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ORIGINAL RESEARCH

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Immune response to SARS-CoV-2 mRNA vaccination and booster dose in patients with multiple myeloma and monoclonal gammopathies: impact of Omicron variant on the humoral response

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ABSTRACT

The humoral and cellular response to SARS-CoV-2 mRNA full vaccination and booster dose as well as the impact of the spike variants, including Omicron, are still unclear in patients with multiple myeloma (MM) and those with pre-malignant monoclonal gammopathies. In this study, involving 40 patients, we found that MM patients with relapsed-refractory disease (MMR) had reduced spike-specific antibody levels and neutralizing titers after SARS-CoV-2 vaccination. The five analyzed variants, remarkably Omicron, had a significant negative impact on the neutralizing ability of the vaccine-induced antibodies in all patients with MM and smoldering MM. Moreover, lower spike-specific IL-2-producing $CD4^+$ T cells and reduced cytotoxic spike-specific IFN-γ and TNF-α-producing $CD8^+$ T cells were found in MM patients as compared to patients with monoclonal gammopathy of undetermined significance. We found that a heterologous booster immunization improved SARS-CoV-2 spike humoral and cellular responses in newly diagnosed MM (MMD) patients and in most, but not all, MMR patients. After the booster dose, a significant increase of the neutralizing antibody titers against almost all the analyzed variants was achieved in MMD. However, in MMR patients, Omicron retained a negative impact on neutralizing ability, suggesting further approaches to potentiating the effectiveness of SARS-CoV-2 vaccination in these patients.

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Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by impairment of both cellular and humoral responses,[1] with high risk of viral and bacterial infections.[2] Alterations of immune response to infections have been also described in the pre-malignant state of monoclonal gammopathies as monoclonal gammopathies of undetermined significance (MGUS) and smoldering MM (SMM); although the risk of infections in these patients is lower as compared to MM patients.[2–4] In MM patients undergone to treatment, vaccination prophylaxis is recommended to prevent the consequences of infections and the risk of death from pneumonia.[5] However, some studies reported a reduced response of MM patients to vaccines, including the influenza vaccine.[6]

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) completely changed the epidemiological scenario of infections also in hematological patients, including MM, due to either their intrinsic immunological defects or to the effect of the immune-suppressive treatments. It has been reported that COVID-19 causes moderate-to-severe acute respiratory disease in approximately 75% to 80% of patients with MM, resulting in death in almost one-third of hospitalized patients.[7,8] Thus, vaccination against SARS-CoV-2 is the most important preventive strategy to protect MM patients from COVID-19. Published data show that MM patients share a reduced antibody production in response to anti-SARS-CoV-2 vaccination.[9,10] In addition, MM patients treated with anti-CD38 or anti–B-cell maturation antigen (BCMA) antibodies, do not develop anti–SARS-CoV-2 antibodies or have insufficient response even after full vaccination.[11,12] However, in monoclonal gammopathies patients, very few data are actually available about the cellular response to SARS-CoV-2 mRNA vaccination[13] and the efficacy of the mRNA vaccine, including the booster dose, to the different SARS-CoV-2 variants characterized by mutations encoding for the spike protein.[14]

Since November 2021, the B.1.1.529 (Omicron) variant has rapidly become dominant globally.[15] Omicron partially evades antibodies induced by infection or vaccination and it raises concerns regarding the effectiveness of current vaccines.[16–18] There are few studies available on the response after full vaccination and booster dose in cancer patients[19,20] and there is little data available on patients with MM and monoclonal gammopathies.[21]

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In this study, we investigated both the humoral and cellular response to SARS-CoV-2 mRNA full vaccination and to the booster dose in a cohort of patients with MGUS, SMM, and MM. In these patients, we quantified SARS-CoV-2 spike IgG antibodies (spike-IgG-Abs), neutralizing antibody (NAb) titers against vaccine homologous spike and five variants of concern (VoC), including Omicron, and SARS-CoV-2 spike-specific $CD4^+$ and $CD8^+$ T cells. Moreover, in MM patients, we also studied the effect of a heterologous booster dose on the SARS-CoV-2 spike-specific immune responses, especially, on the neutralizing capacity against the spike variants.

Materials and methods

Study information and patient clinical characteristics

All patients were followed and treated at the Hematology Unit of Parma Hospital and received vaccination as part of the national COVID-19 vaccination program. From February 25 to July 23, 2021, 40 consecutive patients were enrolled in the study: 6 MGUS, 10 SMM, and 24 MM patients, including either newly diagnosed MM (MMD) or relapsed-refractory MM (MMR). All MMD patients are receiving first-line treatment whereas patients with MMR have received at least two lines of treatment.

Peripheral blood (PB) samples were collected at two time points: before the first dose (PRE) and 14 ± 2 days after the second dose (POST), 35 days after the first dose of the BNT162b2, mRNA vaccine by Pfizer-BioNTech (Figure 1a). In a subset of 16 patients with MM (MMD and MMR), blood samples were also collected after 14 ± 2 days of a heterologous booster dose (BOOSTER) with mRNA-1273 by Moderna, received in November 2021, at least six months (>180 days) after the complete vaccination according to the national COVID-19 vaccination program. (Figure 4a).

Ethics statement

PB samples were obtained according to the criteria of the Declaration of Helsinki and following written informed consent. The study was approved by the Local Ethics Committee.

Detection of SARS-CoV-2-specific IgG antibodies

Heat-inactivated sera samples were tested for SARS-CoV-2-specific IgG antibodies using a commercial quantitative two-step ELISA (COVID-SeroIndex, Kantaro Quantitative SARS-CoV-2 IgG Antibody Kit, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's recommendations. Quantitative results are reported in Arbitrary Units/mL (AU/mL) with the lower limit of detection at <3.20 (AU/mL).

SARS-CoV-2 pseudoviruses generation and neutralization assay against the original viral strain and variants

Lentiviral vector-based SARS-CoV-2 spike pseudoviruses were generated as previously described[22] with minor modifications as described in Supplemental Methods. SARS-CoV-2 spike pseudoviruses displayed on their surface six different spike glycoproteins: Wuhan-Hu-1 (B.1 Lineage; China) Alpha (B.1.1.7. Lineage; United Kingdom), Beta (B.1.351 Lineage; South Africa), Gamma (P.1 Lineage; Brasil), Delta (B.1.617.2 Lineage, India) or Omicron (B.1.1.529 Lineage; Europe).

The neutralization assay was performed on 104 HEK/ACE2/TMPRRS2/Puro cells[22] testing heat inactivated sera samples at the dilution of 1:4–1:8–1:16–1:32–1:64–1:128–1:256–1:512 as described in Supplemental Methods. A negative control was established without serum. The relative luciferase units (RLUs) were compared and normalized to those derived from wells where pseudovirus was added in the absence of sera (100%). Neutralization titer 50 (NT50) was expressed as the maximal dilution of the sera where the reduction of the signal is ≥50%. Each serum was tested in triplicate.

Intracellular cytokine staining flow cytometry (ICS) T cell assay

Patients' PBMCs were thawed, resuspended in RPMI media supplemented with 10% heat-inactivated fetal calf serum (Biochrom, GmbH, Berlin, Deutschland), 2 mM L-glutamine and 1% penicillin–streptomycin (R10), and rested at 37°C for 6 hours (h). After resting, 1×10^6 cells of each sample were supplemented with R10 containing CD107a (cat. 555801, BD Pharmingen, Franklin Lakes, NJ, USA), monensin (cat.554724, BD Golgi Stop, BD Biosciences) and S1 and S2 peptide pools (PepMIX SARS-CoV-2 spike glicoprotein, cat. PM-WCPV-S-3, JPT Peptide Techonologies GmbH, Berlin, Germany) added at a final concentration of 1 µg/mL. For each tested sample, a positive control (*S. enterotoxin B* at 2 µg/mL, Merck KGaA, Darmstadt, Deutschland) and an unstimulated control (stimulation with an equimolar amount of DMSO, Merck KGaA) were also included. Cells were incubated for 2 h at 37°C with 5% CO_2 and then R10 containing brefeldin A (5 µg/mL, Merck KGaA) were added and the samples were incubated for 16 h. PBMCs were washed and stained with BD Horizon Fixable Viability stain 575 V (1:1000). A surface staining cocktail was added containing saturating concentrations of BV480 CD3 (cat.566105, BD Horizon), BV786 CD4 (cat.563877, BD Horizon) and BV711 CD8 (cat.563677, BD Horizon). PBMCs were fixed and permeabilized with FACS Lysins Solution 1x (cat.349202, BD Bioscience) and FACS Permeabilizing Solution2 1x (cat. 340973, BD Biosciences). After washes, PBMCs were stained with a cocktail of anti-human IFN-γ-FITC (cat.554700, BD Pharmingen), IL-2-PerCP-Cy5.5 (cat.560708, BD Pharmingen), and TNF-α-BV421 (cat.562783, BD Horizon). Samples were acquired on a BD Bioscience FACSCelesta flow cytometer using the FACSDiva Software (version 8.02, BD Bioscience). A hierarchical gating strategy was created during assay qualification and was applied for all PRE, POST, and BOOSTER vaccine sample analysis (Supplemental Figure S1A-B). Peptide-specific responses were calculated by subtraction of the unstimulated controls from the paired peptide-stimulated samples, then POST vaccination and BOOSTER responses were subtracted of the paired PRE vaccination responses. Negative values were designated as zero and data are represented as a percentage of total $CD4^+$ or $CD8^+$ T cells with the lower limit of detection at <0.01 of the parental gate.

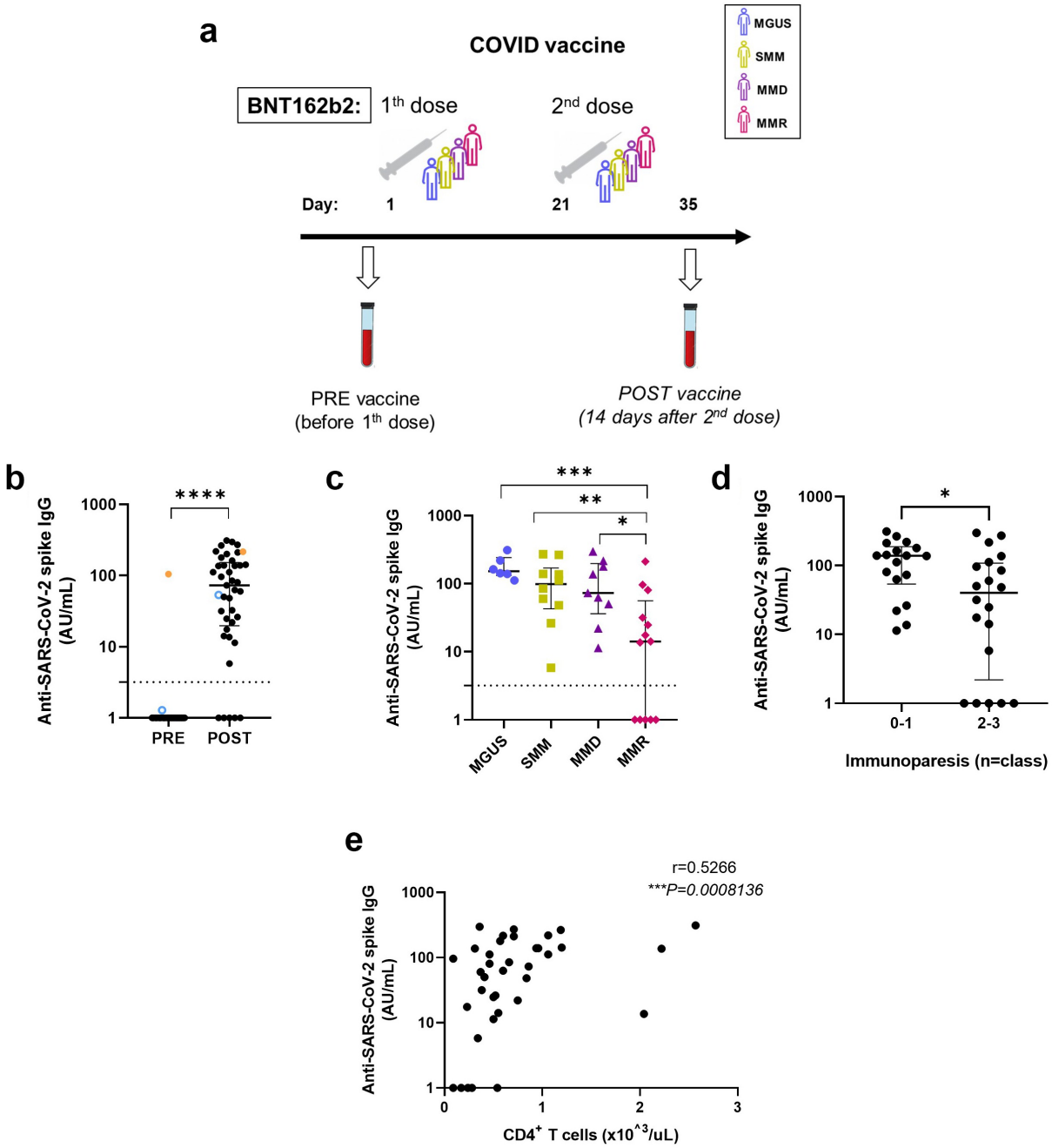

Figure 1. Vaccine-induced SARS-CoV-2 spike IgG antibodies in patients with monoclonal gammopathies at different stages of disease. (a) Study design, vaccine administration scheme and time points collected for patients. (b) SARS-CoV-2 spike IgG antibodies were measured PRE and POST vaccination (n = 40 patients), using the COVID-SeroIndex Kantaro SARS-CoV-2 IgG test. Quantitative results are reported in Arbitrary Units/mL (AU/mL) with the lower limit of detection at <3.20 (AU/mL). Individual data points are shown as scatter dot plot with lines showing the median with IQR. Dotted lines show the lower limit of detection. Orange dots identify a patient with highly suspected pre-vaccination COVID-19 infection and light blue dots a patient with a PCR-proven COVID-19 infection between the two time points. Statistical analysis was performed using a paired, two-tailed, nonparametric Wilcoxon test. (c) SARS-CoV-2 spike IgG antibody levels in patients subdivided in different stages of disease: MGUS (indigo ●) (n = 6); SMM (avocado ■) (n = 10); MMD (purple ▲) (n = 9) and MMR (fuchsia ◆) (n = 13). Significant difference was determined by two-tailed Mann–Whitney U tests. (d) SARS-CoV-2 spike IgG antibody levels in relation to number of Ig classes involved in immunoparesis at the time of vaccination. (e) Correlation between levels of SARS-CoV-2 spike IgG antibodies after vaccination and the baseline (PRE) percentage of total CD4 + T cells. Correlation was calculated using nonparametric Spearman rank correlation. *P* values are shown when $P < .05$ (*$P < .05$, **$P < .01$, ***$P < .001$).

Statistical analysis

Data are presented as medians with IQRs. Unpaired samples were compared using Kruskal-Wallis and Mann–Whitney U tests, and paired samples were compared with the Wilcoxon test. Correlation coefficients were quantified by the Spearman rank correlation coefficient. All tests were performed in a two-sided manner, using a nominal significance threshold of $P < .05$ unless otherwise specified. Data analysis, as well as all graphical representation of the data, were performed in GraphPad Prism v.8.0.1 software. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

Results

Antibody responses to vaccination in patients with MM and pre-malignant monoclonal gammopathies

We evaluated spike-IgG-Abs PRE and POST BNT162b2 mRNA vaccination in 40 patients with monoclonal gammopathies at different stages of disease (Figure 1a-b). Two patients were excluded, one because preexisting antibodies (Figure 1b, orange dots) and the second because having COVID-19 infection between the time points (Figure 1b, light blue circles). Thus, we focused on 38 COVID-naïve patients: 6 low-intermediate risk MGUS, 10 SMM, 9 MMD and 13 MMR, described in Table 1.

The seropositivity (detectable levels of spike-IgG-Abs in patient's sera) rate for spike-IgG-Abs in the total cohort was 86.8% (n. 33) with 5 (13.2%) patients exhibiting no detectable antibodies (Figure 1b). We did not find any significant correlation of the humoral response with patients' age (Supplemental Figure S2A). Looking at the different stages of disease, we found that MMR patients had significantly lower spike-IgG-Abs as compared with MGUS, SMM and MMD patients (Figure 1c).

In order to identify factors affecting humoral responses to SARS-CoV-2 mRNA vaccination, we firstly stratified SMM patients according to 2.20.20 risk stratification score[23] and we did not find any significant differences in spike-IgG-Abs levels (Supplemental Figure S2B). We found the same result, in MM patients stratified according to International Staging System (ISS) risk stratification score (Supplemental Figure S2C).

We also evaluated the possible impact of immunostimulatory anti-MM treatment, but we lacked to find significant differences in the spike-IgG-Abs levels based on the therapy with immunomodulatory drugs (IMiDs) (Supplemental Figure S2D).

Moreover, we found that all five seronegative patients had partial immunoparesis, defined as at least two suppressed uninvolved Ig classes,[24,25] and that, overall, the presence of partial immunoparesis was associated with lower spike-IgG-Abs compared to involvement of only one class of Ig (Figure 1d).

Considering other immune parameters possibly affecting the vaccine-induced humoral responses, we did not find any significant correlation between spike-IgG-Abs responses and the baseline (PRE) total lymphocyte counts although they were significantly reduced in MMR and MMD patients compared with MGUS patients (Supplemental Figure S2E, F). Interestingly, there was a significant correlation between spike-IgG-Abs and the baseline total $CD4^+$ T cells counts (Figure 1e), but not with total $CD8^+$ T cells counts (Supplemental Figure S2G).

To examine the functional quality of vaccine-induced antibodies, we tested patients' sera using a neutralization assay with a pseudovirus displaying on its surface vaccine homologous spike (Wuhan-Hu-1 sequence). Looking at the different stages of disease, we found that MMR patients had significantly lower NAb titers compared with MGUS, SMM and MMD patients (Figure 2a). Notably, 5 out of 6 patients with a neutralizing titer below the detection level were MMR patients. We found a significant correlation between spike-IgG-Abs levels and neutralizing titers against Wuhan-Hu-1 spike (Figure 2b).

We next wanted to determine whether the vaccine-induced antibodies protect patients against VoC of the virus that raise concerns regarding the effectiveness of current vaccines.[26–29] We tested patients' sera samples in the neutralization assay with pseudoviruses displaying Alpha, Beta, Gamma, Delta or the most recent Omicron variants of the spike protein. We found significant correlations between levels of spike-IgG-Abs and NAb titers against the five variants, indicating that vaccine-induced antibodies retained functional characteristics and neutralizing ability against the studied variants, even if we noticed a general reduction in neutralizing titers to variants (Supplemental Figure S3).

We analyzed neutralizing titers to the five variants in comparison to Wuhan-Hu-1 spike, in patients stratified for disease stages (Figure 2c). Interestingly, among MGUS patients, we found that NAb titers to Beta and Omicron variants were significantly lower than Wuhan-Hu-1 spike. On the contrary, among SMM and MMD patients, NAb titers to all variants were significantly lower than original spike (Figure 2c). In MMR patients, already showing a significant reduction in NAb titers to the Wuhan-Hu-1 spike compared with other patients (Figure 2a), we found lower NAb titers to Beta, Delta and Omicron variants than original spike (Figure 2c).

In particular, the neutralization ability against Omicron variant was dramatically reduced in all groups of patients (red dots in Figure 2c): in MMR patients, we did not find any detectable neutralizing titer (0/13) against this variant compared to 4/6 (66.6%), 4/10 (40%), and 3/9 (33.3%) in MGUS, SMM and MMD patients, respectively.

These data overall indicated that, after full vaccination, all the analyzed variants, remarkably the Omicron one, had a significant negative impact on the neutralizing ability of the vaccine-induced antibodies in SMM, MMD and MMR.

Cellular responses to vaccination in patients with MM and pre-malignant monoclonal gammopathies

Clinical studies have suggested a protective role for both humoral and cell-mediated immunity in recovery from SARS-CoV-2 infection[30–32] and the goal of COVID-vaccines is to elicit a coordinated immunological memory including both NAbs and SARS-CoV-2 spike-specific T cells.[33] Today, the size and the quality of T cell responses induced by SARS-CoV-2 vaccination in patients with monoclonal gammopathies are not fully understood. Then, we evaluated vaccine-induced circulating SARS-CoV-2 spike-specific T cells by ICS flow cytometry analysis.

First, we noticed that $CD4^+$ and $CD8^+$ T cell responses to SARS-CoV-2 vaccine were highly variable in our cohort (responder patient defined as patient with detectable spike-specific $CD4^+$ or $CD8^+$ T cell expressing at least one of the three analyzed cytokines: IL-2 or IFN-γ or TNF-α) (Figure 3). We observed that 100% of MGUS patients had a SARS-CoV-2 spike-specific $CD4^+$ T cell response compared to 70% of SMM, 77.7% of MMD, and 58.3% of MMR patients (Figure 3a). In particular, we found that SMM, MMD and MMR had significantly reduced IL-2^+CD4^+ T cells compared to MGUS patients, while we did not find any significant differences for IFN-γ and TNF-α producing $CD4^+$ T cells (Figure 3b). Focusing on $CD4^+$

Table 1. Patients' clinical data.

	MGUS (n = 6)		SMM (n = 10)		MMD (n = 9)		MMR (n = 13)	
Age (years)	74	[61–84]	77	[51–86]	69	[51–83]	80	[63–83]
Female gender	100%	(6)	40%	(4)	16.60%	(2)	53.80%	(7)
Years from diagnosis	7	[3–15]	9.5	[3–15]	2	[1–6]	6	[2–10]
Lymphocyte Absolute counts (x10^{-3}/μl)	2.07	[1.6–4.04]	1.44	[0.67–3.85]	1.2	[0.54–2.09]	0.97	[0.4–3.17]
Cytogenetic risk								
High					0%	0	15.4%	(2)
Standard					100%	(9)	53.8%	(7)
N/A					0%	(0)	30.8%	(4)
Immunoparesis								
None	16.6%	(1)	20%	(2)	0%	(0)	0%	(0)
1 class	66.7%	(4)	20%	(2)	66.7%	(6)	23.1%	(3)
2–3 classes	16.6%	(1)	60%	(6)	33.3%	(3)	76.9%	(10)
ISS								
I					33.3%	(3)	23.1%	(3)
II					55.5%	(5)	30.8%	(4)
III					11.1%	(1)	46.1%	(6)
Mayo Score								
0	0.0%	(0)						
1	33.7%	(2)						
2	66.7%	(4)						
3	0.0%	(0)						
2–20-20 Score								
0			10.0%	(1)				
1			50.0%	(5)				
2–3			40.0%	(4)				
Previous line of therapy (n°)					1	[1–2]	2	[2–6]
Disease reponse status:								
CR or sCR					33.3%	(3)	46.1%	(6)
VGPR or PR					55.5%	(5)	46.1%	(6)
SD or PD					11.1%	(1)	7.8%	(1)
Treatment regimen contains:								
IMiDs					55.5%	(5)	61.5%	(8)
PI					22.2%	(2)	7.8%	(1)
Steroids					44.4%	(4)	69.2%	(9)
Anti-CD38 mAbs					0.0%	(0)	30.8%	(4)
No active treatment					22.2%	(2)	23.1%	(3)

Note: Values are presented as percentage (n) ore median [range]
High Cytogenetic Risk definition: presence of one or more of these cytogenetic alterations: t(4:14), t(14:16), t(14:20),del17p
Abbreviations: MGUS: Monoclonal Gammopathy of Undetermined Significance; SMM: Smoldering Multiple Myeloma; MM: Multiple Myeloma; ND: Newly Diagnosed; R: Relapsed; ISS: International Staging System; CR: Complete Response; sCR: stringent Complete Response; VGPR: Very Good Partial Response; PR: Partial Response; SD: Stable Disease; PD: Progressive Disease; IMiDs: Immunomodulatory Drugs; PI: Proteasome Inhibitors; N/A: not available; mAbs: monoclonal antibodies.

T cells double positive, we found that MMR had significantly reduced IL-2^+TNF-α^+ $CD4^+$ T cells compared to MGUS patients (Figure 3c).

Moreover, the percentages of patients with a SARS-CoV-2 spike-specific $CD8^+$ T cell response were 100%, 80%, 55.5% and 91.6% among MGUS, SMM, MMD and MMR, respectively (Figure 3a). In particular, we found that MMD patients had a significant reduction in IFN-γ^+ $CD8^+$ T cells compared to MGUS but any significant difference for TNF-α production (Figure 3d). Focusing on $CD8^+$ T cells double positive, we evaluated $CD8^+$ T cells expressing the degranulation marker CD107a, indicating cytotoxic ability and we found that both MMD and MMR patients had significantly reduced IFN-$\gamma^+$$CD107a^+$ $CD8^+$ T cell responses compared to MGUS and MMD compared also to SMM patients (Figure 3e). Moreover, MMD patients had significantly reduced TNF-$\alpha^+$$CD107a^+$ $CD8^+$ T cells compared to MGUS (Figure 3e).

Finally, we did not find a significant correlation between levels of spike-IgG-Abs and SARS-CoV-2-specific CD4+ or CD8 + T cells (Supplemental Figure S4). In SMM and MM patients, we also lacked to find significant difference among the risk stratification group of patients (based on 2-2-20 and ISS scores, respectively) (Supplemental Figure S5A and S5B). Moreover, since some of the agents commonly used in the anti-MM therapy could have an impact on vaccine-induced T cell immune responses, we analyzed the T cells cytokine production based on the treatment or not with IMiDs, but we lacked to find significant differences (Supplemental Figure S5C).

Taken together, these data indicate that not only MM but also SMM patients showed a reduced cellular response to SARS-CoV-2 full vaccination compared to MGUS and a reduced percentage of antigen-specific IL-$2^+$$CD4^+$ T cells. MM patients, concomitantly, showed a reduced cytotoxic profile exhibited by the IFN-γ^+ and TNF-$\alpha^+$$CD8^+$ T cells, compared to MGUS.

Effects of the booster dose on spike-specific immune responses in patients with MM

In 16 MM patients of our cohort, 7 MMD and 9 MMR, we also evaluated the humoral and cellular spike-specific responses after a heterologous booster mRNA-1273 vaccine (Figure 4a).

Firstly, MMR patients retained significantly lower anti-spike -IgG-Abs levels compared to MMD patients, even after the booster dose (Figure 4b).

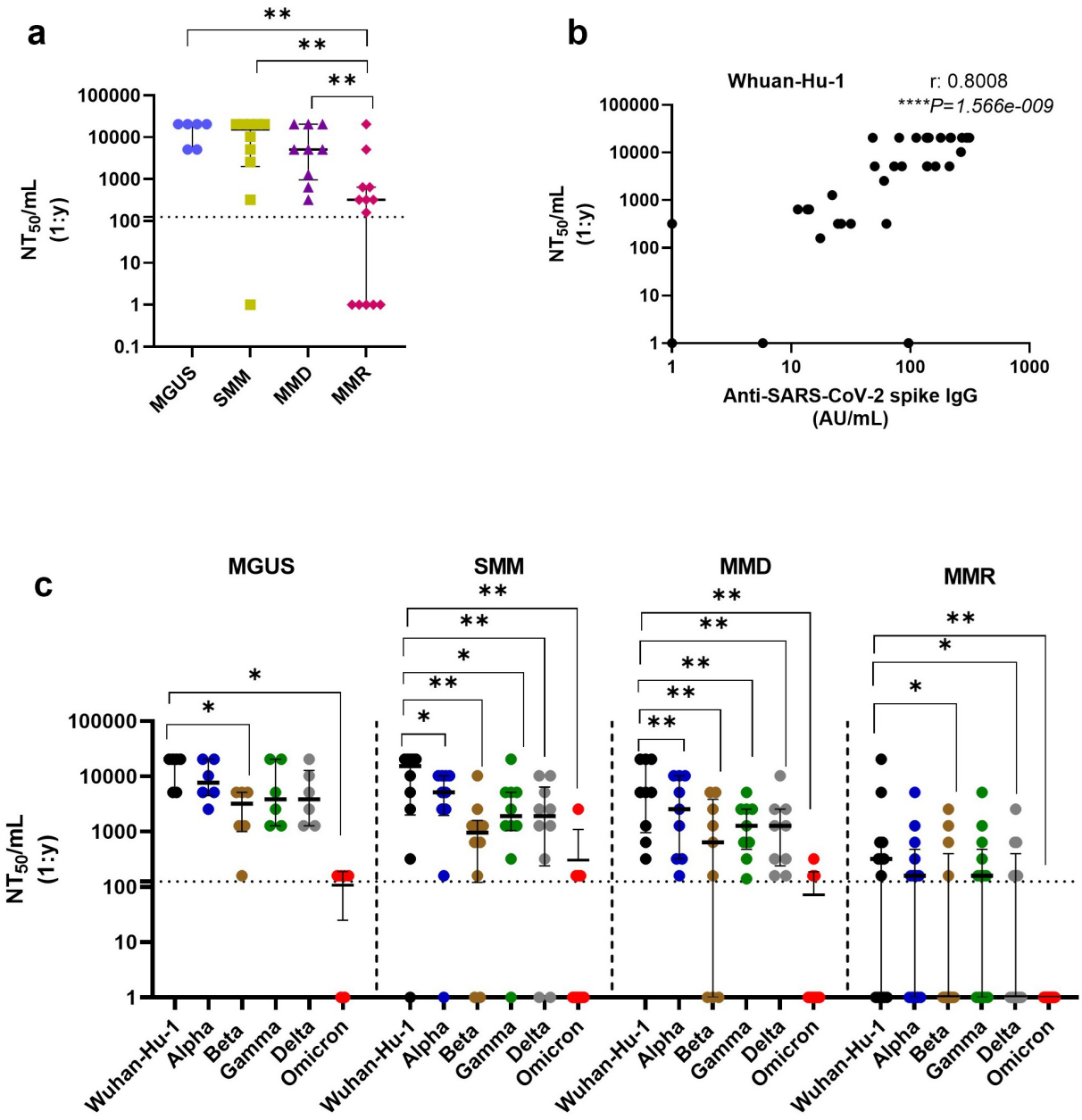

Figure 2. Vaccine-induced SARS-CoV-2 spike neutralizing antibody titers in patients with monoclonal gammopathies at different stages of disease. Neutralizing antibody titers were measured POST vaccination using a neutralization assay with pseudoviruses expressing the Whuan-Hu-1 original spike protein and Alpha, Beta, Gamma, Delta or Omicron variants. Quantitative results are reported in Neutralization titer 50/mL (NT_{50}/mL) as the maximal dilution of the sera where the reduction of the signal is ≥50%, with the lower limit of detection at 160 NT_{50}/mL. (a) Neutralizing antibody titers against the Whuan-Hu-1 original spike protein. Individual data points are shown as scatter dot plot with lines showing the median with IQR. Dotted lines show the lower limit of detection. Patients are subdivided in relation to the different stages of disease MGUS (indigo ●) (n = 6); SMM (avocado ■) (n = 10); MMD (purple ▲) (n = 9) and MMR (fuchsia ◆) (n = 13). Significant difference was determined by two-tailed Mann–Whitney U tests. (b) Correlation between levels of SARS-CoV-2 spike IgG antibodies and neutralizing antibody titers to Whuam-Hu-1 original spike protein. (c) Comparison between neutralizing titers to Whuan-Hu-1 original spike protein and Alpha, Beta, Gamma, Delta and Omicron variants, in patients stratified for stages of disease. Individual data points are shown as scatter dot plot with lines showing the median with IQR. Dotted lines show the lower limit of detection. Statistical analysis were performed using a paired, two-tailed, non-parametric Wilcoxon test. *P* values are shown when $P < .05$ (*$P < .05$, **$P < .01$).

Secondly, comparing spike-IgG-Abs levels between POST and BOOSTER, we did not find a significant increase in both patients with MMD and MMR. However, after the booster dose, 85.7% (6/7) of MMD patients improved spike-IgG-Abs levels and among MMR patients, 50% (2/4) of the seronegative patients, in the POST sample, had a seroconversion and 60% of the seropositive patients (3/5) had an increase in spike-IgG-Abs levels (Figure 4c).

Subsequently, we found a significant increase in the NAb titer to the Whuan-Hu-1 spike in MMD but not in MMR patients, although 6/9 (66.6%) MMR patients improved their titers respect to the POST sample (Figure 4d).

Interestingly, the NAb titers against spike variants, including Omicron, significantly increased after the booster compared to the second dose in MMD patients (Figure 4e). Among MMR patients, we found a significant increase in

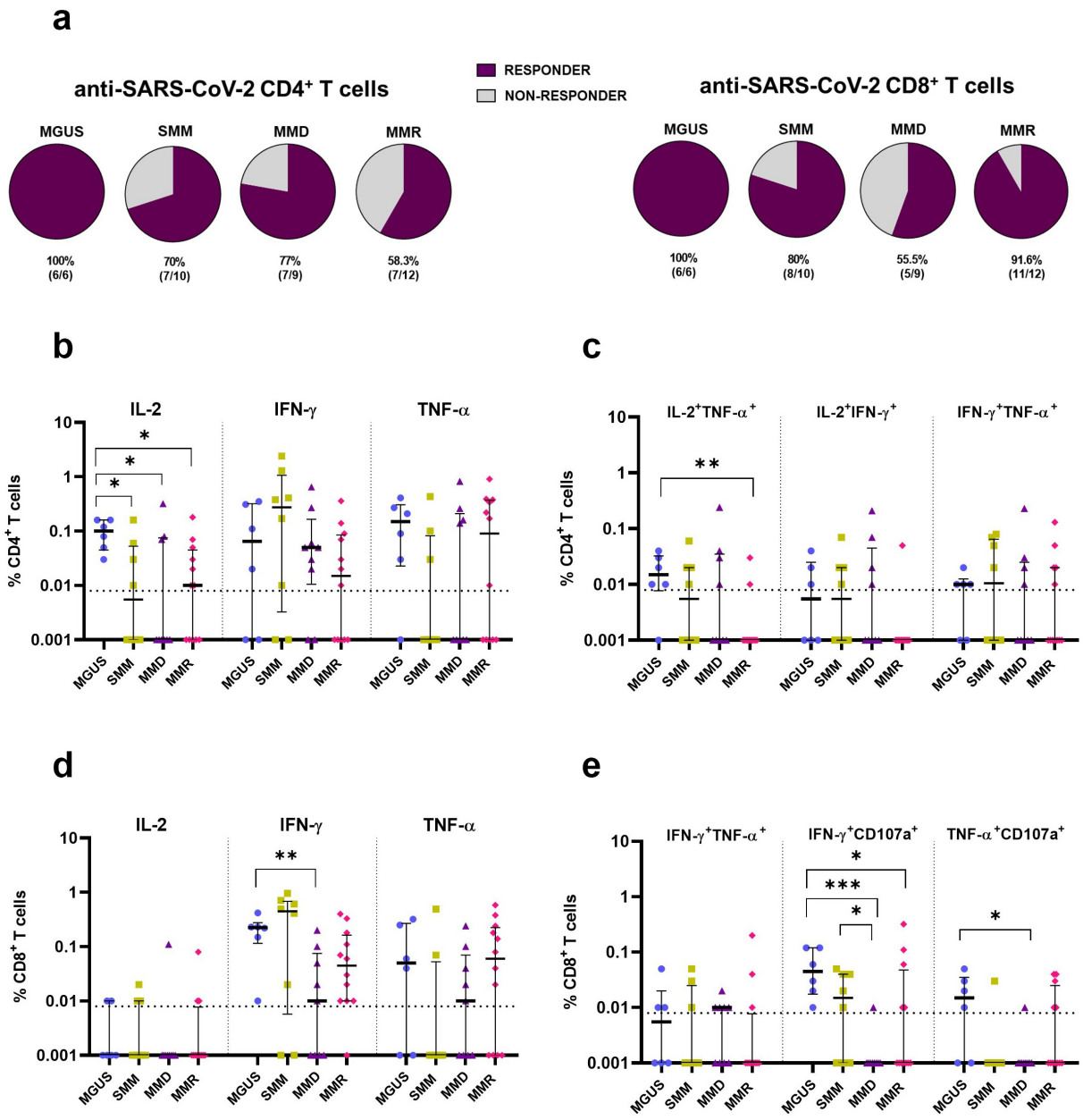

Figure 3. Vaccine-induced SARS-CoV-2 spike-specific T cell responses in patients with monoclonal gammopathies at different stages of disease. Circulating SARS-CoV-2 spike-specific T cells were evaluated by ICS flow cytometry analysis, after overnight stimulation of PBMCs collected PRE and POST vaccination with peptide pools covering the SARS-CoV-2 spike protein (original Wuhan-Hu-1 sequence). Peptide-specific responses were calculated by subtraction of the unstimulated controls from the paired peptide-stimulated samples and subsequently POST vaccination responses were subtracted of the paired PRE vaccination responses. Negative values were designated as zero. Data are represented as a percentage of total $CD4^+$ or $CD8^+$ T cells with the lower limit of detection at <0.01. (a) Pie graphs indicate the frequency of patients with (RESPONDER) or without (NON RESPONDER) detectable SARS-CoV-2 spike-specific $CD4^+$ or $CD8^+$ T cell responses, according to the different stages of disease (MGUS n = 6, SMM n = 10, MMD n = 9 and MMR n = 12). (b) Percentage of SARS-CoV-2 spike-specific $CD4^+$ T cells expressing IL-2, IFN-γ, or TNF-α and (c) percentage of double positive $CD4^+$ T cells for the indicated cytokines in patients at different stages of disease. (d) Percentage of SARS-CoV-2 spike-specific $CD8^+$ T cells expressing IL-2, IFN-γ, or TNF-α and (e) percentage of double positive $CD8^+$ T cells for the indicated cytokines or the degranulation marker CD107a in patients at different stages of disease. Individual data points are shown as scatter dot plot (MGUS (indigo ●); SMM (avocado ■); MMD (purple ▲) and MMR (fuchsia ◆) with lines showing the median with IQR. Dotted lines show the lower limit of detection. Significant difference was determined by two-tailed Mann–Whitney U tests with *P* values shown when $P < .05$ (*$P < .05$, **$P < .01$, ***$P < .001$).

the NAb titer to the Alpha, Gamma, and Delta variants, but not to Beta and Omicron variants (Figure 4e). However, 4/9 (44.4%) of MMR patients reached delectable levels of NAbs against Omicron variant in BOOSTER compared to POST samples, where this titer was undetectable in 100% of the samples.

Despite this last result, MMR patients retained significantly lower titers compared to MMD patients against the Whuan-Hu-1 spike and the five variants, even after the booster dose (figure 4f, solid comparison lines).

Interestingly, after the booster dose, MMD patients lost the negative impact of the spike variants seen with the full vaccination. In fact, we did not observe any significant difference compared to Wuhan-Hu-1 spike in NAb titers to four variants, except for Omicron (figure 4f dotted comparison line); nevertheless, all MMD patients reached detectable levels of NAbs

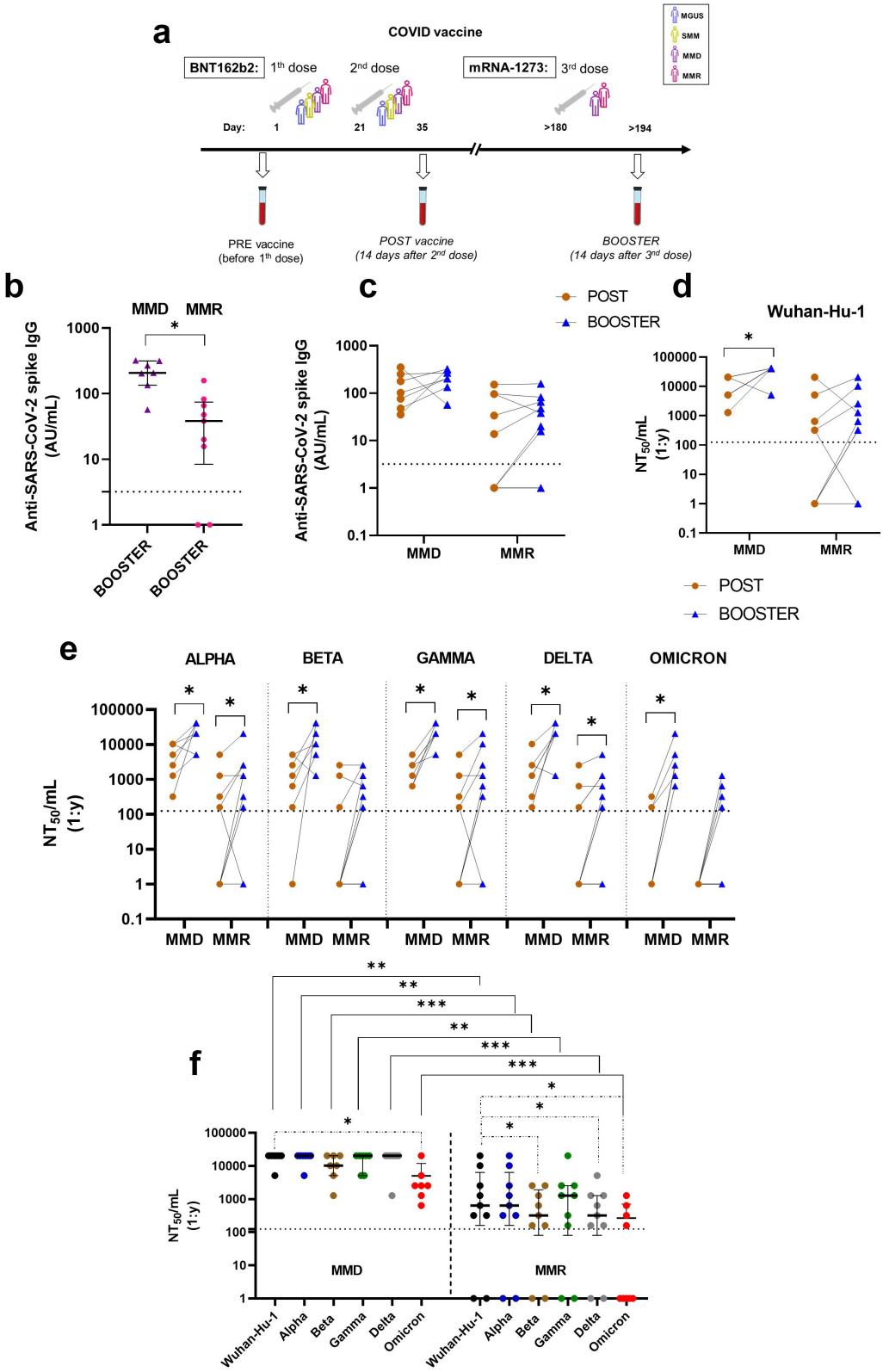

Figure 4. Effects of the booster dose on spike-specific humoral responses in patients with MM. (a) Study design, vaccine administration scheme and time points collected for patients. (b) SARS-CoV-2 spike IgG antibodies were measured after the third vaccine dose (BOOSTER) in 16 patients (MMD = n.7 and MMR = n.9). Individual data points are shown as scatter dot plot with lines showing the median with IQR. Dotted lines show the lower limit of detection. Significant difference was determined by two-tailed Mann–Whitney U tests. *P* values are shown when $P < .05$. (c) SARS-CoV-2 spike IgG antibodies measured after POST (Orange ●) and BOOSTER (blue ▲) in MMD and MMR patients: individual data points are shown, and lines connect the paired samples. Dotted lines show the lower limit of detection. Statistical analysis were performed using a paired, two-tailed, nonparametric Wilcoxon test. (d) Neutralizing antibody titers were measured in the POST (Orange ●) and BOOSTER (blue ▲) samples using a neutralization assay with pseudoviruses expressing the Whuan-Hu-1 original spike protein and (e) Alpha, Beta, Gamma, Delta and Omicron spike protein variants. Quantitative results are reported as individual dot and lines connect paired samples and the y axis reports Neutralization titer 50/mL (NT_{50}/mL) as the maximal dilution of the sera where the reduction of the signal is ≥50%, with the lower limit of detection at 160 NT_{50}/mL. Dotted lines show the lower limit of detection. Statistical analysis were performed using a paired, two-tailed, nonparametric Wilcoxon test. *P* values shown when $P < .05$ (*$P < .05$). (f) Comparison between neutralizing titers to Whuan-Hu-1 original spike protein and Alpha, Beta, Gamma, Delta and Omicron variants, within MMD and MMR groups. Individual data points are shown as scatter dot plot with lines showing the median with IQR. Dotted lines show the lower limit of detection. Statistical analysis were performed using a paired, two-tailed, non-parametric Wilcoxon test. *P* values are shown when $P < .05$ (*$P < .05$, **$P < .01$, ***$P < .001$).

against Omicron. MMR patients retained NAb titers to Beta, Delta and Omicron variants significantly lower than Whuan-Hu-1 spike (figure 4f, dotted comparison lines).

Finally, we explored the impact of the heterologous booster on the cellular responses. We noticed that $CD4^+$ and $CD8^+$ T cell responses to SARS-CoV-2 vaccine were highly variable in our cohort still after the booster dose (Figure 5).

We observed an increase in the percentage of MM patients with detectable SARS-CoV-2 spike-specific CD4 + T cells (at least one cytokine production) (100% of MMD and MMR patients) (Figure 5a). Notably, we found a significant increase in IL-2^+CD4^+ T cells in the BOOSTER compared to the POST among MMD patients. MMR patients did not reach statistical significance as group, but 6/9 (66.6%) patients showed an increase in IL-2^+CD4^+ T cells. Intriguingly, the 4 MMR patients, that reached delectable levels of NAbs against Omicron variant after BOOSTER, had a concomitant increase in the percentage of IL-2^+CD4^+ T cells. In our cohort, we did not find any significant differences between BOOSTER and POST, in terms of IFN-γ^+ or TNF-α^+CD4^+ T cells (Figure 5b).

The percentages of patients with detectable SARS-CoV-2 spike-specific $CD8^+$ T cell responses increased to 85.7% in MMD and, on the other hand, the percentages decreased to 55.5% in MMR patients (Figure 5a). In fact, we found a significant reduction in SARS-CoV-2 spike-specific IFN-γ^+CD8^+ T cells in MMR patients in BOOSTER compared to POST samples (Figure 5c), while we did not find any significant differences between BOOSTER and POST, in terms of IL-2^+ or TNF-α^+CD8^+ T cells both in MMD and MMR patients (Figure 5c).

Taken together, these data demonstrate that booster immunization improved SARS-CoV-2 spike humoral and cellular responses in MMD patients and in most, but not all, MMR patients. After the booster dose, a significant increase of the NAb titers against almost all the analyzed variants was achieved in MMD, but in MMR patients Omicron retained a negative impact on the vaccine efficacy.

Finally, after the booster dose, MM patients were monitored for about four months, until the end of April 2022, when the national vaccination program introduced the possibility of an additional fourth dose. The clinical follow-up showed no patient with disease progression and three breakthrough infections: one infection occurred among MMD patients (1/7, 14.3%) and two infections occurred among MMR patients (2/9, 22.2%). One of these infected MMR patients had undetectable NAbs to Omicron variant and the other one had detectable NAbs.

Discussion

In this study, we performed a comprehensive analysis of immune responses after mRNA SARS-CoV-2 full vaccination among a cohort of patients with monoclonal gammopathies at different stages of disease, that are known to show a impaired or reduced response to vaccination.[7] In addition, we evaluated the impact of five SARS-Cov-2 variants and the effect of a booster dose on the vaccine-induced immune response.

Firstly, consistently with recent literature data, we found that MMR patients had significantly lower spike-IgG-Abs compared to the other groups of patients.[11,12,34] Considering pre-vaccination immune parameters, partial immunoparesis with the involvement of at least two classes of Ig and the reduction of absolute number of $CD4^+$ T cells may affect the production of spike-IgG-Abs after full vaccination. MM patients are known to have a deep reduction of $CD4^+$ T cells with an inverted $CD4^+/CD8^+$ ratio compared to precursors stages of the disease.[35] In fact, $CD4^+$ T cells are closely associated with B cell differentiation into IgG-producing plasma cells and with the development of an adequate humoral immune response.

Only a fraction of the spike-IgG-Abs produced after vaccination are capable of neutralization, resulting in a blunted virus infection. We reported that anti-SARS-CoV-2 NAb titers for the original Wuhan-Hu-1 spike protein were correlated with the spike IgG antibody levels. In particular, MMR patients showed reduced NAb titers compared to all the other groups of patients, especially to MGUS, as previously reported.[12] Several SARS-CoV-2 variants have emerged in the last year with an impact on vaccine efficacy.[36] Delta variant was dominating the pandemic at the time of vaccination of our patients, while the Omicron variant is currently dominating.[15] It is reported in healthy and oncological subjects a reduction of NAb titers for Beta and Delta variants and a highly resistance of Omicron variant against antibody-mediated neutralization after both homologous and heterologous vaccination.[16,37–39] Thus we have analyzed the susceptibility of five variants, including the Omicron one, to vaccine-induced antibody neutralization in our cohort of patients. In this study, after full vaccination, NAb titers against Omicron variant dramatically dropped compared to those against the original strain or the other four variants, with no MMR patients showing detectable levels of NAbs. Interestingly, all the analyzed variants, remarkably Omicron, had a significant negative impact on the neutralizing ability of the vaccine-induced antibodies not only in MMD and MMR but also in SMM, suggesting that this last group of patients already had features of immune system impairment more similar to MMD that MGUS patients. On the other hand, MGUS patients showed a lower neutralizing ability only to the Beta and Omicron variants, as reported in the general population.[17,18,38,40]

Although NAbs are likely to be crucial in vaccine-induced protection, precise correlation to immunity is not completely defined and solid evidences suggest a relevant role for T cells.[30,31,41,42] Until now, only two groups described variable results on the antigen-specific cellular responses, measured using IFN-γ ELISPOT, in the MM setting. Aleman et al.[13] highlighted that fully vaccinated seronegative MM patients had a reduction in the percentage of spike-specific $CD4^+$ T cells compared to the seropositive patients. On the other hand, Salvini et al. reported that MM patients showed a significantly lower percentage of spike-specific INF-γ^+CD8^+T cells in the seronegative group.[43] Our study explores the vaccine-induced cellular responses, measured by ICS, not only in MM, but also in the premalignant monoclonal gammopathies. Our data indicate that 100% of the MGUS patients mounted a SARS-CoV-2 spike-specific $CD4^+$ and $CD8^+$ T cell response while the responder patients percentages decreased in MMD, MMR, and also SMM groups. In particular, a significant reduction of IL-2^+CD4^+ T cell percentage was described in MMD, MMR and SMM compared to MGUS patients. It is

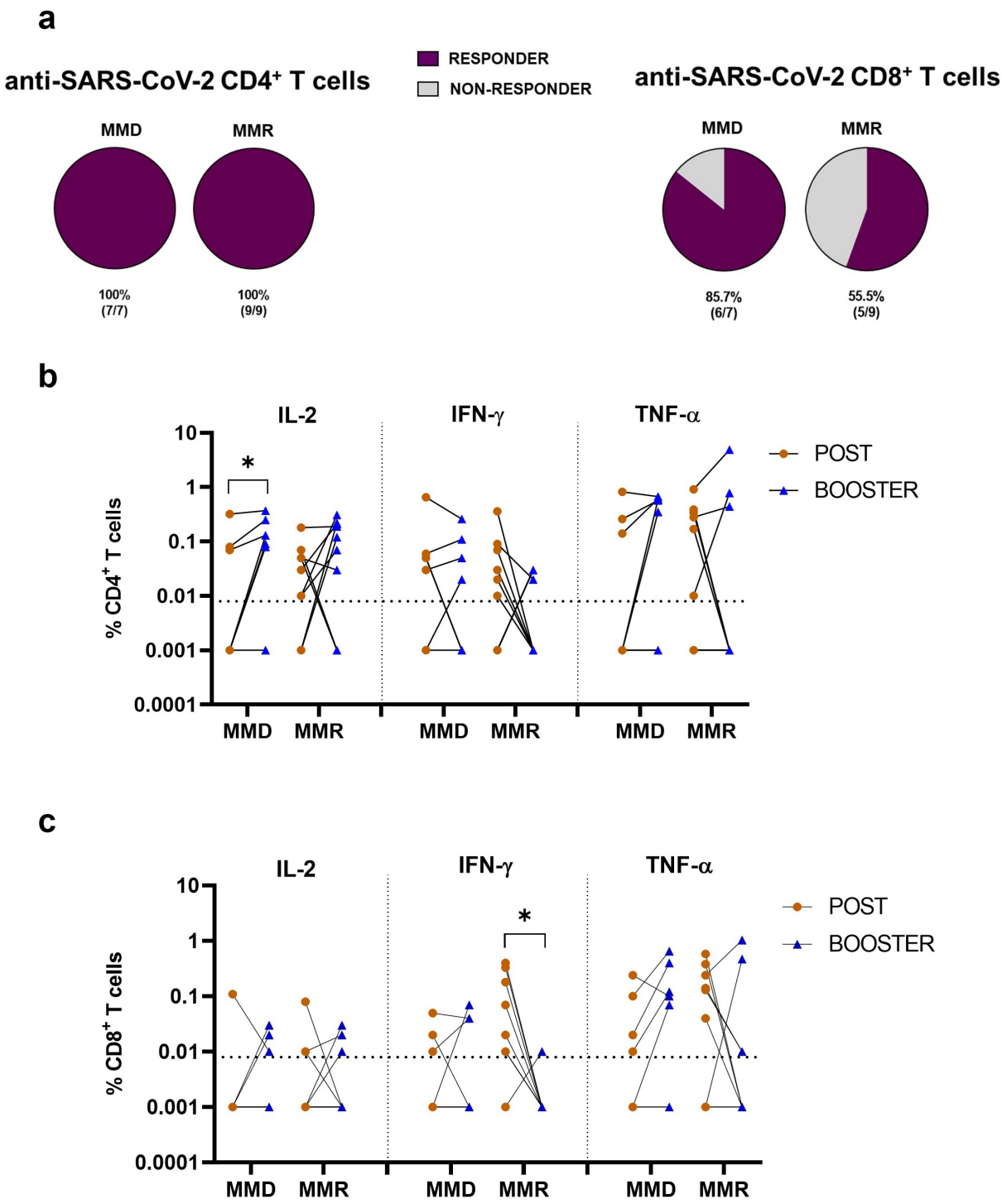

Figure 5. Effects of the booster dose on spike-specific cellular responses in patients with MM. Circulating SARS-CoV-2 spike-specific T cells were evaluated by ICS flow cytometry analysis, after overnight stimulation of PBMCs with peptide pools covering the SARS-CoV-2 spike protein (original Wuhan-Hu-1 sequence). Peptide-specific responses were calculated by subtraction of the unstimulated controls from the paired peptide-stimulated samples and subsequently POST vaccination and BOOSTER responses were subtracted of the paired PRE vaccination responses. Negative values were designated as zero. Data are represented as a percentage of total $CD4^+$ or $CD8^+$ T cells. (a) Pie graphs indicate the frequency of patients with (RESPONDER) or without (NON RESPONDER) detectable SARS-CoV-2 spike-specific $CD4^+$ or $CD8^+$ T cell responses, according to the different stages of disease (MMD n = 7 and MMR n = 9). (b) Percentage of SARS-CoV-2 spike-specific $CD4^+$ T cells expressing IL-2, IFN-γ or TNF-α and (c) percentage of SARS-CoV-2 spike-specific $CD8^+$ T cells expressing IL-2, IFN-γ or TNF-α in POST (Orange ●) and BOOSTER (blue ▲) samples. Individual data points are shown, and lines connect the paired samples. Dotted lines show the lower limit of detection at <0.01. Statistical analysis were performed using a paired, two-tailed, non-parametric Wilcoxon test. *P* values shown when $P < .05$ (*$P < .05$, **$P < .01$).

reported that the development of antigen-specific IL-2^+CD4^+ T cells are essential for the differentiation of IgG-secreting plasma cells in humans.[44] These data, together with the ones of the NAb titers, suggest that SMM patients have already dysregulated immune system which impairs both the humoral and $CD4^+$ T cell responses to vaccination.

Looking at $CD8^+$ T cells, critical for the clearance of virus-infected cells, MM patients showed a reduced cytotoxic profile

exhibited by spike-specific IFN-γ^+ and TNF-α^+ $CD8^+$ T cells compared to MGUS patients.

The decline of vaccine-induced immunity over time and the emergence of new variants led to the administration of a booster dose of SARS-CoV-2 vaccine. Our patients received a heterologous booster dose, according to the national vaccination program; this is a strategy that may offer immunologic advantages to extend the breadth and longevity of protection provided by the full vaccination. Our data demonstrate that booster immunization improved spike-specific humoral responses in MMD patients, as similar reported in a recent study.[45] Interestingly, a significant increase of the NAb titers against all the analyzed variants was achieved after the booster dose compared to full vaccination. The booster dose leads MMD patients to produce enough spike-IgG-Abs, and consequently spike-specific NAbs that erased the negative impact of four spike variants seen after the full vaccination, but not of Omicron one.

On the other hand, MMR benefited from the booster dose, even if they maintained lower levels of spike-IgG-Abs and NAbs compared to MMD patients. In fact, a significant increase of NAb titers was observed against 3 out of 5 analyzed variants. A slightly increase was reported also against Omicron one, where almost half of MMR patient reach detectable levels of NAbs. Overall MMR patients retained a reduction in the vaccine efficacy, with a possible increased susceptibility to Beta, Delta and Omicron variants as reported after full vaccination. The limited number of patients and the introduction of the fourth dose of vaccine after few months did not allow us to assume correlations between vaccine-induced antibodies neutralizing activity and protection from breakthrough infections.

Concerning the impact of the booster dose on antigen-specific T cells, interestingly, both MMD and MMR reached the 100% of responders in terms of SARS-CoV-2 spike-specific $CD4^+$ T cells with an increased percentage of IL-2^+CD4^+ T cells compared to full vaccination. On the other hand, after booster dose, SARS-CoV-2 spike-specific $CD8^+$ T cells had a highly variable trend in MM patients and, notably, almost all MMR patients had undetectable percentage of SARS-CoV-2 spike-specific IFN-γ^+CD8^+ T cells. This evidence suggests the hypothesis that MMR patients were responsive after full vaccination, that induced a primary antigenic response but, probably, they were refractory to a subsequent antigenic stimulation (booster dose). This effect could be ascribed to the exposure to several lines of therapy that characterize only the MMR group. In addition, in MMR, there might be a different kinetic (slower than in MMD) and/or homing of circulating spike-specific CD8 + T cells. In fact, the kinetics of CD8 + T cells after the booster vaccination in both healthy and immunocompromised patients remain sparsely investigated. Therefore, the ideal timepoint for the T cell measurement is yet to be determined. Similarly, no data are available about the homing of these T cells: it could be that two weeks after the booster vaccination, the spike-specific $CD8^+$ memory T cells are tissue-resident more than circulating in peripheral blood.

Our study analyzed a limited number of patients for each group of disease stage and it lacks healthy donors. However, it is important to note that the groups are very homogeneous for age and that all patients underwent the same vaccine administration scheme and blood collection time points, thus excluding potential bias related to age, vaccine type or timing of antigen-specific responses detection. Two previous studies,[12,46] explored the levels of spike-IgG-Abs and the production of NAbs in large cohorts of vaccinated patients with plasma cell neoplasms, including healthy controls. Interestingly, both studies reported that patients with MGUS did not have significant differences compared to healthy controls, suggesting that these patients could be considered proper controls of an optimal response to SARS-CoV-2 vaccination

Finally, we could not explore the spike-specific T cell immunity to Omicron variant after vaccination, but data on healthy subjects demonstrate a durable spike-specific $CD8^+$ and $CD4^+$ T cell responses, with an extensive cross-reactivity against Omicron variant, including in central and effector memory cellular subpopulations.[38,47,48]

In conclusion, by considering different disease stages, our study reveals how the immune response to SARS-CoV-2 vaccination changes as patients with pre-malignant monoclonal gammopathies develop active disease and/or progress. In fact, patients with monoclonal gammopathies share a different and variable cellular and humoral immunity to SARS-CoV-2 vaccination. A significant reduced response was reported in MM patients with relapsed or refractory disease that underwent to at least two lines of treatment compared to MGUS patients. Our study underlines the negative impact of Omicron variant on the neutralizing ability of the vaccine-induced antibodies in MM and also in SMM patients after a full vaccination. Interestingly, the booster dose rescues the negative impact of spike variants on humoral response efficacy in all MMD patients and only partially in MMR patients. These data give the rational to carefully monitor vaccinated MM patients and to consider further prophylactic approach as additional, possibly novel, vaccine dose or administration of mAbs, and preventive measures especially in patients under several lines of treatment.

Author contributions

The study was conceptualized by N.G. and G.D.; P.S., V.M., and R. V. performed ELISA assay and PBMCs stimulation; G.D. prepared the pseudoviruses and performed the neutralization assays supported by V. F. and L.R.; R.V. performed the flow cytometry analysis; V.R., D.T., J.B.G, F.C., and N.T.I. processed the blood samples and collected sera and mononuclear cells; L.N., G.S., B.D.P., and N.G. provide clinical data and enrolled the patients; P.S., V.M., and R.V. analyzed data; P.S., R.V., G.D., and N.G. wrote the manuscript with input from all authors.

Disclosure statement

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflicts with the subject matter or materials discussed in the manuscript.

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Data availability statement

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