μM)				EC ₅₀ ratio				
	B.1.1	BA.2	BA.5	BA.2.75	BA.5/BA.2	BA.2.75/BA.2	BA.2/B.1.1	BA.5/B.1.1	BA.2.75/B.1.1
Remdesivir	1.08	1.89	1.31	0.63	0.70	0.34	1.75	1.21	0.59
EIDD-1931	0.24	0.02	0.21	0.08	8.82	3.53	0.10	0.89	0.36
Nirmatrelvir	0.84	0.85	0.63	0.81	0.74	0.95	1.02	0.75	0.97

1067

Figure legends

Figure 1. Epidemics of BA.2.75 in India

(A) A maximum likelihood tree of Omicron sublineages. Sequences of BA.1–BA.5 sampled from South Africa and BA.2.75 are included. The mutations acquired in the S protein of BA.2.75 are indicated in the panel. Note that R493Q is a reversion [i.e., back mutation from the BA.1–BA.3 lineages (R493) to the B.1.1 lineage (Q493)]. Bootstrap values, *, ≥ 0.8 ; **, ≥ 0.95 .

(B) Amino acid differences among BA.2, BA.2.75, and BA.5. Heatmap color indicates the frequency of amino acid substitutions.

(C) Lineage frequencies of BA.5 (left) and BA.2.75 (right) in each Indian state. SARS-CoV-2 sequences collected from June 15, 2022, to July 15, 2022, were analyzed.

(D) Epidemic dynamics of SARS-CoV-2 lineages in Indian states. Results for BA.2.75 and BA.5 are shown. The observed daily sequence frequency (dot) and the dynamics (posterior mean, line; 95% CI, ribbon) are shown. The dot size is proportional to the number of sequences.

(E) Estimated relative R_e of each viral lineage, assuming a fixed generation time of 2.1 days. The R_e value of BA.2 is set at 1. The posterior (violin), posterior mean (dot), and 95% CI (line) are shown. The average values across India estimated by a Bayesian hierarchical model are shown, and the state-specific R_e values are shown in **Figure S1B**. The dynamics of the top seven predominant lineages in India were estimated. BA.5 sublineages are summarized as “BA.5”, and non-predominant BA.2 sublineages are summarized as “other BA.2”.

See also **Figure S1 and Table S1**.

Figure 2. Immune resistance of BA.2.75

Neutralization assays were performed with pseudoviruses harboring the S proteins of B.1.1 (the D614G-bearing ancestral virus), BA.1, BA.2, BA.2.75. Delta pseudovirus is included only in the experiment shown in **F**. The BA.2 S-based derivatives are included in **H and I**. The following sera were used.

(A–C) BNT162b2 vaccine sera (15 donors) collected at 1 month after 2nd-dose vaccination (A), 1 month after 3rd-dose vaccination (B), and 4 months after 3rd-dose vaccination (C).

(D) Convalescent sera from fully vaccinated individuals who had been infected with BA.1 after full vaccination (16 2-dose vaccinated donors).

(E) Convalescent sera from fully vaccinated individuals who had been infected with BA.2 after full vaccination (9 2-dose vaccinated and 5 3-dose vaccinated. 14 donors in total).

(F) Convalescent sera from unvaccinated individuals who had been infected with Delta (18 donors).

1109 (G–I) Sera from hamsters infected with BA.1 (6 hamsters) (G), BA.2 (6
1110 hamsters) (H), and BA.5 (6 hamsters) (I).
1111 Assays for each serum sample were performed in triplicate to determine the
1112 50% neutralization titer (NT50). Each dot represents one NT50 value, and the
1113 geometric mean and 95% CI are shown. The numbers in the panels indicate the
1114 fold change resistance versus BA.2 (B–E, G and H) or BA.5 (I). The horizontal
1115 dashed line indicates the detection limit (120-fold in other than F, 40-fold in F).
1116 Statistically significant differences were determined by two-sided Wilcoxon
1117 signed-rank tests. The *P* values versus BA.2 (B–E, G and H) or BA.5 (I) are
1118 indicated in the panels. For the BA.2 derivatives and B.1.1 (H and I), statistically
1119 significant differences versus BA.2 (*P* < 0.05) are indicated with asterisks.
1120 Information on the vaccinated/convalescent donors is summarized in Table S2.
1121 See also Table S2.

1122 Figure 3. Virological features of BA.2.75 S *in vitro*

1123 (A) Pseudovirus assay. The percent infectivity compared to that of the virus
1124 pseudotyped with the BA.2 S protein are shown.
1125 (B) Binding affinity of the RBD of SARS-CoV-2 S protein to ACE2 by yeast
1126 surface display. The *K_D* value indicating the binding affinity of the RBD of the
1127 SARS-CoV-2 S protein to soluble ACE2 when expressed on yeast is shown.
1128 (C) Electrostatic potential of B.1.1 S RBD (PDB: 6M17) (Yan et al., 2020), BA.2
1129 S RBD (PDB: 7UB0) (Stalls et al., 2022) and BA.2.75 S RBD. The structure of
1130 BA.2.75 S RBD was prepared using AlphaFold2 (Mirdita *et al.*, 2022).
1131 Electrostatic potential surface depictions calculated by PDB2PQR tool (Dolinsky
1132 et al., 2007) with the positions of BA.2.75 characteristic mutations. The scale bar
1133 shows the electrostatic charge [kT/e].
1134 (D) The binding of BA.2.75 S RBD and human ACE2 (PDB: 6M17) (Yan *et al.*,
1135 2020). Left, the four substitutions in BA.2.75 S RBD compared to BA.2 S RBD
1136 are highlighted. Right, binding of BA.2.75 S RBD (top) and human ACE2
1137 (bottom). The electrostatic potential surface of human ACE2 is shown.
1138 (E and F) S-based fusion assay. (E) S protein expression on the cell surface.
1139 The summarized data are shown. (F) S-based fusion assay in Calu-3 cells. The
1140 recorded fusion activity (arbitrary units) is shown. The dashed green line
1141 indicates the results of BA.2.
1142 Assays were performed in quadruplicate (A and F) or triplicate (B and E), and
1143 the presented data are expressed as the average ± SD. In A and B, the dashed
1144 horizontal lines indicated the value of BA.2. In A, B and E, each dot indicates the
1145 result of an individual replicate. In A, B and E, statistically significant differences
1146 between BA.2 and other variants (*, *P* < 0.05) were determined by two-sided
1147 Student's *t* tests. In F, statistically significant differences between BA.2 and other

1149 variants across timepoints were determined by multiple regression. The FWERs
1150 calculated using the Holm method are indicated in the figures.
1151 See also **Figure S3**.

1152

1153 **Figure 4. Growth capacity of BA.2.75 *in vitro***

1154 **(A–I)** Growth kinetics of B.1.1, Delta, BA.2, BA.5 and BA.2.75. Clinical isolates of
1155 B.1.1 (strain TKYE610670; GISAID ID: EPI_ISL_479681), Delta (B.1.617.2,
1156 strain TKYTK1734; GISAID ID: EPI_ISL_2378732), BA.2 (strain TY40-385;
1157 GISAID ID: EPI_ISL_9595859), BA.5 (strain TKYS14631; GISAID ID:
1158 EPI_ISL_12812500), and BA.2.75 (strain TY41-716; GISAID ID:
1159 EPI_ISL_13969765) were inoculated into Vero cells **(A)**, VeroE6/TMPRSS2
1160 cells **(B)**, HEK293-ACE2/TMPRSS2 cells **(C)**, AO-ALI **(D)**, iPSC-derived airway
1161 epithelial cells **(E)**, iPSC-derived lung epithelial cells **(F)**, and an
1162 airway-on-a-chip system **(G and H)**; the scheme of experimental system is
1163 illustrated in **Figure S3C**). The copy numbers of viral RNA in the culture
1164 supernatant **(A–C)**, the apical sides of cultures **(D–F)**, the top **(G)** and bottom **(H)**
1165 channels of an airway-on-a-chip were routinely quantified by RT-qPCR. The
1166 dashed green line in each panel indicates the results of BA.2. In **I**, the
1167 percentage of viral RNA load in the bottom channel per top channel at 6 d.p.i.
1168 (i.e., % invaded virus from the top channel to the bottom channel) is shown.
1169 **(J)** Plaque assay. VeroE6/TMPRSS2 cells were used for the target cells.
1170 Representative panels (left) and a summary of the recorded plaque diameters
1171 (20 plaques per virus) (right) are shown.
1172 Assays were performed in quadruplicate, and the presented data are expressed
1173 as the average \pm SD. In **A–H**, statistically significant differences between BA.2
1174 and the other variants across timepoints were determined by multiple regression.
1175 The FWERs calculated using the Holm method are indicated in the figures. In **I**
1176 **and J (right)**, statistically significant differences versus BA.2 (*, $P < 0.05$) were
1177 determined by two-sided Mann–Whitney U tests. Each dot indicates the result of
1178 an individual replicate.

1179 See also **Figure S3**.

1180

1181 **Figure 5. Virological characteristics of BA.2.75 *in vivo***

1182 Syrian hamsters were intranasally inoculated with Delta, BA.2, BA.5 and
1183 BA.2.75. Six hamsters at the same age were intranasally inoculated with saline
1184 (uninfected). Six hamsters per each group were used to routinely measure
1185 respective parameters **(A and B)**. Four hamsters per each group were
1186 euthanized at 2 and 5 d.p.i and used for virological and pathological analysis
1187 **(C–G)**.

1188 **(A)** Body weight, Penh, Rpef, BPM, and SpO₂ values of infected hamsters (n = 6
1189 per infection group).

1190 **(B)** Viral RNA loads in the oral swab (n = 6 per infection group).
 1191 **(C)** Viral RNA loads in the lung hilum (left) and lung periphery (right) of infected
 1192 hamsters (n = 4 per infection group) at 2 d.p.i. (top) and 5 d.p.i. (bottom).
 1193 **(D and E)** IHC of the viral N protein in the lungs at 2 d.p.i. (top) and 5 d.p.i.
 1194 (bottom) of all infected hamsters. **(D)** Representative figures. **(E)** Percentage of
 1195 N-positive cells in whole lung lobes (n = 4 per infection group). The raw data are
 1196 shown in **Figure S4B and S4C**.
 1197 **(F and G)** **(F)** H&E staining of the lungs of infected hamsters. Representative
 1198 figures are shown. Uninfected lung alveolar space and bronchioles are also
 1199 shown. **(G)** Histopathological scoring of lung lesions (n = 4 per infection group).
 1200 Representative pathological features are reported in our previous studies
 1201 (Kimura *et al.*, 2022c; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Yamasoba *et al.*,
 1202 2022b).
 1203 **(H)** Type II pneumocytes in the lungs of infected hamsters. The percentage of
 1204 the area of type II pneumocytes in the lung at 5 d.p.i. is summarized. The raw
 1205 data are shown in **Figure S4D**.
 1206 In **A–C, E, G and H**, data are presented as the average \pm SEM. In **C, E and H**,
 1207 each dot indicates the result of an individual hamster.
 1208 In **A, B and G**, statistically significant differences between BA.2 and other
 1209 variants across timepoints were determined by multiple regression. In **A**, the 0
 1210 d.p.i. data were excluded from the analyses. The FWERs calculated using the
 1211 Holm method are indicated in the figures.
 1212 In **C, E and G**, the statistically significant differences between BA.2 and other
 1213 variants were determined by a two-sided Mann–Whitney *U* test.
 1214 In **D and F**, each panel shows a representative result from an individual infected
 1215 hamster. Scale bars, 500 μ m (**D**); 200 μ m (**F**).
 1216 See also **Figure S4**.
 1217
 1218 **Table S1.** Estimated relative R_e values of viral lineages in India, related to
 1219 **Figure 1**
 1220
 1221 **Table S2.** Human sera used in this study, related to **Figure 2**
 1222
 1223 **Table S3.** Primers used for the construction of SARS-CoV-2 S expression
 1224 plasmids, related to **Figures 2 and 3**
 1225
 1226 **Table S4.** Summary of unexpected amino acid mutations detected in the
 1227 working virus stocks, related to **Figures 4 and 5 and Table 1**
 1228
 1229 **Figure S1.** Epidemic dynamics of BA.2.75 in India, related to **Figure 1**

1230 (A) Amino acid differences in B.1.1, Delta, BA.2, BA.5 and BA.2.75 compared to
1231 the SARS-CoV-2 A lineage. Heatmap color indicates the frequency of amino
1232 acid mutations.

1233 (B) Estimated relative R_e of each viral lineage, assuming a fixed generation time
1234 of 2.1 days. The R_e value of BA.2 is set at 1. The posterior (violin), posterior
1235 mean (dot), and 95% CI (line) are shown. The R_e values for respective Indian
1236 states are shown. The dynamics of the top seven predominant lineages in India
1237 were estimated. BA.5 sublineages are summarized as “BA.5”, and
1238 non-predominant BA.2 sublineages are summarized as “other BA.2”. Raw data
1239 are summarized in **Table S1**.

1240 (C) Fold change in R_e values between BA.2.75 and BA.5. Posterior mean (dot)
1241 and 95% CI (line) are shown. Red indicates that the 95% CI does not overlap
1242 with the value of 1.

1243

1244 **Figure S2. Effects of antiviral drugs in AO, related to Table 1**

1245 (A) Antiviral effects of the three drugs in AO culture. The assay of each antiviral
1246 drugs was performed in quadruplicate, and the 50% effective concentration
1247 (EC_{50}) was calculated. The data are summarized in **Table 1**.

1248 (B) Cytotoxic effects of the three drugs in AO culture. The assay of each antiviral
1249 drugs was performed in quadruplicate, and the 50% cytotoxic concentration
1250 (CC_{50}) was calculated. The CC_{50} values are indicated in the panels.

1251

1252 **Figure S3. Virological features of BA.2.75 *in vitro*, related to Figures 3 and** 1253 **4**

1254 (A) Fold increase in pseudovirus infectivity based on TMPRSS2 expression.

1255 (B) The structural effect of the D339H substitution in the BA.2.75 S RBD. The
1256 BA.2 S RBD (PDB: 7UB0) (Stalls *et al.*, 2022) and an AlphaFold2 structural
1257 model of BA.2.75 S RBD (bottom) are shown. The residues 339 and 343 are
1258 indicated in stick. The squared regions are enlarged in the right panel. A dashed
1259 line in the top panel indicates ion-dipole interaction between the D339 and the
1260 N343 residues.

1261 (C) A scheme of airway-on-a-chip system.

1262

1263 **Figure S4. Histological observations in infected hamsters, related to** 1264 **Figure 5**

1265 (A) IHC of the viral N protein in the middle portion of the tracheas of all infected
1266 hamsters at 2 d.p.i (4 hamsters per infection group). Each panel shows a
1267 representative result from an individual infected hamster.

1268 (B and C) IHC of the SARS-CoV-2 N protein in the lungs of infected hamsters at
1269 2 d.p.i. (B) and 5 d.p.i. (C) (4 hamsters per infection group). In each panel, IHC
1270 staining (top) and the digitalized N-positive area (bottom, indicated in red) are

1271 shown. The red numbers in the bottom panels indicate the percentage of the
 1272 N-positive area. Summarized data are shown in **Figure 5E**.
 1273 **(D)** Type II pneumocytes in the lungs of infected hamsters (4 hamsters per
 1274 infection group). H&E staining (top) and the digitalized inflammatory area with
 1275 type II pneumocytes (bottom, indicated in red) are shown. The red numbers in
 1276 the bottom panels indicate the percentage of inflammatory area with type II
 1277 pneumocytes. Summarized data are shown in **Figure 5H**.
 1278 Scale bars, 1 mm (**A**); 5 mm (**B–D**).

1279 **STAR[®]METHODS**

1280

1281 **KEY RESOURCES TABLE**

1282 **RESOURCE AVAILABILITY**

1283 **Lead Contact**

1284 Further information and requests for resources and reagents should be directed
1285 to and will be fulfilled by the Lead Contact, Kei Sato
1286 (KeiSato@g.ecc.u-tokyo.ac.jp).

1287

1288 **Materials Availability**

1289 All unique reagents generated in this study are listed in the Key Resources
1290 Table and available from the Lead Contact with a completed Materials Transfer
1291 Agreement.

1292

1293 **Data and Software Availability**

1294 All databases/datasets used in this study are available from GISAID database
1295 (<https://www.gisaid.org>) and GenBank database (<https://www.gisaid.org>;
1296 EPI_SET ID: EPI_SET_220804hy).

1297 The computational codes used in the present study, the raw data of
1298 virus sequences, and the GISAID supplemental table for EPI_SET ID:
1299 EPI_SET_220804hy are available in the GitHub repository
1300 (https://github.com/TheSatoLab/Omicron_BA.2.75).

1301

1302 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

1303 **Ethics statement**

1304 All experiments with hamsters were performed in accordance with the Science
1305 Council of Japan's Guidelines for the Proper Conduct of Animal Experiments.
1306 The protocols were approved by the Institutional Animal Care and Use
1307 Committee of National University Corporation Hokkaido University (approval ID:
1308 20-0123 and 20-0060). All experiments with mice were also performed in
1309 accordance with the Science Council of Japan's Guidelines for the Proper
1310 Conduct of Animal Experiments. All protocols involving specimens from human
1311 subjects recruited at Kyoto University were reviewed and approved by the
1312 Institutional Review Boards of Kyoto University (approval ID: G1309) and Chiba
1313 University (approval ID: HS202103-03). All human subjects provided written
1314 informed consent. All protocols for the use of human specimens were reviewed
1315 and approved by the Institutional Review Boards of The Institute of Medical
1316 Science, The University of Tokyo (approval IDs: 2021-1-0416 and
1317 2021-18-0617), Kyoto University (approval ID: G0697), Kumamoto University
1318 (approval IDs: 2066 and 2074), and University of Miyazaki (approval ID:
1319 O-1021).

1320

1321 Human serum collection

1322 Vaccine sera of fifteen individuals who had BNT162b2 vaccine
1323 (Pfizer/BioNTech) (average age: 38, range: 24–48; 53% male) (**Figures 2A–2C**)
1324 were obtained at one month after the second dose, one month after the third
1325 dose, and four months after the third dose. The details of the vaccine sera are
1326 summarized in **Table S2**.

1327 Convalescent sera were collected from the following donors: fully
1328 vaccinated individuals who had been infected with BA.1 (16 2-dose vaccinated.
1329 10–27 days after testing; average age: 48, range: 20–76, 44% male) (**Figure**
1330 **2D**), fully vaccinated individuals who had been infected with BA.2 (9 2-dose
1331 vaccinated and 5 3-dose vaccinated. 11–61 days after testing. n=14 in total;
1332 average age: 47, range: 24–84, 64% male) (**Figure 2E**), and unvaccinated
1333 individuals who had been infected with Delta (6–55 days after testing. n=18 in
1334 total; average age: 50, range: 22–67, 78% male) (**Figure 2F**). The SARS-CoV-2
1335 variants were identified as previously described (Kimura *et al.*, 2022c;
1336 Yamasoba *et al.*, 2022b). Sera were inactivated at 56°C for 30 minutes and
1337 stored at –80°C until use. The details of the convalescent sera are summarized
1338 in **Table S2**.

1339

1340 Cell culture

1341 HEK293T cells (a human embryonic kidney cell line; ATCC, CRL-3216),
1342 HEK293 cells (a human embryonic kidney cell line; ATCC, CRL-1573) and
1343 HOS-ACE2/TMPRSS2 cells (HOS cells stably expressing human ACE2 and
1344 TMPRSS2) (Ferreira *et al.*, 2021; Ozono *et al.*, 2021) were maintained in DMEM
1345 (high glucose) (Sigma-Aldrich, Cat# 6429-500ML) containing 10% fetal bovine
1346 serum (FBS, Sigma-Aldrich Cat# 172012-500ML) and 1%
1347 penicillin-streptomycin (PS) (Sigma-Aldrich, Cat# P4333-100ML).

1348 HEK293-ACE2 cells (HEK293 cells stably expressing human ACE2) (Motozono
1349 *et al.*, 2021) were maintained in DMEM (high glucose) containing 10% FBS, 1
1350 µg/ml puromycin (InvivoGen, Cat# ant-pr-1) and 1% PS.

1351 HEK293-ACE2/TMPRSS2 cells (HEK293 cells stably expressing human ACE2
1352 and TMPRSS2) (Motozono *et al.*, 2021) were maintained in DMEM (high
1353 glucose) containing 10% FBS, 1 µg/ml puromycin, 200 ng/ml hygromycin
1354 (Nacalai Tesque, Cat# 09287-84) and 1% PS.

1355 Vero cells [an African green monkey (*Chlorocebus sabaeus*) kidney cell line;
1356 JCRB Cell Bank, JCRB0111] were maintained in Eagle's minimum essential
1357 medium (EMEM) (Sigma-Aldrich, Cat# M4655-500ML) containing 10% FBS and
1358 1% PS.

1359 VeroE6/TMPRSS2 cells (VeroE6 cells stably expressing human TMPRSS2;
1360 JCRB Cell Bank, JCRB1819) (Matsuyama *et al.*, 2020) were maintained in

DMEM (low glucose) (Wako, Cat# 041-29775) containing 10% FBS, G418 (1 mg/ml; Nacalai Tesque, Cat# G8168-10ML) and 1% PS. Calu-3/DSP₁₋₇ cells (Calu-3 cells stably expressing DSP₁₋₇) (Yamamoto et al., 2020) were maintained in EMEM (Wako, Cat# 056-08385) containing 20% FBS and 1% PS. Human airway and alveolar epithelial cells derived from human induced pluripotent stem cells (iPSCs) were manufactured according to established protocols as described below (see “Preparation of human airway and alveolar epithelial cells from human iPSCs” section) and provided by HiLung Inc. Airway organoids (AO) and AO-derived air-liquid interface model (AO-ALI) were generated according to established protocols as described below (see “Airway organoids” and “AO-ALI model” sections).

1373

1374 **METHOD DETAILS**

1375 **Viral genome sequencing**

1376 Viral genome sequencing was performed as previously described (Meng *et al.*,
1377 2022; Motozono *et al.*, 2021; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Yamasoba *et al.*, 2022b). Briefly, the virus sequences were verified by viral RNA-sequencing
1378 analysis. Viral RNA was extracted using a QIAamp viral RNA mini kit (Qiagen,
1379 Cat# 52906). The sequencing library employed for total RNA sequencing was
1380 prepared using the NEB next ultra RNA library prep kit for Illumina (New England
1381 Biolabs, Cat# E7530). Paired-end 76-bp sequencing was performed using a
1382 MiSeq system (Illumina) with MiSeq reagent kit v3 (Illumina, Cat# MS-102-3001).
1383 Sequencing reads were trimmed using fastp v0.21.0 (Chen et al., 2018) and
1384 subsequently mapped to the viral genome sequences of a lineage B isolate
1385 (strain Wuhan-Hu-1; GenBank accession number: NC_045512.2) (Matsuyama
1386 *et al.*, 2020) using BWA-MEM v0.7.17 (Li and Durbin, 2009). Variant calling,
1387 filtering, and annotation were performed using SAMtools v1.9 (Li et al., 2009)
1388 and snpEff v5.0e (Cingolani et al., 2012).

1390

1391 **Phylogenetic analyses**

1392 To construct an ML tree of Omicron lineages (BA.1–BA.5) sampled from South
1393 Africa and BA.2.75 (shown in **Figure 1A**), the genome sequence data of
1394 SARS-CoV-2 and its metadata were downloaded from the GISAID database
1395 (<https://www.gisaid.org/>) (Khare et al., 2021) on July 23, 2022. We excluded the
1396 data of viral strains with the following features from the analysis: i) a lack
1397 collection date information; ii) sampling from animals other than humans, iii) >2%
1398 undetermined nucleotide characters, or iv) sampling by quarantine. From each
1399 viral lineage, 30 sequences were randomly sampled and used for tree
1400 construction, in addition to an outgroup sequence, EPI_ISL_466615,
1401 representing the oldest isolate of B.1.1 obtained in the UK. The viral genome

1402 sequences were mapped to the reference sequence of Wuhan-Hu-1 (GenBank
1403 accession number: NC_045512.2) using Minimap2 v2.17 (Li, 2018) and
1404 subsequently converted to a multiple sequence alignment according to the
1405 GISAID phylogenetic analysis pipeline
1406 (<https://github.com/roblanf/sarscov2phylo>). The alignment sites corresponding to
1407 the 1–265 and 29674–29903 positions in the reference genome were masked
1408 (i.e., converted to NNN). Alignment sites at which >50% of sequences contained
1409 a gap or undetermined/ambiguous nucleotide were trimmed using trimAl v1.2
1410 (Capella-Gutierrez et al., 2009). Phylogenetic tree construction was performed
1411 via a three-step protocol: i) the first tree was constructed; ii) tips with longer
1412 external branches (Z score > 4) were removed from the dataset; iii) and the final
1413 tree was constructed. Tree reconstruction was performed by RAxML v8.2.12
1414 (Stamatakis, 2014) under the GTRCAT substitution model. The node support
1415 value was calculated by 100 times bootstrap analysis.

1416

1417 **Modelling the epidemic dynamics of SARS-CoV-2 lineages**

1418 To quantify the spread rate of each SARS-CoV-2 lineage in the human
1419 population in India, we estimated the relative R_e of each viral lineage according
1420 to the epidemic dynamics, calculated on the basis of viral genomic surveillance
1421 data. The data were downloaded from the GISAID database
1422 (<https://www.gisaid.org/>) on August 1, 2022. We excluded the data of viral
1423 strains with the following features from the analysis: i) a lack of collection date
1424 information; ii) sampling in animals other than humans; or iii) sampling by
1425 quarantine. We analyzed the datasets of the ten states of India, where ≥ 20
1426 sequences of either BA.2.75 or BA.5 are reported (i.e., Himachal Pradesh,
1427 Odisha, Haryana, Rajasthan, and Maharashtra, Gujarat, West Bengal, Delhi,
1428 Tamil Nadu, and Telangana). BA.5 sublineages are summarized as “BA.5”, and
1429 BA.2 sublineages with ≤ 400 sequences are summarized as “other BA.2”.
1430 Subsequently, the dynamics of the top seven predominant lineages in India were
1431 estimated from April 24, 2022, to August 1, 2022, were analyzed. The number of
1432 viral sequences of each viral lineage collected on each day in each country was
1433 counted, and the count matrix was constructed as an input for the statistical
1434 model below.

1435 We constructed a Bayesian hierarchical model to represent relative
1436 lineage growth dynamics with multinomial logistic regression as described in our
1437 previous study (Yamasoba *et al.*, 2022b). In brief, we incorporated a hierarchical
1438 structure into the slope parameter over time, which enabled us to estimate the
1439 global average relative R_e of each viral lineage in India as well as the average
1440 value for each country. Arrays in the model index over one or more indices: $L = 7$
1441 viral lineages l ; $S = 10$ states s ; and $T = 100$ days t . The model is:

$$\beta_{ls} \sim \text{Student_t}(6, \beta_l, \sigma_l)$$

$$\begin{aligned}\mu_{lst} &= \alpha_{ls} + \beta_{ls}t \\ \theta_{.st} &= \text{softmax}(\mu_{.st}) \\ y_{lst} &\sim \text{Multinomial}\left(\sum_l y_{lst}, \theta_{.st}\right)\end{aligned}$$

1442 The explanatory variable was time, t , and the outcome variable was y_{lst} , which
1443 represented the count of viral lineage l in state s at time t . The slope
1444 parameter of lineage l in state s , β_{ls} , was generated from a Student's t
1445 distribution with hyperparameters of the mean, β_l , and the standard deviation,
1446 σ_l . As the distribution generating β_{ls} , we used a Student's t distribution with six
1447 degrees of freedom instead of a normal distribution to reduce the effects of
1448 outlier values of β_{ls} . In the model, the linear estimator $\mu_{.st}$, consisting of the
1449 intercept $\alpha_{.s}$ and the slope $\beta_{.s}$, was converted to the simplex $\theta_{.st}$, which
1450 represented the probability of occurrence of each viral lineage at time t in state
1451 s , based on the softmax link function defined as:

$$\text{softmax}(x) = \frac{\exp(x)}{\sum_i \exp(x_i)}$$

1452 y_{lst} is generated from $\theta_{.st}$ and the total count of all lineages at time t in state s
1453 according to a multinomial distribution.

1454 The relative R_e of each viral lineage in each county (r_{ls}) was calculated
1455 according to the slope parameter β_{ls} as:

$$r_{ls} = \exp(\gamma\beta_{ls})$$

1456 where γ is the average viral generation time (2.1 days)
1457 ([http://sonorouschocolate.com/covid19/index.php?title=Estimating_Generation_](http://sonorouschocolate.com/covid19/index.php?title=Estimating_Generation_Time_Of_Omicron)
1458 [Time_Of_Omicron](http://sonorouschocolate.com/covid19/index.php?title=Estimating_Generation_Time_Of_Omicron)). Similarly, the global average relative R_e of each viral lineage
1459 was calculated according to the slope hyperparameter β_l as:

$$r_l = \exp(\gamma\beta_l)$$

1460 For parameter estimation, the intercept and slope parameters of the BA.2 variant
1461 were fixed at 0. Consequently, the relative R_e of BA.2 was fixed at 1, and those
1462 of the other lineages were estimated relative to that of BA.2.

1463 Parameter estimation was performed via the MCMC approach
1464 implemented in CmdStan v2.28.1 (<https://mc-stan.org>) with CmdStanr v0.4.0
1465 (<https://mc-stan.org/cmdstanr/>). Noninformative priors were set for all
1466 parameters. Four independent MCMC chains were run with 1,000 and 2,000
1467 steps in the warmup and sampling iterations, respectively. We confirmed that all
1468 estimated parameters showed <1.01 R-hat convergence diagnostic values and
1469 >200 effective sampling size values, indicating that the MCMC runs were
1470 successfully convergent. The above analyses were performed in R v4.1.3
1471 (<https://www.r-project.org/>). Information on the relative R_e estimated in the
1472 present study is summarized in **Table S1**.
1473

1474 **Plasmid construction**

1475 Plasmids expressing the codon-optimized SARS-CoV-2 S proteins of B.1.1 (the
1476 parental D614G-bearing variant), BA.2 and BA.5 were prepared in our previous
1477 studies (Kimura *et al.*, 2022a; Ozono *et al.*, 2021; Saito *et al.*, 2022; Suzuki *et al.*,
1478 2022; Yamasoba *et al.*, 2022b). Plasmids expressing the codon-optimized S
1479 proteins of BA.2.75 and the BA.2 S-based derivatives were generated by
1480 site-directed overlap extension PCR using the primers listed in **Table S3**. The
1481 resulting PCR fragment was digested with KpnI and NotI and inserted into the
1482 corresponding site of the pCAGGS vector (Niwa *et al.*, 1991). Nucleotide
1483 sequences were determined by DNA sequencing services (Eurofins), and the
1484 sequence data were analyzed by Sequencher v5.1 software (Gene Codes
1485 Corporation).

1486

1487 **Neutralization assay**

1488 Pseudoviruses were prepared as previously described (Kimura *et al.*, 2022a;
1489 Kimura *et al.*, 2022b; Kimura *et al.*, 2022c; Motozono *et al.*, 2021; Uriu *et al.*,
1490 2022; Uriu *et al.*, 2021; Yamasoba *et al.*, 2022a; Yamasoba *et al.*, 2022b;
1491 Yamasoba *et al.*, 2022c). Briefly, lentivirus (HIV-1)-based, luciferase-expressing
1492 reporter viruses were pseudotyped with the SARS-CoV-2 S proteins. HEK293T
1493 cells (1,000,000 cells) were cotransfected with 1 µg psPAX2-IN/HiBiT (Ozono *et al.*,
1494 2020), 1 µg pWPI-Luc2 (Ozono *et al.*, 2020), and 500 ng plasmids
1495 expressing parental S or its derivatives using PEI Max (Polysciences, Cat#
1496 24765-1) according to the manufacturer's protocol. Two days posttransfection,
1497 the culture supernatants were harvested and centrifuged. The pseudoviruses
1498 were stored at -80°C until use.

1499 Neutralization assay (**Figure 2**) was prepared as previously described
1500 (Kimura *et al.*, 2022a; Kimura *et al.*, 2022b; Kimura *et al.*, 2022c; Saito *et al.*,
1501 2022; Uriu *et al.*, 2022; Uriu *et al.*, 2021; Yamasoba *et al.*, 2022a; Yamasoba *et al.*,
1502 2022b; Yamasoba *et al.*, 2022c). Briefly, the SARS-CoV-2 S pseudoviruses
1503 (counting ~20,000 relative light units) were incubated with serially diluted
1504 (120-fold to 87,480-fold dilution at the final concentration) heat-inactivated sera
1505 at 37°C for 1 hour. Pseudoviruses without sera were included as controls. Then,
1506 a 40 µl mixture of pseudovirus and serum/antibody was added to
1507 HOS-ACE2/TMPRSS2 cells (10,000 cells/50 µl) in a 96-well white plate. At 2
1508 d.p.i., the infected cells were lysed with a One-Glo luciferase assay system
1509 (Promega, Cat# E6130) or a Bright-Glo luciferase assay system (Promega, Cat#
1510 E2650), and the luminescent signal was measured using a GloMax explorer
1511 multimode microplate reader 3500 (Promega) or CentroXS3 (Berthold
1512 Technologies). The assay of each serum was performed in triplicate, and the
1513 50% neutralization titer (NT₅₀) was calculated using Prism 9 software v9.1.1
1514 (GraphPad Software).

1515

Airway organoids

1517 Airway organoids (AO) model was generated according to our previous report
1518 (Sano *et al.*, 2022). Briefly, normal human bronchial epithelial cells (NHBE, Cat#
1519 CC-2540, Lonza) were used to generate AO. NHBE were suspended in 10
1520 mg/ml cold Matrigel growth factor reduced basement membrane matrix
1521 (Corning). 50 µl of cell suspension was solidified on pre-warmed cell-culture
1522 treated multi-dishes (24-well plates; Thermo Fisher Scientific) at 37 °C for 10
1523 min, and then 500 µl of expansion medium was added to each well. AO were
1524 cultured with AO expansion medium for 10 days. To mature the AO, expanded
1525 AO were cultured with AO differentiation medium for 5 days. In experiments
1526 evaluating the antiviral drugs (see “Antiviral drug assay using SARS-CoV-2
1527 clinical isolates and AO” section below), AO were dissociated into single cells,
1528 and then were seeded into 96-well plates.

1529

SARS-CoV-2 preparation and titration

1531 The working virus stocks of SARS-CoV-2 were prepared and titrated as
1532 previously described (Kimura *et al.*, 2022b; Kimura *et al.*, 2022c; Meng *et al.*,
1533 2022; Motozono *et al.*, 2021; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Yamasoba *et al.*,
1534 2022b). In this study, clinical isolates of B.1.1 (strain TKYE610670; GISAID
1535 ID: EPI_ISL_479681) (Suzuki *et al.*, 2022), Delta (B.1.617.2, strain TKYTK1734;
1536 GISAID ID: EPI_ISL_2378732) (Saito *et al.*, 2022), BA.2 (strain TY40-385;
1537 GISAID ID: EPI_ISL_9595859) (Kimura *et al.*, 2022c) and BA.5 (strain
1538 TKYS14631; GISAID ID: EPI_ISL_12812500) (Tamura *et al.*, 2022), and
1539 BA.2.75 (strain TY41-716; GISAID ID: EPI_ISL_13969765) were used. In brief,
1540 20 µl of the seed virus was inoculated into VeroE6/TMPRSS2 cells (5,000,000
1541 cells in a T-75 flask). One h.p.i., the culture medium was replaced with DMEM
1542 (low glucose) (Wako, Cat# 041-29775) containing 2% FBS and 1% PS. At 3
1543 d.p.i., the culture medium was harvested and centrifuged, and the supernatants
1544 were collected as the working virus stock.

1545 The titer of the prepared working virus was measured as the 50%
1546 tissue culture infectious dose (TCID₅₀). Briefly, one day before infection,
1547 VeroE6/TMPRSS2 cells (10,000 cells) were seeded into a 96-well plate. Serially
1548 diluted virus stocks were inoculated into the cells and incubated at 37°C for 4
1549 days. The cells were observed under microscopy to judge the CPE appearance.
1550 The value of TCID₅₀/ml was calculated with the Reed–Muench method (Reed
1551 and Muench, 1938).

1552 To verify the sequences of SARS-CoV-2 working viruses, viral RNA
1553 was extracted from the working viruses using a QIAamp viral RNA mini kit
1554 (Qiagen, Cat# 52906) and viral genome sequences were analyzed as described
1555 above (see "Viral genome sequencing" section). Information on the unexpected

substitutions detected is summarized in **Table S4**, and the raw data are deposited in the GitHub repository (https://github.com/TheSatoLab/Omicron_BA.2.75).

Antiviral drug assay using SARS-CoV-2 clinical isolates and AO

Antiviral drug assay (**Table 1 and Figure S2A**) was performed as previously described (Meng *et al.*, 2022). Briefly, one day before infection, AO (10,000 cells) was dissociated, and then seeded into a 96-well plate. The cells were infected with either B.1.1, BA.2, BA.5 or BA.2.75 isolate (100 TCID₅₀) at 37°C for 2 hours. The cells were washed with DMEM and cultured in DMEM supplemented with 10% FCS, 1% PS and the serially diluted Remdesivir (Clinisciences, Cat# A17170), EIDD-1931 (an active metabolite of Molnupiravir; Cell Signalling Technology, Cat# 81178S), or Nirmatrelvir (PF-07321332; MedChemExpress, Cat# HY-138687). At 24 hours after infection, the culture supernatants were collected, and viral RNA was quantified using RT-qPCR (see “RT-qPCR” section below). The assay of each compound was performed in quadruplicate, and the 50% effective concentration (EC₅₀) was calculated using Prism 9 software v9.1.1 (GraphPad Software).

Cytotoxicity assay

The cytotoxicity of Remdesivir, EIDD-1931 or Nirmatrelvir (**Figure S2B**) was performed as previously described (Meng *et al.*, 2022). Briefly, one day before the assay, AO (10,000 cells) was dissociated and then seeded into a 96-well plate. The cells were cultured with the serially diluted antiviral drugs for 24 hours. The cell counting kit-8 (Dojindo, Cat# CK04-11) solution (10 µl) was added to each well, and the cells were incubated at 37°C for 90 min. Absorbance was measured at 450 nm using the Multiskan FC (Thermo Fisher Scientific). The assay of each compound was performed in quadruplicate, and the 50% cytotoxic concentration (CC₅₀) was calculated using Prism 9 software v9.1.1 (GraphPad Software).

Pseudovirus infection

Pseudovirus infection (**Figure 3A**) was performed as previously described (Ferreira *et al.*, 2021; Kimura *et al.*, 2022a; Kimura *et al.*, 2022b; Kimura *et al.*, 2022c; Motozono *et al.*, 2021; Uriu *et al.*, 2022; Uriu *et al.*, 2021; Yamasoba *et al.*, 2022a; Yamasoba *et al.*, 2022b; Yamasoba *et al.*, 2022c). Briefly, the amount of pseudoviruses prepared was quantified by the HiBiT assay using Nano Glo HiBiT lytic detection system (Promega, Cat# N3040) as previously described (Ozono *et al.*, 2021; Ozono *et al.*, 2020), and the same amount of pseudoviruses (normalized to the HiBiT value, which indicates the amount of p24 HIV-1 antigen) was inoculated into HOS-ACE2/TMPRSS2 cells,

HEK293-ACE2 cells or HEK293-ACE2/TMPRSS2 and viral infectivity was measured as described above (see “Neutralization assay” section). To analyze the effect of TMPRSS2 for pseudovirus infectivity (**Figure S3A**), the fold change of the values of HEK293-ACE2/TMPRSS2 to HEK293-ACE2 was calculated.

Yeast surface display

Yeast surface display (**Figure 3B**) was performed as previously described (Dejnirattisai *et al.*, 2022; Kimura *et al.*, 2022a; Kimura *et al.*, 2022b; Kimura *et al.*, 2022c; Motozono *et al.*, 2021; Yamasoba *et al.*, 2022b; Zahradnik *et al.*, 2021b). Briefly, the RBD genes [“construct 3” in (Zahradnik *et al.*, 2021b), covering residues 330–528] in pJYDC1 plasmid were cloned by restriction enzyme-free cloning and transformed into the EBY100 *Saccharomyces cerevisiae*. Primers are listed in **Table S3**. The expression media 1/9 (Zahradnik *et al.*, 2021a) was inoculated (OD 1) by overnight (220 rpm, 30°C, SD-CAA media) grown culture and cultivated for 24 hours at 20°C. The media was supplemented by 10 mM DMSO solubilized bilirubin (Sigma-Aldrich, Cat# 14370-1G) for expression co-cultivation labeling (pJYDC1, eUnaG2 reporter holo-form formation, green/yellow fluorescence (Ex. 498 nm, Em. 527 nm). Cells (100 ul aliquots) were collected by centrifugation (3000 g, 3 minutes), washed in ice-cold PBSB buffer (PBS with 1 g/L BSA), and resuspended in an analysis solution with a series of CF®640R succinimidyl ester labeled (Biotium, USA, Cat# 92108) ACE2 peptidase domain (residues 18–740) concentrations. The reaction volume was adjusted (1–100 ml) to avoid the ligand depletion effect, and the suspension was incubated overnight in a rotator shaker (10 rpm, 4°C). Incubated samples were washed by PBSB buffer, transferred into 96 well plates (Thermo, USA, Nunc, Cat# 268200), and analyzed by the CytoFLEX S Flow Cytometer (Beckman Coulter, USA, Cat#. N0-V4-B2-Y4) with the gating strategy described previously (Zahradnik *et al.*, 2021b). The eUnaG2 signals were compensated by the instrument CytExpert software (Beckman Coulter). The mean binding signal (FL4-A) values of RBD expressing cells, subtracted by signals of non-expressing populations, were subjected for the determination of binding constant K_D , Y_D by non-cooperative Hill equation fitted by nonlinear least-squares regression using Python v3.7 fitted together with two additional parameters describing titration curve (Zahradnik *et al.*, 2021b).

AlphaFold2

To generate the structure model of BA.2.75 S RBD (**Figures 3C, 3D and S3B**), the AlphaFold2 structural prediction was performed using ColabFold (Mirdita *et al.*, 2022) using the BA.2 S RBD template (PDB: 7UB0) (Stalls *et al.*, 2022). The MMseqs2 and HHsearch parameters were set as default. The models were manually inspected, and those exhibiting poor parameters and models that did

not adopt the classical RBD interface conformation were eliminated. The two highest score models were analyzed in detail. Three-dimensional visualization and analyses were performed using PyMOL v2.1.1 (Schrödinger, <https://pymol.org/2/>). In **Figure 3D**, the wild-type SARS-CoV-2 S RBD of the crystal structure of RBD-ACE2 complex (PDB:6M17) (Yan *et al.*, 2020) was replaced with the BA.2.75 S RBD structure generated by AlphaFold2.

SARS-CoV-2 S-based fusion assay

SARS-CoV-2 S-based fusion assay (**Figures 3E and 3F**) was performed as previously described (Kimura *et al.*, 2022b; Kimura *et al.*, 2022c; Motozono *et al.*, 2021; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Yamasoba *et al.*, 2022b). Briefly, on day 1, effector cells (i.e., S-expressing cells) and target cells (Calu-3/DSP₁₋₇ cells) were prepared at a density of $0.6\text{--}0.8 \times 10^6$ cells in a 6-well plate. On day 2, to prepare effector cells, HEK293 cells were cotransfected with the S expression plasmids (400 ng) and pDSP₈₋₁₁ (Kondo *et al.*, 2011) (400 ng) using TransIT-LT1 (Takara, Cat# MIR2300). On day 3 (24 hours posttransfection), 16,000 effector cells were detached and reseeded into 96-well black plates (PerkinElmer, Cat# 6005225), and target cells were reseeded at a density of 1,000,000 cells/2 ml/well in 6-well plates. On day 4 (48 hours posttransfection), target cells were incubated with EnduRen live cell substrate (Promega, Cat# E6481) for 3 hours and then detached, and 32,000 target cells were added to a 96-well plate with effector cells. *Renilla* luciferase activity was measured at the indicated time points using Centro XS3 LB960 (Berthold Technologies). To measure the surface expression level of S protein, effector cells were stained with rabbit anti-SARS-CoV-2 S S1/S2 polyclonal antibody (Thermo Fisher Scientific, Cat# PA5-112048, 1:100). Normal rabbit IgG (SouthernBiotech, Cat# 0111-01, 1:100) was used as negative controls, and APC-conjugated goat anti-rabbit IgG polyclonal antibody (Jackson ImmunoResearch, Cat# 111-136-144, 1:50) was used as a secondary antibody. Surface expression level of S proteins (**Figure 3E**) was measured using FACS Canto II (BD Biosciences) and the data were analyzed using FlowJo software v10.7.1 (BD Biosciences). To calculate fusion activity, *Renilla* luciferase activity was normalized to the MFI of surface S proteins. The normalized value (i.e., *Renilla* luciferase activity per the surface S MFI) is shown as fusion activity.

AO-ALI model

AO-ALI model (**Figure 4D**) was generated according to our previous report (Sano *et al.*, 2022). To generate AO-ALI, expanding AO were dissociated into single cells, and then were seeded into Transwell inserts (Corning) in a 24-well plate. To promote their maturation, AO-ALI were cultured with AO differentiation medium for 5 days. AO-ALI were infected with SARS-CoV-2 from the apical side.

1679

1680 **Preparation of human airway and alveolar epithelial cells from human iPSC**

1681 The air-liquid interface culture of airway and alveolar epithelial cells (**Figures 4E**
1682 **and 4F**) were differentiated from human iPSC-derived lung progenitor cells as
1683 previously described (Gotoh et al., 2014; Kimura et al., 2022c; Konishi et al.,
1684 2016; Tamura et al., 2022; Yamamoto et al., 2017). Briefly, lung progenitor cells
1685 were stepwise induced from human iPSCs referring a 21-days and 4-steps
1686 protocol (Yamamoto et al., 2017). At day 21, lung progenitor cells were isolated
1687 with specific surface antigen carboxypeptidase M and seeded onto upper
1688 chamber of 24-well Cell Culture Insert (Falcon, #353104), followed by 28-day
1689 and 7-day differentiation of airway and alveolar epithelial cells, respectively.
1690 Alveolar differentiation medium supplemented with dexamethasone
1691 (Sigma-Aldrich, Cat# D4902), KGF (PeproTech, Cat# 100-19), 8-Br-cAMP
1692 (Biolog, Cat# B007), 3-Isobutyl 1-methylxanthine (IBMX) (FUJIFILM Wako, Cat#
1693 095-03413), CHIR99021 (Axon Medchem, Cat# 1386), and SB431542
1694 (FUJIFILM Wako, Cat# 198-16543) was used for induction of alveolar epithelial
1695 cells. PneumaCult ALI (STEMCELL Technologies, Cat# ST-05001)
1696 supplemented with heparin (Nacalai Tesque, Cat# 17513-96) and Y-27632 (LC
1697 Laboratories, Cat# Y-5301) hydrocortisone (Sigma-Aldrich, Cat# H0135) was
1698 used for induction of airway epithelial cells.

1699

1700 **Airway-on-a-chips**

1701 Airway-on-a-chips (**Figure S3C**) were prepared as previously described
1702 (Hashimoto et al., 2022). Human lung microvascular endothelial cells
1703 (HMVEC-L) were obtained from Lonza (Cat# CC-2527) and cultured with
1704 EGM-2-MV medium (Lonza, Cat# CC-3202). To prepare the airway-on-a-chip,
1705 first, the bottom channel of a polydimethylsiloxane (PDMS) device was
1706 pre-coated with fibronectin (3 µg/ml, Sigma, Cat# F1141). The microfluidic
1707 device was generated according to our previous report (Deguchi et al., 2021).
1708 HMVEC-L were suspended at 5,000,000 cells/ml in EGM2-MV medium. Then,
1709 10 µl suspension medium was injected into the fibronectin-coated bottom
1710 channel of the PDMS device. Then, the PDMS device was turned upside down
1711 and incubated for 1 h. After 1 hour, the device was turned over, and the
1712 EGM2-MV medium was added into the bottom channel. After 4 days, AO were
1713 dissociated and seeded into the top channel. The AO was generated according
1714 to our previous report (Sano et al., 2022). AO were dissociated into single cells
1715 and then suspended at 5,000,000 cells/ml in the AO differentiation medium. Ten
1716 microliter suspension medium was injected into the top channel. After 1 hour, the
1717 AO differentiation medium was added to the top channel. In the infection
1718 experiments (**Figures 4G–4I**), the AO differentiation medium containing either
1719 B.1.1, Delta, BA.2, BA.5 or BA.2.75 isolate (500 TCID₅₀) was inoculated from the

1720 top channel (**Figure S3C**). At 2 h.p.i., the top and bottom channels were washed
1721 and cultured with AO differentiation and EGM2-MV medium, respectively. The
1722 culture supernatants were collected, and viral RNA was quantified using
1723 RT-qPCR (see “RT-qPCR” section above).

1724

1725 **Microfluidic device**

1726 The microfluidic device was generated according to our previous report (Deguchi
1727 *et al.*, 2021). Briefly, the microfluidic device consisted of two layers of
1728 microchannels separated by a semipermeable membrane. The microchannel
1729 layers were fabricated from PDMS using a soft lithographic method. PDMS
1730 prepolymer (SYLGARD 184, Dow Corning) at a base to curing agent ratio of
1731 10:1 was cast against a mold composed of SU-8 2150 (MicroChem) patterns
1732 formed on a silicon wafer. The cross-sectional size of the microchannels was 1
1733 mm in width and 330 μ m in height. To introduce solutions into the microchannels,
1734 access holes were punched through the PDMS using a 6-mm biopsy punch (Kai
1735 Corporation). Two PDMS layers were bonded to a PET membrane containing
1736 3.0 μ m pores (Cat# 353091, Falcon) using a thin layer of liquid PDMS
1737 prepolymer as the mortar. PDMS prepolymer was spin-coated (4000 rpm for 60
1738 sec) onto a glass slide. Subsequently, both the top and bottom channel layers
1739 were placed on the glass slide to transfer the thin layer of PDMS prepolymer
1740 onto the embossed PDMS surfaces. The membrane was then placed onto the
1741 bottom layer and sandwiched with the top layer. The combined layers were left
1742 at room temperature for 1 day to remove air bubbles and then placed in an oven
1743 at 60°C overnight to cure the PDMS glue. The PDMS devices were sterilized by
1744 placing them under UV light for 1 hour before the cell culture.

1745

1746 **SARS-CoV-2 infection**

1747 One day before infection, Vero cells (10,000 cells), VeroE6/TMPRSS2 cells
1748 (10,000 cells), and HEK293-ACE2/TMPRSS2 cells were seeded into a 96-well
1749 plate. SARS-CoV-2 [1,000 TCID₅₀ for Vero cells (**Figure 4A**); 100 TCID₅₀ for
1750 VeroE6/TMPRSS2 cells (**Figure 4B**) and HEK293-ACE2/TMPRSS2 cells
1751 (**Figure 4C**)] was inoculated and incubated at 37°C for 1 hour. The infected cells
1752 were washed, and 180 μ l culture medium was added. The culture supernatant
1753 (10 μ l) was harvested at the indicated timepoints and used for RT-qPCR to
1754 quantify the viral RNA copy number (see “RT-qPCR” section below). In the
1755 infection experiments using human iPSC-derived airway and alveolar epithelial
1756 cells (**Figures 4E and 4F**), working viruses were diluted with Opti-MEM (Thermo
1757 Fisher Scientific, 11058021). The diluted viruses (1,000 TCID₅₀ in 100 μ l) were
1758 inoculated onto the apical side of the culture and incubated at 37°C for 1 hour.
1759 The inoculated viruses were removed and washed twice with Opti-MEM. To
1760 collect the viruses, 100 μ l Opti-MEM was applied onto the apical side of the

1761 culture and incubated at 37°C for 10 minutes. The Opti-MEM was collected
1762 and used for RT-qPCR to quantify the viral RNA copy number (see “RT-qPCR”
1763 section below). The infection experiments using an airway-on-a-chip system
1764 (**Figures 4G–4I**) was performed as described above (see “Airway-on-a-chips”
1765 section).

1766 1767 **RT-qPCR**

1768 RT-qPCR was performed as previously described (Kimura *et al.*, 2022b; Kimura
1769 *et al.*, 2022c; Meng *et al.*, 2022; Motozono *et al.*, 2021; Saito *et al.*, 2022; Suzuki
1770 *et al.*, 2022; Yamasoba *et al.*, 2022b). Briefly, 5 µl culture supernatant was mixed
1771 with 5 µl 2 × RNA lysis buffer [2% Triton X-100 (Nacalai Tesque, Cat# 35501-15),
1772 50 mM KCl, 100 mM Tris-HCl (pH 7.4), 40% glycerol, 0.8 U/µl recombinant
1773 RNase inhibitor (Takara, Cat# 2313B)] and incubated at room temperature for
1774 10 min. RNase-free water (90 µl) was added, and the diluted sample (2.5 µl) was
1775 used as the template for real-time RT-PCR performed according to the
1776 manufacturer's protocol using One Step TB Green PrimeScript PLUS RT-PCR
1777 kit (Takara, Cat# RR096A) and the following primers: Forward *N*, 5'-AGC CTC
1778 TTC TCG TTC CTC ATC AC-3'; and Reverse *N*, 5'-CCG CCA TTG CCA GCC
1779 ATT C-3'. The viral RNA copy number was standardized with a SARS-CoV-2
1780 direct detection RT-qPCR kit (Takara, Cat# RC300A). Fluorescent signals were
1781 acquired using QuantStudio 1 Real-Time PCR system (Thermo Fisher Scientific),
1782 QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific), QuantStudio
1783 5 Real-Time PCR system (Thermo Fisher Scientific), CFX Connect Real-Time
1784 PCR Detection system (Bio-Rad), Eco Real-Time PCR System (Illumina),
1785 qTOWER3 G Real-Time System (Analytik Jena) Thermal Cycler Dice Real Time
1786 System III (Takara) or 7500 Real-Time PCR System (Thermo Fisher Scientific).

1787 1788 **Plaque assay**

1789 Plaque assay (**Figure 4J**) was performed as previously described (Kimura *et al.*,
1790 2022b; Kimura *et al.*, 2022c; Suzuki *et al.*, 2022; Yamasoba *et al.*, 2022b).
1791 Briefly, one day before infection, VeroE6/TMPRSS2 cells (100,000 cells) were
1792 seeded into a 24-well plate and infected with SARS-CoV-2 (0.5, 5, 50 and 500
1793 TCID₅₀) at 37°C for 1 hour. Mounting solution containing 3% FBS and 1.5%
1794 carboxymethyl cellulose (Wako, Cat# 039-01335) was overlaid, followed by
1795 incubation at 37°C. At 3 d.p.i., the culture medium was removed, and the cells
1796 were washed with PBS three times and fixed with 4% paraformaldehyde
1797 phosphate (Nacalai Tesque, Cat# 09154-85). The fixed cells were washed with
1798 tap water, dried, and stained with staining solution [0.1% methylene blue
1799 (Nacalai Tesque, Cat# 22412-14) in water] for 30 minutes. The stained cells
1800 were washed with tap water and dried, and the size of plaques was measured
1801 using Fiji software v2.2.0 (ImageJ).

1802

Animal experiments

1804 Animal experiments (**Figure 5**) were performed as previously described (Kimura
1805 *et al.*, 2022b; Kimura *et al.*, 2022c; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Tamura
1806 *et al.*, 2022; Yamasoba *et al.*, 2022b). Syrian hamsters (male, 4 weeks old) were
1807 purchased from Japan SLC Inc. (Shizuoka, Japan). For the virus infection
1808 experiments, hamsters were euthanized by intramuscular injection of a mixture
1809 of 0.15 mg/kg medetomidine hydrochloride (Domitor®, Nippon Zenyaku Kogyo),
1810 2.0 mg/kg midazolam (Dormicum®, FUJIFILM Wako Chemicals) and 2.5 mg/kg
1811 butorphanol (Vetorphale®, Meiji Seika Pharma) or 0.15 mg/kg medetomidine
1812 hydrochloride, 4.0 mg/kg alphaxalone (Alfaxan®, Jurox) and 2.5 mg/kg
1813 butorphanol. The Delta, BA.2, BA.5 and BA.2.75 (1,000 TCID₅₀ in 100 µl), or
1814 saline (100 µl) were intranasally inoculated under anesthesia. Oral swabs were
1815 collected at indicated timepoints. Body weight was recorded daily by 7 d.p.i.
1816 Enhanced pause (Penh), the ratio of time to peak expiratory flow relative to the
1817 total expiratory time (Rpef), and BPM were measured every day until 7 d.p.i.
1818 (see below). Subcutaneous oxygen saturation (SpO₂, see below) was monitored
1819 at 0, 1, 3, 5, and 7 d.p.i. Lung tissues were anatomically collected at 2 and 5 d.p.i.
1820 Viral RNA load in the oral swabs and respiratory tissues were determined by
1821 RT-qPCR. These tissues were also used for IHC and histopathological analyses
1822 (see below).

1823

Lung function test

1825 Lung function test (**Figure 5A**) was routinely performed as previously described
1826 (Kimura *et al.*, 2022b; Kimura *et al.*, 2022c; Saito *et al.*, 2022; Suzuki *et al.*,
1827 2022; Tamura *et al.*, 2022; Yamasoba *et al.*, 2022b). The three respiratory
1828 parameters (Penh, Rpef and BPM) were measured by using a whole-body
1829 plethysmography system (DSI) according to the manufacturer's instructions. In
1830 brief, a hamster was placed in an unrestrained plethysmography chamber and
1831 allowed to acclimatize for 30 seconds, then, data were acquired over a
1832 2.5-minute period by using FinePointe Station and Review softwares
1833 v2.9.2.12849 (STARR). The state of oxygenation was examined by measuring
1834 SpO₂ using pulse oximeter, MouseOx PLUS (STARR). SpO₂ was measured by
1835 attaching a measuring chip to the neck of hamsters sedated by 0.25 mg/kg
1836 medetomidine hydrochloride.

1837

Immunohistochemistry

1839 Immunohistochemistry (IHC) (**Figures 5D, S4A–S4C**) was performed as
1840 previously described (Kimura *et al.*, 2022b; Kimura *et al.*, 2022c; Saito *et al.*,
1841 2022; Suzuki *et al.*, 2022; Tamura *et al.*, 2022; Yamasoba *et al.*, 2022b) using an
1842 Autostainer Link 48 (Dako). The deparaffinized sections were exposed to

EnVision FLEX target retrieval solution high pH (Agilent, Cat# K8004) for 20 minutes at 97°C to activate, and mouse anti-SARS-CoV-2 N monoclonal antibody (clone 1035111, R&D systems, Cat# MAB10474-SP, 1:400) was used as a primary antibody. The sections were sensitized using EnVision FLEX (Agilent) for 15 minutes and visualized by peroxidase-based enzymatic reaction with 3,3'-diaminobenzidine tetrahydrochloride (Dako, Cat# DM827) as substrate for 5 minutes. The N protein positivity (**Figures 5E, S4A and S4B**) was evaluated by certificated pathologists as previously described (Kimura *et al.*, 2022b; Kimura *et al.*, 2022c; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Tamura *et al.*, 2022; Yamasoba *et al.*, 2022b). Images were incorporated as virtual slide by NDP.scan software v3.2.4 (Hamamatsu Photonics). The N-protein positivity was measured as the area using Fiji software v2.2.0 (ImageJ).

H&E staining

H&E staining (**Figures 5F and S4D**) was performed as previously described (Kimura *et al.*, 2022b; Kimura *et al.*, 2022c; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Tamura *et al.*, 2022; Yamasoba *et al.*, 2022b). Briefly, excised animal tissues were fixed with 10% formalin neutral buffer solution, and processed for paraffin embedding. The paraffin blocks were sectioned with 3 µm-thickness and then mounted on MAS-GP-coated glass slides (Matsunami Glass, Cat# S9901). H&E staining was performed according to a standard protocol.

Histopathological scoring

Histopathological scoring (**Figure 5G**) was performed as previously described (Kimura *et al.*, 2022b; Kimura *et al.*, 2022c; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Tamura *et al.*, 2022; Yamasoba *et al.*, 2022b). Pathological features including (i) bronchitis or bronchiolitis, (ii) hemorrhage with congestive edema, (iii) alveolar damage with epithelial apoptosis and macrophage infiltration, (iv) hyperplasia of type II pneumocytes, and (v) the area of the hyperplasia of large type II pneumocytes were evaluated by certified pathologists and the degree of these pathological findings were arbitrarily scored using four-tiered system as 0 (negative), 1 (weak), 2 (moderate), and 3 (severe). The "large type II pneumocytes" are the hyperplasia of type II pneumocytes exhibiting more than 10-µm-diameter nucleus. We described "large type II pneumocytes" as one of the remarkable histopathological features reacting SARS-CoV-2 infection in our previous studies (Kimura *et al.*, 2022b; Kimura *et al.*, 2022c; Saito *et al.*, 2022; Suzuki *et al.*, 2022; Tamura *et al.*, 2022; Yamasoba *et al.*, 2022b). Total histology score is the sum of these five indices.

To measure the inflammation area in the infected lungs (**Figures 5H and S4D**), four hamsters infected with each virus were sacrificed at 5 d.p.i., and all four right lung lobes, including upper (anterior/cranial), middle, lower

(posterior/caudal), and accessory lobes, were sectioned along with their bronchi. The tissue sections were stained by H&E, and the digital microscopic images were incorporated into virtual slides using NDP.scan software v3.2.4 (Hamamatsu Photonics). The inflammatory area including type II pneumocyte hyperplasia in the infected whole lungs was morphometrically analyzed using Fiji software v2.2.0 (ImageJ).

1890

1891 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1892 Statistical significance was tested using a two-sided Mann–Whitney *U*-test, a
1893 two-sided Student's *t*-test or a two-sided paired *t*-test unless otherwise noted.
1894 The tests above were performed using Prism 9 software v9.1.1 (GraphPad
1895 Software).

1896 In the time-course experiments (**Figures 3F, 4A–4H, 5A, 5B, and 5G**),
1897 a multiple regression analysis including experimental conditions (i.e., the types
1898 of infected viruses) as explanatory variables and timepoints as qualitative control
1899 variables was performed to evaluate the difference between experimental
1900 conditions thorough all timepoints. The initial time point was removed from the
1901 analysis. *P* value was calculated by a two-sided Wald test. Subsequently,
1902 familywise error rates (FWERs) were calculated by the Holm method. These
1903 analyses were performed in R v4.1.2 (<https://www.r-project.org/>).

1904 In **Figures 5D, 5F and S4**, photographs shown are the representative
1905 areas of at least two independent experiments by using four hamsters at each
1906 timepoint.









