Impact of reinfection with SARS-CoV-2 Omicron variants in previously infected hamsters

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Running Head: Reinfection of SARS-CoV-2 variant in a hamster model

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ABSTRACT

The diversity of SARS-CoV-2 mutations raises the possibility of reinfection of individuals previously infected with earlier variants, and this risk is further increased by the emergence of the B.1.1.529 Omicron variant. In this study, we used an *in vivo*, hamster infection model to assess the potential for individuals previously infected with SARS-CoV-2 to be reinfected with Omicron variant and we also investigated the pathology associated with such infections. Initially, Syrian hamsters were inoculated with a lineage A, B.1.1.7, B.1.351, B.1.617.2 or a subvariant of Omicron, BA.1 strain and then reinfected with the BA.1 strain 5 weeks later. Subsequently, the impact of reinfection with Omicron subvariants (BA.1 and BA.2) in individuals previously infected with the BA.1 strain was examined. Although viral infection and replication were suppressed in both the upper and lower airways, following reinfection, virus-associated RNA was detected in the airways of most hamsters. Viral replication was more strongly suppressed in the lower respiratory tract than in the upper respiratory tract. Consistent amino acid substitutions were observed in the upper respiratory tract of infected hamsters after primary infection with variant BA.1, whereas diverse mutations appeared in hamsters reinfected with the same variant. Histopathology showed no acute pneumonia or disease enhancement in any of the reinfection groups and, in addition, the expression of inflammatory cytokines and chemokines in the airways of reinfected animals was only mildly elevated. These findings are important for understanding the risk of reinfection with new variants of SARS-CoV-2.

IMPORTANCE

The emergence of SARS-CoV-2 variants and the widespread use of COVID-19 vaccines has resulted in individual differences in immune status against SARS-CoV-2. A decay in immunity over time and the emergence of variants that partially evade the immune response can also lead to reinfection. In this study, we demonstrated that, in hamsters, immunity acquired following primary infection with previous SARS-CoV-2 variants was effective in preventing the onset of pneumonia after reinfection with the Omicron variant. However, viral infection and multiplication in the upper respiratory tract were still observed after reinfection. We also showed that more diverse nonsynonymous mutations appeared in the upper respiratory tract of reinfected hamsters that had acquired immunity from primary infection. This hamster model reveals the within-host evolution of SARS-CoV-2 and its pathology after reinfection, and provides important information for countermeasures against diversifying SARS-CoV-2 variants.

58 INTRODUCTION

59 After the emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-
60 CoV-2), at the end of 2019, various Variants of Concern (VOC) emerged, including the
61 B.1.1.7 (Alpha), B.1.351 (Beta) and B.1.617.2 (Delta) strains, allowing COVID-19
62 infection to spread and persist worldwide (1). At the end of November 2021, the World
63 Health Organization designated a variant of SARS-CoV-2, B.1.1.529, as a VOC (2). The
64 B.1.1.529, Omicron variant, has 30 amino acid substitutions, three deletions, and one
65 insertion site in the spike region compared with the ancestral, Wuhan, SARS-CoV-2 strain,
66 including 15 mutations in the receptor binding region (3). The emergence of Omicron
67 strains with high transmission capacity has changed the infection risk situation for the
68 current COVID-19 pandemic (3, 4). Although several studies indicate that Omicron
69 variants cause less severe disease than the Delta variant (5), it is clear that an individual
70 infected with an earlier VOC or Omicron strain is still at risk of reinfection with the next
71 variant, including Omicron subvariant (6). In addition, infection with Omicron is
72 considered primarily as an upper respiratory tract infection. This differs from the
73 pathophysiology associated with the earlier VOC, which tended to cause lower respiratory
74 tract infection (7-10). Thus, there is increasing concern about the efficacy of immunity of
75 previously infected individuals against the new variants and impact of immunopathology
76 due to reinfection of SARS-CoV-2 (11, 12).

77 The Syrian hamster is more susceptible to SARS-CoV-2 than other animal species (13).
78 Weight loss, clinical signs, pathology, and immune response can be used as indicators of
79 viral infection in hamsters, making them a useful small animal model for development of
80 vaccines and antiviral agents for COVID-19 (13-15). Several research groups have also
81 used this animal model to investigate the phenotype of mutant viruses by testing for

82 changes in infectivity, infectiousness, and antigenicity (16, 17). Hansen et al used a hamster
83 model to conduct reinfection experiments with a homologous, ancestor strain, WA1, and
84 heterologous B.1.1.7 (Alpha) and B.1.351 (Beta) SARS-CoV-2 variants to determine the
85 transmission through reinfection of asymptomatic individuals (18). Reinfection leads to
86 SARS-CoV-2 replication in the upper respiratory tract with the potential for virus shedding,
87 suggesting the risk of transmission through reinfected asymptomatic individuals. On the
88 other hand, another group showed that prior infection with the WA1 strain prevented Delta
89 variant transmission to naïve hamsters (19). In this study, therefore, we used a hamster
90 model to i) evaluate the risk of reinfection with Omicron BA.1 strain, following infection
91 with prior VOC strains, and ii) determine the risk of reinfection with Omicron subvariants
92 in individuals first infected with the Omicron BA.1 strain.
93
94 RESULTS
95 Primary infection of hamsters with SARS-CoV-2 variants.
96 Five isolates of SARS-CoV-2 including the ancestor strain from lineage A, and four isolates
97 of the VOC (B.1.1.7, B.1.351, B.1.617.2 and B.1.1.529; BA.1.18 [referred to as BA.1];
98 Table 1) were used for primary inoculation of Syrian hamsters as depicted in Figure 1A.
99 Because hamsters are highly susceptible to SARS-CoV-2, primary infection was conducted
100 by intranasal inoculation of a low dose of virus (1.0×10^3 $TCID_{50}$ in 8 μL of Dulbecco's
101 Modified Eagle Medium (DMEM)), which reached the local upper respiratory tract, and
102 induced seroconversion. Five weeks after primary infection, the lower respiratory tract of
103 animals was then reinfected with a higher dose (1.0×10^4 $TCID_{50}$ in 50 μL) of virus.
104 After the primary inoculation, animals infected with isolates from lineage A, B.1.1.7, and
105 B.1.617.2 showed significantly lower weight gain rate than the mock-infection group

106 (DMEM in Figure 1B, n = 3–4) in the first week post-inoculation (p.i.) but showed no
107 differences by four weeks p.i. (Figure 1B). At four weeks p.i., all animals, except those
108 inoculated with the B.1.1.529; BA.1 strain, showed high antibody titers against the ancestor
109 strain, lineage A and poor neutralizing activities against B.1.1.529; BA.1 strain (Figure 1C).
110 The B.1.1.529; BA.1 strain-inoculated animals had neutralizing antibodies (1:64) against
111 the homologous strain, but no neutralizing activities against the ancestor strain (detection
112 limit was 1:16). Sera were also used in a multiple assay for antibodies that block the
113 binding of human ACE2 to the spike proteins from variants of SARS-CoV-2 (Figure 1D).
114 Lower inhibition activity against the spike from the B.1.1.529; BA.1 variant was seen in the
115 sera from the A-, B.1.1.7-, and B.1.617.2-inoculated animals than in the sera from the
116 B.1.351- and B.1.1.529 especially when used 1:100 diluted sera; BA.1-infected animals.
117 Significantly, poor inhibition activity against the earlier VOC spike forms was observed in
118 sera from the B.1.1.529; BA.1-infected animals.
119
120 Reinfection of hamsters with an Omicron variant.
121 Five weeks after the primary inoculations, the B.1.1.529; BA.1 strain was inoculated into
122 all animals. No obvious respiratory illness was seen in any reinfected animals in the three
123 days after the second inoculation. The homologous reinfected hamsters (BA.1-BA1 group
124 in Figure 2A) showed transient body weight loss at 1 day post second infection unlike the
125 mock infected- and primary infected-animals (DMEM group and DMEM-BA.1 group,
126 respectively) by day 3 (Figure 2A). Three days after the second inoculation, all animals
127 were euthanized, under overdose anesthesia, and blood, nasal wash fluid, and lung samples
128 were obtained. The reinfected groups (A-BA.1 and B.1.1.7-BA.1 groups) showed
129 significantly higher lung weight/body weight ratios at 3 days p.i. (Figure 2B). Infectious

130 virus was detected in the respiratory samples of some of the primary infected hamsters
131 (DMEM-BA.1 group, one of four in the nasal wash fluid; three of four in the lung
132 homogenate), but no infectious virus was recovered from any of the reinfected animals
133 (Figure 2C; detection limit was $10^{1.5}$ $TCID_{50}$/mL). Interestingly, high viral RNA levels were
134 detected in the nasal wash fluid from almost all animals, even though infectious virus was
135 not isolated from these animals (Figure 2D upper panels). By contrast, no virus-associated
136 RNA was detected in the lungs of animals with prior homologous BA.1 infection (BA.1-
137 BA.1 group), and much lower virus copy numbers of virus-related RNA were detected in
138 the lungs of animals with prior heterologous SARS-CoV-2 infection compared with those
139 in the lungs of animals of DMEM-BA.1 group (Figure 2D lower panels). Thus, both prior
140 homologous and heterologous SARS-CoV-2 infection elicited lower immune protection in
141 the upper respiratory tract than that in the lower respiratory tract in this animal model.
142 The full-genome sequence of each of the Omicron BA.1 variants recovered from the nasal
143 wash fluid samples was determined (Supplementary Table 1). Nsp15 (ORF1b: K2340T)
144 and ORF10 (ORF10: V30L) nonsynonymous mutations appeared in all four hamsters
145 whose primary infection was with BA.1. (Figure 2E, left). Three of the four animals also
146 harbored variants with one or two amino acid substitutions in ORF1a region (nsp1:G180E
147 and/or nsp6:L260F). The same within-host variants could be observed in the animals from
148 the DMEM-BA.1 group. However, in the animals with prior homologous BA.1 infection
149 (BA.1-BA.1 group), nonsynonymous mutations were unevenly distributed among
150 individuals with low or high frequency mutations in nonstructural genes (nsp6:L37F,
151 nsp6:L260F, nsp9:T67A, nsp10:Q36R, nsp15:K289T) and structural genes (S: M1del and
152 ORF10: V30L). Mutations with greater diversity were also detected from animals subjected
153 to prior heterologous SARS-CoV-2 infection (Supplementary Table 1, Figure 2E, right).

154 Common nonsynonymous mutations were observed in the upper respiratory tract of BA.1-
155 primary infected hamsters, but diverse mutations appeared in that of BA.1-reinfected
156 hamsters.
157
158 Pathology in the respiratory tract after reinfection.
159 Histopathological changes in reinfected animals were determined. Three days after
160 reinfection, pathological lesions consisted of mild to moderate rhinitis and focal broncho-
161 interstitial pneumonia in the respiratory tract of the primary infection group (DMEM-BA.1
162 group, Figure 3A, second row). SARS-CoV-2 N antigen-positive cells were observed both
163 in the respiratory and olfactory epithelium of the nasal cavity and in bronchiolar epithelium
164 and alveolar epithelia of the lungs (Figure 3B).
165 In the homologous infection group (BA.1-BA.1), the lungs were hardly damaged by the
166 primary infection and the onset of pneumonia, following reinfection, was prevented (Figure
167 3A-D). By contrast, the heterologous reinfection groups showed moderate lymphocyte
168 infiltration in the respiratory area of nasal cavity in the absence of viral antigens, except in
169 a few animals from the B.1.1.7-BA.1 group (two of four animals) and the B.1.617.2-BA.1
170 group (one of four animal); viral antigens were detected in the epithelia of these animals
171 and marked lymphocytic infiltration was observed from the lamina propria into the
172 epithelium with or without multilayering of respiratory epithelial cells (regeneration) in the
173 nasal cavity (Supplementary Figure 1 and Figure 3A). The lungs from the heterologous
174 reinfection groups showed small clusters of lymphocytes, plasma cells, and macrophages
175 around the bronchioles and blood vessels in the absence of detectable viral antigens
176 (Supplementary Figure 1, Figure 3A, B, and C). Bronchiolar regeneration was also
177 observed in the reinfection groups. In particular, the epithelial regeneration was more

178 pronounced in the lungs of the A-BA.1 group than in the lungs of other groups, indicating
179 the severe damage caused by the primary infection with the lineage A strain
180 (Supplementary Figure 1). In the B.1.617.2-BA.1 group, lymphocyte and macrophage
181 infiltration was predominantly seen around the bronchioles, as reflected in the lung tissue
182 score (Figure 3D). Fibrin deposition in the lungs is one of the main histopathological
183 features of COVID-19 related acute pneumonia (20, 21). Fibrin deposition was often
184 observed in lungs of the primary infected animals but in very few of the reinfected animals
185 (Figure 3E).
186 High levels of cytokines and chemokines, including CXCL10/IP-10 and IL-1β, were
187 observed in the nasal wash fluid of the primary infection group (DMEM-BA.1), but these
188 were lower in reinfected animals (Figure 3F). Neither primary nor secondary infections
189 with the BA.1 strain induced cytokine and chemokine profiles typically associated with
190 pneumonia. Exacerbated cytokine production due to reinfection was not observed in either
191 the upper or lower respiratory tract. Taken together, these data suggest that viral replication
192 after reinfection was more strongly suppressed in the lower respiratory tract than in the
193 upper respiratory tract.
194
195 Experimental hamster infection with Omicron subvariants
196 Next, we evaluated the risk of reinfection with B.1.1.529, Omicron subvariants in hamsters
197 first infected with the BA.1 strain. Hamsters were infected as above, using four isolates of
198 the B.1.1.529 subvariants (BA.1, BA.1.1, BA.2 and BA.2.3; Table 1) were used for primary
199 and/or secondary inoculation of Syrian hamsters as depicted in Figure 4A. After primary
200 inoculation with the BA.1 strain, animals showed slightly lower weight gain rate than the
201 control animals (n = 16) during the first week p.i. but no difference was evident by the

202 second week p.i. (Figure 4B). At four weeks p.i., all BA.1 strain-inoculated animals showed
203 seroconversion (Figure 4C). Clear antigenic differentiation between the BA.1 and BA.2
204 subvariants was shown in the sera from hamsters. While no neutralizing activity against the
205 BA.2 strain was detected by a neutralization assay (Figure 4C), an assay measuring
206 antibodies that block the binding of human ACE2 to spike proteins suggested the BA.1-
207 infected hamster sera had inhibition activities against the spike from Omicron subvariants
208 (Figure 4D).
209 Five weeks after the primary inoculation, B.1.1.529 subvariants (n = 4 per subvariant) were
210 inoculated into the animals. No obvious respiratory illness was seen in any of the animals
211 in the three days following the second inoculation. Body weight graphs did not show any
212 significant difference between the primary and the reinfection groups (Figure 4E). Only
213 animals infected with the BA.2 variant showed a significant difference in the lung/body
214 weight ratio between primary and reinfection groups (Figure 4F). Infectious virus was
215 detected from the respiratory samples of most hamsters after primary infection, but very
216 low or no infectious virus was detected in samples from the reinfected animals (Figure 4G).
217 However, infectious virus was detected in the nasal wash of three of the four animals in the
218 BA.1-BA.1 reinfection group and the lungs of one of the four animals in the BA.1-BA.2
219 reinfection group. Despite the absence of infectious virus, high copy numbers of virus-
220 associated RNA were detected in nasal lavage fluid from both homologous and
221 heterologous reinfected animals. By contrast, significantly lower copy numbers of virus-
222 associated RNA were detected in the lungs of reinfected animals than in those of the
223 primary infected group (Figure 5A). Thus, animals with prior infection with BA.1 showed
224 low protection of the upper respiratory tract against reinfection with the subvariants.
225 The full-genome sequence of each of the B.1.1.529 subvariants recovered from the nasal

226 wash fluid samples was determined (Supplementary Table 2 and 3). As in the earlier
227 infection study, nsp15 (ORF1b: K2340T) and ORF10 (ORF10: V30L) nonsynonymous
228 mutations appeared in all animals whose primary infection was with BA.1. (Figure 5B). In
229 reinfected animals, several mutations were detected in nonstructural and structural genes of
230 the variants, though the mutations were unevenly distributed among the infection groups
231 (Figure 5B). Interestingly, for three of the four infection groups fewer within-host variants
232 were detected following primary infection than were detected following reinfection.
233 In animals from all primary infection groups, pathological lesions consisted of mild to
234 moderate rhinitis and focal broncho-interstitial pneumonia in the respiratory tract associated
235 with virus infection (Figure 6A-D). Animals in the reinfection groups showed very slight to
236 moderate lymphocyte infiltrations in the nasal cavity in the presence or absence of viral
237 antigen (Figure 6A-C). Some animals from the reinfection groups (BA.1-BA.1, one of four;
238 BA.1-BA.1.1, two of four; BA.1-BA.2, two of four in each group) showed focal
239 infiltrations with lymphocytes, plasma cells, and macrophages in the alveoli and around the
240 blood vessels. In reinfected animals, lung pathology occurred even though viral antigen was
241 detected in only one of the animals from the BA.1-BA.2 group (Figure 6A-D). Fibrin
242 deposition was observed in the lungs of most animals after primary infection but in the
243 lungs of very few reinfected animals (Figure 6E). High levels of CXCL10/IP-10 and IL-1β
244 were observed in the nasal wash fluid and lungs of the primary infection groups, but the
245 levels were lower in reinfection animals (Supplementary Figure 2). Thus, exacerbated
246 cytokine production, due to reinfection, was not seen in either the upper or lower
247 respiratory tract. These data suggest that despite the fact that some animals reinfected with
248 subvariants are unable to mount a sufficient immune response against reinfection,
249 especially in the upper respiratory tract, they are still able to eliminate these variants more

250 rapidly, in early phase, than occurs in naïve animals. In addition, there were no findings
251 suggesting disease exacerbation.
252
253 DISCUSSION
254 Plasma from convalescent human cases and from individuals who had been vaccinated
255 against SARS-CoV-2 exhibited marked reductions in neutralizing activity against Omicron
256 than against the ancestral SARS-CoV-2 (22-26). Multiple lineages of the Omicron variants
257 have emerged including BA.1, BA.2, BA.3, BA.4 and BA.5, with the dominant strain being
258 replaced by an emerging variant (27). Initially, BA.1 was the most prolific sublineage
259 detected worldwide; however, BA.2 is overtaking BA.1 as the dominant variant and now
260 BA. 5 has replaced (27). In human cases, lower neutralizing antibody titers against the
261 BA.4 and BA.5 subvariants than against the BA.1 and BA.2 subvariants suggest that the
262 Omicron variant is continuing to evolve with increasing capacity for neutralization escape
263 (28). Consequently, the monitoring of antigenic changes in new SARS-COV-2 variants that
264 emerge should continue. Syrian hamster models are considered to be ideal for determining
265 the antigenic differences of SARS-CoV-2 variants (29, 30) and, as in a previous study (30),
266 our hamster model showed clear antigenic differentiation between the BA.1 and BA.2
267 subvariants. Female hamsters were selected for the infection model used in this study since
268 they show better systemic and local antiviral antibody responses and more prolonged
269 humoral immunity, than male hamsters, following SARS-CoV-2 infection (31).
270
271 Because the strength of the humoral immune response and the duration of neutralizing
272 antibodies may correlate with disease severity, viral shedding from individuals with mild
273 COVID-19 is a concern (32). In hamsters, primary infection induced immune responses

274 with disease-protective capacity against reinfection by homologous or heterologous
275 Omicron subvariants. In this study, the induction of immunity and neutralizing antibodies,
276 during upper respiratory tract infection, was not a sufficient at preventing proliferation in
277 the upper respiratory tract of hamsters, following reinfection, as reported in previous
278 studies (16, 18, 19). The presence of infectious virus in the upper respiratory tract of
279 hamsters, despite a significant decrease in virus levels following reinfection, suggests that
280 even asymptomatic individuals are capable of shedding infectious virus that can cause
281 infection. On the other hand, it appears that the virus was efficiently cleared from the lower
282 respiratory tract in reinfected animals. Neutralizing antibodies in the blood are believed to
283 be effective in preventing reinfection of the lower respiratory tract (33).
284
285 The sequence of variants recovered from hamster nasal wash fluid after reinfection was
286 greater than 99.99% identical to that of the virus used for reinfection. SARS-CoV-2
287 population in a host is not represented by a single dominant sequence, but rather consists of
288 an ensemble of replicating viruses comprised of closely related sequences called
289 quasispecies (34). Consequently, the alteration of functional genes (such as the S gene) is
290 likely to generate SARS-CoV-2 quasispecies that are better adapted for infection and
291 survival in a particular host (35). Interestingly, viral RNA recovered from nasal washes,
292 from all eight hamsters in two, separate, primary BA.1 infection experiments, showed
293 consistent amino acid substitutions at Nsp15 (ORF1b: K2340T) and ORF10 (ORF10:
294 V30L). By contrast, this consistency was less observed in viral RNA recovered after
295 homologous and heterologous reinfection of Omicron variants, with amino acid
296 substitutions occurring in more diverse regions. Nsp 15 of SARS-CoV-2 functions as an
297 endoribonuclease (36). Nsp15 and several other SARS-CoV-2 proteins inhibit primary

298 interferon production and interferon signaling and thus may interfere with the body's
299 defense against infection (36-38). Compared with SARS-CoV and other nidoviruses, the
300 Orf10 protein of SARS-CoV-2 is a unique 38 aa protein (39). However, the role of ORF10
301 is still unclear. Interestingly, the overexpression of ORF10, *in vitro*, markedly suppressed
302 the expression of type I interferon genes (40). In addition, the V30L amino acid substitution
303 in ORF10 correlates with disease severity in COVID-19 patients (41). The significance of
304 these mutations observed in the hamsters remains to be elucidated.
305
306 One concern regarding reinfection with the SARS-CoV-2 subtype is that an abnormal
307 immune response to the primary infection may exacerbate the secondary infection and
308 enhance immunopathology (42), as is seen, for example, in antibody-dependent
309 enhancement (ADE) breakthrough infections such as dengue fever (43). Feline infectious
310 peritonitis (FIP), a coronavirus infection in cats, causes classic ADE disease (44, 45); in
311 contrast to FIP, SARS-CoV-2 infections in humans primarily involve the respiratory tract
312 and other organs but not the reticuloendothelial system. It is clear that the primary target of
313 SARS-CoV-2 infection is alveolar epithelial cells, which are directly injured by the virus
314 (46). Since myeloid cells are not the primary target of infection, it is unlikely that vaccine-
315 derived, non-protective, coronavirus antibodies will cause ADE infection in human (47). In
316 addition, no cases of enhanced disease due to reinfection with Omicron have been reported
317 (48-50). CXCL10/IP-10 has a well-established role in the COVID-19-related cytokine
318 storm and is involved in the development of severe lung impairment (51). Elevated levels
319 of key inflammatory chemokines and cytokines, such as CXCL10/IP-10 and IL-1β, were
320 observed in nasal wash fluid and lung homogenate supernatant of hamsters during primary
321 infection, but not after the reinfection. In addition, reinfected hamsters showed no evidence

322 of the eosinophil-associated lung inflammation observed, previously, in infected mice (52).
323 Thus we concluded that pathological and cytokine/chemokine analyses showed no evidence
324 of disease progression after reinfection in our hamster model. However, as new SARS-
325 CoV-2 variants emerge, their virulence could change and, hence, there is a need to actively
326 monitor variants.
327
328 Overall, the hamster model demonstrated that immunity acquired during primary infection,
329 with a previous SARS-CoV-2 variant or early Omicron sublineage, suppressed the
330 development of pneumonia after reinfection with a subsequently emerged variant.
331 However, infection and multiplication in the upper respiratory tract after reinfection were
332 inevitable, suggesting the likelihood of virus excretion in asymptomatic hamsters. The virus
333 population in the upper respiratory of reinfected hamsters was shown to be more diverse
334 than that seen in hamsters after primary infection. In addition, differences were observed
335 between the BA.1 strain and other subvariants, in the diversity of virus populations
336 generated in infected hamsters, which may reflect alterations in virus infectivity and
337 replication within specific hamsters. These findings could provide a better understanding of
338 pathology after reinfection by new variants of SARS-CoV-2. In addition, the hamster model
339 should provide insight into the within-host evolution of SARS-CoV-2 and provide
340 important information for countermeasures aimed at diversifying SARS-CoV-2 mutant
341 strains.
342

343 MATERIALS AND METHODS

344 Ethics.

345 All procedures involving cells and animals were conducted in a Biosafety Level (BSL) 3

346 laboratory. All animal experiments were approved by the Animal Care and Use Committee
347 of the National Institute of Infectious Diseases in Japan (approval nos. 120108, 120142, and
348 121152), and all experimental animals were handled in BSL3 animal facilities according to
349 the guidelines of this committee (approval nos. 19-53, 20-39, and 20-31). All animals were
350 housed in a facility certified by the Japan Health Sciences Foundation.
351

352 Viruses and cells.

353 Viruses were isolated from anonymized clinical specimens (nasopharyngeal/nasal swabs or
354 saliva) collected from individuals diagnosed with COVID-19 as part of the public health
355 diagnostic activities conducted by National Institute of Infectious Diseases (53, 54).
356 VeroE6/TMPRSS2 cells purchased from the Japanese Collection of Research Bioresources
357 Cell Bank (JCRB1819, the National Institute of Biomedical Innovation, Health and Nutrition,
358 Osaka, Japan) were used for viral isolation and viral titrations (53). Cells were cultured in
359 DMEM, low glucose (Sigma-Aldrich, St. Louis, MO), containing 10% FBS, 50 IU/mL
360 penicillin G, and 50 μg/mL streptomycin (10DMEM). Viral infectivity titers were expressed
361 as $TCID_{50}$/mL in VeroE6/TMPRSS2 cells and were calculated according to the Behrens–
362 Kärber method. Work with infectious SARS-CoV-2 was performed under BSL3 conditions.
363

364 Animal experiments.

365 Five-week-old female Syrian golden hamsters (SLC, Shizuoka, Japan) were used for animal
366 experiments. After anesthesia, animals were inoculated intranasally with 1.0×10^3 $TCID_{50}$ (in
367 8 μL) of one of five isolates of SARS-CoV-2 including the ancestor strain from lineage A,
368 and four isolates of the VOC (lineage B.1.1.7, B.1.351, B.1.617.2, and B.1.1.529 BA.1; Table
369 1) . All mock-infected hamsters were inoculated with DMEM containing 2% (v/v) FCS

370 containing 50 IU/mL penicillin G, and 50 μg/mL streptomycin (2DMEM). Body weight was
371 measured daily for 3 days (*n* = 4 or 16 per group). Five weeks after their first inoculation,
372 animals were then re-inoculated intranasally with 1.0×10^4 $TCID_{50}$ (50 μL) of four isolates of
373 the B.1.1.529 subvariants (BA.1, BA.1.1, BA.2, BA.2.3; Table 1). Body weight was
374 measured daily for 3 days (*n* = 3-4 per group), and animals were sacrificed at 3 days p.i. to
375 analyze viral replication and disease pathology (*n* = 3-4 per group). The humane endpoint
376 was defined as the appearance of clinically diagnostic signs of respiratory stress, including
377 respiratory distress and more than 25% weight loss. Animals were euthanized under
378 anesthesia with an overdose of isoflurane if severe disease symptoms or weight loss was
379 observed.
380
381 RNA extraction and quantification of viral RNA genomes.
382 Total RNA from each lung homogenate and nasal wash was isolated using the Maxwell RSC
383 Maxwell RSC Viral Total Nucleic Acid Purification Kit (Promega Corporation), following
384 the manufacturer's suggested protocol, and quantified by NanoDrop (Thermo Fisher
385 Scientific). The viral RNA copy number in the samples was estimated by real-time RT-PCR.
386 Subgenomic viral RNA transcripts were also detected in N gene transcripts. The primer and
387 probe sets are as follows: NIID_2019-nCOV_N_F2 (5'-AAATTTTGGGGACCAGGAAC-
388 3'), NIID_2019-nCOV_N_R2 (5'-TGGCAGCTGTGTAGGTCAAC-3'), and NIID_2019-
389 nCOV_N_P2 (5'-FAM-ATGTCGCGCATTGGCATGGA-BHQ-3') for targeting the viral
390 RNA; and SARS2-LeaderF60 (5′-CGATCTCTTGTAGATCTGTTCTCT-3′), SARS2-
391 N28354R (5′-TCTGAGGGTCCACCAAACGT-3′), and SARS2-N28313Fam (5′-FAM-
392 TCAGCGAAATGCACCCCGCA-TAMRA-3′) for targeting the subgenomic RNA. The
393 reaction mixtures were incubated at 50°C for 30 min, followed by incubation at 95°C for 15

394 min, and thermal cycling, which consisted of 40 cycles of denaturation at 94°C for 15 s, and
395 annealing and extension at 60°C for 60 s. This assay was performed on a LightCycler 480
396 (Roche, Basel, Switzerland).
397

398 ## SARS-CoV-2 neutralizing assay

399 Blood was obtained from each hamster under anesthesia (55) and when euthanized. Sera
400 were then obtained by centrifugation and were inactivated by incubation at 56°C for 30 min.
401 Aliquots (100 $TCID_{50}$/well) of SARS-CoV-2 were incubated at 37°C for 1 h in the presence
402 or absence of hamster serum (serially diluted two-fold), and then added to confluent
403 VeroE6/TMPRSS2 cell cultures in 96-well microtiter plates. Samples were examined for
404 viral cytopathic effects on Day 5, and the neutralizing antibody titers were determined as the
405 reciprocal of the highest dilution at which no CPEs were observed. The lowest and highest
406 serum dilutions tested were 1:16 and 1:2048, respectively.
407

408 ## ACE2 binding inhibition electrochemiluminescence immunoassay

409 A multiple assay for neutralizing antibodies to spike antigens from variants of SARS-CoV-2
410 using the V-PLEX SARS-CoV-2 (ACE2) kits (K15570U and K15586U, Meso Scale
411 Discovery) was used. 1:10 and 1:100 diluted sera were used for the assay. The assay samples
412 were read on a high-performance electrochemiluminescence immunoassay instrument,
413 MESO QuickPlex SQ 120 (Meso Scale Discovery), as described by the manufacturer.
414

415 ## Detection of inflammatory cytokines and chemokines

416 Homogenized lung tissue samples (10% w/v) and nasal wash samples were diluted 1:1 in cell
417 extraction buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM

418 NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, and 0.5%
419 deoxycholate (BioSource International, Camarillo, CA)), incubated for 10 min on ice with
420 vortexing, irradiated for 10 min with UV-C light to inactivate infectious virus, and tested in
421 the BSL2 laboratory. Cytokine and chemokine levels were measured with a commercial rat
422 cytokine/chemokine magnetic bead panel 96-well plate assay kit (Milliplex MAP kit, Merck
423 Millipore), which detects 5 cytokines and chemokines including IP-10/CXCL10, IL-1α, IL-
424 1β, IL-10, and VEGF (56). The assay samples were read on a Luminex 200 instrument with
425 xPONENT software (Merck Millipore), as described by the manufacturer.
426

427 Histopathology and immunohistochemistry

428 The lungs and head including nasal cavity and brain were harvested and fixed in 10%
429 phosphate-buffered formalin. Fixed tissues were routinely embedded in paraffin, sectioned,
430 and stained with hematoxylin and eosin (H&E). For immunohistochemistry, antigen
431 retrieval of the formalin-fixed tissue sections was performed by autoclaving at 121°C for 10
432 min in retrieval solution at pH 6.0 (Nichirei, Tokyo, Japan). SARS-CoV-2 antigens were
433 detected using a polymer-based detection system (Nichirei-Histofine Simple stain MAX
434 PO; Nichirei Biosciences, Inc., Tokyo, Japan), and an in-house rabbit anti-SARS-CoV-2 N
435 antibody was used as the primary antibody. Nuclei were counterstained with hematoxylin
436 for 10 s.
437 Histopathology scores were determined based on the percentage of lesion area including
438 inflammation, hemorrhage and edema, as determined by HE staining in each group by
439 using the following scoring system: 0, no lesion; 1, focal lesion within 30% or less total
440 area; 2, diffuse lesion involving 30–70% total area; 3, diffuse lesion involving more than
441 70% total area. Scores were also determined based on the percentage of virus antigen-

442 positive cells, as determined by immunohistochemistry in each group by using the
443 following scoring system: 0, no antigen-positive cells; 1, antigen-positive cells were
444 occasionally observed in each cut sections (1–3 antigen-positive areas per section were
445 observed in the high magnification); 2, scattered positive cells were observed (4–9 antigen-
446 positive areas per section were observed in the high magnification); 3, many positive cells
447 were diffusely and widely observed (more than 10 antigen-positive areas per section were
448 observed in the low magnification). Mean scores from all lung sections (four lung
449 sections/animal) in each animal were calculated. Dots in figure indicate mean scores in
450 each animal.
451

452 Next generation sequencing analysis for comparison of SARS-CoV-2 mutations

453 To identify major population of virus in respiratory tract of hamsters after reinfection, a
454 next generation sequencer was used to obtain the entire length of the viral genome. The
455 sequences obtained from the samples were compared with those of the inoculated viruses.
456 The viral RNAs were extracted from the homogenized lung tissue samples (10% w/v) and
457 nasal wash samples using the Maxwell® RSC Viral Total Nucleic Acid Purification Kit
458 (Promega, Madison, WI). The whole genomes of SARS-CoV-2 used in this research were
459 amplified using a modified ARTIC protocol with several primers replaced or added (57,
460 58). The viral cDNAs were synthesized from extracted RNA using the Luna Script RT
461 Super Mix Kit (New England BioLabs, Ipswich, MA), followed by DNA amplification by
462 multiplex PCR in two separated primer pools using ARTIC-N5 primers (59, 60) and Q5
463 Hot Start DNA polymerase (New England BioLabs). The DNA libraries for Illumina NGS
464 were prepared from pooled amplicons using the QIAseq FX DNA Library Kit (QIAGEN)
465 and analyzed using the iSeq 100 and MiSeq (Illumina). The obtained reads were analyzed

466 by the CLC Genomics Workbench (version 21, QIAGEN) with the Wuhan/Hu-1/2019
467 sequence (GenBank accession number MN908947) as a reference. The sequence data have
468 been deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive, under
469 submission (BioProject Accession: PRJDB14262; BioSample accessions:
470 SAMD00523210-SAMD0052326). In the reinfection experiment, the frequencies of gene
471 mutations were calculated with each protein. The average depth of aligned reads was 3132.
472 In the co-infection experiment, the ratio of Delta and BA.1 was calculated as the
473 percentages based on the 10 regions where these two viruses can be distinguished. The
474 nucleotide numbers of these 10 regions are: 2832, 8393, 11537, 21618, 22673-22674,
475 23048, 23063, 23604, 26530, and 28311 of the Wuhan/Hu-1/2019 genome, which
476 correspond to amino acids 856, 2710, 3758 in ORF1a, 19, 371, 496, 501, 681 in the spike
477 protein, 3 in the M protein, and 13 in the N protein, respectively. The ratio of BA.1 and
478 BA.2 was also analyzed based on the 10 regions where these two strains could be
479 identified. The nucleotide numbers in these 10 regions are: 2832, 8393, 11537, 21618,
480 22204-22205, 22673-22674, 22786, 22898, 23048, and 29510 in the Wuhan/Hu-1/2019
481 genome, which correspond to amino acids 856, 2710, 3758 in ORF1a, 19, 214, 371, 408,
482 446, 496 in the spike protein, and 13 in the N protein, respectively. The average depth of
483 aligned reads was 3132. Each read depth at the 20 regions used to calculate the ratios was
484 more than 200.
485
486 Statistical analysis.
487 All data are expressed as the mean and standard error of the mean, except for neutralizing
488 antibodies (Geometric mean titers with 95% confidence interval, GMT+95%Cl). Statistical
489 analyses were performed using GraphPad Prism 9 software (GraphPad Software, La Jolla,

490 CA). Intergroup comparisons were performed using nonparametric analysis. A P value <
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492

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773

774

TABLE 1 SARS-CoV-2 variants in this study

Pango lineage (WHO label)	Sub lineage	Strain (Simplified name)	GISAID Accession no. of original isolate	Note
A		hCoV-19/Japan /TY-WK-521/2020 (WK-521)	EPI_ISL_408667	
B.1.1.7 (Alpha)		hCoV-19/Japan/QK002/2020 (QK002)	EPI_ISL_768526	
B.1.351 (Beta)		hCoV-19/Japan/TY8-612-P1/2021 (TY8-612)	EPI_ISL_1123289	
B.1.617.2 (Delta)		hCoV-19/Japan/TY11-927-P1/2021 (TY11-927)	EPI_ISL_2158617	
B.1.1.529 (Omicron)	BA.1.18	hCoV-19/Japan/TY38-873P0/2021 (TY38-873)	EPI_ISL_7418017	BA.1 + ORF1a:T1822I
B.1.1.529 (Omicron)	BA.1.1	hCoV-19/Japan/TY38-871P0/2021 (TY38-871)	EPI_ISL_7571618	
B.1.1.529 (Omicron)	BA.2	hCoV-19/Japan/TY40-385-P1/2022 (TY40-385)	EPI_ISL_9595859	
B.1.1.529 (Omicron)	BA.2.3	hCoV-19/Japan/TY40-816-P1/2022 (TY40-816)	EPI_ISL_9595861	BA.2 + S:A688V

779 FIGURE LEGENDS

780 **FIG 1 Experimental infection of hamsters with SARS-CoV-2 variants.** Study design
781 outlining experimental infections using 5-week-old female Syrian hamsters and SARS-
782 CoV-2 **(A)**. Body weight curve for 4 weeks after primary infection with SARS-CoV-2
783 variants. Dunnett's multiple comparison test compared with DMEM-inoculated animals (n
784 = 3 or 4). *, P < 0.05; **, P < 0.01 **(B)**. Neutralizing (NT) antibody titers against WK-521
785 (lineage A, black circle) or TY38-873 (lineage B.1.1.529, BA.1, **(C,** red circle)) strains in
786 the sera from hamsters at 4 weeks post-inoculation (p.i.). Data from the same animal are
787 connected with lines. *, P < 0.05 by Dunn's multiple comparison test following the
788 Kruskal–Wallis test **(C)**. Blocking activities of hamster sera between ACE2 and SARS-
789 CoV-2 spike was tested by the ACE2 binding inhibition ELISA using a V-Plex COVID-19
790 ACE2 neutralization kit (K15570U, Meso Scale Discovery) (n = 3 or 4). Each dot
791 represents data from an individual animal. Sera were diluted 1:10 (black dot) or 1:100
792 (black circle) for the analysis. Data from the same animal are connected with lines. *, P <
793 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 by Sidak's multiple comparison test
794 following two-way ANOVA **(D)**.

795

796 **FIG 2 Reinfection of hamsters with an Omicron variant.** Body weight curve during 3
797 days after second infection with an isolate of the TY38-873, B. 1.1.529 (Omicron)
798 subvariant BA.1 (n = 3 or 4) **(A)**. Bar graph showing the ratio of the chest organ weights
799 including lungs, trachea, heart, and thymus per body weight at 3 days p.i. (n = 3 or 4) **(B)**.
800 Bar graph showing virus titers **(C)** and virus-related RNA copies **(D)** in the nasal wash fluid
801 (upper panels) and supernatant from 10% lung tissue homogenates (lower panels) (n = 3 or
802 4). *, P < 0.05; **, P < 0.01; ***, P < 0.001; by Dunn's multiple comparison test following

803 the Kruskal–Wallis test. Percentage of animals with mutations involving amino acid
804 substitutions in each region **(E)**. The full-genome sequences of the Omicron BA.1 strains
805 recovered from the nasal wash fluid samples (n = 3 or 4). Brown bars in each panel indicate
806 the same data for the DMEM-BA.1 group.
807
808 **FIG 3 Histopathology and immunopathology of respiratory tract after reinfection**
809 **with an Omicron BA.1 variant.** Representative histopathological findings for the nasal
810 cavity epithelia and lungs of hamsters 3 days after second infection with an isolate of
811 TY38-873, B. 1.1.529 (Omicron) subvariant BA.1 (n = 4). H&E, hematoxylin and eosin
812 staining; IHC, immunohistochemistry for SARS-CoV-2 antigen detection. Br, bronchi.
813 Viral antigen-positive cells were mainly detected in the nasal epithelium on the cranial side.
814 Scale bars, 50 μm **(A)**. Distribution of viral antigens in nasal cavity and lungs by
815 immunohistochemistry. Heat map shows percentages of viral antigen-positive animals. SE;
816 squamous epithelium, RE; respiratory epithelium, OE; Olfactory epithelium, OB; Olfactory
817 bulb **(B)**. Viral antigen scores and pathological severity scores of lungs from hamsters (**C**
818 **and D**). Fibrin deposition scores of lungs by immunohistochemistry. Four lung lobes were
819 taken from each individual animal and scored to evaluate comprehensive pathological
820 changes. *$p < 0.05$ by one-way ANOVA **(E)**. Cytokine and chemokine levels in the nasal
821 wash fluid (upper panels) and supernatant from 10% lung tissue homogenates (lower
822 panels) of hamsters at 3 days p.i. (n = 3 or 4). *, $P < 0.05$ by Dunn's multiple comparison
823 test following the Kruskal–Wallis test **(F)**.
824
825 **FIG 4 Experimental infection of hamsters with Omicron subvariants.** Study design
826 outlining experimental infections using 5-week-old female Syrian hamsters and Omicron

827 subvariants **(A)**. Body weight curve for 4 weeks after primary inoculation with an isolate of
828 TY38-873, B. 1.1.529 (Omicron) subvariant BA.1. Control groups were inoculated with
829 cell culture medium (DMEM). No significant difference compared with DMEM-inoculated
830 animals (n = 16) was detected by Sidak's multiple comparison test following two-way
831 ANOVA **(B)**. Neutralizing (NT) antibody titers against TY38-873 (BA.1**,** left dots and
832 triangles) or TY40-385 (BA. 2, right dots and triangles) strains in the sera from hamsters on
833 4 weeks p.i. The dashed line indicates the limit of detection (<16). Each dot represents and
834 triangles data from an individual animal (n = 16). Data from the same samples are
835 connected with lines **(C)**. Blocking of interactions between ACE2 and SARS-CoV-2 spike
836 was tested by ACE2 binding inhibition ELISA using a V-Plex COVID-19 ACE2
837 neutralization kit (K15586U, Meso Scale Discovery) (n = 16). Sera were diluted 1:10 or
838 1:100 for the analysis. **, P < 0.01; ****, P < 0.0001 by Sidak's multiple comparison test
839 following two-way ANOVA **(D)**. Body weight curve during 3 days after primary or
840 reinfection with subvariants of B. 1.1.529. (n = 4). No significant differences were detected
841 between the primary (black lines) and reinfection (red lines) groups by two-way ANOVA
842 **(E)**. Bar graph showing the ratios of the weights of the chest organ including lungs, trachea,
843 heart, and thymus to body weights at 3 days p.i *, P < 0.05 by Mann-Whitney test **(F)**. Bar
844 graph of virus titers in the nasal wash fluid (upper panels) and supernatant from 10% lung
845 tissue homogenates (lower panels) **(G)**. Dot line indicates detection limit. *, P < 0.05 by
846 Mann-Whitney test., Black lines/bars indicate the primary infection groups and red
847 lines/bars are from the reinfection groups (E-G).
848
849 **FIG 5 Impact of reinfection in hamsters with Omicron subvariants.** Bar graph showing
850 virus-related RNA copies in the nasal wash fluid (upper panels) and supernatant from 10%

851 lung tissue homogenates (lower panels). Dot line indicates detection limit. *, $P < 0.05$ by
852 Mann-Whitney test. B-D, black bars indicate the primary infection groups and red bars the
853 reinfection groups **(A)**. Percentage of animals with mutations involving amino acid
854 substitutions in each region. Comparison of mutations detected in animals reinfected with
855 SARS-CoV-2. The full-genome sequences of the B. 1.1.529 subvariants recovered from the
856 nasal wash fluid samples (n = 4 per group) were determined using NGS analyses **(B)**.
857
858 **FIG 6 Histopathology of respiratory tract tissues from hamsters following primary- or**
859 **reinfection with Omicron subvariants.** Representative histopathological findings of the
860 nasal cavity and lungs from hamsters after primary or second infection with subvariants of
861 B. 1.1.529 (Omicron) (n = 4) **(A)**. H&E, hematoxylin and eosin staining; IHC,
862 immunohistochemistry for SARS-CoV-2 antigen detection. Br, bronchi. Viral antigen-
863 positive cells were mainly detected in the nasal epithelium on the cranial side. Scale bars,
864 50 μm. Distribution of viral antigen in respiratory tract from hamsters **(B)**. Heat map shows
865 percentage of viral antigen-positive animals. SE; squamous epithelium, RE; respiratory
866 epithelium, OE; Olfactory epithelium, OB; Olfactory bulb. Viral antigen scores and
867 pathological severity scores of lungs in hamsters **(C and D)**. Fibrin deposition scores of
868 lungs from hamsters by immunohistochemistry. Four lung lobes were taken from each
869 individual animal and scored. *$p < 0.05$ by one-way ANOVA **(E)**.
870
871 **Supplementary Figure 1. Histopathology of respiratory tract tissues after reinfection**
872 **with an Omicron BA.1 variant.** Representative histopathological findings of the nasal
873 cavity and lungs from hamsters 3 days after second infection with an isolate of TY38-873,
874 B. 1.1.529 (Omicron) subvariant BA.1 (n = 3 or 4). H&E, hematoxylin and eosin staining;

875 IHC, immunohistochemistry for SARS-CoV-2 antigen detection. Br, bronchi. Viral antigen-
876 positive cells were mainly detected in the nasal epithelium on the cranial side. Scale bars,
877 50 μm.
878
879 **Supplementary Figure 2. Cytokine and chemokine levels in the respiratory airway of**
880 **hamsters 3 days post primary or reinfection.** The nasal wash fluid **(A)** and supernatant
881 from 10% lung tissue homogenates **(B)** of hamsters at 3 days p.i. (n = 4) *, $P < 0.05$ by
882 Mann-Whitney test. Black bars indicate the first infection group; red bars, reinfection
883 group. Dashed line indicates mean values in the nasal wash fluid or lung homogenates from
884 mock-infected hamsters (n = 4, at 3 days p.i. with DMEM).

Figure 1

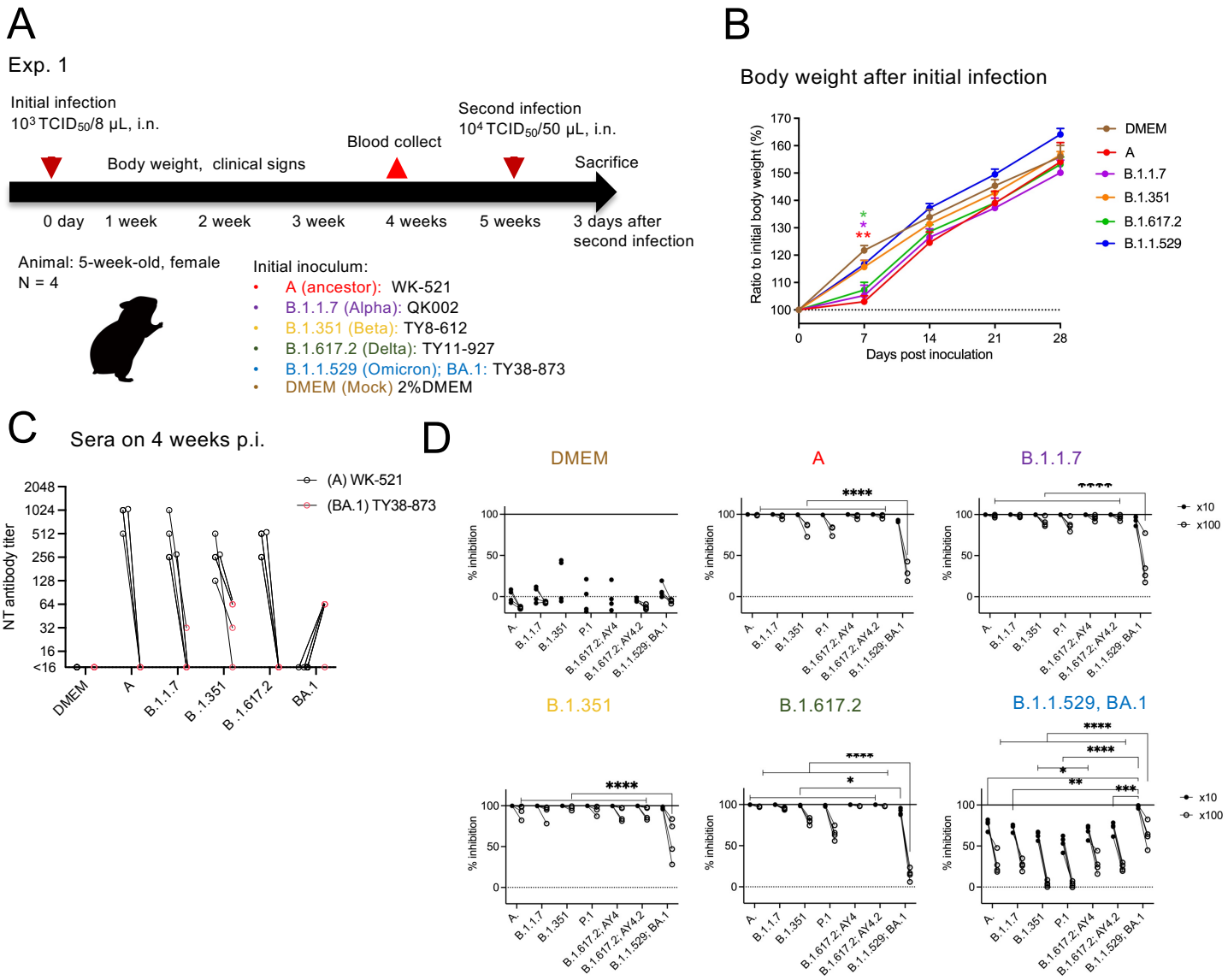

A
Exp. 1
Initial infection
10³ TCID50/8 μL, i.n.
Second infection
10⁴ TCID50/50 μL, i.n.
Blood collect
Body weight, clinical signs
Sacrifice
0 day
1 week
2 week
3 week
4 weeks
5 weeks
3 days after second infection
Animal: 5-week-old, female
N = 4
Initial inoculum:
A (ancestor): WK-521
B.1.1.7 (Alpha): QK002
B.1.351 (Beta): TY8-612
B.1.617.2 (Delta): TY11-927
B.1.1.529 (Omicron); BA.1: TY38-873
DMEM (Mock) 2%DMEM
B
Body weight after initial infection
Ratio to initial body weight (%)
Days post inoculation
DMEM
A
B.1.1.7
B.1.351
B.1.617.2
B.1.1.529
C
Sera on 4 weeks p.i.
NT antibody titer
(A) WK-521
(BA.1) TY38-873
DMEM
A
B.1.1.7
B.1.351
B.1.617.2
BA.1
D
DMEM
A
B.1.1.7
B.1.351
B.1.617.2
B.1.1.529, BA.1
% inhibition
x10
x100

Figure 2

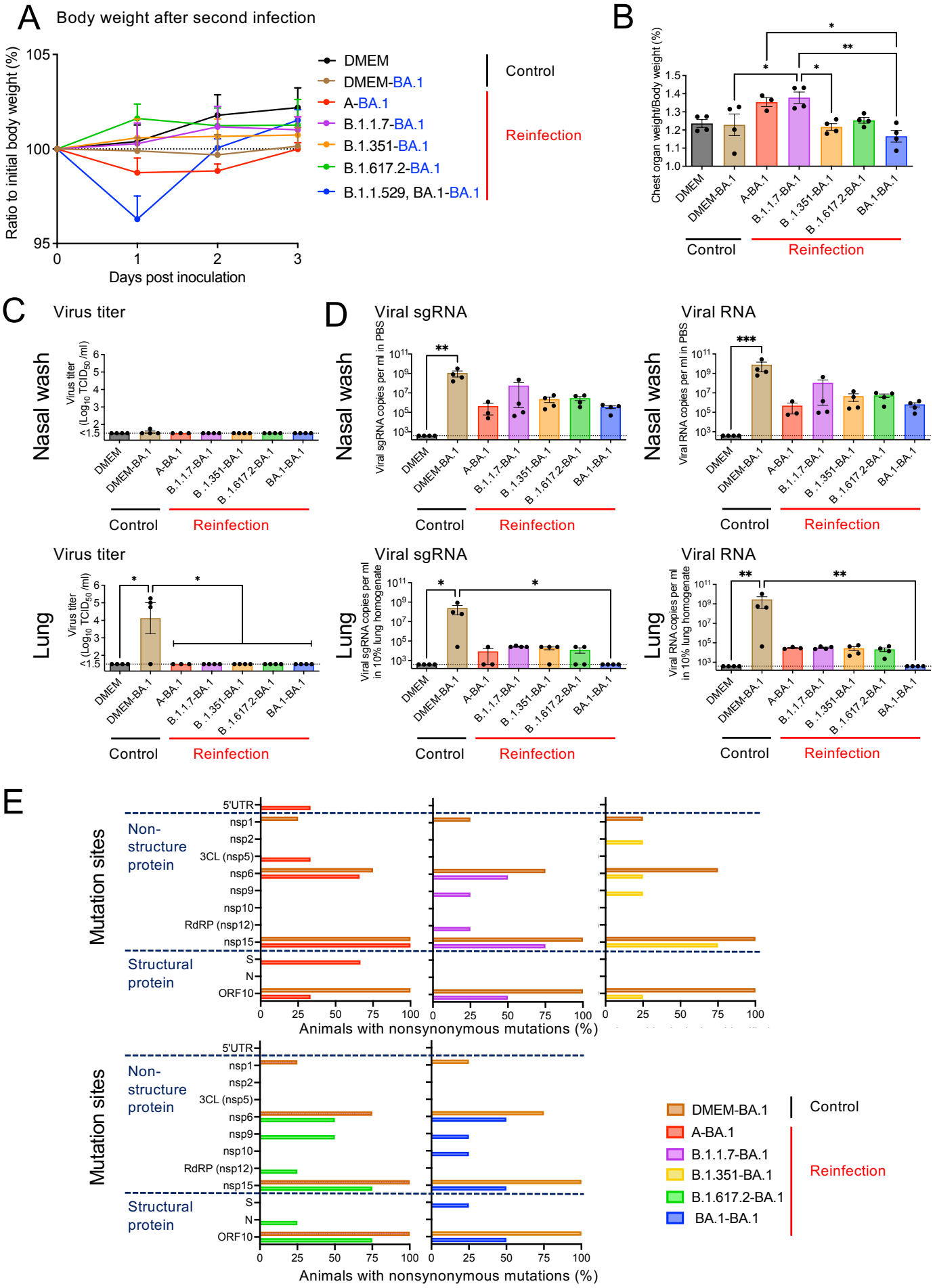

A
Body weight after second infection
Ratio to initial body weight (%)
Days post inoculation
DMEM
DMEM-BA.1
A-BA.1
B.1.1.7-BA.1
B.1.351-BA.1
B.1.617.2-BA.1
B.1.1.529, BA.1-BA.1
Control
Reinfection
B
Chest organ weight/Body weight (%)
C
Virus titer
Nasal wash
Lung
D
Viral sgRNA
Viral RNA
E
Mutation sites
Non-structure protein
Structural protein
5'UTR
nsp1
nsp2
3CL (nsp5)
nsp6
nsp9
nsp10
RdRP (nsp12)
nsp15
S
N
ORF10
Animals with nonsynonymous mutations (%)

Figure 3

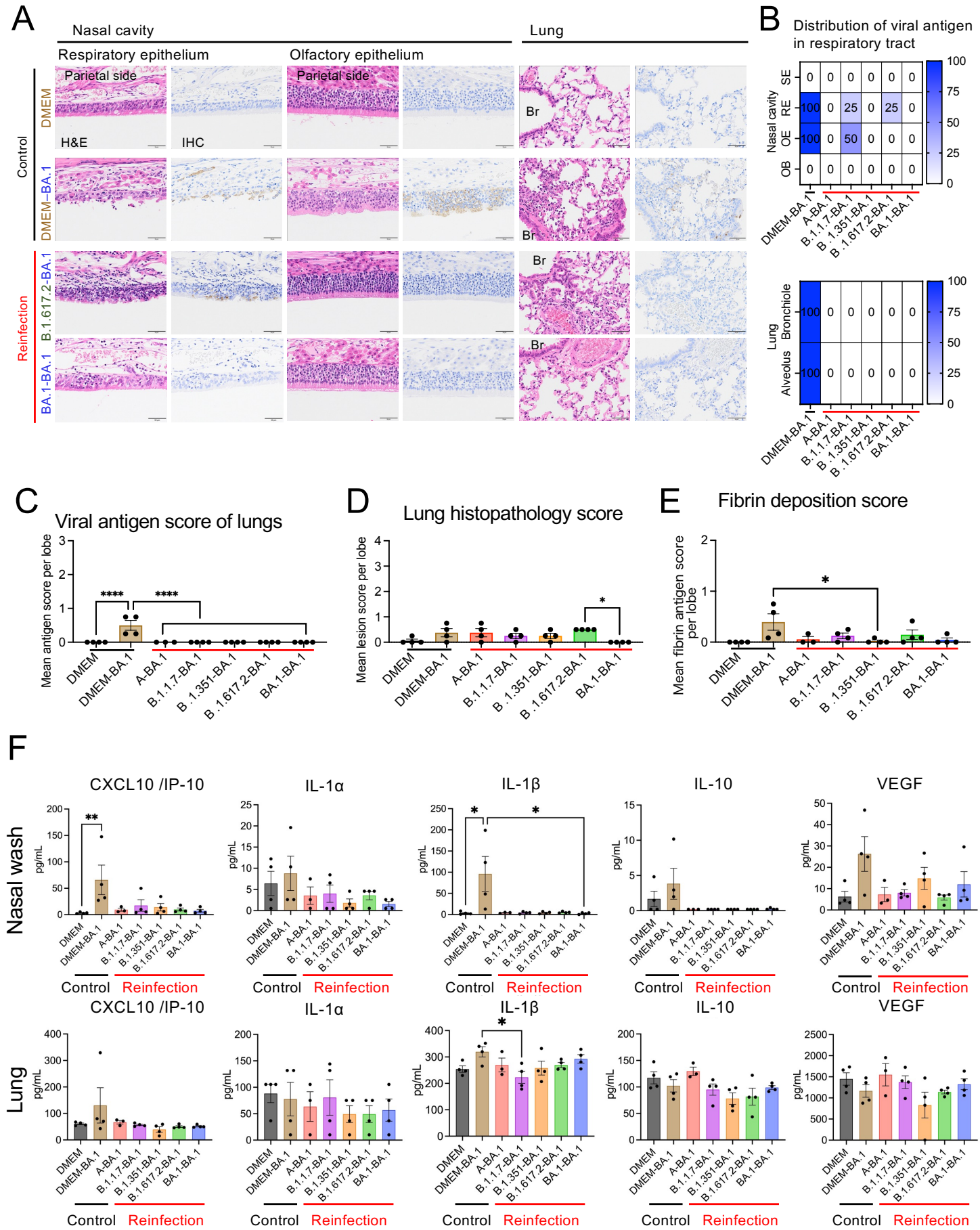

A
Nasal cavity
Lung
Respiratory epithelium
Olfactory epithelium
Parietal side
Parietal side
Control
DMEM
DMEM–BA.1
Reinfection
B.1.617.2-BA.1
BA.1-BA.1
H&E
IHC
Br
B
Distribution of viral antigen in respiratory tract
Nasal cavity
SE
RE
OE
OB
Lung
Bronchiole
Alveolus
DMEM-BA.1
A-BA.1
B.1.1.7-BA.1
B.1.351-BA.1
B.1.617.2-BA.1
BA.1-BA.1
C
Viral antigen score of lungs
Mean antigen score per lobe
D
Lung histopathology score
Mean lesion score per lobe
E
Fibrin deposition score
Mean fibrin antigen score per lobe
F
Nasal wash
Lung
CXCL10 /IP-10
IL-1α
IL-1β
IL-10
VEGF
pg/mL
Control
Reinfection

Figure 4

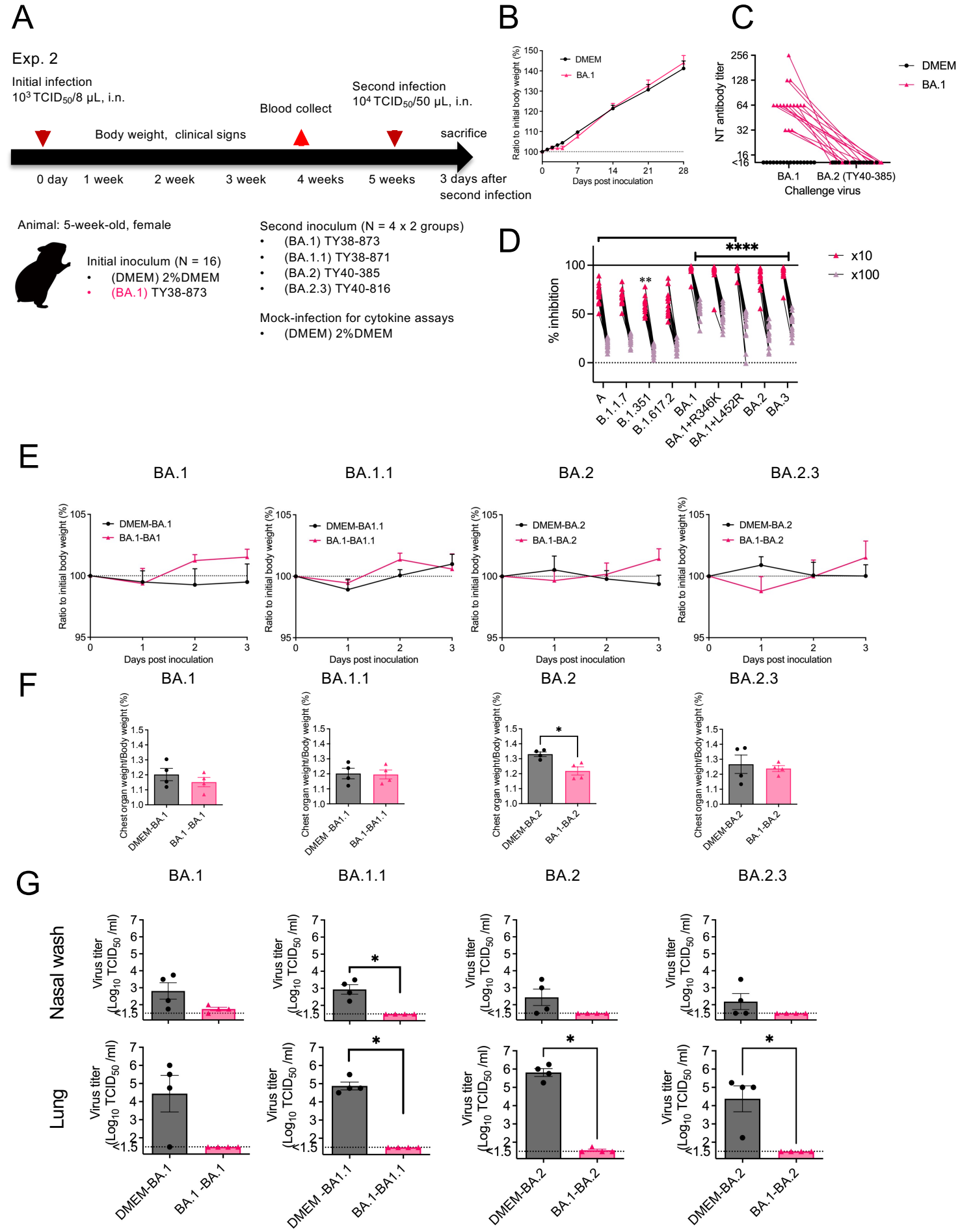
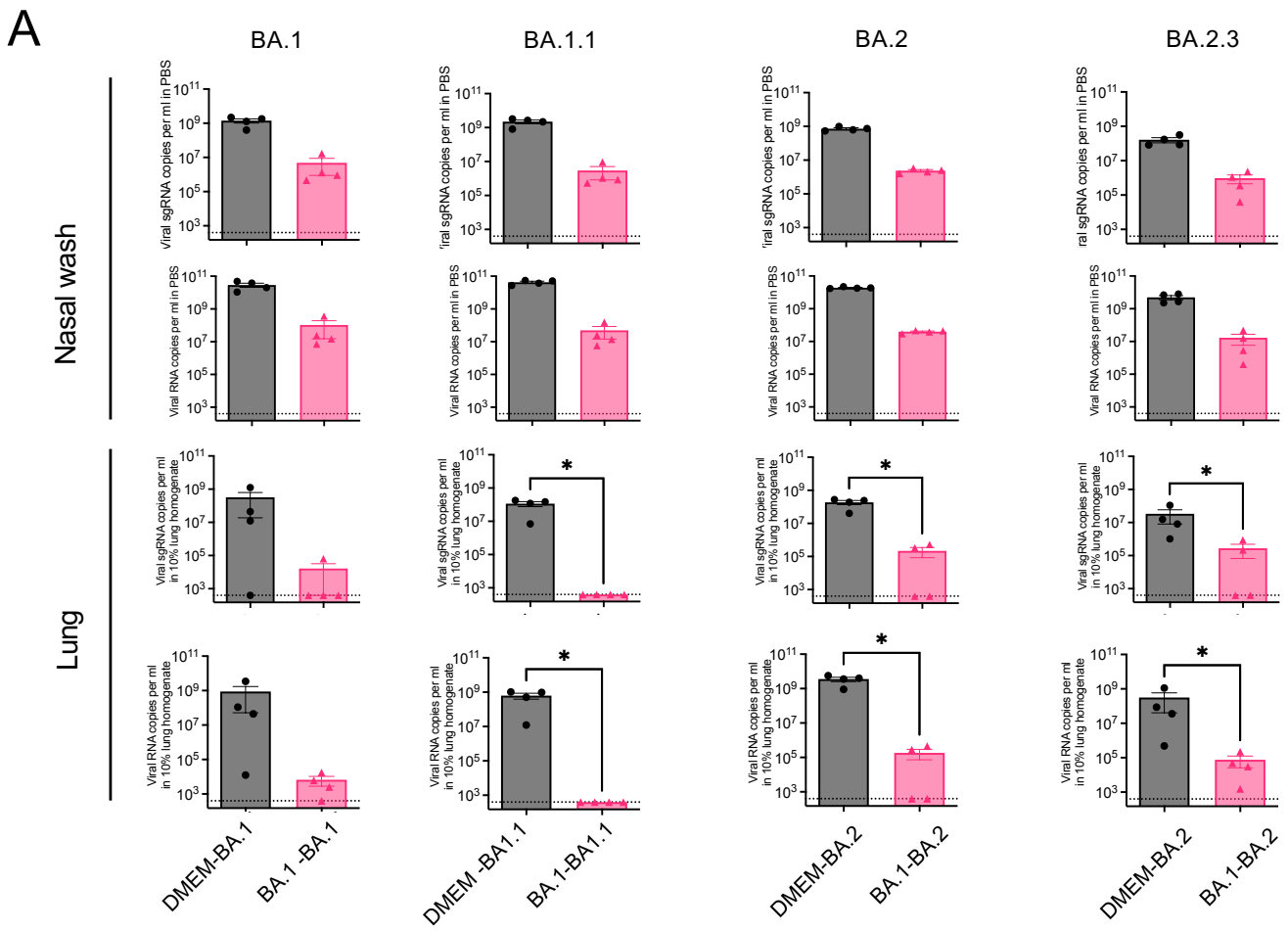

A
Exp. 2
Initial infection
10³ TCID50/8 μL, i.n.
Blood collect
Second infection
10⁴ TCID50/50 μL, i.n.
Body weight, clinical signs
sacrifice
0 day
1 week
2 week
3 week
4 weeks
5 weeks
3 days after second infection
Animal: 5-week-old, female
Initial inoculum (N = 16)
(DMEM) 2%DMEM
(BA.1) TY38-873
Second inoculum (N = 4 x 2 groups)
(BA.1) TY38-873
(BA.1.1) TY38-871
(BA.2) TY40-385
(BA.2.3) TY40-816
Mock-infection for cytokine assays
(DMEM) 2%DMEM
B
Ratio to initial body weight (%)
Days post inoculation
DMEM
BA.1
C
NT antibody titer
Challenge virus
BA.1
BA.2 (TY40-385)
D
% inhibition
x10
x100
A
B.1.1.7
B.1.351
B.1.617.2
BA.1
BA.1+R346K
BA.1+L452R
BA.2
BA.3
E
BA.1
BA.1.1
BA.2
BA.2.3
DMEM-BA.1
BA.1-BA1
DMEM-BA1.1
BA.1-BA1.1
DMEM-BA.2
BA.1-BA.2
F
Chest organ weight/Body weight (%)
G
Nasal wash
Lung
Virus titer (Log10 TCID50 /ml)

Figure 5


A
BA.1
BA.1.1
BA.2
BA.2.3
Nasal wash
Lung
Viral sgRNA copies per ml in PBS
Viral RNA copies per ml in PBS
Viral sgRNA copies per ml in 10% lung homogenate
Viral RNA copies per ml in 10% lung homogenate
*
DMEM-BA.1
BA.1 -BA.1
DMEM -BA1.1
BA.1-BA1.1
DMEM-BA.2
BA.1-BA.2
DMEM-BA.2
BA.1-BA.2

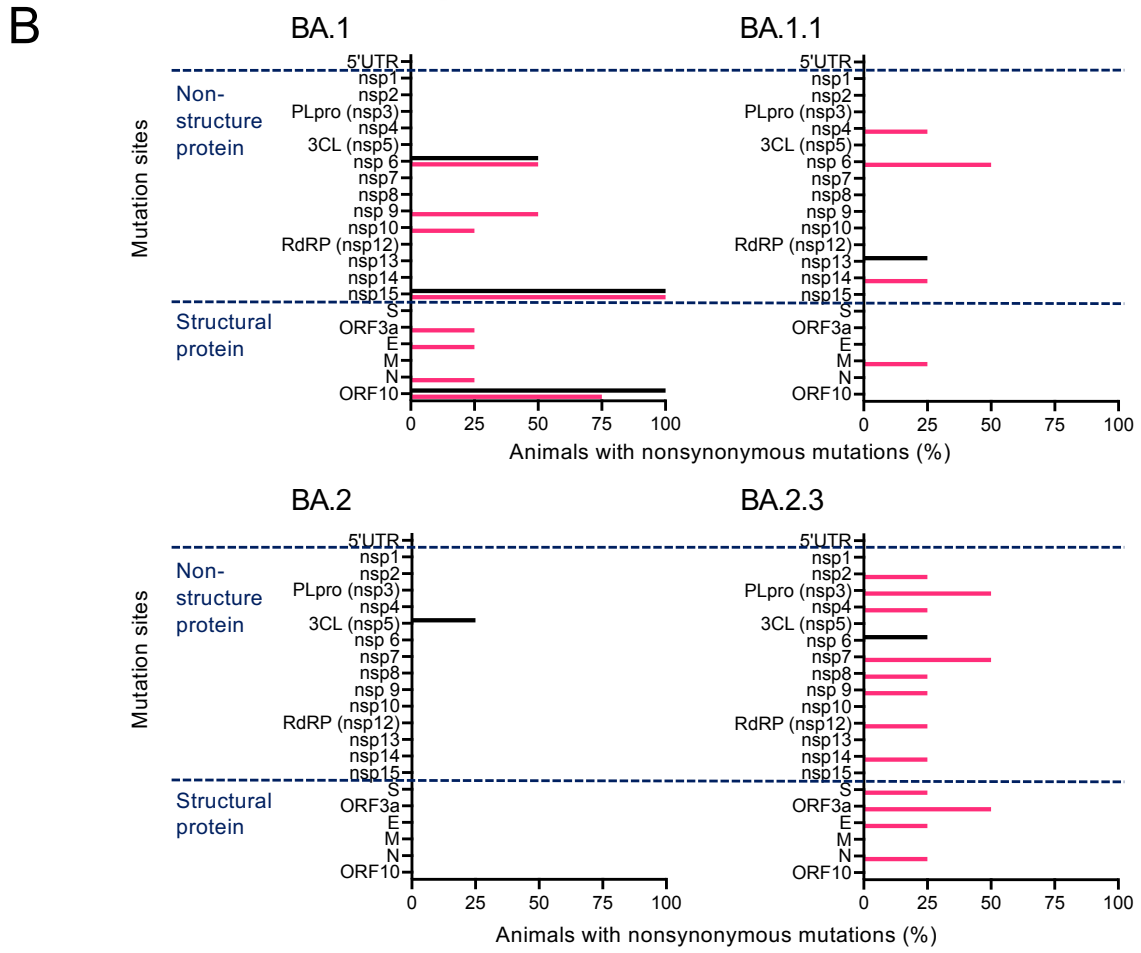
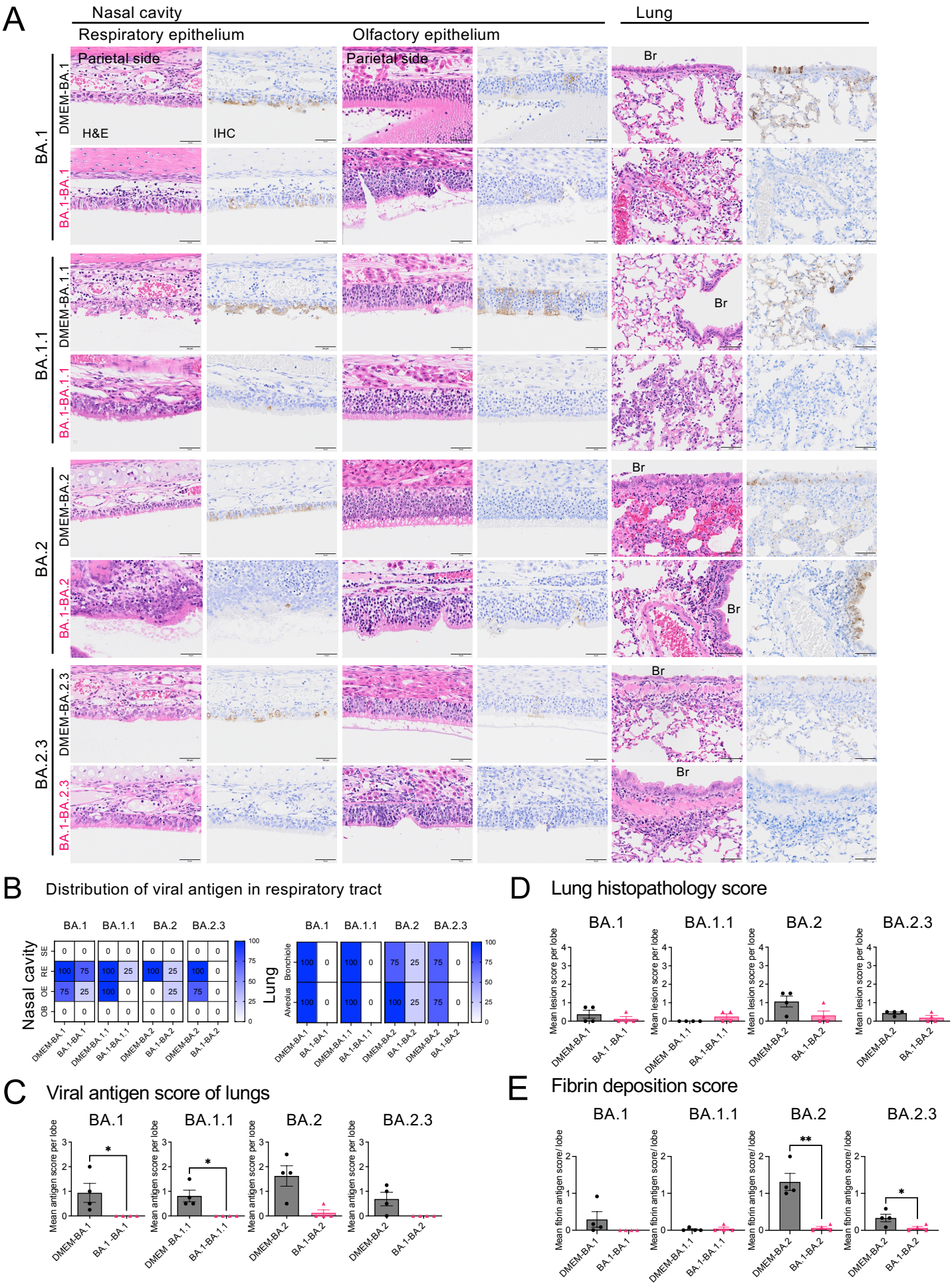
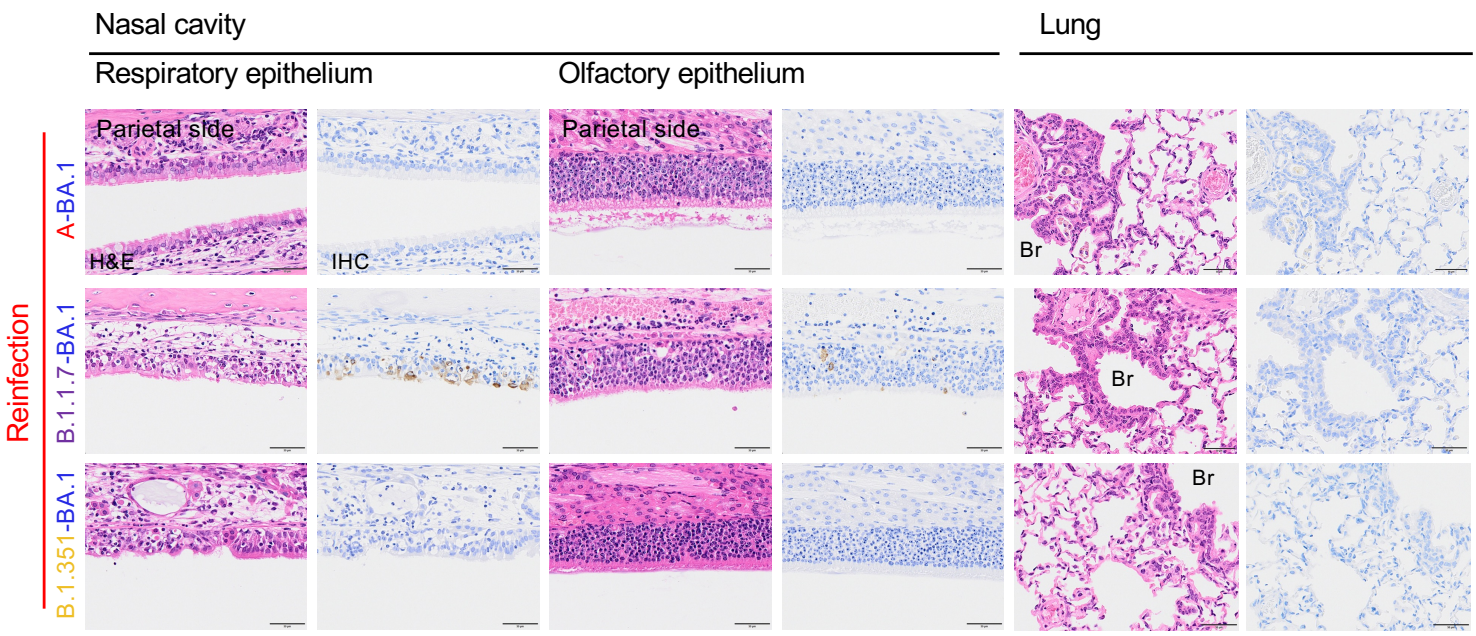

B
BA.1
BA.1.1
BA.2
BA.2.3
Mutation sites
Non-structure protein
Structural protein
5'UTR
nsp1
nsp2
PLpro (nsp3)
nsp4
3CL (nsp5)
nsp 6
nsp7
nsp8
nsp 9
nsp10
RdRP (nsp12)
nsp13
nsp14
nsp15
S
ORF3a
E
M
N
ORF10
0
25
50
75
100
Animals with nonsynonymous mutations (%)

Figure 6


A
Nasal cavity
Lung
Respiratory epithelium
Olfactory epithelium
Parietal side
Parietal side
Br
H&E
IHC
BA.1
DMEM-BA.1
BA.1-BA.1
BA.1.1
DMEM-BA.1.1
BA.1-BA.1.1
BA.2
DMEM-BA.2
BA.1-BA.2
BA.2.3
DMEM-BA.2.3
BA.1-BA.2.3
B
Distribution of viral antigen in respiratory tract
Nasal cavity
Lung
SE
RE
OE
OB
Bronchiole
Alveolus
C
Viral antigen score of lungs
Mean antigen score per lobe
D
Lung histopathology score
Mean lesion score per lobe
E
Fibrin deposition score
Mean fibrin antigen score/ lobe

Supplementary Figure 1



Supplementary Figure 2

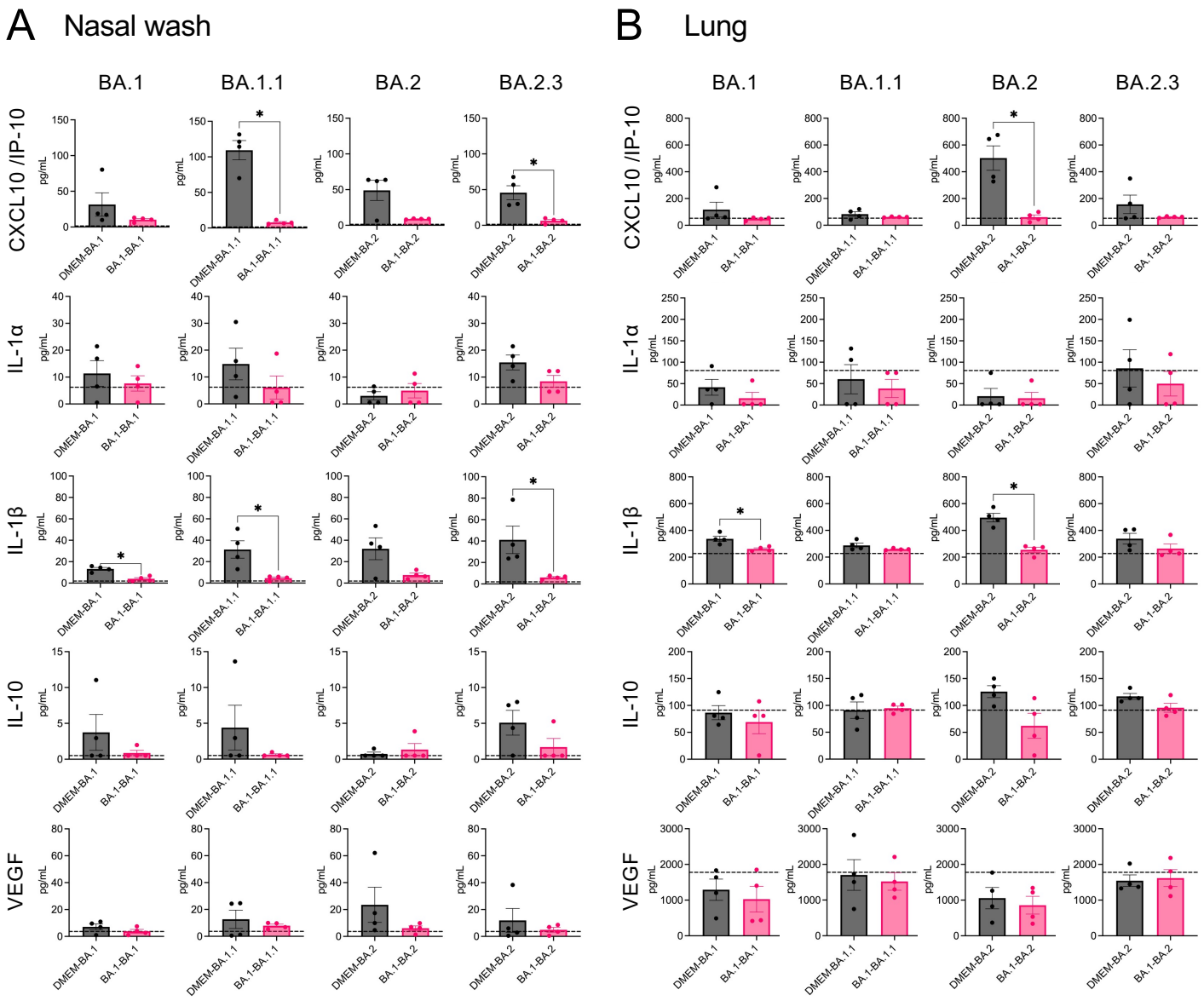


Table S1

Variant			DMEM-BA1				A-BA.1				B.1.1.7-BA.1				B.1.351-BA.1				B.1.617.2-BA.1				B.1.1.529, BA.1-BA.1			
Nuc no. in Wuhan	AA substitution	protein	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
209T	Silent	-						Y																		
								C:44.2%																		
804G	ORF1a: G180E	nsp1:G180E		R																						
				A:12.8%																						
921T	ORF1a: L219Q	nsp2:L39Q													W											
															A:33.1%											
1915C	Silent	nsp2									Y															
											T:13.0%															
7051C	Silent	PLpro (nsp3)							Y																	
									T:24.9%																	
7945C	Silent	PLpro (nsp3)												Y												
														T:33.3%												
9451C	Silent	nsp4													Y											
															T:17.3%											
9970A	Silent	nsp4									R															
											G:45.1%															
10934T	ORF1a: F3557L	3CL (nsp5): F294L						Y																		
								C:37.6																		
11020C	Silent	nsp6							Y																	
									T:28.7%																	
11083G	ORF1a: L3606F	nsp6:L37F																					K			
																							T:11.4%			
11355C	ORF1a: A3697V	nsp6:A128V														Y										
																T:33.0%										
11674C	Silent	nsp6										Y														
												T:21.5%														
11750C	ORF1a: L3829F	nsp6:L260F	Y	Y	Y		Y		Y			Y							Y			Y	Y		Y	
			T:15.5%	T:36.4%	T:12.5%		T:13.7%		T:39.4%			T:51.3%							T:45.4%			T:10.5%	T:12.0%		T:19.3%	
12747C	ORF1a: T4161I	nsp9:T21I									Y						Y									
											T:13.6%						T:23.6%									
12884A	ORF1a: T4207A	nsp9:T67A																		R		R		R		
																				G:20.6%		G:10.5%		G:73.1%		
13131A	ORF1a: Q4289R	nsp10:Q36R																							R	
																									G:35.2%	
15211A	ORF1b: T582A	RdRP (nsp12): T591A												R								R				
														G:10.5%								G:36.6%				
19206C	Silent	nsp14																							Y	
																									T:28.9%	
20486A	ORF1b: K2340T	nsp15:K289T	M	M	M	M	M	M	M			M	C	M		M	M	M	M		C	M	M		M	
			C:39.6%	C:35.8%	C:46.3%	C:40.9%	C:38.4%	C:12.0%	C:77.2%			C:30.1%	C:95.6%	C:26.5%		C:46.8%	C:24.2%	C:69.1%	C:24.5%		100%	C:16.4%	C:50.8%		C:24.0%	
21561C	Silent	-																						Y		
																								T:10.2%		
21562A	Silent	-																						W		
																								T:10.2%		
21565G	S: M1 del	S: M1 del																						R		
																								A:10.1%		
21925T	Silent	S													Y											
															C:56.7%											
22151A	S: I197L	S: I197L						M																		
								C:40.9%																		
22194A	S: N211del, L212I	S:N211I, L212del					A/del																			
							A:15.1%																			
22195T							T/del																			
							T:15.1%																			
22196T							T/del																			
							T:15.1%																			
23788T	Silent	S																							Y	
																									C:34.9%	
28971A	N: K233R	N: K233R																				R				
																						G:12.1%				
29645G	ORF10: V30L	ORF10: V30L	K	K	K	K	K				K	K					K		K	K		K		K	K	
			G:28.4%	G:23.1%	G:22.1%	G:22.4%	T:74.2%				T:16.8%	T:78.1%					G:50.3%		G:13.3%	G:21.5%		G:21.6%		G:10.7%	G:50.3%	

Table S2

Variant			Inoculum		Group/Animal No.				Inoculum		Group/Animal No.			
			1st	2nd	DMEM-BA.1				1st	2nd	BA.1-BA.1			
Nuc no. in Wuhan	AA substitution	protein	DMEM	TY38-873	1	2	3	4	TY38-873	TY38-873	17	18	19	20
11048G	ORF1a: V3595F	nsp6:V26F		G					G	G				K
														T:11.1%
11750C	ORF1a: L3829F	nsp6:L260F		C	Y			Y	C	C		Y		
					T:12.9%			T:15.9%				T:37.7%		
12747C	ORF1a: T4161I	nsp9:T21I		C					C	C	Y	Y		
											T:17.8%	T:13.0%		
13131A	ORF1a: Q4289R	nsp10:Q36R		A					A	A	R			
											G:29.0%			
20486A	ORF1b: K2340T	nsp15:K289T		A	M	M	M	M	A	A	M	M	M	M
					C:46.5%	C:45.0%	C:49.9%	C:46.1%			C:34.5%	C:24.3%	C:89.8%	C:44.7%
25487C	ORF3a: T32I	ORF3a:T32I		C					C	C		Y		
												T:11.8%		
26263G	E: E7K	E: E7K		G					G	G		R		
												A:10.4%		
28391C	N: R40C	N: R40C		C					C	C	Y			
											T:23.1%			
29645G	ORF10: V30L	ORF10: V30L		T	K	K	K	K	T	T	K	K		K
					G:19.3%	G:22.5%	G:16.2%	G:19.6%			G:32.6%	G:52.8%		G:30.0%
PANGO				BA.1.18	BA.1.18	BA.1.18	BA.1.18	BA.1.18	BA.1.18	BA.1.18	BA.1.18	BA.1.18	BA.1.18	BA.1.18

Variant			Inoculum		Group/Animal No.				Inoculum		Group/Animal No.			
			1st	2nd	DMEM-BA.1.1				1st	2nd	BA.1-BA.1.1			
Nuc no. in Wuhan	AA substitution	protein	DMEM	TY38-871	5	6	7	8	TY38-873	TY38-871	21	22	23	24
9005G	ORF1a: V2914I	nsp4:V151I		G					G	G	R			
											A:13.6%			
11355C	ORF1a: A3697V	nsp6:A128V		C					C	C				Y
														T:35.8%
11516G	ORF1a: V3751I	nsp6:V182I		G					G	G	R			
											A:31.4%			
17977C	ORF1b: L1504F	nsp13:L581F		C		Y			C	C				
						T:21.6%								
19284T	ORF1b: D1939E	nsp14:D415E		T					T	T				W
														A:34.8%
26536A	M: N5S	M:N5S		A					A	A			R	
													G:31.5%	
PANGO			-	BA.1.1	BA.1.1	BA.1.1	BA.1.1	BA.1.1	BA.1.18	BA.1.1	BA.1.1	BA.1.1	BA.1.1	BA.1.1

Variant			Inoculum		Group/Animal No.				Inoculum		Group/Animal No.			
			1st	2nd	DMEM-BA.2				1st	2nd	BA.1-BA.2			
Nuc no. in Wuhan	AA substitution	protein	DMEM	TY40-385	9	10	11	12	TY38-873	TY40-385	25	26	27	28
10512A	ORF1a: D3416G	3CLpro:D153G	-	A				R	A	A				
								G:10.3%						
28471C	Silent	N		C					C	C				Y
														T:10.7%
PANGO				BA.2	BA.2	BA.2	BA.2	BA.2	BA.1.18	BA.2	BA.2	BA.2	BA.2	BA.2

Variant			Inoculum		Group/Animal No.				Inoculum		Group/Animal No.			
			1st	2nd	DMEM-BA.2.3				1st	2nd	BA.1-BA.2.3			
Nuc no. in Wuhan	AA substitution	protein	DMEM	TY40-816	13	14	15	16	TY38-873	TY40-816	29	30*	31	32
3784C	Silent	nsp3		C					C	C	Y			
											T:18.3%			
7093C	Silent	nsp3		C					C	C				Y
														T:13.0%
7317T	ORF1a: F2351S	nsp3: F1533S		T					T	T	Y			
											C:44.6%			
11750C	ORF1a: L3829F	nsp6:L260F		C			Y		C	C				
							T:21.4%							
12060A	ORF1a: E3932A	nsp7:G73A		A					A	A				M
														C:37.3%
26198C	ORF3a: T269M	ORF3a:T269M		C					C	C				Y
														T:14.2%
PANGO				BA.2.3	BA.2.3	BA.2.3	BA.2.3	BA.2.3	BA.1.18	BA.2.3	BA.2.3	BA.2.3	BA.2.3	BA.2.3

*Animal No.30 had other mutations. See Supplementary Table3

Table S3

			Inoculum		Group/Animal No.
14-3, ham#29-32 1st: TY38-873, 2nd: TY40-816			1st	2nd	BA.1-BA.2.3
Nuc no. in Wuhan	AA substitution	Protein	TY38-873	TY40-816	30
2086G	ORF1a: Q607H	nsp2: Q427H	G	G	K T:10.9%
4250T	ORF1a: Y1329H	nsp3: Y511H	T	T	Y C:15.2%
5514T	ORF1a: V1750A	nsp3: V932A	T	T	Y C:15.9%
5763G	ORF1a: C1833F	nsp3: C1015F	G	G	K T:10.9%
8600G	ORF1a: V2779L	nsp4: V16L	G	G	S C:12.8%
12054T	ORF1a: C3931fs	nsp7: C72fs	T	T	del 10.3%
12467G	ORF1a: A4068T	nsp8: A126T	G	G	R A:10.8%
12708C	ORF1a: A4148V	nsp9: A8V	C	C	Y T:10.1%
14460T	ORF1b: F331L	nsp12: F340L	T	T	K G:22.3%
18352T	ORF1b: L1629del, P1630del	nsp14: L105del, P106del	T	T	del 13.4%
18353T			T	T	del 13.4%
18354A			A	A	del 13.4%
18355C			C	C	del 13.4%
18356C			C	C	del 13.4%
18357T			T	T	del 13.4%
18786T	Silent	nsp14	T	T	Y C:10.3%
21575C	S: L5F	S: L5F	C	C	Y T:11.4%
23766C	S: S735L	S: S735L	C	C	Y T:11.4%
25613C	ORF3a: S74F	ORF3a: S74F	C	C	Y T:13.0%
25714C	ORF3a: L108F	ORF3a: L108F	C	C	Y T:12.3%
26355T	Silent	E	T	T	Y C:13.2%
26413T	E: Y57H	E: Y57H	T	T	Y C:12.8%
28864A	Silent	N	A	A	W T:18.9%
29409C	N: T379N	N: T379N	C	C	M A:16.1%
29708C	Silent	3'UTR	C	C	Y T:11.2%
PANGO			BA.1.18	BA.2.3	BA.2.3