

Humoral- and T-Cell–Specific Immune Responses to SARS-CoV-2 mRNA Vaccination in Patients With MS Using Different Disease-Modifying Therapies

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Abstract

Background and Objectives

To evaluate the immune-specific response after full severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccination of patients with multiple sclerosis (MS) treated with different disease-modifying drugs by the detection of both serologic and T-cell responses.

Methods

Healthcare workers (HCWs) and patients with MS, having completed the 2-dose schedule of an mRNA-based vaccine against SARS-CoV-2 in the past 2–4 weeks, were enrolled from 2 parallel prospective studies conducted in Rome, Italy, at the National Institute for Infectious Diseases Spallanzani–IRCSS and San Camillo Forlanini Hospital. Serologic response was evaluated by quantifying the region-binding domain (RBD) and neutralizing antibodies. Cell-mediated response was analyzed by a whole-blood test quantifying interferon (IFN)- γ response to spike peptides. Cells responding to spike stimulation were identified by fluorescence-activated cell sorting analysis.

Results

We prospectively enrolled 186 vaccinated individuals: 78 HCWs and 108 patients with MS. Twenty-eight patients with MS were treated with IFN- β , 35 with fingolimod, 20 with cladribine, and 25 with ocrelizumab. A lower anti-RBD antibody response rate was found in patients treated with ocrelizumab (40%, $p < 0.0001$) and fingolimod (85.7%, $p = 0.0023$) compared to HCWs and patients treated with cladribine or IFN- β . Anti-RBD antibody median titer was lower in patients treated with ocrelizumab ($p < 0.0001$), fingolimod ($p < 0.0001$), and cladribine ($p = 0.010$) compared to HCWs and IFN- β -treated patients. Serum neutralizing activity was present in all the HCWs tested and in only a minority of the fingolimod-treated patients (16.6%). T-cell–specific response was detected in the majority of patients with MS (62%), albeit with significantly lower IFN- γ levels compared to HCWs. The lowest frequency of T-cell response was found in fingolimod-treated patients (14.3%). T-cell–specific response

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INMI COVID-19 Vaccine Study Group coinvestigators are listed in the appendix at the end of the article.

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Glossary

COVID-19 = coronavirus disease 2019; **CPE** = cytopathic effect; **DMT** = disease-modifying treatment; **FBS** = fetal bovine serum; **HCW** = health care worker; **IFN** = interferon; **IgG** = immunoglobulin G; **INMI** = National Institute for Infectious Diseases; **IQR** = interquartile range; **PBMC** = peripheral blood mononuclear cell; **RBD** = region-binding domain; **SARS-CoV-2** = severe acute respiratory syndrome coronavirus 2.

correlated with lymphocyte count and anti-RBD antibody titer ($\rho = 0.554, p < 0.0001$ and $\rho = 0.255, p = 0.0078$ respectively). IFN- γ T-cell response was mediated by both CD4⁺ and CD8⁺ T cells.

Discussion

mRNA vaccines induce both humoral and cell-mediated specific immune responses against spike peptides in all HCWs and in the majority of patients with MS. These results carry relevant implications for managing vaccinations, suggesting promoting vaccination in all treated patients with MS.

Classification of Evidence

This study provides Class III data that SARS-CoV-2 mRNA vaccination induces both humoral and cell-mediated specific immune responses against viral spike proteins in a majority of patients with MS.

Multiple sclerosis (MS) is an inflammatory autoimmune disease of the CNS and is a leading cause of disability in young adults¹ in Western countries. Most people with MS are treated with immunomodulatory or immunosuppressive medications, which might increase the risk of opportunistic infections, infection-related hospitalization, and infection-related mortality rates.²⁻⁴

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has emerged as a human-to-human transmissible disease with a severe global health impact⁵ and difficult clinical management.^{6,7}

Large-scale vaccination is the single most effective public health measure for controlling the COVID-19 pandemic and a global effort to develop and distribute an effective vaccine produced several effective options. Several data are now available about the efficacy of the mRNA platform vaccines, namely BNT162b2 and mRNA-1273 vaccines, in inducing strong antibody and cell-mediated immune responses in naive healthy individuals.⁸⁻¹² The ability of vaccines to induce a coordinated induction of both humoral- and cell-mediated arms is fundamental for a more effective fighting of SARS-CoV-2 infection^{13,14}; this is particularly crucial in people with MS treated with immunotherapy targeting pathogenetic inflammatory processes.^{15,16}

Disease-modifying treatments (DMTs) used in MS act at different levels of the immune system. Based on their mechanism of action, they can be divided into: (1) immunomodulators: interferon (IFN)- β , glatiramer acetate, dimethyl fumarate, and teriflunomide; (2) cell trafficking alteration molecules like S1P receptor modulators (i.e., fingolimod) and α 4-integrin antibody (natalizumab); (3) depletive drugs (ocrelizumab, an anti-CD20 antibody; cladribine, a purine analog that interferes with

DNA synthesis inducing a prolonged lymphocyte depletion; and alemtuzumab, an anti-CD52 antibody).

The overall effects of these DMTs in affecting the humoral and cell-mediated immune responses to SARS-CoV-2 vaccine is unknown. Preliminary data have been published suggesting that the antibody response to BNT162b2 vaccine is impaired in people with MS treated with fingolimod and ocrelizumab, whereas it is preserved in those treated with cladribine.¹⁷⁻¹⁹ More recently, Guerrieri et al.²⁰ in a real-world study on 32 people with MS have shown a higher frequency of the humoral response (62.5%) in patients treated with fingolimod. These data are essential for health decision and need to be confirmed and supplemented by the evaluation of the T-cell-specific response.

The aim of this study was to evaluate the anti-region-binding domain (RBD) neutralizing antibodies and spike (S)-specific T-cell response after the full SARS-CoV-2 vaccination of patients with MS treated with different DMTs.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

Human study protocols were approved by the Lazzaro Spallanzani National Institute for Infectious Diseases (INMI) Ethical Committee (approvals 297/2021 and 319/2021). The study protocols followed the ethics principles for human experimentation in agreement with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Study Population

Participants were enrolled from 2 parallel prospective studies conducted at the INMI Lazzaro Spallanzani. In detail, the studies evaluated the immune response to SARS-CoV-2

vaccination in both health care workers (HCWs) enrolled at INMI and in patients with MS enrolled at the MS Centre of the Department of Neurosciences of San Camillo Forlanini Hospital (Rome, Italy).

Patients With MS

A total of 108 participants were enrolled. Inclusion criteria for the enrollment of patients with MS were (1) diagnosis of MS according to McDonald 2017 criteria,²¹ (2) ongoing DMT treatment with IFN- β , fingolimod, ocrelizumab, or cladribine for at least 6 months before study entry, and (3) completed vaccination cycle (both doses) of an mRNA vaccine within the previous 2–4 weeks. In patients undergoing pulsed therapy (ocrelizumab and cladribine), the timing of vaccination after the last DMT administration was scheduled following the recommendation of both the Italian and European Academy of Neurology for COVID-19 vaccination. In particular, in patients with MS, the drugs were provided with a delay of 3 months for ocrelizumab and of at least 4 weeks for cladribine. The therapies with IFN- β and fingolimod were not interrupted when vaccination was scheduled.²² Blood tests and lymphocyte count were performed within 1 week from the time when the samples were taken for the immune-based assays. Percentage and absolute count of CD19⁺ B cells and serum immunoglobulin G (IgG) levels were collected in patients treated with ocrelizumab within 1 month from the study enrollment.

Health Care Workers

A convenient sample of 78 HCWs from the cohort of vaccinated HCWs at INMI Lazzaro Spallanzani was included as healthy control group.^{12,23} Blood sampling and handling were performed following a standardized written protocol. Blood samples from all patients with MS were collected at the MS Center of San Camillo Forlanini Hospital, transported to INMI, and processed within 2 hours from collection. The same researchers' group at INMI processed all HCW samples.

Peptide Pools for the T-Cell–Based Tests

SARS-CoV-2 PepTivator Peptide Pools (Miltenyi Biotec) covering the sequence of SARS-CoV-2 spike protein (PepTivator SARS-CoV-2 Prot_S1, Prot_S, and Prot_S+) were used.^{24–26} The PepTivator Peptide Pools are constituted by peptides of 15 amino acid length with 11 amino acid overlap.

IFN- γ Whole Blood Assay

Whole blood (600 μ L) was stimulated with SARS-CoV-2 spike peptide pool in a 48-well flat-bottom plate according to the concentrations reported²⁴ and incubated at 37°C (5% CO₂). Plasma was harvested after 20–24 hours of stimulation and stored at –80°C until use. IFN- γ levels were quantified in the plasma samples using an automatic ELISA (ELLA, Protein Simple). IFN- γ values of the stimulated samples were subtracted from the unstimulated control. The detection limit of this assay is 0.17 pg/mL.

Peripheral Blood Mononuclear Cells and in Vitro Stimulation

Peripheral blood mononuclear cells (PBMCs) from a small subset of the vaccinated individuals (8 patients with MS and 7 HCWs) were isolated on density gradient centrifugation (SepMate-50 cat#85460 or SepMate-15 cat#85420, Stem-Cell Technologies) according to manufacturer's procedure. The 7 HCWs, used as control group, were employed as controls in another publication.²⁷ All samples were frozen in heat-inactivated fetal bovine serum (FBS; Euroclone SpA) with 10% DMSO and stored in liquid nitrogen. PBMCs were thawed, counted, assessed for viability, and rested for 2–4 hours at 37°C in RPMI+10% FBS prior to further use. Complete medium was freshly prepared as follows: RPMI-1640, 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin (Euroclone SpA). Cells were seeded at a concentration of 2.5×10^6 cells/mL in a 96-multiwell flat-bottom plate (COSTAR; Sigma Aldrich) and stimulated with spike peptide pool at 1 μ g/mL or staphylococcal enterotoxin B (SEB) at 200 ng/mL, as a positive control. Anti-CD28 and anti-CD49d monoclonal antibodies (BD Biosciences) were added at 2 μ g/mL to costimulate cells. After 1 hour of incubation at 37°C (5% CO₂), 1 μ L/mL of Golgi Plug (BD Biosciences) was added to cell cultures to inhibit cytokine secretion. Following an incubation of 16–24 hours, cells were stained as described below.

T-Cell Subpopulations and Intracellular IFN- γ Detection

PBMCs were stained with an appropriate combination of fluorochrome-conjugated antibodies prepared in Brilliant Stain Buffer (BD Biosciences). Cytofix/Cytoperm solution kit (BD Biosciences) was used for intracellular staining of IFN- γ , according to manufacturer's instructions (see eTable 1, links. [lww.com/WNL/B668](http://www.com/WNL/B668) for a complete list of antibodies and reagents). Dead cells were excluded from the analysis by side/forward scatter gating and then by Fixable Viability stain 700 (BD Biosciences). At least 100,000 gated events on living cells were analyzed for each sample, whenever possible. Samples were acquired on a BD Lyric (BD Biosciences) cytometer. Data were analyzed with FlowJo software, version 10 (Tree Star). Cytokine background was subtracted to the stimulated conditions. The T-cell response was considered positive when SARS-CoV-2 spike stimulated PBMCs contained at least twofold higher frequencies of CD4⁺ or CD8⁺ T cells compared to the unstimulated control and at least 10 events were present in the IFN- γ gate.²⁸

Anti-SARS-CoV-2-Specific IgG Evaluation

Humoral response to vaccination was assessed by quantifying the anti-nucleoprotein IgG and the anti-RBD IgG (Architect i2000sr; Abbott Diagnostics). Anti-N-IgG were expressed as arbitrary units/mL and values ≥ 1.4 were considered positive. Anti-RBD-IgG were expressed as binding arbitrary units/mL and values ≥ 7.1 were considered positive.

Microneutralization Assay

Neutralizing antibodies to SARS-CoV-2 were assessed by a microneutralization assay with live SARS-CoV-2 virus (strain 2019-nCoV/Italy-INMI1; GISAID accession ID: EPI_ISL_412974). The assay has been described in detail²⁹ and is based on inhibition of Vero E6 cells infection by serum dilution curves, with cytopathic effect (CPE) determination at 48 hours postinfection. Briefly, heat-inactivated and titrated sera (duplicate 2-fold serial dilutions, starting dilution 1:10) were mixed with equal volumes of 100 TCID₅₀ SARS-CoV-2 and incubated at 37°C, 5% CO₂ for 30 minutes. Subsequently, 96-well tissue culture plates with subconfluent Vero E6 cell monolayers were infected with 100 μL/well of virus-serum mixtures and incubated at 37°C and 5% CO₂. To standardize the interassay procedures, positive control samples showing high (1:160) and low (1:40) neutralizing activity were included in each MNA session. After 48 hours, microplates were observed by light microscope for the presence of CPE and then stained with crystal violet solution containing 2% formaldehyde. Cell viability was measured by photometer at 595 nm (Synergy HTX Multi-Mode Microplate Reader, BioTek). The highest serum dilution inhibiting at least 90% of the CPE was indicated as the neutralization titer and expressed as the reciprocal of serum dilution (MNA₉₀).

Statistical Analysis

Data were analyzed using Graph Pad (GraphPad Prism 8 XML Project). Categorical variables were reported as count and proportion; continuous variables, including IFN-γ levels and anti-RBD, anti-N, and MNA₉₀ titers, were reported as median and interquartile range (IQR). All data were investigated by nonparametric statistical inference tests. The Kruskal-Wallis test was used for between-group comparisons, Mann-Whitney *U* test with Bonferroni correction for pairwise comparisons, and χ^2 test for categorical variables. Correlations of demographic, clinical, and laboratory variables with serologic and spike-specific T-response in mRNA-vaccinated individuals, as well as between-assay correlations, were assessed by nonparametric Spearman rank test before and after multivariable adjustment. Spearman $\rho > 0.7$ was considered high correlation, $0.7 < \rho > 0.5$ moderate correlation, and $\rho < 0.5$ low correlation.

Two-tailed *p* values < 0.05 were considered significant, with except for subgroup analyses by type of MS-specific treatment, where a correction for multiplicity was applied according to Bonferroni method, yielding a significant 2-tailed *p* value threshold of 0.0125 ($\alpha/4$).

Results

Demographic and Clinical Characteristics of the Enrolled Participants

We prospectively enrolled 186 vaccinated participants: 108 patients with MS and 78 HCWs. Demographic and clinical data were collected at enrollment (Table 1). No significant

Table 1 Demographic and Clinical Characteristics of the 186 Enrolled Participants

Characteristics	Patients with MS	Health care workers	<i>p</i> Value
Total	108 (58.1)	78 (41.9)	
Age, y	47 (39–54)	44 (33–53)	0.098 ^a
Male	34 (31.5)	20 (25.6)	0.408 ^c
Origin			
Western Europe	105 (97.2)	76 (97.4)	0.661 ^c
Eastern Europe	2 (1.9)	2 (2.6)	
South America	1 (0.9)	0 (0)	
BMI, kg/m²	23.2 (20.9–26.5)	—	
MS duration, y	13 (7–20)	—	
MS course			
Relapsing-remitting	98 (90.7)	—	
Primary progressive	10 (9.3)	—	
EDSS score	2.0 (1.0–3.5)	—	
MS treatment			
Ocrelizumab	25 (23.2)	—	
Fingolimod	35 (32.4)	—	
Cladribine	20 (18.5)	—	
IFN-β	28 (25.9)	—	
Lymphocytes count available	87 (80.5)	0 (0)	
Lymphocytes count, n (%); median ×10³/μL (IQR)			
Ocrelizumab	25 (28.7); 1.46 (1.27–1.86)	—	$< 0.0001^b$
Fingolimod	34 (39.1); 0.66 (0.57–0.95)	—	
Cladribine	20 (23); 1.11 (0.87–1.47)	—	
IFN-β	8 (9.2); 1.60 (1.42–1.99)	—	

Abbreviations: BMI = body mass index; EDSS = Expanded Disability Status Scale; IFN = interferon; IQR = interquartile range; MS = multiple sclerosis. Values are n (%) or median (IQR).

^a Mann-Whitney *U* statistic test.

^b Kruskal-Wallis test performed only on patients with MS.

^c χ^2 test.

differences were found regarding age, sex, or country of origin between the 2 groups.

Twenty-eight patients with MS were treated with IFN-β, 35 with fingolimod, 20 with cladribine, and 25 with ocrelizumab. The median treatment duration at the first vaccine dose was 8.9 years (IQR 6.9–13.5) for IFN-β, 6.5 years (IQR 3.6–8.1) for fingolimod, and 1.7 years (IQR 1.1–2.3) for ocrelizumab.

The median time elapsed from the first administration of cladribine to the first vaccine dose was 1.7 years (IQR 1.2–2.0); 16 out of 20 patients (80%) completed the second year treatment cycle. The median time elapsed since the last drug assumption was 8.9 months (IQR 7.7–12.7) for cladribine and 3.8 months (IQR 2.8–4.3) for ocrelizumab.

HCWs as well as 103 patients with MS received the BNT162b2 vaccine; 5 patients with MS received the mRNA-1273 vaccine. The median time elapsed from the second vaccine dose and the blood sample collection was 23 days (IQR 21–26), without any difference across treatment subgroups.

As expected, lymphocyte count at the time of immune-based assays sampling was significantly decreased in patients treated with fingolimod compared to those treated with other DMTs ($p < 0.0001$). Patients treated with ocrelizumab showed a very low percentage of CD19⁺ B cells (median 0.04%; IQR 0.03%–0.09%; normal range 6%–20%) and CD19⁺ absolute count (median 0.89 cells/ μ L; IQR 0.38–1.67 cells/ μ L; normal range 90–520 cells/ μ L, respectively). In these patients, the median IgG level, obtained within 1 month from the study enrollment, was 900 mg/dL (IQR 829–1,100 mg/dL), except for 2 patients with IgG levels below the lowest limit of the normal range (700–1,600 mg/dL). No correlation was found between IgG levels and anti-RBD titer ($r_s = 0.26$, $p = 0.19$).

Most of the enrolled HCWs were healthy ($n = 64$ [82%]); 93.5% ($n = 73$) were untreated, 4% ($n = 3$) were treated with corticosteroids for a history of allergic diseases, whereas no clinical data were available for 2.5% ($n = 2$) of the HCWs (see eTable 2, links.lww.com/WNL/B668).

Serologic-Specific Response in Vaccinated Individuals

Anti-N antibodies were undetectable in both patients with MS and HCWs, confirming the absence of SARS-CoV-2 natural infection in the study population (eFigure 1, links.lww.com/WNL/B668).

A detectable anti-RBD antibody response was observed in all HCWs (100%). The majority of patients with MS ($n = 87$ [80.5%]) showed anti-RBD antibody response, although the percentage of seropositive patients and the quantitative specific response varied according to the ongoing DMTs. A detectable anti-RBD response was found in 10/25 (40%) patients treated with ocrelizumab, in 30/35 (85.7%) patients treated with fingolimod, in 27/28 (96.4%) patients treated with IFN- β , and in all patients (100%) treated with cladribine (Table 2). Ocrelizumab- and fingolimod-treated patients showed lower response rates compared to HCWs ($p < 0.0001$ and $p = 0.0023$, respectively).

The anti-RBD antibody median titer was significantly lower in patients with MS treated with ocrelizumab ($p < 0.0001$),

fingolimod ($p < 0.0001$), and cladribine ($p = 0.01$) compared to HCWs. No differences in the serologic median titer in comparison to HCWs were found in patients treated with IFN- β ($p = 0.359$) (Figure 1A). In ocrelizumab-treated patients, a longer treatment duration was significantly associated with reduced anti-RBD antibody titers ($\rho = -0.529$, $p = 0.007$), whereas age, BMI, and disease duration did not show any effect. Furthermore, none of these variables was associated with reduced anti-RBD antibody titers in patients treated with fingolimod or cladribine (Table 3).

In patients treated with cladribine and ocrelizumab, no correlation was found between the anti-RBD antibody titer and the time elapsed since the last treatment cycle ($\rho = 0.111$, $p = 0.640$ and $\rho = -0.014$, $p = 0.946$, respectively). Moreover, in those treated with ocrelizumab, the anti-RBD titer did not correlate with serum IgG levels ($p = 0.19$).

IFN- γ T-Cell-Specific Response in Vaccinated Individuals

All HCWs showed an IFN- γ spike-specific T-cell response (78/78 [100%]) as compared with 67 (62%) in the MS cohort. Different proportions of T-cell-specific responses were found among patients with MS: 92% (23/25 patients) in the ocrelizumab-treated group, 89.3% (25/28 patients) in the IFN- β -treated group, 70% (14/20 patients) in the cladribine-treated group, and 14.3% (5/35 patients) in the fingolimod-treated group ($p < 0.0001$). Cladribine- and fingolimod-treated patient response rates were significantly lower compared to HCWs ($p < 0.0001$) (Table 2).

The IFN- γ T-cell-specific response levels were significantly lower in MS-vaccinated individuals undergoing any DMTs than in HCWs ($p < 0.0001$) (Table 2 and Figure 1B). In patients with MS, sex, age, BMI, disease duration, and DMT treatment duration at the time of vaccination did not affect the IFN- γ T-cell-specific response. No association was found between the IFN- γ T-cell-specific response and the above-mentioned variables in the single MS-treated group (Table 3).

In patients treated with cladribine and ocrelizumab, the IFN- γ T-cell-specific response was not related to time elapsed since the last treatment cycle ($\rho = -0.353$, $p = 0.127$ and $\rho = -0.271$; $p = 0.189$, respectively).

IFN- γ Response Is Mediated by CD4⁺ T Cells and CD8⁺ T Cells

To evaluate whether the IFN- γ T-cell-specific response was due to the CD4⁺ or CD8⁺ T-cell subset, we evaluated the IFN- γ -specific T-cell frequency in stimulated PBMCs of 8 patients with MS (4 treated with IFN- β and 4 with cladribine) and 7 HCWs. We selected IFN- β - and cladribine-treated patients since they showed, as reported in Figure 1, good specific antibody and T-cell responses. T cells were gated as described in eFigure 2, links.lww.com/WNL/B668. In HCWs, IFN- γ -T-cell-specific response was mediated by CD4⁺ (Figure 2A) and CD8⁺ T cells (Figure 2B) with a different magnitude of

Table 2 Serologic and T-Cell-Specific Responses

Characteristics	Patients with MS	Health care workers	p Value	
Total	108 (58.1)	78 (41.9)		
Antibody response				
Qualitative response				
Anti-RBD Abs responders	87 (80.5)	78 (100)	<0.0001 ^a	
Anti-RBD Abs responders within the subgroups				
Ocrelizumab	10/25 (40)	—	<0.0001 ^a	<0.0001 ^{a,d}
Fingolimod	30/35 (85.7)	—		0.0023 ^{a,d}
Cladribine	20/20 (100)	—		>0.9999 ^a
IFN-β	27/28 (96.4)	—		0.264 ^a
Quantitative response				
Anti-RBD Abs, BAU/mL	284.5 (18.8–1,497)	2,395 (1,445–4,089)	<0.0001 ^b	
Ocrelizumab	3.40 (0.45–21.85)	—	<0.0001 ^c	<0.0001 ^{b,d}
Fingolimod	48 (20.60–166.70)	—		<0.0001 ^{b,d}
Cladribine	1,360 (967.5–2,177)	—		0.010 ^{b,d}
IFN-β	2,164 (1,047–3,504)	—		0.359 ^b
Spike-specific IFN-γ T-cell response				
Qualitative response				
Anti-spike responders	67 (62)	78 (100)	<0.0001 ^a	
Anti-spike responders within the subgroups				
Ocrelizumab	23/25 (92)	—	<0.0001 ^a	0.057 ^a
Fingolimod	5/35 (14.3)	—		<0.0001 ^{a,d}
Cladribine	14/20 (70)	—		<0.0001 ^{a,d}
IFN-β	25/28 (89.3)	—		0.017 ^a
Quantitative response				
Anti-spike IFN-γ, pg/mL	53.09 (3.47–135.3)	343.8 (167–703)	<0.0001 ^b	
Ocrelizumab	128.9 (49.5–268.7)	—	<0.0001 ^c	<0.0001 ^{b,d}
Fingolimod	1.75 (0.18–5.3)	—		<0.0001 ^{b,d}
Cladribine	60 (14.6–138.9)	—		<0.0001 ^{b,d}
IFN-β	84 (51.2–385.6)	—		0.0004 ^{b,d}

Abbreviations: Abs = antibodies; BAU = binding arbitrary unit; IFN = interferon; IQR = interquartile range; MS = multiple sclerosis; RBD = receptor-binding domain.

Values are n (%) or median (IQR).

^a Chi-square test.

^b Mann-Whitney *U* statistic test.

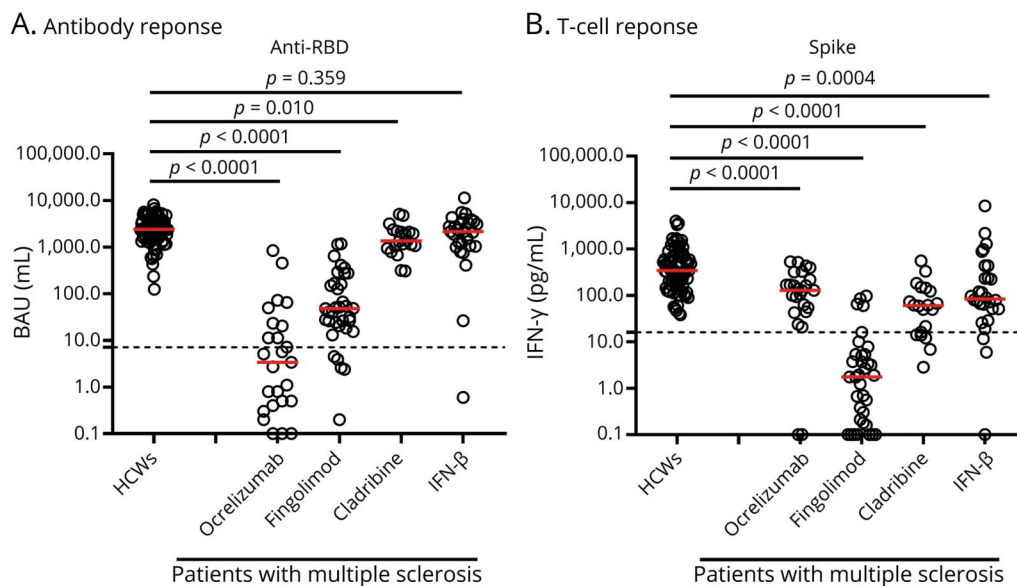
^c Kruskal-Wallis.

^d Significant after multiplicity correction by the Bonferroni method ($\alpha/4 = 0.0125$).

response (median CD4: 0.279%, IQR 0.193–0.427 vs median CD8: 0.058%, IQR 0.00–0.140) (Figure 2C). In patients with MS, the IFN-γ response was mediated only by CD4⁺ T cells (IFN-β: median 0.16%, IQR 0.109–0.192 and cladribine: median 0.13%, IQR 0.117–0.163) (Figure 2C). The frequency of antigen-specific CD4⁺ or CD8⁺ T cells was lower in patients

with MS compared to HCWs, although this difference was not significant (Figure 2C). A positive T-cell response to SEB, used as a positive control, was found in all subjects, and the percentages of CD3⁺, CD4⁺, and CD8⁺ T cells were comparable between HCWs and patients with MS (data not shown).

Figure 1 Antibody and T-Cell Responses After SARS-CoV-2 Vaccination in Patients With Multiple Sclerosis



(A) Evaluation of antibody (Ab) response in 78 health care workers (HCWs) and 108 patients with multiple sclerosis stratified according to drug treatment in 4 groups: ocrelizumab ($n = 25$), fingolimod ($n = 35$), cladribine ($n = 20$), and interferon (IFN)- β ($n = 28$). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-specific anti-receptor-binding domain (RBD) Abs were quantified in plasma or sera samples. Anti-RBD immunoglobulin G (IgG) was expressed as binding arbitrary units (BAU)/mL and values ≥ 7.1 were considered positive. (B) Evaluation of IFN- γ response to spike antigen. IFN- γ was measured by automatic ELISA in plasma harvested from stimulated whole blood samples and shown as median after subtracting the background. Dashed lines identify the cutoff of each test (spike 16 pg/mL and anti-RBD 7.1 BAU/mL). Each black dot represents 1 sample. The red horizontal lines represent the median; statistical analysis was performed using the Mann-Whitney test and p value was considered significant if ≤ 0.0125 .

Correlation Between Anti-RBD Antibody Titer, IFN- γ T-Cell-Specific Response, and Lymphocyte Count

A significant slight correlation was observed in patients with MS between anti-RBD antibody titer and IFN- γ -spike-specific T-cell response ($\rho = 0.255$, $p = 0.0078$) (Figure 3A), persisting after adjusting for sex, age, BMI, and disease duration ($\rho = 0.234$, $p = 0.017$). No significant correlations were found within the differently treated MS groups (data not shown). There was no correlation between the lymphocyte count and the anti-RBD antibody titer ($\rho = 0.132$, $p = 0.211$) (Table 3), whereas quantitative IFN- γ -T-cell-specific response correlated with lymphocyte count in the whole MS group ($\rho = 0.569$, $p < 0.001$), but not in the single DMTs-treated subgroup (Figure 3B and Table 3).

Correlation Between Anti-RBD Antibody Titer and Neutralization Activity

We evaluated the neutralization activity in the sera of 69 HCWs (88.5%). All the enrolled HCWs showed detectable neutralizing antibodies, whose titer significantly correlated with anti-RBD titers ($\rho = 0.754$, $p < 0.001$) (Figure 4A). Among patients with MS, the neutralization test was performed only in 24 (68.6%) patients treated with fingolimod due to the low antibody titers, to characterize the neutralizing capacity of the specific antibodies elicited by vaccination. Only 4/24 (16.6%) patients showed a neutralizing activity, although at low titer (Figure 4B), with a significant correlation

between the neutralizing antibody and anti-RBD antibody titers ($\rho = 0.591$, $p = 0.0024$).

This study provides Class III data that SARS-CoV-2 mRNA vaccination induces both humoral and cell-mediated specific immune responses against viral spike proteins in a majority of patients with MS.

Discussion

This study combines analysis of humoral- and cell-mediated immunity responses to SARSCoV-2 vaccination in people with MS treated with different DMTs.

Mass vaccination against SARS-CoV-2 is crucial for control of the pandemic that is ongoing in large populations all over the world. A coordinated humoral- and cell-mediated response induced by specific vaccination is the only tool available for more effective prevention of SARS-CoV-2 infection, symptom onset, and severe disease outcome.^{13,14} In particular, the humoral response blocks viral replication itself, whereas the viral-specific T-cell response kills viral-infected cells.³⁰

Recently, Achiron et al.¹⁷ demonstrated, in a cohort of 125 patients with MS, the development of COVID-19 humoral response to the mRNA-based vaccine BNT162b2 in all untreated

Table 3 Factors Associated With Antibody and T-Cell-Specific Responses in Patients With Multiple Sclerosis

	All (n = 108)	Ocrelizumab (n = 25)	Fingolimod (n = 35)	Cladribine (n = 20)	IFN- β (n = 28)
Age					
Anti-RBD Abs, BAU/mL	$\rho = -0.145$; $p = 0.134$	$\rho = -0.118$; $p = 0.574$	$\rho = -0.059$; $p = 0.734$	$\rho = -0.471$; $p = 0.036$	$\rho = -0.002$; $p = 0.990$
Anti-spike IFN- γ , pg/mL	$\rho = -0.103$; $p = 0.287$	$\rho = 0.025$; $p = 0.906$	-0.190 ; $p = 0.267$	$\rho = -0.168$; $p = 0.478$	$\rho = -0.196$; $p = 0.327$
BMI					
Anti-RBD Abs, BAU/mL	$\rho = 0.060$; $p = 0.535$	$\rho = 0.361$; $p = 0.076$	$\rho = -0.174$; $p = 0.310$	$\rho = 0.026$; $p = 0.915$	$\rho = -0.249$; $p = 0.211$
Anti-spike IFN- γ , pg/mL	$\rho = -0.162$; $p = 0.095$	$\rho = -0.073$; $p = 0.0727$	$\rho = -0.044$; $p = 0.798$	$\rho = -0.217$; $p = 0.359$	$\rho = -0.031$; $p = 0.880$
Disease duration					
Anti-RBD Abs, BAU/mL	$\rho = 0.097$; $p = 0.319$	$\rho = 0.159$; $p = 0.447$	$\rho = 0.083$; $p = 0.631$	$\rho = -0.535$; $p = 0.015$	$\rho = 0.192$; $p = 0.337$
Anti-spike IFN- γ , pg/mL	$\rho = -0.066$; $p = 0.495$	$\rho = 0.229$; $p = 0.271$	$\rho = -0.170$; $p = 0.323$	$\rho = -0.179$; $p = 0.450$	$\rho = -0.120$; $p = 0.550$
Lymphocyte count					
Anti-RBD Abs, BAU/mL	$\rho = 0.132$; $p = 0.211$	$\rho = -0.261$; $p = 0.466$	$\rho = -0.012$; $p = 0.944$	$\rho = 0.185$; $p = 0.435$	—
Anti-spike IFN- γ , pg/mL	$\rho = 0.569^a$; $p < 0.001^a$	$\rho = -0.316$; $p = 0.374$	$\rho = 0.099$; $p = 0.564$	$\rho = -0.095$; $p = 0.691$	—
Treatment duration					
Anti-RBD Abs, BAU/mL	$\rho = 0.193$; $p = 0.045$	$\rho = -0.529^a$; $p = 0.007^a$	$\rho = -0.220$; $p = 0.198$	$\rho = 0.289$; $p = 0.217$	$\rho = 0.230$; $p = 0.249$
Anti-spike IFN- γ , pg/mL	$\rho = -0.160$; $p = 0.099$	$\rho = 0.005$; $p = 0.983$	$\rho = -0.313$; $p = 0.063$	$\rho = -0.384$; $p = 0.095$	$\rho = 0.189$; $p = 0.344$

Abbreviations: Abs = antibodies; BAU = binding arbitrary unit; BMI = body mass index; IFN = interferon; RBD = receptor-binding domain.
^a Significant after multiplicity correction by the Bonferroni method ($\alpha/4 = 0.0125$).

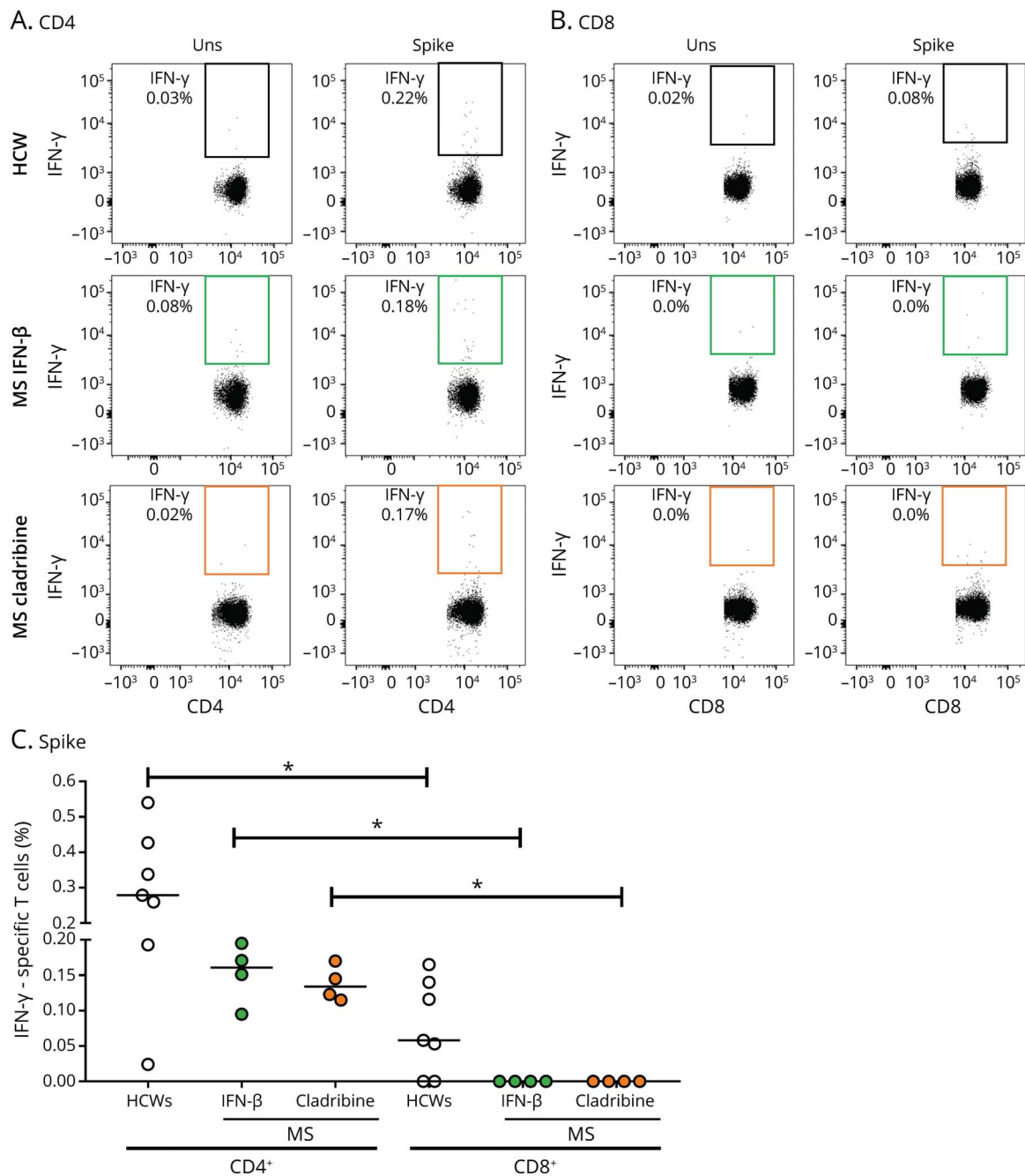
patients with MS and in all patients treated with cladribine. In the same study, only 22.7% of patients treated with ocrelizumab and 3.8% of patients treated with fingolimod developed SARS-CoV-2 antibodies. These results were confirmed in a very small French cohort,³¹ and, only partially, in an Italian series of 32 patients with MS showing the humoral response in 32.5% (6/16) patients treated with ocrelizumab, but also in 62.5% (10/16) of patients treated with fingolimod.²⁰

In this study, we demonstrate a normal qualitative and quantitative humoral response to COVID-19 vaccination in patients treated with IFN- β . We confirm that 100% of patients treated with cladribine developed a humoral response to mRNA-based vaccines, although the antibody titer was slightly lower than HCWs. We found a detectable humoral response in 85.7% of the patients treated with fingolimod and in 40% of those treated with ocrelizumab, both with an anti-RBD-antibody titer significantly reduced compared to HCWs. These findings are in agreement with previous results on vaccines other than COVID-19, showing a humoral response similar to healthy people in IFN- β -treated patients,³² and a reduced antibody titer in patients treated with ocrelizumab³³ and fingolimod.³⁴ The higher proportion of positive specific antispikes serologic response in patients with MS treated with fingolimod and ocrelizumab compared to that reported by Achiron et al.¹⁷ might be due to the more accurate serologic tests used here to detect the specific response.

Few data are available regarding the T-cell-specific response induced by COVID-19 vaccination in treated or untreated patients with MS. Recently, Apostolidis et al.³⁵ showed that anti-CD20 agents significantly reduced spike- and RBD-specific antibody and memory B-cell responses in most patients with MS. This effect was dependent on the time from the last anti-CD20 treatment and from the extent of the B-cell reconstitution.³⁵ Compared to this work, here, we report evidence of quantitative and qualitative SARS-CoV-2-spike-specific T-cell response in a larger cohort of patients with MS (n = 108 vs n = 20) treated not only with anti-CD20 drugs, but also with other different DMTs. In the current study, T-cell-specific response was observed in 92% of patients treated with ocrelizumab, 89.3% of the patients treated with IFN- β , and 70% of the patients treated with cladribine, but only in 14% of the fingolimod-treated patients. IFN- γ -T-cell-specific response was lower in all treated patients with MS compared to HCWs, in agreement with Apostolidis et al.³⁵ describing the results from only 1 cohort under anti-CD20 treatment. Moreover, we analyzed the T-cell response by an easy-to-perform assay on whole blood, quantifying the IFN- γ produced by T cells after specific stimulation.^{24,36} Accordingly, these results correlated with the number of lymphocytes.

Ocrelizumab is an anti-CD20 monoclonal antibody that depletes B lymphocytes and interferes with the process of antibody production.³⁷ This mechanism leads to a reduced

Figure 2 Evaluation of Interferon- γ -Spike-Specific T-Cell Response by Flow Cytometry

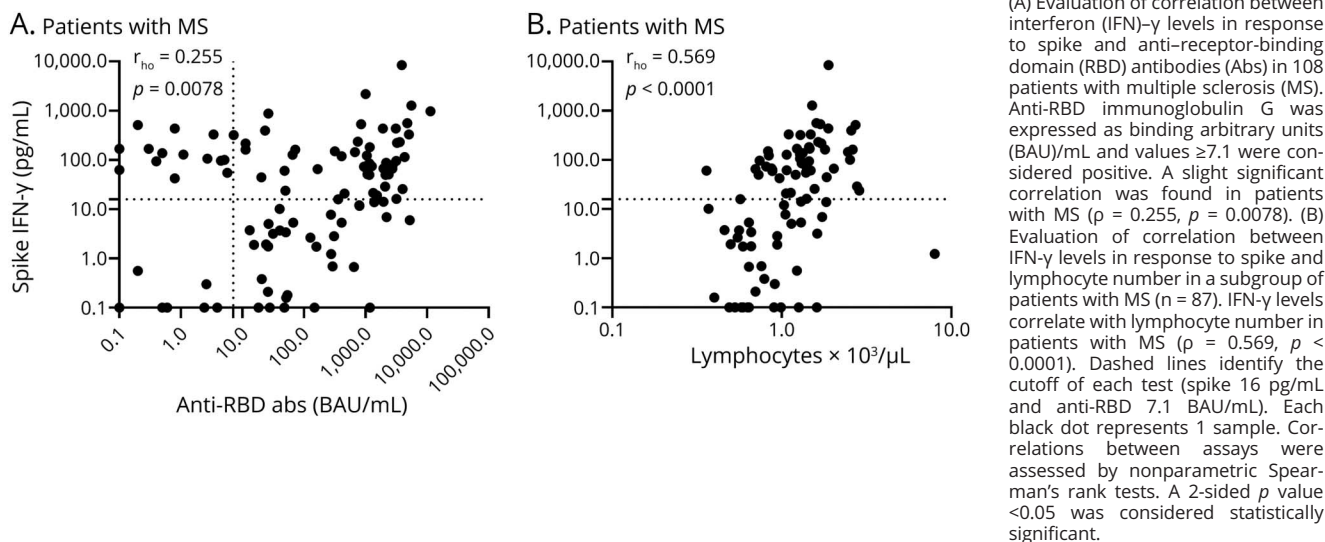


Health care workers (HCWs) ($n = 7$) and patients with multiple sclerosis (MS) (interferon [IFN]- β -treated $n = 4$; cladribine-treated $n = 4$) were stimulated for 24 hours with spike peptide pool and the frequency of IFN- γ -specific T cells was evaluated by flow cytometry. Plots show the frequency of IFN- γ -specific T cells in a representative HCW, patient with MS treated with IFN- β , and patient with MS under cladribine within the CD4⁺ subset (A) and CD8⁺ T-subset (B). (C) Frequency of the CD4⁺ and CD8⁺ T-cell responses (after subtraction of the unstimulated condition value) is shown in HCWs and patients with MS. Each dot represents a different HCW or patient with MS and black lines represent medians. Statistical analysis was performed using the Mann-Whitney test and p value was considered significant if ≤ 0.05 .

humoral response to vaccination, a higher risk of severe COVID-19,^{2,4,19} and the possibility of persistent SARS-CoV-2 infection in ocrelizumab-treated patients despite the induction of a SARS-CoV-2-specific T-cell response.³⁸ It is well known that serum IgG and IgM levels decrease with ocrelizumab treatment duration³⁹; this is confirmed by our results showing a relationship between treatment duration

and the entity of the humoral response to SARS-CoV-2 vaccine. We show that ocrelizumab-treated patients mount a spike-specific T-cell response comparable to that developed in patients treated with IFN- β or cladribine. Whether the presence of a T-cell response, associated to an impaired humoral immunity, might be sufficient to control SARS-CoV-2 infection is a matter of debate³⁸ and outside the

Figure 3 Correlations Across Humoral and Cell-Mediated Immunity and Lymphocyte Count



purpose of this study. However, preservation of the T-cell response might explain why ongoing treatment with ocrelizumab is not always associated with an increased severity of SARS-CoV-2 infection,⁴⁰ and why in a small cohort of anti-CD20-treated patients a low frequency of vaccine failure has been reported.³¹ Lastly, the long-term potential beneficial effect of the SARS-CoV-2-specific T-cell response might contribute to reduce COVID-19 disease severity.

Fingolimod is a sphingosine 1-phosphate modulator that prevents T cell egress from lymph nodes, reducing the number of circulating lymphocytes. This mechanism supports our results showing both reduced T-cell-specific and humoral responses in fingolimod-treated patients. We demonstrate also that only a minority of patients treated with fingolimod have an antiviral neutralizing capacity.

In vitro T-cell response to SARS-CoV-2 spike glycoprotein is mediated by both CD4⁺ and CD8⁺ T cells^{11,24} and was confirmed in our vaccinated HCWs cohort.²⁷ CD8⁺ T-cell response was not found in patients treated with cladribine or IFN- β . It is known that these treatments may have an effect on both CD4⁺ and CD8⁺ T-cell populations^{41,42} and that spike-specific response by CD8⁺ T cells is only one-tenth of the CD4⁺ T-cell response^{24,36} and therefore difficult to detect if present. These results need to be confirmed in larger cohorts of patients.

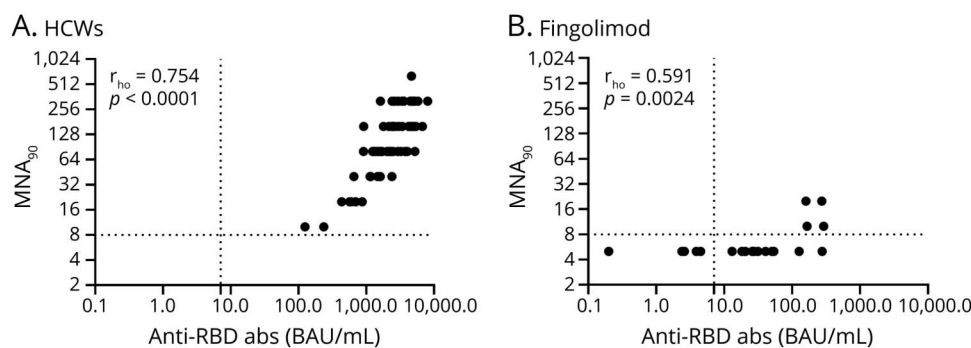
This study has some limitations. First, the small size of the cohort restricts the power of the study, especially for the comparison of the effects of vaccination between different DMTs. Nevertheless, the enrolled participants are representative of patients with MS and are well characterized, both clinically and immunologically. Second, the evaluation of immune responses was done at a single time point post-vaccination and the methodology used to detect the T-cell

response was based on the measurement of a single cytokine (IFN- γ), differently from published studies evaluating additional T-helper 1 cytokines.^{12,23} However, as we have shown, the IFN- γ T-cell response correlates with RBD antibody titers,¹² therefore IFN- γ may be considered as a robust parameter to measure the T-cell-specific response induced after vaccination.

One of the main strengths of this study, compared to prior works,^{17,18,20} is the evaluation of the humoral immune response using both specific anti-RBD IgG and SARS-CoV-2 neutralization tests, in addition to characterization of the T-cell response in terms of both CD4⁺ or CD8⁺ T-cell involvement. The assays used in this study to detect SARS-CoV-2-specific response are easy and highly reproducible,^{24,36} and therefore, compatible with the routine monitoring of vaccinated people. Indeed, the T-cell response was detected using a whole blood assay, whose platform is similar to current tests measuring the T-cell-specific responses against *Mycobacterium tuberculosis*.⁴³

This is the first study demonstrating the development of a T-cell-specific response to SARS-CoV-2 in the majority of DMT-treated patients with the lowest rate in patients treated with fingolimod. Together with the observation concerning the humoral response, these data carry relevant implications for managing vaccinations in people with MS, suggesting to promote vaccination in all treated patients with MS. Future studies are needed to evaluate the longevity of the humoral and T-cell responses following COVID-19 vaccination in patients with MS and the effect of different time window vaccination on immunity development in patients treated with ocrelizumab. These data will be key for defining the best vaccination strategy to balance the risk of MS disease progression and protection against SARS-CoV-2 infection.

Figure 4 Correlations Within Humoral Levels (Anti-Receptor-Binding Domain Immunoglobulin G and MNA₉₀)



The correlation between anti-receptor-binding domain (RBD) immunoglobulin G (IgG) levels and neutralizing antibodies (Abs) was evaluated in a subgroup of the (A) enrolled health care workers (HCWs) (n = 69) and (B) patients with multiple sclerosis (MS) under fingolimod therapy (n = 24). Anti-RBD IgG was expressed as binding arbitrary units (BAU)/mL and values ≥ 7.1 were considered positive; neutralizing antibodies were expressed as the reciprocal of dilution and values ≥ 10 were considered positive. A strong significant correlation was found in HCWs ($p = 0.754$, $p < 0.0001$), whereas a moderate correlation was found in patients with MS ($p = 0.591$, $p = 0.0024$). Dashed lines identify the cutoff of each test (anti-RBD 7.1 BAU/mL and MNA₉₀ 8). Each black dot represents 1 sample. Correlations between assays were assessed by nonparametric Spearman's rank tests. A 2-sided p value < 0.05 was considered statistically significant.

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Continued

Appendix (continued)

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Appendix (continued)

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