Supplemental Material

SARS-CoV-2 mRNA vaccinations fail to elicit humoral and cellular immune responses in multiple sclerosis patients receiving fingolimod

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Supplemental Methods

SARS-CoV-2 pseudovirus neutralization assay

SARS-CoV-2 pseudoviruses were generated by co-transfection of plasmids encoding HIV Tat, HIV Gag/Pol, HIV Rev, luciferase followed by an IRES and ZsGreen, and the SARS-CoV-2 spike protein (Wu01 spike, EPI_ISL_406716) into HEK 293T cells using FuGENE 6 Transfection Reagent (Promega). Virus culture supernatant was harvested at 48 h and 72 h post transfection and stored at -80°C till use. Harvested virus was titrated by infecting 293T expressing ACE2[42] and after a 48-hour incubation at 37°C and 5% CO2, luciferase activity was determined after addition of luciferin/lysis buffer (10 mM MgCl2, 0.3 mM ATP, 0.5 mM Coenzyme A, 17 mM IGEPAL (all Sigma-Aldrich), and 1 mM D-Luciferin (GoldBio) in Tris-HCL) using the Tristar microplate reader (Berthold). Neutralization assays were performed as described before. Briefly, 3-fold serial dilutions of serum (1:10 starting dilution) were co-incubated with pseudovirus supernatants for 1 h at 37°C, following which 293T-ACE-2 cells were added. After 48 h at 37°C and 5% CO_2, luciferase activity was determined using the luciferin/lysis buffer. Background relative light units (RLUs) of non-infected cells was subtracted and 50% inhibitory dilution (ID_{50}) were calculated as the serum dilution resulting in a 50% reduction in RLU compared to the untreated virus control wells. ID_{50} values were calculated by plotting a non-linear fit dose response curve in GraphPad Prism 7.0.

SARS-CoV-2 spike epitope-specific peptide microarray

Peptides were synthesized and immobilized on peptide microarray slides as described previously. In brief, the peptides were synthesized using SPOT synthesis, cleaved from the solid support and chemoselectively immobilized on functionalized glass slides. Each peptide was deposited on the microarray in triplicates. The peptide microarrays were incubated with human sera (applied dilution 1:200) in a 96-well microarray incubation chamber for one hour at 30°C, followed by incubation with 0.1 µg/mL fluorescently labelled anti human IgG detection antibody (Jackson Immunoresearch). Washing steps were performed after each incubation step with 0.1 % Tween-20 in 1x TBS. After the final incubation step the microarrays were washed and dried. Each microarray slide was scanned using a GenePix Scanner 4300 SL50 (Molecular Devices). Signal intensities were evaluated using GenePix Pro 7.0 analysis software (Molecular Devices). For each peptide, the MMC2 value of the three triplicates was calculated. The MMC2 value was equal to the mean value of all three instances on the microarray except when the coefficient of variation (CV) – standard-deviation divided by the mean value – was larger than 0.5. In this case the mean of the two values closest to each other (MC2) was assigned to MMC2. Further data analysis and generation of the heatmaps was performed using the statistical computing and graphics software R (Version 4.1.1, www.r-project.org).

Ex vivo T cell stimulations

In short, PBMC were stimulated with PepMix™ SARS-CoV-2 spike glycoprotein pool 1 (JPT) covering the N-terminal aa residues 1-643 (abbreviated to "S-I") and with PepMix™ SARS-CoV-2 spike glycoprotein pool 2 covering the C-terminal part (aa residues 633-1273, abbreviated to "S-II") at a final concentration of 1 µg/ml per peptide, respectively. Stimulation controls were performed with equal concentrations of DMSO in PBS (unstimulated control) and 1 µg/ml per peptide of CEFX Ultra SuperStim pool (JPT) as positive control. All approaches contained 1 µg/ml purified anti-CD28 (clone CD28.2; BD Biosciences). Incubation was performed at 37˚C, 5% CO_2 for 16 h in the presence of 10 µg/ml brefeldin A (Sigma-Aldrich) during the last 14h. T cell stimulations were stopped by incubation in 20mM EDTA for 5 min.

B cell and T cell flow cytometry

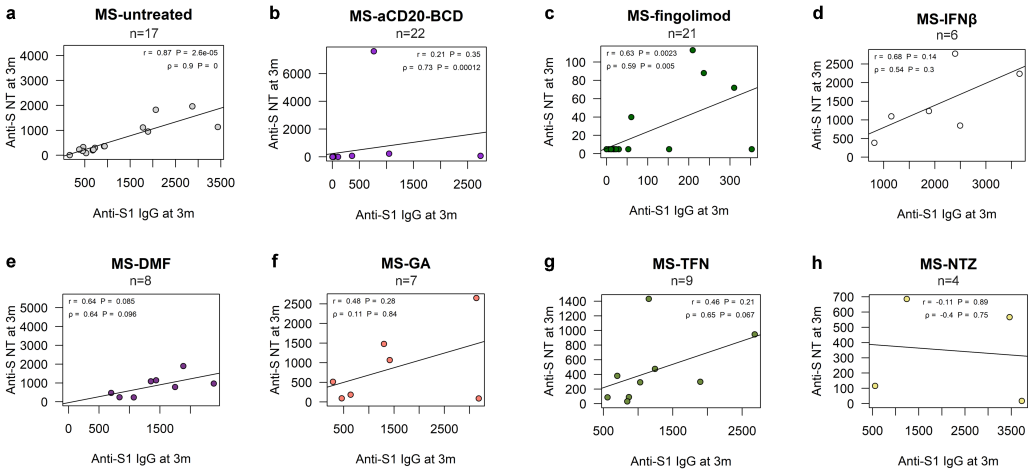
For S-I- and S-II-specific T cell analysis, antibodies were used as described before: CD3-FITC (REA613, Miltenyi), CD4-VioGreen (REA623, Miltenyi), CD8-VioBlue (REA734, Miltenyi), CD38-APC (REA671, Miltenyi), HLA-DR-PerCpVio700 (REA805, Miltenyi). For B cell status analysis: CD8 Vioblue (REA734, Miltenyi), IgD BV510 (IA6-2, Biolegend), CD14 BV570 (M5E2, Biolegend), CD21 BV605 (1048, BD), CD3 FITC (REA613, Miltenyi), SLAMF7 PE (162.1, Biolegend), CD27 PE-Dazzle594 (O323, Biolegend), HLADR Percp-Vio770 (REA780, Miltenyi), CD20 PE-

Vio770 (REA780, Miltenyi), CD38 APC (REA572, Miltenyi), CD4 AlexaFluor700 (RPA-T4, Biolegend) and CD19 APC-Vio770 (REA675, Miltenyi). For intracellular staining of stimulated T cells, fixation and permeabilization were performed with eBioscience™ FoxP3 fixation and PermBuffer (Invitrogen) according to the manufacturer's protocol. Intracellular staining was carried out for 30 min in the dark at room temperature with 4-1BB-PE (REA765, Miltenyi) and CD40L-PeVio770 (REA238, Miltenyi).

Peripheral blood B and T cell subsets were quantified using the following staining: 50µl of blood were stained for 20 minutes at room temperature in the presence of 1mg/ml Beriglobin (CSL Behring) and the following antibodies at their determined optimum titration: CD4-VioBlue (REA623, Miltenyi), CD3-VioGreen (REA641, Miltenyi), HLA-A2-FITC (REA517, Miltenyi), CCR7-PE (REA108, Miltenyi), CD8-PerCP (REA734, Miltenyi), CD31-PE-Vio770 (REA730, Miltenyi), CD19-APC (REA675, Miltenyi), CD16-AF700 (REA423, Miltenyi), CD45RA-APC-Vio770 (REA1047, Miltenyi). 500µl of cold Erythrocyte lysis buffer (Buffer EL, Quiagen) were added and incubated 30 minutes on ice. 400µl of cold PBS, BSA, 2mM EDTA were then added.

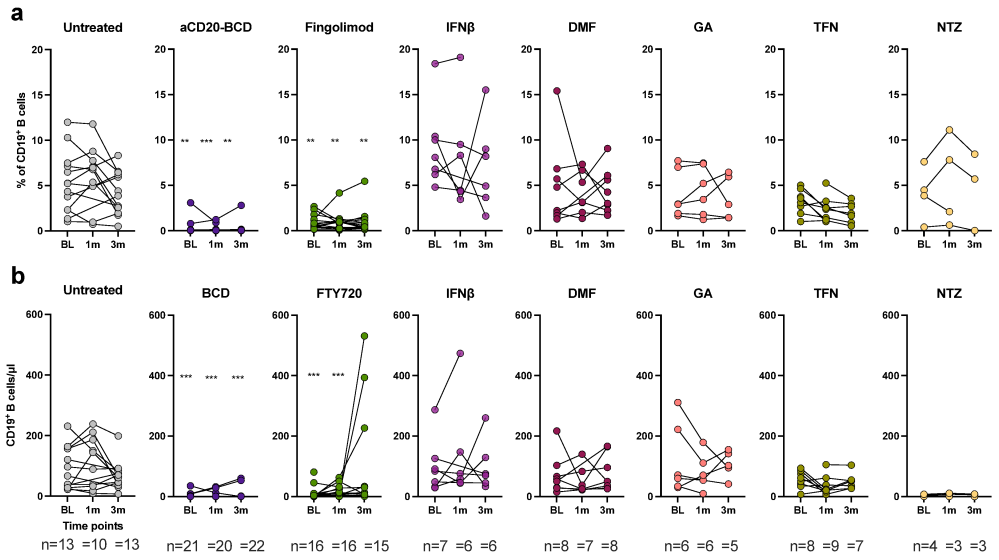
Supplemental Results

Supplemental Fig. 1



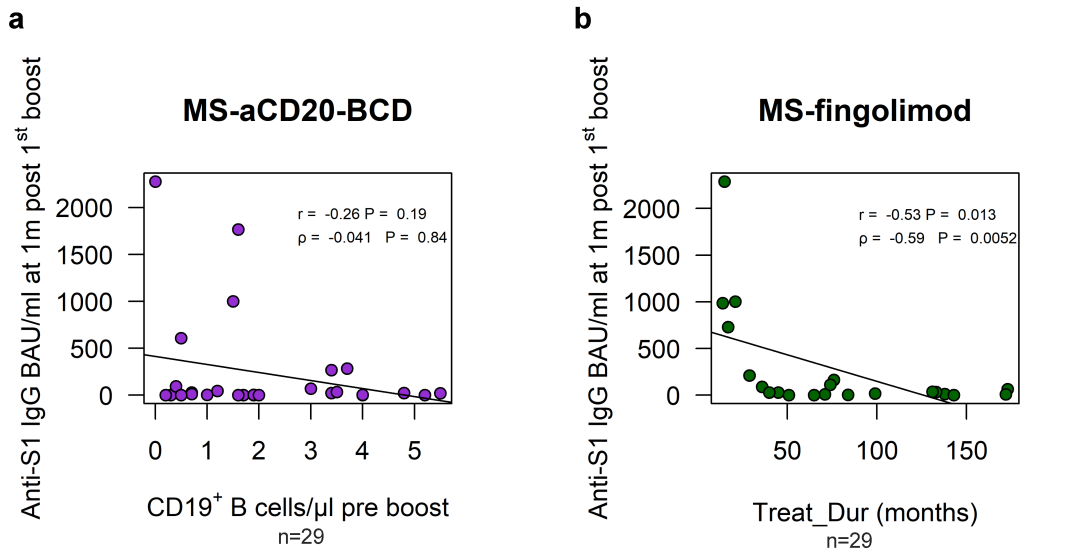
Supplemental Fig. 1. Association of anti-S1 IgG levels and neutralizing capacity. Correlation of anti-S1 IgG and neutralizing capacity in serum at 3m for untreated MS patients (MS-untreated) **(a)**, MS-aCD20-BCD **(b),** and MS-FTY720 **(c)**, MS-IFNβ **(d)**, MS-DMF **(e)**, MS-GA **(f)**, MS-TFN **(g)**, and MS-NTZ **(h)**. Simple linear regression tests performed per treatment; correlation coefficients depicted as lines and reported as r (for Pearson correlation) and rho (for Spearman rank correlation), respectively, with corresponding p-values: Spearman r value of 0.87 for untreated MS patients, 0.68 for IFNβ, 0.64 for DMF, 0.48 for GA, 0.46 for TFN, -0.11 for NTZ, 0.2 for aCD20-BCD, and 0.64 for fingolimod.

Supplemental Fig. 2



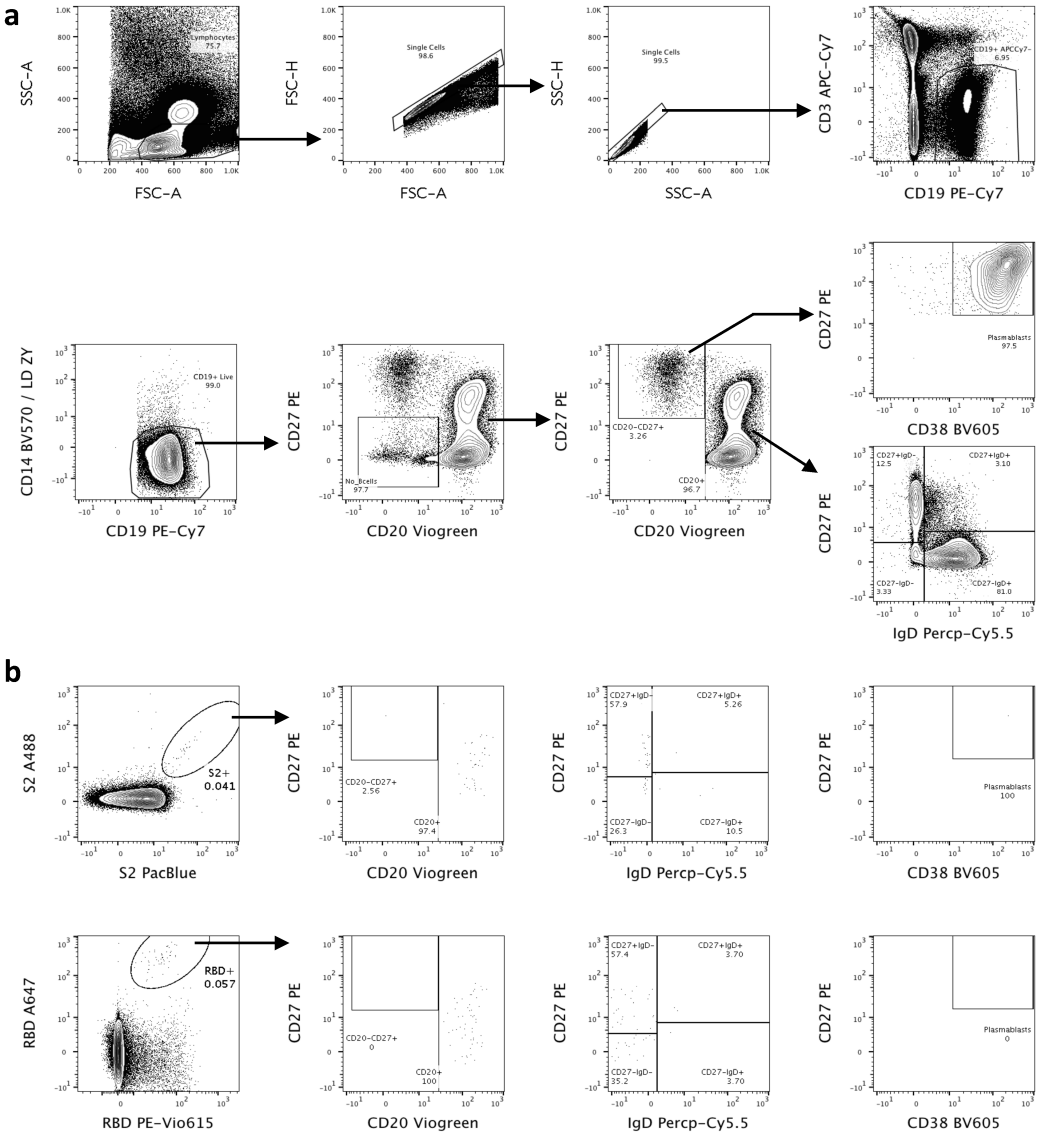
Supplemental Fig. 2. Total number and relative frequencies of peripheral blood CD19⁺ B cells. a) Frequencies of CD19⁺ B cells in total lymphocytes and **b)** total number of CD19⁺ B cells per µl per treatment group. Kruskal-Wallis test followed by Dunn's multiple comparisons test performed to test between treatment groups at the respective time point were reported for comparison to untreated patients as: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Supplemental Fig. 3



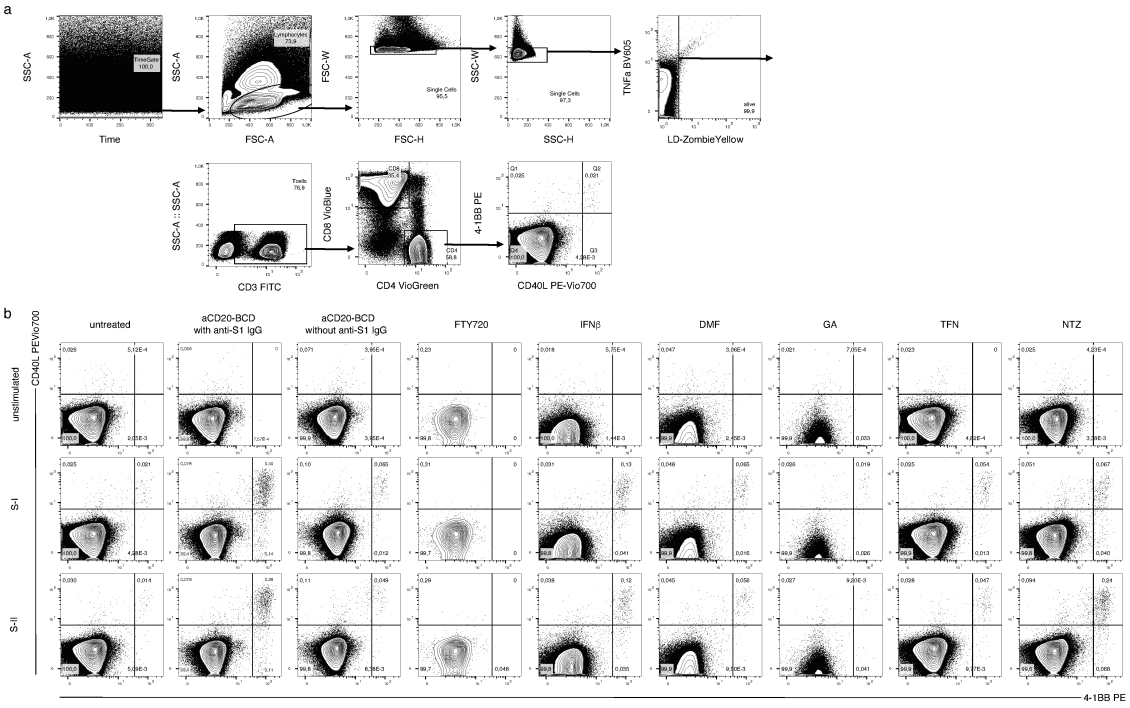
Supplemental Fig. 3. Associations of anti-S1 IgG levels 1m post 1st boost for aCD20-BCD and fingolimod. Correlation of anti-S1 IgG at 1m post 1st boost with CD19+ B cells/µl pre boost (aCD20-BCD; **a**) and fingolimod treatment duration in months (**b**) respectively. Simple linear regression tests performed per treatment; correlation coefficients depicted as lines and reported as r (for Pearson correlation) and rho (for Spearman rank correlation), respectively, with corresponding p-values.

Supplemental Fig. 4



Supplemental Fig. 4. Gating strategy of RBD- and S2-specific B cells. a, B cells are gated from peripheral blood mononuclear cells (PBMCs; FSC-A vs SSC-A), by first excluding doublets (FSC-A vs FSC-H and SSC-A vs SSC-H), gating on $CD19^{+}CD3^{-}$ B cells, excluding both dead cells (LiveDead Zombie Yellow) and $CD14^{+}$ cells, and finally by excluding $CD20^{-}$ $CD27^{-}$ cells. Among $CD19^{+}CD3^{-}$ B cells, plasmablasts were identified as $CD20^{-/low}CD27^{++}CD38^{++}$ cells. $CD20^{+}$ B cells were further classified into subpopulations based on their CD27 and IgD expression. $CD20^{+}CD27^{+}IgD^{-}$ were classified as classical memory B cells (mBC), $CD20^{+}CD27^{+}IgD^{+}$ were classified as double positive mBC (dp mbC), $CD20^{+}CD27^{-}IgD^{-}$ were classified as double negative mBC (dn mBC), and $CD20^{+}CD27^{-}IgD^{+}$ were classified as naïve B cells (nB). **b**, exemplary contour plots showing gating of S2-specific B cells and RBD-specific B cells, and again their subsequent subpopulation classification based on their CD27 and IgD expression.

Supplemental Fig. 5



Supplemental Fig. 5. Gating strategy of antigen-reactive CD4⁺ T cells and differences between DMT treatment groups. a, antigen-reactive CD40L⁺4-1BB⁺CD4⁺ T cells were gated by controlling for measurement-dependent artefacts (Time vs SSC-a) from antigen-stimulated PBMC (FSC-A vs SSC-A), by first excluding doublets (FSC-H vs FSC-W and SSC-H vs SSC-W), dead cells (LiveDead Zombie Yellow), then gating on CD3⁺ T cells and CD4⁺ double positive cells. **b**, exemplary contour plots showing CD40L and 4-1BB expression in unstimulated control, S-I-, and S-II-stimulated CD4⁺ T cells, displayed for the seven different DMT treatment groups at around two months after secondary SARS-CoV-2 mRNA vaccination.

Supplemental Table 1

aCD20-BCD treatment	Days since last treatment before 1st vaccination	CD19⁺ B cells per µl before 1st vaccination	Days since last treatment before booster	CD19⁺ B cells per µl before booster
Ocrelizumab	68	0	65	0.5
Ocrelizumab	133	0	87	0.3
Ocrelizumab	91	0.1	125	1.9
Ocrelizumab	93	0.3	143	5.5
Ocrelizumab	64	0	105	1.7
Ocrelizumab	154	35		
Ocrelizumab	162	9.2	96	3.7
Ocrelizumab	56	0.1	100	1.2
Ocrelizumab	269	8.2		
Ocrelizumab	114	0.1	360	3.4
Ocrelizumab	114	0	102	1
Ocrelizumab	144	0	116	4.8
Ocrelizumab	45	0.2	58	1.9
Ocrelizumab	114	2.5		
Ocrelizumab	94	0	84	5.2

Ocrelizumab	92	0.1		
Ocrelizumab	130	0	82	4
Ocrelizumab	97	0.7	120	0.7
Rituximab	103	0.1		
Rituximab	120	0.1	103	3.5
Rituximab	101	2.3	111	1.5
Ocrelizumab	112		179	1.6
Ocrelizumab	104			
Ocrelizumab	117		149	3.4
Ocrelizumab	92		105	0.2
Ocrelizumab	77		99	0.7
Ocrelizumab	94		314	378.1
Ocrelizumab			83	4
Ocrelizumab			99	0.2
Ocrelizumab			129	0
Ocrelizumab			45	1.5
Ocrelizumab			68	2
Ocrelizumab			92	0.5
Ocrelizumab			93	3
Ocrelizumab				
Ocrelizumab			94	1.6

Supplemental Table 1. Days since last aCD20-BCD treatment administration and absolute B cell numbers before primary and booster vaccinations. 22 patients treated with aCD20-BCD therapies were enrolled prior to primary vaccination, another 5 after secondary vaccination (before 3m) and another 9 before booster vaccinations who received either rituximab or ocrelizumab (dosage per administration: 1000 mg rituximab or 600 mg ocrelizumab) in 6-month intervals (except for one patient whose next treatment interval was delayed due to low lymphocyte counts).