Activation of Macrophages by Lysophosphatidic Acid through the Lysophosphatidic Acid Receptor 1 as a Novel Mechanism in Multiple Sclerosis Pathogenesis

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Abstract

Multiple sclerosis (MS) is a neuroinflammatory disease which pathogenesis remains unclear. Lysophosphatidic acid (LPA) is an endogenous phospholipid involved in multiple immune cell functions and dysregulated in MS. Its receptor LPA_1 is expressed in macrophages and regulates their activation, which is of interest due to the role of macrophage activation in MS in both destruction and repair.

In this study, we studied the genetic deletion and pharmaceutical inhibition of LPA_1 in the mice MS model, experimental autoimmune encephalomyelitis (EAE). LPA_1 expression was analyzed

in EAE mice and MS patient immune cells. The effect of LPA and LPA_1 on macrophage activation was studied in human monocyte-derived macrophages.

We show that lack of LPA_1 activity induces milder clinical EAE course, and that *Lpar1* expression in peripheral blood mononuclear cells (PBMC) correlates with onset of relapses and severity in EAE. We see the same over-expression in PBMC from MS patients during relapse compared to progressive forms of the disease, and in stimulated monocyte-derived macrophages. LPA induced a proinflammatory-like response in macrophages through LPA_1, providing a plausible way in which LPA and LPA_1 dysregulation can lead to the inflammation in MS.

These data show a new mechanism of LPA signaling in the MS pathogenesis, prompting further research into its use as a therapeutic target biomarker.

Keywords: Lysophosphatidic acid, LPA_1 receptor, macrophages, multiple sclerosis, experimental autoimmune encephalomyelitis, inflammation.

Abbreviations

CNS: central nervous system

EAE: experimental autoimmune encephalomyelitis

HD: healthy donors

IMNC: infiltrating mononuclear cell

KO: knock-out

LPA: lysophosphatidic acid

MS: multiple sclerosis

OPC: oligodendrocyte precursor cell

PBMC: peripheral blood mononuclear cell

PP-MS: primary progressive multiple sclerosis

RR-MS: relapsing-remitting multiple sclerosis

SP-MS: secondary progressive multiple sclerosis

WT: wild-type

Introduction

Multiple sclerosis (MS) is one of the most widespread neurological diseases in young adults affecting approximately 2.3 million people worldwide [1]. MS pathogenesis consists of inflammation of the central nervous system (CNS), oligodendrocyte death and myelin damage, with many different immune cells exercising significant influence. Macrophages play a dual role in MS pathology; they can contribute to tissue damage and inflammation, but also exert a neuroprotective and regenerative effect [2]. Accordingly, classically activated ("M1") macrophages show proinflammatory characteristics, whilst alternatively activated ("M2"), macrophages display an anti-inflammatory phenotype [3]. During remyelination, M1 macrophages phagocytize myelin debris and induce oligodendrocytes precursor cells (OPC) to proliferate and migrate to the lesion site. Next, a switch from the M1 to the M2 phenotype triggers the secretion of trophic factors that foster OPC differentiation into new myelin-forming oligodendrocytes [4]. However, it remains unclear to what extent macrophages intrinsically contribute to myelin destruction and repair in MS.

Lysophosphatidic acid (LPA) is a bioactive phospholipid that influences numerous cell responses and acts through a family of G-protein coupled receptors (LPA_{1-6}), widely distributed in peripheral organs and brain [5-7]. Among all LPA receptors, LPA_1 is the most studied in relation to biological action through of LPA [5,6].

LPA dysregulation has been implicated in different inflammatory diseases, such as atherosclerosis [8], cancer [9], and MS [10] because of its effect on immune cells, of both the innate and adaptive immune systems [11-13]. Although some studies showed a dysregulation of serum LPA levels in MS patients [10, 14-15] and suggested a role of LPA_2 in T cells homing [10], the role of LPA_1 in MS, in particular through macrophage function, remains unclear.

LPA constitutes a major serum survival factor for murine macrophages [16], in which it has been shown to upregulate the expression of proinflammatory cytokines, such as IL-1β and TNF-α [13], while its role in human macrophages is largely unexplored. Interestingly, LPA_1 is expressed in peripheral blood monocytes and/or tissue macrophages in both mice and humans, and influences their activation, migration and infiltration in different murine disease models [17-19].

Following these findings, we here aim to elucidate the role of LPA_1 in MS, by analyzing the disease course of experimental autoimmune encephalomyelitis (EAE) using both a genetic model (maLPA_1-null) and a pharmacological model (LPA_1 antagonist). We provide evidence of a milder symptomatology in absence or antagonism of LPA_1, suggesting a role of this receptor in the pathogenesis of the disease. This role was further strengthened with the analysis of LPA_1 expression levels in PBMC from EAE mice and from patients with relapsing-remitting (RR-), primary progressive (PP-) or secondary progressive (SP-) MS. We demonstrated that the

initiation of relapses is accompanied by an increase of LPA_1 transcripts (*Lpar1*) in mouse PBMC. Finally, we provide *in vitro* data demonstrating that proinflammatory activation of human monocyte-derived macrophages includes increased expression of human LPA_1 transcripts (*LPAR1)* and that LPA is involved in LPA_1-driven polarization of human macrophages towards a M1-like phenotype. These results evidence a role of LPA in the initiation of the inflammatory process during MS relapses via LPA_1.

In short, our studies unveil a novel mechanism for LPA in the classical activation of macrophages through LPA_1 and suggest for the first time that targeting LPA_1 receptors represents a promising therapeutic strategy in MS as well as for other immune-related diseases.

Material and Methods

Mice

All experiments were performed on the *Málaga* variant of the LPA_1-null mouse derived from the original colony of Contos et al [20] on a mixed C57BL/6J x 129X1/SvJ background, as previously described [21,22]. Experiments were conducted on age-matched female wild-type and maLPA_1-null homozygous littermates that were approximately 7 weeks old (n total =103). All mice were housed in pathogen-free conditions on a 12-h light/dark cycle (lights on at 07:00 h) with water and food provided *ad libitum*. Experiments were conducted in accordance with the European guidelines and national laws on laboratory animal welfare and approved by the Experimentation Ethics Committees of the University of Malaga.

Induction of EAE.

Seven-week-old female mice were immunized according to a standard protocol [23] with subcutaneous injection of incomplete Freund's adjuvant containing 4 mg/mL *Mycobacterium tuberculosis* (strain H37Ra; Difco Laboratories, Detroit, MI, USA) and 200 µg of encephalitogenic myelin oligodendrocyte glycoprotein peptide 35–55 ($MOG_{35–55}$). The mice received intraperitoneal injections with 200 ng pertussis toxin (Sigma-Aldrich, St. Louis, Missouri, USA) at the time of immunization and 48 hours later. After 7 days, the mice received a half booster immunization with MOG, complete Freund's adjuvant (CFA) and pertussis toxin. Control mice received identical injections without $MOG_{35–55}$. Clinical disease usually commenced around 15 days postimmunization (dpi). The mice were scored four times per week in the mornings as follows: limp tail or waddling gait with tail tonicity, defined score 1; waddling gait with limp tail (ataxia) as score 2; ataxia with partial limb paralysis as score 2.5; full paralysis of one limb as score 3; full paralysis of one limb with partial paralysis of second limb as score 3.5. Animals that maintained a score of at least 3.5 more than 3 days were euthanized with overdose of pentobarbital to minimize animal suffering. A total of 103 animals were immunized

for this study.. DietGel® Recovery was put at their disposal on the bedding to provide a nutritionally fortified water gel and avoid dehydration during their clinical course.

LPA_1 antagonist administration.

VPC 32183 (S), (S)-Phosphoric acid mono-(2-octadec-9-enoylamino-3-[4-(pyridine-2-ylmethoxy)-phenyl]-propyl) ester (Ammonium Salt) (CAS number 799268-75-0;Avanti Polar Lipids, Alabaster, AL, USA) was dissolved in 3% fatty-free acid BSA (FFA-BSA; Sigma-Aldrich) in saline. VPC32183 was diluted to a concentration of 5 µM and a volume of 100 µl was injected intravenously in the tail vein at the time-points described in the text. Non-treated control mice only received vehicle injections (3% FFA-BSA in saline).

Subjects

RNA analysis of total PBMC was performed on samples provided by the Biobank of our hospital, as part of the Andalusian Public Health System Biobank. All patients participating in the study gave their informed consent and protocols were approved by institutional ethical committees (Comite de Ética de la Investigación provincial de Málaga). The study was conducted according to international ethical principles contained in the Declaration of Helsinki, Spanish regulations on biomedical research (Law 14/2007, of July 3, on biomedical research) and the provisions of the European General Personal Data Protection Regulation (Royal Decree-Law 5/2018, of 27 July, and Regulation (EU) 2016/679, of April 27, 2016). Patient selection was based on the criteria of first diagnosis and under no MS treatment. For the RNA sequencing analysis, 22 MS patients (11 pairs of siblings from a cohort established for a parallel study) and 9 healthy controls were recruited for the macrophage experiments. The study was approved by the French Ethics committee and the French ministry of research (DC-2012-1535 and AC-2012-1536). Written informed consent was obtained from all study participants. All patients fulfilled diagnostic criteria for MS, and individuals (MS patients and healthy donors) with any other inflammatory or neurological disorders were excluded from the study. The study was not pre-registered. No randomization was performed to allocate subjects in the study.

Isolation of PBMC for RNA extraction

PBMC were isolated from whole blood obtained from mice during the first minute of PBS perfusion (n = 42) by standard Ficoll®-Paque (Sigma-Aldrich) density gradient centrifugation. Briefly, heparinized blood was diluted with saline solution (1:1 dilution). Then, Ficoll®-Paque was covered with a layer of diluted blood. After 30 min of centrifugation (600 x g, room temperature (RT), without break), the PBMC could easily be collected. After two washing steps and counting, cells were resuspended in 1 ml of TRIzol™ Reagent (Invitrogen™, Thermo Fisher Scientific Inc., Waltham, MA, USA) to extract RNA.

Isolation of brain infiltrating mononuclear cells for RNA extraction

Infiltrated mononuclear cells (IMNC) were isolated from CNS of EAE mice according to Beeton and Chandy [24]. After dissecting and weighing brain and spinal cord from individual animals (n = 27), these were minced finely in phosphate buffer saline, centrifuged, and resuspended in 37% Percoll® (Sigma-Aldrich). This suspension was laid on a 70% Percoll® cushion and spun at 600 x g at room temperature for 25min. CNS IMNC were obtained from the 37-70% Percoll® interface, washed twice, and cell counted. Finally, cells were resuspended in 1 ml of TRIzol™ for RNA extraction.

RT-PCR from PBMC and IMNC

Total RNA was extracted from PBMC and IMNC using the TRIzol™ reagent as originally described by Chomczynski and Sacchi [25] . cDNA was synthesized using 1 µg of total RNA by the enzyme reverse transcriptase MMLV (Sigma-Aldrich) and random primers. Real-time PCR was performed by the LightCycler® System (Roche Molecular Systems, Inc., Pleasanton, CA, USA) following the manufacturer's specifications. The 10 µl final reaction volume consisted of 5.4 µl of RNAase-free distilled water, 1.3 µL of $MgCl_2$, 0.2 µl of each forward and reverse primers, 1 µl of Fast SYBR™ Green Master Mix and 2 µl of cDNA. Reaction conditions were as follows: polymerase activation at 95°C for 15 min, 40 denaturation cycles of 95°C for 30 s, and annealing/elongation at 68°C (*Lpar1* and GAPDH) for 5 s (*Lpar1*) or 10 s (GAPDH).

The primer sequences used in the amplification of *Lpar1* and *Lpar2* have been described previously [26] (*Lpar1* forward: GAGGAATCGGGACACCATGAT; and reverse: ACATCCAGCAATAACAAGACCAATC, Gapdh forward: GCCAAGGTCATCCATGACAACT, and reverse: GAGGGGCCATCCACAGTCTT). A melting curve analysis was performed to assess primer specificity and product quality by step-wise denaturation of the PCR product at a rate of 0.1°C / sec to 98°C. The relative levels of receptor expression were quantified using the standard curve method.

Isolation of human primary monocytes and macrophage culture and activation.

Blood was sampled from all participants in acid citrate dextrose (ACD) tubes. PBMC were isolated using Ficoll®-Paque Plus and centrifugation (600 x g, 20 min without brake). Cells were washed in PBS (2x10 min at 1500 rpm) and RPMI 1640 with 10% foetal bovine serum (FBS) (Thermo Fisher). Monocytes were isolated with anti-CD14 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and plated in 12-well plates (500 000 cells/well) or in 24-well plates (200 000 cells/well) in RPMI 1640 with 10% FBS and granulocyte macrophage colony-stimulating factor (GM-CSF) (500 U/ml, ImmunoTools GmbH, Friesoythe, Germany). After 72h, media was replaced with fresh media and one of the following: GM-CSF (500 U/ml); IFNβ (100 U/ml, ImmunoTools); IL4 (1000 U/ml, ImmunoTools); or combined IFNγ (200 U/ml, ImmunoTools) and ultra-pure LPS (10 ng/ml, InvivoGen, Inc., San Diego, CA, USA). Cell lysis

and RNA extraction were performed 24h post-activation using Nucleospin RNA extraction kit (Macherey-Nagel GmbH & Co. KG, Dueren, Germany). Quality of RNA (RINe>8) was confirmed on Agilent TapeStation (Agilent Technologies, Inc., Santa Clara, CA, USA).
1-Oleoyl lysophosphatidic acid sodium salt, LPA (CAS number 325465-93-8; Tocris, Bio-Techne, Minneapolis, MN, USA 3854) and the LPA_1 antagonist Ki16425 (CAS number 355025-24-0; Sigma-Aldrich) were dissolved in 3% FFA-BSA and added to the medium at a final concentration of 1µM LPA and 400nM Ki16425 during 24h.

RNA sequencing

Transcriptome sequencing cDNA libraries of macrophage RNA were prepared using a stranded mRNA polyA selection (Truseq stranded mRNA kit, Illumina, Inc., San Diego, CA, USA). For each sample, we performed 60 million single-end, 75 base reads on a NextSeq 500 sequencer (Illumina). RNA-Seq data analyses were performed by GenoSplice technology(www.genosplice.com, Paris, France). Sequencing, data quality, reads repartition, and insert size estimation were performed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), Picard-Tools (https://broadinstitute.github.io/picard/), Samtools (http://www.htslib.org/) and rseqc (http://rseqc.sourceforge.net/). Reads were mapped using STARv2.4.0 [27] on the hg19 Human genome assembly. Gene expression regulation study was performed as previously described [28]. Briefly, for each gene present in the FAST DB v2018_1 annotations, reads aligning on constitutive regions (that were not prone to alternative splicing) were counted. Based on these read counts, normalization was performed using DESeq2 in R (v.3.2.5) [29]. Genes were considered as expressed if their RPKM value was greater than 97.5% of the background RPKM value based on intergenic regions. The normalized data were used for all subsequent analysis.

RT-PCR of monocyte-derived macrophages

RNA obtained from differentially activated macrophages were used as templates to synthetize cDNA using the Quantitect Reverse Transcription kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. Quantitative RT-PCR was performed with the LightCycler® 1536 Instrument (Roche Molecular Systems), and the following primers:

Hs_CD86_1_SG QuantiTect Primer Assay (200) Cat No./ID: QT00033915
Hs_TLR2_1_SG QuantiTect Primer Assay (200) Cat No./ID: QT00236131
Hs_CCL2_1_SG QuantiTect Primer Assay (200) Cat No./ID: QT00212730
Hs_CCL5_1_SG QuantiTect Primer Assay (200) Cat No./ID: QT00090083
Hs_CCL20_1_SG QuantiTect Primer Assay (200) Cat No./ID: QT00012971
Hs_*LPAR1*_1_SG QuantiTect Primer Assay (200) Cat No./ID: QT00021469
Hs_MRC1_1_SG QuantiTect Primer Assay (200) Cat No./ID: QT00012810
Hs_CD163_1_SG QuantiTect Primer Assay (200) Cat No./ID: QT00074641

Hs_CD180_1_SG QuantiTect Primer Assay (200) Cat No./ID: QT00203574
Hs_PDGFC_1_SG QuantiTect Primer Assay (200) Cat No./ID: QT00026551

Statistical methods

The sample size calculation was performed by the resource equation method, trying to minimize the sample size to follow the ARRIVE guidelines for reporting animal research. Each n represents one animal or sample in the experiment. All mouse studies were repeated a minimum of 3 times, and each experimental group included at least 4 samples. The values are expressed as mean ± SEM. The statistical analysis was done with GraphPad Prism (GraphPad Software, San Diego, CA, USA). Normality in the variable distributions was assessed by the D'Agostino & Pearson omnibus test and Grubbs' test was used to detect and exclude possible outliers. If the normality test was passed, the means were compared by two-tailed Student's t test. When one or both groups did not follow a normal distribution, means were compared by two-tailed Mann-Whitney U test. One-way ANOVA plus Bonferroni posthoc tests was used to compare different independent groups. Statistical significance was determined using the appropriate statistical test mentioned in each experiment. Values were statistically significant when $p < 0.05$. No blinding was performed in the analysis of the results.

Results

LPA_1 deletion leads to milder EAE clinical course.

Recently, a role of LPA in the pathogenesis of MS and its animal model EAE has been suggested [10], focusing on the contribution of LPA_2-expressing T cells. Here, we question whether LPA_1, also present in immune cells, could have a role in EAE. To answer this question, we first compared the EAE clinical course in presence and absence of LPA_1 using the *Malaga* variant of LPA_1-null mouse (maLPA_1-null mouse) [21].

Analysis of MOG_{35-55} induced-EAE clinical courses showed a relapsing-remitting clinical course in both wild-type and maLPA_1-null animals but also highlighted important differences between the two genotypes. Notably, maLPA_1-null mice showed a less severe clinical course compared to wild-type mice (Fig. 1a). Accordingly, maLPA_1-null mice reduced the number of relapses and exhibited a significantly lower average clinical score and maximal clinical score reached during relapses, as well as a better recovery during remission (Fig. 1b).

Intravenous injection of an LPA_1 antagonist ameliorates EAE clinical score in wild-type mice.

LPA_1 is also expressed in oligodendrocytes and we previously demonstrated that its absence perturbs developmental myelination in maLPA_1-null mice [22]. To exclude any interference of deficient myelin patterns in the EAE outcome in maLPA_1-null animals, we analyzed EAE in

wild-type mice that were intravenously injected with VPC32183, an LPA_1 antagonist that primarily blocks LPA_1 (10-100nM range), and partially blocks LPA_3 (10-fold lower; 100-1000nM range) [30].

To maintain the levels of the antagonist, immunized mice were treated with repeated doses of VPC32183 every 5 days (Fig. 2a). Recurrent intravenous administration of the LPA_1 antagonist resulted in a milder EAE disease course characterized by a lower average clinical score, milder relapses, and better remissions (Fig. 2a,b), corroborating the requirement of LPA_1 activation to develop a normal EAE clinical course.

***Lpar1* expression increases when mononuclear cells invade the CNS**

Under normal physiological conditions, mononuclear cells are rarely found in the CNS. However, in MS and EAE, activated immune cells infiltrate the CNS. Due to the reported role of LPA_1 in immune cell infiltration [31] and its obvious impact in the clinical course, we analyzed whether the number of infiltrating mononuclear cells (IMNC) was altered in EAE-mice lacking LPA_1. We quantified the number of IMNC from brain and spinal cord of wild type and maLPA_1-null mice with similar clinical scores. We did not find significant differences in the number of infiltrates (Fig. 3a), suggesting that LPA_1 modulation of EAE course might intervene at another step of immune cell activation beside infiltration.

Despite LPA_1 not being essential for cell infiltration, we found that its expression still appeared to be related to immune presence in the CNS. When analyzing the *Lpar1* expression in circulating immune cells (PBMC) and CNS-IMNC from wild-type EAE mice using RT-PCR, our results showed an increase of *Lpar1* expression in immune cells in the CNS compared to the periphery (Fig. 3b). These data suggest that an increase of *Lpar1* expression reflects immune cell activation.

Onset of EAE relapses correlates with increase expression of *Lpar1* in PBMC.

Knowing that *Lpar1* is expressed by immune cells, and that immune cells are critical for EAE development, we wondered whether *Lpar1* expression in PBMC reflects disease activity. To this end, the expression of *Lpar1* in PBMC along the EAE clinical course was evaluated in wild-type mice.

MOG-immunized animals showed a two-fold significant increase of *Lpar1* expression compared to control animals (Fig. 4a). However, no significant correlation was found between *Lpar1* expression and clinical score after sacrifice (EAE score 1: 0,459818 ± 0,123361 (n=9); EAE score 2: 0,681039 ± 0,151682 (n=7); EAE score 3: 0,648933 ± 0,144792 (n=8)).

To decipher whether *Lpar1* expression in PBMC might reflect a different phase of the disease, *Lpar1* expression was analyzed according to mice stratification based on whether animals were initiating a relapse or in remission/progressive course of the disease at the moment of the

sampling. There was a significantly increased expression of *Lpar1* during relapses when compared to control animals or animals in remission or progressive episodes (Fig. 4b). Of note, *Lpar1* expression and clinical symptoms during the EAE relapses were significantly positively correlated during the clinical course of the disease (Fig. 4c).

***LPAR1* expression increases during relapses in RR-MS patient PBMC**.

The above observations in EAE mice suggest a modulation of LPA_1 in the first stages of the relapses during the inflammatory clinical course. To corroborate this observation in the context of MS, we compared the expression of *LPAR1* in PBMC from RR-MS patients at the time of first relapse and compared with its expression in healthy donors (HD), matched in age and gender (Fig. 5a), and patients with progressive form of the disease (SP-MS and PP-MS). Like in EAE, *LPAR1* expression was significantly higher in RR-MS patient PBMC than in HD or progressive patients (Fig. 5b). Thus, we provide evidence that alterations in *LPAR1* expression associates with the inflammatory phase of MS.

***LPAR1* expression correlates with a proinflammatory phenotype of human monocyte-derived macrophages**

Circulating PBMC are mainly composed of lymphocytes and monocytes. We focused on the role of LPA_1 on monocytes/macrophages because of their dual role in MS pathology [3], being both deleterious when endorsing a proinflammatory phenotype and beneficial under pro-regenerative activation [2, 4].To elucidate the role of LPA_1 in macrophages polarization, we obtained naïve circulating monocytes from HD and RR-MS patients in remission. Naïve monocytes remain circulating in the blood stream for a short period before infiltrating the tissues [32], reducing the impact of other circulating factors before blood extraction. Blood CD14+ monocytes were differentiated into macrophages using GM-CSF. Then, monocyte-derived macrophages were exposed to: i) LPS+IFNγ, to activate a proinflammatory (M1) state; ii)IFNβ or IL4, to activate a pro-regenerative (M2) state; or iii) GM-CSF, to remain in a neutral state. We next evaluated the expression of membrane LPA receptors LPA_1 and LPA_2, and the nuclear LPA-receptor PPARγ. Although the patients were recruited from a cohort of siblings with MS, the siblings did not show higher similarity in expression than random pairs of patients and, in consequence, they were considered as independent samples (data not shown).

Interestingly, while we could not detect a difference of *LPAR2* expression (Fig. 6a) in any activation state, *LPAR1* and *PPARγ* receptors were differentially regulated, displaying an inverse pattern of across the macrophage activation states (Figs. 6b, c). In both MS and HD, *LPAR1* expression was up-regulated in the M1 proinflammatory state, while PPARγ expression was increased in the pro-regenerative state. This observation underlines the possible role of LPA_1 in the proinflammatory activation of human macrophages.

LPA mediates human macrophage polarization.

LPA levels are altered along the course of MS [10, 14] suggesting an important role of this phospholipid in the course of the disease. We therefore tested whether, in addition to observed M1-associated *LPAR1* overexpression, LPA could promote an M1-phenotype in macrophages, as has been observed in murine microglia [33].

LPS mediates time-dependent macrophage M1/M2 polarization, promoting the M1 phenotype over time [34]. We examined transcripts specific for proinflammatory or pro-regenerative profiles in human macrophages after an LPA treatment of 24h and compared the expression of different markers with the canonical M1 polarization by LPS (Fig. 7).

The levels of different M1 markers (CCL2, CCL20, CCL5, and TLR2), though to a lesser extent than after LPS treatment, increased after LPA incubation, indicating a role of LPA in the proinflammatory activation of human macrophages. Moreover, this M1 polarization was partially inhibited by addition of an LPA_1 inhibitor (Ki16425) revealing the mediation of LPA_1 in this LPA-induced response (Fig. 7). No significant alterations in the expression of M2 markers were observed after LPA incubation.

Discussion

In this study, we present evidence of a role of the LPA_1 receptor in the pathogenesis of the neuroinflammatory disease MS and its animal model, EAE. We also propose a mechanism through which LPA may exert this effect via macrophage activation.

After the discovery of the first receptor for LPA, the LPA_1, in 1996 [35], this receptor has been implicated in a numerous process, with an outstanding importance in the physiology and pathology of the CNS [36]. In this context, the importance of LPA in MS pathogenesis has been suggested [10, 14, 15]. However, the role of LPA_1 in the MS pathogenesis remains unclear. In the present study, we unveil a new aspect of LPA through the LPA_1 receptor in this neuroinflammatory disease.

Our results show, for the first time, the importance of the receptor LPA_1 in the EAE clinical course. The lack of LPA_1, or its pharmacological inhibition by the repetitive intravenous injections of a LPA_1 antagonist (VPC32183), reduces the severity of the disease. The milder symptoms observed in the absence of LPA_1 signaling indicates that this pathway is involved in EAE pathogenesis. This is in contrast with a study of another LPA receptor, LPA_2, of which reduction led to more severe disease [10]. This indicates a complex role of LPA in MS and EAE, and that potential treatment strategies should target specific receptor pathways rather than LPA itself. In our study, the administration of VPC32183 confirmed the clinical course observed in LPA_1-null EAE, albeit infiltrating mononuclear cells were only analyzed in LPA_1-null mice. In this

sense, we cannot ignore that VPC32183 is a selective LPA_1/LPA_3 antagonist and the involvement of LPA_3 receptor in the endothelial cell interactions with monocytes during inflammation processes [18, 37]. For this reason, the analysis of infiltrating mononuclear cells in EAE-mice was restricted to an LPA_1-null scenario, to exclude any other receptor that could interfere the study.

Previous studies have described a role for LPA and autotaxin, its main synthesis enzyme, in inflammatory processes [8, 9, 37, 38]. In line with this, we found that the expression of LPA_1 was high during relapses – a period generally associated with high inflammatory activity – in immune cells from both EAE and MS. The differential expression of *LPAR1* in the different clinical forms of MS indicates a direct role of the LPA-LPA_1 pathway in the inflammatory component of the disease and suggests a potential use of *LPAR1* expression as a biomarker of disease activity. These results are consistent with previous studies showing increased levels of LPA in blood or cerebrospinal fluid of RR-MS patients during relapse compared to HD or RR-MS patients in remission [14, 15] and suggest a broader dysregulation of LPA signaling than previously thought.

We also found a significant positive correlation between the levels of *Lpar1* expression during relapses and the severity of the EAE clinical course, encouraging future analysis of RR-MS patient clinical disability and *LPAR1* expression. Preliminary results showed that *LPAR1* expression in patients when first diagnosed and after two years of treatment decreased. Despite these promising observations, more clinical data and treatment-based sorting would strengthen the implication of LPA_1 in the disease course and potentially enable the use of its expression to estimate the individual patient's prognosis. Nevertheless, large cohorts and the inclusion of immune-modulatory treatments would be necessary to extract meaningful statements.

To understand how LPA_1 exerts its influence on the inflammatory component of MS and EAE, we examined infiltration and activation of immune cells. In the case of LPA_2, its effect on the EAE disease course appears to be reliant on its capacity to increase T-cell homing, thus reducing infiltration. While some studies have indicated a detrimental role of LPA_1 in blood-brain-barrier (BBB) integrity [39-41] and potential to increase extravasation through induction of chemokine expression [42], our results did not indicate a significant impact of the LPA_1 deletion on PBMC infiltration into the CNS. While this does not exclude an effect below statistical significance or an effect of BBB leakage independent of PBMC infiltration, we cannot explain the amelioration of clinical scores through reduced infiltration. Instead, the observation that infiltrating cells express *Lpar1* to a higher degree than peripheral cells in EAE wild-type mice suggests that LPA_1 is involved in immune cell activation without necessarily affecting infiltration.

Following the hypothesis that LPA_1 correlates with immune cell activation, we examined its expression in activated human macrophages. Our results show an increase of *LPAR1* expression, but not *LPAR2*, in both HD and RR-MS patient macrophages when activated

towards a proinflammatory phenotype. On the other hand, expression of the LPA nuclear receptor *PPARγ* not only decreases when macrophages acquire M1 polarization but also shows a trend towards increasing after pro-regenerative activation. We did not identify a difference between MS patients and healthy controls, but this could be due to the fact that MS samples were taken during a remission phase. In this case, the modular expression of different LPA receptors after differential activation hints a complex role of LPA signaling in the homeostasis of macrophages during the disease and suggests that modulating the expression or saturating the activation of one receptor or another could be a mechanism of trans-differentiation of human macrophages. Although this aspect requires further study, the fact that LPA_1 is related to glycolysis [43-45] (main source of energy for proinflammatory polarized macrophages) and PPARγ induces oxidative phosphorylation [46], and that these two metabolic processes are central in pro- and anti-inflammatory macrophage activation respectively [47], suggests that the modulation of these LPA receptors could have major implications in the macrophage physiology and activation.

Knowing that LPA is dysregulated in MS relapses [14, 15] and that *LPAR1* expression is increased in macrophage activation, we hypothesized that LPA_1 could mediate LPA-induced proinflammatory activation in macrophages. This was confirmed through increased expression of M1 markers following LPA incubation with partial correction by exposure to the LPA_1 antagonist Ki16425. Increased expression of LPA_1 in EAE and MS PBMC during relapse thus suggests both an activated state as well as a predisposition to further proinflammatory activation. The coordinated responses between the induced LPA_1 expression and the proinflammatory activation of LPA via the LPA_1 would promote a positive feedback loop that grants to LPA the role of boosting the inflammatory response and maintain the classical activation of macrophages. The milder EAE clinical course observed in LPA_1-null and LPA_1-antagonized mice, which present lower maximal and minimal scores in relapses and remissions respectively, could therefore be explained by a milder activation of immune cells, whereas the number or relapses and the onset of the disease unaffected as infiltration still occurs to the same extent.

Although this work focused on peripheral immune cells, a similar effect of LPA on microglia and non-parenchymal macrophages could be predicted in view of reported LPA-dependent effects on microglia including chemokinesis, activation, oxidative stress response or proinflammatory cytokine production [48]. Moreover, LPA_1 has been associated to proinflammatory microglial activation and their TNF-α production [49]. Similarities between microglia and non-CNS macrophages are extensive, and their final phenotype is largely dependent on ontogeny [50]. It is thus possible that the hypothesized feedback loop of LPA in inflammation is further exacerbated by resident immune cells.

In short, our study unveils for the first time a role of LPA_1 in the pathogenesis of MS and its animal model, EAE, and the importance of the regulation of the LPA signaling in the

development of the disease. In addition to opening up to new avenues for immuno-modulatory treatment, this research also indicates a potential for LPA_1 as a biomarker of disease activity. Further research on LPA in MS should therefore consider the exact pathways being targeted and the current level of disease activity in the patient, in order to develop strategies to better follow and treat these neurological patients.

Declarations

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Conflicts of interest/Competing interests. The authors declare no conflict of interest.

Ethics approval. Animal experiments were conducted in accordance with the European guidelines (European Council Directives 2010/63/UE and 90/219/CEE, Regulation (EC) No. 1946/2003) and Spanish national laws on laboratory animal welfare (Royal Decrees 53/2013 and 1386/2018, Law 32/2007) and approved by the Experimentation Ethics Committees of the University of Malaga and the Biomedical Research Institute of Málaga. In relation to the human samples, the study were approved by institutional ethical committees (Comite de Ética de la Investigación provincial de Málaga), French Ethics committee and the French ministry of research, and conducted according to international ethical principles contained in the Declaration of Helsinki (Fortaleza, 2013), Spanish regulations on biomedical research (Law 14/2007, of July 3, on biomedical research), and the provisions of the European General Personal Data Protection Regulation (Royal Decree-Law 5/2018, of 27 July, and Regulation (EU) 2016/679, of April 27, 2016)

Consent to participate The samples were provided by the Biobank of our hospital, as part of the Andalusian Public Health System Biobank, and the Pitié-Salpêtrière Hospital All patients participating in the study gave their informed consent and protocols were approved by institutional ethical committees (Comite de Ética de la Investigación provincial de Málaga), and French Ethics committee and the French ministry of research.

Consent for publication Not applicable

Availability of data and material Not applicable

Code availability Not applicable

Authors' contributions: J.F. performed human experimental work, analyzed and interpreted the results, and revised the manuscript. AI.G. assisted with animal experiments and J.R-I analyzed the clinical data. O.F., L.L., F.R.F., J.C. and A.B-V. critically reviewed the manuscript. C.L. provided the human cell samples. V.Z. helped with the design of the project and revised the manuscript. G.E-T. conceived the project, handled the funding, supervised and interpreted the results, and edited the manuscript. B.G-D. conceived the project, handled the funding, performed animal experimental work, analyzed and interpreted the results, and wrote the manuscript. All of the authors approved the manuscript.

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Figure legends

Fig. 1. LPA_1 null mice exhibit a less severe EAE disease course than wild-type (WT).

a) EAE disease progression in wild-type (n = 7) and maLPA$_1$-null (n = 7) mice. Graph presents mean values with error bars (SEM) indicating data from one representative experiment out of three independent experiments b) Comparison of clinical parameters of EAE. Student's *t* test for the mean clinical score p = 0.01; for the cumulative clinical score p = 0.03; for the max score p = 0.03; and for the min score p = 0.04. * p < 0.05.

Fig. 2. LPA_1 antagonist treatment lowers clinical scores in EAE mice.

a) EAE disease progression in wild-type mice with three doses of LPA_1 antagonist given at 11, 16 and 21dpi (arrows) compared to vehicle. The graph summarizes data from three independent experiments (n = 10 per condition and experiment). Graphs present mean values with error bars indicating + SEM. b) Comparison of clinical parameters of EAE in vehicle- and LPA_1 antagonist-treated wildtype animals; Student's *t* test: mean clinical score p = 0.001, cumulative clinical score p = 0.003, max score of relapses p = 0.001, min score of remissions p = 0.002. ** p < 0.01

Fig. 3. *Lpar1* increases in CNS infiltrating macrophages compared to circulating PBMC but does not participate to macrophage recruitment.

a) No differences between genotypes were observed in the number of brain and spinal cord IMNC of two sets of wild-type and maLPA$_1$-null EAE mice with similar clinical courses. Clinical course average of wild-type mice (n = 13) was 1.36 ± 0.19 and maLPA$_1$-null mice (n = 14) was 1.43±0.17. IMNC number was normalized against CNS weight (g): wild-type = 845276 ± 124261 cells/g; maLPA$_1$-null = 658148 ± 125219 cells/g. Unpair *t*-test, p=0.29. b) *Lpar1* expression (normalized to *Gapdh*) was higher in CNS IMNC when compared to the *Lpar1* levels in PBMC (n = 6 per group). Clinical course average was 1.56 ± 0.4. Wilcoxon matched-pair test p = 0.031 * p < 0.05, n.s., not significant.

Fig. 4. *Lpar1* expression in mouse PBMC during EAE development

a) Relative RT-PCR analysis of *Lpar1* expression in PBMC normalized to *Gapdh* in control (n=12) and EAE-induced (n = 30) animals regardless the moment of the disease. The expressions of *Lpar1* in EAE-induced mice were ~2-fold higher to those in control animals. Student's *t*-test p = 0.0022. b) *Lpar1* expression of the EAE mice in relapses (n = 10) was significantly higher than in controls (Student's *t* test p = 0.0024) and reduced during remission (n = 12) (Student's *t* test between relapses and remissions groups p = 0.016). C) Positive

correlation between the clinical symptoms and the expression of *Lpar1* in EAE mice during relapses. * $p < 0.05$, ** $p < 0.01$.

Fig. 5. Up regulation of LPA_1 expression in MS patient PBMC during relapses.

A) Demographic data of the studied groups. B) Relative expression of *LPAR1* normalized to *GAPDH* in healthy donor (HD), RR-MS patients during relapses, SP-MS and PP-MS patients . Analyses were performed by one-way ANOVA followed by Bonaferroni posthoc test ($p = 0.04$). * $p < 0.05$.

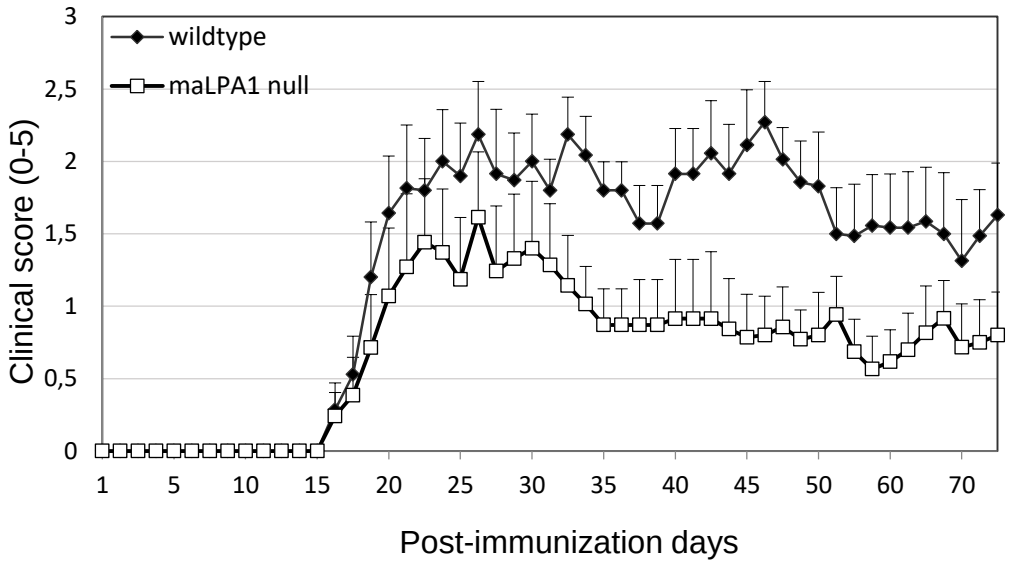
Fig. 6. LPA receptor LPA_1 and PPARɣ are differentially regulated in human macrophages after proinflammatory and pro-regenerative differentiation.

RNA sequencing analysis. Comparison of macrophage expression profiles in naïve (GM-CSF), classically activated (LPS+INFɣ, proinflammatory) or alternatively (pro-regenerative, IFNβ or IL4) human macrophages, from HD (circles, n = 9) and RR-MS patients (triangles, n = 22). While *LPAR2 expression* did not change after activation (A), *LPAR1* expression was significantly increased in both HD and RR-MS patients after proinflammatory activation (B). In contrast, the nuclear LPA receptor *PPARɣ* was increased in the pro-regenerative state and significantly reduced in proinflammatory macrophages in HD but not in RR-MS patients (C). One-way ANOVA (LPAR1: $p < 0.0001$; LPAR2: $p = 0.06$; PPARG: $p < 0.0001$) followed by Bonferroni-corrected Tukey posthoc pairwise tests (*$p < 0.15$; **$p < 0.01$;***$p < 0.001$;****$p < 0.0001$). Significant differences between two different activation states in two different disease groups have been masked for legibility.

Fig. 7. LPA_1 antagonist directs human macrophages toward a more pro-regenerative phenotype.

Heatmap visualization of the expression of specific markers of proinflammatory or pro-regenerative phenotypes after macrophage activation with LPS. LPA and LPA+Ki16425, are expressed as ratio to the non-activated condition (GM-CSF). LPA treatment (1µM) increased the expression of the M1-like marker genes in a milder manner as compared to LPS. Addition of Ki16425 (400nM) reduced the M1-like polarization effect of LPA, indicating that this response is mediated (at least partially) by LPA_1. Data were normalized to the housekeeping gene HPRT and represented as mean of three different individuals.

a

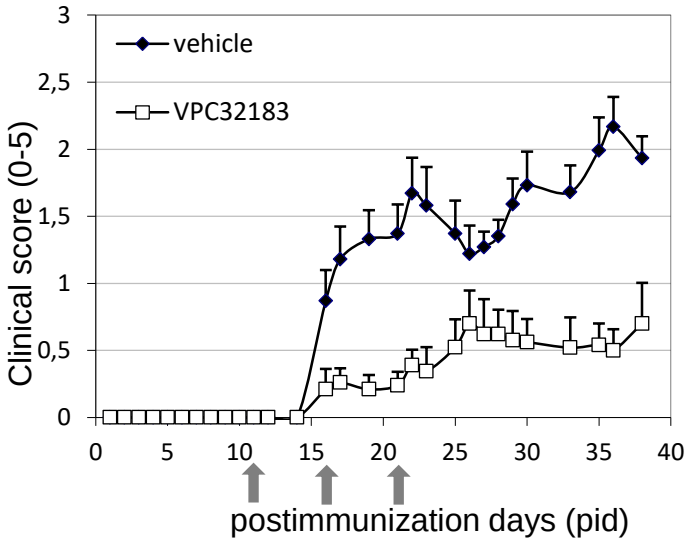


b

	wild-type	maLPA1 null
Mean Clinical Score	1.70 ± 0.13	0.93 ± 0.11*
Cumulative clinical score	62.38 ± 4.8	32.65 ± 11.47*
Onset day	16.35 ± 1.18	16.84 ± 0.94
Number of relapses	8.37 ± 1.18	5.37 ± 0.53*
Max score of relapses	2.27 ± 0.17	1.61 ± 0.24*
Min score of remissions	1.31± 0.26	0.56 ± 0.12*

Fig. 1

a



b

	vehicle	VPC32183
Mean Clinical Score	1.51 ± 0.04	0.46 ± 0.08**
Cumulative clinical score	22.66± 3.11	5.95 ± 2.22**
Onset day	14.05 ± 1.50	15.02 ± 1.80
Number of relapses	3.46 ± 0.26	2.03 ± 0.11**
Max score of relapses	2.42 ± 0.28	0.72 ± 0.44**
Min score of remissions	1.34 ± 0.15	0.28 ± 0.17**

Fig. 2.

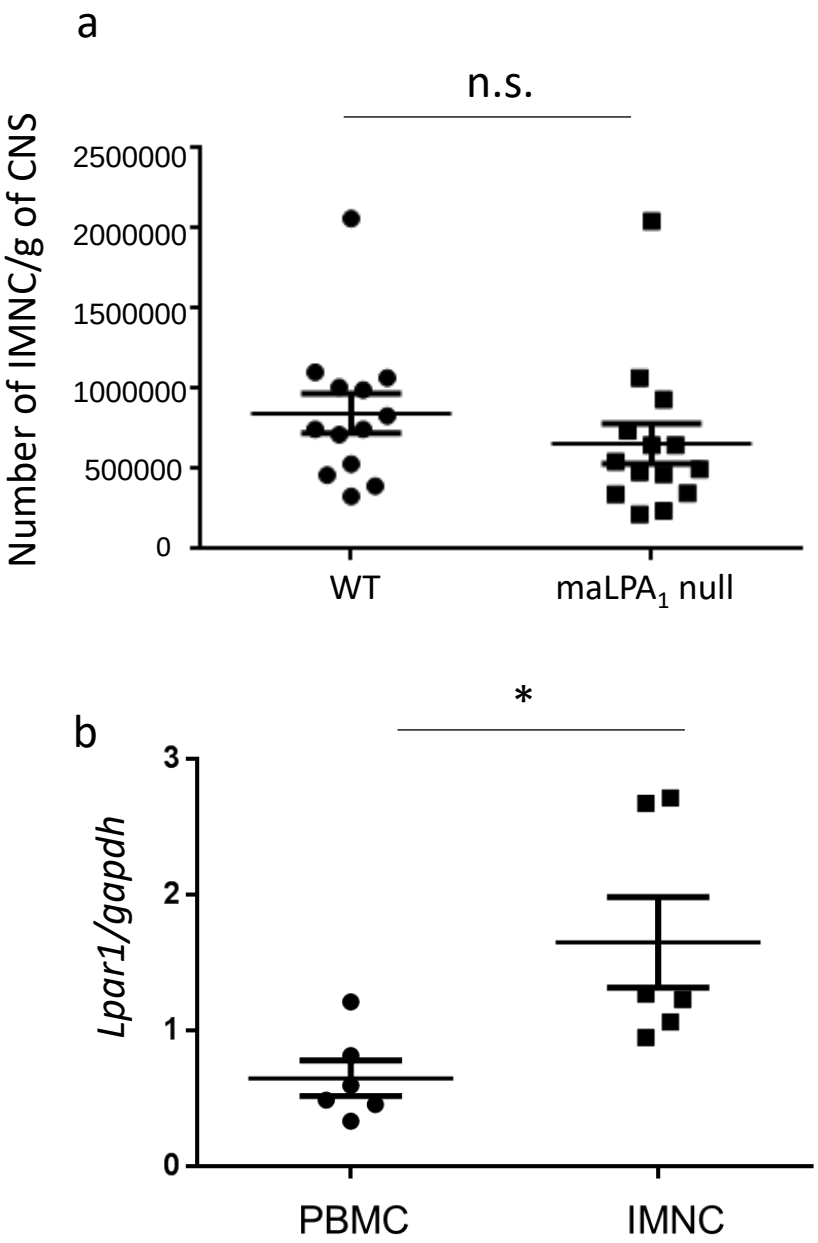

a
n.s.
Number of IMNC/g of CNS
2500000
2000000
1500000
1000000
500000
0
WT
maLPA1 null
b
*
Lpar1/gapdh
3
2
1
0
PBMC
IMNC

Fig.3.

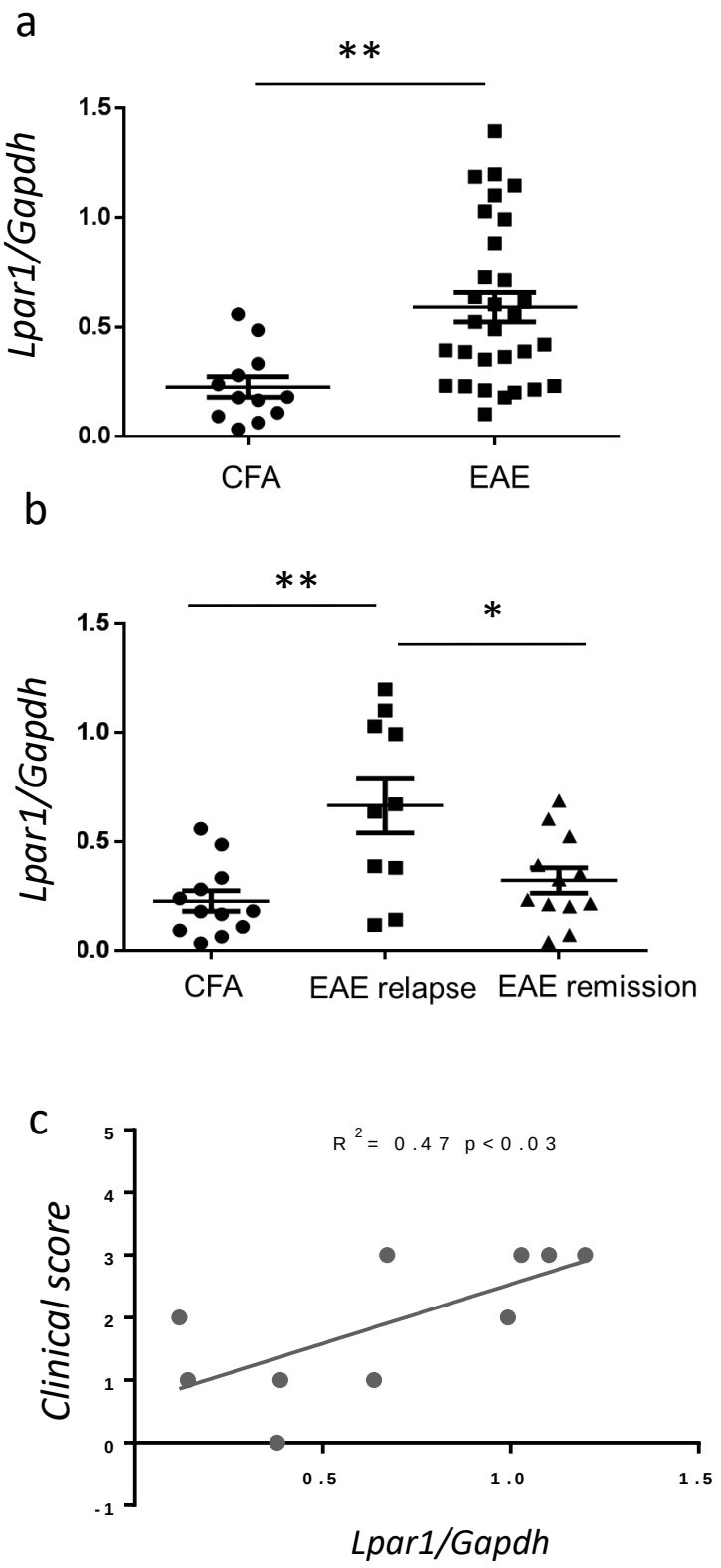

a
**
1.5
1.0
0.5
0.0
Lpar1/Gapdh
CFA
EAE
b
**
*
1.5
1.0
0.5
0.0
Lpar1/Gapdh
CFA
EAE relapse
EAE remission
c
R 2 = 0.47 p < 0.03
5
4
3
2
1
0
-1
Clinical score
0.5
1.0
1.5
Lpar1/Gapdh

Fig. 4.

a

	HD	RR-MS	SP-MS	PP-MS
Number	27	56	20	5
Age (range)	29,8 (21-37)	30,3 (18-55)	48,2 (32-62)	52,8 (50-71)
Onset age	-	26,5	33,1	46,3
Gender(F/M)	58.6 % (17/12)	57,8% (33/23)	85% (17/3)	60% (3/2)
Initial EDSS	-	1,17	3,85	6,37

b

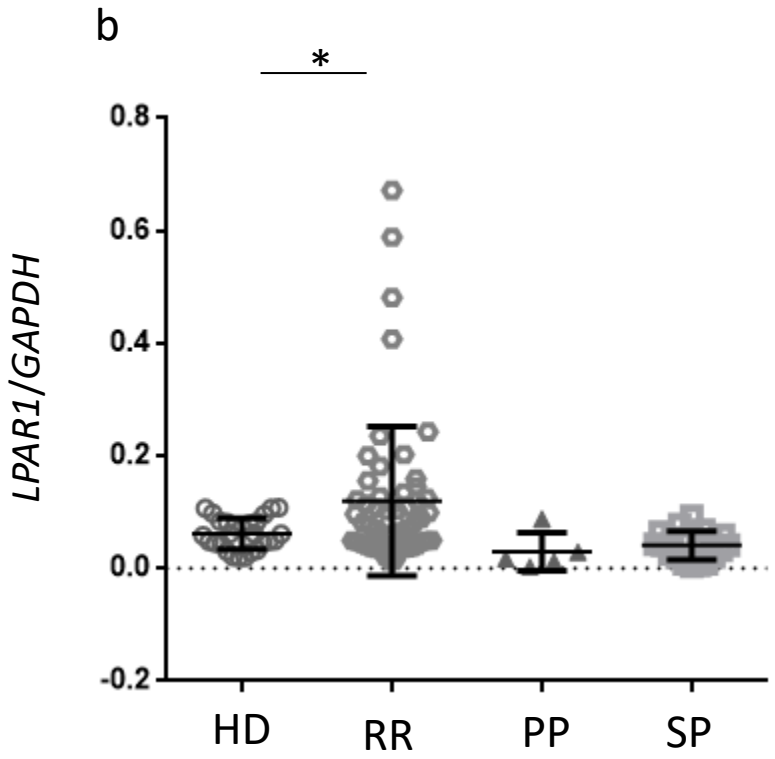


Fig. 5.

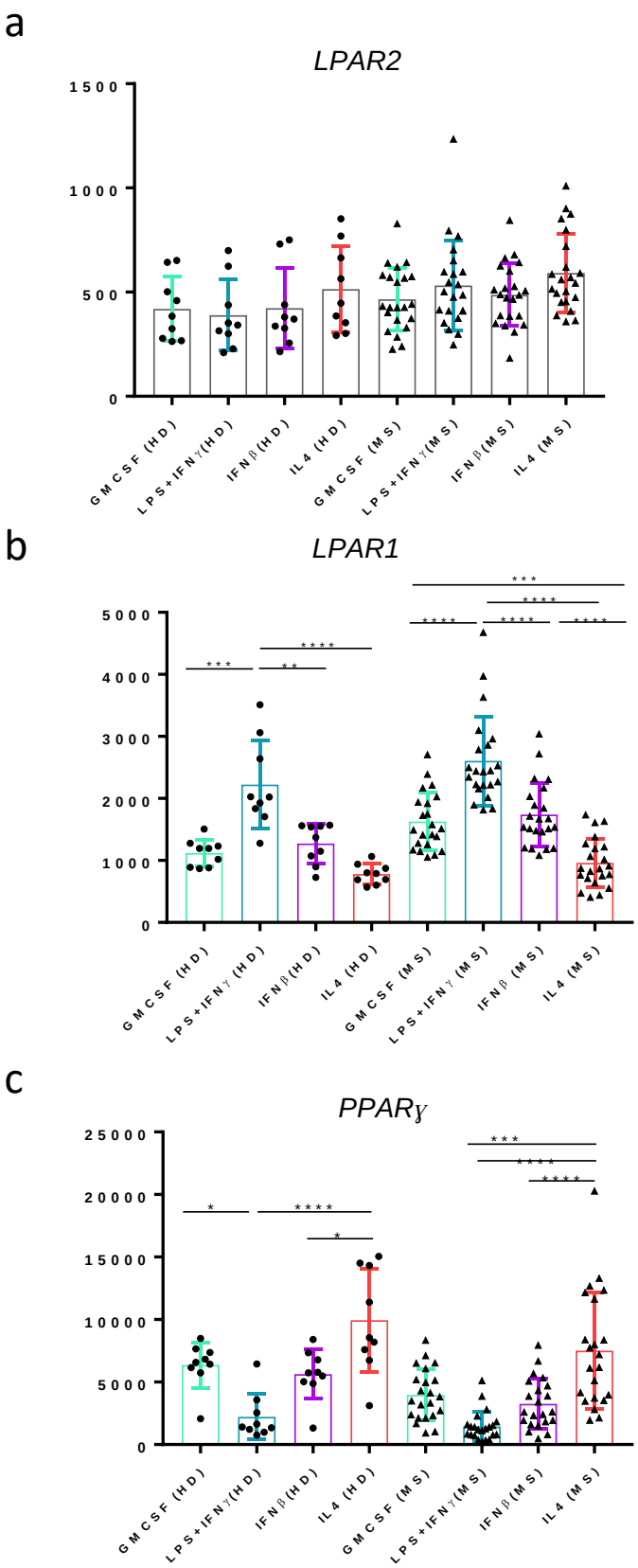

a
LPAR2
1500
1000
500
0
GMCSF (HD)
LPS+IFNγ(HD)
IFNβ(HD)
IL4 (HD)
GMCSF (MS)
LPS+IFNγ(MS)
IFNβ(MS)
IL4 (MS)
b
LPAR1
5000
4000
3000
2000
1000
0
***
****
**
c
PPARγ
25000
20000
15000
10000
5000
0
*

Fig. 6.

	Genes	GM CSF	LPS	LPA	LPA+Ki
M1	CCL2	1	2,22	1,68	1
	CCL20	1	40,76	4,28	2,95
	CCL5	1	12,46	1,73	1,51
	CD68	1	2,26	1,06	1,12
	TLR2	1	1,85	1,92	1,63
	LPAR1	1	1,68	1,35	1,23
M2	CD68	1	0,54	0,99	1,43
	CD180	1	0,83	1,01	1,08
	MRC1	1	0,86	0,98	1,01
	PDGFC	1	0,98	0,93	0,96

Fig. 7.