**Supplemental material**

**Supplemental methods**

*Criteria for patient recruitment*

Inclusion criteria were: age 18-80 years; MS diagnosis according to the 2010 McDonald’s criteria[e1]; relapsing-remitting or progressive course according to Lublin’s criteria[e2]; disease duration ≤15 years for RRMS patients (no restriction with respect to disease duration for the PMS group). Exclusion criteria were: use of corticosteroids in the last 30 days, a relapse in the month prior to inclusion, impossibility to perform blood draw, chronic diseases other than MS, pregnancy during the course of the study. Use of DMTs was allowed. For untreated patients who had been treated previously, a washout of at least 3 months was required (6 months for ocrelizumab/rituximab; 1 year following alemtuzumab). Patients needed to be stable in their DMT for at least 1 year when treated with interferon (IFN)-beta or glatiramer acetate (GA) or least 6 months for other treatments.

*Standardization of the flow cytometry data acquisition and analysis*

**- *Isolation of peripheral blood mononuclear cells (PBMC) from blood****.* Mononuclear cells were isolated from 20 ml peripheral blood by Ficoll-gradient centrifugation (Lympholyte-H, Cedarlane), as per the SOP followed by all centers. Briefly, the undiluted blood was laid over 20 ml density gradient medium in a 50 ml Falcon tube which was centrifuged for 20 mins at 800x*g* with brakes off. The PBMC were collected from the plasma/Ficoll interface, transferred to 40 ml Ca2+- and Mg2+-free PBS, and pelleted at 420x*g* for 10 mins with centrifuge brakes on. This wash was repeated twice and the cells resuspended at a concentration of 1 x 107 cells/ml.

***- LyotubesTM and flow cytometry acquisition.*** The flow cytometry analysis was conducted on freshly isolated PBMCs (1 x 106 cells/tube) using LyotubesTM prepared by Becton Dickinson (BD, La Jolla CA, USA) specifically for this study. Ready-made tubes containing a cocktail of the relevant selected antibodies were designed by BD to enable lyophilisation so that the cocktails could be stored at room temperature where they have a shelf-life of 18 months. These LyotubesTM were “custom made” for the Sys4MS study by BD according to the selected antibody panels, with the appropriate choice of fluorophores to optimize the detection of even rare populations, allowing the identification and quantification of several populations in 3 tubes, effector T cells (T-eff), regulatory T cells (T-reg), and effector and regulatory B cells and NK cells (B/NK) tubes (see below and Fig. S1). The right combinations for the tubes were tested and optimized by BD. The LyotubesTM provided a single test, ready-to-use format that essentially only needed the addition of the cells, overcoming the unavoidable inconsistencies resulting from dispensing each antibody singly. The LyotubesTM were prepared at the beginning of the study by BD in quantities sufficient for the study (one single batch for all partners), thereby removing any possible risk of variation between centers due to type and quantity of the antibodies used. The three types of tubes (see Fig. S1) prepared to assess each PBMC sample were as follows:

i) T-eff tube to detect CD4+ and CD8+ effector T cells and broad CD4+ effector T-cell subpopulations as per the following markers: CD3+CD4+ CCR6+CXCR3hiCCR4lowCD161+ (Th17.1 cells); CD3+CD4+CCR6-CD161-CXCR3+ (classic Th1 cells); CD3+CD4+CCR6-CD161+CXCR3+ (non-classic Th1 cells); and CD3+CD4+CCR6+CD161+CCR4+ (Th17 cells)

ii) T-reg tube to detect CD4+ and CD8+ regulatory T cells: CD3+CD4+CD25highCD127- cells and CD45RA as marker to follow Treg activation; and CD3+CD8+CD28- CD127- cells

iii) NK/B tube to detect effector and regulatory NK and B cells: CD3-CD16+CD56dim (effector NK cells); CD3-CD16+CD56bright (regulatory NK cells); CD3-CD19+CD38int,CD24int (mature B cells); CD3-CD19+CD38negCD24hi (memory B cells); CD3-CD19+CD38hiCD24hi (regulatory B cells). The panels of fluorochrome-conjugated antibodies is shown in Fig. S1.

All centers used a FACSCanto II platform (BD) working with Diva version 6.1.3 or higher to ensure a consistent standardized procedure for acquisition of flow cytometry data from PBMC of MS patients and control individuals that will minimize possible differences in the different laboratories. The instruments were standardized, and standardization was maintained over time taking advantage of BD OneFlow Setup Beads, according to manufacturer’s procedures. the acquisition of the data was done in each center, the consistency of the study was further increased by centralizing the data analysis which was performed at the Genoa center, on acquired raw data sent by the other centers through appropriate channels to ensure privacy of the individual. The analyses were performed real-time, to understand if any drift was occurring in any of the centers with regard to the standardization of the machine and, therefore, to correct this in good time.

**Supplemental information related to Fig. 1**

Table: Weights of PC1 and PC2 in the PCA analyses

|  |  |  |
| --- | --- | --- |
|   | PC1 | PC2 |
| Total CD3+ | -0.4519 | 0.1959 |
| CD3+CD4+ | -0.501 | 0.1076 |
| CD3+CD8+ | 0.0142 | 0.0762 |
| CD4+CD25+CD127-(CD4+T-reg) | 0.0545 | -0.0172 |
| CD3+CD8+CD28-CD127-(CD8+T-reg) | 0.6336 | -0.0706 |
| Total CD19+ | -0.0362 | -0.0867 |
| CD19+CD24highCD38low (B-memory) | -0.1046 | 0.3132 |
| CD19+CD24lowCD38low (B-mature) | -0.1755 | -0.692 |
| CD19+CD24highCD38high (B-reg) | 0.2146 | -0.0615 |
| CD19+CD24-CD38high ( B-plasma) | 0.0491 | 0.065 |
| CD19+CD24highCD38-34 (B-memory-atypical) | -0.0259 | 0.2712 |
| CD56+ CD16 low (CD56 dim) | 0.2139 | 0.522 |
| CD56+ CD16 high (CD 56 bright) | -0.0305 | -0.0211 |
| CD3+CD4+CCR6-CD161-CXCR3+ (Th1) | 0.0344 | -2.00E-04 |
| CD3+CD4+CCR6+CD161+CXCR3-CCR4+ (Th17) | 0.0066 | 0.0017 |
| CD3+CD4+CCR6+CD161+CxCR3highCCR4low (Th1/Th17) | -0.0024 | 0.0081 |

**Supplemental references**

e1. Polman, C.H., et al., *Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria.* Ann Neurol, 2011. **69**(2): p. 292-302.

e2. Lublin, F.D., et al., *Defining the clinical course of multiple sclerosis: the 2013 revisions.* Neurology, 2014. **83**(3): p. 278-86.