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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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Running title: resolving mediators are altered in multiple sclerosis

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Abstract

Chronic inflammation is a key pathological hallmark of multiple sclerosis and suggests that resolution of inflammation, orchestrated by specialized pro-resolving lipid mediators, is impaired. Here, through targeted-metabololipidomics in peripheral blood of patients with multiple sclerosis, we revealed that each disease form was associated with distinct lipid mediator profiles that significantly correlated with disease severity. In particular, relapsing and progressive multiple sclerosis patients were associated with high eicosanoids levels, whereas the majority of pro-resolving lipid mediators were significantly reduced or below limits of detection and correlated with disease progression. Furthermore, we found impaired expression of several pro-resolving lipid mediators biosynthetic enzymes and receptors in blood-derived leukocytes of MS patients. Mechanistically, differentially expressed mediators like LXA₄, LXB₄, RvD1 and PD1 reduced multiple sclerosis-derived monocyte activation and cytokine production and inhibited inflammation-induced blood-brain barrier dysfunction and monocyte transendothelial migration. Altogether, these findings reveal peripheral defects in the resolution pathway in multiple sclerosis, suggesting pro-resolving lipid mediators as novel diagnostic biomarkers and potentially safe therapeutics.

Article Summary: Persistent peripheral inflammation is a pathological hallmark of multiple sclerosis (MS) and suggests that the resolution of inflammation, which is mediated by the recently identified “specialized pro-resolving mediators” (SPMs), is impaired. Here we show that MS patients show significant and severity-associated impairment in SPM production from peripheral blood leukocytes and that specific and differentially expressed SPMs reduced MS-derived monocyte activation and cytokine production and inhibited inflammation-induced blood-brain barrier dysfunction and monocyte transendothelial migration.
Introduction

Multiple sclerosis (MS) is the most common chronic inflammatory demyelinating disease of the central nervous system (CNS) associated to uncontrolled/excessive neuro-inflammation and autoimmunity (1,2). The underlying immunopathogenesis of the disease has been extensively studied and is currently thought to involve an initial alteration of peripheral and brain immune responses, as well as a disruption of the blood-brain barrier (BBB). Subsequently, this leads to a substantial infiltration of autoreactive lymphocytes and innate immune cells causing demyelination, axonal loss and ultimately neurodegeneration (3-6). Nevertheless, there is still an unmet need for new diagnostic and therapeutic options, especially for the progressive forms of MS for which almost no drugs are available (with the exception of the off-label rituximab and the recently approved ocrelizumab for the management of primary progressive MS). Recent studies suggest that chronic inflammation and autoimmunity could be a consequence of failure to resolve inflammation, and this resolution of inflammation is mediated by newly discovered metabolites termed specialized pro-resolving lipid mediators (SPMs) (7), temporally and spatially synthesized from \( \omega-3 \) polyunsaturated fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) (7,8).

During the process of resolution of inflammation, the very same cells recruited to the inflammatory milieu and that produce inflammatory mediators (mainly innate immune cells) undergo a temporal lipid mediator class switch, whereby they stop producing classical eicosanoids (prostaglandins, leukotrienes, thromboxanes) from \( \omega-6 \) arachidonic acid and start to biosynthesize SPMs mainly from \( \omega-3 \) EPA and DHA (7-8), through the stereoselective and concerted action of the same enzymes engaged in classical eicosanoids production, namely cyclooxygenase (COX) COX-2, lipoxygenases (LOX) LOX-5, LOX-12 and LOX-15 as well as cytochrome P450 and several pathway specific epoxide hydrolases (7). These lipid mediators are potent and extinguish the eicosanoid-induced inflammation by activating local resolution programs (7-9), also by directly modulating oxidative stress (10) and T cell responses (11), via five separate G protein-coupled receptors (e.g. ALX/FPR2, GPR32/DRV1, ChemR23/ERV, BLT1 and GPR18/DRV2) (9), without evoking unwanted side effects as opposed to the immunosuppressive agents that are most currently used as disease-modifying treatments. Despite accumulating data suggest that SPM metabolism and functions are differentially altered in several chronic peripheral and brain inflammatory diseases (7,10,12,13), research on these lipid mediators in MS and
how they attribute to disease is of high interest. On the basis of this scenario, we sought to determine whether MS patients at different phases of disease, compared to healthy subjects, displayed different levels and abilities to endogenously synthesize and respond to ω-6- and ω-3-derived pro-inflammatory (eicosanoids) and pro-resolving lipid mediators (lipoxins, resolvins, protectins and maresins) by means of targeted lipid metabololipidomics in human plasma and by evaluating the expression of their enzymes and key target receptors in peripheral blood mononuclear cells. Finally, we investigated whether specific SPMs could modulate the inflammatory response of MS patients-derived monocytes and whether they could attenuate inflammation-induced BBB dysfunction as well as monocyte-transendothelial migration, which all represent key pathological hallmarks of MS pathogenesis (14).
Methods

**MS Patients**
Peripheral blood was collected from two different cohorts. The first cohort was admitted to the neurological clinic of the University Hospital Tor Vergata (14 females and 6 males, mean 34.51±3.35 years) and the second cohort to the San Camillo Hospital of Rome (13 females and 5 males, mean 38.24±2.76 years). Both cohorts were diagnosed as suffering from relapsing–remitting MS (RR-MS, n=26) or primary progressive (P-MS, n=12). Fifteen age-matched healthy subjects (HS, n=15) were used as controls. See online methods and Table 1 for diagnosis details. 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.

**LC-MS-MS-based LM metabololipidomics and analysis**
Total lipids were extracted from plasma samples with solid phase C18 cartridges. LC-MS-MS was used to perform absolute quantifications of all lipid mediators (15,16). Lipidomics data were analyzed by PCA using SIMCA 13.0.3 software (MKS Data Analytics Solution Umea, Sweden) and by volcano plots using MetaboAnalyst (http://www.metaboanalyst.ca).

**Human Leukocyte and brain endothelial cell treatments**
Freshly isolated PBMCs from HS or MS patients were left untreated or pretreated with SPMs and then stimulated with Imiquimod and ssRNA40 for 5 hours in presence of brefeldin A. Human brain endothelial cell line hCMEC/D3 cells were grown and treated with TNF-α in the presence or absence of SPMs.

**Flow Cytometry**
PBMCs were assayed for surface immunophenotype (CD14, CD16, CD69) and intracellular cytokine production (TNF-α, IL-1β, IL-6 and IL-12) by multiple fluorochrome-conjugated antibody staining. hCMEC/D3 were assayed for anti-ICAM-1 (REK-1) and SPM receptors (GPR32, ALX/FPR2 and GPR18) through primary specific antibodies followed by fluorochrome-conjugated secondary antibodies.
**Real-time quantitative polymerase chain reaction**

Total RNA was extracted from PBMCs and hCMEC/D3, and retro-transcribed to cDNA. Specific probes for SPM receptors and SPM biosynthetic enzymes were used to assess relative mRNA abundance for each gene in respect with beta actin or GAPDH expression.

**Electric cell-substrate impedance sensing (ECIS)**

hCMEC/D3 cells were seeded on collagen-coated 96W10idf ECIS arrays (Ibidi). Trans-endothelial electrical resistance (TEER) of hCMEC/D3 cells was measured at multiple frequencies, and TNF-α was added as maximum barrier resistance was reached, in the presence or absence of different SPM. Subsequently, TEER was measured over time and finally analyzed.

**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**

In vitro monocyte transendothelial cell migration assay was performed using a collagen 1-coated Transwell system. Briefly, hCMEC/D3 cells were cultured alone or with TNF-α upon which resting or SPM-treated purified human monocytes were added to the transwell filters. Transmigrated cells were determined through flow Cytometry.

**Statistical analysis**

All data were expressed as means ± 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.
Results

*MS patients show altered lipid mediators profiles in the blood*

To address potential differences in lipid mediator (LMs) profiles between MS patients and healthy donors, involving both pro-inflammatory and specialized pro-resolving lipid mediators (SPMs), we performed targeted lipid mediator metabololipidomics on human plasma samples using LC-MS-MS in two different cohorts of MS patients, analyzing 42 distinct LMs from the endogenous substrates AA, DHA and EPA based on published criteria for each LMs (i.e. matching chromatographic retention times (RTs), fragmentation patterns, and six characteristic and diagnostic ions) (15,16). This analysis revealed a pronounced biosynthesis of 27 of these lipid mediators in the blood of healthy donors and MS patients (Table 2), and the identification of key lipid mediators, including leukotriene B4 (LTB4), resolvin (Rv) D1, RvD5 and protectin D1, as shown in Figure 1. Quantitation of lipid mediators was performed using signature ion pairs via multiple reaction monitoring (MRM) and revealed marked differences in several lipid mediators of each metabolome between MS patients and healthy subjects (Figure 2). In particular, total MS patients showed significantly higher blood levels of many AA-derived pro-inflammatory lipid mediators, such as prostaglandins (PG) PGE2, PGD2 (Figure 2A) and hydroxyeicosatetraenoic acids (HETE) 12-HETE and 15-HETE (Figure S1A) as well as increases, yet not significant, in leukotriene B4 and in AA-derived pro-resolving mediators lipoxins (LX)A4 and LXb4 (Figure 2A). Furthermore, as for lipid mediators derived from the DHA metabolome, we observed that MS patients displayed significantly higher levels of pathway markers 17-HDHA and 14-HDHA (Figure S1B); however, among the 10 possible DHA-derived SPMs (D-series resolvins, protectins and maresins), only 4 were detected, e.g. RvD1, RvD5, PD1 and PDX, and these were all generally increased in MS patients, with PD1 and PDX being significant (Figure 2B). Of note, other DHA-derived SPMs, such as RvD2, RvD3, RvD4 RvD6, AT-RvD1, AT-RvD3 and maesin (MaR) 1 were undetectable (Table 2). Among the EPA-derived lipid mediators, only 12-HEPE was significantly higher and 18-HEPE was slightly lower in MS patients; levels of E-series resolvins (RvE 1-3) were not identified in these patient samples (Figure 2C and Table 2). Of interest, the total levels of AA and DHA were almost unchanged between MS patients and healthy subjects, while those of EPA were significantly reduced (Figure S1). We next observed that all the lipid mediators
that were increased in MS significantly correlated with disease severity, evaluated as EDSS, except for LTB\(_4\) and EPA-derived 12-HEPE and 5-HEPE (Figure 3). In contrast, RvD1 and PD1 showed a negative correlation, inasmuch as their levels progressively decreased with clinical score (Figure 3B). Interestingly, we did not observe any correlation with the age and gender of patients, whereby levels of SPMs were fairly constant in both males and females (data not shown).

**SPMs, their biosynthetic enzymes and receptors are differentially expressed in MS patients according to disease phase.**

Since MS is characterized by different and independent forms of the disease (1-3), we next stratified the metabololipidomics analysis according to disease clinical subtype. Using unbiased principal component analysis (PCA, Figure 4A), we observed that each form of disease and healthy subjects were associated with distinct lipid mediator profiles. Indeed, relapse patients were associated with a cluster characterized by few lipid mediators, including PD1 and TXB\(_2\) and remitting patients with a cluster that included several HEPEs and HETEs as well as LTB\(_4\) and RvD1, while progressive patients gave a cluster that included the most abundant and diversified lipid mediators, from pro-inflammatory PGE\(_2\) and PGD\(_2\) to anti-inflammatory LXA\(_4\) and LXB\(_4\). In particular, ANOVA analysis showed that many AA-derived pro-inflammatory and pro-resolving mediators, including PGD\(_2\), PGE\(_2\), LXA\(_4\), LXB\(_4\) (Figure 4B) as well as TXB\(_2\) (Figure S2A) followed a similar trend, being generally increased in both relapsing MS and progressive MS patients compared to healthy subjects (with PGD\(_2\) and PGE\(_2\) being also significant for relapsing MS and LXA\(_4\) and LXB\(_4\) for progressive MS), while showing reduced levels in remitting MS patients compared to clinically active forms. For all of these lipid mediators, progressive patients consistently displayed higher levels compared to relapsing patients (Figure 4B-C). Of note, levels of their precursor AA moved in the opposite direction (Figure S2A), suggesting an active metabolic conversion into such lipid mediators associated to disease-phase. Other key metabolites of AA, including HETEs, PGF\(_{2\alpha}\) and LTB\(_4\), appeared with specific trends of expression, with 5-HETE being mostly found in progressive MS and 12-HETE in remitting MS, while 15-HETE and PGF\(_{2\alpha}\) steadily increased along disease forms (Figure S2A). Levels of aspirin-triggered (AT) lipoxins were detected only for AT-LXA\(_4\), which was particularly present in progressive patients (Figure 4B and Table 2). As for the DHA metabolome, pro-
resolving lipid mediators RvD5 and PDX were slightly increased in relapsing MS and significantly increased in progressive MS, with remitting MS showing similar levels compared to healthy subjects (Figure 4C). Furthermore, RvD1 and PD1 were increased in relapsing MS, with the latter being also significant and showing a reduction in both remitting and progressive patients, while the former being reduced in remitting MS and undetectable in progressive MS (Figure 4C). Of interest, the levels of their precursor DHA, although showing an initial, yet not significant, increase in relapsing MS, were progressively reduced along disease forms (Figure S2B). Other pathway makers and metabolites of DHA were significantly increased, especially in remitting (14-HDHA and 17-HDHA) or progressive (17-HDHA and 4-HDHA) patients (Figure S2B). As for EPA metabolome, levels of 15-HEPE and 18-HEPE were reduced in relapsing and progressive MS, returning back to control levels in remitting MS, showing a similar trend as their precursor EPA (Figure 4D, and Figure S2C). However, 12-HEPE was particularly high in both relapsing and remitting MS (Figure 4E). Specific LM fingerprints are also shown by volcano plots when comparing every two groups against each other, with SPMs like RvD1 and PD1 being reduced along disease progression and others like LXB4 and pro-inflammatory prostaglandins being produced during the active phases of disease, especially in progressive patients (Figure S3).

Of note, both cohorts of healthy donors and MS patients displayed an almost identical LM profile (Figure S4), with pro-inflammatory AA-derived prostanoids being induced in relapsing and progressive patients and reduced during remission, whereas lipoxins being slightly induced during the relapsing phase and even more during the progressive phase. As for the DHA- and EPA-derived SPMs, both cohorts showed an induction of RvD1 and PD1 during the relapsing phase, which were both reduced or even undetected along disease progression, and showed a significant induction RvD5 in progressive patients. The only SPM that was discordant was PDX, being significantly induced during the relapsing phase in the first cohort of MS patients, whereas in the second cohort PDX was markedly induced in progressive patients (Figure S4).

Having observed that each clinical form of MS is characterized by differential profiles in the levels of SPMs, we next sought to evaluate whether this was associated to contradistinctive capacities to produce them and/or to respond to them. Thus, we further characterized the different forms of MS by investigating the mRNA
expression of the main SPM biosynthetic enzymes and their known receptors in peripheral blood leukocytes. While COX-2 and 5-LOX were particularly induced in relapsing MS to be then strongly reduced in both remitting and progressive patients, 15-LOX was consistently found in all MS forms (Figure 5A). On the contrary, 12-LOX expression was antithetical, inasmuch as its levels were substantially reduced in relapsing and remitting MS and started to recover in progressive MS patients (Figure 5A). As for SPM receptors expression, ALX/FPR2, DRV1 and ERV all displayed a similar pattern, with their expression levels being induced in relapsing MS, reaching their highest expression in remitting MS and then exhibiting a reduction in progressive MS (Figure 5B). In addition, while BLT1 receptor expression was induced only in the active phases of the disease, DRV2 was strongly induced in relapsing MS and markedly reduced in both remitting and progressive patients, to expression levels much lower than healthy subjects (Figure 5B).

Specific specialized pro-resolving mediators attenuate monocyte inflammatory responses in MS patients.

The observed differences of MS patients in producing distinct SPMs profiles prompted us to examine whether peripheral blood leukocytes of MS patients were responsive to the immunomodulatory activity of disease-affected SPMs. Accordingly, we tested the ability of SPMs that showed an initial induction in relapsing MS patients and are subsequently diminished along disease progression (RvD1 and PD1) and SPMs that showed a higher expression in relapsing MS patients and no induction in remitting MS (LXA4, LXB4) to evaluate their potential to affect the activation and cytokine production of activated monocytes obtained from RRMS patients, i.e. in the disease phase where such mediators were initially increased and then decreased. To do so, we analyzed the expression of activation markers and inflammatory cytokines in SPM-treated monocytes that were then challenged with two different viral Toll-like receptors (TLR) agonists, i.e. TLR7 and TLR8 (see Figure S5A for gating strategy). As expected, the simultaneous stimulation of monocytes of relapsing MS patients with selective agonists of viral Toll-like receptors (TLR) 7 and TLR8 induced a strong upregulation of the activation marker CD69 on their cell surface compared to resting monocytes (Figure 6A). Treatment of activated monocytes with LXA4, LXB4, RvD1 or PD1 caused a significant reduction of CD69 surface expression (Figure 6A), indicating an overall ability of these SPMs to attenuate the general activation of
myeloid cells. More specifically, all these tested SPMs were equally able to significantly reduce the intracellular production of several pro-inflammatory cytokines. Indeed, the high levels of TNF-α, IL-1β, IL-6 and IL-12 production from activated monocytes of relapsing MS patients were equivalently and significantly reduced by LXA₄, LXB₄, RvD₁ and PD₁, although RvD₁ and PD₁ showed a higher vigor in reducing IL-6 production (Figure 6B and Figure S5B). The immunomodulatory activity of these SPMs was even more evident in activated monocytes of healthy donors (Figure S6A-B) inasmuch as all tested SPMs induced an even stronger reduction of CD69 and of all pro-inflammatory cytokines, suggesting that although their pro-resolving actions are equally functional in health and disease, cells of MS patients are likely less responsive to SPMs. Of note, besides reducing pro-inflammatory cytokines production, LXA₄, LXB₄, RvD₁ and PD₁ all equally enhanced the production of the typical anti-inflammatory cytokine IL-10 produced from TLR7/8-activated monocytes (Figure 6C). Incidentally, the SPM-induced effect on cytokine reduction was not observed with monocytes treated with the pro-inflammatory lipid mediator LTB₄ (Figure S6C).

Specific specialized pro-resolving mediators counteract blood-brain barrier dysfunction and attenuate monocyte transendothelial migration.

A key pathological feature of MS is BBB dysfunction that ultimately leads to monocytes transmigration into the CNS by crossing the inflamed and disrupted BBB. Therefore, we addressed the question whether the differentially expressed SPMs were able to counteract inflammation-induced BBB dysfunction, by using human brain endothelial cells (BECs) as a BBB model. We first assessed if these cells responded to SPMs by looking at the expression pattern of specific SPMs receptors. We found that BECs mainly express ALX/FPR2 and to a lesser extent GPR18/DVR2 and GPR32/DVR1 (Figure 7A-B). Interestingly, such marked ALX/FPR2 expression was even more evident when BECs were stimulated with TNF-α, inasmuch as inflamed cells underwent a significant upregulation of ALX/FPR2 mRNA, while only showed a slight increase in GPR18/DVR2 and GPR32/DVR1 expression (Figure 7C). Next, we assessed whether LXA₄, LXB₄, RvD₁ and PD₁ were capable to counteract inflammation-induced BBB dysfunction by measuring the trans-endothelial electrical resistance (TEER) in real-time. We found that these SPMs were able to rescue the TNF-α-mediated decrease in TEER in a time-dependent manner, at both a 10 nM
(Figure 7D-E) and 100 nM concentration (Figure S7), with LXB₄ starting to have a significant effect as early as 24h (at 10 nM but not at 100 nM) and with all SPMs significantly rescuing TEER after 72h (at both concentrations). Of note, no dose-dependency in SPM potency in rescuing TEER was observed, except for 100 nM LXA₄ that showed a significant impact at 48h. In view of these results, we next sought to investigate the SPM effect on monocyte transendothelial migration by using this human BBB model. As shown in Figure 7F, treatment with LXA₄, LXB₄, RvD1 or PD1 significantly inhibited the migration of monocytes across BECs and this action was associated with a significant reduction in the expression of endothelial adhesion molecule ICAM-1 and chemokine CCL2 (Figure 7G-H), thereby accounting for a potent anti-inflammatory action of the pro-resolving lipid mediators in preventing inflammation-induced BBB dysfunction.

Discussion

This study provides an unprecedented comprehensive overview of the lipid mediator (LMs) signature in plasma from MS patients with different clinical forms of the disease compared to healthy controls. Targeted LM metabololipidomics using LC-MS-MS with subsequent analyses revealed that relapsing MS patients display most of AA-derived prostaglandins (i.e. PGD₂ and PGE₂) as well as of DHA-derived SPMs like PD1 and RvD1 (and partly PDX), which were all reduced in remitting MS patients. In addition, progressive MS patients not only were characterized by the co-presence of all pro-inflammatory mediators (all of them even in higher levels than relapsing patients) but also on the appearance of some AA-derived or DHA-derived SPMs, such as LXA₄ and LXB₄, RvD5 and PDX. Of note, the levels of PD1 and RvD1 where significantly reduced or even undetected along disease progression.

In line with the general concepts that bioactive lipid mediators undergo temporal and spatial production during inflammation, that SPMs appear at the peak of acute inflammation in order to later reduce inflammation by activating endogenous resolution programs (7,10) and that chronic inflammation may result from failed resolution mechanisms (12,13), our results display that during the acute phase of the disease (relapsing form) there is an imbalance between pro-inflammatory and pro-resolving lipid mediators, in favor of the former, and an insufficient or lack of expression of many key SPMs (including E-resolvins, maresins and the rest of D-resolvins), which may in turn affect the outcome of remission and thereby, in theory
and yet to be fully elucidated in further studies, even lead to disease progression. Indeed, over 80% of individuals with MS initially develop a clinical pattern with periodic relapses that reflect acute inflammation in the CNS and myelin disruption as well as induced activation of innate immune cells and pro-inflammatory mediators in peripheral blood, followed by continuous remissions during which self-remyelination occurs and symptoms decrease or temporarily disappear (6,17). Repeated relapses and remissions lead to less and less effective remyelination, appearance of scar-like plaques (scleroses) and thus after 10 to 20 years, patients might evolve into a progressive form of the disease, characterized by an irreversible disruption of peripheral and central immune tolerance, neurodegeneration and permanent cortical and subcortical grey matter atrophy (18). More than a dozen disease-modifying (and mostly anti-inflammatory) agents are available to reduce the frequency of transient episodes of neurologic disability and limit the accumulation of CNS lesions, but these systemically applied agents not only are exclusive for RR-MS patients and not for progressive patients, but also result in severe side-effects and none of these prevents nor reverses the neurological deterioration (19). Therefore, we set out to investigate whether impairments of endogenous processes to resolve inflammation correlate with MS progression to ultimately provide tools to either slow down inflammatory activation and simultaneously promote neuroprotection or prevent disease progression. The primary objective of our study was to identify pro-inflammatory and pro-resolving lipid mediators and validate their structures in peripheral blood of MS patients and healthy controls and secondarily, to correlate levels with clinical outcomes. To this aim, targeted metabololipidomics allowed us to unveil the full spectrum of lipid mediators in plasma samples of healthy donors and of MS patients with different clinical disease forms and to find that acute MS patients are able to produce only very few SPMs (i.e. lipoxins, RvD1 and PD1). This is suggestive of a defective resolution program during MS that could not only result in a partial recovery, but could also eventually increase the probability to evolve into the progressive form, as substantiated by an inverse correlation of such SPMs with clinical severity. Our observed presence of high levels of few SPMs (LXA₄, LXB₄, RvD5 and PDX) in progressive MS, which instead positively correlate with clinical severity, is indicative of a last, yet insufficient, attempt of the body to respond to an even higher inflammatory status, where all pro-inflammatory lipid mediators are consistently produced in high amounts and are also associated to disease severity.
This is particularly relevant for LXA₄ and LXB₄, that are metabolically derived from arachidonic acid and that are the actual initiators of the metabolic switch from the omega 6 pro-inflammatory eicosanoids to the omega 3 SPMs. Indeed, SM patients attempt to induce a compensatory boost of these two lipoxins in order to promote the subsequent production of all SPMs, which is reflected only in an increased production of RvD5 and PDX (whose potency is much lower than its stereoisomer PD1) and not by an induction of all other SPMs. Of note, typical SPMs that are usually produced later in the inflammatory process and that appear also during chronic stages, namely RvD3 and RvD4, are undetected in all MS phases, further suggesting that although progressive patients endeavor in a last attempt to boost a lipoxin-mediated metabolic switch towards SPMs, it’s not followed by an actual SPM production.

Our findings are in line with the only other study that analyzed few of such lipid mediators in MS, whereby RvD1 and PD1 were induced in highly active MS patients (20). However, this study, which was performed on cerebrospinal fluid samples, not only analyzed a smaller cohort of patients and did not take into considerations healthy subjects, but was only able to detect only one third of the lipid mediators that were, instead, measured herein. Furthermore and most importantly, our metabololipidomics analysis was performed on three clinically distinct MS forms, which included not only MS with active relapse phases but also patients with clear signs of remission or progression, allowing us to have a complete overview of a vast array of lipid mediators and how they vary along disease phases and progression.

The recent evidence that several chronic inflammatory diseases are associated with altered SPM metabolism also support our findings. Indeed, decreased production of lipoxins and resolvins (especially RvD1) have been linked to the pathogenesis of chronic obstructive pulmonary disease (21), type-2 diabetes and obesity (22,23), inflammatory bowel disease (24,25) and rheumatoid arthritis (26). Also, an imbalance between pro-inflammatory leukotrienes and pro-resolving SPMs was observed in atherosclerosis (27). Of note, the notion that also neuroinflammatory and neurodegenerative diseases might be linked to a dysfunctional resolution of inflammation is very recent and an impaired pro-resolution pathway, involving both specific SPMs (i.e. LXA₄ and RvD1) and their receptors was found in post-mortem brain tissues of Alzheimer’s disease patients (28,29), where clinical trials with DHA show a reduced peripheral inflammation associated with increases in specific SPMs (30). Accordingly, our observed significant and progressive reduction of DHA during
MS, reaching very low levels in progressive patients, once again support a defect in producing its SPM derivatives; in line with this, Holmann and colleagues described deficiencies in PUFAs and subsequent replacement by non-essential fatty acids in MS (31). Along these lines, untargeted metabolomics analysis of plasma samples derived from mice with experimental autoimmune encephalomyelitis (EAE), the most commonly used animal model for MS, revealed similar profound alterations in the omega-3 and omega-6 PUFA pathways, with several metabolites of PUFAs being significantly lower EAE mice including RvD1 (32). Importantly, RvD1 supplementation ameliorated clinical signs of EAE, illustrating *in vivo* efficacy of SPMs during neuro-inflammation (32). Epidemiological studies suggest that in particular omega-3 PUFA supplementation is linked with improved clinical outcomes in patients with MS (19,33,34). However, although the levels of AA and DHA could be restored by supplementation in MS patients, the efficacy of PUFA supplementation remains to be established.

Next, we further investigated the profile of peripheral blood leukocytes and our analysis revealed distinctive expressions patterns of SPM biosynthetic enzymes and receptors in each clinical form of MS, with relapsing MS patients showing increased expression of COX-2 and 5-LOX as well as of all 5 identified receptors (ALX/FPR2, GPR2/DRV1, GPR18/DRV2, ChemR23/ERV and BLT1). Interestingly, 12-LOX, which is responsible for maresins production, was consistently lower in all MS phases compared to healthy donors. Furthermore, the expression of SPM enzymes and receptors decreased along disease progression, with the exception of 15-LOX (that remained constant in all MS phases) and of ALX/FPR2, GPR32/DRV1 and ChemR23/ERV that further increased only during remission, thus suggesting their possible involvement in promoting pro-resolution programs and neuroprotection, but subsequently dropped in progressive MS patients.

It is worth mentioning that the levels of SPMs observed in the different disease phases might also be a consequence of a differential utilization and/or degradation as well as a different expression of their target receptors. Indeed, progressive patients bear the lowest amount of all SPM target receptors, yet they keep on expressing high levels of the proinflammatory BLT1 receptor, whose action is only blocked by E-series resolvins, that are never to be found in all MS phases.

Although many types of leukocytes are involved in disease progression, activated monocytes are believed to be one of the first to arrive to the brain and initiate
inflammation (35). In MS, the majority of monocytes display a classical inflammatory phenotype and are hyperactive (36). Herein, we found that monocytes isolated from RR-MS patients not only displayed a more activated and pro-inflammatory status, since their expression of CD69 and cytokines were indeed much higher than monocytes of healthy subjects, but also that specific SPMs significantly inhibited such inflammatory responses in both healthy monocytes and those of RR-MS patients. However, the ability of SPMs to modulate the inflammatory response of these peripheral cells was more evident in cells of healthy subjects, suggesting that, despite expressing comparable levels of pro-resolving receptors, MS patients-derived monocytes are less susceptible to SPMs. These findings confirm and extend earlier reports in which such SPMs were shown to reduce the inflammatory profile of human monocytes upon a pro-inflammatory stimulus (37-39). Of note, such SPM-induced effect is of crucial importance in preventing priming and activation of autoreactive T cells (especially Th1 and Th17 cells) and NK cells, whose pathogenicity are strictly dependent on monocyte-derived cytokines.

Of note, the onset of MS starts when activated and autoreactive peripheral immune cells cross the BBB and start to damage myelin. In this process, BBB endothelial cells are key regulators of the neuroinflammatory response, inasmuch as when inflamed lead to BBB disruption, upregulation of several adhesion molecules and production of chemokines, ultimately favoring leukocyte transmigration and subsequent MS lesion development (40). However, BBB endothelial cells also play an important role during the resolution phase of inflammation, via the secretion of pro- and anti-inflammatory mediators that coordinate both leukocyte traffic and barrier function (41). In this context, despite a great deal of studies have shown the anti-inflammatory and pro-resolving effect of SPMs on various cell types of the immune system, their potential impact on inflamed BBB has never been reported and our results show for the first time not only that BBB endothelial cells express several pro-resolving receptors, which are increased upon inflammation, but also that specific SPMs (LXA₄, LXB₄, RvD1 and PD1) can prevent inflammation-induced BBB dysfunction, reduce monocyte transmigration as well as expression of ICAM-1 adhesion molecule and production of CCL2 chemokine. Our results confirm and extend previous findings in which SPMs have been shown to positively regulate endothelial barrier functions through different mechanisms of action. Indeed, it has been shown that LXA₄ and RvD1 were able to protect LPS-induced barrier integrity and function via suppression
of reactive oxygen species (ROS) production (42), inhibition of the NF-kB pathway (43) or induction of the antioxidant protein Nrf2 (44). Furthermore, SPMs like (AT)-LXA₄, RvD1, RvD2, and MaR1 were reported to reduce monocyte/macrophage infiltration and chemotaxis both in vitro and in vivo (45,46), with RvD1 also being able to induce a switch to the anti-inflammatory M2 phenotype on monocyte-derived brain macrophages in the murine model of MS (32). Although several studies report the anti-inflammatory role of different SPMs (LXA₄ in particular) on vascular endothelial cells or monocytes/macrophages, in terms of reduction of ICAM-1 expression (47,48) and CCL2 production (49), we are the first to reveal potent SPM effects on the BBB, therefore providing novel tools to counteract inflammation-induced BBB dysfunction.

In conclusion, we provide herein a comprehensive profiling of the lipid mediator (LM) signature in plasma from MS patients with different clinical forms of the disease compared to healthy controls. Importantly, our data indicate that key SPMs are lacking in different disease stages which not only indicates a failed resolution response in these individuals, but also may provide an explanation why the disease progresses and may hint at novel therapeutic strategies aimed at boosting their endogenous production or at activating their target receptors. At a functional level, we here show that LXA₄, LXB₄, RvD1 and PD1 significantly reduce the inflammatory profile of MS patient derived monocytes and potently inhibit inflammation-induced BBB dysfunction and monocyte-BBB traversal, which are key pathological hallmarks of MS lesion development. Although further investigations are needed to verify whether SPM impairment is also associated to demyelination and behavior/motor functions in MS patients, this study highlights the potential to use SPMs as novel blood biomarkers for MS diagnosis and provides novel tools to ultimately limit MS pathogenesis in several disease clinical stages.
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Conflicts of interest
CNS is an inventor on patents [resolvins, protectins and maresins] assigned to B.W.H. and licensed for clinical development.

Author contributions
VC, CNS and GK designed research studies; VC, GK, CDT, AL and SL conducted experiments, acquired and analyzed data; PN and IR conducted metabololipidomics analyses and PCA analysis; MA, SR, CG, GG, NBM, FB and DC provided blood samples of healthy donors and MS patients; SVDP, BVHH, YS helped collecting data; VC, GK, LB, HEV and CNS provided reagents; LB and HEV provided scientific suggestions; VC, GK and CDT wrote the manuscript; CNS revised the manuscript. We thank Dr. Xavier de la Rosa for helping in PCA analysis.
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Table 1. Demographic data of patients with MS and control subjects

|                              | Healthy | Relapsing MS | Remitting MS | Progressive MS |
|------------------------------|---------|--------------|--------------|----------------|
| No. of subjects              | n=14    | n=14         | n=12         | n=12           |
| Mean age                     | 36.12±1.77 | 36.82±2.91 | 37.67±2.23 | 38.82±2.54     |
| Female/male                  | 9/6     | 10/4         | 9/3          | 8/4            |
| Disease duration* (years)    | -       | 3.6          | 4.2          | 6.1            |
| Mean EDSS                    | -       | <3 (1.5–3)   | <3 (1–1.5)   | >3 (4–6)       |
| Corticosteroids† (yes/no)    | 0       | 0/0          | 0/0          | 0/0            |
| DMT‡ (yes/no)                | 0       | 0/0          | 0/0          | 0/0            |
| No. of Gd+ T2 brain MRI lesions (%) | 0     | 10-20 (65%) | 10-20 (45%) | >20 (50%)      |

*Disease duration was defined as the time from disease onset to the time of sampling (in years)

‡At time of sampling

Expanded Disability Status Scale scores, EDSS; disease modifying treatments, DMT; gadolinium, Gd; magnetic resonance imaging, MRI.
### Table 2. Human plasma LMs (pro-inflammatory in red and pro-resolving in green) (pg/ml)

| AA bioactive metabolome                  | HS       | MS       | p value |
|------------------------------------------|----------|----------|---------|
| AA                                       | 303       | 259      | 13940 ± 1942 | 12480 ± 811.1 | 0.4127 |
| LTB4                                     | 335       | 195      | 32.79 ± 11.55 | 63.16 ± 16.25 | 0.2958 |
| 20-OH-LTB4                               | 351       | 195      | —           | —            | —      |
| 20-COOH-LTB4                             | 365       | 195      | —           | —            | —      |
| 5S,12S-diHETE                            | 335       | 195      | 21.04 ± 11.04 | 64.11 ± 40.07 | 0.2406 |
| 5S,15S-diHETE                            | 335       | 115      | 10.78 ± 5.02 | 47.30 ± 14.48 | 0.1336 |
| PGD2                                     | 351       | 233      | 1.69 ± 0.44  | 8.42 ± 1.63  | 0.0211 |
| PGE2                                     | 351       | 189      | 3.84 ± 1.22  | 16.37 ± 2.68 | 0.0098 |
| PGF2a                                    | 353       | 193      | 8.55 ± 3.14  | 17.44 ± 1.69 | 0.0768 |
| TBX2                                     | 369       | 169      | 63.14 ± 20.03| 209.60 ± 40.96| 0.0289 |
| 5-HETE                                   | 319       | 115      | 199.6 ± 39.91| 345.20 ± 97.29| 0.1588 |
| 12-HETE                                  | 319       | 179      | 657.10 ± 145.50| 1472.00 ± 180.20| 0.0095 |
| 15-HETE                                  | 319       | 219      | 56.98 ± 14.84| 102.70 ± 11.85| 0.0348 |
| LXA4                                     | 351       | 115      | 0.65 ± 0.56  | 1.85 ± 0.88  | 0.4077 |
| LXB4                                     | 351       | 221      | 1.64 ± 0.44  | 4.71 ± 1.66  | 0.2705 |
| AT-LXA4                                  | 351       | 115      | 3.56 ± 1.69  | 6.57 ± 1.59  | 0.2797 |
| AT-LXB4                                  | 351       | 221      | —           | —            | —      |
| DHA bioactive metabolome                  |          |          |           |           |       |
| DHA                                      | 327       | 283      | 253 ± 30.00 ± 3090.00 | 22690.00 ± 3133.00 | 0.6247 |
| RvD1                                     | 375       | 121      | 0.19 ± 0.09  | 0.68 ± 0.32  | 0.2735 |
| RvD2                                     | 375       | 215      | —           | —            | —      |
| RvD3                                     | 375       | 147      | —           | —            | —      |
| RvD4                                     | 375       | 101      | —           | —            | —      |
| RvD5                                     | 359       | 199      | 0.38 ± 0.15  | 1.37 ± 0.43  | 0.1794 |
| RvD6                                     | 359       | 101      | —           | —            | —      |
| AT-RvD1                                  | 375       | 121      | —           | —            | —      |
| AT-RvD3                                  | 375       | 147      | —           | —            | —      |
| PD1                                      | 359       | 153      | 0.02 ± 0.01  | 0.14 ± 0.03  | 0.0325 |
| AT-PD1                                   | 359       | 153      | —           | —            | —      |
| PDX                                      | —         | —        | 0.43 ± 0.15  | 2.08 ± 0.65  | 0.1182 |
| Maresin 1                                | 359       | 221      | —           | —            | —      |
| 17-HDHA                                  | 343       | 245      | 71.47 ± 17.66| 114.70 ± 12.40| 0.0588 |
| 14-HDHA                                  | 343       | 205      | 310.20 ± 91.26| 784.90 ± 130.90| 0.0333 |
|        |       |               |               |               |       |
|--------|-------|---------------|---------------|---------------|-------|
| 4-HDHA | 359   | 101           | 30.19 ± 6.94  | 69.51 ± 18.37 | 0.1810|
| 7-HDHA | 359   | 250           | 5.68 ± 1.29   | 7.23 ± 1.06   | 0.4185|

**EPA bioactive metabolome**

|        |       |               |               |               |       |
|--------|-------|---------------|---------------|---------------|-------|
| EPA    | 301   | 259           | 6605.00 ± 1296.00 | 4606.00 ± 341.80 | 0.0472|
| RvE1   | 349   | 195           | –             | –             | –     |
| RvE2   | 333   | 253           | –             | –             | –     |
| RvE3   | 333   | 201           | –             | –             | –     |
| 18-HEPE| 317   | 259           | 75.50 ± 28.23 | 49.89 ± 8.10  | 0.2445|
| 15-HEPE| 317   | 219           | 18.54 ± 5.76  | 17.85 ± 2.32  | 0.6215|
| 12-HEPE| 317   | 179           | 244.70 ± 66.11| 393.70 ± 49.88| 0.0895|
| 5-HEPE | 317   | 115           | 27.07 ± 7.79  | 30.16 ± 6.90  | 0.7989|
Figure legends

**Fig.1. Identification of lipid mediators.** Lipid mediators were isolated from plasma of healthy subjects (n=15) and MS patients (n=38) and analyzed by LC-MS-MS. Representative MRM of arachidonic acid (AA)-derived prostaglandins, leukotrienes and lipoxins, docosahexaenoic acid (DHA)-derived resolvins and protectins, and eicosapentaenoic acid (EPA)-derived resolvins, and representative MS-MS of AA-derived PGE2 and LXA4, DHA-derived RvD1, RvD5 and PD1.

**Fig.2. 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) Schematic representation of the AA-derived respective lipid mediator biosynthetic pathways and their selected values between HS and MS. (B) Schematic representation of the DHA-derived respective lipid mediator biosynthetic pathways and their selected values between HS and MS. (C) Schematic representation of the EPA-derived respective lipid mediator biosynthetic pathways and their selected values between HS and MS. Data are presented as means pg/ml ± S.E.M. *p<0.05 or **p<0.01 compared to HS, determined by Student’s t test.

**Fig.3. Correlations between EDSS of patients and lipid mediators.** Correlation plots between Expanded Disability Status Scale scores (EDSS) values and levels (pg/ml) of specific lipid mediators of the AA metabolome (A), DHA metabolome (B) and EPA metabolome (C) in the entire cohort of patients with MS. Data were compared by Spearman’s rank correlation coefficient (p<0.05).

**Fig.4. 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. A) Principal component analysis (PCA) of the lipid mediator profile. Upper panel: 3D score plot; lower panel: 3D loading plot. B) heat map of lipid mediators fingerprint. Pro-inflammatory lipid mediators are shown in red, anti-inflammatory/pro-resolving lipid mediators are shown in green and pathway intermediates in black (C-E) Selected values of lipid mediators of AA metabolome (C), DHA metabolome (D) and EPA metabolome (E) from healthy...
subjects, relapsing, remitting and progressive MS patients. Data are presented as means pg/ml ± 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.5. SPMs biosynthetic enzymes and receptors are differentially expressed in MS patients according to the clinical disease phase. Peripheral blood mononuclear cells (PBMCs, 2x10^6 cells) from healthy subjects (n=5), relapsing MS (n=7), remitting MS (n=5) and progressive MS (n=5) patients were quantified for their mRNA content by qRT-PCR of lipid mediator biosynthesizing enzymes COX-2, 5-LOX, 12-LOX and 15-LOX (A) and of SPMs receptors ALX/FPR2, GPR32/DRV1, GPR18/DRV2, ChemR23/ERV and BLT1 (B). Data are means ± SEM of 5-7 independent experiments. *p<0.05, **p<0.01 determined by one-way ANOVA followed by Bonferroni’s multiple comparison test.

Fig.6. SPMs reduce monocyte activation and cytokine production in MS patients. Peripheral blood mononuclear cells (PBMCs, 2x10^6 cells) from relapsing MS patients (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 representative flow cytometry histograms and as means of fluorescence intensity (M.F.I.) ± SEM of 5 independent experiments. **p<0.01 compared to control cells and ^p<0.05 compared to TLR7/TLR8 agonists, determined by one-way ANOVA followed by Bonferroni’s multiple comparison test. (B) Cytofluorimetric plots and percentages of intracellular pro-inflammatory cytokine production (IL-6, IL-12, IL1-β and TNF-α) from CD14⁺ monocytes. Data are presented as means ± SEM of 5 independent experiments. **p<0.01 and ***p<0.001 compared to control cells, ^p<0.05 and #<0.001 compared to TLR7/TLR8 agonists, determined by one-way ANOVA followed by Bonferroni’s multiple comparison test. (C) Cytofluorimetric plots and percentages of intracellular IL-10 production from CD14⁺ monocytes. Data are presented as means ± SEM of 4 independent
**Fig.7. SPMs improve BBB function and reduce monocyte transmigration and activation.**

(A) Representative scatter plots of forward scatter vs side scatter from human BECs and representative overlays histogram plot gated on live cells for GPR18/DRV2, GPR32/DRV1 and ALX/FPR2 surface expression. (B) Quantification of surface expression. Data are means ± SEM of 4 independent experiments. (C) SPMs receptors mRNA content in resting or TNF-α-activated BECs. Data are means ± SEM of 3 independent experiments. Statistical analysis was carried out using Student’s t-test. ***p < 0.001. (D,E) The functional effect of TNF-α (5ng/ml) in the presence or absence of LXA, LXB₄, RvD1 or PD1 on BBB function was assessed by measuring the TEER of BECs. Confluent BECs monolayer was treated as described and TEER was measured over time. Data are shown as representative TEER curves of 3 independent experiments. Graphs showing the TNF-α effect at selected time-points, plotted as % TNF-α effect of control BECs ± SEM of 3 independent experiments. Statistical analysis was carried out using Student’s t-test. *p<0.05, **p<0.01, ***p < 0.001. (F-H) Confluent BECs were stimulated for 24 hours with TNF-α in the presence or absence of LXA, LXB₄, RvD1 or PD1. Human monocytes (1X10⁵ cells/well) were left untreated or treated with LXA, LXB₄, RvD1 or PD1 prior plated on BECs. Cells were incubated for 8 hours before harvesting the transmigrated cells. (F) Percentage of monocyte transmigration evaluated by flow cytometry. Data are shown as means ± SEM of 3 independent experiments. ***p<0.001 compared to control cells and # p<0.001 compared to TNF-α stimulated cells, determined by one-way ANOVA followed by Bonferroni’s multiple comparison test. ICAM-1 expression by flow cytometry (G) and CCL2 secretion was measured by ELISA (H). Data are means ± SEM of 3 independent experiments. ***p<0.001 compared to control cells, ^p<0.05 and #p<0.001 compared to TNF-α stimulated cells, determined by one-way ANOVA followed by Bonferroni’s multiple comparison test.
Fig. 3

A
AA metabolome

B
DHA metabolome

C
EPA metabolome
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 ± 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 ± 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.
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=8 from Cohort II), and progressive (n=6 from Cohort I, n=8 from Cohort II) MS patients.
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 ± 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, 2x10^6 cells) from healthy subjects (n=5) were left untreated or pre-treated with LXA, LXB, 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, 2x10^6 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.) ± SEM of 5 independent experiments. **p<0.01 compared to control cells and ^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 ± SEM of 5 independent experiments. **p<0.01 and ***p<0.001 compared to control cells, ^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 ± 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.
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,9Z,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±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 (11).

**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 (15,16). 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 (15,16). 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 (50-52) 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 (53) (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 Cytofix/Cytoperm
reagents (BD Biosciences), and then stained intracellularly with phycoerythrin (PE)-Cy7-conjugated anti-TNF-α (1:100, eBioscience), Alexa 647-conjugated anti-IL-1β (1:30, Biolegend), PE-conjugated anti-IL-16 (1:80, Pharminen) Brilliant Violet 421–conjugated IL-12 p40/p70 (1:100, Pharminen) 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 (11,50).

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 ReverseTranscriptase (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’-cttttgtgacaggagtgctc-3’, reverse 5’-gggagtacgttaacgcaagtct-3’; GPR32 forward 5’-catgaatggggtctcgaggg-3’, reverse 5’-
ataaccacagtctgggcg-3'; FPR2 forward 5’-acagggaatgtgaggatggg-3’, reverse 5’-gctggaactgggattagggt-3'; GAPDH forward 5’-ccatgttcgtcatgggtgtg-3’, reverse 5’-ggtgctaagcagtgggtg-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 (3.7x10^4) 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 Zθ instrument (Applied BioPhysics, NY, USA) (54). 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 (55).

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 µm, 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 (56) 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 (1x105 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 (57). All experiments were performed in triplicate with three different human donors.

Statistical analysis
All data were expressed as means ± 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).