

2 Detailed experimental protocol

2.1 Cell isolation

The blood from 40 naïve RRMS patients and from 20 healthy donors will be collected in heparinized tubes (Greiner Bio-One cat.no. 455051).

PBMCs will be isolated by density gradient centrifugation using Ficoll-Paque (Sigma cat. no.H8889)

2.2 Cell culture and activation

PBMC will be cultured in RPMI-1640 growth medium containing L-glutamine (Sigma R8758) supplemented with 1% penicillin, 1% streptomycin (Sigma A, cat.no. P4333) and 10% Fetal Bovine Serum (FBS Sigma cat. no. F7524) at a density of $1-2 \times 10^6$ cells/ml. PBMC will be incubated with soluble NA/LE™ format of CD3 mAb (clone BD cat. no. 555336) at 1 µg/ml final concentration in culture for 3 days. The cells will be washed than re-suspended in the culture medium at 10^6 cells/ml and distributed 96-well round bottom plate at 2×10^5 cells/well. Soluble NA/LE™ format of CD28.2 mAb (BD cat. no. 555725) will be add at 5 µg/ml final concentration.

2.3 The cytotoxic effect of the Cladribine on the target cells

Further analyses of *ex vivo* stimulated PBMC will be performed to evaluate the cytotoxic effect of Cladribine, meaning the depletion of T helper cell subsets exposed to Cladribine in their culture medium. For this purpose, we will initiate short-term cultures, the cells being incubated for additional 72 hours in the presence of Cladribine (2-Chloro-2'-deoxyadenosine-Sigma Aldrich cat. no. C4438) at a concentration range between 1×10^{-8} and 5×10^{-7} M or in the absence of the Cladribine. In this step, an important purpose will be to establish the optimal concentration of Cladribine to use in cell culture medium [14].

The immediate cytotoxicity effect of the Cladribine (at a single-cell level) will be assessed by flow cytometry in BD FACSAria III using Apoptosis, DNA Damage and Cell Proliferation Kit (BD Pharmingen cat. no. 562253).

2.4. The immunomodulatory effect of Cladribine

After the removal of Cladribine, we will analyse the immunophenotyping changes as well as the changes in the profile of cytokines secreted by the surviving T helper cells, in long-term cultures, over 21 days. We are expecting to see an anti-inflammatory shift in the cytokine profile of surviving peripheral blood mononuclear cells after the exposure to Cladribine.

The surviving cells will be transferred in Cladribine free-medium with recombinant human IL-2 (10 ng/ml; BD Pharmingen cat. no. 554603). The re-stimulation of the cells will be performed at the day 7, 14 and 21. 20 hours before re-stimulation of the cells, rhIL-2 will be removed and the viability and proliferation of the surviving cells will be re-evaluated [14].

2.4.1. The phenotypic profile assessment

The phenotypic profile of the T helper cell subsets includes cell surface markers, lineage-specific transcription factors and the intracellular cytokines. For the **assessment of their phenotype**, the cells are harvested and re-stimulated for 6 hr with PMA (5 ng/ml; Sigma, cat. no. P-8139), ionomycin (500 ng/ml; Sigma, cat. no. I-0634) in the presence of the protein transport inhibitor Monensin (GolgiStop, BD Pharmingen cat. no. 554724).

Multiparametric flow cytometry analysis with BD FACS Aria III will be performed in order to identify the phenotypic profile of T helper cells which contribute to the immunological signature in MS patients, using a first panel of cell surface markers (CD3 CD4 CD161 CCR6 CXCR3 CCR4) and lineage-specific transcription factors (T-bet and ROR- γ t) [9,14-16].

Therefore, this **first panel** will include: PE Mouse Anti-Human CD3 clone UCHT1, BD Pharmingen cat.no.555333; Pacific Blue™ Mouse Anti-Human CD4 clone RPA-T4, BD Pharmingen cat. no. 558116; PerCP-Cy™5.5 Mouse Anti-Human CD183, BD Pharmingen cat. no. 560832; APC Mouse Anti-Human CD196 (CCR6) clone 11A9 BD Pharmingen, cat. no. 560619; BV605 Mouse Anti-Human CD194 clone 1G1 BD Horizon, cat. no. 562906; BV711 Mouse Anti-Human CD161 clone DX12 BD Horizon, cat.no. 563865; BV786 Mouse Anti-T-bet clone O4-46 BD Horizon, cat.no. 564141 and Alexa Fluor® 488 Mouse Anti-Human ROR γ t clone Q21-599, BD Pharmingen cat. no. 563621. Mouse isotype controls will be also purchased. For the data acquisition, PMT voltages settings and compensation will be performed using cell surface staining controls.

Surface staining will be performed firstly, using BD Horizon Brilliant Stain Buffer (BD Horizon cat. no. 563794) more suitable for staining with panels designed with antibodies conjugated with BD Horizon Brilliant fluorescent dyes. Cells will be fixed and permeabilized and washed using BD Pharmingen™ Transcription Factor Buffer set (cat. no. 562574) which is designed for the optimal permeabilization for further staining of the proteins with intranuclear localization. The staining of the transcription factors (T-bet and ROR- γ t) will be done with BD Horizon Brilliant Stain Buffer and the fluorochrome-conjugated antibodies mentioned above.

The frequencies of cytokine IFN- γ and IL-17- producing cells will be measured with the second panel consisting of fluorochrome-conjugated antibodies which will recognize the cell surface markers and intracellular cytokines [14-16].

The **second panel** will include: PE Mouse Anti-Human CD3 clone UCHT1, BD Pharmingen cat.no.555333; Pacific Blue™ Mouse Anti-Human CD4 clone RPA-T4, BD Pharmingen cat. no. 558116; PerCP-Cy™5.5 Mouse Anti-Human CD183, BD Pharmingen cat. no. 560832; APC Mouse Anti-Human CD196 (CCR6) clone 11A9 BD Pharmingen, cat. no. 560619; BV605 Mouse Anti-Human CD194 clone 1G1 BD Horizon, cat. no. 562906; BV711 Mouse Anti-Human CD161 clone DX12 BD Horizon, cat.no. 563865; Alexa Fluor® 488 Mouse anti-Human IL-17A clone N49-653 BD Pharmingen, cat. no. 560488 and BV605 Mouse anti-Human IFN- γ clone B27 BD Horizon, cat. no. 562974.

We will perform firstly the **multicolor staining of cell surface antigens** in order to provide controls for setting proper compensation of the brightest fluorescent signals and to get the proper recognition and binding of cell surface markers which may not bind to fixed/denatured antigens. Cells will be then fixed, permeabilized and washed using BDCytofix/ Cytoperm™ Fixation/ Permeabilization Kit (BD cat. no. 554714). We will proceed with the staining of the intracellular cytokines with fluorochrome-conjugated anti-cytokine antibodies. The staining buffer will be BD Horizon Brilliant Stain Buffer (cat. no. 563794). The protocol will include negative controls, a combination of unstained cells and the isotype controls.

The **BD FACSAria III analysis** performance is comparable to state-of-the-art highly sensitive analyzers. With 4 laser beams 375/405nm, 488 nm and 633nm, flow cell with gel-coupled cuvette, octagon and trigon detection system which includes 20 detectors, the system achieves in high sensitivity and resolution, giving superior multicolor performance.

Data will be acquired through **BD FACSDiVa™** digital software. Data analysis will be performed with **FlowJo software**.

2.4.2. The secreted cytokines profile evaluation

The cytokine signature associated to T helper cells will be analysed after *in vitro* re-stimulation with CD3mAb/CD28.2 mAb at day 7,14 and 21. The supernatant will be removed after 48 h of re-stimulation and stored at -80°C until tested. The secreted cytokines profile associated to T helper cells will be measured using the FlexMap 3D Luminex Multiplex Technology and an 8-plex human customized cytokine panel including IFN- γ , IL-4, IL-5, IL-10, IL-17A, IL-21, IL-23, IL-27 built with ProcartaPlex™ Multiplex Kits from Invitrogen (Th1/Th2 Cytokine Panel (11-plex) A Cat. No. EPX110-10810-901, Th9/Th17/Th22/Treg Cytokine Panel (7-plex) B Cat. No. EPX070-10817-901) [17].

Data will be acquired and analysed with Invitrogen™ ProcartaPlex™ Analyst software. FLEXMAP 3 Luminex Technology, the most innovative flow cytometry based system which includes two class I lasers and a high-speed processor for analyzing the acquired data. The device allows multiplexing by determining up to 500 parameters of a single sample quickly and precisely through the combination of differentially dyed fluorescent microsphere sets and an innovative instrument design. The new xPONENT® 4.0 software controls the FLEXMAP 3D system and offers interfacing options for laboratory information systems as well as automation. FLEXMAP 3D can process a large number of information in a very short time. For a 96- and 384-well plate and 500 parameters the required time is 45 minutes, the final number of results being 144,000/hour with high accuracy and reproducibility. Using multiplex arrays for cytokines detection in MS patients is permitting not only to quantify in a small sample volume up to 15 cytokines, but to evaluate each parameter in the context of multiple others. The information on the research infrastructure are available at <http://erris.gov.ro/CCAMF>.

2.5. Algorithm of the protocol design

