

1 **Vaccination-infection interval determines cross-neutralization potency to SARS-CoV-2 Omicron**
2 **after breakthrough infection by other variants**

3

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30

31 **SUMMARY**

32 **Background**

33 The immune profile against SARS-CoV-2 has dramatically diversified due to a complex combination of
34 exposure to vaccines and infection by various lineages/variants, likely generating a heterogeneity in
35 protective immunity in a given population. To further complicate this, the Omicron variant, with numerous
36 spike mutations, has emerged. These circumstances have created the need to assess the potential of
37 immune evasion by the Omicron in individuals with various immune histories.

38 **Methods**

39 The neutralization susceptibility of the variants including the Omicron and their ancestor was comparably
40 assessed using a panel of plasma/serum derived from individuals with divergent immune histories. Blood
41 samples were collected from either mRNA vaccinees or from those who suffered from breakthrough
42 infections by the Alpha/Delta with multiple time intervals following vaccination.

43 **Findings**

44 The Omicron was highly resistant to neutralization in fully vaccinated individuals without a history of
45 breakthrough infections. In contrast, robust cross-neutralization against the Omicron were induced in
46 vaccinees that experienced breakthrough infections. The time interval between vaccination and infection,
47 rather than the variant types of infection, was significantly correlated with the magnitude and potency of
48 Omicron-neutralizing antibodies.

49 **Conclusions**

50 Immune histories with breakthrough infections can overcome the resistance to infection by the Omicron,
51 with the vaccination-infection interval being the key determinant of the magnitude and breadth of
52 neutralization. The diverse exposure history in each individual warrants a tailored and cautious approach

53 to understanding population immunity against the Omicron and future variants.

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58 **KEYWORDS**

59 SARS-CoV-2; Omicron variant; COVID-19 vaccine; BNT162b2 mRNA vaccine; Breakthrough infection;

60 Neutralizing antibody

61

62

63 **Introduction**

64 Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2
65 (SARS-CoV-2), continues to cause significant morbidity and mortality globally. Since the first detection
66 of a new SARS-CoV-2 variant belonging to the Pango lineage B.1.1.529 in South Africa, it has spread
67 rapidly worldwide, especially in African countries (Scott et al., 2021; The Lancet Infectious Diseases,
68 2021). The World Health Organization (WHO) classified SARS-CoV-2 variant B.1.1.529 as a Variant of
69 Concern (VOC), due to possible changes in viral characteristics, and designated it as “Omicron” (WHO,
70 <https://www.who.int/>). The Omicron variant is characterized by approximately 30 amino acid mutations,
71 three small deletions, and one insertion in the spike protein compared to the vaccine strain. Among these,
72 15 mutations are located in the receptor-binding domain (RBD), which induces antibody evasion.
73 Compared to previous VOCs, the Omicron variant contains a larger number of mutations in its spikes,
74 which can dramatically alter its infectivity and immune evasion capabilities compared to any other variant
75 to date, raising a serious global public health concern (Dejnirattisai et al., 2021; Leung and Wu, 2021; Lu
76 et al., 2021).

77 A retrospective study using routine epidemiological surveillance data taken from several countries
78 demonstrated that the Omicron variant was associated with a substantial ability to evade immunity from
79 prior infection (Eggink et al., 2021; Goga et al., 2021; Pulliam et al., 2021). In addition, a number of
80 studies have reported that the Omicron variant evades neutralization by sera collected from vaccinated or
81 convalescent patients (Carreno et al., 2021; Dejnirattisai et al., 2021; Liu et al., 2021; Lu et al., 2021). In
82 contrast, it has also been reported that booster immunization with an mRNA vaccine significantly increases
83 the serum neutralizing potency against the Omicron variant (Doria-Rose et al., 2021; Edara et al., 2021;
84 Gruell et al., 2021) and also provides a significant increase in protection against mild and severe disease

85 (Andrews et al., 2021; Hansen et al., 2021), indicating that an enhancement of pre-existing immunity
86 induced by the ancestral virus antigens could overcome the antigenic shift of the Omicron variant and
87 confer cross-protection against it. Moreover, it has been reported that sera from individuals who
88 experienced COVID-19 vaccine breakthrough infections have improved cross-neutralization potency
89 against SARS-CoV-2 variants, especially Delta variant breakthrough infections (Bates et al., 2021).
90 Currently, the cumulative number of COVID-19 cases, seroprevalence of SARS-CoV-2, types of vaccines
91 available, primary vaccination coverage, booster vaccine availability, and number of COVID-19 vaccine
92 breakthrough infections vary in different regions of the world, and the immunity status of populations in
93 each region is also diverse. To properly assess the risk of the spread of the Omicron variant in each region,
94 it is necessary to understand the ability of the variant to evade immunity induced in various settings.
95 Therefore, there is a need to better understand the susceptibility of the Omicron variant to neutralizing
96 antibodies elicited by other variant breakthrough infections, as well as sera from vaccine recipients, to
97 thoroughly assess the risk of immune evasion by the Omicron variant in populations with diverse immune
98 histories to SARS-CoV-2. Here, we determined the neutralization susceptibility of the Omicron variant to
99 antibodies from COVID-19 mRNA vaccine recipients with or without breakthrough infections and
100 compared it to the ancestral SARS-CoV-2 lineage A virus, D614G, Beta, and Delta variants.

101

102 **Results**

103 *High resistance of SARS-CoV-2 Omicron variant to neutralizing antibodies from mRNA vaccinees*

104 First, we tested the *in vitro* neutralization activity of plasma samples obtained from individuals
105 vaccinated twice with BNT162b2 (Pfizer/BioNTech mRNA vaccine) against SARS-CoV-2 Pango lineage
106 A (as a reference for ancestral strains), D614G (B.1), Beta variant (B.1.351), Delta variant (B.1.617.2),
107 and Omicron variant (B.1.1.529) by vesicular stomatitis virus (VSV) pseudovirus-based and live-virus
108 neutralization assays. Twenty health care workers who received two doses of mRNA vaccine were enrolled
109 for blood donation at the early (median = 52.5 days) and late (median = 171.5 days) time points after the
110 second dose (Supplemental Table 1). All volunteers were confirmed to be negative for anti-nucleocapsid
111 antibodies in the pre-vaccinated plasma. Both VSV pseudovirus-based and live-virus neutralization assays
112 with plasma samples from fully vaccinated individuals demonstrated a significant reduction in
113 neutralization activity against the Omicron variant at early and late time points compared to the ancestral
114 virus (Figure 1). Almost all samples lost all neutralizing activity against the Omicron variant in both VSV
115 pseudovirus-based and live-virus neutralization assays, and the reduction in neutralization for the Omicron
116 variant was greater than 18.5, or approximately 8-fold, at the early time point and 5.4, or 3.0-fold, at the
117 late time point by VSV pseudovirus or live-virus neutralization assay, respectively, which was greater than
118 that for the Beta variant, which has the most pronounced *in vitro* escape phenotype to date, or for the Delta
119 variant (Figure 1).

120

121 *Cross-neutralization of the Omicron variant by sera from individuals with mRNA vaccine breakthrough*
122 *Alpha or Delta variant infections*

123 While sera from individuals completing two doses of mRNA vaccination showed only limited

124 neutralizing activity, breakthrough infections after full vaccination induced elevated immune responses
125 and cross-neutralization potency against SARS-CoV-2 variants (Bates et al., 2021). To investigate the
126 neutralizing activity against the Omicron variant using sera from individuals who had mRNA vaccine
127 breakthrough infections due to non-Omicron variants, convalescent sera (obtained 10-22 days after
128 infection with Alpha or Delta variant; Table 1) were examined in VSV pseudovirus-based and live-virus
129 neutralization assays. In the pseudovirus-based neutralization assay, neutralizing activity against the Delta
130 variant was elevated compared to that against the ancestral virus and was particularly pronounced in those
131 with breakthrough infection with the Delta variant, suggesting that breakthrough infection preferentially
132 induces antibodies with high neutralizing activity against the infected variant (Figure 2A, 2B, 2C, and 2D).
133 In contrast, the neutralizing activity against Beta or Omicron variants was markedly lower than that against
134 the ancestral virus in the VSV pseudovirus-based neutralization assay, with a 3.8-fold or 9.7-fold decrease
135 for Beta or Omicron variants, respectively (Figure 2A). In the live-virus neutralization assay, the
136 neutralizing activity of the two isolates of the Omicron variant was also reduced by approximately 10-fold
137 compared to that against the ancestral virus (Figure 2B). In contrast to the sera of mRNA vaccinees without
138 breakthrough infections, we detected neutralizing activity against the Omicron variant in most sera of
139 individuals with breakthrough infections in both assays, and there were large individual variations in the
140 degree of reduction in neutralizing activity compared to the ancestral virus. Notably, some sera from
141 individuals with breakthrough infections showed high cross-neutralizing activity and neutralized the
142 Omicron variant to a level comparable to that of the other variants. In addition, the cross-neutralizing
143 activity against the Omicron variant tended to be higher in sera from individuals with breakthrough
144 infections than that against the Delta variant (Figure 2C, 2D). In the breakthrough cases included in this
145 study, there was variation in the interval between vaccination and breakthrough infection, as epidemics

146 caused by the Alpha variant and those caused by the Delta variant occurred during different periods.
147 Specifically, half of the breakthrough infections due to the Delta variant, which occurred later than the
148 Alpha epidemic, occurred >60 days after vaccination (Figure 2E).

149

150 *Positive correlation between cross-neutralization activity against SARS-CoV-2 variants and interval*
151 *between vaccination and breakthrough infection*

152 To understand the factors contributing to the high heterogeneity of cross-neutralizing activity against
153 variants in sera from breakthrough cases, we evaluated the relationship between neutralizing activity
154 against each variant and time between vaccine completion and breakthrough infection in each case.
155 Intriguingly, in both pseudovirus-based and live-virus assays, the neutralizing activity against the ancestral
156 virus and each variant, including Omicron, increased as the time between vaccination and breakthrough
157 infection increased (Figure 3A–3K). Notably, the degree of correlation between neutralizing activity and
158 the vaccination-to-infection interval tended to be stronger for Beta and Omicron variants, which were
159 antigenically shifted from the ancestral virus (Figure 3C, 3E, 3H, 3J, and 3K). These trends did not differ
160 significantly between sera after breakthrough infection with Alpha and Delta variants, except for
161 neutralizing activity against the Delta variant. The neutralizing activity against the Delta variant by the
162 sera obtained after Delta variant breakthrough infection showed a completely different trend from that of
163 other serum-virus combinations (Figure 3D and 3I). Considering the effect of age on the cross-neutralizing
164 activity against SARS-CoV-2, we excluded elderly patients whose sera were obtained only with a short
165 interval between vaccination and breakthrough infection, and evaluated only sera obtained from patients
166 younger than 60 years. We found a positive correlation between cross-neutralization activity against
167 SARS-CoV-2 variants and the interval between vaccination and breakthrough infection, similar to the

168 results described above (Figure S1A–S1G). In addition, to evaluate the effect of the presence or absence
169 of symptoms on cross-neutralizing activity, we compared the neutralizing activity between subjects with
170 and without symptoms, but there was no significant difference in the neutralizing activity for each variant
171 between subjects with and without symptoms (Figure S1H and S1I).

172

173 *Enhanced neutralizing potency index of anti-Omicron variant antibody in sera from individuals with*
174 *breakthrough infections*

175 To assess the quality of neutralizing activity by distinguishing the antibodies with a high neutralizing
176 potency from those with a lower potency which might be present in abundance, the neutralization potency
177 index (NPI), which represents the average of the neutralization potencies of individual antibodies
178 (Moriyama et al., 2021), was estimated in the sera from individuals who had breakthrough infections. The
179 IgG titer of antibody against the RBD of the Omicron variant was greatly reduced by more than 40-fold
180 compared to that of the ancestral virus (Figure 4A). Notably, as the interval between vaccination and
181 infection increased, the IgG titer of antibody recognizing the RBD of the ancestral virus did not change
182 whereas the IgG titer of antibodies against the Omicron variant RBD increased, suggesting that IgG
183 antibodies recognizing the Omicron RBD were readily induced as this period extended (Figure 4B). The
184 NPI against the ancestral virus, Beta, Delta, and Omicron variants was then calculated. The NPI was
185 calculated using the neutralizing activity determined in pseudovirus-based and live-virus assays, and the
186 NPI calculated in both assays demonstrated a significant increase in the Omicron variant compared with
187 the ancestral virus (Figure 4C and 4D). Furthermore, evaluation of the relationship between NPI against
188 the Omicron variant and vaccination-to-infection interval showed that, as with neutralizing activity, NPI
189 was positively correlated with vaccination-to-infection interval: the longer the interval, the higher the NPI

190 and the higher the quality of antibodies produced (Figure 4E, 4F, and 4G). These results suggest that high-
191 quality antibodies play a role in cross-neutralization against the Omicron variant in sera from individuals
192 with breakthrough infections.

193

194 **Discussion**

195 The Omicron variant is a highly divergent SARS-CoV-2 variant with approximately 30 amino acid
196 mutations in the spike protein, causing a severe evasion of humoral immunity induced by vaccines and
197 previous infections (Carreno et al., 2021; Cele et al., 2021; Dejnirattisai et al., 2021; Lu et al., 2021). In
198 this study, we demonstrated that sera from individuals with BNT162b2 mRNA vaccine breakthrough
199 infection cases by SARS-CoV-2 Alpha or Delta variants showed robust cross-neutralizing potency against
200 the Omicron variant, even though sera from vaccinees without breakthrough infection had greatly reduced
201 neutralizing potency against the Omicron variant. Furthermore, a longer interval between vaccination and
202 breakthrough infection was favorable for better antibody responses against the Omicron variant in serum
203 from individuals who had non-Omicron variant breakthrough infections.

204 The Omicron variant is spreading rapidly in regions where the population has already been immunized
205 by vaccines or previous infections. Elucidating the underlying factors that have led to marked immune
206 evasion in immunized populations is urgently needed to mitigate the rapid global spread of the Omicron
207 variant. It remains unclear whether the observed rapid growth rate of the Omicron variant can be attributed
208 to immune evasion, increased intrinsic transmissibility, or a combination of the two. A recent analysis of
209 data estimated that the risk of reinfection with the Omicron variant was significantly higher than that with
210 the Delta variant (Pulliam et al., 2021). In addition, several reports have shown that after the primary series
211 (two doses) of COVID-19 vaccines, the vaccine effectiveness against infection with the Omicron variant

212 was significantly reduced compared to that with the Delta variant (Andrews et al., 2021; Hansen et al.,
213 2021). In contrast, studies using serum samples from individuals who had been previously infected and
214 subsequently vaccinated with the mRNA vaccine and those who had received two doses of the mRNA
215 vaccine followed by an mRNA vaccine booster found high levels of neutralization against the Omicron
216 variant (Doria-Rose et al., 2021; Edara et al., 2021; Gruell et al., 2021). Furthermore, it was also reported
217 that vaccine effectiveness against the Omicron variant after mRNA vaccine booster dramatically increased
218 against SARS-CoV-2 infection (Andrews et al., 2021; Hansen et al., 2021). Taken together, these results
219 suggest that part of the waning immunity to the Omicron variant in populations immunized with the
220 primary vaccination series or previous infection due to other variants may be explained by a reduced serum
221 neutralizing activity against the Omicron variant. Notably, enhanced neutralization of the Omicron variant
222 by the ancestral virus-based booster vaccine indicated that the spike protein of the Omicron variant still
223 shares some neutralizing epitopes with the ancient virus. Supporting this, multiple neutralizing monoclonal
224 antibodies have been found to overcome the antigenic shift of the Omicron variant and cross-neutralize
225 any SARS-CoV-2 variant, as well as their ancestral strain (Cameroni et al., 2021).

226 In this study, we demonstrated that longer intervals between vaccination and breakthrough infection
227 improved cross-neutralizing potency against the Omicron variant. COVID-19 vaccine breakthrough
228 infections elicited robust cross-neutralizing antibody responses against several SARS-CoV-2 variants,
229 which were largely recalled from memory B cells induced by previous vaccinations (Bates et al., 2021).
230 Longer prime-boost intervals have been reported to result in higher antibody responses in both adenoviral
231 vectors and mRNA vaccines (Grunau et al., 2021; Payne et al., 2021; Voysey et al., 2021), and mRNA
232 vaccines induce a persistent germinal center B cell response in the draining lymph nodes at least 12 weeks
233 after the second dose, which enables the generation of robust humoral immunity (Turner et al., 2021).

234 Notably, affinity maturation of IgG antibodies to the spike protein-conserved region, which is highly
235 correlated with serum neutralization potency against antigenically drifted variants, persists for more than
236 3 months after SARS-CoV-2 infection, presumably owing to the supply of long-lived plasma cells
237 (Moriyama et al., 2021). These observations in vaccinees and convalescents suggest that longer intervals
238 between priming and boosting might be favorable for improving the potency and breadth of neutralizing
239 antibodies against SARS-CoV-2 through continued affinity maturation of variant-resistant IgG antibodies
240 expressed on memory B cells and plasma cells. Furthermore, our observations also imply that the primary
241 vaccination series with mRNA vaccines (2 doses) induces memory B cells to produce cross-neutralizing
242 antibodies against Omicron variants, despite limited induction of serum anti-Omicron neutralizing
243 antibodies.

244 SARS-CoV-2 variants that emerge repeatedly with no clear seasonality expand globally and replace
245 existing variants in a few months, making it very difficult to develop a vaccine that is fully antigenically
246 matched to the upcoming variants and has greatly impeded the employment of strategies similar to those
247 used for seasonal influenza. Therefore, it is important to encourage research on vaccine development with
248 enhanced cross-protection against not only the Omicron variant but also unidentified variants that are
249 expected to appear in the future. This can be achieved by designing vaccine antigens with high cross-
250 neutralizing antibody induction capacity against various variants, optimizing the vaccination interval,
251 modifying the vaccination route, and elaborating adjuvants. Furthermore, it has been reported that booster
252 immunization with an mRNA vaccine provides a significant increase in protection against mild and severe
253 disease but is still less effective than immunization for other variants, highlighting the need to develop
254 next-generation vaccines.

255 This study had several limitations. First, the number of samples evaluated was small. Second, since

256 sera from vaccine recipients without breakthrough infection at 6 months after vaccination had a low
257 neutralization titer even against the ancestral virus in our assay, an accurate fold-reduction in neutralizing
258 antibody titer could not be determined. Third, we did not include serum samples collected immediately
259 (<10 days) after breakthrough infection. It is expected that the longer the interval between the vaccine and
260 infection, the lower the antibody titer at the time of breakthrough infection. The possibility that reduced
261 neutralizing activity at the time of breakthrough infection results in efficient viral replication in the upper
262 respiratory tract, which may contribute to a better antibody response, was not evaluated in the present
263 study. Fourth, we did not assess T cell immunity against SARS-CoV-2, including the Omicron variant,
264 which contributes to protection when antibody titers are low in non-human primate models (McMahan et
265 al., 2021) and may correlate with protection against severe disease. Finally, our investigation did not
266 evaluate the actual risk of reinfection by the Omicron variant in individuals with a history of breakthrough
267 infection.

268 In conclusion, breakthrough sera demonstrated improved cross-neutralization against the Omicron
269 variant, and the time from vaccination to breakthrough infection was a key determinant of the magnitude
270 and breadth of neutralizing activity against variants after breakthrough infection. These results suggest
271 that population immunity is becoming increasingly diverse against Omicron and future variants depending
272 on different settings, including varying degrees of exposure to existing variants, types of vaccines available,
273 primary vaccination coverage, availability of booster vaccines, and the magnitude of COVID-19 vaccine
274 breakthrough infections. Therefore, a tailored and cautious approach is warranted to understand the
275 population immunity against Omicron and future variants.

276

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340

341 **Author contributions**

342 Conceptualization, SF, YT, TS; Methodology, SMi, TA, SF, YA, SMo, HKi, KM, HKa, SI; Investigation,
343 SMi, TA, YA, SMo, HKi, TK, SS, AA, RK, SY, YKu, TY, KI, EP, YI, YKa, MT, HKa, SI, NI, NS, KT,

344 SO, TH, AU, NK, SW, KN, YS, TM, KM; Writing – Original Draft, SMi, TA, YA, SMo, HKi, SF, YT,
345 TS; Writing – Review & Editing, SMi, TA, YA, SMo, HKi, TK, SS, HKa, SI, AA, RK, SY, YKu, TY, KI,
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348 Acquisition, KM, YT, TS. SMi performed statistical analyses. SF, YT and TS had unrestricted access to
349 all data. SMi, TA, YA, SMo, HKi, SF, YT, and TS prepared the first draft of the manuscript, which was
350 reviewed and edited by all other authors. All authors agreed to submit the manuscript, read and approved
351 the final draft, and take full responsibility of its content including the accuracy of the data and statistical
352 analysis.

353

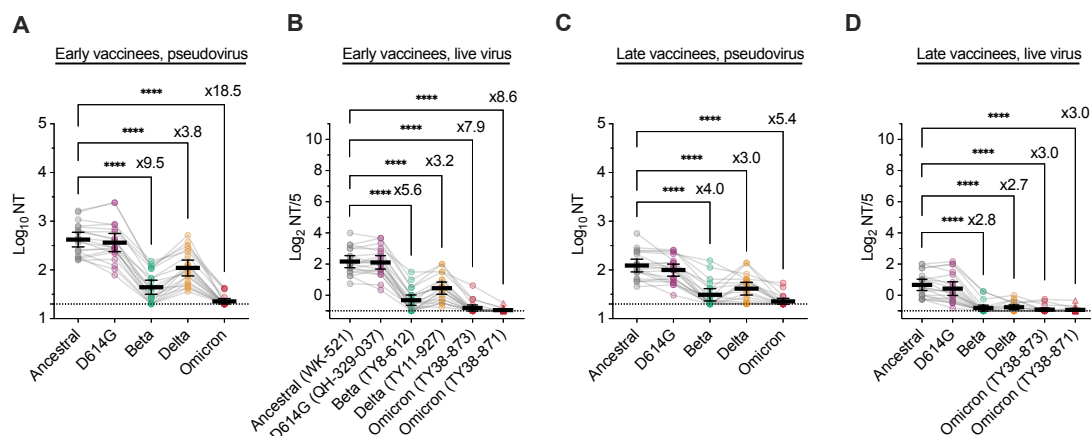
354 **Declaration of interests**

355 The authors declare no competing interests.

356

357

358 **Figures**



359

360 **Figure 1.** High resistance of SARS-CoV-2 Omicron variant to neutralizing antibodies from mRNA

361 vaccinees (A, B) Neutralization titers (NTs) of early vaccinee serums against variants of SARS-CoV-2

362 pseudoviruses (A) and live viruses (B). (C, D) Neutralization titers of late vaccinee serums against variants

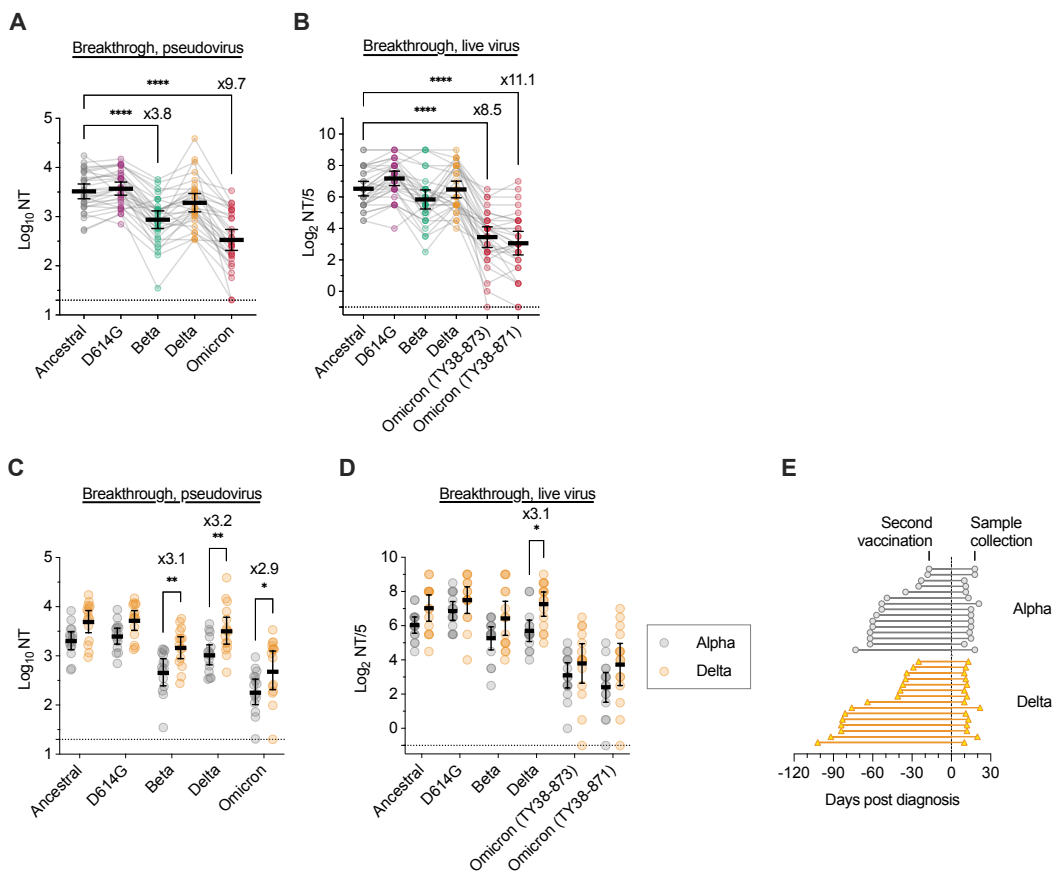
363 of SARS-CoV-2 pseudoviruses (C) and live viruses (D). Data from the same serum are connected with

364 lines, and the mean ± 95% CI of each serum titer is presented. The titers between the ancestral and variants

365 were compared using the one-way ANOVA with Dunnett's test; *****p* < 0.0001. Fold-reductions are

366 indicated above columns in statistically significant cases.

367



368

369 **Figure 2.** Cross-neutralization to the Omicron variant by sera from individuals who experienced mRNA

370 vaccine breakthrough Alpha or Delta variant infections (A, B) Neutralization titers of vaccine-

371 breakthrough case serums against variants of SARS-CoV-2 pseudoviruses (A) and live viruses (B). Data

372 from the same serum are connected with lines, and the mean \pm 95% CI of each serum titer is presented.

373 The titers between the ancestral and variants were compared using the one-way ANOVA with Dunnett's

374 test; **** $p < 0.0001$. Fold-reductions are indicated above columns in statistically significant cases. (C, D)

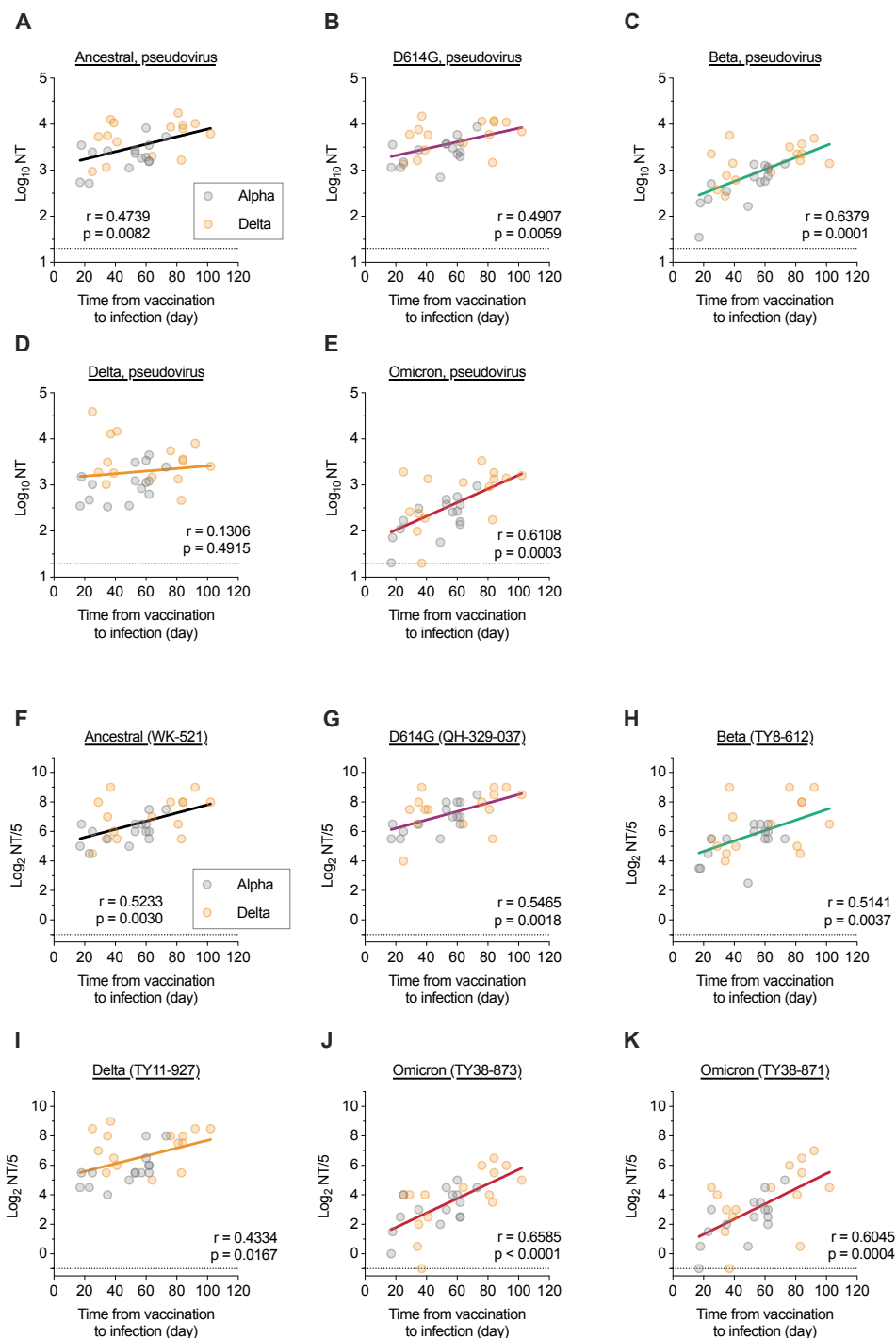
375 Comparison of the neutralization titers between background variant types of breakthrough infection

376 against variants of SARS-CoV-2 pseudo-viruses (C) and live viruses (D). The titers between the

377 background variants types were compared using the two-way ANOVA with Sidak's test; * $p < 0.05$, ** p

378 < 0.01 . Fold-increases are indicated above columns in statistically significant cases. (E) Timeline of sample

379 collection in vaccine breakthrough individuals: Alpha (n=15) and Delta (n=15). Timing from second
380 vaccination to sample collection are indicated as line-connected circles. Day 0 (dotted line) indicates the
381 day of COVID-19 diagnosis.
382

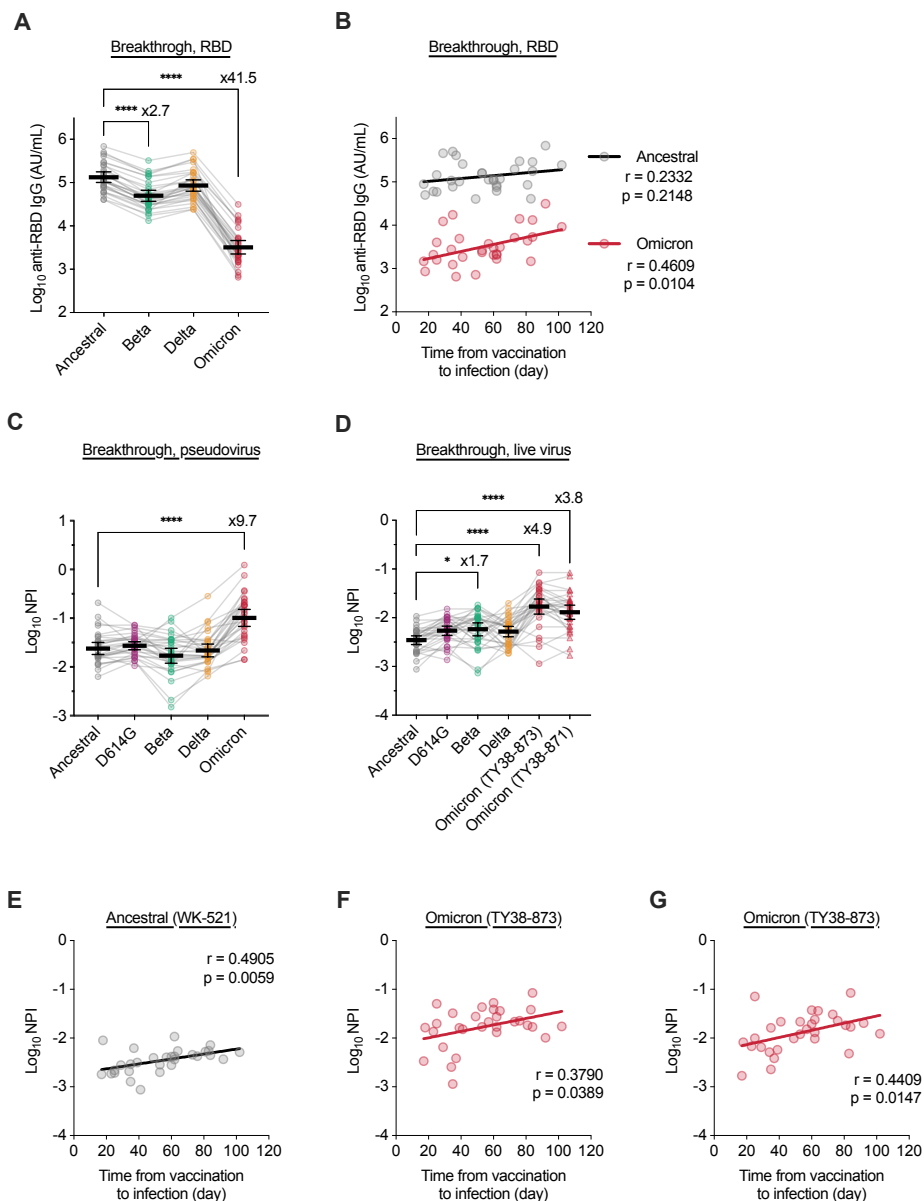


383

384 **Figure 3.** Positive correlation between cross-neutralization activity against SARS-CoV-2 variants and

385 interval between vaccination and breakthrough infection (A-K) Correlation between interval from

386 vaccination to breakthrough infection and the neutralization titers of the serums against variants of SARS-
387 CoV-2 pseudoviruses (A-E) and live viruses (F-K). The correlation plots represent against ancestral (A,
388 F), D614G (B, G), Beta (C, H), Delta (E, I), and Omicron (F, J, K) pseudo and live viruses, respectively.
389 The regression line, Pearson correlation r value, and p value are shown.
390



391

392 **Figure 4.** *Enhanced neutralizing potency index of anti-Omicron variant antibody in breakthrough*

393 *infection sera (A) IgG titers of vaccine-breakthrough case sera against ancestral RBD and RBD mutants*

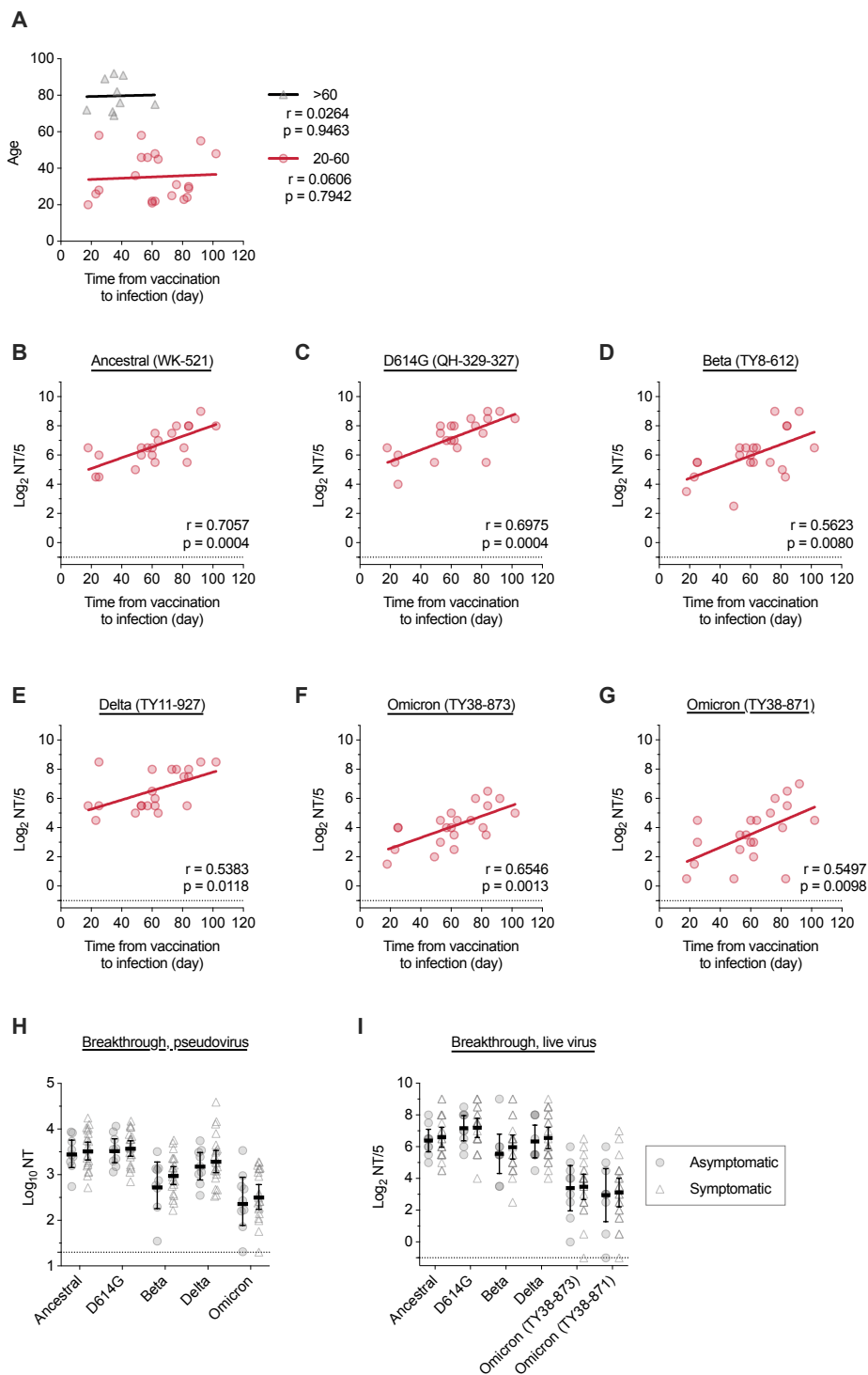
394 *of SARS-CoV-2 variants. Data from the same serum are connected with lines, and the mean ± 95% CI of*

395 *each serum titer is presented. The titers between the ancestral and variants were compared using the one-*

396 *way ANOVA with Dunnett's test; ****p < 0.0001. Fold-reductions are indicated above columns in*

397 statistically significant cases. (B) Correlation between interval from vaccination to breakthrough infection
398 and the IgG titers of the serums against the ancestral (gray) and Omicron (red) RBDs. The regression line,
399 Pearson correlation r value, and p value are shown. (C, D) Neutralization potency index (NPI) of vaccine-
400 breakthrough case serums against variants of SARS-CoV-2 pseudoviruses (C) and live viruses (D). Data
401 from the same serum are connected with lines, and the mean \pm 95% CI of each serum titer is presented.
402 The titers between the ancestral and variants were compared using the one-way ANOVA with Dunnett's
403 test; * $p < 0.05$, **** $p < 0.0001$. Fold-increases are indicated above columns in statistically significant cases.
404 (E-G) Correlation between interval from vaccination to breakthrough infection and the NPI of the serums
405 against ancestral and Omicron variants of SARS-CoV-2 live viruses. The correlation plots represent
406 against ancestral (E) and Omicron (F, G) live viruses, respectively. The regression line, Pearson correlation
407 r value, and p value are shown.
408

409 Supplemental information



411 **Figure S1.** *Effect of age and symptoms in vaccine breakthrough individuals on the neutralization titers,*
412 *Related to Figure 3 (A) Correlation of age of the individuals with time interval from vaccination to*
413 *breakthrough infection. Data were stratified by age (20-60 or >60 years). (B-F) Correlation between*
414 *interval from vaccination to breakthrough infection and the neutralization titers of the serums against*
415 *variants of SARS-CoV-2 live viruses in the 20-60 years individuals. The correlation plots represent against*
416 *ancestral (B), D614G (C), Beta (D), Delta (E), and Omicron (F, G) live viruses, respectively. The*
417 *regression line, Pearson correlation r value, and p value are shown. (H, I) Comparison of the neutralization*
418 *titers between the asymptomatic and symptomatic cases against variants of SARS-CoV-2 pseudoviruses*
419 *(H) and live viruses (I). The titers between the asymptomatic and symptomatic cases were compared using*
420 *the two-way ANOVA; $p = 0.1598$ (H), $p = 0.4710$ (I).*

421

422

423 **Supplemental Table 1. Characteristics of participants in this study**

	Vaccinated only	Breakthrough infection (All)	Breakthrough infection (Alpha variant infection)	Breakthrough infection (Delta variant infection)
Number of subjects	20	30	15	15
Age, y	42 (33-48)	46 (26-71)	36 (22-58)	55 (30-76)
Male sex	4 (20.0)	10 (33.3)	1 (6.7)	9 (60.0)
Vaccine	BNT162b2 (2 doses)	BNT162b2 (2 doses)	BNT162b2 (2 doses)	BNT162b2 (2 doses)
Severity				
Asymptomatic	N/A	9 (30.0)	8 (53.3)	1 (6.7)
Mild	N/A	17 (56.7)	7 (46.7)	10 (66.7)
Moderate*	N/A	4 (13.3)	0 (0.0)	4 (26.7)
Dose interval (days)	21 (21-21)	21 (21-21)	21 (20-21)	21 (21-21)
Infection to sera collection (days)	N/A	13 (11-15)	15 (11-18)	12 (10-13)
Dose 2 to infection (days)	N/A	55 (35-74)	53 (25-62)	64 (35-84)
Dose 2 to sera collection (days)	Early: 31.5 (29-34) Late: 150.5 (148.25-152.75)	72 (46-91)	72 (36-75)	74 (46-96)
Period of infection	N/A	April 30-August 31, 2021	April 30-August 14, 2021	June 18-August 31, 2021

424 Median (interquartile range) or n (%)

425 *Moderate includes individuals who required oxygen

426

427

428 **Methods**

429 *Human subjects and sampling*

430 Human plasma samples obtained from vaccinated health care workers without infection, who received
431 two doses of BNT162b2 (Pfizer/BioNTech) mRNA vaccine, were collected on approximately day 51 and
432 day 161 after the first vaccination, with written informed consent prior to enrollment and ethics approval
433 by the medical research ethics committee of the National Institute of Infectious Diseases (NIID) for the
434 use of human subjects (#1321). Blood was collected in Vacutainer CPT tubes (BD Biosciences) and
435 centrifuged at $1800 \times g$ for 20 min. Peripheral blood mononuclear cells (PBMCs) were suspended in
436 plasma and harvested into conical tubes, followed by centrifugation at $300 \times g$ for 15 min. The plasma
437 was transferred into another conical tube, centrifuged at $800 \times g$ for 15 min, and the supernatant transferred
438 into another tube to completely remove PBMCs. Human serum samples obtained from breakthrough cases
439 were also included in this study. Demographic information, vaccination status, and respiratory samples to
440 determine the type of variant infected among breakthrough cases in this report were collected as part of
441 the public health activity led by NIID under the Infectious Diseases Control Law and were published on
442 the NIID website to meet statutory requirements. Sera were collected concurrently for clinical testing
443 provided by NIID with patient consent, and neutralization assays for this report were performed using
444 residual samples as a research activity with ethics approval by ethics approval from the medical research
445 ethics committee of NIID for the use of human subjects (#1275) and informed consent. To examine
446 neutralization, plasma and serum samples were heat-inactivated at 56°C for 30 min before use.

447

448 *SARS-CoV-2 virus*

449 The SARS-CoV-2 ancestral strain WK-521 (lineage A, GISAID ID: EPI_ISL_408667), D614G strain QH-

450 329-037 (lineage B.1, GISAID: EPI_ISL_529135), Beta variant TY8-612 (lineage B.1.351, GISAID:
451 EPI_ISL_1123289), Delta variant TY11-927 (lineage B.1.617.2, GISAID:EPI_ISL_2158617), and
452 Omicron variant TY38-873 (lineage BA.1, GISAID: EPI_ISL_7418017) and TY38-871 (lineage BA.1,
453 GISAID: EPI_ISL_7571618) were used in this study, which were isolated using VeroE6/TMPRSS2 cells
454 at NIID with ethics approval by the medical research ethics committee of NIID for the use of human
455 subjects (#1178). To isolate viruses belonging to the Omicron variant (TY38-873; GISAID ID:
456 EPI_ISL_7418017, and TY38-871; GISAID: EPI_ISL_7571618), respiratory specimens, which were
457 collected from individuals being screened at airport quarantine stations in Japan and then transferred to
458 NIID for whole genome sequencing, were subjected to viral isolation using VeroE6/TMPRSS2 cells at
459 NIID. The TY38-871 virus harbors an additional R346K mutation.

460

461 *Cells*

462 293T cells, obtained from American Type Culture Collection, were cultured in Dulbecco's modified Eagle
463 medium (DMEM; FUJIFILM Wako Chemical, Kanagawa, Japan) supplemented with 10% fetal bovine
464 serum at 37°C and 5% CO₂. Expi293F cells were maintained in the Expi293 expression medium (Thermo
465 Fisher Scientific). VeroE6/TMPRSS2 cells (JCRB1819, Japanese Collection of Research Bioresources
466 Cell Bank) were maintained in low glucose DMEM (Fujifilm) containing 10% heat-inactivated fetal
467 bovine serum (Biowest), 1 mg/mL geneticin (Thermo Fisher Scientific), and 100 U/mL
468 penicillin/streptomycin (Thermo Fisher Scientific) at 37°C supplied with 5% CO₂.

469

470 *Recombinant RBD antigens*

471 Human codon-optimized sequences coding the SARS-CoV-2 RBD (amino acids: 331-529) with the

472 following mutations were cloned into the mammalian expression vector pCAGGS: Beta, K417N / E484K
473 / N501Y; Delta, L452R / T478K; Omicron, G339D / S371L / S373P / S375F / K417N / N440K / G446S /
474 S477N / T478K / E484A / Q493R / G496S / Q498R / N501Y / Y505H. Recombinant Avi-tagged His-
475 tagged proteins were produced using Expi293F cells, according to the manufacturer's instructions
476 (Thermo Fisher Scientific), in the presence of the secreted BirA-Flag plasmid (Addgene) and biotin.
477 Recombinant proteins were purified from the culture supernatant using Ni-NTA agarose (Qiagen).

478

479 *Electrochemiluminescence immunoassay (ECLIA)*

480 IgG titers for variant RBDs were measured using multiplex kits (Meso Scale Discovery) according to the
481 manufacturer's instructions. Briefly, plates were coated with biotinylated RBDs premixed with linkers at
482 4°C overnight. The plates were washed with phosphate-buffered saline supplemented with 0.05% Tween-
483 20 and incubated with the MSD Blocker A reagent (Meso Scale Discovery) at room temperature for 1 h
484 with shaking. Plasma samples diluted with MSD Diluent 100 (Meso Scale Discovery) were added to the
485 plates after washing, and the plates were incubated at room temperature for 2 h with shaking. The plates
486 were washed and incubated with sulfo-tag-conjugated anti-human IgG (Meso Scale Discovery) at room
487 temperature for 1 h with shaking. Finally, the plates were measured for electrochemiluminescence using
488 MESOQuickPlex SQ 120 (Meso Scale Discovery) after washing and adding MSD Gold read buffer B
489 (Meso Scale Discovery).

490

491 *VSV pseudovirus production*

492 The VSV pseudovirus bearing SARS-CoV-2 spike proteins was generated as previously described (Tani
493 et al., 2021). Briefly, the spike genes of the SARS-CoV-2 ancestral strain, D614G, Beta, and Delta variants

494 were obtained from viral RNA extracted from SARS-CoV-2 strain WK-521 for ancestral strain, TY4-920
495 for D614G strain (lineage B.1.1, GISAID ID: EPI_ISL_2931303), TY8-612 for Beta, and TY11-927 for
496 Delta, respectively, by RT-PCR using PrimeScript II High Fidelity One Step RT-PCR kit (Takara-Bio,
497 Shiga, Japan). The spike gene of the SARS-CoV-2 Omicron variant was obtained from RNA extracted
498 from nasopharyngeal swab specimens of patients infected with the SARS-CoV-2 Omicron variant (TY38-
499 873) by RT-PCR, as described above. The cytoplasmic 19 aa-deleted SARS-CoV-2 spike genes were
500 cloned into the CAGGS/MCS expression vector. The spike sequence in the expression plasmids was
501 confirmed by Sanger sequencing. 293T cells transfected with expression plasmids encoding the spike
502 genes of SARS-CoV-2 ancestral strain, D614G, Beta, Delta, and Omicron variants were infected with G-
503 complemented VSV Δ G/Luc. After 24 h, the culture supernatants containing VSV pseudoviruses were
504 collected and stored at -80°C until use.

505

506 *VSV pseudovirus-based neutralization assay*

507 Neutralization of SARS-CoV-2 pseudovirus was performed as previously described (Moriyama et al.,
508 2021). Briefly, serially diluted plasma (five-fold serial dilution of the plasma from vaccinated participants,
509 or eight-fold serial dilution of the plasma from breakthrough infected patients, starting at 1:10 dilution)
510 was mixed with equal volume of the VSV pseudoviruses bearing SARS-CoV-2 spike protein and incubated
511 at 37°C for 1 h. The mixture was then inoculated into VeroE6/TMPRSS2 cells seeded on 96 well solid
512 white flat-bottom plates (Corning). At 24 h post-infection, the infectivity of VSV pseudovirus was assessed
513 by measuring luciferase activity using the Bright-Glo Luciferase Assay System (Promega, Madison, WI,
514 USA) and GloMax Navigator Microplate Luminometer (Promega). The half-maximal inhibitory
515 concentration (IC₅₀) is presented as the neutralization titer.

516

517 *Live virus neutralization assay*

518 Live virus neutralization assays were performed as previously described (Moriyama et al., 2021; Onodera
519 et al., 2021). Briefly, plasma or serum samples were serially diluted (2-fold dilutions starting from 1:5) in
520 high-glucose DMEM supplemented with 2% fetal bovine serum and 100 U/mL penicillin/streptomycin
521 and were mixed with 100 TCID₅₀ SARS-CoV-2 viruses, WK-521 (ancestral strain), QH-329-037 (D614G
522 strain), TY8-612 (Beta variant), TY11-927 (Delta variant), TY38-873 (Omicron variant), and TY38-871
523 (Omicron variant), followed by incubation at 37°C for 1 h. The virus-plasma mixtures were placed on
524 VeroE6/TMPRSS2 cells (JCRB1819) seeded in 96-well plates and cultured at 37°C with 5% CO₂ for 5
525 days. After culturing, the cells were fixed with 20% formalin (Fujifilm Wako Pure Chemicals) and stained
526 with crystal violet solution (Sigma-Aldrich). The mean cut-off dilution index with > 50% cytopathic effect
527 from two to four multiplicate series is presented as the neutralizing titer. Assays on sera from vaccinated
528 individuals without infection were performed in multiple independent laboratories within NIID to confirm
529 the findings, with adjustments made using rabbit sera immunized with RBD (ancestral strain). Since sera
530 from individuals who suffered from breakthrough infections were limited in quantity, the assay was
531 performed once. All experiments using live viruses were performed in a biosafety level 3 laboratory at
532 NIID, Japan.

533

534 *Ethical statement approval*

535 All samples, protocols, and procedures were approved by the Medical Research Ethics Committee of NIID
536 for the use of human subjects (Approval numbers 1178, 1275, 1316, and 1321).

537

538 *Statistical analysis*

539 Data analysis and visualization were performed using GraphPad Prism software. For statistical analysis,
540 one-way ANOVA with Dunnett's test and two-way ANOVA with Sidak's test were used to compare the
541 titers. The Pearson correlation coefficient was used to assess correlations between titers and time intervals.
542 Statistical significance was set at $p < 0.05$.

543

544

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