Supplementary Appendix

Supplement to: Davis-Gardner ME, Lai L, Wali B, et al. Neutralization against BA.2.75.2, BQ.1.1, and XBB from mRNA bivalent booster. N Engl J Med 2023;388:183-5. DOI: 10.1056/NEJMc2214293

This appendix has been provided by the authors to give readers additional information about the work.

Supplementary Appendix

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Supplemental Methods

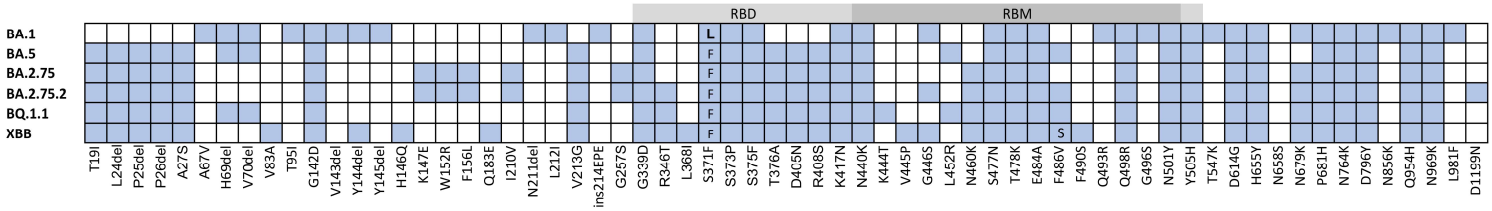
Serum samples. All samples were collected at Emory University. Collection and processing were performed under approval from the University Institutional Review Board (#00002061 and #00058271). Adults ≥18 years were enrolled who met eligibility criteria under these protocols, were at least 1 week post-COVID booster vaccination, and provided informed consent. All patient samples were de-identified prior to inclusion in the study.

Cells and Viruses. Vero-TMPRSS2 cells were cultured in complete DMEM medium consisting of 1x DMEM (VWR, #45000-304), 10% FBS, 2mM L-glutamine, and 1x antibiotic as previously described[1]. nCoV/USA_WA1/2020 (WA/1), closely resembling the original Wuhan strain, was propagated from an infectious SARS-CoV-2 clone as previously described[2]. icSARS-CoV-2 was passed once to generate a working stock. The BA.1 isolate has been previously described (Edara Cell Reports 2022). Omicron subvariants were isolated from residual nasal swabs: BA.5 isolate (EPI_ISL_13512579), provided by Dr. Richard Webby (St Jude Children's Research Hospital), BA.2.75.2 (EPI_ISL_15146622), BQ.1.1 isolate (EPI_ISL_15196219), BA.2.75 isolate (EPI_ISL_14393635), and XBB isolate (EPI_ISL_15509864) provided by Dr. Benjamin Pinsky (Stanford University). All variants were plaque purified and propagated once in VeroE6-TMPRSS2 cells to generate working stocks. Viruses were deep sequenced and confirmed as previously described[7].

Focus Reduction Neutralization Assay. FRNT assays were performed as previously described[3]. Briefly, samples were diluted at 3-fold in 8 serial dilutions using DMEM in duplicates with an initial dilution of 1:10 in a total volume of 60 μl. Serially diluted samples were incubated with an equal volume of SARS-CoV-2 (100-200 foci per well) at 37° C for 1 hour in a round-bottomed 96-well culture plate. The antibody-virus mixture was then added to Vero cells and incubated at 37° C for 1 hour. Post-incubation, the antibody-virus mixture was removed and 100 μl of prewarmed 0.85% methylcellulose overlay was added to each well. Plates were incubated

at 37° C for 18 to 40 hours, and the methylcellulose overlay was removed and washed six times with PBS. Cells were fixed with 2% paraformaldehyde in PBS for 30 minutes. Following fixation, plates were washed twice with PBS, and permeabilization buffer (0.1% BSA, 0.1% Saponin in PBS) was added to permeabilize cells for at least 20 minutes. Cells were incubated with an anti-SARS-CoV spike primary antibody directly conjugated to Alexa Fluor-647 (CR3022-AF647) overnight at 4°C. Cells were then washed twice with 1x PBS and imaged on an ELISPOT reader (CTL Analyzer).

Quantification and Statistical Analysis. Antibody neutralization was quantified by counting the number of foci for each sample using the Viridot program[4] The neutralization titers were calculated as follows: 1 – (ratio of the mean number of foci in the presence of sera and foci at the highest dilution of the respective sera sample). Each specimen was tested in duplicate. The FRNT-50 titers were interpolated using a 4-parameter nonlinear regression in GraphPad Prism 9.2.0. Samples that do not neutralize at the limit of detection at 50% are plotted at 20 and used for geometric mean and fold-change calculations.



62 **Supplemental Figure S1.** Alignment of Spike protein sequence for variants included in this study.

63 White boxes indicate wild-type sequence, colored box indicates amino acid substitution labeled

64 below. Receptor binding domain (RBD) and receptor binding motif (RBM) are indicated above.

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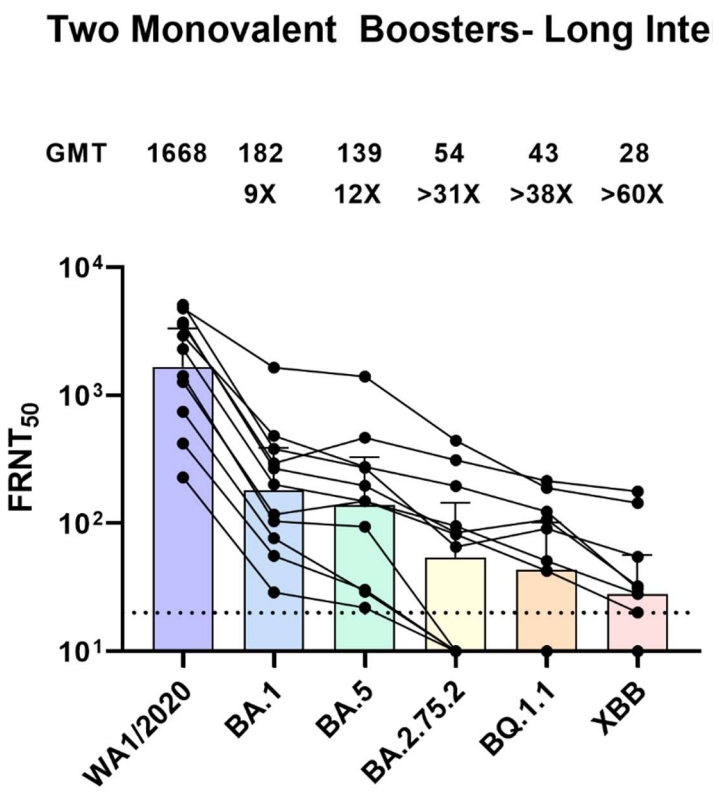
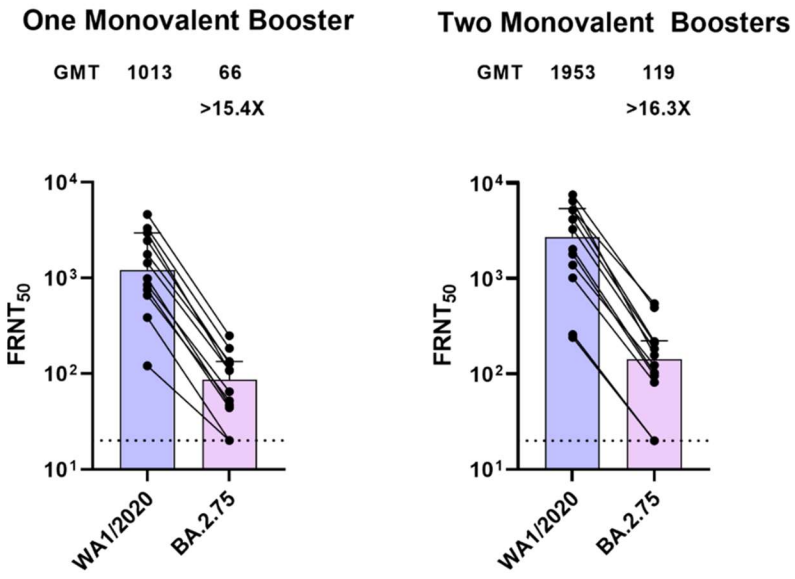


Supplemental Figure S2. Neutralizing responses against WA1/2020, BA.1, BA.5, BA.2.75.2, BQ.1.1, and XBB 70-100 days after two monovalent boosters. Shown is the neutralization activity against SARS-CoV-2 variants among 11 individuals who received two monovalent boosters 70-100 days prior to sample collection. The focus reduction neutralization test ($FRNT_{50}$ [the reciprocal dilution of serum that neutralizes 50% of the input virus]) geometric mean titers for each variant are shown above each panel along with ratios of GMT compared to WA1/2020. The connecting lines between the variants represent matched serum samples. The horizontal lines represent the limit of detection of the assay ($FRNT_{50}$ GMT 20). Red symbols in panels B and C indicate individuals who self-reported prior SARS-CoV-2 infection. The connecting lines represent matched samples, colored bars represent geometric mean, error bars represent 95% confidence intervals that are not adjusted for multiplicity and may not be used for hypothesis testing.



Supplemental Figure S3. Neutralizing responses against WA1/2020 and BA.2.75. Shown is the neutralization activity against SARS-CoV-2 variants among 12 individuals who received one monovalent booster (Table S2) and 12 individuals who received two monovalent boosters (Table S5). The focus reduction neutralization test ($FRNT_{50}$ [the reciprocal dilution of serum that neutralizes 50% of the input virus]) geometric mean titers for each variant are shown above each panel along with ratios of GMT compared to WA1/2020. The connecting lines between the variants represent matched serum samples. Colored bars represent geometric mean, error bars represent 95% confidence intervals that are not adjusted for multiplicity and may not be used for hypothesis testing. The horizontal lines represent the limit of detection of the assay ($FRNT_{50}$ GMT 20).

Supplemental Table S1: Amino acid substitutions in spike protein of variants used in the study.

Variant	Virus Name	GISAID	Amino Acid Substitutions
BA.1	hCoV-19/USA/GA-EHC-2811C/2021	EPI_ISL_7171744	A67V, H69del, V70del, T95I, G142D, V143del, Y144del, Y145del, N211del, L212I, ins214EPE, S371L, S373P, S375F, G339D, K417N, N440K, G446S, Q493R, Q498R, S477N, T478K, E484A, G496S, N501Y, T547K, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F
BA.5	hcov-19/USA/MD/HP30386/2022	EPI_ISL_13512579	T19I, L24del, P25del, P26del, A27S, H69del, V70del, T76I, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, L452R, S477N, T478K, E484A, F486V, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K
BA.2.75	hCoV-19/USA/CA-Stanford-94_S13/2022	EPI_ISL_14393635	T19I, L24del, P25del, P26del, A27S, G142D, K147E, W152R F157L, I210V, V213G, G257S, G339H, S371F, S373P, S375F, T376A, D405N R408S, K417N, N440K, G446S, N460K, S477N, T478K, E484A, Q498R, , N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K
BA.2.75.2	hCoV-19/USA/CA-Stanford-105_S27/2022	EPI_ISL_15181486	T19I, L24del, P25del, P26del, A27S, G142D, K147E, W152R, F157L, I210V, V213G, G257S, G339H, R346T, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, G446S, N460K, S477N, T478K, E484A, F486S, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K, D1199N

BQ.1.1	hCoV-19/USA/CA-Stanford-106_S04/2022	EPI_ISL_15196219	T19I, L24del, P25del, P26del, A27S, H69del, V70del, G142D, V213G, G339D, R346T, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, K444T, L452R, N460K, S477N, T478K, E484A, F486V, Q498R, N501Y, Y505H, D614G, H655Y, P681H, N679K, N764K, D796Y, Q954H, N969K
XBB	hCoV-19/USA/CA-Stanford-109_S21/2022	EPI_ISL_15509864	T19I, L24del, P25del, P26del, A27S, V83A, G142D, Y144del, H146K, Q183E, V213E, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, N460K, S477N, T478K, E484A, F486S, F490S, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K,

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Supplemental Table S2: Demographic information for individuals in single monovalent booster group in Figure 1A.

Sample #	Age	Sex	Exposure status	Vaccine used	Time from vaccination
1	40	Male	Naive	Pfizer	1 week after booster dose
2	28	Male	Naive	Pfizer	4 weeks after booster dose
3	33	Female	Naive	Pfizer	4 weeks after booster dose
4	31	Female	Naive	Pfizer	4 weeks after booster dose
5	29	Female	Naive	Pfizer	4 weeks after booster dose
6	54	Male	Naive	Pfizer	4 weeks after booster dose
7	51	Male	Naive	Moderna	4 weeks after booster dose
8	26	Female	Naive	Moderna	4 weeks after booster dose
9	27	Male	Naive	Pfizer	1 week after booster dose
10	26	Female	Naive	Moderna	4 weeks after booster dose
11	33	Female	Naive	Moderna	1 week after booster dose
12	27	Female	Naive	Pfizer	1 week after booster dose

Supplemental Table S3: Demographic information for double monovalent booster group in Figure 1B.

Sample #	Age	Sex	Exposure status	Vaccine used	Time from vaccination
13	59	Male	Naïve	Pfizer	14 days after 2nd booster
14	64	Female	Naïve	Pfizer	32 days after 2nd booster
15	47	Female	Naïve	Moderna	33 days after 2nd booster
16	43	Male	Naïve	NA	42 days after 2nd booster
17	41	Female	Naïve	Moderna	30 days after 2nd booster
18	35	Male	Recovered	Moderna	31 days after 2nd booster
19	21	Female	Recovered	Moderna	6 days after 2nd booster
20	59	Female	Recovered	Moderna	30 days after 2nd booster
21	58	Female	Naïve	Moderna	12 days after 2nd booster
22	62	Male	Naïve	Pfizer	54 days after 2nd booster
23	59	Female	Naïve	Pfizer	57 days after 2nd booster

NA: Data not available

Supplemental Table S4: Demographic information for bivalent booster group in Figure 1C.

Sample #	Age	Sex	Exposure status	Vaccine used	Time from vaccination
24*	70	F	Naïve	Moderna	20 days after booster
24	47	M	Naïve	NA	20 days after booster
25	46	F	Naïve	Moderna	17 days after booster
26	41	F	Naïve	NA	21 days after booster
27	36	F	Naïve	Pfizer	17 days after booster
28	34	F	Naïve	Moderna	16 days after booster
29	46	F	Recovered	NA	28 days after booster
30	38	M	Naïve	NA	19 days after booster
31**	23	M	Naïve	NA	16 days after booster
32	32	M	Naïve	NA	17 days after booster
33	33	F	Naïve	NA	42 days after booster
34	40	M	Recovered	NA	36 days after booster

NA: Data not available

*This individual received two monovalent boosters prior to bivalent booster.

**This individual received a primary vaccination of Johnson and Johnson followed by one monovalent booster and one bivalent booster.

Supplemental Table S5: Demographic information for double monovalent booster group with longer interval since vaccination in Supplemental Figure 2.

Sample #	Age	Sex	Exposure status	Vaccine used	Time from vaccination
35	68	Female	Naive	Moderna	100 days after 2nd booster
36	72	Female	Naïve	Moderna	92 days after 2nd booster
37	69	Male	Naive	Moderna	92 days after 2nd booster
38	72	Female	Naïve	Moderna	73 days after 2nd booster
39	60	Female	Naïve	Moderna	100 days after 2nd booster
40	64	Male	Naïve	Moderna	83 days after 2nd booster
41	71	Male	Naïve	Moderna	70 days after 2nd booster
43	60	Male	Naïve	Moderna	71 days after 2nd booster
43	67	Female	Naïve	Moderna	71 days after 2nd booster
44	70	Female	Naïve	Moderna	104 days after 2nd booster
45	73	Male	Naïve	Moderna	74 days after 2nd booster

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4. Katzelnick LC, Coello Escoto A, McElvany BD, et al. Viridot: An automated virus plaque (immunofocus) counter for the measurement of serological neutralizing responses with application to dengue virus. PLoS Negl Trop Dis 2018;12:e0006862.