SUPPLEMENTAL APPENDIX

Supplement to:

third vaccinations in patients with myelodysplastic syndromes and acute myeloid

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MATERIALS AND METHODS

Study background, patient characteristics, vaccines

 Patients with myeloid malignancy, including MDS and AML, are routinely vaccinated on the presumption that the potential benefits outweigh any risk, although the response to such vaccinations are not well documented. Moreover, such patients were prioritized for vaccination against the emergent pandemic viral infection caused by SARS-CoV-2. Despite these efforts, there remains a fundamental gap in clinical knowledge about whether patients with myeloid malignancy respond to routine vaccination or if the therapies we use for these diseases impact these antibody responses.

 To address this gap in knowledge, we designed a clinical study to assess the immunologic response following administration of the yearly influenza vaccination in patients with myeloid malignancies who are receiving different types of chemotherapy compared to healthy age-matched controls. The study was registered with clinicaltrials.gov (#NCT04484532) and is conducted in accordance with the Declaration of Helsinki, approved by the Roswell Park Cancer Institute Review Board. All patients provided written informed consent. Consent on this study included agreement for the use of remnant material for additional immunological assays at the time of study enrollment. Eligible patients carried a diagnosis of myeloid malignancy and were receiving standard of care treatment for their disease, including watchful waiting, growth factor support, or hypomethylating agents (including azacitidine or decitabine) either alone or in combination with venetoclax. Additional enrollment criteria included willingness to undergo seasonal influenza vaccination, and estimated survival of at least 8 weeks following study enrollment. Patients enrolled to this study received seasonal influenza vaccination for the year of enrollment and underwent

 peripheral blood sampling prior to the vaccine and at serial time points following the influenza vaccination (baseline, 0-3m after vaccination, 3-6 months after vaccination).

 Given the emergence of pandemic viral illness with SARS-CoV-2, the etiological agent of COVID-19, we assessed whether these patients with myeloid disorders demonstrated responses to COVID-19 vaccines received as standard of care. Medical records for these patients were reviewed and the types of vaccine, dates of vaccine administration and clinical characteristics of the patients were extracted. Table 1 lists the summary of patient characteristics for this cohort. Supplementary Table S1 lists the extended clinical characteristics, treatments summary, vaccine/booster types, and the time point of sampling relative to the most recent COVID-19 vaccination given.

 Post-SARS-Cov-2 mRNA vaccination serum samples after the second vaccination and third vaccination were also obtained from SARS-CoV-2 naïve 16 healthy adult healthcare workers who work at research institution and are not exposed to COVID-19 patients as a comparative control group.

Neutralization assay

 Sera were evaluated in a qualified SARS-CoV-2 pseudovirion neutralization assay (PsVNA) using SARS-CoV-2 WA-1 strain and the five variants of concern (VOCs): Alpha variant (B.1.1.7; with spike mutations H69-V70del, Y144del, N501Y, A570D, D614G, P681H, T716I, S982A, and D1118H), Beta variant (B.1.351; with spike mutations L18F, D80A, D215G, L242-244del, R246I, K417N, E484K, N501Y, D614G, and A701V), Gamma variant (P.1; with spike mutations L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, H655Y, T1027I, D614G, V1176F), Delta variant (B.1.617.2; with spike mutations

 T19R,G142D,E156del,F157del,R158G,L452R,T478K,D614G,P681R,D950N) and Omicron variant (B.1.1.529; with spike mutations A67V, H69-70del, T95I, G142D, V143-145del, Y145D, N211del, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F). SARS-CoV-2 neutralizing activity measured by PsVNA correlates with PRNT (plaque reduction neutralization test with authentic SARS-CoV-2 virus) in previous studies $1-3$

Pseudovirions were produced as previously described³. Briefly, human codon-optimized cDNA encoding SARS-CoV-2 spike glycoprotein of the WA-1 and variant strains were synthesized by GenScript and cloned into eukaryotic cell expression vector pcDNA 3.1 between the *BamH*I and *Xho*I sites. The plasmid vector encoding spike for Omicron variant was a gift from Vaccine Research Center, NIAID, NIH. Pseudovirions were produced by co- transfection Lenti-X 293T cells with psPAX2(gag/pol), pTrip-luc lentiviral vector and pcDNA 3.1 SARS-CoV-2-spike-deltaC19, using Lipofectamine 3000. The supernatants were harvested at 48h post transfection and filtered through 0.45µm membranes and titrated using 88 293T-ACE2-TMPRSS2 cells (HEK 293T cells that express ACE2 and TMPRSS2 proteins)³.

89 Neutralization assays were performed as previously described $14,5$. For the neutralization 90 assay, 50 µL of SARS-CoV-2 S pseudovirions (counting ~200,000 relative light units) were pre-incubated with an equal volume of medium containing serial dilutions (20-, 60-, 180-, 540- , 1,620-, 4,860-, 14,580- and 43,740-fold dilution at the final concentration) of heat-inactivated serum at room temperature for 1h. Then 50 µL of virus-antibody mixtures were added to 293T-94 ACE2-TMPRSS2 cells (10⁴ cells/50 μ L)³ in a 96-well plate. The input virus with all SARS-95 CoV-2 strains used in the current study were the same $(2 \times 10^5 \text{ relative light units}/50 \text{ }\mu\text{L/well}).$

 After a 3 h incubation, fresh medium was added to the wells. Cells were lysed 24 h later, and **Iuciferase activity was measured using One-Glo luciferase assay system (Promega, Cat#** E6130). The assay of each serum was performed in duplicate, and the 50% neutralization titer was calculated using Prism 9 (GraphPad Software). Controls included cells only, virus without any antibody and positive sera. The limit of detection for the neutralization assay is 1:20. Two independent biological replicate experiments were performed for each sample and variation in PsVNA50 titers was <9% between replicates.

Seroreactivity of post-vaccination samples to SARS-CoV-2 receptor binding domain by ELISA

 96 well Immulon plates were coated with 50 ng/100 µL of recombinant spike-RBD either 107 from vaccine-homologous WA1/2020 or the Omicron variant in PBS overnight at 4° C. Starting at a 1:100 dilution, serum samples were serially diluted 5-fold and applied to the coated well for 1 hr at ambient temperature. Serum samples were assayed in duplicate. After three washes with PBS/0.05% Tween 20, bound human IgG antibodies were detected with 1:5000 dilution of HRP-conjugated anti-human IgG Fc-specific antibody (Jackson Immuno Research). After 1 hr, plates were washed PBST followed by PBS, and o-Phenylenediamine dihydrochloride (OPD) was added for 10 min. Absorbance was measured at 492 nm. End titer was determined as 2-fold above the average of the absorbance values of the binding of serum samples to blank control wells. The end-point titer is reported as the serum dilution that was above this cutoff and was calculated using Prism 9 (GraphPad Software).

Quantification and statistical analysis

 Descriptive statistics were performed to determine the geometric mean titer values and were calculated using GraphPad. All experimental data to compare differences among groups were analyzed using lme4 and emmeans packages in R (RStudio version 1.1.463).

 The demographic characteristics of these study participants are shown in Supplementary Table 1. Since age and sex can be biologically plausible confounders, data were analyzed for statistical significance between groups to control for age and sex as covariates (predictor variables) using a multivariate linear regression model. To ensure robustness of the results, absolute measurements were log2-transformed before performing the analysis. For comparisons between the vaccine groups (factor variable), pairwise comparisons were extracted using 'emmeans' and Tukey-adjusted p values were used for denoting significance to reduce Type 1 error due to multiple testing. The tests were two-sided tests. The differences were considered statistically significant with a 95% confidence interval when the p value was 131 Iess than 0.05. (* ≤0.05, ** ≤0.01, *** ≤ 0.001, **** ≤0.0001).

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Supplementary References

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Supplementary Figure S1

Supplementary Figure S1: Relationship of post-vaccination SARS-CoV-2 serum neutralizing antibodies in AML/MDS patients and healthy controls with SARS-CoV-2 RBD binding antibodies. Correlation analysis between serum PsVNA50 neutralization antibody titers generated following second (panels A & C) and third (panels B & D) vaccination of 48 AML/MDS patients (in red) or 16 healthy controls (in blue) against vaccine-matched SARS-CoV-2 WA1 or the Omicron variant, and binding antibodies against either SARS-CoV-2 WA1 RBD (panels A-B) or Omicron RBD (panels C-D). Correlation analysis was performed using non-linear regression model and associated Spearman's correlation coefficients (r) and regression significance (p) are shown.