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Supplemental information

Augmentation of humoral and cellular immune responses after third-dose SARS-CoV-2 vaccination and viral neutralization in myeloma patients

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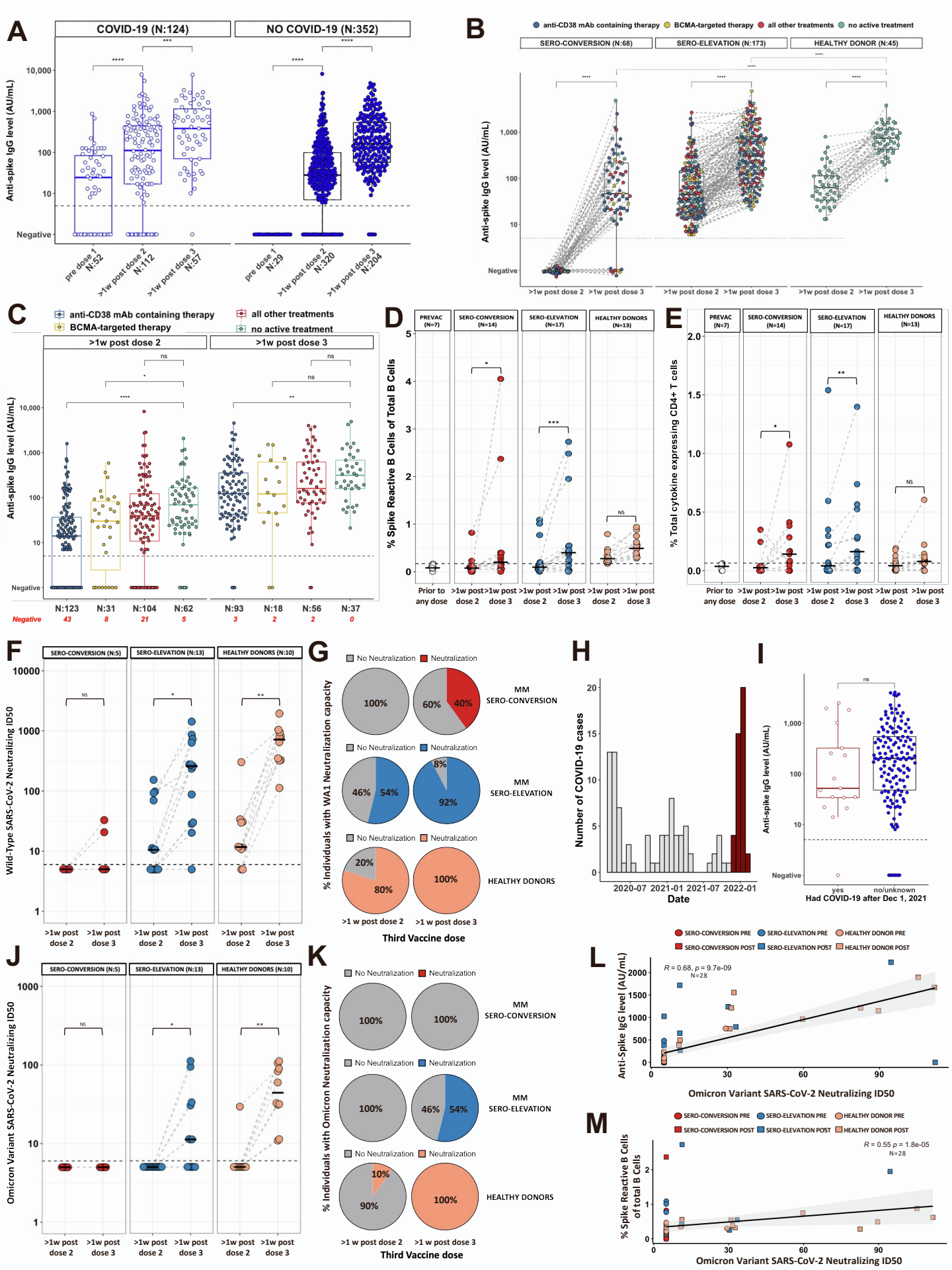
A
COVID-19 (N:124)
NO COVID-19 (N:352)
Anti-spike IgG level (AU/mL)
Negative
pre dose 1 N:52
>1w post dose 2 N:112
>1w post dose 3 N:57
pre dose 1 N:29
>1w post dose 2 N:320
>1w post dose 3 N:204
B
anti-CD38 mAb containing therapy
BCMA-targeted therapy
all other treatments
no active treatment
SERO-CONVERSION (N:68)
SERO-ELEVATION (N:173)
HEALTHY DONOR (N:45)
Anti-spike IgG level (AU/mL)
Negative
>1w post dose 2
>1w post dose 3
C
anti-CD38 mAb containing therapy
BCMA-targeted therapy
all other treatments
no active treatment
>1w post dose 2
>1w post dose 3
Anti-spike IgG level (AU/mL)
Negative
N:123
N:31
N:104
N:62
N:93
N:18
N:56
N:37
Negative
43
8
21
5
3
2
2
0
D
PREVAC (N=7)
SERO-CONVERSION (N=14)
SERO-ELEVATION (N=17)
HEALTHY DONORS (N=13)
% Spike Reactive B Cells of Total B Cells
Prior to any dose
>1w post dose 2
>1w post dose 3
E
PREVAC (N=7)
SERO-CONVERSION (N=14)
SERO-ELEVATION (N=17)
HEALTHY DONORS (N=13)
% Total cytokine expressing CD4+ T cells
Prior to any dose
>1w post dose 2
>1w post dose 3
F
SERO-CONVERSION (N:5)
SERO-ELEVATION (N:13)
HEALTHY DONORS (N:10)
Wild-Type SARS-CoV-2 Neutralizing ID50
>1w post dose 2
>1w post dose 3
G
No Neutralization
Neutralization
100%
60%
40%
MM SERO-CONVERSION
46%
54%
8%
92%
MM SERO-ELEVATION
20%
80%
100%
HEALTHY DONORS
% Individuals with WA1 Neutralization capacity
>1 w post dose 2
>1 w post dose 3
Third Vaccine dose
H
Number of COVID-19 cases
2020-07
2021-01
2021-07
2022-01
Date
I
Anti-spike IgG level (AU/mL)
Negative
yes
no/unknown
Had COVID-19 after Dec 1, 2021
J
SERO-CONVERSION (N:5)
SERO-ELEVATION (N:13)
HEALTHY DONORS (N:10)
Omicron Variant SARS-CoV-2 Neutralizing ID50
>1w post dose 2
>1w post dose 3
K
No Neutralization
Neutralization
100%
100%
MM SERO-CONVERSION
46%
54%
MM SERO-ELEVATION
10%
90%
100%
HEALTHY DONORS
% Individuals with Omicron Neutralization capacity
>1 w post dose 2
>1 w post dose 3
Third Vaccine dose
L
SERO-CONVERSION PRE
SERO-ELEVATION PRE
HEALTHY DONOR PRE
SERO-CONVERSION POST
SERO-ELEVATION POST
HEALTHY DONOR POST
R = 0.68, p = 9.7e-09
N=28
Anti-Spike IgG level (AU/mL)
Omicron Variant SARS-CoV-2 Neutralizing ID50
M
SERO-CONVERSION PRE
SERO-ELEVATION PRE
HEALTHY DONOR PRE
SERO-CONVERSION POST
SERO-ELEVATION POST
HEALTHY DONOR POST
R = 0.55 p = 1.8e-05
N=28
% Spike Reactive B Cells of total B Cells
Omicron Variant SARS-CoV-2 Neutralizing ID50

Supplemental Figure S1. Humoral and cellular responses to SARS-CoV-2 vaccination in patients with multiple myeloma. (A) Time course of anti-SARS-CoV-2 spike (S) IgG antibody levels in multiple myeloma (MM) patients split by COVID-19 infection status. Antibody concentrations measured in artificial units per mL (AU/mL) and are depicted on a log-10 scale. The horizontal dotted line indicates the lower limit of detection (5 AU/mL). **(B)** Effect of SARS-CoV-2 third vaccination on anti-S IgG antibody levels in MM patients and age-matched healthy donors (HD). Dots are colored to indicate treatment regimen at the time of vaccination. Antibody concentrations measured in artificial units per mL (AU/mL) and are depicted on a log-10 scale. The horizontal dotted line indicates the lower limit of detection (5 AU/mL). **(C)** Anti-S IgG antibody levels at least 7 days after receiving two doses of SARS-CoV-2 mRNA vaccine and at least 7 days after receiving three doses of SARS-CoV-2 mRNA vaccine in MM patients split according to major treatment groups. None of the depicted patients in this panel developed COVID-19 at any point during the pandemic. Antibody concentrations measured in artificial units per mL (AU/mL) and are depicted on a log-10 scale. The horizontal dotted line indicates the lower limit of detection (5 AU/mL). **(D)** Frequencies of SARS-CoV-2 spike-reactive B cells in different cohorts within the $CD19^+$ gate. The horizontal dotted line indicates the highest observed frequency of total spike-reactive B cells in the unvaccinated HD control cohort. The bold horizontal line indicates the median for each group. **(E)** SARS-CoV-2 specific $CD4^+$ T cell responses in MM patients and HD. Total cytokine-expressing $CD4^+$ T cells were estimated by aggregating activated $CD4^+$ T cells producing GM-CSF, IFN-γ, IL-2, IL-4, IL-17, and TNF-α. Frequencies were calculated by subtracting water control frequencies from the $CD4^+$ T cell response for each subject. The horizontal dotted line

indicates highest observed frequency of total cytokine response in the unvaccinated HD control cohort. The bold horizontal line indicates the median for each group. **(F)** Neutralizing antibody ID50 to WA1 wild-type SARS-CoV-2 strain for MM subject groups and HD. The bold horizontal line indicates the median for each group. **(G)** Quantification of MM patients and HD that achieve neutralization to the WA1, wildtype strain >1 week post dose 2 and >1 week post dose 3. **(H)** Histogram representing COVID-19 infection cases in MM patients at Mount Sinai Hospital between March 2020 and January 2021. Dark red overlay indicates cases during the period when the Omicron variant was dominant in New York. **(I)** Anti-S IgG antibody levels in MM patients that contracted COVID-19 during the period where the Omicron variant was dominant in New York compared to non-infected MM patients. Antibody concentrations measured in artificial units per mL (AU/mL) and are depicted on a log-10 scale. The horizontal dotted line indicates the lower limit of detection (5 AU/mL). **(J)** Neutralizing antibody ID50 to Omicron SARS-CoV-2 strain for MM subject groups and HD. The bold horizontal line indicates the median for each group. **(K)** Quantification of MM patients and HD that achieve neutralization to Omicron strain >1 week post dose 2 and >1 week post dose 3. **(L)** Spearman's rank correlation between anti-S IgG antibody levels and WA1, wildtype neutralizing ID50. **(M)** Spearman's rank correlation between anti-S IgG antibody levels and Omicron variant neutralizing ID50. The lower and upper hinges of the boxplot correspond to the first and third quartiles (the 25^{th} and 75^{th} percentiles) with a bold horizontal line indicating the median. Vertical whiskers are extended up to 1.5 times the interquartile range (IQR). P-values represent comparison using the non-parametric

Mann-Whitney U test. P-values for contingency outcomes represent comparison using Fisher's exact test; (ns) $p>0.05$, (*) $p<0.05$, (**) $p<0.01$, (***), $p<0.001$, (****) $p<0.0001$.

Supplemental Table S1. Clinical characteristics of patients with multiple myeloma and healthy donor controls.

VARIABLE	MM COHORT (N = 476)		HD (N=45)	
Age (y)	67	[38-96]	58	[49-71]
Male gender	56.7%	(270)	24%	(11)
Vaccine Type Initial Dose				
Pfizer-BioNTech	70.6%	(336)	73%	(33)
Moderna	29.4%	(140)	27%	(12)
Received ≥2 documented doses	99.2%	(472)	100%	(45)
Received ≥3 documented doses	72.5%	(345)	100%	(45)
Timing of dose 3 after dose 2 (d)	207	[41-360]	280	[208-361]
Heterologous vaccination regimen	5.8%	(20/345)	4%	(2)
Had documented COVID-19	26.1%	(124)	44%	(20)
Disease Isotype				
IgG	60.1%	(286)		
IgA	20.2%	(96)		
LC	18.9%	(90)		
Other	0.8%	(4)		
SMM	8.4%	(40)		
Time since diagnosis (mo)	64.9	[0-254]		
> 3 previous lines of treatment	28.2%	(134)		
> 5 previous lines of treatment	16.2%	(77)		
Disease response status				
CR or sCR	40.5%	(193)		
VGPR	17.6%	(84)		
PR or MR	8.2%	(39)		
SD or PD	19.1%	(91)		
Unable to assess	14.3%	(68)		
Treatment regimen at initial vaccination contains:				
Immunomodulatory drug	46.2%	(220)		
Proteasome inhibitor	6.1%	(129)		
Anti-CD38 mAb	40.3%	(192)		
Anti-SLAMF7 mAb	5.0%	(24)		
BCMA-targeted therapy	10.7%	(51)		
BCMA-targeted bispecific	3.4%	(16)		
CAR T cell therapy	6.1%	(29)		
Other BCMA-targeted therapy	1.3%	(6)		
Other bispecific (non-BCMA)	4.2%	(20)		
Other therapy (incl. venetoclax, selinexor, alkylators)	8.2%	(39)		
Previous ASCT	49.8%	(237)		
ASCT < 12 mo before dose 1	6.3%	(30)		
No active treatment	19.3%	(92)		

Note: values are presented as percentage (n) or median [range]. Disease response status and treatment regimen were registered at the date of administration of the first dose of mRNA vaccine.

Abbreviations: y, years; mo, months; COVID-19, coronavirus disease 2019; Ig, immunoglobulin; MM, multiple myeloma; SMM, smoldering multiple myeloma; HD, healthy donor; CR, complete response; sCR, stringent complete response; VGPR, very good partial response; PR, partial response; MR, minimal response; SD, stable disease; PD; progressive disease; ASCT, autologous stem cell transplant; mAb, monoclonal antibody; BCMA, B-cell maturation antigen; CAR, chimeric antigen receptor

Supplemental Materials and Methods:

Study information and patient selection. Multiple myeloma (MM) patients: The serology study cohort consisted of 476 patients with and without previously documented COVID-19 pooled from two different non-interventional Institutional Review Board (IRB) approved study protocols at The Icahn School of Medicine at Mount Sinai. A total of 279 MM patients were enrolled after obtaining written informed consent for the MARS study, an ongoing longitudinal study at our institution (IRB-16-00791). Patients had blood and saliva taken for analysis at multiple time points before or after administration of the SARS-CoV-2 mRNA vaccine. All specimens were coded prior to processing and antibody testing for all serum specimen was performed in a blinded manner. All participants with, at least, one post vaccine antibody data point available at the time of writing this report were included in the analysis. The remaining 197 MM patients were identified under a retrospective study (IRB: GCO#: 11-1433) by conducting a chart review for patients at our MM clinic who had SARS-CoV-2 spike IgG results at various time points around SARS-CoV-2 mRNA vaccine administration. Chart review was conducted to retrieve patient clinical characteristics.

All 31 MM patients used in cellular and neutralization assays consented to enrollment in the MARS clinical trial IRB: 16-00791.The study was approved by the Program for Protection of Human Subject an Institutional Review Board approved research study. Peripheral blood was collected in heparin green tops (Cat#362761), BD Vacutainer CPT (Cat#367985) and BD SST™ Serum Separation Tubes (Cat#0268396) via venipuncture according to trial schedule. Peripheral blood mononuclear cells (PBMC) were Ficoll density separated and cryopreserved by the MARS processing team. Cryopreserved PBMC samples were used to Flow Cytometry analysis. Sera isolated from blood was used to SARS-CoV-2 antibody ELISA and neutralizing assay.

Healthy donors (HD) group: 13 participants of the PARIS (Protection Associated with Rapid Immunity to SARS-CoV-2) study were selected as controls to best match the demographics of the 31 MM patient population. The PARIS cohort follows health care workers longitudinally to assess the durability and effectiveness of SARS-CoV-2 immune responses. The study was reviewed and approved by the Mount Sinai Hospital Institutional Review Board (IRB-20-03374). All participants provided written informed consent prior to collection of data and specimen.

Both studies were carried out in compliance with the Declaration of Helsinki and International Conference on Harmonization Guidelines for Good Clinical Practice. Chart review was conducted to retrieve patient clinical characteristics. Anti-SARS-CoV-2 antibody testing was performed using an anti-IgG assay developed at Mount Sinai Health System Department of Pathology in collaboration with the Icahn School of Medicine at Mount Sinai Department of Microbiology under a Food and Drug Administration (FDA) Emergency Use Authorization.

SARS-CoV-2 antibody ELISA. Antibodies to SARS-CoV-2 spike were detected using an established quantitative two-step ELISA termed Mount Sinai Antibody test described in detail in the referenced manuscripts.(Stadlbauer et al., 2020; Stadlbauer et al., 2021) The assay shows a performance of 100% specificity and 95% sensitivity in in-house evaluation.

Flow cytometry assay to detect SARS-CoV-2 spike-reactive B cells. SARS-CoV-2 spike-reactive B cells were detected with a in house antibody panel (Panel A) developed to simultaneously detect spike-reactive B cells along with immunophenotyping of myeloid and lymphoid cells in peripheral blood. Recombinant spike protein used is known as OptSpike1 which is cloned into the mammalian expression vector pCAGGS and includes the majority of the ectodomain (OptSpike1: AAs 1–1208). Spike protein purification and production described in detail in the referenced manuscript. (Herrera et al., 2021) Strategy to detect spike-reactive B

cells have been described in our previous report (Aleman et al., 2021). Thawed PBMC were initially stained with Live/Dead Fixable Blue Dead Cell Stain Kit (L23105, Thermofisher Scientific) for 15 minutes at room temperature. Viability dye stained PBMC were further stained with Panel A in multiple staining steps at different temperatures. PBMC were stained at room temperature for 15 minutes with a cocktail of 14 antibodies, washed and further stained with spike protein for 30 mins on ice. Post spike protein staining PBMC were washed and stained on ice for 30 minutes with equal amounts of anti-Strep II-FITC and anti-Strep II-Biotin antibodies (A01736, A01737, GeneScript) at a dilution of 1:150. Washed PBMC were further stained with a cocktail of remaining antibodies in Panel A including APC labeled Streptavidin (BioLegend) for 30 minutes on ice. Antibodies in Panel A stained at room temperature include CCR6-BUV496 (clone 11Ag), CD45RA-BUV563 (clone HI100), CD28-BUV737 (clone 28.2) (all from BD Biosciences), TCR gamma-delta-PerCP-eFluor710 (clone B1.1, Thermofisher), CCR7-BV421 (clone G043H7), CXCR3-BV510 (clone G025H7), CD27-BV570 (clone O323), CXCR5-BV605 (clone J25D4), CRTH2-BV711(clone BM16),PD-1-BV750 (clone EH12.1H7),CD25-PE (clone M-A251), CD66b-PE-Dazzzle 594 (clone QA17A51), CCR4-PE-FIRE 810 (clone L291H4), CD11c-Alexa700 (clone Bu15) (all from BioLegend). Antibodies in Panel A stained on ice include CD4-BUV395 (clone SK3), CD56-BUV615 (clone NCAM16.2), HLA-DR-BUV661 (clone G46-6), CD3-BUV805 (clone UCHT1), CD20-BV480 (clone 2H7) (all from BD Biosciences), CD1c-SuperBright 436 (clone L161), CD123-eFluor450 (clone 6H6), CD8-NFB555 (clone OKT8), CD19-NFB610-70S (clone HIB19), CD14-NFB660-40S (clone MEM-15), CD127-PE-Cy5.5 (clone eBioRDR5), CD16-NFR685 (clone 3G8) (all from Thermofisher), IgM-BV650 (clone MHM-88), IgD-BV785(IA6-2), CD11b-PerCP (clone M1/70), CD57-PerCP-Cy5.5 (clone HNK-1), CD24-PE-Cy5 (clone ML5), IgG Fc-PE-Cy7 (clone M1310G05) CD38-APC-FIRE810 (clone HIT2) (all from BioLegend), IgA-APC-VIO770 (clone IS11-8E10, Miltenyi Biotec) Anti-Strep II-FITC, Anti-Strep II-Biotin. Each antibody was used at a dilution of 1:25. All antibody cocktail preparations included True-Stain Monocyte buffer (Biolegend), CellBlox Monocyte and

Macrophage blocking buffer (Thermofisher) and Super Bright Complete Staining buffer (Thermofisher) at a dilution of 1:20 to avoid nonspecific dye-dye and dyes to cell interaction. Cells were acquired on Cytek Aurora Flow Cytometer (Cytek Biosciences). Flow data was compensated on Cytek Aurora acquisition software SpectroFlo and compensated .fcs files were exported to Flowjo software (BD Biosciences) for analysis. Supervised hierarchal gating was employed to delineate major cell types and identify spike-reactive B cells in PBMC. Total cells were initially gated to remove dead cells, doublets and CD66b+ cells. From the live CD66b negative cell gate monocytes were identified based on expression of markers CD16 and CD14 (CD16hi/-CD14-/+). Monocyte-negative cells were sequentially gated for markers CD1c, CD123 and CD19 (CD1c vs CD123 followed by CD1c vs CD19 on CD123- cells) to identify plasmacytoid dendritic cells (pDC, CD123+CD1c-) and conventional dendritic cells (cDC, CD123-CD19-CD1c+). pDC and cDC negative cell fraction was gated for CD3 and CD38 to identify total T cells (CD3+CD38+/-). Subsequently B cells were identified from the CD3 negative gate as cells expressing HLADR and CD19 (CD19+HLADR+/-). As described in our previous report (Aleman *et al.*, 2021), B cells showing fluorescent signals for both Strep-II-FITC and Strep-II-Biotin-Streptavidin-APC were classified as spike-reactive B cells. Finally, B cell-negative cells were plotted as CD56 vs CD16 to identify NK cells (CD56hiCD16- and CD56dimCD16+ NK cells). PBMC from healthy donors prior to any SARS-CoV-2 vaccination or SARS-CoV-2 exposure were stimulated similarly and were used as a control group.

Intracellular cytokine staining flow cytometry (ICS-Flow) T cell assay. T cell assays were carried out in RPMI supplemented with 10% Human Ab serum (R&D Systems), 1x glutamax (Lonza) 1x Penicillin-Streptomycin. PBMC were stimulated for 6 hours with a pool of spike peptides (15-mer sequences with 11 amino acids overlap spanning the entire spike protein, Miltenyi Biotec) at concentrations recommended by the manufacturer or with water as control along with co-stimulators for CD28 and CD49d (i.e., anti-CD28 clone CD28.1 and anti-CD49d

clone 9F109, both from Biolegend). Culture conditions also included antibodies to detect CD4 activation marker CD154 and CD8 degranulation marker CD107 (CD154-PE, clone 24-31 and CD107a-FITC, clone H4A3 both from BioLegend) and Monensin (BioLegend). Stimulations with Staphylococcal enterotoxin B (SEB) were used as positive control. Post stimulation cells were washed and stained with Live/Dead Fixable Blue Dead Cell Stain Kit for 15 minutes at room temperature followed by surface staining with a cocktail of antibodies comprising of CD3- BUV805 (clone UCHT1), CD4-BUV395 (clone SK3), CD8-BUV496 (clone RPA-T8), CD45RA-BUV563 (clone HI100), PD-1-BUV615 (clone EH12.1), HLA-DR-BUV661 (clone G46-6) (all from BD Biosciences,) CCR7-BV510 (clone G043H7), CD27-BV570 (clone O323), CD69-BV605 (clone FN50),CD200-BV711 (clone OX-104), CXCR5-BV785 (clone J252D4), ICOS-PE-Dazzle594 (clone QA17A51), OX40-PE-Cy5 (clone Ber-ACT35) 4-1bb-APC-Fire750 (clone 4B4-1) (all from BioLegend) and CD19-NFB610-70S (cloneHIB19). After surface marker staining cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with BD perm buffer (BD Biosciences) and stained with a cocktail of antibodies to cytokines IL-4 (IL-4-BUV737, clone MP4-25D2), IL-17 (IL-17-BV650, clone N49-653) (both from BD Biosciences), IFN-g (IFN-g-BV421, clone B27), TNF-a (TNF-a-PE-Cy7, clone MaB11), IL-2 (IL-2-APC, clone MQ1-17H12) and GM-CSF (GM-CSF-PerCP-Cy5.5) (all from BioLegend). The cells were acquired on Cytek Aurora Flow Cytometer. Flow data was compensated on Cytek Aurora acquisition software SpectroFlo and compensated .fcs files were exported to Flowjo software (BD Biosciences) for analysis. Data was gated to exclude dead cells and doublets and then further gated on forward scatter (FSC-A) vs side scatter (SSC-A) plot to identify lymphocytes. CD3 vs CD19 plots on lymphocytes were used to identify total T cells (CD3+). Total T cells were further gated to identify CD4+ and CD8+ T cells. Activated CD4+ T cell population were identified by the expression of activation markers CD154 or CD69 as described in our previous report (Aleman *et al.*, 2021). Total cytokine responses in CD4+ T cells were quantified by performing Boolean gating for each cytokine on activated CD4+ T cells. Events from each cytokine combination were pooled and divided by total CD4 T cells events to

calculate the frequency of total cytokine positive CD4+ T cells. Finally, SARS-CoV-2 spike-specific CD4+ T cell response was calculated by subtracting water control total cytokine frequencies from SARS-CoV-2 spike peptide-stimulated conditions. Negative values were designated as zero. PBMC from healthy donors prior to any SARS-CoV-2 vaccination or SARS-CoV-2 exposure were stimulated similarly and were used as a control group.

Cells and SARS-CoV-2 isolates. Vero-E6-TMPRSS2 cells were cultured in Dulbecco's modified Eagles medium (DMEM; Corning) supplemented 10% heat-inactivated fetal bovine serum (FBS; GeminiBio) and 1% minimum essential medium (MEM) amino acids solution (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), 100 µg/ml normocin (InvivoGen, #ant-nr), and 3 µg/ml puromycin (InvivoGen). The authentic SARS-CoV-2 virus (USA-WA1/2020; GenBank: MT020880) was obtained from BEI resources. (BEI resources, NR-52281). The B.1.1.529 isolate USA/NY-MSHSPSP-PV44488/2021 (BA.1, EPI_ISL_7908059) was previously described.(Carreno et al., 2022) Viruses were grown and tittered on Vero-E6-TMPRSS2 cells.

SARS-CoV-2 multi-cycle microneutralization assay. Serum samples from study participants were used to determine the neutralization of wild type (WA1), and B.1.1.529 (Omicron) SARS-CoV-2 isolates. All procedures were performed in a biosafety level 3 (BSL-3) facility at the Icahn School of Medicine at Mount Sinai following standard safety guidelines. The day before infection, Vero-E6-TMPRSS2 cells were seeded in 96-well high binding cell culture plates (Costar, #07620009) at a density of 20,000 cells/well in complete Dulbecco's modified Eagle medium (cDMEM) one day prior to the infection. After heat inactivation of sera (56°C for 1 hour), serum samples were serially diluted (3-fold) in minimum essential media (MEM; Gibco, #11430-030) supplemented with 2 mM L-glutamine (Gibco, #25030081), 0.1% sodium bicarbonate (w/v, HyClone), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 /ml penicillin, 100 µg/ml streptomycin (Gibco) and 0.2% bovine serum albumin (BSA, MP Biomedicals, Cat#.

810063) starting at 1:10. Remdesivir (Medkoo Bioscience Inc.) was included to monitor assay variation. Serially diluted sera were incubated with 10,000 $TCID_{50}$ of WT USA-WA1/2020 SARS-CoV-2, or USA/NY-MSHSPSP-PV44488/2021 (B.1.1.529, Omicron) for one hour at RT, followed by the transfer of 120µl of the virus-sera mix to Vero-E6-TMPRSS2 plates. Infection proceeded for one hour at 37°C and inoculum was removed. 100 µl/well of the corresponding antibody dilutions plus 100µl/well of infection media supplemented with 2% fetal bovine serum (FBS; Gibco, #10082-147) were added to the cells. Plates were incubated for 48h at 37°C followed by fixation overnight at 4°C in 200 µl/well of a 10% formaldehyde solution. For staining of the nucleoprotein, formaldehyde solution was removed, and cells were washed with PBS (pH 7.4) (Gibco) and permeabilized by adding 150 µl/well of PBS, 0.1% Triton X-100 (Fisher Bioreagents) for 15 min at RT. Permeabilization solution was removed, plates were washed with 200 µl/well of PBS (Gibco) twice and blocked with PBS, 3% BSA for 1 hour at RT. During this time the primary antibody was biotinylated according to manufacturer protocol (Thermo Scientific EZ-Link NHS-PEG4-Biotin). Blocking solution was removed and 100 µl/well of biotinylated mAb 1C7C7, a mouse anti-SARS nucleoprotein monoclonal antibody generated at the Center for Therapeutic Antibody Development at The Icahn School of Medicine at Mount Sinai ISMMS (Millipore Sigma) at a concentration of 1µg/ml in PBS, 1% BSA was added for 1 hour at RT. Cells were washed with 200 µl/well of PBS twice and 100 µl/well of HRP-conjugated streptavidin (Thermo Fisher Scientific) diluted in PBS, 1% BSA were added at a 1:2,000 dilution for 1 hour at RT. Cells were washed twice with PBS, and 100 µl/well of o-phenylenediamine dihydrochloride (Sigmafast OPD; Sigma-Aldrich) were added for 10 min at RT, followed by addition of 50 µl/well of a 3 M HCl solution (Thermo Fisher Scientific). Optical density (OD) was measured (490 nm) using a microplate reader (Synergy H1; Biotek). Analysis was performed using Prism 9 software (GraphPad). After subtraction of background and calculation of the percentage of neutralization with respect to the “virus only” control, a nonlinear regression curve fit analysis was performed to calculate the 50% inhibitory dilution (ID_{50}), with top and bottom constraints set to 100% and 0%

respectively. All samples were analyzed in a blinded manner. Viral isolates composition and methods are described in more detail in our previous publication (Carreno *et al.*, 2022).

Statistical Analysis. The Mann-Whitney U test was used to determine significance for all continuous variables that were non-parametrically distributed. Fisher's exact test was used to determine significance in outcome measures. A two-sided alpha < 0.05 was considered statistically significant. Differences between continuous variables and contingency variables were done using R (v4.0.2). All statistical tests were run with R (v4.0.2).

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