

## Neutralization and Stability of SARS-CoV-2 Omicron Variant

Cong Zeng<sup>1, 2#</sup>, John P. Evans<sup>1, 2, 3#</sup>, Panke Qu<sup>1, 2#</sup>, Julia Faraone<sup>1, 2, 3</sup>, Yi-Min Zheng<sup>1, 2</sup>,  
Claire Carlin<sup>4</sup>, Joseph S. Bednash<sup>5</sup>, Tongqing Zhou<sup>6</sup>, Gerard Lozanski<sup>7</sup>,  
Rama Mallampalli<sup>5</sup>, Linda J. Saif<sup>8, 9, 10</sup>, Eugene M. Oltz<sup>11</sup>, Peter Mohler<sup>4, 12, 13, 14</sup>,  
Kai Xu<sup>1, 2</sup>, Richard J. Gumina<sup>4, 13, 14</sup>, and \*Shan-Lu Liu<sup>1, 2, 10, 11</sup>

<sup>1</sup>Center for Retrovirus Research, The Ohio State University, Columbus, OH 43210, USA

<sup>2</sup>Department of Veterinary Biosciences, The Ohio State University, Columbus, OH 43210, USA

<sup>3</sup>Molecular, Cellular, and Developmental Biology Program, The Ohio State University, Columbus, OH 43210, USA

<sup>4</sup>Department of Internal Medicine, Division of Cardiovascular Medicine, The Ohio State University, Columbus, OH 43210, USA

<sup>5</sup>Department of Internal Medicine, Division of Pulmonary, Critical Care, and Sleep Medicine, The Ohio State University, Columbus, OH 43210, USA

<sup>6</sup>Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

<sup>7</sup>Department of Pathology, The Ohio State University, Columbus, OH 43210, USA

<sup>8</sup>Center for Food Animal Health, Animal Sciences Department, OARDC, College of Food, Agricultural and Environmental Sciences, The Ohio State University, Wooster, OH 44691, USA

<sup>9</sup>Veterinary Preventive Medicine Department, College of Veterinary Medicine, The Ohio State University, Wooster, OH 44691, USA

<sup>10</sup>Viruses and Emerging Pathogens Program, Infectious Diseases Institute, The Ohio State University, Columbus, OH 43210, USA

<sup>11</sup>Department of Microbial Infection and Immunity, The Ohio State University, Columbus, OH 43210, USA

<sup>12</sup>Frick Center for Heart Failure and Arrhythmia Research, The Ohio State University Wexner Medical Center, Columbus, OH 43210, USA

<sup>13</sup>Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Wexner Medical Center, Columbus, OH, 43210, USA

<sup>4</sup>Department of Physiology and Cell Biology, College of Medicine, The Ohio State University Wexner Medical Center, Columbus, OH 43210, USA

Wexner Medical Center, Columbus, OH, 43210, USA

\*Corresponding Author:

Liu.6244@osu.edu

## #Authors Contributed Equally to this Work

39         **The SARS-CoV-2 B.1.1.529/Omicron variant was first characterized in South Africa**  
40         **and was swiftly designated a variant of concern<sup>1</sup>. Of great concern is its high number of**  
41         **mutations, including 30-40 mutations in the virus spike (S) protein compared to 7-10 for**  
42         **other variants. Some of these mutations have been shown to enhance escape from**  
43         **vaccine-induced immunity, while others remain uncharacterized. Additionally, reports of**  
44         **increasing frequencies of the Omicron variant may indicate a higher rate of transmission**  
45         **compared to other variants. However, the transmissibility of Omicron and its degree of**  
46         **resistance to vaccine-induced immunity remain unclear. Here we show that Omicron**  
47         **exhibits significant immune evasion compared to other variants, but antibody**  
48         **neutralization is largely restored by mRNA vaccine booster doses. Additionally, the**  
49         **Omicron spike exhibits reduced receptor binding, cell-cell fusion, S1 subunit shedding,**  
50         **but increased cell-to-cell transmission, and homology modeling indicates a more stable**  
51         **closed S structure. These findings suggest dual immune evasion strategies for Omicron,**  
52         **due to altered epitopes and reduced exposure of the S receptor binding domain, coupled**  
53         **with enhanced transmissibility due to enhanced S protein stability. These results highlight**  
54         **the importance of booster vaccine doses for maintaining protection against the Omicron**  
55         **variant, and provide mechanistic insight into the altered functionality of the Omicron spike**  
56         **protein.**

57         Since the introduction of SARS-CoV-2 into the human population in late 2019, adaptive  
58         evolution of the virus has resulted in increased transmissibility and resistance to vaccine- or  
59         infection-induced neutralizing antibodies<sup>2,3</sup>. Indeed, the initial D614G mutation in the virus spike  
60         (S) protein enhanced virus stability, infectivity, and transmission<sup>4,5</sup>. This initial adaptation was  
61         followed by the emergence of several SARS-CoV-2 variants of concern, including Alpha (B.1.1.7),  
62         which rapidly spread from Europe to become the global dominant variant in other parts of the  
63         world<sup>6</sup>. Subsequently, the Beta (B.1.351) variant exhibited substantial resistance to neutralization<sup>7</sup>,  
64         although failed to disseminate as widely. The most recent wave of the pandemic is attributed to

65 the Delta (B.1.617.2) variant characterized by moderate neutralization resistance combined with  
66 enhanced transmissibility, driving its dominance worldwide<sup>8</sup>. Despite these evolutionary leaps,  
67 vaccine-mediated protection from severe disease and hospitalization remained high<sup>9</sup>.

68 The emergence of the Omicron (B.1.1.529) variant has generated serious concern about  
69 the continued efficacy of vaccines and the future course of the pandemic<sup>10,11</sup>. In addition to its  
70 sheer number of mutations<sup>12</sup>, Omicron contains specific alterations that have previously been  
71 shown to impact vaccine resistance, namely in the receptor-binding domain (RBD), a primary  
72 target of host neutralizing antibodies to the S protein, as well as a number of other non-RBD  
73 mutations, including some in the S2 subunit (**Fig. 1a**). Moreover, when present in a geographic  
74 region, the Omicron variant constitutes a rapidly increasing proportion of COVID-19 cases<sup>13,14</sup>,  
75 suggesting a further enhancement of transmissibility.

76

77 **Resistance of Omicron to neutralizing antibodies in two-dose vaccinees, booster-dose  
78 recipients and COVID-19 patients**

79 Given heightened concerns about Omicron's transmissibility and vaccine resistance, we  
80 examined the its S protein compared to other major SARS-CoV-2 variants using a previously  
81 reported pseudotyped lentivirus system<sup>15</sup>. We first examined infectivity using lentivirus  
82 pseudotypes and HEK293T-ACE2 as target cells. As shown in **Fig. 1b**, the infectivity of Omicron  
83 was largely comparable to the other major variants, all of which were lower than the ancestral  
84 D614G. However, in a more physiologically relevant host, human lung epithelia-derived CaLu-3  
85 cells, Omicron exhibited reduced infectivity relative to D614G (**Fig. 1c**).

86 We next examined the ability of Omicron to escape vaccine-induced neutralizing  
87 antibodies, a critical measure of protection from SARS-CoV-2 infection<sup>16</sup>. To address this, we  
88 collected sera from 48 health care workers (HCWs) 3-4 weeks post-second dose of either  
89 Moderna mRNA-1273 (n = 20) or Pfizer/BioNTech BNT162b2 (n = 28). Having previously  
90 examined this cohort for the ability of the D614G, Alpha, Beta, and Delta variants to escape serum

91 antibody neutralization (nAb)<sup>17</sup>, we compared the neutralization resistance of Omicron to these  
92 variants of concern. We found that the Omicron variant exhibited significantly more neutralization  
93 resistance, i.e., 22.9-fold ( $p < 0.001$ ), compared to ancestral D614G, with the Alpha, Beta, and  
94 Delta variants exhibiting a 1.2-fold, 4.4-fold ( $p < 0.001$ ), and 2.0-fold ( $p < 0.01$ ) decrease in nAb  
95 titers, respectively (**Fig. 1d**). In total, only 27.1% (13/48) of HCWs exhibited nAb titers against  
96 Omicron above the detection limit ( $NT_{50} < 80$ ); however, several individuals (1-3) exhibited strong  
97 nAb titers that were maintained against Omicron (**Fig. 1d and h**). Moderna mRNA-1273 in HCWs  
98 slightly outperformed Pfizer/BioNTech BNT162b2 (**Fig. S1a**), as we have reported previously<sup>17,18</sup>.

99 We further sought to examine resistance to vaccine-induced immunity for Omicron along  
100 with D614G, Alpha, Beta, and Delta for 23 HCWs 1-11 weeks post-booster vaccination. We found  
101 that booster vaccination not only increased the nAb  $NT_{50}$  titer against all variants, including D614G,  
102 but also significantly restored neutralization of Omicron, with only 3.3-fold reduced  $NT_{50}$  relative  
103 to D614G, which was even less than the 4.3-fold reduction for the Delta variant (**Fig. 1e**). These  
104 data indicate that booster dose administration not only enhances nAb titers, but also enhances  
105 the breadth of the nAb response, especially against Omicron. For 18 HCWs, we analyzed the  
106 post-second dose and post-booster dose samples, which showed significantly higher nAb titers  
107 following booster vaccination (**Fig. S1b-f**). Pfizer/BioNTech BNT162b2-vaccinated HCWs  
108 exhibited slightly higher nAb titers than Moderna mRNA-1273-vaccinated HCWs post-booster  
109 (**Fig. S1g**).

110 We further examined nAb resistance of Omicron and other major variants in ICU ( $n = 9$ )  
111 and hospitalized non-ICU ( $n = 9$ ) patient serum samples collected during the 2020/D614G wave  
112 of the pandemic prior to vaccination. We found that Omicron was completely resistant to D614G-  
113 wave patient serum samples, with only 22.2% (2/9) of ICU and 11.1% (1/9) of hospitalized patients  
114 exhibiting a threshold of nAb titers (**Fig. 1f and h**). We further examined ICU patient samples ( $n$   
115 = 19) collected during the Delta wave of the pandemic, including 5 confirmed as Delta infections  
116 by sequencing of virus from nasal swabs. Serum from these infected patients exhibited potent

117 neutralization of Delta, as would be expected, while Omicron still exhibited strong resistance (**Fig.**  
118 **1g and h**). Although mean titers from these individuals were comparable to boosted HCWs, a  
119 more significant proportion, 47.4% (9/19), exhibited no detectable nAb titers against Omicron (**Fig.**  
120 **1g and h**). Further, this population contained 5 patients fully vaccinated with two doses, 1 fully  
121 vaccinated with 1 Johnson & Johnson dose, and 1 partially vaccinated (1 dose of Pfizer), which  
122 dramatically out-performed unvaccinated patients against Omicron and other variants, except  
123 Delta (**Fig. S1h**).

124

125 **Omicron spike exhibits reduced ACE2 binding, furin cleavage and cell-cell fusion**

126 To better understand the impact of the Omicron mutations on binding to the virus receptor  
127 ACE2, we transfected HEK293T cells with variant spike constructs and determined S surface  
128 expression, as well as their capacity to bind to soluble ACE2 (sACE2), using flow cytometry (**Fig.**  
129 **S2a-d**). We observed comparable levels of S expression for Alpha, Beta, and Delta relative to  
130 D614G, with Omicron showing a slight reduction (**Fig. 2a**, **Fig. S2a-c and e**), as detected by a  
131 polyclonal antibody T62 against S1 or by anti-FLAG antibody specific for the N-termini of each  
132 recombinant spike. However, by normalizing for S surface expression in five independent  
133 experiments, we observed that Omicron variant exhibited a 2.4-fold reduced binding to sACE2  
134 compared to the ancestral D614G (**Fig. 2b-c**, **Fig. S2d-f**).

135 We further sought to examine the processing and incorporation of the Omicron S protein  
136 into viral particles. Pseudotyped virus was purified and probed for the S1 subunit of S alongside  
137 virus producer cell lysate. We found that Omicron S, similar to that of Alpha, exhibited a reduced  
138 furin processing into S1 compared to D614G and Beta; consistent with prior reports<sup>8,19</sup>, the Delta  
139 variant showed increased furin cleavage in the spike (**Fig. 2d**). Notably, substantially less S1 was  
140 detectable in purified Omicron pseudotyped virus (**Fig. 2d**), despite a modestly low level of p24,  
141 which may reflect reduced incorporation of the Omicron S into pseudotyped virions and accounts  
142 for its decreased infectivity compared to D614G.

143 We next examined the efficiency of Omicron S protein-mediated cell-cell fusion. HEK293T  
144 cells were transfected with GFP and S protein constructs, and then co-cultured with HEK293T  
145 cells stably expressing ACE2 (HEK293T-ACE2) for 24 hrs. Cell-cell fusion was imaged using  
146 fluorescence microscope. We observed a marked reduction in the size of syncytia induced by  
147 Omicron S compared to other variants, especially by Delta and Beta (**Fig. 2e**). These results were  
148 confirmed using a more quantitative Tet-Off-based fusion assay, where spike and Tet-Off-  
149 expressing HEK293T cells were co-cultured with HEK293FT-mCAT-Gluc cells expressing ACE2.  
150 Upon cell-cell fusion, Tet-Off would induce the expression of *Gaussia* luciferase driven by a  
151 tetracycline-controlled transcription factor<sup>20</sup>. The Omicron variant exhibited 1.7-fold lower cell-cell  
152 fusion compared to D614G ( $p < 0.001$ ) (**Fig. 2f**), consistent with reduced efficiency in mediating  
153 fusion at the plasma membrane.

154

#### 155 **Omicron spike has enhanced capacity for cell-to-cell transmission**

156 We reported the ability of SARS-CoV-2 spike to mediate cell-to-cell transmission<sup>20</sup>. To  
157 measure the efficiency of this process by Omicron S, we co-cultured with HEK293T-ACE2 target  
158 cells with HEK293T pseudotyping virus-producer cells and assessed cell-to-cell transmission  
159 after 24 hrs. Unexpectedly, we found that Omicron drastically increased the efficiency of cell-to-  
160 cell transmission, with 4.8-fold higher levels than D614G and other variants (**Fig. 2g**);, despite  
161 comparable levels of cell-free infection (**Fig. 1b**), reduced ACE2 binding (**Fig. 2c**), and reduced  
162 cell-cell fusion (**Fig. 2e**).

163

#### 164 **Omicron spike exhibits less S1 shedding**

165 Given the reduced ACE2 binding of Omicron, we speculated that its spike may be less  
166 stable. We therefore sought to explore the stability of the Omicron S in parallel with other VOC  
167 spikes. First, we examined ACE2-induced S1 subunit shedding by transfecting HEK293T cells  
168 with variant S constructs. Cells treated with or without sACE2 and S1 containing-media were

169 collected, and S1 subunit was immune-precipitated using anti-Flag beads. We observed reduced  
170 S1 shedding in the Omicron variant for both sACE2-treated and untreated cells (**Fig. 3a**), despite  
171 relatively comparable levels of S expression in the cells, with the exception of Delta (**Fig. 3a**).  
172 While this may be related to the low ACE2 binding of Omicron variant, distinct mutations unique  
173 to Omicron spike, especially those in the furin cleavage site, likely contribute to their differential S1  
174 shedding.

175 To provide additional molecular insights, we created a structural model of the Omicron  
176 spike protein using the cryo-EM structure of D614G strain spike (PDB 7KRR) as a homology  
177 modeling template (**Fig. 3b**). Structural analysis of the inter-protomer interface indicated an  
178 increment in the Omicron spike ( $6397 \text{ \AA}^2$ ) compared to the G614 spike ( $6017.4 \text{ \AA}^2$ ). The overall  
179 solvation free energy gain ( $\Delta G$ ) shifts from  $-59.6 \text{ kcal/mol}$  to  $-60.4 \text{ kcal/mol}$ , reflecting the  
180 enhanced hydrophobic interaction at the Omicron S trimer interface (**Table S1**). Moreover,  
181 additional hydrophilic interactions are introduced by Omicron mutations that stabilize the spike  
182 trimer formation, including the N856K mutation, which enables formation of a salt-bridge and a  
183 hydrogen bond with residues D568 and T572, respectively, on the neighboring protomer.  
184 Moreover, the N764K mutation, jointly with the residue D737, forms hydrogen bonds with residue  
185 N317 on the neighboring protomer. These inter-protomer interactions enhance association  
186 between protomers, as well as between subunits S1 and S2 (**Fig. 3b inset**). The structural model  
187 also revealed two critical conformation-stabilizing mutations, T547K and L981F, located close to  
188 the base of the spike RBD. When RBD is in the “down” conformation, K547 is in close proximity  
189 to both residue 981 in the core of S trimer and residue D389 on the base of RBD (**Fig. 3c**). The  
190 hydrophobic interaction formed by K547 and F981, together with the salt-bridge formed by K547  
191 and D389, would lock RBD in the “down” conformation. The “up” conformation of RBD disrupts  
192 the interaction among these three residues (**Fig. 3c inset**).

193

194 **Discussion**

195 In this work, we examined the efficacy of vaccine-induced immunity against the Omicron  
196 variant, along with molecular and virological features of Omicron spike, in parallel with other  
197 variants. We found that, while recipients of two-doses of mRNA vaccines showed minimal  
198 neutralization of the Omicron variant, recipients of a booster dose, either Moderna or Pfizer,  
199 exhibited much stronger neutralizing capacity against Omicron. The fold-difference between nAb  
200 titers against D614G and Omicron in the boosted group (3.8-fold) vs. that in the two-dose vaccine  
201 group (22.9-fold) revealed that a booster dose not only raises nAb levels, but also increases the  
202 breadth of circulating nAbs, a finding that is similar to a recent report by Pfizer<sup>21</sup>. While underlying  
203 mechanisms for this remain unclear, enhanced breadth of protection likely is due to additional  
204 affinity-maturation following a third antigen exposure. As the Omicron variant may have evolved  
205 from a lineage distinct from Delta<sup>22</sup>, it is unsurprising that Omicron exhibited strong resistance to  
206 nAb from unvaccinated Delta-infected ICU patients, with Omicron exhibiting ~8-fold lower nAb  
207 sensitivity than Delta for this group.

208 One surprising finding is that, distinct from Alpha, Beta, and Delta, the Omicron spike  
209 exhibited reduced binding to soluble ACE2, which likely accounts for, at least in part, its lower  
210 cell-cell fusion efficiency. This may indicate a fitness cost following an accumulation of RBD  
211 mutations while under selective pressure for nAb escape. Additionally, reduced cell-cell fusion  
212 would reduce cytotoxicity and could contribute to a lower virulence for the Omicron variant, which  
213 has been tentatively reported by anecdotal evidence<sup>23</sup>.

214 Alternatively, reduced ACE2 binding could be reflective of a strategy to reduce premature  
215 S inactivation that would enhance virus transmissibility. This possibility is supported by the low  
216 S1 shedding observed for Omicron, and confirmed by enhanced stability of the RBD-closed  
217 conformation in homology modeling. Notably, infectivity of Omicron in HEK293T-ACE2 cells was  
218 not impaired, indicating that it retains functional receptor utilization. However, Omicron exhibited  
219 reduced infection in CaLu-3 cells. SARS-CoV-2 is thought to predominantly utilize a TMPRSS2-  
220 mediated plasma membrane entry route in CaLu-3 cells, while a cathepsin-B/L-mediated

221 endosomal entry route dominates in HEK293T-ACE2 cells<sup>24,25</sup>. Thus, Omicron S may have  
222 evolved to be less fusogenic and utilizes plasma membrane fusion less efficiently in order to  
223 minimize potential cytopathogenic effect.

224 Indeed, the Omicron variant exhibited enhanced cell-to-cell transmission, which would  
225 facilitate virus spread. Cell-to-cell transmission is commonly used by many viruses, including  
226 SARS-CoV-2, and is a highly efficient mechanism of virus spread within a host<sup>20,26,27</sup>. Enhanced  
227 cell-to-cell transmission may help compensate for other observed defects in the Omicron S protein,  
228 such as reduced ACE2 binding and fusogenicity. Notably, cell-to-cell transmission of SARS-CoV-  
229 2 does not absolutely require ACE2, and extended cell-cell fusion by its spike impairs cell-to-cell  
230 transmission<sup>20</sup>. Additionally, cell-to-cell transmission is resistant to neutralizing antibodies,  
231 implicating another potential mechanism of Omicron immune evasion<sup>20,26,27</sup>.

232 Overall, our report highlights the need for booster dose administration to better combat  
233 the emerging Omicron variant, and that reformulation of existing mRNA vaccines to target  
234 Omicron may not be necessary with a three-dose vaccine regimen. However, the emergence of  
235 future divergent variants may further compromise even boosted immunity. Evidence to date  
236 suggests no significant enhancement of virulence for Omicron; however, a definitive conclusion  
237 will require population level studies as this variant spreads to new locales. Indeed, continued  
238 monitoring of emerging SARS-CoV-2 variants is vital to allow for rapid investigation of their  
239 transmissibility, neutralization resistance, and pathogenicity.

240

## 241 **Figure Legends**

242

243 **Figure 1. The Omicron SARS-CoV-2 variant exhibits strong immune-escape that is**  
244 **overcome by booster vaccination.** (a) Diagrams of SARS-CoV-2 S variants used for  
245 pseudotyping, which indicate the location of specific mutations as well as the S1 and S2 subunits  
246 of S, the N-Terminal Domain (NTD), Receptor Binding Domain (RBD), Fusion Peptide (FP), and

247 Trans-membrane domain (TM). **(b)** Infectivity of pseudotyped viruses produced in parallel for  
248 infection of HEK293T cells stably expressing ACE2. **(c)** Infectivity of pseudotyped lentivirus in  
249 human lung epithelia-derived CaLu-3 cell line. Bars in b and c represent means +/- standard  
250 deviation, and significance is determined by one-way ANOVA with Bonferroni's multiple testing  
251 correction. Results of at least 3 independent experiments are averaged and shown. **(d)** Sera from  
252 48 HCWs collected 3-4 weeks after second mRNA vaccine dose was used to neutralize  
253 pseudotyped virus for variants, and the resulting 50% neutralization titers (NT<sub>50</sub>) are displayed.  
254 **(e)** Sera from 23 HCWs following homologous mRNA booster vaccination were assessed for nAb  
255 titers. **(f)** Sera from 9 ICU COVID-19 patient samples and 9 hospitalized non-ICU COVID-19  
256 patient samples collected in 2020 prior to the approval of any SARS-CoV-2 vaccines were  
257 assessed for nAb titers. **(g)** Sera from 19 ICU COVID-19 patient samples collected during the  
258 Delta-wave of the pandemic were assessed for nAb titers. Mean NT<sub>50</sub> values in panels d-g are  
259 displayed at the top of plots along with relative neutralization sensitivity with D614G set to 100%;  
260 bars represent mean +/- standard error, and significance relative to D614G is determined by one-  
261 way ANOVA with Bonferroni's multiple testing correction. **(h)** Heat maps showing patient/vaccinee  
262 NT<sub>50</sub> values against each variant. Patient/vaccinee numbers are identified as P for  
263 Pfizer/BioNTech BNT162b2 vaccinated/boosted HCW, M for Moderna mRNA-1273  
264 vaccinated/boosted HCW, I for ICU patient samples collected during the 2020 D614G-wave, H  
265 for hospitalized non-ICU patient samples collected during the 2020 D614G-wave, or D for ICU  
266 patient samples collected during the Delta-wave. P-values are represented as \*p < 0.05, \*\*p <  
267 0.01, \*\*\*p < 0.001; ns, not significant.

268

269 **Figure 2. The Omicron S exhibits reduced ACE2 binding, processing, and fusion, but**  
270 **enhanced cell-to-cell transmission. (a-c)** HEK293T cells were transfected with S expression  
271 constructs and stained for FACS with anti-S1 (T62) **(a)** or soluble ACE2-Fc fusion protein (sACE2)  
272 **(b). (c)** Mean fluorescence intensity for sACE2 signal was normalized with S1 signal to determine

273 relative ACE2 binding; n=5. (d) Pseudotyped virus producer cells were lysed and pseudotyped  
274 virus was purified by ultracentrifugation and blots were probed for S1 subunit, lentivirus capsid  
275 (p24), and  $\beta$ -actin loading control; S cleavage was quantified using NIH ImageJ and by setting  
276 the ratio of S1/S of D614G to 1.00. (e) HEK293T cells transfected with GFP and S constructs  
277 were co-cultured with HEK293T-ACE2 cells for 24 hrs and imaged to visualize cell-cell fusion. (f)  
278 HEK293T cells transfected with S constructs and Tet-Off were co-cultured with HEK293FT-  
279 mCAT-Gluc cells transfected with an ACE2-GFP plasmid, and fusion-induced *Gaussia* luciferase  
280 signal was assessed 24 hrs after co-culture. (g) HEK293T cells transfected with S constructs to  
281 pseudotype *Gaussia* luciferase bearing lentivirus and were co-cultured with HEK293T-ACE2 cells  
282 and secreted *Gaussia* luciferase was assayed 24 hrs after co-culture. Bars in panels c, f and g  
283 represent means +/- standard deviation, and significance was determined by one-way ANOVA  
284 with Bonferroni's multiple testing correction. Results were from at least three independent  
285 experiments. P-values are represented as \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

286

287 **Fig. 3. The Omicron S protein exhibits low S1 shedding, consistent with predicted increase**  
288 **in stability.** (a) HEK293T cells were transfected with S constructs and treated with or without  
289 sACE2. Cell lysate and cell culture media were collected after sACE2 treatment, and shed S1  
290 subunit in cell culture media was immune-precipitated with anti-Flag beads. Blots were probed  
291 with anti-S1 and anti-GAPDH, and S1 shedding was quantified by NIH ImageJ by setting D614G  
292 to 1.0. (b) Structure of Omicron spike protein viewed from side. Mutations of Omicron are  
293 highlighted by red sticks and semi-transparent red surfaces. RBD of the yellow protomer is in an  
294 "up" conformation. Upper inset: the mutation N856K enables formation of salt-bridge and  
295 hydrogen bond with the residues D568 and T572, respectively on the neighboring protomer  
296 (green). Lower inset: the mutation N764K enables formation of hydrogen bond with residue N317  
297 on the neighboring protomer (grey) and salt-bridge with residue D737 on the same protomer  
298 (yellow). (c) Omicron mutations T547K and L981F stabilize RBD in "down" conformation.

299 Structure of Omicron spike protein by homology modeling was illustrated as surfaces (yellow and  
300 grey protomer) and ribbon (rainbow protomer). Upper inset: When RBD is in “down” conformation,  
301 Omicron mutation T547K enables formation of salt-bridge with residue D389 located on the base  
302 of RBD. Mutation T547K and L981F together enhance the hydrophobic interaction between the  
303 neighboring protomers (green and grey). Lower inset: When RBD is in “up” conformation, the  
304 interactions between these residues are disrupted.

305

306 **Figure S1. Neutralization of SARS-CoV-2 Omicron variant by vaccination status and**  
307 **vaccine type.** (a) NT<sub>50</sub> values for HCWs who received two doses of Moderna mRNA-1273 (n =  
308 20) or Pfizer/BioNTech BNT162b2 (n = 28) are plotted by vaccine type. (b) NT<sub>50</sub> values for  
309 recipients of Moderna mRNA-1273 (n = 6) or Pfizer/BioNTech BNT162b2 (n = 17) booster doses  
310 are plotted by vaccine type. (c-g) Post second vaccine dose and post booster dose NT<sub>50</sub> values  
311 are plotted pairwise for HCWs for which both time points were analyzed (n = 18) against the  
312 D614G (c), Alpha (d), Beta (e), Delta (f), and Omicron (g) variants. (h) NT<sub>50</sub> values for  
313 unvaccinated (n = 12) and vaccinated (n = 7) Delta-wave COVID-19 ICU-patients are plotted  
314 according to vaccination status. Bars in panels a, b and h represent means +/- standard error and  
315 statistical significance in all cases was determined by two-tailed t-test with Welch’s correction.  
316 Dashed lines indicate the threshold NT<sub>50</sub>, which was set to 80. P-values are represented as \*\*p <  
317 0.01.

318

319 **Figure S2. Omicron spike surface expression and sACE2 binding:** (a-d) The gating strategy  
320 for one representative experiment is shown for determine the single cell population (a), the S1  
321 positive population by N-terminal Flag tag (b), the S1 positive population by anti-S1 antibody (c),  
322 and the sACE2 positive population (d). (e) HEK293T cells were transfected with S expression  
323 constructs and stained for FACS with anti-Flag or soluble ACE2-Fc fusion protein (sACE2). (f)

324 Mean fluorescence intensity for sACE2 signal was normalized with anti-Flag S1 signal to  
325 determine relative ACE2 binding.

326

327 **Methods**

328 **Vaccinated and ICU patient cohorts**

329 Vaccinated HCW samples were collected under approved IRB protocols (2020H0228 and  
330 2020H0527). Sera were collected 3-5 weeks post second vaccine dose for 48 HCWs which  
331 included 20 Moderna mRNA-1273 and 28 Pfizer/BioNTech BNT162b2 vaccinated HCWs. In the  
332 study group, 23 HCWs received homologous vaccine booster doses 34-42 weeks post second  
333 dose, these included. Sera was then collected from these 23 HCWs 1-11 weeks post vaccine  
334 booster dose. These samples included 6 Moderna mRNA-1273 and 17 Pfizer/BioNTech  
335 BNT162b2 boosted HCWs.

336 D614G-wave ICU and hospitalized non-ICU patient samples were collected under an  
337 approved IRB protocol (OSU 2020H0228) as previously described<sup>15</sup>. Delta-wave ICU patient  
338 samples were collected under an approved IRB protocol (2020H0175). Plasma samples were  
339 collected 3 days after hospitalization. Where detectable, the strain of SARS-CoV-2 infecting the  
340 ICU patients was determined by viral RNA extraction on nasal swabs with QIAamp MinElute Virus  
341 Spin kit followed by RT-PCR (CDC N1 F: 5'-GACCCCAAAATCAGCGAAAT-3'; CDC N1 R: 5'-  
342 TCTGGTTACTGCCAGTTGAATCTG-3'; CDC N2 F: 5'-TTACAAACATTGGCCGCAA-3'; CDC  
343 N2 R: 5'-GCGCGACATTCCGAAGAA-3') and Sanger sequencing to identify virus strain. Due to  
344 risk of infection, Delta-wave ICU patient plasma samples were treated with 1% Triton X100 to  
345 inactivate virus. A starting dilution for virus neutralization assays of 1:640 was found to not exhibit  
346 Triton X100 mediated cell toxicity.

347 **Cell lines and maintenance**

348 HEK293T (ATCC CRL-11268, CVCL\_1926), HEK293T-ACE2 (BEI NR-52511), and  
349 HEK293FT-mCAT-Gluc (a gift from Marc Johnson, University of Missouri) cells were maintained

350 in DMEM (Gibco, 11965-092) supplemented with 10% FBS (Sigma, F1051) and 1% penicillin-  
351 streptomycin (HyClone, SV30010). CaLu-3 cells were maintained in EMEM (ATCC, 30-2003)  
352 supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were maintained at 37°C  
353 and 5% CO<sub>2</sub>.

### 354 **Plasmids**

355 We utilized a previously reported pNL4-3-inGluc lentivirus vector which is based on ΔEnv  
356 HIV-1 and bears a *Gaussia* luciferase reporter gene that is expressed in virus target cells but not  
357 virus producing cells<sup>15,28</sup>. Additionally, SARS-CoV-2 variant spike constructs with N- and C-  
358 terminal flag tags were produced and cloned into a pcDNA3.1 vector by GenScript Biotech  
359 (Piscataway, NJ) using Kpn I and BamH I restriction enzyme cloning. A pLenti-hACE2-GFP (a gift  
360 from Jacob Yount, The Ohio State University) was used transient expression of ACE2. For  
361 transient expression of the Tet-Off (tTA) transcription factor, a pQCXIP-Tet-Off expression  
362 plasmid was used (a gift from Marc Johnson, University of Missouri).

### 363 **Pseudotyped lentivirus production and infectivity**

364 Lentiviral pseudotypes were produced as previously reported<sup>17</sup>. Briefly, HEK293T cells  
365 were transfected with pNL4-3-inGluc and spike construct in a 2:1 ratio using polyethylenimine  
366 transfection. Virus was harvested 24, 48, and 72 hrs after transfection.

367 HEK293T-ACE2 or CaLu-3 cells were infected with pseudotyped virus for each strain,  
368 produced in parallel. *Gaussia* luciferase activity was assessed 48 hrs after infection by combining  
369 cell culture media with *Gaussia* luciferase substrate (0.1M Tris pH 7.4, 0.3M sodium ascorbate,  
370 10 μM coelenterazine). Luminescence was immediately measured by a BioTek Cytation5 plate  
371 reader.

### 372 **Virus neutralization assay**

373 Pseudotyped lentivirus neutralization assays were performed as previously  
374 described<sup>15,17,18,29</sup>. Briefly, HCW serum or ICU patient plasma was 4-fold serially diluted and equal  
375 amounts of infectious SARS-CoV-2 variant pseudotyped virus was added to the diluted serum.

376 Final dilutions of 1:80, 1:320, 1:1280, 1:5120, 1:20480, and no serum control for vaccinated HCW  
377 samples; while final dilutions of 1:1280, 1:2560, 1:5120, 1:10240, and no serum control were used  
378 for ICU patient plasma to avoid Triton X100 toxicity. Virus and serum were incubated for 1 hr at  
379 37°C and then transferred to HEK293T-ACE2 cells for infection. *Gaussia* luciferase activity was  
380 determined 48 and 72 hrs after infection by combining 20 µL of cell culture media with 20 µL of  
381 *Gaussia* luciferase substrate. Luminescence was immediately measured by a BioTek Cytation5  
382 plate reader. NT<sub>50</sub> values were determined by least-squares-fit, non-linear regression in  
383 GraphPad Prism 5 (San Diego, CA). Heat maps with NT<sub>50</sub> generated by GraphPad Prism 9.

#### 384 **Spike binding sACE2 and anti-Flag and detection by flow cytometry**

385 Virus producing HEK293T cells were harvested 72 hrs after transfection. Cells were  
386 dissociated by incubation at 37°C in PBS + 5mM EDTA for 30 min. Then, cells were fixed in 4%  
387 formaldehyde in PBS, and stained with sACE2-humanFc fusion protein (construct is a gift of  
388 Jason McLellan, University of Texas at Austin) or mouse anti-Flag antibody (Sigma, F3165). Cells  
389 were then stained with anti-human-IgG-FITC (Sigma, F9512) or anti-mouse-IgG-FITC (Sigma,  
390 F0257) and processed by a Life Technologies Attune NxT flow cytometer. Results were  
391 processed using FlowJo v7.6.5 (Ashland, OR). Relative ACE2 binding was then determined by  
392 dividing sACE2 signal by anti-Flag signal measured by mean fluorescence intensity.

#### 393 **Syncytia formation assay**

394 HEK293T cells were co-transfected with a plasmid encoding GFP along with that a variant  
395 spike of interest. The transfected HEK293T cells were co-cultured with HEK293T-ACE2 cells 24  
396 hrs after transfection. Cells were imaged 24 hrs after co-culture at 4x magnification on a Leica  
397 DMi8 confocal microscope. Representative images were selected.

#### 398 **Tet-off-based cell-cell fusion assay**

399 Donor HEK293T cells were transfected with an S construct of interest and pQCXIP-Tet-  
400 Off in a 1:1 ratio. Target HEK293FT-mCAT-Gluc cells stably expressing tetracycline-responsive  
401 element (TRE)-driven *Gaussia* luciferase were transfected with pLenti-hACE2-GFP. 24 hrs after

402 transfection, HEK293FT-mCAT-Gluc and HEK293T cells were dissociated with PBS-EDTA, and  
403 cocultured at a 1:1 ratio. Fusion between S and Tet-Off expressing HEK293T cells and ACE2  
404 expressing HEK293FT-mCAT-Gluc cells would drive expression of *Gaussia* luciferase. Thus, at  
405 24 hrs and 48 hrs after co-culture, cell culture media were sampled and assayed for *Gaussia*  
406 luciferase activity.

#### 407 **Spike incorporation into pseudotyped virus**

408 Pseudotyped virus particles were purified by ultracentrifugation through a 20% sucrose  
409 cushion. Virus was resuspended in SDS-PAGE loading buffer. Cell lysate from virus producer  
410 cells was collected by 30 min incubation of cells on ice in RIPA lysis buffer (50 mM Tris pH 7.5,  
411 150 mM NaCl, 1 mM EDTA, Nonidet P-40, 0.1% SDS) supplemented with protease inhibitor  
412 (Sigma, P8340). Samples were run on a 10% acrylamide SDS-PAGE gel and transferred to a  
413 PVDF membrane. Membranes were probed with anti-Flag (Sigma, F3165), anti-p24 (Abcam,  
414 ab63917; NIH ARP-1513), anti-S1 (Sino Biological, 40150-T62), and anit- $\beta$ -actin (Sigma, A1978).  
415 Anti-mouse-IgG-Peroxidase (Sigma, A5278) and anti-rabbit-IgG-HRP (Sigma, A9169) were used  
416 as secondary antibodies where appropriate. Blots were imaged with Immobilon Crescendo  
417 Western HRP substrate (Millipore, WBLUR0500) on a GE Amersham Imager 600.

#### 418 **S1 shedding**

419 HEK293T cells were transfected with S expression constructs. Then, 24 hrs after  
420 transfection, cells were treated with or without sACE2 (25  $\mu$ g/mL) for 4 hrs at 37°C. Cell lysate  
421 and cell culture media was harvested. S1-containig cell culture media was incubated with 10  $\mu$ L  
422 of anti-Flag beads (Sigma, F2426) to precipitate S1 subunit. Following immune-precipitation, cell  
423 lysate and shed S1 were run on 10% SDS-PAGE gel, transferred, and probed with anti-S1 (Sino  
424 Biological, 40150-T62) and anit-GAPDH (Santa Cruz, sc-47724). Anti-mouse-IgG-Peroxidase  
425 (Sigma, A5278) and anti-rabbit-IgG-HRP (Sigma, A9169) were used as secondary antibodies.

#### 426 **Structural modeling**

427 Homology modeling of Omicron spike protein was conducted on SWISS-MODEL server  
428 with cryo-EM structure of SARS-CoV2 G614 strain spike (PDB 7KRR) as template. The resulting  
429 homo-trimer spike structure has one RBD in up conformation and the other two RBD in down  
430 conformation. Residue examination and renumbering were carried out manually with program  
431 Coot.

#### 432 **Molecular contact analysis**

433 Inter-protomer interaction analysis was performed with PDBePISA server. The Omicron  
434 spike inter-protomer interface was compared to that of G614 strain in both overall assembly and  
435 individual residue levels. Intra-protomer contacts of Omicron mutants were examined with the  
436 programs PyMOL and chimeraX. All structural figures were generated with PyMOL. The detail  
437 analysis results were listed in Table S1 and Supplementary Table 1. Homology modeling  
438 was performed in PyMOL using the cryo-EM structure of D614G strain spike (PDB 7KRR) as  
439 homology modeling template to predict Omicron spike protein structure.

#### 440 **Statistics**

441 Comparisons between multiple groups were made using a one-way ANOVA with  
442 Bonferroni post-test. Comparisons between two-groups were made using a two-tailed student's  
443 t-test with Welch's correction.

444

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515

516

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518 S.-L.L. conceived and directed the project. C.Z., J.P.E., and P.Q. contributed the majority of the  
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524

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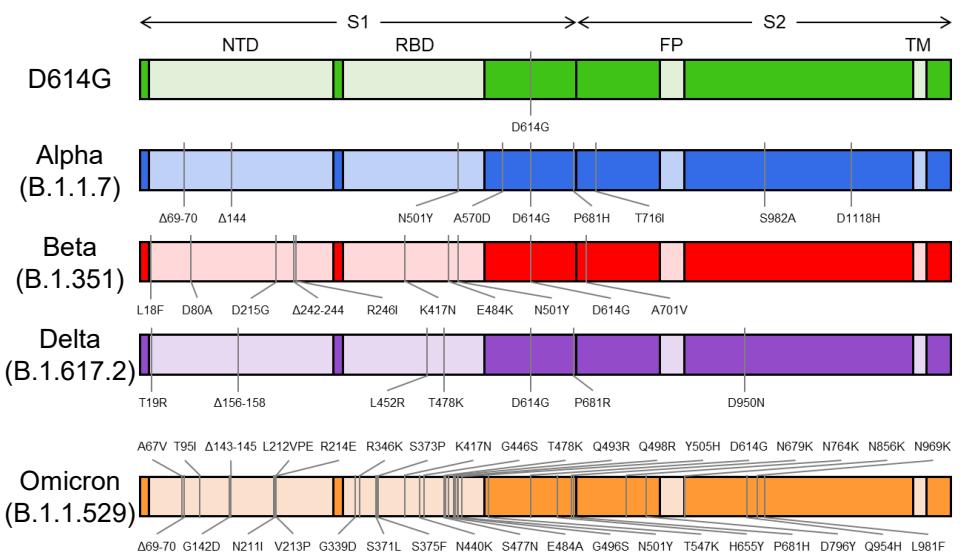
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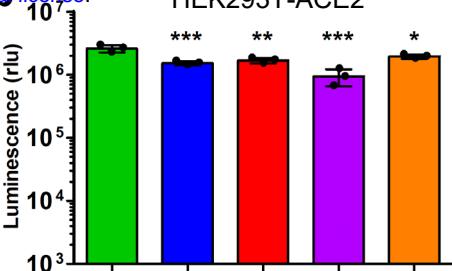
538 **Competing Interest Declaration:**

539 The authors have no competing interests to disclose

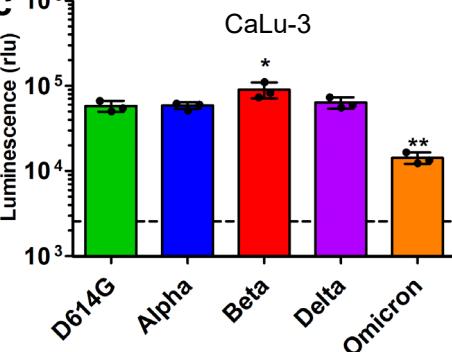
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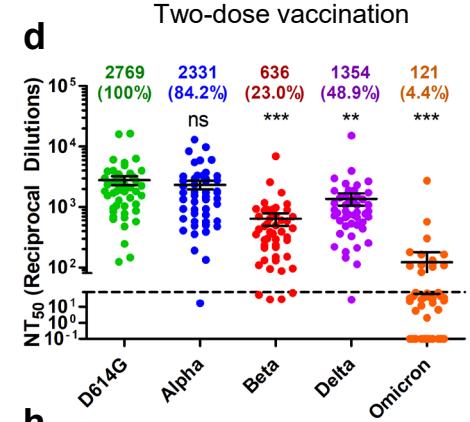
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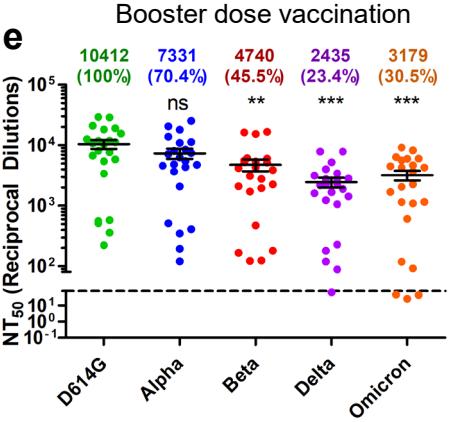
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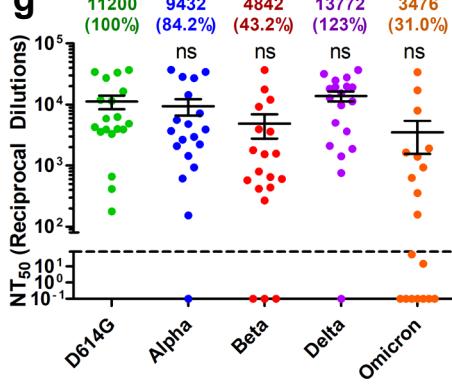
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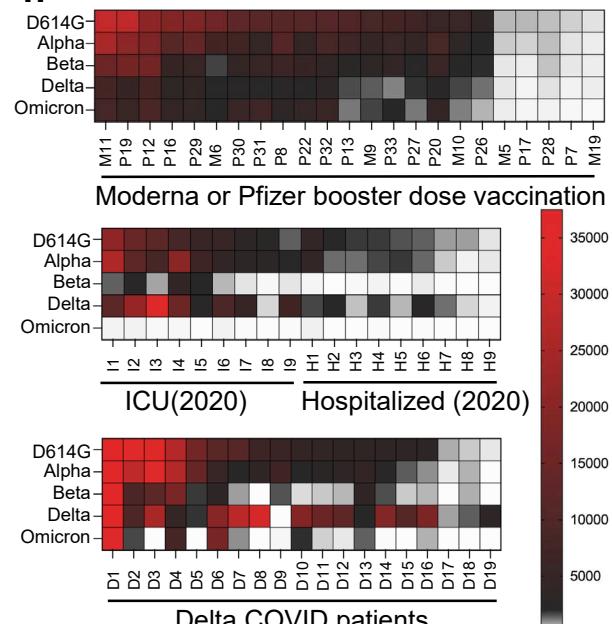
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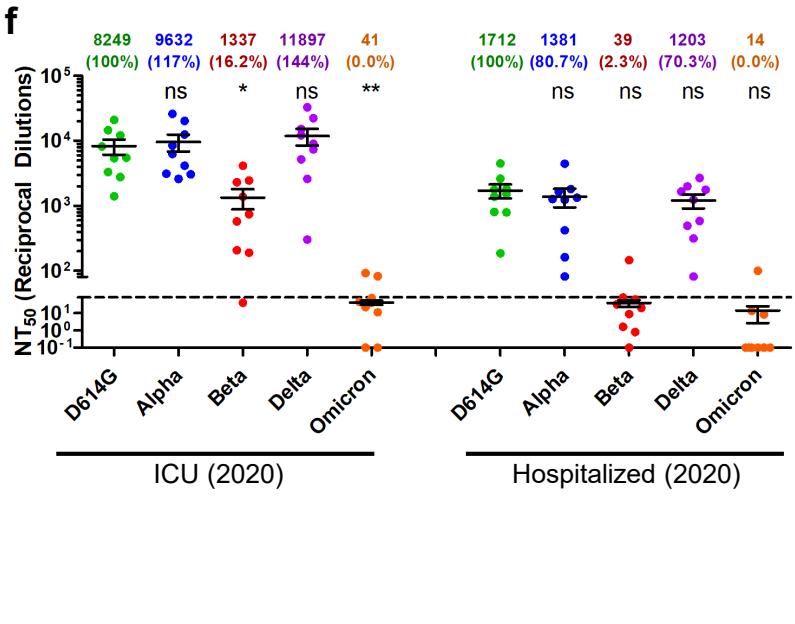
Delta COVID patients



**h**



**f**



**g**

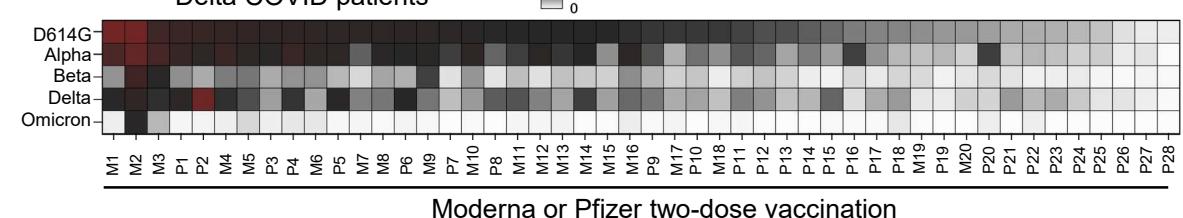
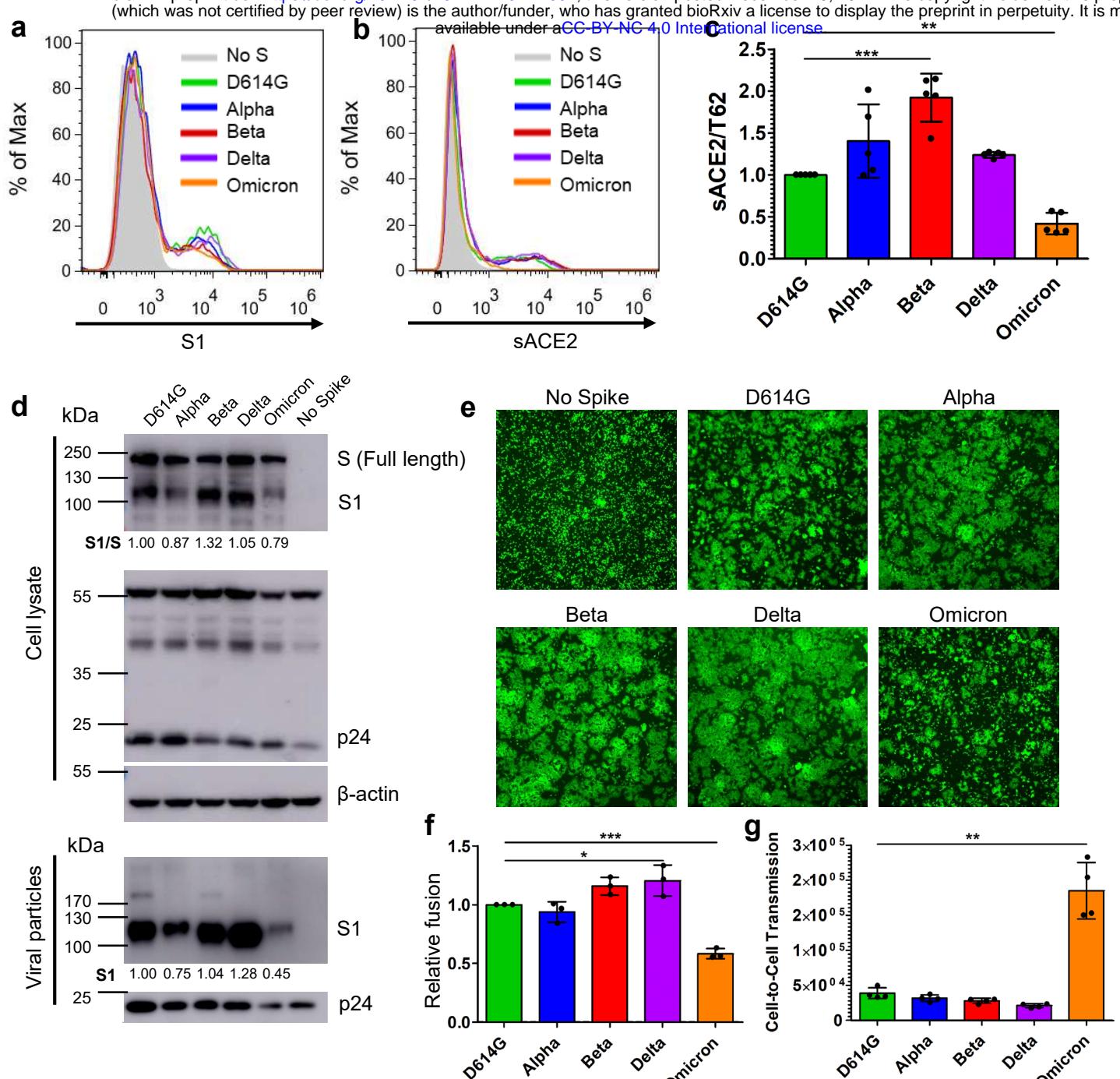
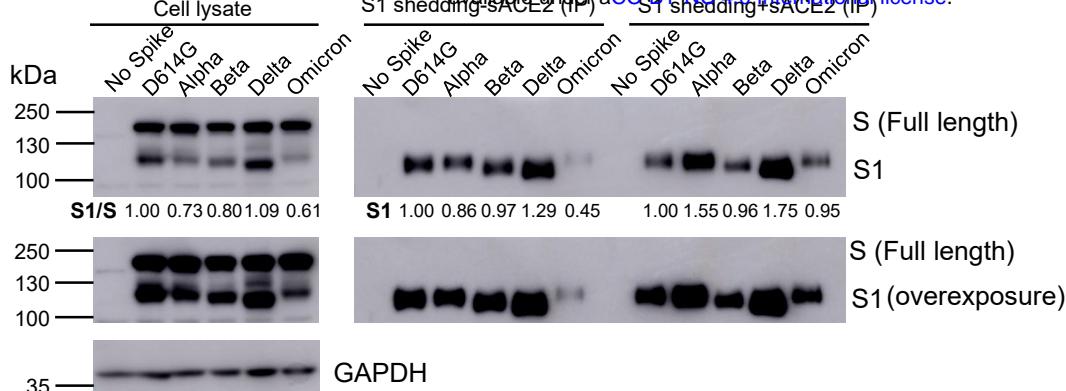
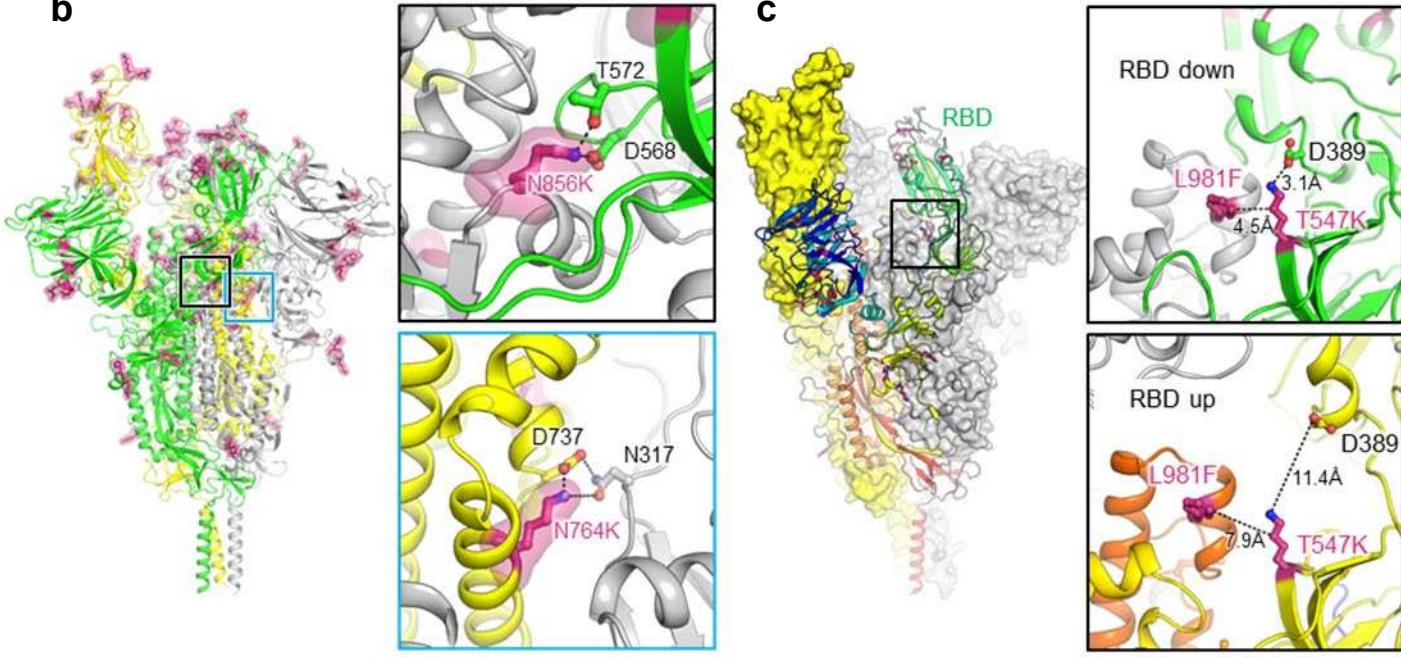
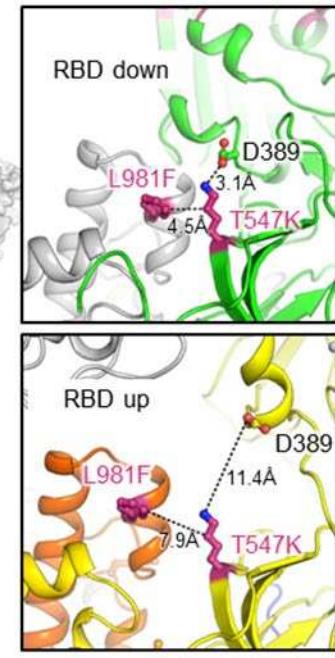


Figure 1

**Figure 1. The Omicron SARS-CoV-2 variant exhibits strong immune-escape that is overcome by booster vaccination.** (a) Diagrams of SARS-CoV-2 S variants used for pseudotyping, which indicate the location of specific mutations as well as the S1 and S2 subunits of S, the N-Terminal Domain (NTD), Receptor Binding Domain (RBD), Fusion Peptide (FP), and Trans-membrane domain (TM). (b) Infectivity of pseudotyped viruses produced in parallel for infection of HEK293T cells stably expressing ACE2. (c) Infectivity of pseudotyped lentivirus in human lung epithelia-derived CaLu-3 cell line. Bars in b and c represent means +/- standard deviation, and significance is determined by one-way ANOVA with Bonferroni's multiple testing correction. Results of at least 3 independent experiments are averaged and shown. (d) Sera from 48 HCWs collected 3-4 weeks after second mRNA vaccine dose was used to neutralize pseudotyped virus for variants, and the resulting 50% neutralization titers ( $NT_{50}$ ) are displayed. (e) Sera from 23 HCWs following homologous mRNA booster vaccination were assessed for nAb titers. (f) Sera from 9 ICU COVID-19 patient samples and 9 hospitalized non-ICU COVID-19 patient samples collected in 2020 prior to the approval of any SARS-CoV-2 vaccines were assessed for nAb titers. (g) Sera from 19 ICU COVID-19 patient samples collected during the Delta-wave of the pandemic were assessed for nAb titers. Mean  $NT_{50}$  values in panels d-g are displayed at the top of plots along with relative neutralization sensitivity with D614G set to 100%; bars represent mean +/- standard error, and significance relative to D614G is determined by one-way ANOVA with Bonferroni's multiple testing correction. (h) Heat maps showing patient/vaccinee  $NT_{50}$  values against each variant. Patient/vaccinee numbers are identified as P for Pfizer/BioNTech BNT162b2 vaccinated/boosted HCW, M for Moderna mRNA-1273 vaccinated/boosted HCW, I for ICU patient samples collected during the 2020 D614G-wave, H for hospitalized non-ICU patient samples collected during the 2020 D614G-wave, or D for ICU patient samples collected during the Delta-wave. P-values are represented as \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001; ns, not significant.



**Figure 2. The Omicron S exhibits reduced ACE2 binding, processing, and fusion, but enhanced cell-to-cell transmission.** (a-c) HEK293T cells were transfected with S expression constructs and stained for FACS with anti-S1 (T62) (a) or soluble ACE2-Fc fusion protein (sACE2) (b). (c) Mean fluorescence intensity for sACE2 signal was normalized with S1 signal to determine relative ACE2 binding; n=5. (d) Pseudotyped virus producer cells were lysed and pseudotyped virus was purified by ultracentrifugation and blots were probed for S1 subunit, lentivirus capsid (p24), and  $\beta$ -actin loading control; S cleavage was quantified using NIH ImageJ and by setting the ratio of S1/S of D614G to 1.00. (e) HEK293T cells transfected with GFP and S constructs were co-cultured with HEK293T-ACE2 cells for 24 hrs and imaged to visualize cell-cell fusion. (f) HEK293T cells transfected with S constructs and Tet-Off were co-cultured with HEK293FT-mCAT-Gluc cells transfected with an ACE2-GFP plasmid, and fusion-induced *Gaussia* luciferase signal was assessed 24 hrs after co-culture. (g) HEK293T cells transfected with S constructs to pseudotype *Gaussia* luciferase bearing lentivirus and were co-cultured with HEK293T-ACE2 cells and secreted *Gaussia* luciferase was assayed 24 hrs after co-culture. Bars in panels c, f and g represent means +/- standard deviation, and significance was determined by one-way ANOVA with Bonferroni's multiple testing correction. Results were from at least three independent experiments. P-values are represented as \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

**a****b****c**

**Figure 3. The Omicron S protein exhibits low S1 shedding, consistent with predicted increase in stability.** (a) HEK293T cells were transfected with S constructs and treated with or without sACE2. Cell lysate and cell culture media were collected after sACE2 treatment, and shed S1 subunit in cell culture media was immune-precipitated with anti-Flag beads. Blots were probed with anti-S1 and anti-GAPDH, and S1 shedding was quantified by NIH ImageJ by setting D614G to 1.0. (b) Structure of Omicron spike protein viewed from side. Mutations of Omicron are highlighted by red sticks and semi-transparent red surfaces. RBD of the yellow protomer is in an “up” conformation. Upper inset: the mutation N856K enables formation of salt-bridge and hydrogen bond with the residues D568 and T572, respectively on the neighboring protomer (green). Lower inset: the mutation N764K enables formation of hydrogen bond with residue N317 on the neighboring protomer (grey) and salt-bridge with residue D737 on the same protomer (yellow). (c) Omicron mutations T547K and L981F stabilize RBD in “down” conformation. Structure of Omicron spike protein by homology modeling was illustrated as surfaces (yellow and grey protomer) and ribbon (rainbow protomer). Upper inset: When RBD is in “down” conformation, Omicron mutation T547K enables formation of salt-bridge with residue D389 located on the base of RBD. Mutation T547K and L981F together enhance the hydrophobic interaction between the neighboring protomers (green and grey). Lower inset: When RBD is in “up” conformation, the interactions between these residues are disrupted.