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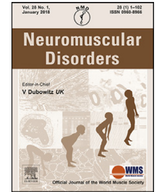

Immunological response after SARS-CoV-2 infection and mRNA vaccines in patients with myasthenia gravis treated with Rituximab

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ABSTRACT

In this study we employed a comprehensive immune profiling approach to determine innate and adaptive immune response to SARS-CoV-2 infection and mRNA vaccines in patients with myasthenia gravis receiving rituximab. By multicolour cytometry, dendritic and natural killer cells, B- and T-cell subsets, including T-cells producing IFN-γ stimulated with SARS-CoV-2 peptides, were analysed after infection and mRNA vaccination. In the same conditions, anti-spike antibodies and cytokines' levels were measured in sera. Despite the impaired B cell and humoral response, rituximab patients showed an intact innate, CD8 T-cell and IFN-γ specific CD4+ and CD8+ T-cell response after infection and vaccination, comparable to controls. No signs of cytokine mediated inflammatory cascade was observed. Our study provides evidence of protective immune response after SARS-CoV-2 infection and mRNA vaccines in patients with myasthenia gravis on B cell depleting therapy and highlights the need for prospective studies with larger cohorts to clarify the role of B cells in SARS-CoV-2 immune response.

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1. Introduction

The COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has had a profound public health impact due to the absence of protective immunity to the disease [1]. Vaccines have been rapidly developed, and both BNT162b2 and mRNA-1273 vaccines have been effective in inducing humoral and cell-mediated response against SARS-CoV-2 in healthy individuals [2,3]. In this scenario, the management of patients with chronic conditions and those receiving immunosuppressive treatments has been challenging, given the higher risk of COVID-19-associated morbidity and poorer characterization of the response to mRNA vaccines. [4,5] Real-world data has confirmed that mRNA vaccines are effective and well-tolerated in most patients with autoimmune neurological conditions [6–8], including myasthenia gravis (MG) [9–12]. However, in patients on B cell-depleting therapies, uncertainty remains around the robustness of protective immunity after vaccination. Several reports showed that multiple sclerosis (MS) patients treated with anti-CD20 therapies were able to mount spike-specific T-cell responses comparable to patients not under B cell-depleting therapies [13,14]. To date, a single study reported the humoral and cell-mediated response (by an interferon gamma (IFN-γ) release assay in plasma) to mRNA vaccines in MG patients under different immunotherapies, including rituximab (RTX) [15], an anti-CD20 monoclonal antibody used as second-line treatment in MG. Here we employed a comprehensive immune profiling approach to determine the innate and adaptive immune response, including humoral and cellular response, to SARS-CoV-2 infection and mRNA vaccines in MG patients receiving RTX.

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2. Materials and methods

2.1. Study population and sample collection

Clinical characteristics of the cohort are summarised in Table 1. In this longitudinal study, we prospectively enrolled 3 MG patients (pt1, pt2, pt3) from our MG referral clinic, who underwent RTX treatment in October 2020, along with 2 MG patients as disease-controls (pt4, pt5) who did not receive RTX and 2 healthy controls (HC1, HC2). During the study period of 2019–2021 a total of 5 patients from our MG clinic were under RTX treatment (2 patients with MuSK-MG and 3 patients with AChR-MG), only one of whom contracted SARS-CoV-2 infection (pt1). The subjects received 2 doses of mRNA vaccine for SARS-COV-2. Pt1, pt5, HC1 contracted the SARS-COV-2 infection before being vaccinated. Blood samples for serum and peripheral blood mononuclear cells (PBMCs) collection were drawn before/after 6 months from RTX, before/after one month from SARS-CoV-2 infection resolution and/or vaccination. RTX was administered with the 375 mg/m2/weekx4 regimen, and a single dose of 375 mg/m2 after 6 months.

2.2. Antibody response

Humoral response was assessed by using a chemiluminescence immunoassay detecting immunoglobulin G (IgG) specific for the S1/S2 viral spike protein, according to the manufacturer's instructions (Diasorin LIAISON, Saluggia, Italy). Acetylcholine receptor (AChR) and muscle-specific tyrosine kinase (MuSK) IgGs positivity, and end-point titrations were assessed by live cell-based assay, as previously described [16].

Flow cytometry for innate immune cells' and T cell subsets' phenotyping

PBMCs were isolated on density gradient centrifugation using a cell separation media according to manufacturer's procedure (Lympholite, Euroclone). Before analysis with a Cytoflex LX cytometer (Beckman coulter), PBMCs were incubated with normal mouse serum (NMS) and stained with a combination of fluorochrome-conjugated antibodies prepared in Brilliant Stain Buffer (BSB, BrandBD Horizon). A complete list of the antibodies used in the panels is available in Supplementary Table 1.

2.3. In vitro stimulation of T cell subsets and intracellular IFN-γ detection

For the in vitro stimulation of SARS-CoV-2–specific CD4+ and CD8+ T cells, we employed PepTivator SARS-CoV-2 Prot N, S and S1 (Miltenyi Biotec 130–126–698; 130–126–700; 130–127–311 respectively). PBMCs were seeded at a density of 5 $\times$ 10^6 cells/cm² in a 96-multiwell flat-bottom plate and stimulated with PepTivators at a final concentration of 0.6 nmol/peptide (approximately 1 μg/mL). A negative control (without antigen) and a positive control (polyclonally stimulated with CytoStim, Miltenyi Biotec, 130–092–172), were included. After 2 h of incubation at 37 °C (5% CO2), Brefeldin A (1:1000, Sigma Aldrich) was added to cell cultures. After 4 further hours of incubation, cells were harvested and stained with a combination of fluorochrome-conjugated antibodies prepared in BSB (Supplementary Table 1 for the panel used). Cytofix/Cytoperm solution kit (BD Biosciences) was used for intracellular staining of IFN-γ according to manufacturer's instructions. Dead cells were excluded from the analysis by side/forward scatter gating and Fixable Viability dye 808 (Viakrome, Beckman Coulter). At least 100,000 gated events on living cells were analysed for each sample, whenever possible.

2.4. Cytokine, chemokine and growth factor quantification

A Bio-Plex ProTM Human Cytokine 27-plex Immunoassay 96-well kit (Bio-Rad Laboratory, Hercules) was used to measure the serum concentration of pro- and anti-inflammatory cytokines, chemokines and growth factors. The assay was performed according to the manufacturer's guidelines and the plates were read on the BioPlex 200 system (Bio-Rad), powered by Luminex xMAP technology. Twenty-two Italian sex/age matched HC, 19 females and 3 males, mean age ± SD: 30.73 ± 7.98 years, without autoimmune disorders or infections at the time of blood collection, were tested and the average and standard deviations for each analyte were used as cut-off, as previously described [17]. Sera from patients and HCs were diluted 1:4, tested in duplicate and mixed in all plates to reduce the variability. Data are expressed as a concentration (pg/mL). The concentration of analyte bound to each bead was proportional to the median fluorescence intensity of the reporter signal and was corrected by the standards provided in the kit.

2.5. Data analysis and graphical representation

Flow cytometric data were manually gated and analysed using FlowJo software 10.6.2 (BD Biosciences). Gating strategies are presented in Supplementary Figure 1–2. Gating was performed using single stains and fluorescence minus one control and adjusted according to the HC for each individual sample. Spontaneous IFN-γ production was assessed following incubation with the unstimulated control and subtracted from participant-and timepoint-matched data. GraphPad Prism v9 and Adobe Illustrator were used for graphical representation.

3. Results

The study subjects were all early-onset MG, with a median age at disease onset of 29 years (range:18–51) and a female proportion of 3/5 patients (60%). Two/5 patients were MuSK-

Table 1
Clinical characteristics of the cohort.

	Sex/age at disease onset (yrs)	MG antibody	Max MGFA	pre RTX therapy	RTX therapy	Age at sampling	SARS-Cov2 infection	mRNA vaccination	PIS at last FU
pt1	F/51	MuSK	IIIB	Steroids	yes	52	yes	BNT162b2	MM
pt2	M/27	AChR	IIIA	Steroids, AZA	yes	31	no	mRNA-1273	MM
pt3	F/29	AChR	IIIA	Steroids, AZA	yes	39	no	BNT162b2	I
pt4	F/18	MuSK	IIIB	Steroids	no	18	no	BNT162b2	I
pt5	M/41	AChR	IIA	Steroids	no	50	yes	mRNA-1273	MM
HC1	F/34	–	–	No therapy	–	34	yes	mRNA-1273	–
HC2	M/29	–	–	No therapy	–	29	no	BNT162b2	–

AChR= acetylcholine receptor; AZA= azathioprine; *I*=improved; MG= myasthenia gravis; MGFA=myasthenia gravis foundation of America; MM= minimal manifestation; MuSK= muscle tirosin kinase; PIS= post-intervention status; RTX=rituximab.

antibody positive, while 3/5 patients were treated AChR antibodies. They were all treated with RTX as second-line treatment, after partial or no response to steroids in 3/5 (60%) patients or steroids and azathioprine in 2/5 (40%) (Table 1).

3.1. Effect of RTX treatment on Immune response to SARS-CoV-2 infection

We examined the effect of RTX on immune response to SARS-CoV-2 infection in pt1 compared to pt5 and HC1, sampled at different time points (T1, T2, T3) as shown in Fig. 1A.

Pt1 contracted SARS-Cov-2 one month after the last RTX infusion. The infection was asymptomatic despite a delayed virus clearance, with no change in MG status. Both pt5 and HC1 were COVID positive for 2 weeks and developed only mild symptoms during the infection. In pt1 circulating B cells were undetectable both at 1 and 3 months after RTX (T2-T3, Fig 1B). This was reflected in the humoral response to SARS-CoV-2 infection, with anti-spike IgGs being under the cut-off value for positivity (Fig 1C). Interestingly, the end-point titre of anti-MuSK antibodies was not influenced by either RTX or the infection (Fig 1D). Unexpectedly, pt5 displayed a marked anti-spike IgGs increase parallel to anti AChR-IgG titre at T3, with no changes in the MG clinical course (Fig 1C).

While the level of plasmacytoid and myeloid dendritic cells (PDC, MDC, Supplementary Figure 1A-B) and of natural killer (NK, Supplementary Figure 1C) cells did not differ before/after SARS-CoV-2 infection in pt5 and HC1, in pt1 the frequency of PDC and NK cells increased after RTX and even further after the infection (Fig 1E-F). The frequency of CD3+CD4+ total and naïve T cells, the activation of which depends upon B cells, decreased over time in pt1 compared to pt5 and HC1(Fig. 1H-I) [18], while the level of activated T regulatory cells (Tregs) increased after the infection in pt1 but not in the non-RTX study subjects (Fig. 1J). We found that, while total and naïve CD3+CD8+*T* cells had similar dynamics over time in all study subjects (Fig. 1K-L), effector memory CD8+*T* cells increased at higher magnitude in pt1 than HC1 at T3 (Fig. 1M). Finally, we assessed the antigen-induced IFN-γ T-cell within the CD4+ and CD8+ T-cell subsets (Supplementary Fig. 2B). A positive T-cell response to Cytostim was found in all study subjects. Upon N stimulation, IFN-γ CD4+ and CD8+*T*-cell responses were undetectable in pt1. By contrast, both CD4+ and CD8+ T cells produced IFN-γ in pt5 upon SARS-CoV2 infection, whereas in HC1 only CD8+ T cells did so (Fig. 1N–O). After stimulation with *S* + S1 peptides pooled together, IFN-γ production was markedly low in all study subjects at any time point (data not shown).

3.2. Effect of RTX treatment on immune response to SARS-CoV-2 mRNA vaccination

To examine the effect of RTX on the immune response to SARS-CoV-2 mRNA vaccination, we compared 3 MG patients treated with RTX (pt1, pt2, pt3) with 2 non-RTX MG patients (pt4, pt5) and 2 HCs (Fig. 2A). First, we confirmed that B cells were depleted in RTX-treated patients (Fig 2B). Spike-IgG seroconversion was observed in 1/3 RTX patients (Fig 2B-C). Neither RTX nor the vaccination had any effect on MG pathologic antibodies over time (Fig. 2D). While there was no clear difference in the frequency of DC response between RTX and non-RTX subjects, except for a reduced level of MDC cells in 1/3 RTX patients (Fig. 2E-F), NK cell frequency increased at equal or higher magnitude in the RTX patients compared to the other subjects after vaccination (Fig. 2G). Importantly, we found no difference in the frequency of total CD4+ and CD8+ T cells between the RTX and non-RTX study subjects (Fig. 2H-I), though 2/3 RTX patients had a lower frequency of CD4+ naïve T cells than the control group at all time-points. Similarly, all RTX patients had a lower frequency of activated Tregs compared to the control groups (Fig. 2J-K), probably due to a more refractory MG status in these patients rather than to an effect of RTX or the vaccine. Upon stimulation with peptides *S* + S1 and N, IFN-γ producing CD4+ and CD8+ T cells expanded in all patients, with the exception of pt1 (Fig. 2L-O). A possible asymptomatic SARS-CoV-2-infection in pt2, pt3, pt4, HC2, which could reflect the observed IFN-γ production after nucleoprotein stimulation, couldn't be ruled out in this study.

3.3. Cytokine response after SARS-CoV-2 infection and mRNA vaccination

The cytokine levels of the study subjects were compared with the mean value obtained in the control group of 22 HC. Serum concentration analysis of cytokines showed levels of IL-1β, IL-6, IL-10 within range in the cohort, while IL-2, IL-5, IL-12, IL-13, TNF-α, IFN-γ and IP-10 were lower than the normal range in all subjects, both after SARS-CoV-2 infection and vaccination (Fig. 3A, 3G). Although levels of IL-7 were generally decreased in the cohort, we observed increased levels after infection in pt1 compared to the non-RTX controls (Fig. 3B). Similarly, levels of IL-17 and IL-4 increased after infection in pt1, but not in the non-RTX group (Fig. 3C-D), while the level of these cytokines were decreased or within normal limits after the vaccination both in RTX and non RTX study subjects (Fig. 3H-J). Finally, elevated values of IL-9 were present in 2/3 RTX patients regardless of the time point considered (Fig. 3E, 3K). G-CSF levels were higher than the normal range in all the subjects (Fig. 3F, 3L).

4. Discussion

The purpose of this pilot study was to evaluate the impact of RTX on the immune response to SARS-CoV-2 infection and mRNA vaccines in MG patients. First, we showed that, despite the impaired B cells and humoral anti-spike response, the patient under RTX who contracted the SARS-CoV-2 infection mounted a robust innate and CD8-effector memory T cell response. These findings are in agreement with reports showing that B cells are not required for recovering from SARS-CoV-2 in MS patients under immunosuppressants [19,20], and that, innate and CD8 T cell response are most relevant in fighting SARS-CoV2 infection [21,22]. After vaccination, we found comparable innate and adaptive responses between patients with no circulating B cells and controls, as previously shown in patients with MS under anti-CD20 therapies [10]. NK cells increased in RTX patients in accordance with previous reports after mild SARS-Cov2 infection and vaccination in immunocompromised patients [20,21]. One unexpected finding was the lack of IFN-γ production in one of the RTX-treated patients after both infection and vaccinations. Given that T cells from the other RTX patients analysed here produced IFN-γ, this finding may be related to a delayed immune response - not captured at the time points we analysed - rather than to B cell depletion. [22] Overall, based on the results of our Bio-plex immunoassays, no signs of aggressive inflammatory cascade ("cytokine storm") were detected.

4.1. Study limitations

This study has some limitations to address. First, the rarity of the disease, and the even rarer proportion of MG patients under RTX is responsible for the low number of patients enrolled, which precluded any statistical analysis and stratification of patients based on the antibody status. The lack of T follicular helper cell analysis presents a second limitation, being abundant after mRNA immunization and associated with the magnitude of the neutralizing antibodies [22,23]. Third, despite the longitudinal nature of the study, our observations are limited by the short

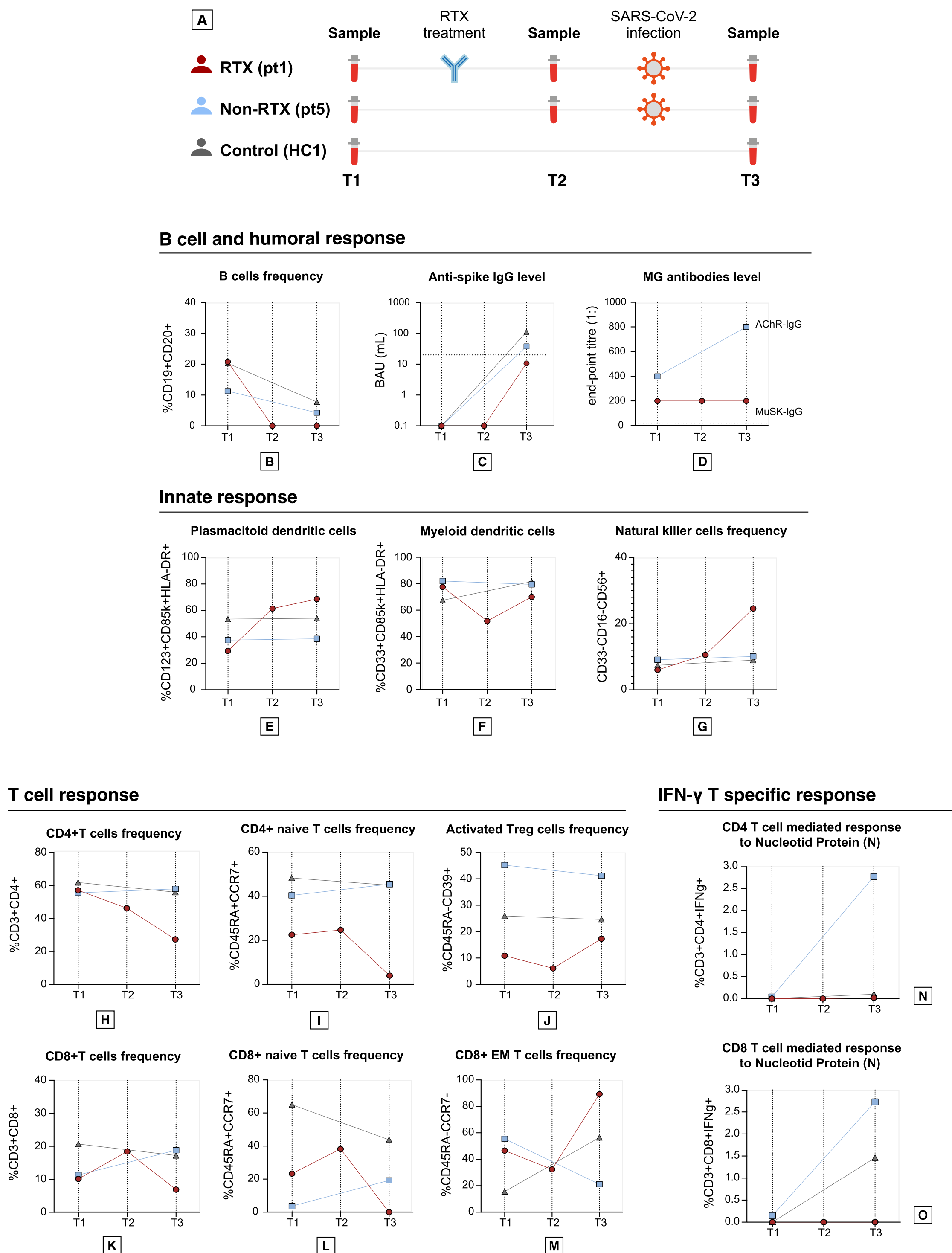


Fig. 1. Immune response to SARS-CoV-2 infection Timeline of samples collection in pt1 (in red), pt5 (in blue), HC1 (in gray). T1=baseline of pt1, PT5, HC1; T2= 1 month after RTX in pt1; T3= 1 month after SARS-CoV2 infection in pt1, pt5, HC1 and 3 months after RTX in pt5 (A). B cell frequency (%CD19+CD20+) (B); anti-Spike antibody level calculated in BAU with a suggested cut-off of >20.33 BAU/mL (C); end-point titration of MuSK antibodies in pt1 and AChR antibodies in pt5 before/after the infection (D). Frequency of plasmacytoid DC cells (%CD123+CD85k+) (E); myeloid DC cells (%CD33+CD85k+) (F) and NK cells (%CD14-CD16-CD56+) (G). Frequency of CD4 T cells (%CD3+CD4+) (H); naive CD4 T cells (CD3+CD4+CD45RA+CCR7+) (I); activated Tregs (CD3+CD4+CD25+CD127-CD45RA+CD39+) (J). The level of central and effector memory CD4 T cells (% CD3+CD4+CD45RA+CCR7- and% CD3+CD4+CD45RA-CCR7-) and Tregs (CD3+CD4+CD25+CD127-) didn't show any change at the timepoints analysed and between the subjects (data not shown). Frequency of CD8 T cells (%CD3+CD8+) (K), naive CD8 T cells (%CD3+CD8+CD45RA+CCR7+) (L), effector memory CD8 T cells (CD3+CD8+CD45RA-CCR7-) (M). The levels of central memory CD8 T cells (%CD3+CD8+CD45RA+CCR7) were very low at all the timepoints analysed and therefore data were not shown. Frequency of CD4 T cells and CD8 T cells producing IFN-γ after nucleoprotein (N) stimulation (N–O).

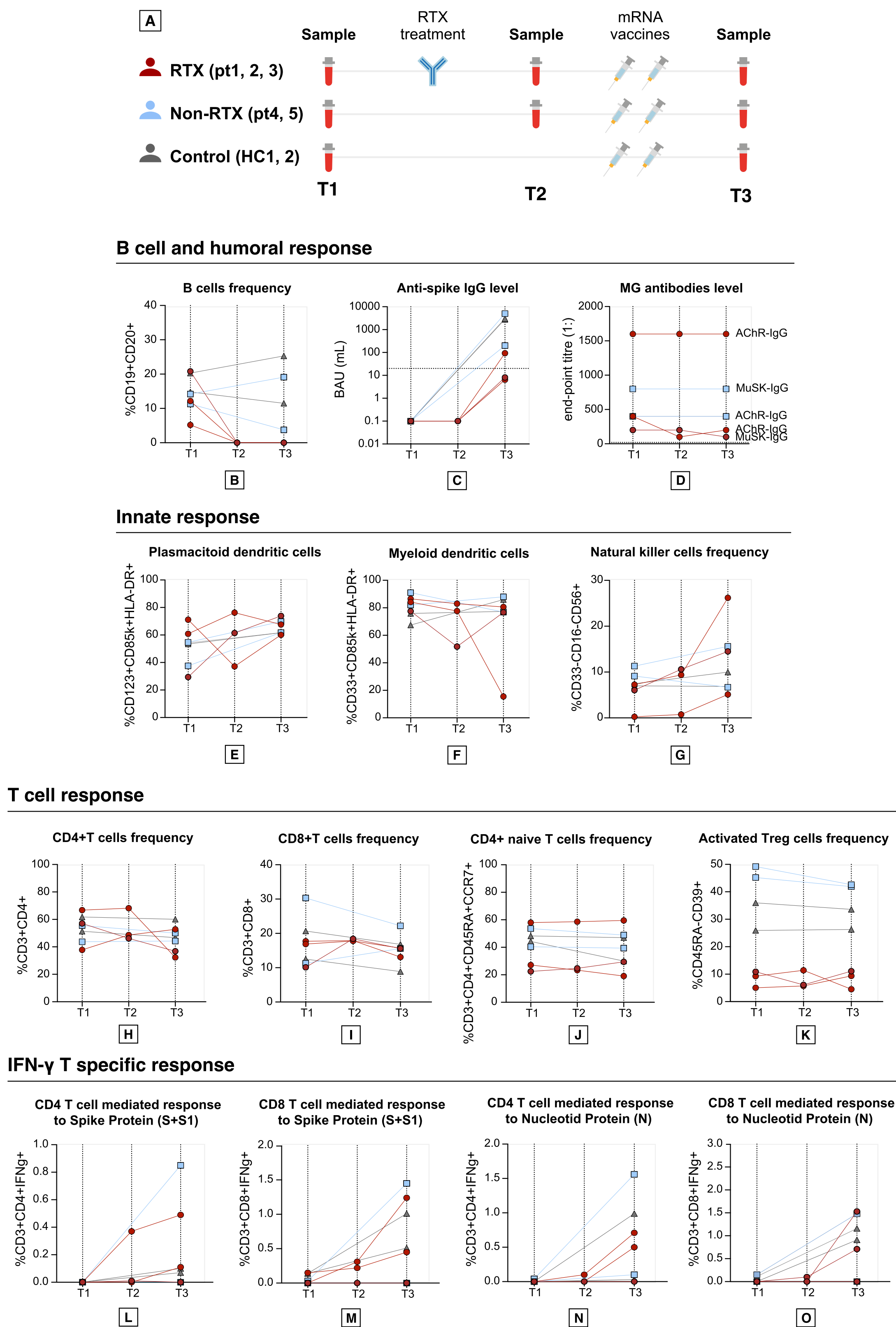
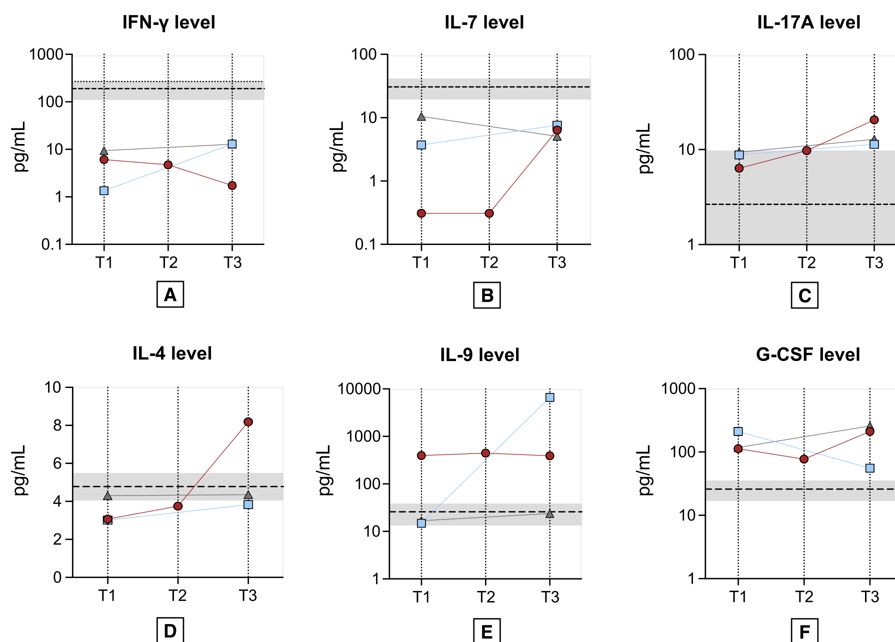


Fig. 2. Immune response toSARS-Cov-2 infection and mRNA vaccines Timeline of samples collection in pt1,2,3 (in red), pt4,5 (in blue), HC1, HC2 (in gray). T1=baseline of the cohort; T2= 1 month after RTX; T3= 1 month after vaccination, and 6 months after RTX in pt1,2,3 (A). B cell frequency (%CD19+CD20+) (B); anti-Spike antibody level calculated in BAU with a suggested cut-off of >20.33 BAU/mL (C); end-point titration of MuSK antibodies in pt1, pt4 and AChR antibodies in pt2,3,5 before/after the vaccine (D). Frequency of plasmacytoid DC cells (%CD123+CD85k+) (E); myeloid DC cells (%CD33+CD85k+) (F) and NK cells (%CD14-CD16-CD56+) (G). Frequency of CD4 T cells (%CD3+CD4+) and CD8 T cells (%CD3+CD8+) (H-I); naive CD4 T cells (CD3+CD4+CD45RA+CCR7+) (J); activated Tregs (CD3+CD4+CD25+CD127-CD45RA+CD39+) (K). The level of Tregs (CD3+CD4+CD25+CD127-) didn't show any change at the timepoints analysed and between the subjects (data not shown). Frequency of CD4 T cells and CD8 T cells producing IFN-γ after S + S1 (L-M) and nucleoprotein stimulation (N–O).



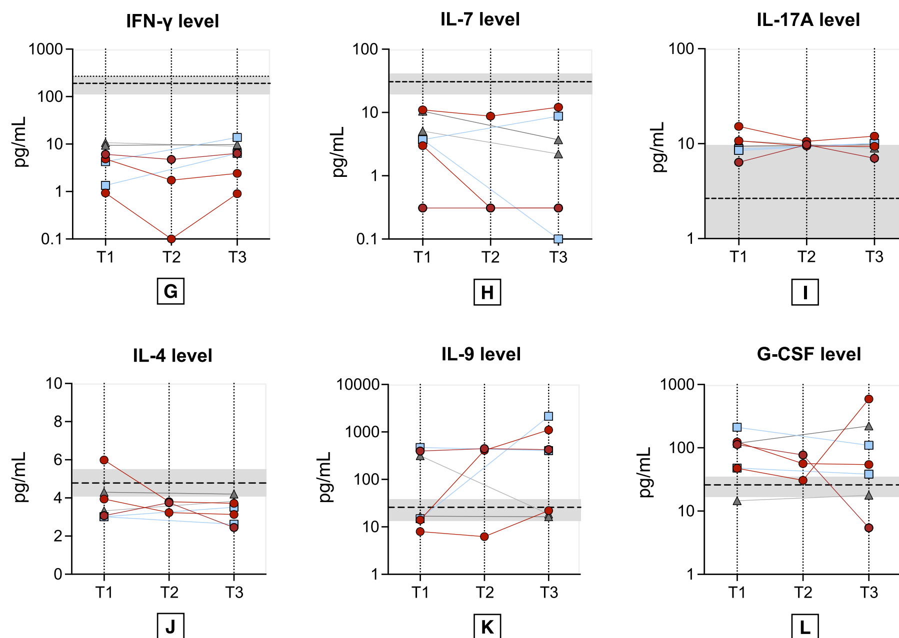


Fig. 3. Cytokine response to SARS-Cov-2 infection and mRNA vaccines The serum concentration of 27 immune mediators was analysed after SARS-CoV-2 infection (A-F) and mRNA vaccines (G-L) at the same timepoints described in Fig. 1-2 (T1-T3). The levels of 6 molecules (IFN-γ, IL-7, IL-17A, IL-4, IL-9, G-CSF) differed from the normal range in the MG population, while only the level of IFN-γ, IL-7 and G-CSF was abnormal at T1 in HC1–2, probably due to the high variability of these molecules in the general population. The serum concentration in range is represented by the gray area corresponding to the mean and standard deviation of the control group.

follow-up after vaccination and the lack of data after the third dose of vaccination.

5. Conclusions

Our pilot study suggests that RTX-treated MG patients might present protective immune response after SARS-CoV-2 infection and vaccination and confirms the utility of specific cell assays to monitor vaccine efficacy and response to infection. This study highlights the need for long-term prospective studies with larger MG cohorts to clarify the role of B cells in SARS-CoV-2 immune response and randomized trials to determine the vaccine efficacy in patients with rare conditions, especially those under immunosuppression.

Author contributions

VD, AF, AE designed the work. VD, GS, GM, SF selected the patients and blood samples. VD, GS, LS, PC, NZ, MF performed the experiments and acquired the data. VD, AE wrote the manuscript. VD, GS, PC, RM, AB, AF, AE critically revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional review board statement

The study was conducted according to the Helsinki declaration and approved by the Ethic Committee of the Università Cattolica del Sacro Cuore (Rome, Italy) with E.C. protocol number 49,886/18 (9024/19, ID:2327).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability statement

Data available upon request to the corresponding author.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2023.02.005.

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