

Specialized pro-resolving lipid mediators are differentially altered in peripheral blood of patients with multiple sclerosis and attenuate monocyte and blood-brain barrier dysfunction

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Materials and Methods

Materials

RvD1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid), PD1 (4Z,7Z,10R,11E,13E,15Z,17S,19Z)-10,17-dihydroxydocosa-4,7,11,13,15,19-hexaenoic acid), LXA4 (5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid) and LXB4 (5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid) and all other LM-SPM used herein were purchased from Cayman Chemical. TRL agonists were purchased from InvivoGen and recombinant human (rh)TNF- α from Peprotech.

MS Patients

The diagnosis of MS, for each patient was established at the end of the diagnostic protocol by clinical, laboratory, and magnetic resonance imaging (MRI) parameters, and matched published criteria. The Expanded Disability Status Scale scores (EDSS) were always < 3 for RR-MS and between 4 and 6.5 for P-MS. All patients were naïve from any first-line or second-line disease-modifying treatment, and blood collection was performed at least 1 month after the last corticosteroid therapy. In total, 14 subjects were studied during exacerbations (relapse), 12 during remissions and 12 during primary progressive phase. Duration phase for each was comparable: 3.8 years for RR-MS and 4.1 years for P-MS. Exacerbation was defined as the development of new symptoms or worsening of a pre-existing symptom, confirmed by neurological examination (number of T2 brain lesions at MRI were 10-20), lasting at least 48 hours, and occurring after a period of stability of about 30 days. Remission was defined by MS patients who were clinically stable for at least 3 months prior to enrollment and who did not present Gd-enhancing lesions on MRI. Primary progressive patients were defined as dissemination of lesions in space and time in 2 T2 lesions and a number of total lesions >20). Fifteen healthy subjects (HS, n=14), matched for age with MS patients (9 females and 6 males, mean age 36.12 \pm 1.77 years), with no history of any autoimmune or degenerative diseases of the central or peripheral nervous system, were also enrolled in this study (see Table 1 for all patient demographics). All the subjects gave their written informed consent to the study. The ethics committees of Tor Vergata Hospital and of San Camillo Hospital approved the study.

Plasma collection and cell preparation

Plasma collection was performed by centrifuging MS and HS samples at 300 RCF for 10 minutes. All plasmas were immediately stored at -80°C for LC-MS-MS-based LM metabololipidomics. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), according to standard procedures (1).

LC-MS-MS-based LM metabololipidomics

The plasma samples were transferred to 2 ml of ice-cold methanol containing the deuterium-labeled internal standards d8-5S-HETE, d4-LTB₄, d5-LXA₄, d5-RvD₂, and d4-PGE₂ (500 pg, each purchased from Cayman Chemical) to facilitate quantification and sample recovery. Samples were then centrifuged (1200 × g, 4 °C, 10 min) and solid phase C18 cartridges were equilibrated with 6ml methanol before the addition of 6ml H₂O (15). Next, 9 ml acidified H₂O (pH 3.5, HCl) was added to the samples, and loaded onto conditioned C18 columns that were washed once with 6 ml H₂O, followed by 6ml hexane. The products were eluted with 6 ml of methyl formate. Samples were brought to dryness using an evaporation system (TurboVap LV, Biotage) and immediately suspended in methanol–water (50/50 vol/vol) for LC–MS–MS automated injections.

The LC–MS–MS system employed was equipped with a Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector (Shimadzu, Kyoto, Japan), coupled with a QTrap 5500 (ABSciex, Framingham, MA). An Eclipse Plus C18 column (100 × 4.6 mm × 1.8 μm; Agilent) was kept in a column oven maintained at 50 °C (ThermaSphere TS-130; Phenomenex, Torrance, CA), and LMs were eluted with a mobile phase consisting of methanol–water–acetic acid of 55:45:0.01 (vol/vol/vol) that was ramped to 85:15:0.01 (vol/vol/vol) over 10 min and then to 98:2:0.01 (vol/vol/vol) for the next 8 min. This was subsequently maintained at 98:2:0.01 (vol/vol/vol) for 2 min, and the flow rate was maintained at 0.4 ml/min. The QTrap 5500 was operated in negative ionization mode using scheduled MRM coupled with information-dependent acquisition (IDA) and an enhanced product ion scan. The scheduled MRM window was 90 s, and each LM parameter was optimized individually. To monitor each LM and their respective pathways, an MRM method was used with diagnostic ion fragments and identification using published criteria including matching retention times to those of synthetic and authentic materials as well as at least six diagnostic ions for each LM for positive identification (2,3). Calibration curves were obtained for each using authentic compound mixtures and deuterium-labeled LM at 3.12, 6.25, 12.5, 25, 50, 100, and 200 pg (e.g., d8-5S-HETE, d4-LTB₄, d5-LXA₄, and d5-RvD₂). Linear calibration curves were obtained for each LM, which gave r² values of 0.98–0.99. Internal standard recoveries, interference of the matrix, and

limit of detection (range of 20–220 fg for the QTrap 5500 in tissue and in biological matrix) were determined.

Analysis of lipid mediators

PCA was performed using SIMCA 13.0.3 software (MKS Data Analytics Solution Umea, Sweden) following mean centering and unit variance scaling of LM amounts (2,3). PCA serves as an unbiased, multivariate projection designed to identify the systematic variation in a data matrix (the overall bioactive LM profile of each sample) with lower dimensional plane using score plots and loading plots. The score plot shows the systematic clusters among the observations (closer plots presenting higher similarity in the data matrix). Loading plots describe the magnitude and the manner (positive or negative correlation) in which the measured LM/SPM contribute to the cluster separation in the score plot. Further bioinformatic analysis was carried out by using MetaboAnalyst (<http://www.metaboanalyst.ca>), including several statistical analyses to create Volcano plots and to perform hierarchical clustering of the LM profiles.

Human Leukocyte and brain endothelial cell treatments

The PBMCs isolated from HS or MS patients were left untreated or incubated for 30 minutes with RvD1 or PD1 (10 nM) or with LXA₄ or LXB₄ (100 nM) (the most efficient working dose on immune cells, as reported (4-7) prior to be stimulated with 0,5 µg/ml Imiquimod (Toll-like receptor 7 agonist) and 0,5 µg/ml ssRNA40 (Toll-like receptor 8 agonist) for 5 hours to allow cytokine synthesis and in presence of 10 µg/ml brefeldin A to inhibit cytokine secretion. The human brain endothelial cell line hCMEC/D3 was kindly provided by Dr. Couraud (8) (Institute Cochin, Université Paris Descartes, Paris, France). hCMEC/D3 cells were grown in EBM-2 medium supplemented with hEGF, hydrocortisone, GA-1000, FBS, VEGF, hFGF-B, R3-IGF-1, ascorbic acid and 2.5% fetal calf serum (FCS) (Lonza, Switzerland) and were cultured in a 37 °C humidified atmosphere containing 5% CO₂. Cells were grown to confluency and washed two times with human endothelial serum free medium (SFM) (Invitrogen, The Netherlands) prior the treatments with TNF-α (5 ng/ml) in the presence or absence of different LXA₄, LXB₄, RvD1 and PD1 (10 nM or 100 nM) in SFM. Ethanol vehicle-treated hCMEC/D3 cells in SFM were used as control.

Flow cytometry

For leukocytes analyses, total PBMCs were stained at the cell surface with APC-e780-conjugated anti-CD14 (1:100, eBioscience), FITC-conjugated anti-CD16 (1:100, Miltenyi Biotec) and PerCP5.5-conjugated anti-CD69 (1:30, Biolegend), and made permeable with Cytfix/Cytoperm

reagents (BD Biosciences), and then stained intracellularly with phycoerythrin (PE)-Cy7-conjugated anti-TNF- α (1:100, eBioscience), Alexa 647-conjugated anti-IL-1 β (1:30, Biolegend), PE-conjugated anti-IL-16 (1:80, Pharmingen) Brilliant Violet 421-conjugated IL-12 p40/p70 (1:100, Pharmingen) and PE-conjugated IL-10 (1:50, Biolegend) in 0.5% saponin at room temperature for 30 min. Intracellular cytokines were analyzed by Cytoflex flow cytometer (Beckman Coulter, CA, USA). For each analysis, at least 100,000 live cells were acquired by gating on Pacific Orange-conjugated Live/Dead negative cells, as reported (1).

For hCMEC/D3 analyses, cells were washed with PBS and detached using collagenase type I solution (Sigma-Aldrich, The Netherlands) (1 mg/ml in PBS/0.1% BSA). Collected cells were centrifuged (1500 rpm, 5 min) and re-suspended in PBS/0.1% BSA and stained with anti-ICAM-1 (REK-1). Streptavidin-APC (BD Pharmingen, CA, USA) was used as secondary antibody. Fluorescence intensity was measured using a FACS Calibur flow cytometer (BD Bioscience, CA, USA). For SPM receptor flow cytometry analysis, Fc receptor mediated non-specific antibody binding was blocked by using Human TruStain FcX solution (Biolegend, CA, USA). hCMED/D3 were incubated with rabbit anti-DRV2 (GPR18), rabbit anti-DVR1 (GPR32), PE ERV/CMKLR1 (ChemR23), or mouse anti-ALX/FLR2, followed by non-immune rabbit IgG or non-immune mouse IgG for 30 minutes. Cells were analyzed using FACSCanto II (BD Bioscience, NJ, USA).

Real-time quantitative polymerase chain reaction

Total RNA from PBMCs was extracted with ReliaPrep RNA Cell Miniprep System (Promega, USA). A mixture containing random hexamers, oligo(dT)15 and SuperScript III Reverse Transcriptase (Invitrogen, USA) was used for complementary DNA synthesis. Transcripts were quantified by qRT-PCR on an ABI PRISM 7900 sequence detector (Applied Biosystems, USA) with Applied Biosystems predesigned TaqMan Gene Expression Assays and Absolute QPCR ROX Mix (Thermo Fisher Scientific, USA). The following probes were used (Applied Biosystems; assay identification numbers are in parentheses): GPR32 (Hs01102536_s1), FPR2 (Hs02759175_s1) GPR18 (Hs01921463_s1), ChemR23 (Hs01081979_s1), BLT1 (Hs01938704_s1), ALOX-5 (Hs00167536_m1), ALOX-12 (Hs00167524_m1), ALOX-15 (Hs00993765_g1) and COX-2/PTGS2 (Hs00153133_m1). For each sample, mRNA abundance was normalized to the amount of ribosomal protein L34 (Hs00241560_m1). For the brain endothelial cell experiments, RNA was isolated using the TRIzol® method (Life Technologies, The Netherlands) and cDNA was synthesized with the Reverse Transcription System kit (Promega, USA). The following primer sequences were used: GPR18 forward 5'-cttttgatggacaggaggtgc-3', reverse 5'-gggagtagcgtaaagccaagtct-3'; GPR32 forward 5'-catgaatgggggtctcggagg-3', reverse 5'-

ataaccacagtcagtggcg-3'; FPR2 forward 5'-acaggaatgtgaggatggg-3', reverse 5'-gctggaactgggattagggt-3'; GAPDH forward 5'-ccatgttcgtcatgggtgtg-3', reverse 5'-ggtgctaagcagttggtgtg-3'. qRT-PCR reactions were performed in an ABI7900HT sequence detection system using the SYBR Green method (Applied Biosystems, NY, USA). mRNA abundance was normalized to the amount of GAPDH.

Electric cell-substrate impedance sensing (ECIS)

hCMEC/D3 cells (3.7×10^4) were seeded on collagen-coated 96W10idf ECIS arrays (Ibidi). Trans-endothelial electrical resistance (TEER) of hCMEC/D3 cells was measured at multiple frequencies in real-time with an ECIS Z θ (theta) instrument (Applied BioPhysics, NY, USA) (9). When maximum barrier resistance was reached, cells were washed with PBS and subsequently treated with TNF- α (5 ng/ml) in the presence or absence of different SPMs (10 nM or 100 nM). Ethanol-vehicle treated cells were used as control. Subsequently, TEER was measured over time and finally analyzed to calculate the barrier resistance at each time point measured as described before (10).

ELISA

hCMEC/D3 culture supernatants harvested 24 hours after TNF- α treatment in the presence or absence of SPMs were measured for the levels of CCL2/MCP-1 by its commercial ELISA Kit (Invitrogen, The Netherlands) according to the manufacturer's instructions.

Transwell migration of monocytes

The assay was performed by using a Transwell system (Costar, The Netherlands) with polycarbonate filters pore size of 5 μ m, which were coated with collagen type 1 (Sigma-Aldrich, The Netherlands). hCMEC/D3 cells were cultured until confluence and subsequently left untreated or stimulated with TNF- α alone or in combination with different SPMs for 24 hours in SFM. Human monocytes were isolated from buffy coat as previously described (11) and resuspended in SFM in the presence or absence of different SPMs (10 nM). Ethanol-vehicle treated monocytes were used as control. Subsequently, monocytes were added to the transwell filters (1×10^5 cells/well) and incubated for 8 hours at 37°C and in 5% CO₂. To determine the number of migrated cells, transmigrated cells were transferred to FACS tubes, and 20 000 beads (Beckman Coulter, USA) were added to each sample. Samples were analyzed using a FACS Calibur (Becton Dickinson, Belgium) and the number of migrated monocytes was determined based on 5000 gated beads. The absolute number of migrated monocytes is presented compared to the total number of

monocytes added in the upper chamber as described (12). All experiments were performed in triplicate with three different human donors.

Statistical analysis

All data were expressed as means \pm SEM. Differences between groups were compared using Student's t test (two groups) or one-way ANOVA (multiple groups) followed by a post hoc Bonferroni test. The criterion for statistical significance was $P < 0.05$ or less. All statistical analyses were performed with GraphPad Prism. Flow cytometry analysis was performed using the FlowJo software (Tree Star, OR, USA).

Supplementary material

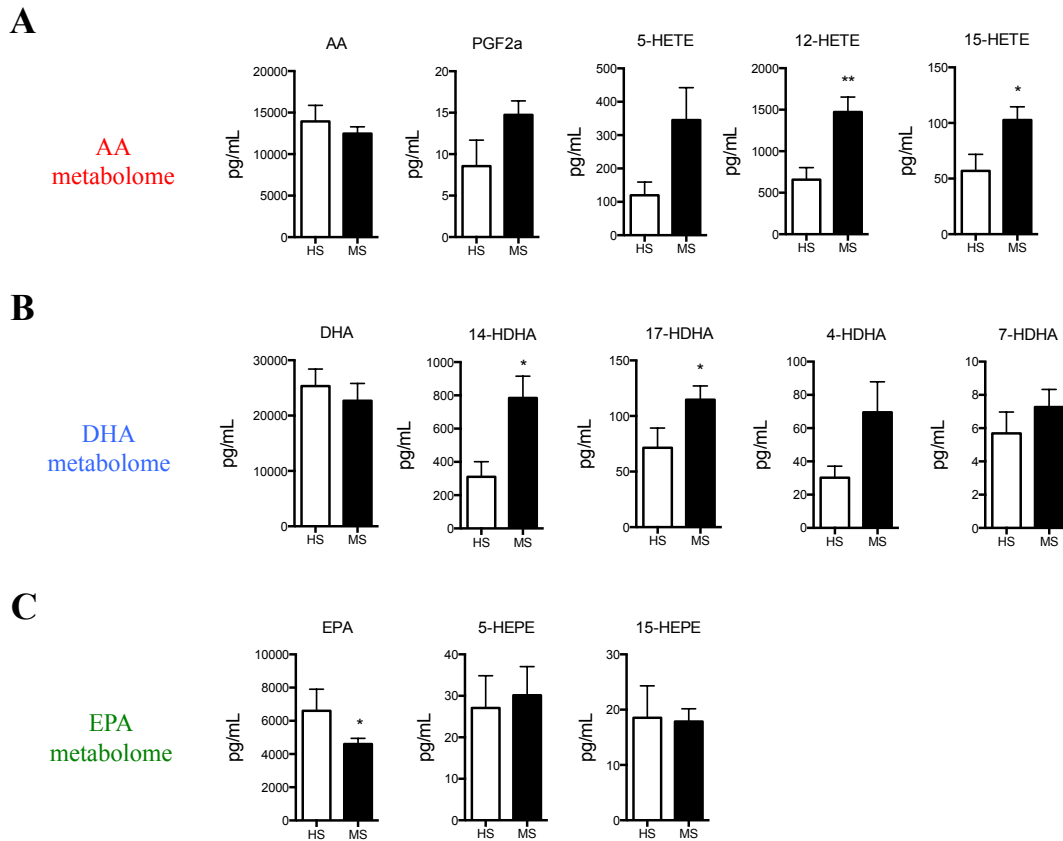


Fig.S1. MS patients show altered lipid mediators profiles in blood. Lipid mediators were isolated from plasma of healthy subjects (HS, n=15) and MS patients (n=38) and analyzed by LC-MS-MS. (A) Levels of AA-derived lipid mediators between HS and MS. (B) Levels of DHA-derived lipid mediators between HS and MS. (C) Levels of EPA-derived lipid mediators between HS and MS. Data are presented as means pg/ml \pm S.E.M. * $p < 0.05$ compared to HS, determined by Student's t test.

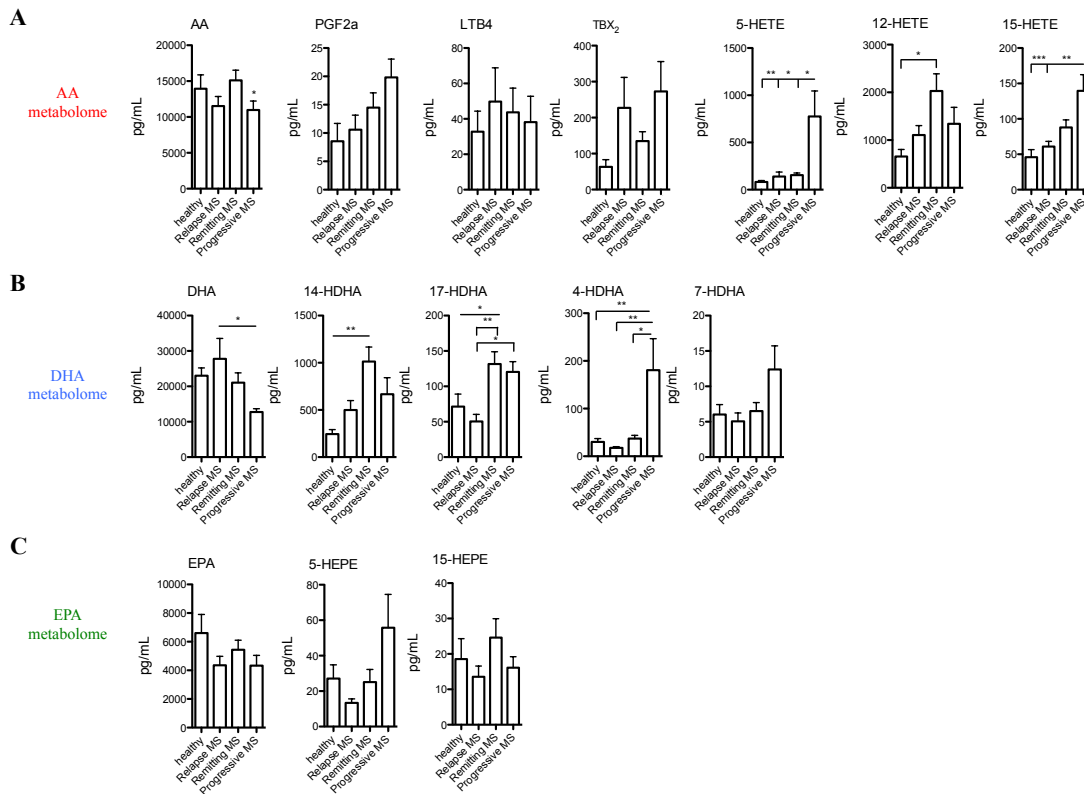


Fig.S2. Lipid mediators are differentially altered in MS patients according to clinical disease phase. Lipid mediators were isolated from plasma of healthy subjects (n=15), relapsing MS (n=14), remitting MS (n=12) and progressive MS (n=12) patients and analyzed by LC-MS-MS. Levels of lipid mediators of AA metabolome (**B**), DHA metabolome (**C**) and EPA metabolome (**D**) from healthy subjects, relapsing, remitting and progressive MS patients. Data are presented as means pg/ml \pm S.E.M. *p<0.05, **p<0.01, ***p<0.001 determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

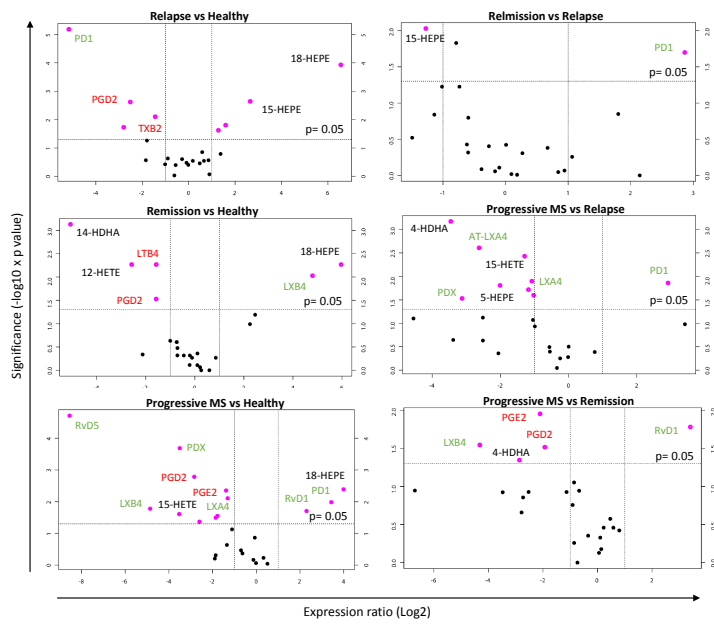


Fig. S3. *Volcano reveal lipid mediator expression levels between different subgroups.* Pink dots represent lipid mediators that exceed the minimum FDR-corrected threshold for significance ($P < 0.05$ by Bonferroni multiple correction) and fold change ($1 >$ or < 1). Pro-inflammatory lipid mediators are shown in red, anti-inflammatory/pro-resolving lipid mediators are shown in green and pathway intermediates in black.

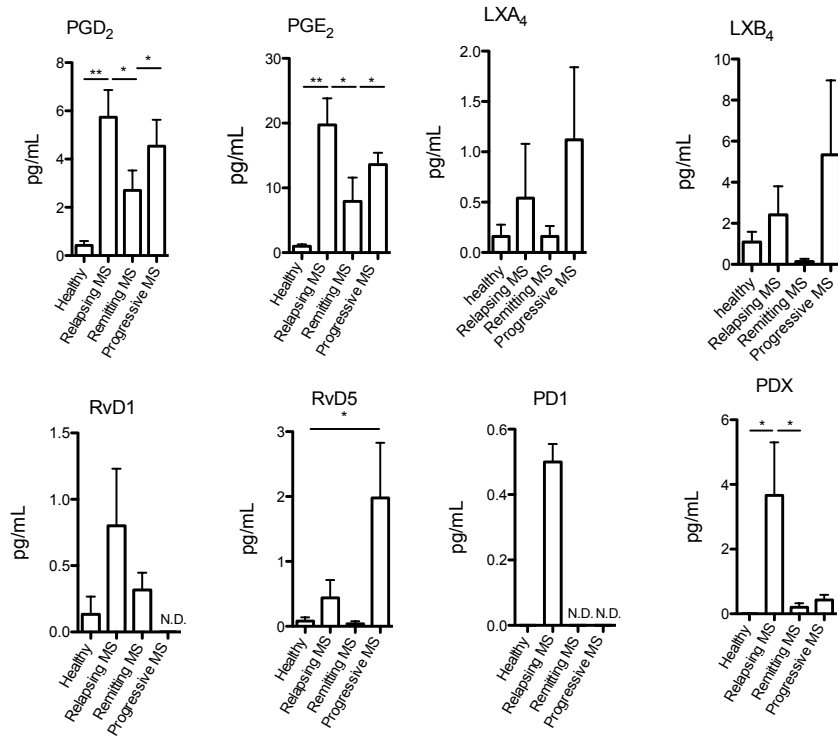
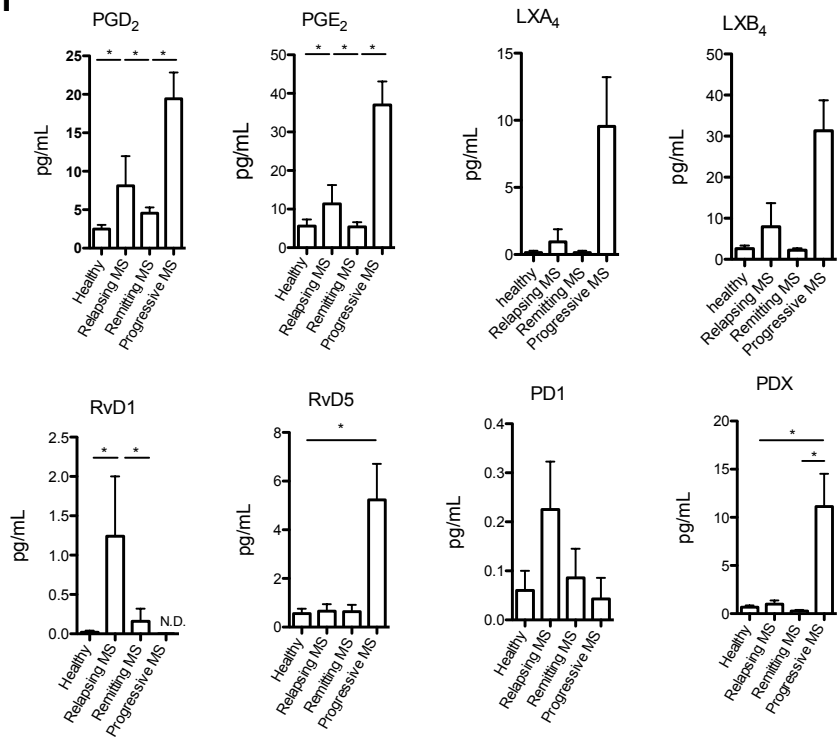
A**Cohort I****B****Cohort II**

Fig.S4. Lipid mediators profile comparison in two different cohorts of MS patients. Levels of the main pro-inflammatory and pro-resolving lipid mediators from healthy subjects (n=6 from Cohort I, n=8 from Cohort II), relapsing (n=8 from Cohort I, n=6 from Cohort II), remitting (n=6 from Cohort I, n=6 from Cohort II) and progressive (n=6 from Cohort I, n=6 from Cohort II) MS patients analyzed by LC-MS-MS. Data are presented as means pg/ml \pm S.E.M. *p<0.05, **p<0.01, determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

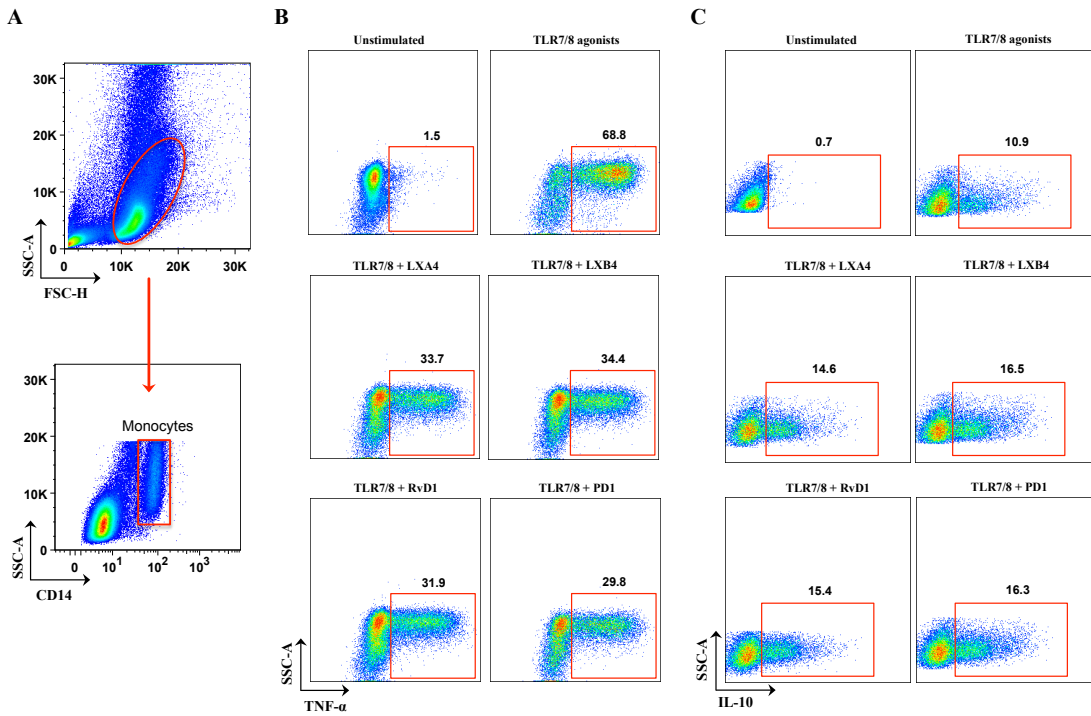


Fig.S5. SPMs reduce monocyte activation and cytokine production in healthy subjects. Peripheral blood mononuclear cells (PBMCs, 2×10^6 cells) from healthy subjects ($n=5$) were left untreated or pre-treated with LXA, LXB₄, RvD1 or PD1 for 30 min. Cells were then stimulated with Imiquimod (Toll-like receptor 7 agonist) and ssRNA40 (Toll-like receptor 8 agonist) for 5 hours in absence or presence of Brefeldin A, stained at the cell surface and intracellularly, and analyzed by flow cytometry. **(A)** Gating strategy **(B)** Representative plots of intracellular pro-inflammatory cytokine TNF- α production from CD14⁺ monocytes. **(C)** Representative plots of intracellular anti-inflammatory cytokine (IL-10) production from CD14⁺ monocytes.

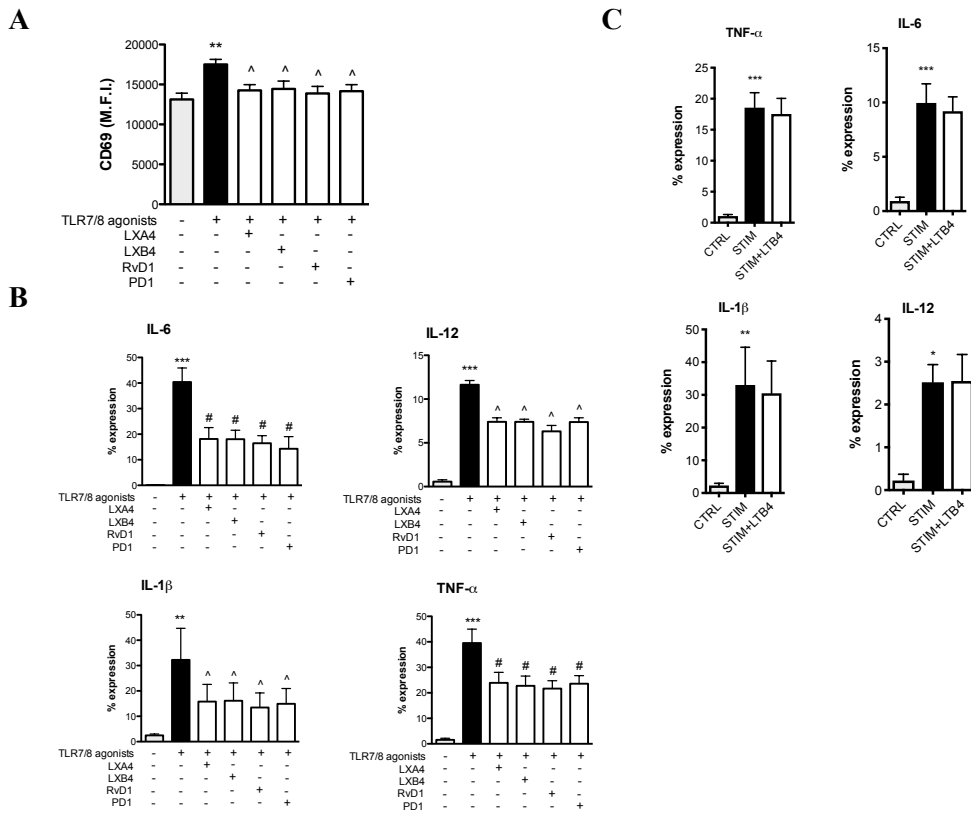


Fig.S6. SPMs reduce monocyte activation and cytokine production in healthy subjects. Peripheral blood mononuclear cells (PBMCs, 2×10^6 cells) from healthy subjects ($n=5$) were left untreated or pre-treated with LXA, LXB₄, RvD1 or PD1 for 30 min. Cells were then stimulated with Imiquimod (Toll-like receptor 7 agonist) and ssRNA40 (Toll-like receptor 8 agonist) for 5 hours in absence or presence of Brefeldin A, stained at the cell surface and intracellularly, and analyzed by flow cytometry by gating on CD14⁺ monocytes. **(A)** Surface expression of CD69 positive monocytes. Data are shown as means of fluorescence intensity (M.F.I.) \pm SEM of 5 independent experiments. ** $p < 0.01$ compared to control cells and $^{\wedge}p < 0.05$ compared to TLR7/TLR8 agonists, determined by one-way ANOVA followed by Bonferroni's multiple comparison test. **(B-C)** Percentages of intracellular cytokine production from CD14⁺ monocytes. Data are presented as means \pm SEM of 5 independent experiments. ** $p < 0.01$ and *** $p < 0.001$ compared to control cells, $^{\wedge}p < 0.05$ and # < 0.001 compared to TLR7/TLR8 agonists, determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

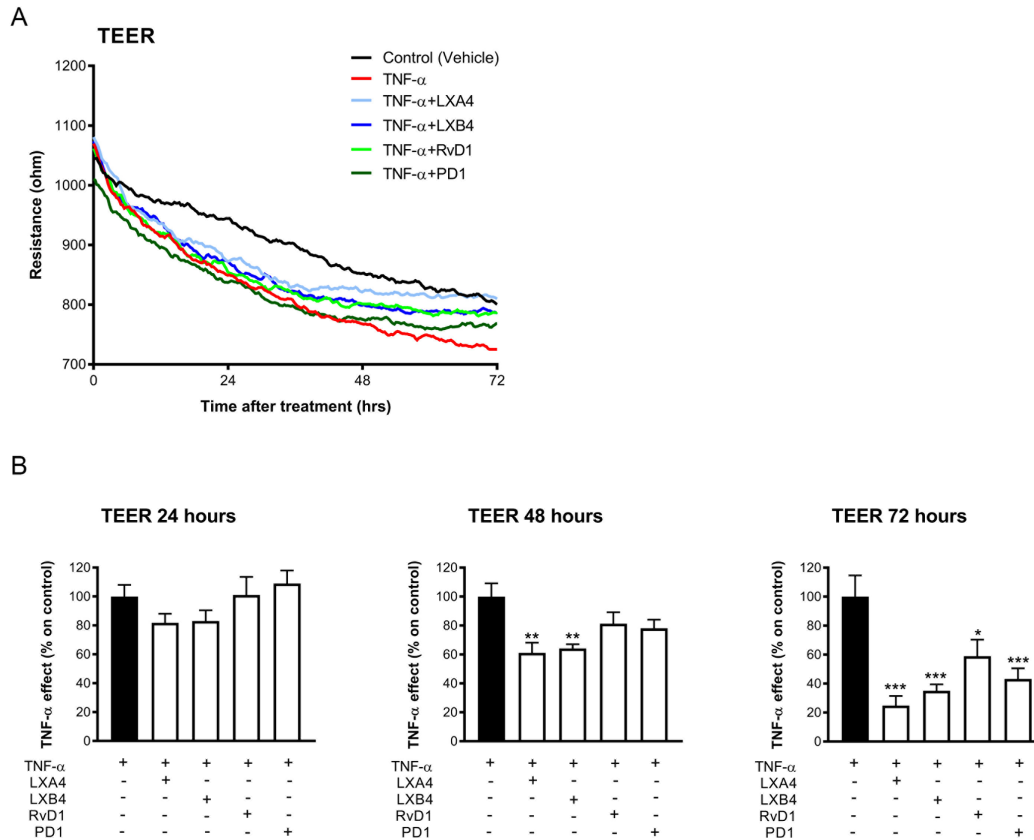


Fig.S7. SPMs improve BBB function. The functional effect of TNF- α in the presence or absence of different SPMs (100 nM) on BBB function was assessed by measuring the TEER of the BECs. Confluent BECs monolayer was treated as described and TEER was measured over time. **(A)** Data are shown as representative TEER curves of 3 independent experiments. **(B)** Graphs showing the TNF- α effect at specific selected time-points, plotted as % TNF- α effect of control BECs \pm SEM of 3 independent experiments. Statistical analysis was carried out using Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ of 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$ determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

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