Supplementary Material

MATERIALS AND METHODS

Study design and participants

This longitudinal prospective study involved the enrolment of PwMS and health care 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]). The 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.

The 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**).

Blood samples of both PwMS and HCWs were collected in heparinized tubes and processed by the same researchers' group at INMI within 2 hours from collection using a standardized protocol [24]. 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.

Anti-SARS-CoV-2 antibody assays

Antibody response was evaluated by measuring anti-nucleoprotein-immunoglobulin G (Anti-N-IgG) and anti-Receptor-Binding Domain (RBD)-IgG using commercial kits (Architect® i2000sr Abbott Diagnostics, Chicago, IL, USA). Anti-N-IgG were reported as index value (sample/Cut-off) and considered positive if ≥1.4. Anti-RBD-IgG were expressed as binding antibody units (BAU)/mL and indicated as positive when ≥7.1.

IFN-γ release assay (IGRA)

To evaluate the IFN-γ-specific T-cell response, whole blood was stimulated with a peptide mix (0.1 μg/mL of each peptide pool) covering the whole SARS-CoV-2 spike protein (PepTivator® Prot_S1, Prot_S, and Prot_S+, Miltenyi Biotec, Germany) or with the staphylococcal enterotoxin B (SEB) (Merck Life Science, cat. S4881) at 200 ng/mL, as positive control, and incubated for 20-24h at 37°C [24,25]. After incubation, plasma was harvested and IFN-γ levels were measured using an automatic ELISA (ELLA, protein simple, R&D Systems, MN, USA) as per manufacturer's instructions. IFN-γ values of the stimulated samples were subtracted from the unstimulated-control value. The detection limit of the assay is 0.17 pg/mL. IFN-γ levels ≥16 pg/mL were considered positive.

Th1 intracellular cytokines and phenotype evaluation by flow cytometry analysis

Fresh whole blood (600 µL) was overnight stimulated with spike protein or SEB together with a-CD28 and a-CD49d (1 µg/mL each). After incubation, Brefeldin A (10 µg/mL) was added for the last 5h to inhibit cytokine release. Afterwards, samples were stained with Fixable viability stain 700, fixed and then frozen [12, 23].

Stimulated cells were thawed and stained with antibodies as reported [12, 23] (see **Supplementary Table 1** for the complete list of antibodies/reagents and **Supplementary Figure 1** for gating strategy). Samples were acquired on a DxFlex cytometer (Beckman Coulter) and analysed with FlowJo software (version 10.8.1, Tree Star). We evaluated the T-cell specific response as frequency of $CD4^+$ and $CD8^+$ T cells producing only IFN-γ, IL-2 or TNF-α, or total Th1 (IFN-γ and/or IL-2 and/or TNF-α). T-cell response was defined positive if the following conditions were satisfied: 1) the percentage of SARS-CoV-2-spike stimulated cells was at least 2-fold higher than the unstimulated control; 2) at least 10 events were detected within the cytokine gate. Cytokine production of the unstimulated condition was subtracted to each stimulated condition. T-cell phenotype of total $CD4^+$ and $CD8^+$ T cells and antigen-specific T cells producing any Th1 cytokine was evaluated according to the expression of CD45RA and CCR7.

Statistical analysis

Data were analyzed using GraphPad software (GraphPad Prism v.9.3.1). Continuous and categorical variables were reported respectively as median and interquartile range (IQR) and count and proportion. The following non-parametric statistical inference tests were performed: Friedman test for comparisons among groups of paired data followed by Dunn's multiple comparisons test, Mann–Whitney and Wilcoxon signed-rank tests for pairwise comparisons (for unpaired and paired data, respectively) and Chi-square and McNemar tests for proportions (for unpaired and paired data, respectively). Two-tailed p values <0.05 were considered significant.

Supplementary Tables

Supplementary Table 1. List of antibodies and reagents for flow cytometry analysis.

	Antibodies/Reagents	Cat#	From
T-cell Panel	Fixable Viability stain 700 APC-R700	564997	BD Bioscience
	CD3 V450	560365	BD Bioscience
	CD4 ECD	6604727	Beckman Coulter
	CD8 APC-H7	611400	BD Bioscience
	CD45RA PE-CY7	337186	BD Bioscience
	TNFα FITC	552889	BD Bioscience
	IFNγ BV510	563287	BD Bioscience
	IL2 PerCp-Cy5.5	560708	BD Bioscience
	CCR7 AF647	560816	BD Bioscience
***in vitro* stimulation and flow cytometry reagents**	purified human antiCD28	555726	BD Bioscience
	purified human antiCD49d	555502	BD Bioscience
	Brefeldin A	B7450	Sigma-Aldrich
	BD FACS Lysing Solution	349202	BD Bioscience
	BD Horizon Brilliant Stain buffer	563794	BD Bioscience
	Perm Wash Buffer (Cytofix/Cytoperm solution kit)	51-2091KZ	BD Bioscience
	Formaldehyde	415666	Carlo Erba

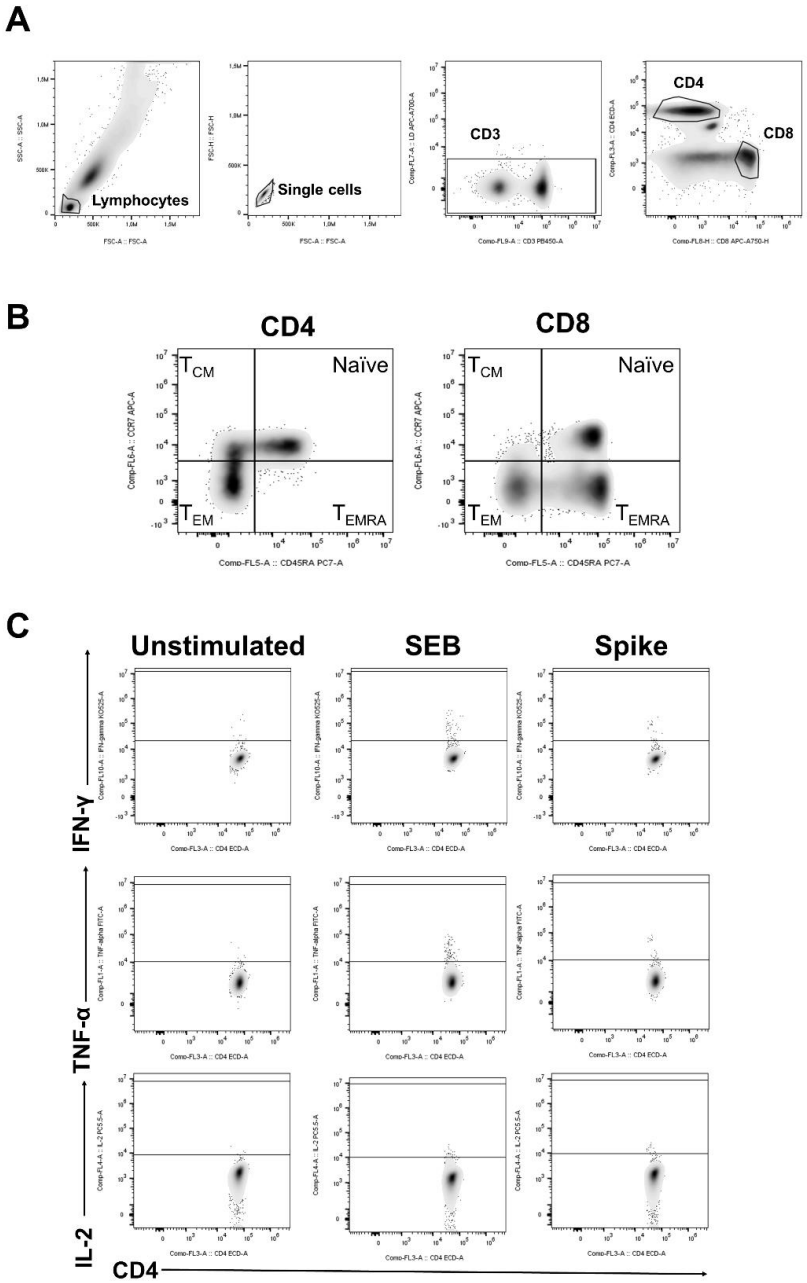
Supplementary Table 2. Impact of the characteristics of MS patients and HCWs on humoral and IFN-γ-T cell responses

		T2				T3			
	Characteristics	**Anti-RBD Abs**		**Spike IFN-γ**		**Anti-RBD Abs**		**Spike IFN-γ**	
		rho	**p**	**rho**	**p**	**rho**	**p**	**rho**	**p**
PwMS	**Age**	0.071	0.752	-0.359	0.100	0.0139	0.951	-0.041	0.853
	Male vs Female	na	0.584	na	0.05	na	0.437	na	0.05
HCWs	**Age**	-0.311	0.094	-0.238	0.241	0.022	0.907	0.061	0.749
	Male vs Female	na	0.188	na	>0.999	na	0.327	na	0.588

Footnotes: Mann-Whitney test for categorical variables; Spearman's correlation was performed for continuous variables; rho: Spearman's correlation coefficient. Na, not applicable.

Supplementary Figures

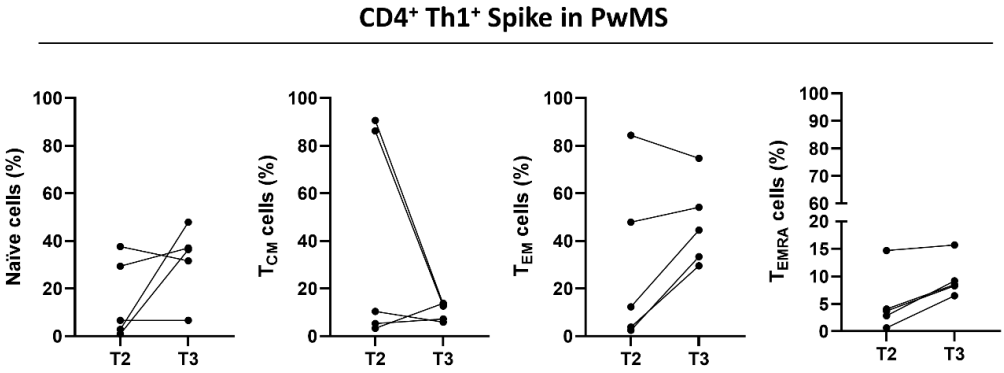
Supplementary Figure 1



Supplementary Figure 1. Gating strategy to detect SARS-CoV-2 reactive $CD4^+$ and $CD8^+$ T cells shown in a representative HCW. (**A**) Lymphocytes were gated based on FSC-A and SSC-A parameters, doublets were excluded (FSC-A/FSC-H), and $CD4^+$ and $CD8^+$ T cells were gated

within $CD3^+$ T cells. (**B**) The expression of CD45RA and CCR7 in total $CD4^+$ and $CD8^+$ T cells was used to evaluate T-cell phenotype. (**C**) Representative cytokine panels used for both $CD4^+$ and $CD8^+$ T cells are shown for unstimulated, SEB and spike conditions. Abbreviations: HCWs, health care workers; T_{CM}, central memory; T_{EM}, effector memory; T_{EMRA}, terminally differentiated effector memory; FSC, forward scatter; SSC, side scatter; SEB, staphylococcal enterotoxin B; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

Supplementary Figure 2



Supplementary Figure 2. $CD4^+$ $Th1^+$ T-cell phenotype in PwMS longitudinally sampled at T2 and T3. $CD4^+$ T-cell memory subsets were evaluated in PwMS after 6 months from the first vaccine dose (T2) and after 4-6 weeks from the booster dose (T3) in Th1-responders found at T3. Data were analysed using Wilcoxon matched pairs signed rank test and p values <0.05 were considered significant. Each dot represents a different PwMS. Abbreviations: PwMS, patients with multiple sclerosis; T_{CM}, central memory; T_{EM}, effector memory; T_{EMRA}, terminally differentiated effector memory.