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# Longitudinal characterisation of B and T-cell immune responses after the booster dose of COVID-19 mRNA-vaccine in people with multiple sclerosis using different disease-modifying therapies

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## ABSTRACT

**Background** The decline of humoral response to COVID-19 vaccine led to authorise a booster dose. Here, we characterised the kinetics of B-cell and T-cell immune responses in patients with multiple sclerosis (PwMS) after the booster dose.

**Methods** We enrolled 22 PwMS and 40 healthcare workers (HCWs) after 4–6 weeks from the booster dose (T3). Thirty HCWs and 19 PwMS were also recruited 6 months (T2) after the first dose. Antibody response was measured by anti-receptor-binding domain (RBD)-IgG detection, cell-mediated response by an interferon (IFN)-γ release assay (IGRA), Th1 cytokines and T-cell memory profile by flow cytometry.

**Results** Booster dose increased anti-RBD-IgG titers in fingolimod-treated, cladribine-treated and IFN-β-treated patients, but not in ocrelizumab-treated patients, although antibody titres were lower than HCWs. A higher number of fingolimod-treated patients seroconverted at T3. Differently, T-cell response evaluated by IGRA remained stable in PwMS independently of therapy. Spike-specific Th1-cytokine response was mainly $CD4^+$ T-cell-mediated, and in PwMS was significantly reduced ($p<0.0001$) with impaired IL-2 production compared with HCWs at T3. In PwMS, total Th1 and IFN-γ $CD4^+$ T-cell responders to spike protein were increased from T2 to T3.

Compared with HCWs, PwMS presented a higher frequency of $CD4^+$ and $CD8^+$ terminally differentiated effector memory cells and of $CD4^+$ effector memory ($T_{EM}$) cells, independently of the stimulus suggesting the association of this phenotype with MS status. $CD4^+$ and $CD8^+$ $T_{EM}$ cell frequency was further increased at T3 compared with T2.

**Conclusions** COVID-19 vaccine booster strengthens humoral and Th1-cell responses and increases $T_{EM}$ cells in PwMS.

### WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Most of the studies have investigated the serological and/or cell-mediated response to SARS-CoV-2 vaccination focusing only on anti-CD20-treated or fingolimod-treated patients. Other studies investigating a wide range of disease-modifying therapies (DMTs) have evaluated only one of the adaptive responses and/or involved subjects before the booster dose. There is a lack of studies focusing on a longitudinal characterisation of both serological and T-cell response in patients with multiple sclerosis before and after the COVID-19 booster dose. The few studies available did not perform any in-depth analysis of the cytokine response or the memory profile of T cells in patients treated with an array of different immunotherapies, which are important to understand the vaccine-induced immunity in the MS population.

### WHAT THIS STUDY ADDS

⇒ To the best of our knowledge, this is the first longitudinal prospective study characterising the humoral response and deeply investigating the T-cell response, in terms of cytokine and memory profile, before and after the COVID-19 vaccine booster dose in patients with multiple sclerosis (PwMS) using different DMTs.

## INTRODUCTION

The COVID-19 pandemic represents a serious concern for human global health, particularly for patients with multiple sclerosis (PwMS). MS is a chronic inflammatory autoimmune disease causing neuroinflammation and myelin neurodegeneration.[1] Most PwMS are treated with immunomodulatory or immunosuppressive disease-modifying therapies (DMTs), including interferon (IFN)-β, fingolimod, ocrelizumab and cladribine. Considered their mechanism of action, DMTs might be to some extent associated with an increased risk of infection or COVID-19 severity and mortality.[2–4]

**HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY**

⇒ Our results highlight the beneficial effects of the booster dose as it strengthens the humoral and the T-cell response, in terms of Th1 cytokine production, in most of the PwMS.

⇒ PwMS showed a significant increase of effector memory ($T_{EM}$) and terminally differentiated effector memory ($T_{EMRA}$) cells after the booster dose. The demonstration of these kinetic changes is important to understand the memory response induced by COVID-19 vaccination in this vulnerable population.

To date, large-scale vaccination represents the most powerful tool to control COVID-19 pandemic and to prevent a severe outcome. Several studies have demonstrated the immunogenicity of mRNA vaccines after the first vaccination cycle in healthy individuals.[5 6]

In PwMS, mRNA vaccines induce both humoral and T-cell specific immune responses with a lower magnitude than healthy subjects according to DMTs.[7 8] Particularly, T-cell and antibody responses are both reduced in fingolimod-treated patients whereas ocrelizumab reduces the antibody response; differently, they are mostly preserved in those under cladribine or IFN-β.[7–11]

The waning of the humoral response led public health services to authorise the administration of a booster dose to restore the protection against COVID-19.[12–14]

In healthy subjects, the efficacy of the booster dose has been largely demonstrated.[15 16] In PwMS, the SARS-CoV-2 vaccine booster dose increases antibody titers according to therapy.[17 18] Indeed, evidence show that the booster does not improve the already low serological response in those under fingolimod or ocrelizumab treatments, and neither the impaired cellular responses of patients under fingolimod therapy.[13 19 20] Similarly, in ocrelizumab-treated patients SARS-CoV-2 booster dose has no additive effect on the maximal T-cell response observed after the first vaccination cycle.[21] To date, in PwMS there is a lack of longitudinal characterisation of both serological and T-cell response before and after the COVID-19 vaccine booster dose. The few studies available did not perform any in-depth analysis of the cytokine or memory profile of T cells,[19 20] which is important to understand the vaccine-induced immunity in PwMS. Therefore, we aimed to assess the adaptive immune response after the booster dose in PwMS treated with different DMTs, deeply investigating the cytokine and memory profiles within the T-cell compartment.

## MATERIALS AND METHODS

The extended version of materials and methods is included as online supplemental information.

### Study design and participants

This longitudinal prospective study involved the enrolment of PwMS and healthcare workers (HCWs) at the MS Centre of the Department of Neurosciences of San Camillo Forlanini Hospital (Rome, Italy) and at the National Institute for Infectious Diseases (INMI)-Lazzaro Spallanzani-IRCCS (Rome, Italy). For PwMS, the inclusion criteria were: (1) diagnosis of relapsing-remitting MS based on McDonald 2017 criteria[22]; (2) ongoing DMTs with ocrelizumab, fingolimod, cladribine or IFN-β for at least 6 months prior to the study entry; (3) completion of the first vaccination cycle of mRNA vaccines (BNT162b2 or mRNA-1273), and booster dose performance within the previous 4–6 weeks and (4) absence of relapses and/or steroids treatment during the last 3 months before study entry.

For PwMS undergoing ocrelizumab and cladribine therapy, the vaccination timing after the last DMT administration was established according to the European Academy of Neurology for COVID-19 vaccination guidelines. In detail, ocrelizumab was provided after 3 months, while cladribine with at least 4 weeks of delay. IFN-β and fingolimod therapies were not interrupted at the time of vaccination.

HCWs were used as healthy control group (some were included in our previous study).[23] Inclusion criteria for their enrolment were: no immunosuppression condition and having received the completed SARS-CoV-2 vaccination cycle and the booster dose as reported above for PwMS.

Exclusion criteria for both cohorts were: previous SARS-CoV-2 infection, HIV infection, age <18 years.

The follow-up study was performed on 30 HCWs and 19 PwMS providing blood samples after both 6 months from the first vaccine dose (T2) and 4–6 weeks from the booster dose (T3) (figure 1A).

### Experimental design

Antibody response was evaluated by measuring antinucleoprotein-immunoglobulin G (Anti-N-IgG) and anti-receptor-binding domain (RBD)-IgG. Anti-RBD-IgG were indicated as positive when ≥7.1 BAU/mL. For the T-cell response evaluated by IFN-γ release assay (IGRA), whole blood was overnight stimulated with a peptide mix (0.1 µg/mL of each peptide pool) covering the SARS-CoV-2 spike protein (Miltenyi Biotec, Germany) or with the staphylococcal enterotoxin B (SEB) at 200 ng/mL, as positive control.[24 25] Plasma IFN-γ levels were measured using an automatic ELISA (ELLA, protein simple, R&D Systems, Minnesota, USA). IFN-γ levels ≥16 pg/mL were considered positive.

For flow cytometry, fresh whole blood (600 µL) was overnight stimulated with spike protein or SEB together with a-CD28 and a-CD49d (1 µg/mL each). Cells were then stained for intracellular cytokines and T-cell phenotype as previously described[12 23] (online supplemental table 1 and figure 1).

### Statistical analysis

Data were analysed using GraphPad software (GraphPad Prism V.9.3.1). Continuous and categorical variables were reported, respectively, as median and IQR and count and proportion. The following non-parametric statistical inference tests were performed: Friedman test for comparisons among groups followed by Dunn's multiple comparisons test, Mann-Whitney and Wilcoxon signed-rank tests for pairwise comparisons, $\chi^2$ and McNemar tests for proportions. Two-tailed p values <0.05 were considered significant.

## RESULTS

### Characteristics of the enrolled subjects

We prospectively enrolled 62 vaccinated subjects: 22 PwMS and 40 HCWs. Demographical and clinical characteristics are summarised in table 1.

Age significantly differed between the two cohorts (p=0.0013). Nevertheless, CD4:CD8 T-cell ratio, which is inverted in people older than 60 years,[26] was comparable between HCWs and PwMS (p=0.842). Among PwMS, five were treated with ocrelizumab, nine with fingolimod, three with cladribine and five with IFN-β. Fourteen PwMS have been previously treated with other DMTs (nine underwent IFN-β, three glatiramer acetate, one with dymethil fumarate and one with azathioprine). All enrolled

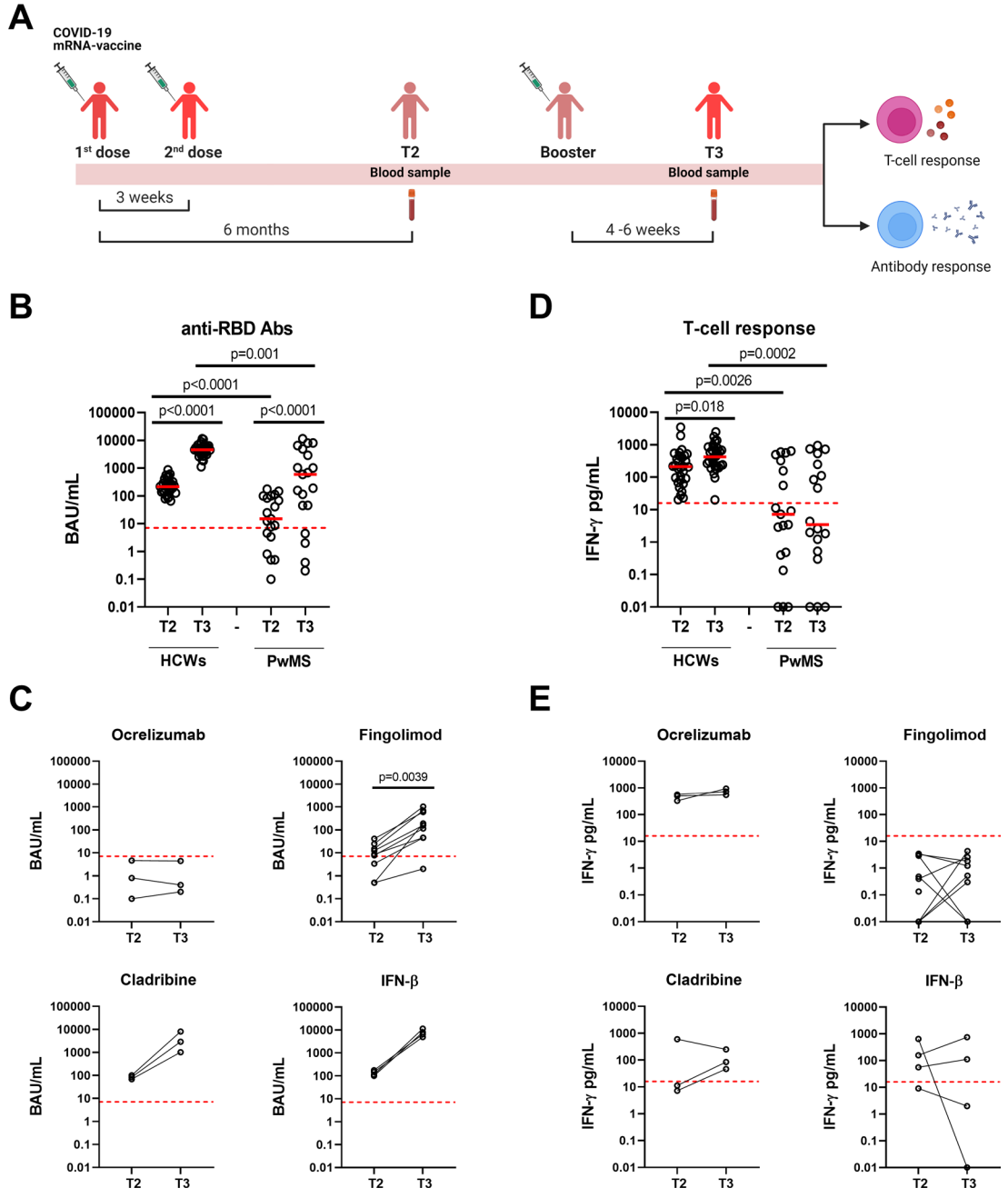



**Figure 1** Kinetic of the antibody and T-cell responses to COVID-19 vaccine in HCWs and PwMS. (A) Timeline of COVID-19 vaccination and study enrolment. For the analyses, blood samples of both HCWs and PwMS were collected after 6 months from the first vaccine dose (T2) and after 4–6 weeks from the booster dose (T3). Anti-RBD antibodies (B, C) and T-cell response (D, E) were evaluated in HCWs (n=30) and PwMS followed over time (n=19). (C, E) Antibody and T-cell responses were stratified according to DMTs: ocrelizumab (n=3), fingolimod (n=9), cladribine (n=3) and IFN-β (n=4). (B, C) Anti-RBD-IgG were measured in sera samples and expressed as binding antibody units (BAU)/mL. The cut-off was set at 7.1 BAU/mL (red dashed line). (D, E) For T-cell response, IFN-γ levels were quantified using an automatic ELISA and reported after subtracting the unstimulated control value. The cut-off was set at 16 pg/mL (red dashed lines). Data were analysed using Mann-Whitney and Wilcoxon matched pairs signed rank test and p values <0.05 were considered statistically significant. Each dot represents a different individual. Abs, antibodies; BAU, binding antibody units; DMTs, disease modifying therapies; HCWs, healthcare workers; IFN-γ, interferon gamma; IFN-β, interferon beta; PwMS, patients with multiple sclerosis; RBD, receptor-binding domain.

subjects were naïve for SARS-CoV-2 infection as confirmed by undetectable anti-N antibodies (data not shown). For the kinetic study, a proportion of PwMS (n=19) and HCWs (n=30) was longitudinally sampled at T2 and T3 (figure 1A).

## Kinetic of humoral and T-cell-specific responses in PwMS

Humoral and IFN-γ-spike-specific T-cell responses were monitored in HCWs (n=30) and PwMS (n=19) sampled at T2 and T3. We found that the booster dose significantly increased anti-RBD-IgG titers in both HCWs and PwMS (p<0.0001 for both), and in PwMS induced a higher seroconversion rate compared with T2 (T3: 15/19, 78.9% vs T2: 13/19, 68.4%), although not significant (figure 1B). However, compared with HCWs, PwMS showed significant lower anti-RBD titers at both T2 (p<0.0001) and T3 (p=0.001). Stratifying patients according to DMTs, we observed that antibody titers significantly differed from

**Table 1** Demographic and clinical characteristics of the enrolled participants

| Characteristics | HCWs | PwMS | P value |
|---|---|---|---|
| Total, N (%) | 40 (100) | 22 (100) | |
| Age, years (IQR) | 43 (29–51) | 51 (47–58) | 0.0013* |
| Male, N (%) | 11 (27.5) | 5 (22.72) | 0.681† |
| Origin | | | |
| Western Europe | 39 | 22 | 0.445† |
| Asia | 1 | 0 | |
| BMI kg/m$^2$ | | 23.90 | |
| Treatment duration, years (IQR) | | 5.26 (2.1–9.2) | |
| Time to last relapse, y (IQR) | | 6.5 (2.4–10.3) | |
| CD4:CD8 T cell ratio‡ | 2.1 (0.8–3.4) | 1.9 (1.1–2.6) | 0.842* |
| MS treatment | | | |
| Ocrelizumab, N (%) | | 5 (22.72) | |
| Fingolimod, N (%) | | 9 (40.9) | |
| Cladribine, N (%) | | 3 (13.63) | |
| IFN-β, N (%) | | 5 (22.72) | |

*Mann-Whitney U statistic test.
†$\chi^2$ test
‡CD4:CD8 T cell ratio is a marker of immune-senescence.[26]
BMI, body mass index; HCWs, healthcare workers; IFN-β, interferon beta; N, Number; PwMS, patients with multiple sclerosis.

**Table 2** Number of CD4$^+$ and CD8$^+$ T-cell responders among HCWs and PwMS at T3

| Stimulation and type of T-cell response evaluation | Cytokine produced | T3 responders over total N (%) | | |
|---|---|---|---|---|
| | | HCWs 15 (100) | PwMS 22 (100) | P value* |
| Spike-specific CD4$^+$ T cells | Any Th1 | 13 (86.6) | 5 (22.7) | **0.0001** |
| | IFN-γ | 7 (46.6) | 5 (22.7) | 0.1267 |
| | TNF-α | 9 (60) | 0 (0) | **<0.0001** |
| | IL-2 | 9 (60) | 0 (0) | **<0.0001** |
| Spike-specific CD8$^+$ T cells | Any Th1 | 4 (26.6) | 2 (9.1) | 0.1544 |
| | IFN-γ | 4 (26.6) | 2 (9.1) | 0.1544 |
| | TNF-α | 1 (6.6) | 0 (0) | 0.2195 |
| | IL-2 | 1 (6.6) | 0 (0) | 0.2195 |
| CD4$^+$ T cells response to SEB | Any Th1 | 15 (100) | 22 (100) | n/a |
| | IFN-γ | 15 (100) | 22 (100) | n/a |
| | TNF-α | 15 (100) | 21 (91.3) | 0.4025 |
| | IL-2 | 12 (80) | 12 (54.5) | **<0.0001** |
| CD8$^+$ T cells response to SEB | Any Th1 | 15 (100) | 22 (100) | n/a |
| | IFN-γ | 15 (100) | 22 (100) | n/a |
| | TNF-α | 13 (86.7) | 21 (95.5) | 0.3363 |
| | IL-2 | 14 (93.3) | 3 (13.6) | **<0.0001** |

In bold are indicated the significant values.
*$\chi^2$ test.
HCWs, healthcare care workers; N, Number; n/a, not available; PwMS, patients with multiple sclerosis; SEB, staphylococcal enterotoxin B.

T2 to T3 in fingolimod-treated patients (T2: 8.60 BAU/mL, IQR: 1.95–19.85 and T3: 157.1 BAU/mL, IQR: 45.45–640.1, p=0.0039). Conversely, no significant differences were observed for cladribine- or IFN-β-treated patients, likely due to the small sample size; however, an increasing trend of anti-RBD-IgG titers was found. To note, patients under ocrelizumab did not present an antibody response (figure 1C).

In contrast, in PwMS the T-cell response, evaluated by IGRA, remained stable over time without significant differences between T2 and T3, although persisted significantly lower than in HCWs (T2: p=0.0026 and T3: p=0.0002) (figure 1D). This result was consistent across all treatments (figure 1E). Differently, in HCWs a significant increase was observed from T2 to T3 (p=0.018) (figure 1D). Sex and age did not show any effect on antibody or T-cell response in either of our cohorts (online supplemental table 2).

## CD4$^+$ and CD8$^+$ T-cell responses after COVID-19 booster

To characterise the T-cell response to SARS-CoV-2 vaccine at T3, we analysed the frequency of CD4$^+$ and CD8$^+$ T cells producing IFN-γ, IL-2, or TNF-α in the 22 PwMS and a portion of HCWs (n=15).

First, we assessed the cytokine response to spike. In the CD4$^+$ T-cell compartment of both cohorts, we found a higher response rate for either total or single Th1 cytokines compared with CD8$^+$ T cells (table 2).

PwMS showed the highest number of CD4$^+$ T-cell responders for IFN-γ compared with TNF-α or IL-2. Compared with HCWs, the proportions of TNF-α-, IL-2- and total Th1-specific-CD4$^+$ T-cell responders were significantly reduced in PwMS (p<0.0001, p<0.0001 and p=0.0001, respectively) (table 2). Particularly, CD4$^+$ T cells from PwMS failed to produce IL-2. Analysing the quantitative response, significant lower frequencies of IFN-γ, TNF-α-, IL-2 and Th1-specific CD4$^+$ T cells were observed in PwMS (p=0.0129, p<0.0001, p=0.0002 and p<0.0001, respectively) (figure 2A).

Regarding the cytokine response within the CD8$^+$ T-cell compartment, the highest number of responders was observed for IFN-γ. However, no significant differences were observed in terms of magnitude or response rate, neither for total nor for single Th1 cytokine response between the two cohorts, likely due to the low number of responders (figure 2B and table 2).

Then, we investigated the ability of T cells to produce cytokines in response to SEB, a non-specific stimulus. Compared with spike, both cohorts showed a higher number of responders in terms of total and single Th1 cytokine response in both CD4$^+$ and CD8$^+$ T-cell subset (table 2).

Within the SEB response, all subjects showed a CD4 and CD8 Th1 cytokine response (table 2); however, PwMS presented a frequency of both CD4$^+$ and CD8$^+$ Th1 response significantly lower than HCWs (p<0.0001 and p=0.0417, respectively) (figure 2C,D). Evaluating the single cytokine response, we confirmed with SEB that the highest response rate in both cohorts and T-cell compartments was observed for IFN-γ. No significant difference was found in the number of TNF-α-specific CD4$^+$ or CD8$^+$ T-cell responders between HCWs and PwMS. Differently, the proportion of IL-2-specific CD4$^+$ and CD8$^+$ T-cell responders was significantly reduced in PwMS compared with HCWs (p<0.0001 for both) (table 2). Regarding the magnitude of the response, CD4$^+$ T cells of PwMS showed lower frequencies of IFN-γ (p=0.0027), TNF-α (p<0.0001) and IL-2 (p=0.0002) producing cells than controls (figure 2C). Similar results were found within the CD8$^+$ T-cell compartment for TNF-α (p=0.034) and IL-2 (p<0.0001), whereas the IFN-γ-specific CD8$^+$ T cells showed a trend of reduction, although not significant (figure 2D). Within the PwMS cohort, the IFN-γ T-cell frequency was significantly higher than that of IL-2 and TNF-α in both CD4$^+$ and CD8$^+$ T-cell compartments (figure 2C,D). Similar results were found in the CD8$^+$ T-cell compartment of HCWs.

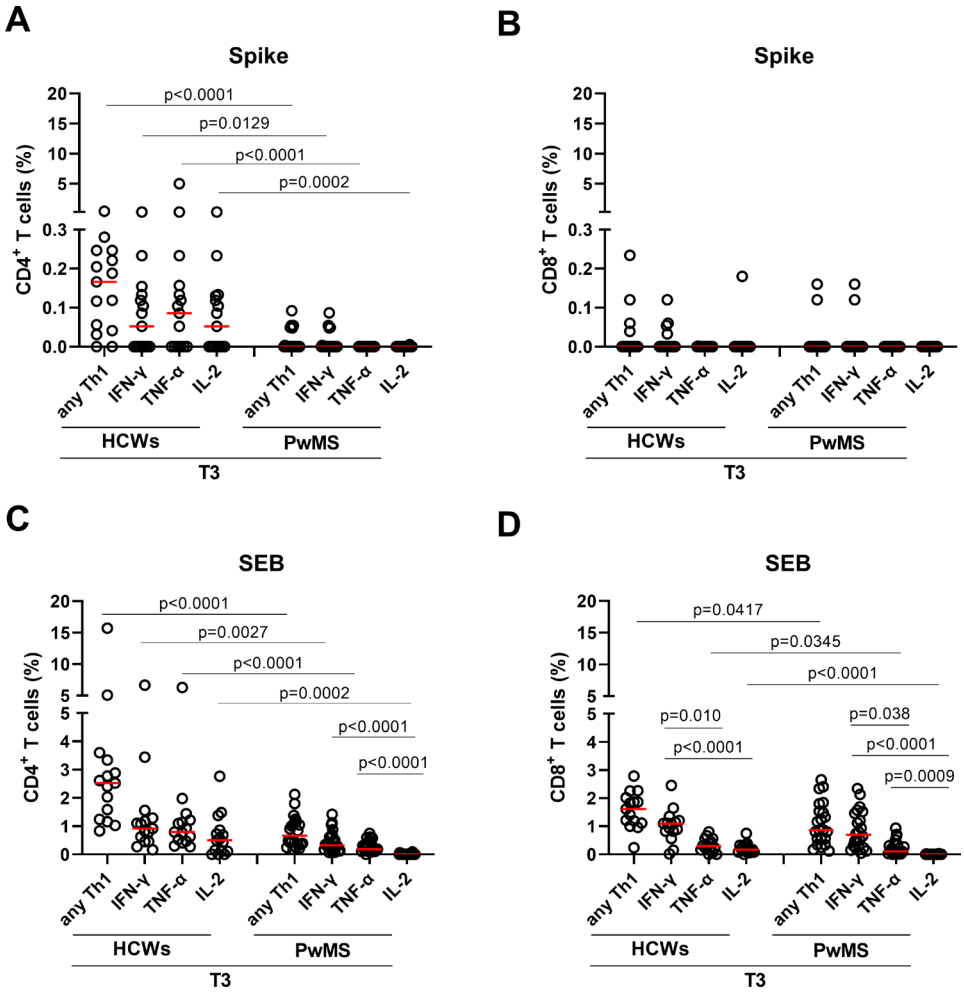



**Figure 2** CD4$^+$ and CD8$^+$ T cell cytokine profiles in response to spike and SEB after the booster dose (T3). (A, B) CD4$^+$ and CD8$^+$ T-cell cytokine responses to spike at T3. Graphs show cytokine production, in terms of total Th1 and single cytokines (IFN-γ, TNF-α and IL-2) in HCWs (n=15) and PwMS (n=22) within the CD4$^+$ (A) and CD8$^+$ (B) T-cell compartments. (C, D) CD4$^+$ and CD8$^+$ T-cell responses to the non-specific stimulus of SEB. Graphs show total Th1 and single cytokine (IFN-γ, TNF-α and IL-2) producing cells in HCWs (n=15) and PwMS (n=22) within the CD4$^+$ (C) and CD8$^+$ (D) T-cell compartments. Frequencies were reported after subtracting the cytokine production of the unstimulated condition. Statistical analysis was performed using Mann-Whitney U test to compare the frequency of the T cells expressing IFN-γ, IL-2 or TNF-α between HCWs and PwMS and Friedman test followed by Dunn's multiple comparison test for intragroup analyses. P values <0.05 were considered significant. Red lines indicate medians and each dot represents a different HCW or PwMS. HCWs, healthcare workers; IFN-γ, interferon gamma; IFN-β, interferon beta; PwMS, patients with multiple sclerosis; SEB, staphylococcal enterotoxin B.

## Cd4$^+$ and CD8$^+$ T-cell memory phenotype after booster

We characterised the CD4$^+$ and CD8$^+$ memory T cells at T3 according to the expression of CD45RA and CCR7.

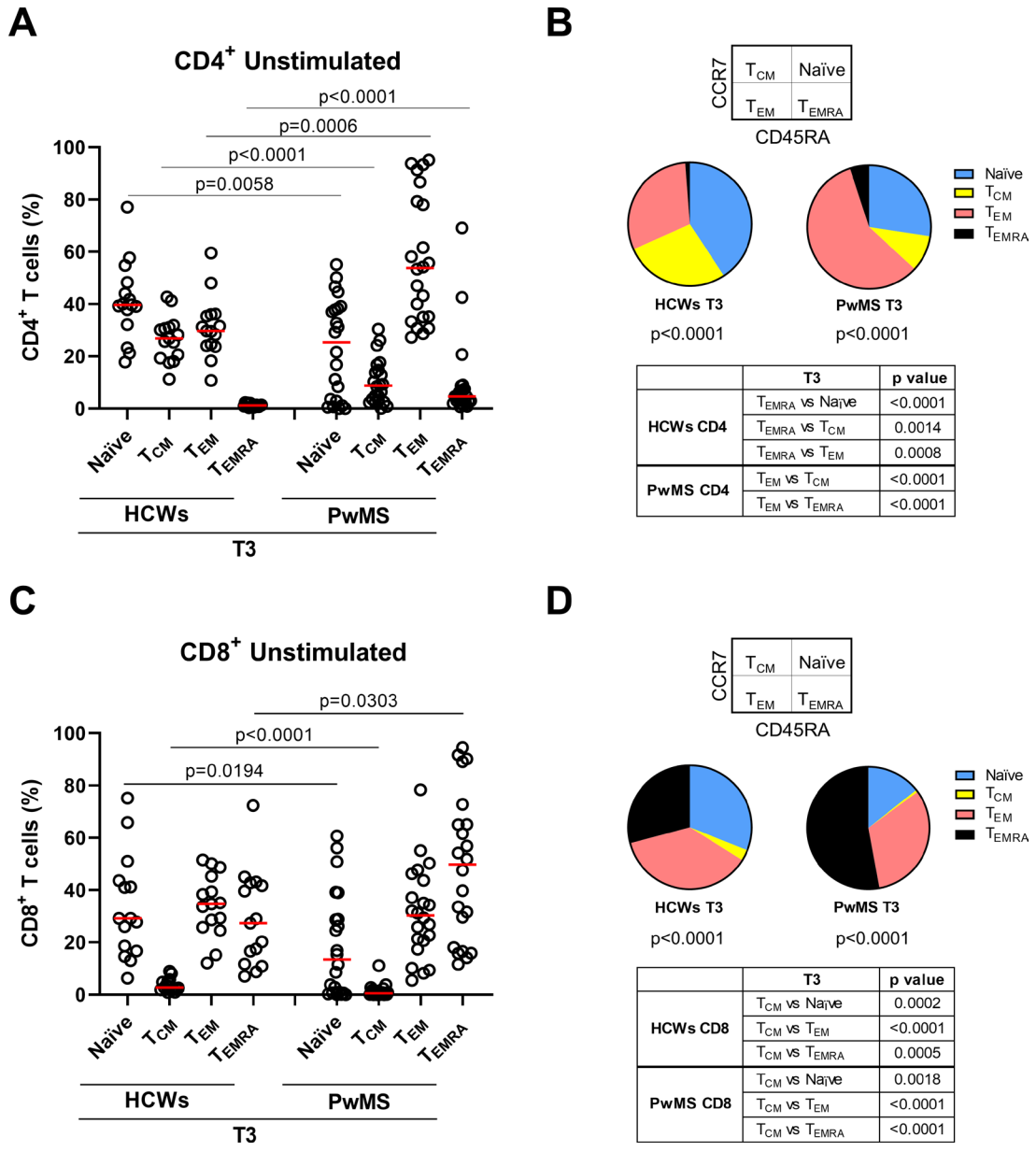
In the CD4$^+$ T-cell subset, naïve (CD45RA$^+$CCR7$^+$) and central memory ($T_{CM}$, CD45RA$^-$CCR7$^+$) cells were significantly reduced in PwMS compared with HCWs (p=0.0058 and p<0.0001, respectively). Differently, effector memory ($T_{EM}$, CD45RA$^-$CCR7$^-$) and terminally differentiated effector memory T cells ($T_{EMRA}$, CD45RA$^+$CCR7$^-$) increased in PwMS (p=0.0006 and p<0.0001, respectively) (figure 3A,B). Within the CD4$^+$ T cells of PwMS, $T_{EM}$ cells were the most represented; differently, the naïve population was more represented in HCWs. In both cohorts, the lowest proportion was represented by the $T_{EMRA}$ subpopulation (figure 3B).

Similarly, within the CD8$^+$ T-cell compartment, significant reduction of both naïve and $T_{CM}$ subset (p=0.0194 and p<0.0001) and significant increase of $T_{EMRA}$ cells (p=0.0303) were observed in PwMS compared with HCWs (figure 3C,D). The frequency of $T_{EM}$ cells was comparable between the two cohorts. Within each cohort, the proportion among memory T-cell subsets significantly differed (p<0.0001). In PwMS, $T_{EMRA}$ and $T_{EM}$ populations were the most represented within the CD8$^+$ T cells, whereas in HCWs $T_{EM}$ and naïve populations showed the highest frequency (figure 3D). In both cohorts, the lowest proportion was represented by the $T_{CM}$ population.

## Temporal evolution of the T-cell response in PwMS

To monitor the over time evolution of the Th1 cytokine response to SARS-CoV-2 vaccination in PwMS, 19 subjects were analysed at T2 and T3.

In response to spike, comparing T2 and T3 no significant differences were observed in terms of magnitude or response rate neither for total nor for single Th1 cytokine responses in both T-cell subsets (figure 4A,B). However, higher proportions of Th1 and IFN-γ-specific CD4$^+$ T-cell responders were observed from T2 to T3 (table 3). Differently, no IL-2-specific CD4$^+$ or CD8$^+$ T- cell responders were observed in either T2




| | T3 | p value |
|---|---|---|
| HCWs CD4 | $T_{EMRA}$ vs Naïve | <0.0001 |
| | $T_{EMRA}$ vs $T_{CM}$ | 0.0014 |
| | $T_{EMRA}$ vs $T_{EM}$ | 0.0008 |
| PwMS CD4 | $T_{EM}$ vs $T_{CM}$ | <0.0001 |
| | $T_{EM}$ vs $T_{EMRA}$ | <0.0001 |

| | T3 | p value |
|---|---|---|
| HCWs CD8 | $T_{CM}$ vs Naïve | 0.0002 |
| | $T_{CM}$ vs $T_{EM}$ | <0.0001 |
| | $T_{CM}$ vs $T_{EMRA}$ | 0.0005 |
| PwMS CD8 | $T_{CM}$ vs Naïve | 0.0018 |
| | $T_{CM}$ vs $T_{EM}$ | <0.0001 |
| | $T_{CM}$ vs $T_{EMRA}$ | <0.0001 |

**Figure 3** Memory T-cell phenotype in $CD4^+$ and $CD8^+$ T cells after the booster dose (T3). (A–D) T-cell phenotype in HCWs (n=15) and PwMS (n=22) in unstimulated condition. Frequency of $CD4^+$ (A) and $CD8^+$ T cells (C) gated according to CD45RA and CCR7 expression. Pie charts show the proportion of $CD4^+$ (B) and $CD8^+$ (D) T-cell subpopulations in both cohorts. Statistical analyses were performed using Mann-Whitney U test and p values <0.05 were considered significant. Friedman test followed by Dunn's multiple comparison test was performed for pie charts analyses and pie graphs were generated using the median frequency. Red lines indicate medians. Each dot represents a different HCW or PwMS. HCWs, healthcare workers; PwMS, patients with multiple sclerosis; $T_{CM}$, central memory; $T_{EM}$, effector memory; $T_{EMRA}$, terminally differentiated effector memory.

or T3. The few TNF-α-specific responders at T2 were lost at T3.

In response to SEB, we also found that Th1, IFN-γ and TNF-α $CD4^+$ T-cell responses were stable over time without significant differences in terms of magnitude or number of responders (figure 4C). Differently, from T2 to T3 we observed a significant reduction of the frequency of $CD4^+$ T cells producing IL-2 (p=0.0093) (figure 4C). Moreover, also the number of IL-2-specific $CD4^+$ T-cell responders decreased, although the difference was not significant (table 3).

Similarly, $CD8^+$ T cells in response to SEB showed a significant reduction of the proportion of IL-2 responders (p=0.026) from T2 to T3 (table 3). No significant differences were found in terms of magnitude or response rate for total Th1 and IFN-γ cytokine responses, whereas the proportion of TNF-α responders significantly increased (p=0.045).

## Kinetic of $CD4^+$ and $CD8^+$ T-cell memory phenotype within PwMS

To evaluate whether the COVID-19 vaccine booster could influence the memory T-cell compartment in PwMS, we compared the T-cell phenotype of the patients longitudinally sampled at T2 and T3.

$CD4^+$ memory T-cell subpopulations significantly differed in frequency at both time points (T2: p=0.0043 and T3: p<0.0001) (figure 5A,B). Particularly, at T3 $T_{EM}$ population frequency was significantly increased compared with T2 (p=0.006), thus

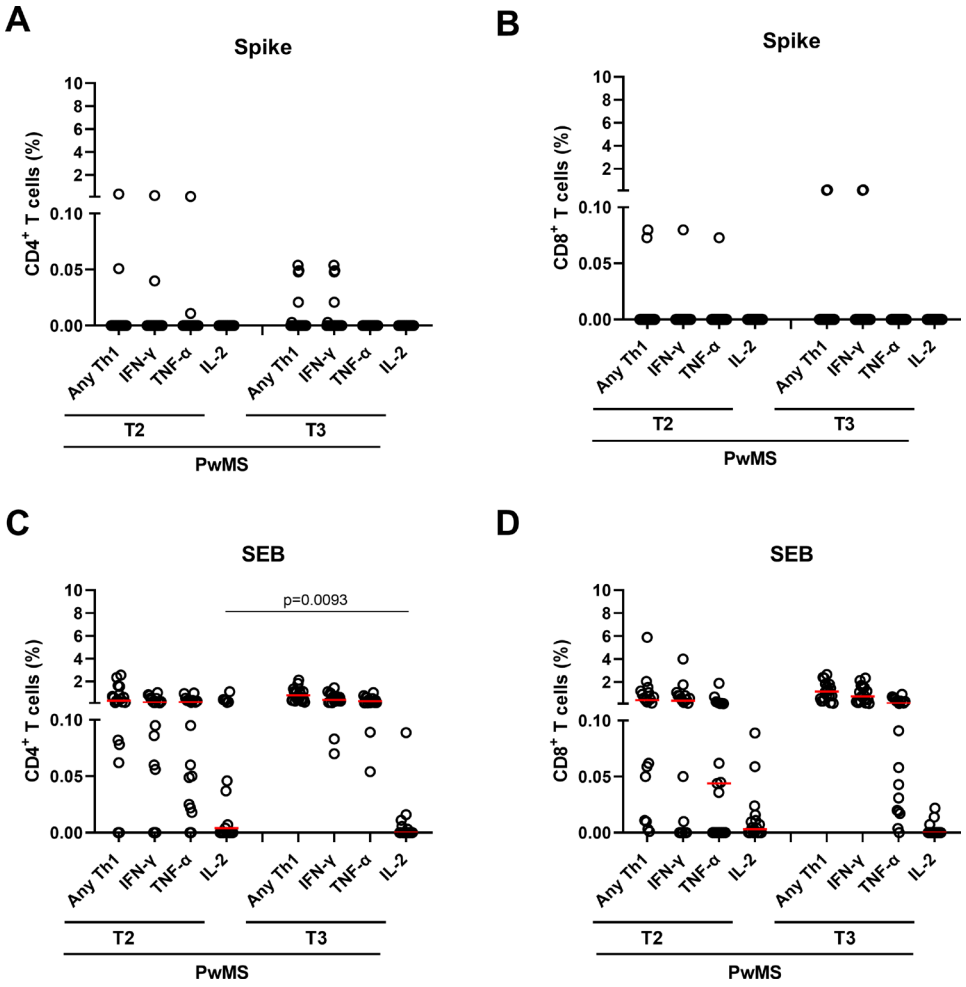



**Figure 4** Temporal evolution of CD4$^+$ and CD8$^+$ T cell cytokine profiles in response to spike and SEB stimuli. (A, B) CD4$^+$ and CD8$^+$ T-cell cytokine responses to spike in the PwMS (n=19) longitudinally sampled after 6 months from the first vaccine dose (T2) and after 4–6 weeks from the booster dose (T3). Graphs show cytokine production, in terms of total Th1 and single cytokines (IFN-γ, TNF-α and IL-2) within the CD4$^+$ (A) and CD8$^+$ (B) T-cell compartments. (C, D) CD4$^+$ and CD8$^+$ T-cell responses to SEB in the PwMS followed over time. Graphs show the total Th1 and single cytokine (IFN-γ, TNF-α and IL-2) producing cells within the CD4$^+$ (C) and CD8$^+$ (D) T-cell compartments. Frequencies were reported after subtracting the cytokine production of the unstimulated condition. Statistical analysis was performed using Wilcoxon matched pairs signed rank test to compare the frequency of the T cells expressing IFN-γ, IL-2 or TNF-α between T2 and T3. P values <0.05 were considered significant. Red lines indicate the medians and each dot represents a different PwMS. IFN-γ, interferon gamma; IFN-β, interferon beta; PwMS, patients with multiple sclerosis; SEB, staphylococcal enterotoxin B.

making $T_{EM}$ cells the most represented among all CD4$^+$ memory T cells. By contrast, no differences were found for naïve, $T_{CM}$ or $T_{EMRA}$ cells between T2 and T3 (figure 5A).

Within the CD8$^+$ T-cell compartment, memory T-cell subpopulations significantly differed from each other in terms of frequency at both time points (T2: p=0.0013 and T3: p<0.0001) and all of them significantly varied over time. Indeed, the frequency of $T_{EM}$ and $T_{EMRA}$ subsets increased (p=0.016 and p=0.023), whereas $T_{CM}$ cells decreased (p=0.0062) from T2 to T3 (figure 5C,D). Also, naïve cells showed a reduction trend, although not significant. Consequently, while at T2 the naïve population presented the highest frequency among all CD8$^+$ memory cells, after the booster dose $T_{EM}$ and $T_{EMRA}$ populations were predominant.

Considering only the CD4$^+$ Th1-specific responders (n=5), we observed a similar increasing trend for $T_{EM}$ and $T_{EMRA}$ populations at T3, although not significant (online supplemental figure 2). These results confirmed what was observed in the whole population independently of the positive response.

A similar memory T-cell phenotype was observed also after spike or SEB stimulation (data not shown), thus demonstrating that the memory profile was independent of the stimulus used.

## DISCUSSION

To the best of our knowledge, this is the first longitudinal prospective study characterising the humoral response and deeply investigating the T-cell response and memory profile, before and after the COVID-19 vaccine booster dose in PwMS treated with different DMTs. Our results highlight the beneficial effects of the booster dose showing increased anti-RBD-IgG titers in fingolimod, cladribine and IFN-β-treated patients, and an increased seroconversion rate for fingolimod-treated patients, although the magnitude of the response is lower than in healthy controls. As expected, no seroconversion was observed for ocrelizumab-treated patients due to CD20$^+$ B cells depletion.[27] Differently, the T-cell response evaluated by IGRA remained mostly stable over time in PwMS, whereas an increasing trend was observed in HCWs. Spike-specific T-cell response analysis by flow cytometry

**Table 3** Number of CD4$^+$ and CD8$^+$ T-cell responders among PwMS at T2 and T3

| Stimulation and type of T-cell response evaluation | Cytokine produced | Responders over total N (%) | | |
|---|---|---|---|---|
| | | T2 19 (100) | T3 19 (100) | P value* |
| Spike-specific CD4$^+$ T cells | Any Th1 | 2 (10.5) | 5 (26.31) | 0.248 |
| | IFN-γ | 2 (10.5) | 5 (26.31) | 0.248 |
| | TNF-α | 2 (10.5) | 0 (0) | 0.479 |
| | IL-2 | 0 (0) | 0 (0) | n/a |
| Spike-specific CD8$^+$ T cells | Any Th1 | 2 (10.5) | 2 (10.5) | n/a |
| | IFN-γ | 1 (5.2) | 2 (10.5) | >0.9999 |
| | TNF-α | 1 (5.2) | 0 (0) | >0.9999 |
| | IL-2 | 0 (0) | 0 (0) | n/a |
| CD4$^+$ T cells response to SEB | Any Th1 | 17 (89.5) | 19 (100) | 0.479 |
| | IFN-γ | 16 (84.2) | 19 (100) | 0.248 |
| | TNF-α | 17 (89.5) | 19 (100) | 0.479 |
| | IL-2 | 10 (60.1) | 5 (26.3) | 0.182 |
| CD8$^+$ T cells response to SEB | Any Th1 | 19 (100) | 19 (100) | n/a |
| | IFN-γ | 16 (84.2) | 19 (100) | 0.248 |
| | TNF-α | 11 (57.9) | 18 (94.7) | **0.045** |
| | IL-2 | 11 (57.9) | 3 (15.8) | **0.026** |

In bold are indicated the significant values.
*McNemar's test.
N, Number; n/a, not available; PwMS, Patients with Multiple Sclerosis; SEB, staphylococcal enterotoxin B.

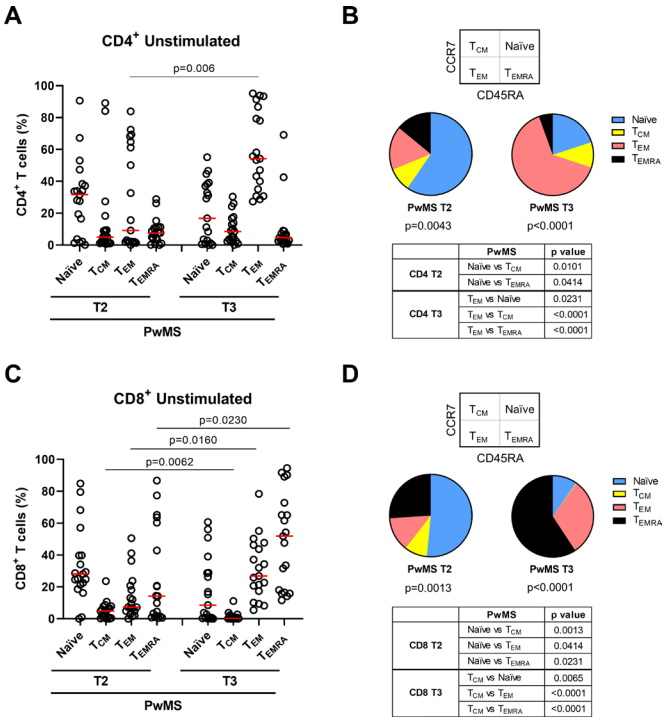



**Figure 5** Over time evolution of memory T-cell phenotypes in CD4$^+$ and CD8$^+$ T cells. (A–D) T-cell phenotype in PwMS (n=19) longitudinally sampled after 6 months from the first vaccine dose (T2) and after 4–6 weeks from the booster dose (T3) in unstimulated condition. Frequency of CD4$^+$ (A) and CD8$^+$ T cells (C) gated according to CD45RA and CCR7 expression. Pie charts show the proportion of CD4$^+$ (B) and CD8$^+$ (D) T-cell subpopulations at both time points. Statistical analyses were performed using Wilcoxon matched pairs signed rank test and p values <0.05 were considered significant. Friedman test followed by Dunn's multiple comparison test was used for pie charts analyses and pie graphs were generated using the median frequency. Red lines indicate the medians. Each dot represents a different PwMS. PwMS, patients with multiple sclerosis; $T_{CM}$, central memory; $T_{EM}$, effector memory; $T_{EMRA}$, terminally differentiated effector memory.

confirmed that the response is mainly CD4$^+$ T cell-mediated.[7] Moreover, after the booster dose, we showed that the magnitude of the specific CD4$^+$ T-cell response was significantly reduced in PwMS compared with controls and characterised by an impaired IL-2 production. Regarding the T-cell memory phenotype, within the CD4$^+$ T-cell compartment PwMS showed a significant reduction of naïve and $T_{CM}$ cells compared with HCWs, while $T_{EM}$ and $T_{EMRA}$ cells increased. A similar memory profile was observed within the CD8$^+$ T-cell compartment, except for $T_{EM}$ cells whose frequency was comparable between the two cohorts. Overall, the booster dose increases the humoral response and the T-cell response in terms of Th1 cytokine production. These effects can be particularly relevant to prevent COVID-19 in the MS population.

Evaluation of both humoral and cell-mediated response is pivotal to accurately estimate the immune response to SARS-CoV-2 vaccines. Several studies demonstrated that antibody titers rapidly decrease over time.[12 14] Differently, CD4$^+$ T cells may contribute to SARS-CoV-2 long-term protection being recalled after antigen re-exposure even after a long period from the booster dose.[28] Moreover, T-cell response cross-recognises also SARS-CoV-2 variants.[29 30] Due to the immune response waning over 6 months following the second vaccine dose,[10 13] the booster dose administration was authorised. Several studies have investigated the adaptive immune responses to SARS-CoV-2 vaccination focusing only on anti-CD20-, fingolimod-treated patients[9 11 13] or on a wide range of DMTs but before the booster dose.[17 31 32] This study confirms that fingolimod and anti-CD20-therapies elicit reduced cellular and humoral responses also after SARS-CoV-2 booster dose. Similarly, in ocrelizumab-treated patients no additive effect was observed on the maximal T-cell response, despite the presence of a boost response.[21]

IL-2 production was impaired in PwMS independently of the booster dose, whereas HCWs showed an IL-2 specific T-cell response. IL-2 is involved in the cell activation associated with a worsening of the MS disease status[33]; therefore, the IL-2 production impairment may indirectly explain the lack of MS worsening symptoms after SARS-CoV-2 vaccination. Interestingly, an impaired IL-2 production was also found in COVID-19 vaccinated patients with other autoimmune diseases, including rheumatoid arthritis.[34]

After the booster, an increased number of spike-specific Th1 T-cell responders, particularly IFN-γ responders, was observed, as previously shown.[35] Despite the small number of spike responders, the greatest response to the non-specific stimulus of SEB supports the specificity and value of our data. The low number of responders could be partially explained by the highest number of PwMS under fingolimod, which reduces T lymphocytes bioavailability by retaining T cells in lymph nodes, thus affecting the T-cell response.[36]

In PwMS, the CD4$^+$ T-cell memory phenotype presented a significant reduction of naïve and $T_{CM}$ cells, while $T_{EM}$ and $T_{EMRA}$ cells increased compared with HCWs in unstimulated conditions or after spike/SEB stimulation, suggesting an association with MS condition independently of COVID-19 vaccination, as reported.[37] The chronic autoantigen exposure in MS favours the development of $T_{EM}$ rather than $T_{CM}$ cells.[38] Moreover, fingolimod acts by reversibly retaining CD4$^+$ naïve and $T_{CM}$ cells in

lymph nodes.[39] These might explain the differences observed. Interestingly, the booster dose in PwMS further increases both $CD4^+$ and $CD8^+$ $T_{EM}$ cells, making them particularly represented within memory T cells. We also confirmed that the booster dose increases $CD8^+$ $T_{EMRA}$ cells, as reported.[16] Memory T cells expand if re-challenged and contribute to prompt immune responses limiting initial viral replication, including that of the current variants,[40 41] and dissemination in the host, thereby preventing severe disease. Indeed, reduced rates of hospitalisation and complications following extensive vaccination were reported in immunocompetent and immunocompromised individuals.[42]

Some limitations are acknowledged. Although PwMS were well characterised both immunologically and clinically, the robustness of the data might be limited by the small sample size that did not allow to study in detail the impact of the different DMTs. The lower number of responders observed in the flow cytometry analysis compared with other studies[43] can be ascribed to the different experimental setting, including SARS-CoV-2 peptide composition/concentrations and the use of whole blood samples instead of peripheral blood mononuclear cells.[44] Furthermore, the limited number of HCWs analysed at T2 by flow cytometry did not allow us to perform comparison analysis with PwMS. Finally, the lacking of untreated PwMS did not allow us to verify whether the memory phenotype might be affected by DMTs. The significantly different age between HCWs and PwMS did not represent a bias for the study, as we already demonstrated that the immune impairment is associated with ongoing DMTs and/or the MS disease status more than with age.[7] Furthermore, other studies reported an age impact on the immune response in MS patients older than 60 years,[45 46] whereas our cohort is younger than 60 years.

The main strength of this study is the longitudinal observation of both T-cell and humoral response as well as the extensive characterisation of the T-cell memory phenotype before and after COVID-19 mRNA-vaccine booster dose in PwMS and HCWs.

In conclusion, this study demonstrates the benefits of the booster dose in most PwMS as it strengthens the humoral response and the T-cell response in terms of Th1 cytokine production. The increase of effector memory T cells after the booster dose highlights the value of implementing COVID-19 vaccine booster strategies in the MS population. Accordingly, COVID-19 vaccination should be strongly recommended for PwMS, and clinicians should evaluate the appropriate timing for vaccine administration and additional protective measures, particularly in anti-CD20 and fingolimod-treated patients, who may have reduced immune responses, potentially limiting vaccine efficacy.

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**Contributors** DG and CG conceived and designed the study. AA, AC and DG analysed, interpreted data and wrote the manuscript. AC, CF, AMGA, AS, VV and LP processed samples for IGRA test and/or flow cytometry analysis. SM, DL and AB performed serologic tests. CT, GC, SH, LP, SG, MEQ, SR, CG, CA and VP enrolled the subjects and collected clinical data. All the authors critically revised the article and approved the final version of the manuscript. AA and AC equally contributed to this work and shared the first authorship with SR. DG acts as the guarantor.

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**Patient consent for publication** Not applicable.

**Ethics approval** The study was approved by the Ethical Committee of INMI 'L. Spallanzani'-IRCCS (approval numbers 247/2021, 297/2021 and 319/2021). Study protocols followed the ethics principles for human experimentation in agreement with the Declaration of Helsinki. A written informed consent was signed by all participants before the study procedures. Participants gave informed consent to participate in the study before taking part.

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**Data availability statement** All data relevant to the study are included in the article or uploaded as online supplementary information.



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