MATERIALS AND METHODS

Study Population and Design

Healthy controls analyzed for this study were enrolled in our prospective, observational cohort study in patients with hematologic malignancies who received care at the University of Maryland Medical Center (UMMC) and had either received or were scheduled to receive any of the two SARS-CoV-2 mRNA vaccines approved by the FDA (IRB HP-00095016). We collected 40ml of heparinized blood from 9 consecutive COVID-19 patients, 21 patients with B cell lymphomas, and 9 healthy controls (Supplemental Table 1). Blood from the lymphoma patient described in this report was collected under a different IRB, HP-00057785. The University of Maryland's Institutional Review Board had approved both studies. Informed consent was obtained, blood samples were collected and plasma was generated from peripheral blood samples after centrifugation at 400g for 10min and frozen immediately at -80C. Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation density gradient and immediately frozen in liquid nitrogen. Our patients with hematologic malignancies and the healthy controls enrolled received COVID-19 vaccination per the treating physician’s discretion.

Measurement of absolute immunoglobulin levels

Absolute serum concentrations of the different immunoglobulins were measured using Human IgG, IgM, and IgA Enzyme-linked Immunosorbent Assay (ELISA) Kits (Invitrogen, Cat. No. BMS2091, BMS2098, BMS2096) as per the manufacturer’s instructions. Absorbance was read at 450nm with a reference wavelength of 620nm in a microtiter plate reader (Tecan, Morrisville, NC).

Levels of anti- SARS-CoV-2 S1 antibodies

For repeated measurement of anti-SARS-CoV-2 S1 antibody levels at the Center for Cancer Research, quantitative measurements by luciferase immunoprecipitation assay systems (LIPS)

were performed at Dr. Jeffrey I. Cohen's lab at the National Institutes of Health, Bethesda, Maryland as previously described [1].

Analysis of SARS-CoV-2-specific antibodies

For the comprehensive immunomonitoring after 5 doses of the vaccine performed at the University of Maryland, serum antibody responses against recombinant, full-length SARS-CoV-2 proteins (Supplemental Table 2) or viral control proteins (Supplemental Table 1) were determined by ELISA as previously described. Briefly, high-binding ELISA plates (Thermo Fisher, Cat. No. 44-2404-21) were coated with 5µg/mL of the respective proteins in PBS (Gibco, Cat. No. 10010-023) overnight at 4ºC. The next day plates were washed twice with PBS and twice with 0.1% PBS-T (VWR, Cat. No. M147-1L). Plates were then blocked with 5% non-fat dry milk (Santa Cruz, Cat. No. sc2325) in PBS (MPBS) for 1h at room temperature (RT), then washed again as described above. Serum was diluted 1:40 for screening assays and for titration 1:100/1:400/1:1,600/1:6,400 and if necessary 1:25,000 and 1:100,000 in MPBS. Diluted sera were added to plates and incubated for 3H at RT. Plates were washed as described above before incubation with secondary antibodies against pan-human IgG (Southern Biotech, Cat. No. 2040-04) or IgA (Southern Biotech, Cat. No. 2050-04). Secondary antibodies were diluted according to the manufacturers' instructions and plates incubated for 1h at RT. Plates were then washed as described above, PNPP tablets (Southern Biotech, Cat. No. 0201-01) dissolved in diethanolamine (Thermo, Cat. No. 34064) and PNPP substrate solution added to each well for 10min in the dark. 15uL of 3N NaOH (VWR, Cat. No. BDH7472-1) stop solution was added to each well and absorbance was read at 405nm with a reference wavelength of 620nm in a microtiter plate reader (Tecan, Morrisville, NC). Endpoint titers were calculated using serum titration curves for positive samples and pooled sera of 5 healthy donors. For non-SARS-CoV-2 antigens, serum dilutions for anti-GST (glutathione-S-transferase) antibodies (Supplemental Table 1) were used as a negative control.

SARS-CoV-2 neutralization assay

Neutralizing activity of patient sera was assessed using the cPass Neutralization Antibody Detection Kit (GenScript, Cat. No. L00847-A) which is a surrogate test detecting circulating neutralizing antibodies against SARS-CoV-2 that block the interaction between the receptor binding domain (RBD) of the viral spike glycoprotein with the ACE2 cell surface receptor. Briefly, samples and controls were diluted with sample dilution buffer and pre-incubated with the Horseradish peroxidase (HRP) conjugated recombinant SARS-CoV-2 RBD fragment (HRP-RBD) or one of its variants listed in Supplemental Table 2 to allow the binding of the circulating neutralization antibodies to HRP-RBD. The mixture was then added to the capture plate, which was pre-coated with the hACE2 protein. The unbound HRP-RBD as well as any HRP-RBD bound to non-neutralizing antibody was captured on the plate, while the circulating neutralization antibodies HRP-RBD complexes remained in the supernatant and were removed during washing. Following a wash cycle, TMB substrate solution was added followed by the Stop Solution. The absorbance of the final solution was read at 450 nm in a microtiter plate reader (Tecan, Morrisville, NC).

Analysis of B cell phenotypes

Approximately 500,000 were stained using the antibody cocktail shown in Supplemental Table 3. B cell subpopulations were analyzed using the BD LSRII flow cytometer.

Analysis of SARS-CoV-2-specific B cells

B cells specific for SARS-CoV-2 were identified using the Spike B Cell Analysis Kit (Miltenyi Biotec, cat no. 130-128-022). Briefly, S protein-tetramers were prepared by incubating SARS-CoV-2 Spike protein (HEK)-Biotin with streptavidin (PE or PE-Vio 770) for 15 minutes at room temperature. For each test 2 µL of each fluorochrome-conjugated antibody (Supplemental Table 4), 5 µL of 7-AAD Staining Solution, 5 µL of spike-tetramer-PE, and 10 µL of spike-tetramer-PE-Vio 770 were used. PBMCs (5-10 × 10^6 were incubated in antibody staining mix for 30 minutes at

4°C. Cells were washed and analyzed by flow cytometry using a MACSQuant® Analyzer 10 (Miltenyi Biotec).

Analysis of SARS-CoV-2-specific T cells

B cells specific for SARS-CoV-2 were identified using different versions of the SARS-CoV-2 T Cell Analysis Kit (Miltenyi Biotec) and pools of lyophilized peptides, consisting mainly of 15-mer sequences with 11 amino acids (aa) overlap, covering the complete sequence of the S protein ("S complete") or sequence domains 1-692 ("S1") or aa 689-895 ("S plus"). For the analysis of T cell responses against the omicron variant of the SARS-CoV-2 Spike Glycoprotein a peptide pool consisting of 315 peptides (15mers with 11 aa overlap) covering the entire sequence of the S protein was used (GenScript, cat. no. RP30121). For the analysis of T cell responses against microbial antigens other than SARS-CoV-2 a pool of 32 MHC class I-specific peptides of 8–12 aa in length derived from cytomegalovirus (HCMV), Epstein-Barr virus (EBV), and influenza virus was used (CEF MHC Class I Plus, Milteny Biotec, cat no. 130-098-426). Briefly, PBMC were thawed, plated cells at a density of 5×10^6/mL in fresh cell culture medium in a 24-well cell culture plate at 37°C and 5% CO_2 overnight. The next morning, cells were resuspended in culture medium at a density of 1×10^7 viable cells per mL and 100 µL of cell suspension per well was plated in a flat-bottom 96-well plate resulting in a total number of 1×10^6 cells per well. For antigen stimulation 2 µL of peptide stock solution was added to the respective wells and mixed by pipetting up and down. As a positive control 2 µL of the CytoStim crosslinking agent were used and as a negative control 2 µL sterile water/10% DMSO solution were added to the respective wells. Cells were incubated at 37 °C and 5% CO_2 for 2 hours, 2 µL of Brefeldin A were added to each well, and cells were incubated at 37 °C and 5% CO_2 for an additional 4 hours. Cells were then resuspended in 100 µL reconstituted Viobility 405/452 Fixable Dye master mix and incubated for 10 minutes at room temperature. Fixation was performed by adding 100 µL Inside Fix to each well followed by incubation for 20 minutes at room temperature. The supernatant was removed

and permeabilization was performed by adding 100 µL Inside Perm to each well. The supernatant was removed and 100 µL of antibody staining mix (rSupplemental Table 4) was added to each well followed by incubation for 10 minutes at room temperature. Cells were washed and analyzed by flow cytometry using a MACSQuant® Analyzer 10 (Miltenyi Biotec).

Statistical analyses

Statistical analyses for serological analyses were performed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA). Groups were compared using the Mann–Whitney U test and paired analyses were performed using the Wilcoxon signed-rank test. For the analysis of clinical characteristics, groups were compared using a student's t-test.

References

1. Burbelo, P.D., et al., *Detection of Nucleocapsid Antibody to SARS-CoV-2 is More Sensitive than Antibody to Spike Protein in COVID-19 Patients.* medRxiv, 2020: p. 2020.04.20.20071423.