Supplementary Materials for

Omicron mutations enhance infectivity and reduce antibody neutralization of
SARS-CoV-2 virus-like particles

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Materials and Methods

Cloning for plasmids encoding structural proteins: pcDNA3.1 backbone plasmids were generated encoding N, and M-IRES-E. Sequences for E, M and N were PCR amplified from codon optimized plasmids were gifts from Nevan Krogan (Addgene plasmid # 141385, 141386, 141391, ). pcDNA3.1-SARS2-Spike was a gift from Fang Li (Addgene plasmid # 145032). Site directed mutagenesis (NEB) was used to remove the C9-tag and introduce the D614G mutation. Delta and Omicron structural protein were cloned ligating eBlocks (IDT) gene fragments following NEBuilder HiFi DNA (NEB E2621L) Assembly Reaction Protocol.

SC2-VLP production: For a 24-well, plasmids CoV2-N (0.67), CoV2-M-IRES-E (0.33), CoV-2- Spike (0.006) and Luc-PS9 (1.0) at indicated mass ratios for a total of 1 μg of DNA were diluted in 50 μL Opti-MEM. 3 μg PEI was diluted in 50 μL Opti-MEM and added to plasmid dilution quickly to complex the DNA. Transfection mixture was incubated for 20 minutes at room temperature and then added dropwise to 293T cells in 0.5 mL of DMEM containing fetal bovine serum and penicillin/streptomycin. Media was changed after 24 hours of transfection and At 48 hours post-transfection, VLP containing supernatant was collected and filtered using a 0.45 μm syringe filter. For other culture sizes, the mass of DNA used was 1 μg for 24-well, 4 μg for 6-well, 20 μg for 10-cm plate and 40 μg for 15-cm plate. Opti-MEM volumes were 100 μL, 400 μL, 1 mL and 3 mL respectively and PEI was always used at 3:1 mass ratio.

Western blot: For western blots of lysates, media was removed and cells were rinsed with PBS. Cells were then lysed for 20 minutes in N-PER lysis buffer containing Halt protease and phosphatase inhibitor cocktail. For western blots of ultracentrifuge concentrated VLPs, 10 mL of VLP supernatant from a 10-cm plate was pelleted (28 000 RPM, 2hrs, SW41 Ti, 1mL 20% sucrose cushion), the supernatant was discarded and VLPs were resuspended in 50 µL of PBS. 15 µL of concentrated VLPs were used to western blot. Laemmli loading buffer (1x final) and dithiothreitol (DTT, 40 mM final) was added to lysates or VLP solution and heated for 95°C for 5 minutes to lyse VLPs and denature proteins. Samples were loaded on to 4-20% gradient gels (Biorad) and transferred to a PVDF membrane (Biorad). Membrane was blocked in 3% BSA (N-staining) or 10% NFDM (all others) and stained with primary antibody: anti-N (abcam ab273434, 1:500 dilution), anti-S (abcam ab272504, 1:1000), anti-GAPDH (Santa Cruz sc-365062, 1:1000), anti-p24 (Sigma, 1:2000) for 2 hours at room temperature. Blots were rinsed with TBS-T three times for 10 minutes each and stained with secondary (abcam ab205719 (mouse), 1:5000). Imaged using pierce chemiluminescence kit.

Northern Blot: VLPs collected from a 10-cm plate were concentrated by ultracentrifugation through a 20% sucrose cushion (28 000 RPM, 2hrs, SW41 Ti). The supernatant was discarded and VLPs were resuspended in 50 µL of PBS. 20 µL of concentrated VLPs were used for Northern blotting. VLPs were lysed by adding 500 µL of Trizol (Sigma) and RNA was extracted by phase separation, precipitated with isopropanol with GlycoBlue and washed with 75% ethanol. RNA was resuspended in 30 µL of water, added to 30 µL 2x RNA Loading Dye (NEB) and denatured at 65°C for 15 minutes then loaded onto a 1% agarose gel containing 1X MOPS and 4% formaldehyde. Samples were run at room temperature for 12hrs at 20V and transferred by capillary action to Nylon membrane. The membrane was hybridized with a 32P-labeled luciferase DNA probe (Prime-a-Gene Labeling System, Promega) and visualized using a phosphoscreen on a Typhoon imager (GE).

Quantification of Western and Northern Blot: Images were analyzed using FIJI. First the background was subtracted using a 50 pixel radius rolling ball and then total intensities were measured for each band. In the case of S protein, intensity of the bands corresponding to complete S and S2 were added together. Intensities of each band were first normalized based on the brightness of each blot and then normalized to the “All B.1” condition. Data were analyzed in Graphpad Prism using one-way ANOVA with repeated measures comparing each condition to the “All B.1” condition and using Dunnett’s correction.

Luciferase readout: In each well of a clear 96-well plate 50 μL of SC2-VLP containing supernatant was added to 50 μL of cell suspension containing 50 000 receiver cells (293T ACE2/Tmprss2). Cells were allowed to attach and take up VLPs overnight. Next day, supernatant was removed and cells were lysed in 20 μL passive lysis buffer (Promega) for 15 minutes at room temperature with gentle rocking. Lysates were transferred to an opaque white 96-well plate and 30 μL of reconstituted luciferase assay buffer was added and mixed with each lysate. Luminescence was measured immediately after mixing using a TECAN plate reader with no attenuation and a luminescence integration time of 1 s.
Cell lines: Cells were maintained in a humidified incubator at 37°C in 5% CO2 in the indicated media and passaged every 3-4 days. 293T cells were obtained from ATCC and maintained in DMEM with 10% FBS and 1% penicillin/streptomycin. 293T cells stably co-expressing ACE2 and TMPRSS2 were generated through sequential transduction of 293T cells with TMPRSS2-encoding (generated using Addgene plasmid #170390, a gift from Nir Hacohen and ACE2-encoding (generated using Addgene plasmid #154981, a gift from Sonja Best) lentiviruses and selection with hygromycin (250 µg/mL) and blasticidin (10 µg/mL) for 10 days, respectively. ACE2 and TMPRSS2 expression was verified by western blot. Vero stably co-expressing human ACE2 and TMPRSS2 cells (gifted from A. Creanga and B. Graham at NIH) were maintained at 37°C and 5% CO2 in Dulbecco's Modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal calf serum, 100ug/mL penicillin and streptomycin (Gibco) and 10µg/mL of puromycin (Gibco).

VLP Neutralization Assay: Each heat inactivated serum sample was serially diluted from 1:20 to 1:20480 dilution in complete DMEM media prior to incubation (1hr at 37°C) with 40µL VLP with total volume of 50µL, then plated onto receiver cells (50000 293T ACE2-TMPRSS2 cells). 24hr later luciferase readout was taken. Neutralization (NT50) was estimated by interpolating the dilution of serum at which 50% infectivity was reduced.

Serum samples: Serum samples from individuals not exposed to SARS-CoV-2 (pre-COVID, control), exposed to SARS-CoV-2 (post-COVID), and those vaccinated with either two doses of elasomeran (Moderna), two doses of tozinameran (Pfizer/BioNTech) vaccine or one dose of Johnson & Johnson vaccine were collected through a clinical trial led by Curative (Supplementary Table 5). Post-COVID samples reflect non vaccinated participant samples that were collected within 4-6 weeks of the original positive test and were negative by PCR at the time of serum collection. Serum from vaccinated participants was collected 4-6 weeks post vaccination following final dose. The clinical trial protocol was approved by Advarra under Pro00054108 for a study designed to investigate immune escape by SARS-CoV-2 variants. The trial has been submitted to clinicaltrials.gov registry (NCT05171803, Unique Protocol ID: PTL-2021-0007). Sample specimens were collected from adult individuals aged 18 to 50 years who either had been vaccinated for COVID-19 and/or had a history of COVID-19. Vulnerable populations were excluded from enrollment. Patients signed consent forms held by Curative. Participants were enrolled from individuals that tested with Curative in Los Angeles County and were sent an IRB-approved email enrollment script. Those who were interested were contacted by the Curative Clinical Trials research team (CITI trained) and those who consented to the study were scheduled for sample collection by a clinician who went to their residence. Participants underwent a standard venipuncture procedure. Briefly, licensed phlebotomists collected a maximum of 15 ml whole blood. Once collected, the sample was left at ambient temperature for 30–60 min to coagulate, then was centrifuged at 2200–2500 rpm for 15 min at room temperature. Samples were then placed on ice until delivered to the laboratory site where the serum was aliquoted to appropriate volumes for storage at ~80 ºC until use. A quantitative SARS-CoV-2 IgG ELISA was performed on serum specimens (EuroImmun, Anti-SARS-CoV-2 ELISA (IgG), 2606–9621G, New Jersey). To quantify SARS-CoV-2 IgG antibodies, an S1-specific monoclonal IgG antibody with no known cross-reactivity to the S2 domain of the spike protein was used as a reference antibody. A standard curve was developed using a monoclonal IgG antibody targeting the S1 antigen of SARS-CoV-2 at different concentrations with a polynomial regression curve-fitting model. The standard curve was used to calculate the sample IgG antibody concentration. Serum samples were heat inactivated at 56°C for 30 mins prior to use in VLP assays. Pre-COVID sera was pooled into one sample.

SARS-CoV-2 culture: SARS-CoV-2/human/USA/USA-WA1/2020 (WA1) (BEI NR-52281), B.1.617.2 (BEI NR-55611) and B.1.1.529 (California Department of Health) were used for serum virus neutralization. The virus infection experiments were performed in a Biosafety Level 3 laboratory. Working stocks of SARS-CoV-2 were made in Vero-TMPRSS2 cells and were stored at -80°C until used.

Virus neutralization assay: Serum dilutions (50µL) 1:5, 1:15, 1:45, 1:135, 1:405, 1:1215 were prepared in serum-free DMEM. The dilutions were separately added with 50 PFU (50µL) of SARS-CoV-2 WA1, B.1.617.2, or B.1.1.529. The mixture was mixed gently, incubated at 37°C for 30 mins, followed by a plaque assay.

Plaque assays: Vero-TMPRSS2 were seeded and incubated overnight. The cells were inoculated with the neutralized inoculum in serum-free DMEM. After the 1 hour absorption period, the media in the wells was overlaid with 2.5% Avicel (Dupont, RC-591). After 72 hours, the overlay was removed, the cells were fixed in 10% formalin for one hour, and stained with crystal violet for visualization of plaque forming
units.

A  S-Cell lysate

B  S-VLPs

C  N-Cell lysate

D  N-VLPs

E  Luc RNA

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0.0131

0.0099

<0.0001

<0.0001

<0.0001

<0.0001

0.0021

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0.0001

0.008

<0.0001

0.003

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0.0016

0.0016

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**Fig. S1. Quantification of Western and Northern blot intensities.** A-E) Quantification of Figure Western and Northern blots shown in 1E and F. p-values calculated from one way ANOVA with Dunnett’s correction for multiple comparisons. n=3 in all cases. Error bars indicate standard deviation.

**Fig. S2. Comparison of S-variants using pseudotyped lentivirus and VLPs.** Luciferase signal from cells transduced using S-pseudotyped lentiviruses or VLPs. Delta and Omicron have opposite trends in infectivity in these two systems.

**Fig. S3. Comparison of 50% neutralization titers (NT50s) of VLPs vs live SARS-CoV-2.** Neutralization titers correlate between live virus and VLP assays with $r^2$ of 0.9839 and $p<0.0001$. 
Fig. S4. Neutralization titers and S-protein ELISA measurements of subjects vaccinated against SARS-CoV-2. The binding antibodies detected by ELISA do not correlate with measured neutralization titers measured using S-B.1 (A) $r^2 = 0.4010$, $p = 0.6319$ or S-Omicron (B) $r^2 = 0.4432$ and $p = 0.4476$. 
Fig. S5. Neutralization titers of subjects vaccinated and boosted against SARS-CoV-2. (A-D) Neutralization titers of subjects vaccinated with Pfizer/BioNTech, Moderna, or J&J or subjects recovered from infection. Neutralization evaluated against the ancestral B.1 lineage S protein, S-Omicron or a modified S-Omicron containing mutations targeting Class 1 and Class 3 antibodies targeting the receptor binding domain. (E-G) Effect of third dose booster for subjects vaccinated using the Pfizer/BioNTech vaccine evaluated against B.1, Delta or Omicron at ~16 days post booster or 21 days post booster.
Table S1.
List of mutations used in each S variant divided by domains.

|       | NTD                                      | RBD                     | CTD                                      |
|-------|------------------------------------------|-------------------------|------------------------------------------|
| B.1   | B.1.1                                    | D614G                   |                                          |
| Delta | A67V, G142D, E156G, Δ157-158              | L452R, T478K            | D614G, P681R, D950N                      |
| Omicron | A67V, Δ69-70, T95I, G142D, Δ143-145, Δ211, L212I, ins214-EPE | G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493K, G496S, Q498R, N501Y, Y505H | T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F |
| OmC1  | A67V, Δ69-70, T95I, G142D, Δ143-145, Δ211, L212I, ins214-EPE | K417N, G496S, Q498R, N501Y | T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F |
| OmC3  | A67V, Δ69-70, T95I, G142D, Δ143-145, Δ211, L212I, ins214-EPE | N440K, G446S, G496S, Q498R | T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F |

Table S2.
List of mutations used in each N variant divided by domains.

|       | NTD                                      | SR                  | linker                   | CTD                                      |
|-------|------------------------------------------|---------------------|--------------------------|------------------------------------------|
| B.1   |                                          |                     |                          |                                          |
| B.1.1 |                                          |                     |                          |                                          |
| Delta | D63G                                     | R203K, G204R        |                          |                                          |
| Omicron | P13L, Δ31-33, D63G                      | R203K, G204R        | G215C, D377Y             |                                          |

Table S3.
List of mutations used in each M variant.

|       |                                           |                     |                          |                                           |
|-------|------------------------------------------|---------------------|--------------------------|------------------------------------------|
| B.1   |                                          |                     |                          |                                          |
| B.1.1 |                                          |                     |                          |                                          |
| Delta | I82T                                     |                     |                          |                                          |
| Omicron | D3G, Q19E, A63T                          |                     |                          |                                          |

Table S4.
List of mutations used in each E variant.

|       |                                           |                     |                          |                                           |
|-------|------------------------------------------|---------------------|--------------------------|------------------------------------------|
| B.1   |                                          |                     |                          |                                          |
| B.1.1 |                                          |                     |                          |                                          |
Table S5.
Serum samples from clinical trial participants used in VLP assays in this study.

| Subject ID | ELISA Status | Total IgG (ug/ml) | Sample Type                      |
|------------|--------------|------------------|----------------------------------|
| CUR01      | Negative     | /                | pre-COVID serum                  |
| CUR02      | Negative     | /                | pre-COVID serum                  |
| CUR03      | Negative     | /                | pre-COVID serum                  |
| CUR04      | Negative     | /                | pre-COVID serum                  |
| CUR05      | Negative     | /                | pre-COVID serum                  |
| PC0002     | Positive     | 4.45             | post-COVID serum                 |
| PC0003     | Positive     | 0.44             | post-COVID serum                 |
| PC0006     | Positive     | 2.29             | post-COVID serum                 |
| PC0007     | Positive     | 1.19             | post-COVID serum                 |
| PC0008     | Positive     | 2.16             | post-COVID serum                 |
| PC0009     | Positive     | 1.19             | post-COVID serum                 |
| PC0011     | Positive     | 39.8             | post-COVID serum                 |
| PC0013     | Positive     | 1.03             | post-COVID serum                 |
| PF0002     | Positive     | 9.67             | Pfizer vaccinee serum - 2 doses   |
| PF0004     | Positive     | 9.32             | Pfizer vaccinee serum - 2 doses   |
| PF0005     | Positive     | 9.36             | Pfizer vaccinee serum - 2 doses   |
| PF0006     | Positive     | 5.05             | Pfizer vaccinee serum - 2 doses   |
| PF0007     | Positive     | 8.85             | Pfizer vaccinee serum - 2 doses   |
| PF0009     | Positive     | 8.21             | Pfizer vaccinee serum - 2 doses   |
| PF0011     | Positive     | 9.66             | Pfizer vaccinee serum - 2 doses   |
| PF0012     | Positive     | 7.01             | Pfizer vaccinee serum - 2 doses   |
| PF0013     | Positive     | 6.41             | Pfizer vaccinee serum - 2 doses   |
| PF0016     | Positive     | 1.79             | Pfizer vaccinee serum - 2 doses   |
| PF0017     | Positive     | 7.72             | Pfizer vaccinee serum - 2 doses   |
| M0002      | Positive     | 91.77            | Moderna vaccinee serum - 2 doses  |
| M0003      | Positive     | 14.5             | Moderna vaccinee serum - 2 doses  |
| M0004      | Positive     | 71.94            | Moderna vaccinee serum - 2 doses  |
| M0005      | Positive     | 9.88             | Moderna vaccinee serum - 2 doses  |
| M0006      | Positive     | 8.5              | Moderna vaccinee serum - 2 doses  |
| M0007      | Positive     | 10.5             | Moderna vaccinee serum - 2 doses  |
| M0008      | Positive     | 21.38            | Moderna vaccinee serum - 2 doses  |
| M0009      | Positive     | 10.2             | Moderna vaccinee serum - 2 doses  |
| M0010      | Positive     | 15.65            | Moderna vaccinee serum - 2 doses  |
| M0011      | Positive     | 15.08            | Moderna vaccinee serum - 2 doses  |
| JJ0002     | Positive     | 1.09             | J+J vaccinee serum - 1 dose       |
| JJ0003     | Positive     | 1.63             | J+J vaccinee serum - 1 dose       |
|   |   |   |   |
|---|---|---|---|
| JJ0005 | Positive | 1.29 | J+J vaccinee serum - 1 dose |
| JJ0006 | Positive | 2.09 | J+J vaccinee serum - 1 dose |
| JJ0007 | Positive | 1.19 | J+J vaccinee serum - 1 dose |
| JJ0008 | Positive | 1.84 | J+J vaccinee serum - 1 dose |
| JJ0009 | Positive | 0.57 | J+J vaccinee serum - 1 dose |
| JJ0010 | Positive | 0.55 | J+J vaccinee serum - 1 dose |
| JJ0011 | Positive | 1.68 | J+J vaccinee serum - 1 dose |
