Pro-resolution lipid mediator maresin-1 ameliorates inflammation, promotes neuroprotection, and prevents disease progression in experimental models of multiple sclerosis

Insha Zahoor[1], Mohammad Nematullah[1], Mohammad Ejaz Ahmed[1], Mena Fatma[1], Sajad Mir[1], Kamesh Ayasolla[1], Mirela Cerghet[1], Suresh Palaniyandi[2,3], Yang Mao-Draayer[4], Ramandeep Rattan[5], Shailendra Giri[1*]

[1]Department of Neurology, Henry Ford Health, Detroit, MI 48202, USA
[2]Division of Hypertension and Vascular Research, Department of Internal Medicine, Henry Ford Health, Detroit, MI 48202, USA
[3]Department of Physiology, Wayne State University, Detroit, MI 48201, USA
[4]Michigan Institute for Neurological Disorders, Farmington Hills, MI 48334, USA
[5]Women's Health Services, Henry Ford Health, Detroit, MI 48202, USA

*Corresponding Author

Keywords: Inflammation, Resolution, DHA, Maresin1, MS, EAE, IL10, Therapeutics

Running title: Maresin1 as a therapeutic strategy in EAE

Corresponding Author:

Shailendra Giri, Ph.D.,
Department of Neurology,
Education & Research Building, Room 4051,
Henry Ford Health
2799 West Grand Boulevard
Detroit, MI 48202, USA

Office: (313) 916-7725
Email: sgiri1@hfhs.org

Abstract

Multiple sclerosis (MS) is the most common inflammatory neurodegenerative disease in young adults, resulting in neurological defects and disability. The endogenous mechanisms to resolve inflammation are intact but become defective in patients, resulting in lack of resolution mediators and unresolved chronic inflammation. Docosahexaenoic acid (DHA) metabolism being impaired in MS, we hypothesize that supplementing its downstream metabolite maresin 1 (MaR1) will alleviate inflammation and demyelination in preclinical mouse model of MS; experimental allergic encephalomyelitis (EAE). Restoration of MaR1 by its exogenous administration in EAE mice propagated inflammatory resolution and had a protective effect on neurological deficits, prevented disease progression, and reduced disease severity by reducing immune cell infiltration (CD4+IL17+ and CD4+IFN-γ+) into the CNS. It significantly reduced the proinflammatory cytokine IL17 and promoted an anti-inflammatory response via IL10 and IL4. Neutralization of IL10 abolished the protective effect of MaR1 in EAE confirming IL10 is mediating MaR1 effect in EAE. Furthermore, it improved the pathophysiology and exerted neuroprotective effects by mitigating disease signs in EAE as evidenced by lower levels of NFL in the plasma of treated group compared to control and higher MBP expression in the brain from the MaR1 treated mice, decreased inflammatory infiltrates, and less demyelination and vacuolization in the spinal cord tissue sections of treated mice. SCENITH data confirmed that MaR1 maintains myelin by regulating oligodendrocyte metabolism. Also, it induces metabolic reprogramming in infiltrating CD4 cells and macrophages, which modulate their phenotype. Metabolic changes induced macrophages by MaR1 restores the impaired efferocytosis in EAE, promoting clearance of damaged myelin and dead cells; thereby lowering the disability with disease course. Overall, MaR1 supplementation has anti-inflammatory and neuroprotective effects in preclinical animal models and induces metabolic reprogramming in disease associated cell-types, promotes efferocytosis, implying that it could be a new therapeutic molecule in MS and other autoimmune diseases.

Highlights

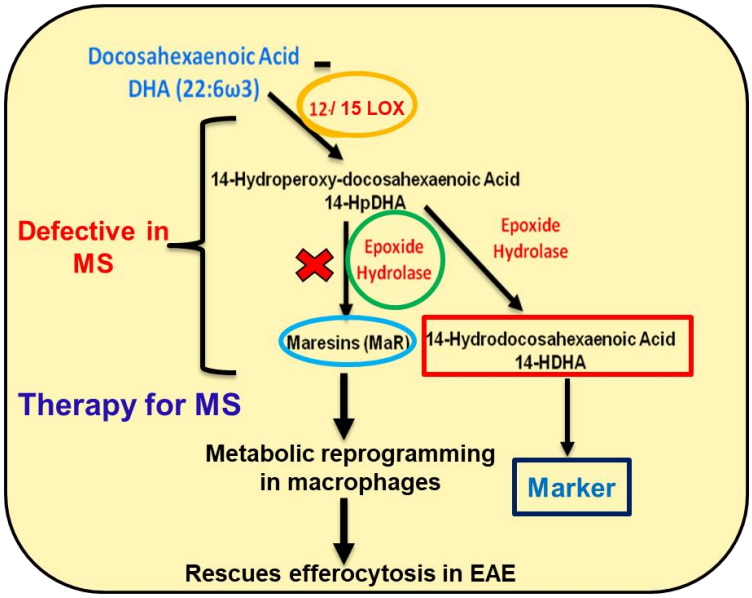
- Inflammation is dysregulated in EAE due to impaired synthesis of DHA derived proresolving lipid mediator MaR1.
- Administration of the resolution agonist MaR1 propagates resolution processes and improves neurological outcome in RR model of EAE.
- MaR1 ameliorates clinical signs of EAE by attenuating pro-inflammatory cytokine IL17 mediated response and promoting anti-inflammatory response through IL10.
- MaR1 supplementation improves the pathophysiology in EAE and shows neuroprotection as indicated by the lower levels of NFL in the plasma and higher expression of MBP in the brain of treated mice.
- MaR1 induces metabolic reprogramming in disease-associated cell types.
- MaR1 promotes efferocytosis in EAE through metabolic reprogramming of macrophages.

Significance

Inflammatory process is a protective response to several challenges like injury or infection. However, it must resolve over time to maintain tissue homeostasis. Impaired or delayed resolution leads to damaging effects, including chronic inflammation, tissue damage, and disease progression as occurs in multiple sclerosis (MS). We report that inflammation is dysregulated in preclinical animal model of MS, experimental autoimmune encephalomyelitis (EAE), partially due to impaired synthesis of proresolving lipid mediators. We show that the administration of the resolution agonist known as maresin 1 (MaR1) in EAE actively propagates resolution processes and improves neurological outcome. We conclude that

MaR1 is a potential interventional candidate to attenuate dysregulated inflammation and to restore neurological deficits in EAE.

Graphical abstract



Introduction

Multiple sclerosis (MS) is one of the most common inflammatory and neurodegenerative diseases in young adults leading to a build-up of neurological defects with an irreversible disability (Pugliatti et al., 2006; Wallin et al., 2019). While the relapsing phenotype of MS is treatable, overall, the disease remains largely incurable and results in progressive disability. Patients commonly suffer from relapses accompanied by damage-inducing inflammation, and current immunomodulatory medications suppress the whole immune system, resulting in ill-tolerated side effects and subpar tolerability (Galetta et al., 2002). It is known that inflammation plays a detrimental role in several autoimmune diseases, including MS, making it important to investigate the endogenous inflammatory pathways (Dendrou et al., 2015). During disease process, the human body is pushed into remission or chronic disease by an imbalance of inflammation and endogenous defence mechanisms. The endogenous mechanisms to combat the inflammation in MS are not sufficient to resolve it, resulting in chronic inflammation. Unresolved inflammation represents the pathological hallmark of MS and several other autoimmune diseases, however current therapeutic options fail to adequately suppress the ongoing inflammation, resulting in inflammatory attacks that gradually increase in severity causing continued neuronal damage and failed or inadequate remyelination, consequently leaving axons demyelinated and vulnerable to degeneration (Mahad et al., 2015; Nakahara et al., 2012). Therefore, it is highly important to investigate the pathways that can reduce excessive inflammation and promote a quick resolution.

Inflammation resolution is an active process mediated by the endogenously derived metabolites of dietary polyunsaturated omega fatty acids (PUFA), collectively termed as specialized pro-resolving lipid mediators (SPM). Dietary factors are modifiable environmental contributors in MS (Olsson et al., 2017; Fitzgerald et al., 2018). A large prospective study has found a significant inverse association between polyunsaturated fatty acid (PUFA) intake and the risk of MS (Bjornevik et al., 2017). Thus, low PUFA intake may be a modifiable risk factor for MS. This reflects requirement of in-depth research on dietary factors to understand the definite mechanisms of disease etiopathogenesis. There is compelling evidence to suggest defects in docosahexaenoic acid (DHA) metabolism which result in the deficiency of downstream metabolites (resolvins, protectins, and maresins), leading to chronic inflammation and a delay in the healing/repair process (Dalli et al., 2013). However, limited information is available regarding the presence or abundance of these metabolites in MS patients (Zahoor and Giri, 2020).

We have previously demonstrated through metabolipidomics in the serum samples from 29 MS patients (compared to 29 age and gender matched) that the level of DHA derived metabolite 14-HDoHE, pathway marker for resolution mediator Maresin (macrophage mediator in resolving inflammation (MaR)) biosynthesis is significantly lower in patients, suggestive of defective MaR1 biosynthesis (Zahoor et al., 2022). It is already known that MaR1 mitigates inflammatory damage in other inflammatory disease models with promising neurological recovery by promoting resolution and neuroprotection, and attenuation of neuroinflammation and neurocognitive dysfunction (Xian et al., 2016; Yang et al., 2019; Francos-Quijorna et al., 2017; Munir et al., 2019). As a new family of anti-inflammatory and proresolving lipid mediators, MaR are a class of 14S-dihydroxyl-containing molecules with conjugated triene double bonds, which are synthesized from DHA through an oxidative (e.g., lipoxygenase-related) pathway during inflammation regression. They are highly conserved resolution mediators with potent anti-inflammatory and pro-resolving properties with role in tissue regeneration in acute or chronic inflammatory related disease models (Tang et al., 2018; Li et al., 2020). Therefore, MaR1 as a potent inflammatory self-limiting factor is expected to become highly promising anti-inflammatory intervention drug target. Briefly, MaR1 is synthesized from DHA by 12 lipoxygenase (12-LOX) with 14-HpDHA as its precursor and 14-HDHA a by-product (pathway marker). It binds to receptor leucine rich repeat containing G protein-coupled receptor 6 (LGR6) and further studies are needed to elucidate if 12-LOX/14-HDHA/LGR6 pathway is crucial metabolic signalling axis in the regulation of MaR1 activity in

peripheral inflammation and neuroinflammation and if it may provide us attractive therapeutic target(s) with potential clinical application for MS.

In the present study, we aimed to further explore the prophylactic and therapeutic role of MaR1 in mitigating inflammation and demyelination in preclinical animal model of MS experimental allergic encephalomyelitis (EAE) with novel pathway/mechanism of action. We hypothesize that during EAE, MaR1 biosynthetic pathway is diminished which augments disease progression in MS. The present study shows that exogenous administration of MaR1 in a preclinical animal model of EAE mediates the resolution of inflammation by promoting anti-inflammatory response through IL10, resulting in improved neurological outcomes in EAE, suggesting that MaR1 could be a novel therapeutic approach to treat MS in humans. Our study is the first to report the protective effects of MaR1 are partly mediated through metabolic reprogramming of disease-associated cell types, regulating their phenotype towards inflammation resolution and myelin repair.

Materials and Methods

EAE induction and functional evaluation

Female SJL mice, 10-12 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in the pathogen-free animal facility of Henry Ford Health, Detroit, MI, according to the animal protocols approved by the Animal Care and Use Committee of Henry Ford Hospital. Mice were immunized on day 0 by subcutaneous injections in the flank region with a total 200 µl of antigen $PLP_{139-151}$ peptide (100 µg/mouse) emulsified in Complete Freund's Adjuvant CFA (Sigma Chemicals, St. Louis, MO, USA) supplemented with 4 mg/ml heat-killed Mycobacterium tuberculosis H37Ra (400 µg; Becton, Dickinson and Company, Sparks, MD, USA) as described previously (Mangalam et al., 2013; Mangalam et al., 2016; Poisson et al., 2015). One set of mice were injected with CFA without antigen/peptide to serve as control.

All the mice were housed with standard food and water ad libitum at a room temperature of 22 ± 2ºC under 12:12 h light-dark cycle. Clinical score was monitored daily till the duration of study in a blinded fashion by measuring paralysis according to the conventional grading system as described previously (Mangalam et al., 2013; Mangalam et al., 2016; Poisson et al., 2015). Clinical signs of EAE appeared 12–14 days after immunization, whereas control mice did not show any neurological deficits. EAE onset was considered on the day animals showed the first signs of disease. Disease peak was considered on the day when EAE score was maximum and did not increase from the previous day (day 16–21 post-immunization). Chronic phase of the disease was considered after 21 days post-immunization if EAE score decreased or was maintained with respect to disease peak.

EAE mice, treated with PBS or MaR1 were euthanized at different stages of the disease (70 days pdi). SJL mice were anesthetized with CO_2 and transcardially perfused with 1X chilled PBS and in some cases, followed by perfusion with 4% paraformaldehyde for histopathological analysis and confocal imaging. The secondary method of euthanasia adopted was either cervical dislocation or needle induced pneumothorax. For recall response, cells (4×10^6/ml) isolated from spleens were cultured in the presence or absence of antigen PLP (20 µg/ml). Cell proliferation and the production of various cytokines (IFNγ, GM-CSF, IL17, IL10, and IL4) were examined as described before (Mangalam et al., 2013; Mangalam et al., 2016; Poisson et al., 2015).

All the animal experiments in this study were performed as per the policies and guidelines of IACUC committee at Henry Ford Health under the animal welfare assurance number D16-00090.

MaR1 administration

The mice were randomly assigned to the treatment and control experimental groups (CFA, EAE, EAE treated with MaR1, will be mentioned as MaR1 hereafter; N=8). MaR1 solution was freshly prepared from the stock solution and administered right away to prevent its degradation. Daily intraperitoneal injection (i.p.) of 1ug and 300 ng/kg bw of MaR1 (7R,14S-dihydroxy-4Z, 8E, 10E,12Z, 16Z, 19Z-DHA; Cayman Chemicals, Ann Arbor, MI, USA) in 200 µL of PBS or vehicle were given to each individual mouse from day 6 post-disease induction until the end of the study after induction. The dose of MaR1 was chosen based on the efficacy and bioactivity of this SPM in other disease models like spinal cord injury (Serhan et al., 2015; Marcheselli et al., 2003; Svensson et al., 2007). After dose optimizations with 1 ug and 300 ng, we selected 300 ng as optimal dose for our studies based on its efficacy in EAE under our experimental conditions.

To confirm if MaR1 exerts its protective effects in EAE through IL10, we performed IL10 neutralizing experiments. SJL mice were immunized as mentioned above (in section EAE induction and functional evaluation), followed by ip injection with 200 µg of anti-IL10 (BioXCell # BE0049) twice a week and daily ip treatment with 300 ng of MaR1. Controls received an isotype-matched rat IgG1 (BioXCell # BE0088) following the same protocol Clinical readout was taken as clinical score followed by euthanizing mice at 21 days pdi.

Behavioural test

Locomotor activity was measured to assess the effect of MaR1 treatment on EAE. We employed infrared (IR) based technology called, novel infrared-based automated activity-monitoring system (IRAMs) developed by Columbus Instruments International (Columbus, OH). This instrument is supported by several electrodes embedded in an electric sensing board spaced 4 inches apart with the beam diameter of 0.125 inches and a beam scan rate of 160 Hz. Sensors were properly screwed on a bracket to make it more flexible and adjustable. Acrylic cages were placed between those brackets. The locomotor activity in form of diurnal horizontal (X-axis) and nocturnal vertical activity (Z-axis) was measured and quantitative analysis was done by the MDI software (Columbus Instruments International, Columbus Ohio) based on the system calculating number of emitted infrared interruptions by mice at a specific time interval.

Macrophage and CD4 cell isolation

At day 18 post-immunization, we isolated F4/80 macrophages from the spleens of treated and untreated RR-EAE using a Miltenyi biotech kit (#130-110-443; 92% purity). Similarly, CD4 cells were isolated from spleens using CD4 isolation kit (Biolegend # 480033).

Single Molecule Array (SIMoA) Assay

To assess the effect of MaR1 treatment on CNS in EAE, we measured neural markers (NfL and GFAP) in plasma samples of CFA and EAE mice with commercially available SiMoA™ Neuro 2-Plex B Advantage Kit (Product Number: 103520) (Quanterix, MA) on the SR-X analyser according to manufacturer's instructions. Also, the effect on inflammation was examined by cytokine profiling. We used Mouse Cytokine 5-Plex Kit for IL6, TNFA, IL17, IL12, IL10 (Cat # 107-178-1-AB; Product Number: 85-0441) (Quanterix, MA) on the SP-X analyzer according to manufacturer's instructions. We applied SIMOA as this is the emerging ultrasensitive technology with potential to detect the analytes which are present in low levels and below detection limits for other conventional assays.

RNA isolation and qRT-PCR

Total RNA was isolated from cells with Qiazol reagent using RNeasy kit (Qiagen), 1 ug of RNA was used for cDNA synthesis using iScript cDNA synthesis kit (Biorad), according to the manufacturer's guidelines. The quantitative-PCR analysis was performed using the SYBR Green (Bio-Rad) on the Bio-Rad CFX384 apparatus and software (CFX Maestro, BioRad). The gene expression was normalized to the expression of the control housekeeping gene ribosomal L27 using CFX Maestro software (BioRad).

Enzyme-linked immunosorbent assay (ELISA)

The protein levels of the following cytokines IL-4, IL-10, IFN-γ, IL-17A, and GMCSF were quantified using standard enzyme-linked immunosorbent assays (ELISA), using commercially available kits provided by Biolegend and BD Biosciences.

Immunohistology

Briefly, brain and spinal cord tissues were harvested from mice under deep anaesthesia and post fixed in 4% paraformaldehyde at 4°C for 48 h, and cryoprotected with 30% sucrose until sank. Tissues were later embedded in OCT compound and frozen sections (10-20 µm) were cut to check the infiltration of immune cells and demyelination in CNS. Histopathology evaluation using Hematoxylin and Eosin (H&E) and Luxol Fast Blue (LFB) staining methods were used based on our previously described protocols (Zahoor et al., 2022; Mangalam et al., 2016; Khan et al., 2015). Specifically, immunohistochemistry (IHC) was performed for CD4, and F4/80 in spinal cord samples to assess the effect of MaR1 treatment on immune landscape inside CNS. All images were captured by using a light microscope. For immunofluorescence staining, sections were washed 20 min with 0.1% PBS-TritonX followed by incubation with a blocking solution containing 1% BSA for 1 h at room temperature, sections were incubated with mouse anti-MBP monoclonal antibody (1:500 Cell Signalling Technology) overnight at 4°C. Subsequently sections were washed followed by incubation with Alexa Fluor (AF) 568 or AF 488-coupled secondary antibodies (Life Technologies). Nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole) H-1200; Vector Laboratories, Inc., CA, USA) and were cover slipped. Images were taken by laser scanning confocal microscope (LSCM, Olympus). Images were quantified using Image-J software (National Institutes of Health, Bethesda, MD).

Flow cytometry

Immune cell profile was determined in spleen, lymph nodes, brain, and spinal cord of EAE mice at different time points for respective experiments using the standard staining methods and analysis protocols previously described in our published work (Zahoor et al., 2022; Mangalam et al., 2016; Poisson et al., 2015). All data were processed using FlowJo® software and analysis program (Treestar, Ashland, OR).

Detection of reactive oxygen and nitrogen species (ROS and RNS)

The intracellular levels of ROS/RNS in different immune cells of CNS were evaluated using the Cellular ROS/RNS Detection Assay Kit (Abcam, USA; catalog number: ab139473) as per manufacturer's instructions. Briefly, Brain Single Cell suspensions were prepared using Percoll density gradient, and cells were then treated with mixture of two different fluorescent dye reagents in Phenol free culture media that allows the detection of ROS and RNS. After ROS/RNS dyes treatment, cells were washed, and Fc receptors were blocked with anti-mouse CD16/32 (Biolegend). Cells were then stained with Fluorochrome conjugated Antibodies; anti-mouse-BV421-CD45, anti-mouse-BV510-CD11b, anti-mouse-BV786-CD4, anti-mouse-PE-Cy7-F4/80 (all purchased from Biolegend) and anti-mouse-PE-O4 (Milentyl Biotech)

antibodies for characterizing Microglial cells, CD4T cells, macrophages, and Oligodendrocytes respectively for 40min at 4C. Cells were washed twice and resuspended in FACS stain buffer. Samples were run on Attune NxT Cytometer (Thermo) and analyzed using FlowJo software (BD Biosciences).

Bioenergetics

Seahorse Bioscience XFe96 Extracellular Flux Analyzer (Agilent) was used to monitor the mitochondrial oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in intact cells. XF mitochondrial Stress Test was performed in CD4+CD25- cells (purity ~95%) isolated from CFA, EAE treated or untreated with MaR1 (300 ng/mouse daily) to examine mitochondrial respiration. The cells were seeded at a concentration of 50 x 10^4 cells per well in Poly-Lysine coated XFe 96-well seahorse culture microplate in 100µl of complete DMEM media and incubated overnight at 37^0C in 5% CO_2 before the treatment. After 24hrs of indicated treatments, the growth media was aspirated, washed twice, and replenished with 175 µl of pre-warmed bicarbonate-free DMEM (basal) assay media. The plate was incubated for 45 minutes at 37^0C in a CO_2-free incubator for degassing. After incubation, OCR and ECAR were measured as per the manufacturer's protocol. For the measurement of ATP levels, the cells were grown in 96 well plate (25 x 10^4/well), and level of ATP was determined with fluorometric ATP determination kit following the manufacturer's protocol (Invitrogen). Data presented as unit/number of cells.

Single-cell energetic metabolism by profiling translation inhibition (SCENITH)

We used the SCENITH assay, a FACS-based, quantitative analysis of mRNA translation in single cells (Adamik et al., 2022; Arguello et al., 2020; Lopes et al., 2021). It takes advantage of cells being exposed to a short pulse of puromycin (PURO), which is incorporated into polypeptides that are being translated. Fixed cells are permeabilized and stained with anti-PURO antibody. Translation being an energy consuming process (Piques et al., 2009), SCENITH is a proxy for the metabolic fitness of the cell and its metabolic outcome correlates well with Seahorse (Arguello et al., 2020; Lopes et al., 2021), requiring only one-tenth of the cell number. It provides information on the use of energetic pathways (glucose dependence, mitochondrial dependence, glycolytic capacity, and fatty acids and glutaminolysis capacity) (Arguello et al., 2020; Lopes et al., 2021). This method allowed us for the analysis of the metabolic activities of specific cell populations, to examine the metabolic alterations in the CD4, macrophages, and O4+ oligodendrocyte population, using the previously described protocol and optimised assay conditions (Suhail et al., 2023).

RNA sequencing

For transcriptome analysis, spinal cords of three mice from each group CFA, EAE, and MaR1 were sent for RNA-Seq using the services of CD-Genomics, NY. The final library size was about 400bp with an insert size of about 250bp. Illumina® 8-nt dual-indices were used. Equimolar pooling of libraries was performed based on QC values and sequenced on an Illumina® NovaSeq 6000 (Illumina, California, USA) with a read length configuration of 150 PE for 40 M PE reads per sample (20M in each direction). To assess the sequencing quality, quality check (QC) was performed using FastQC during data analysis. All samples passed QC, performed using FastQC. Using the STAR aligner, paired-end reads of 150bp were uniquely aligned to the GRCm38 - mm10 with a mapping rate around 80% STAR was used to count the reads for each sample. The limma-Voom package in R was used to assess differential expression, with a padj cutoff of 0.05 and logFC of 0.34. The threshold for filtering out low expressed genes was set to 1 CPM in at least 80% of the samples. The trimmed mean of M-values (TMM) method was used to normalize the counts. Significantly dysregulated genes were analysed for Gene Ontology (GO) biological processes with FDR cutoff of <0.05 using shinyGO.

Data and statistical analysis

All values are presented as Mean ± SEM, and statistically significant differences were assessed by Mann-Whitney test, Student's t-test, and one-way ANOVA. Statistical significance in terms of the P-value is described in the figure legends for a particular experiment. All statistical analysis were performed with GraphPad Prism.

Results

MaR1 ameliorates clinical signs of disease in EAE, improves pathophysiology and promotes neuroprotection

Since we have previously demonstrated that production of MaR1 is impaired in MS patients (Zahoor et al., 2022), we proceeded to investigate whether its exogenous administration attenuates inflammation in EAE. To understand the effect of MaR1 on inflammation and clinical outcome, we assessed its effects on both models of EAE (RR-SJL and Chronic-B6) given at day 6 post-immunization (**Fig. 1A**). We proceeded with RR model for in-depth experiments due to its higher relevance and translational value to human MS as majority of the patients have RRMS. We observed that mice treated with MaR1 showed significant protection against neurological impairments (**Fig. 1A-F**). Indeed, MaR1-treated mice reached an average EAE score of ~ 1-2 at the peak of the disease course, the score was maintained between 0-1 by the end of the follow-up until day 70, while EAE mice reached ~ 3.5-4 at the peak and reflected classical pattern of relapse and remission (**Fig. 1B**). Moreover, mice treated with MaR1 had significantly lower cumulative ($p<0.01$) and maximum scores ($p<0.001$) than EAE mice (**Fig. 1C, D**). The disease severity ($p<0.001$) and incidence of disease symptoms ($p<0.0001$) were also markedly lower in treated mice compared to control EAE mice (**Fig. 1E, F**). Overall, the clinical readout indicated enhancement of neurological functions in EAE with MaR1 treatment and prevented disease progression as mice were maintained in remission stage until the end of study.

Moreover, the locomotor activity of mice was measured based on nocturnal and diurnal activity of mice in horizontal and vertical axis using IRAMS monitoring system confirmed the improvement in neurological outcome with MaR1 treatment as treated mice showed higher activity compared to untreated EAE mice, indicating that MaR1 improved overall behavioural activity in EAE (**Fig. 2**). Collectively, average hourly counts for horizontal and vertical activity during nocturnal and diurnal were higher in MaR1 treated group compared to CFA and EAE ($p<0.01$) (**Fig. 2C**). MaR1 group showed significant increase in average daily counts for horizontal activity compared to EAE, indicating that the decline in activity was restored with MaR1 treatment (average hourly counts diurnal EAE~200 and MaR1~400; nocturnal EAE~400 and MaR1~>400). Further, the histopathology analysis showed a marked decline in inflammatory cell infiltrates in the CNS of mice treated with MaR1 as revealed by HE staining ($p<0.01$) (**Fig. 3G**). We also observed improvement in the demyelination status of MaR1 treated mice compared to EAE as reflected by LFB staining (**Fig. 3H**). There was less demyelination and vacuolization in the spinal cord tissue sections of treated mice ($p<0.05$).

Blood NFL is a marker of neuro-axonal injury and associated with relapses, EDSS worsening, lesions on MRI scans and atrophy of both the brain and spinal cord in MS (Barro et al., 2018). Accordingly, we checked the effect of MaR1 on NFL levels in the plasma as the reports show its association with impairment of blood-brain barrier integrity, immune cell extravasation, and CNS inflammation following the first demyelinating event in MS (Uher et al., 2021). SIMOA showed markedly lower levels of NFL ($p<0.01$) in the plasma of treated mice compared to EAE (**Fig. 3F**), thus indirectly reflecting protective effect of MaR1 on leaky blood brain barrier by an unknown mechanism. This somehow indicates that MaR1 could preserve neuronal and axonal integrity that prevents release of CNS contents into peripheral circulation.

Taken together, these data suggest that the reduced neurological deficits observed in EAE mice treated with MaR1 was due to the ability of MaR1 to reduce the accumulation of inflammatory infiltrates in the CNS and improve the myelination status, thus attenuating neuroinflammation and preventing further damage by imparting neuroprotection, which resulted in the improvement of clinical signs of the disease. Overall, MaR1 treatment reduced the disease severity, prevented disease progression, and improved the neurological outcome in both models of EAE.

MaR1 attenuates infiltration of immune cells into the CNS during EAE and promotes anti-inflammatory response

We next investigated if MaR1 treatment modulates the number of immune cells in the CNS of EAE mice. Flow cytometry revealed that MaR1 strongly reduced the infiltration of immune cells into CNS, including mononuclear cells ($p<0.01$), dendritic cells ($p<0.01$), and T cells ($p<0.001$). Owing to the role of CD4 cells in disease pathology of MS (Peeters et al., 2017), we assessed the effect of MaR1 treatment on CD4 infiltration into CNS. We found that MaR1 reduced the number of CD4 T cells expressing IL17 and IFNγ in the spinal cord of EAE mice, suggesting that the number of pathogenic T cells was reduced (CD4+IL17+ and CD4+IFN+ $p<0.001$) (**Fig. 3**). Overall, IHC provided further evidence confirming that the reduced accumulation and or infiltration of macrophages F4/80 and CD4 cells into CNS in treated mice compared to EAE ($p<0.01$) (**Fig. 3**).

Further, we investigated whether MaR1 modulates immune cell response by monitoring its effect through recall response on pro and anti-inflammatory cytokines in the splenocytes isolated from EAE and MaR1 treated mice. MaR1 treatment showed contrasting effect as it increased IFNy and decreased IL17 significantly ($p<0.01$) (**Fig. 3**). We observed this effect both in gene expression through qPCR and by quantifying protein levels using ELISA. At the same time, it promoted IL4 and IL10 production ($p<0.001$), however, did not alter the regulatory lymphocytes Treg cells (CD3+, CD4+, FoxP3+) (data not shown).

MaR1 imparts protection by promoting anti-inflammatory phenotype through IL10

MaR1 tended to increase the percentage of IL10 producing cells ($p<0.01$) in MaR1 treated group as shown by flow cytometry plots (**Fig. 4A**). To further interrogate, we profiled all cell types expressing IL10 and found that MaR1 treatment significantly induced the expression of IL10 in all cell types including CD4, CD8, B cells, mDC and macrophage; however, it was more profound in CD4 and macrophages ($p<0.001$; ~4.5 folds) (**Fig. 4B**). SIMOA showed markedly higher levels of IL10 ($p<0.01$) in the plasma of treated mice compared to EAE (**Fig. 4C**), thus indirectly reflecting protective effect of MaR1 through IL10 by an unknown mechanism. These results indicate that the effect of MaR1 on immune cell responses observed in the brain and spinal cord of MaR1-treated mice might be, in part, due to its actions in the periphery; however, these findings majorly highlight its role in promoting anti-inflammatory response through IL10. To confirm if MaR1 imparts protection through IL10, anti-IL-10 Ab reversed/neutralised the protective effects of MaR1 as assessed by clinical score (**Fig. 5A**), histology damage, and flow cytometry data for intracellular cytokines IFN-γ and IL17 (**Fig. 5B**). The protective effect of MaR1 was lost compared to group treated with MaR1-IgG. Further, we did adoptive transfer that showed higher disease severity in mice where anti-IL10 ab was used to neutralise the protective effects of MaR1 (**Fig. 6C**). These data confirm that MaR1 imparts protective effect by promoting IL10 phenotype in EAE, indicating that IL10 mediated signaling is involved in protective actions of MaR1 that needs to be further evaluated in studies using IL10-KO mice.

MaR1 shows therapeutic potential in RR-EAE model, impeding the encephalitogenic property of CD4 effector cells.

To examine the therapeutic effect of MaR1 in EAE, its treatment was started on day 20 post-immunization when all mice recovered from paralysis in RR-EAE group (**Fig. 6A**). MaR1

treatment significantly blocked the relapse in RR-EAE ($p<0.01$). Further to investigate the effect of MaR1 on effector function of CD4 cells, we isolated cells from lymph nodes at day 38 (from **Fig. 6B**) and cultured them in Th17 inducing conditions with antigen ($PLP_{139-151}$) and post 3 days, CD4 cells were isolated and adoptively transferred (AdT) into naïve SJL mice and clinical score was monitored daily. CD4 cells from EAE were able to induce EAE, however, CD4 cells from MaR1-treated group lost encephalitogenicity to induce EAE disease ($p<0.01$) (one mouse out of 5 showed clinical score 0.5). CD4 cells significantly induced higher IL17 in AdT-EAE groups compared to AdT-EAE-MaR1 (Data not shown). This data suggest that MaR1 has therapeutic potential, and its treatment impacted the encephalitogenic property of effector CD4 cells.

MaR1 induces metabolic reprogramming in effector CD4 cells

It is becoming clear that metabolic reprogramming is important in T cell activation, differentiation, and function (Dominguez-Andres et al., 2019). To support proliferation and function, activated CD4+T cells require high rates of glucose uptake and glycolysis, glutaminolysis, and lipid synthesis (Chang et al., 2013; Kidani et al., 2013; Lochner et al., 2015; Macintyre et al., 2014; Papadopoulou et al., 2021; Puleston et al., 2021; Wagner et al., 2021). To see if MaR1 affects CD4 metabolism *in vivo*, we examined the energy phenotype of CD4 cells ($CD4^{+}CD25^{-}$) isolated from EAE mice treated with MaR1 or vehicle. CD4 cells isolated from MaR1-treated mice had significantly higher basal (not shown) and spare respiratory capacity compared to EAE or vehicle (CFA) groups, whereas CD4 cells from the EAE group had lower mitochondrial respiration (**Fig. 7A**). Interestingly, MaR1 increased glycolysis in CD4 cells resulting these CD4 being a 'higher metabolic state,' whereas vehicle EAE CD4 had a 'lower metabolic state' (**Fig. 7C**). Overall, these findings indicate that MaR1 treatment alters the metabolic and bioenergetic state of CD4 cells *in vivo*, and that these cells lost their encephalitogenic properties to induce disease after adoptive transfer (**Fig. 6**). To investigate the source of cellular ATP in MaR1-CD4 cells, we exposed CD4 cells from all groups to oligomycin (OM), an inhibitor of the F1F0-ATP synthase that also inhibits OCR. In the presence of OM, the cellular respiration rate was dramatically reduced in all groups of CD4 cells, while the glycolysis rate was simultaneously but differentially increased (**Fig. 7**). When mitochondrial respiration was inhibited, the CD4 from the EAE group demonstrated the greatest flexibility in switching energy sources to glycolysis when compared to other groups. No change in ATP levels was observed in CD4 cells from EAE or vehicle (**Fig. 7D**), but a significant increase in ATP levels was observed in MaR1-CD4 cells (**Fig. 7D**) when mitochondrial respiration was inhibited, while switching to glycolysis was like that observed in CD4 cells from CFA, implying that increased ATP in MaR1-EAE may be due to an alternate energy source (s). Also, we studied the metabolic fitness of infiltrated CD4 cells using SCENITH and found a similar pattern to the seahorse (data not shown).

MaR1 regulates metabolic reprogramming to induce macrophage efferocytosis

We found that MaR1 causes significantly higher metabolic changes in macrophages (F4/80) than untreated macrophages (**Fig. 8**). Splenic macrophages isolated from the EAE group had significantly lower mitochondrial respiration, glycolysis, and fatty acid oxidation (FAO), which was significantly reversed in macrophages treated with MaR1 (**Fig. 8A-B**). Macrophages from vehicle-treated mice had a 'higher metabolic state,' whereas EAE macrophages had a 'lower metabolic state,' which was reversed by MaR1 treatment in the same way that healthy control macrophages were (**Fig. 8D**). Although there was no difference in total cellular ATP levels between EAE and CFA, MaR1 significantly increased ATP levels between CFA and EAE (**Fig. 8E**), implying that an enhanced energetic state in macrophages of the MaR1 group is possibly contributing to energy production (**Fig. 8D**). Overall, these findings suggest that MaR1 treatment alters the metabolic and bioenergetic state of macrophages *in vivo*, with the underlying mechanisms yet to be determined. Further, SCENITH data showed similar metabolic fitness as revealed by seahorse (**Fig. 8F**).

It is worthwhile to mention here that the clearance of dead cells by macrophages, known as "efferocytosis," drives inflammation resolution, limits necrosis, and restores homeostasis. Many neurodegenerative diseases are caused by efferocytosis defects (Taheri et al., 2022). MaR1 promotes efferocytosis, a feature of anti-inflammatory macrophages that is required for inflammation resolution and tissue repair (Serhan et al., 2007; Barden et al., 2016; Serhan et al., 2005). Using flow cytometry and confocal analysis, we discovered that MaR1-treated macrophages had higher efferocytosis than vehicle-treated (**Fig. 9Ai-ii**). Recent studies have shown that metabolism plays an important role in the efficient efferocytic function of macrophages, more specifically efferocytosis-induced macrophage glycolysis maintains continuous efferocytosis (Morioka et al., 2018; Schilperoort et al., 2023; Zhang et al., 2019). We used a seahorse bioanalyzer to examine the metabolic state of MaR1-treated macrophages. MaR1 treatment induces mitochondrial respiration and glycolysis in apoptotic cell (AC) treated macrophages. AC (50K/well) by itself does not demonstrate any metabolic function (data not shown). These data suggest a possible link between increased metabolism and efferocytosis in MaR1-treated macrophages. Furthermore, we also found similar effect of MaR1 treatment under *in vivo* conditions (**Fig. 9B**). The macrophages in spinal cord tissues derived from MaR1 treated EAE mice showed enhanced phagocytic index compared to untreated EAE. However, further studies are needed to study a detailed mechanism of how MaR1-induced metabolism will impact this function.

MaR1 improves myelination by regulating O4 oligodendrocyte metabolism

We evaluated whether the neurological improvement in EAE mice treated with MaR1 was related to myelination status within the CNS. Representative IF analysis confirmed better MBP expression in the brain slices of MaR1 treated mice when compared to EAE (**Fig. 10A**). Further, MaR1 treatment resulted in decrease in ROS and RNS levels in O4+ populations compared to untreated EAE (**Fig. 10B**). Based on metabolic SCENITH parameters calculated using PURO MFI (**Fig. 10C**), we discovered that EAE oligodendrocytes (O4) are dependent on glycolysis, but mitochondrial respiration and FAO are significantly reduced, which were significantly restored by MaR1 (**Fig. 10D**). It's worthwhile to mention that SCENITH data clearly shows that MaR1 protects myelin by controlling the O4 oligodendrocyte metabolism.

MaR1 rescues EAE-induced spinal cord transcriptomic alterations

RNA-seq data in the spinal cord of all the 3 groups, CFA, EAE and MAR1 and functional association of the dysregulated genes with GO analysis revealed the transcriptomic changes associated with MaR1 treatment. Across the groups, we found 631 genes which were significantly upregulated in EAE, whereas MaR1 treatment downregulated their expression (**Fig. 11**). Interestingly, these repressed genes were enriched in inflammatory response, leukocyte activation and other immune/defence related GO terms. MaR1 significantly upregulated a small subset of genes enriched in axonogenesis and related processes, most of which were downregulated in EAE (**Fig. 11**). We also checked if MaR1 furthered the gene expression dysregulation in EAE. Notably, MaR1 didn't share any up or downregulated genes in the same direction as EAE, hence it did not extend gene expression changes in EAE.

Discussion

Despite the availability of numerous immunomodulatory drugs, there are no entirely satisfactory treatments for MS as most drugs have substantial side effects with less-than-optimal tolerance and do not provide complete remission or stabilization of disease. Given the fact that unresolved inflammation is a hallmark of the MS pathogenesis, it is imperative to study the endogenous mechanisms that can curtail excessive inflammation and promote its timely resolution. In MS, disease progression might partly be a consequence of failed inflammation resolution associated with metabolic dysfunction of resolution mediators (Zahoor

and Giri, 2020). However, there is a gap in knowledge about the role of endogenous resolution mediators in MS. Identifying a therapeutic option that can modulate the adaptive and innate immune responses without generally suppressing the immune system has been a key barrier to improve the treatment for patients with MS. Using metabolic profiling, we have identified in our previous work that precursor of MaR1, a pro-resolving lipid metabolite of omega-3 polyunsaturated fatty acids, is significantly altered in the plasma of patients with MS. Consistent with this finding, MS patients have lower levels of omega-3 metabolites, which are precursors of maresins, compared to healthy controls, a finding that has been replicated in animal models of the disease, EAE (Zahoor et al., 2022). Till date protective action of MaR1 has not been reported anywhere in MS; however, there is only one recent report on the effect of MaR1 in chronic EAE with limited findings on its mechanism of action (Sánchez Fernández et al., 2022), thus making our findings novel in the field of MS.

In the present study, we investigated the effect of intraperitoneal administration of resolution agonist MaR1 on inflammation and disease severity in EAE mice. Notably, we found that daily supplementation with MaR1 at the dose of 300ng/mouse significantly attenuated clinical symptoms in both chronic and RR-EAE. These data are provocative for translational potential of maresins, particularly because the immune system is not supressed by MaR1 treatment as it is with steroids and most other MS therapies, making it a novel and potent non-immunosuppressive therapeutic option for MS (Fattori et al., 2020). MaR1 treatment attenuated IL17, however, significantly induced IL4 and IL10 production. These findings highlight the immunomodulatory role of MaR1 in EAE by attenuating the pro-inflammatory environment of the CNS and by modulating T cell responses towards anti-inflammatory phenotype. Nonetheless, it is pertinent to mention that the protective actions of MaR1 on neuroinflammation in EAE involve significant protection against neurological decline and myelin loss despite starting treatment on day 6 post-immunization. Overall, we demonstrated that MaR1 accelerated inflammation resolution in EAE and prevented disease progression. The immunomodulatory effect of MaR1 is believed to be mediated through its receptor, LGR6, leading to modulation of T cell responses. However, the mechanism modulating EAE disease course mediated by MaR1-LGR6 cascade remains elusive.

Multiple studies have shown the role of DHA in neuroprotection, although the underlying mechanisms are still unfolding, which could possibly involve myelin sheath maintenance, resulting in axonal integrity and hence an effective means of promoting remyelination in brain diseases (Mayurasakorn et al., 2011; Kim et al., 2014). Remyelination is mediated by oligodendrocyte precursor cells (OPCs) and these cells respond to injuries in form of inflammation/trauma/hypoxia/demyelination by activation, proliferation, migration and finally differentiation into remyelinating mature oligodendrocytes (Franklin et al., 2008). Interestingly, it is the last of these processes that is most likely to fail in MS and leave areas of demyelination containing oligodendrocyte-lineage cells that are unable to fully differentiate (Franklin et al., 2008; Patel et al., 2012). Moreover, DHA-derived metabolites have neuritogenic, synaptogenic, and neurogenic properties; and treatment with DHA per se and some of its downstream metabolites has been shown to ameliorate or resolve inflammation; thus, acting as a nutritional armour for brain (Bazan et al., 2011; Orr et al., 2013; Kong et al., 2011; Hong et al., 2003; Kim et al., 2011; Rashid et al., 2013; Mukherjee et al., 2004). Previously, our group was the first to test the therapeutic potential of any SPM (resolvin D1; RvD1) on disease progression in preclinical animal model of MS experimental autoimmune encephalomyelitis (EAE) model (Poisson et al., 2015). Daily oral and intraperitoneal administration of RvD1 (100 ng per mouse) decreased disease progression to a greater extent and attenuated clinical symptoms in EAE by suppressing peripheral as well as central inflammation. This study provides the first experimental proof that the application of small endogenous mediator (i.e., RvD1) is an effective therapy in a mouse model of MS. Further, there is another recent report confirming the protective actions of DHA metabolites (RvD1 and protectin D1) on the inflammation-induced blood brain barrier (BBB) dysfunction using the human brain endothelial cell line hCMEC/D3 as a model system (Kooij et al., 2020).

Collectively, these studies provide the crucial evidence about the therapeutic potential of DHA-metabolites in MS.

MaR1 is an understudied resolution agonist from the perspective of MS, given there is only one study so far that has shown its beneficial effect in MS (Sánchez Fernández et al., 2022). MaR1 not only enhanced inflammatory resolution after spinal cord injury and reduced myelin loss and neurological deficits (Francos-Quijorna et al., 2017), but also promoted neuroprotection in spinal muscular atrophy (Ohuchi et al., 2018) and cerebral ischemia (Xian et al., 2019). Recently, we have shown that metabolic programming through pyruvate dehydrogenase regulates divergent metabolic needs of oligodendrocyte development affecting its maturation and remyelination property (Sajad et al., 2023). Given the critical role of oligodendrocyte in remyelination, there is a limited information to understand MaR1 induced metabolic changes during oligodendrocyte development and how that contributes to maintenance of myelination status. Accordingly, in the present study we have demonstrated in-depth beneficial role of MaR1 treatment in EAE mice that has expanded the role of maresin biology in MS. We found that MaR1 acts as an immunomodulator as its administration altered the immune landscape within CNS of EAE mice. Contrary to the findings by Sánchez Fernández *et al.,* 2022, we show that MaR1 significantly increased the RNA and protein levels of anti-inflammatory cytokines, such as IL4 and IL10, thus promoting anti-inflammatory phenotype of CD4 T cells and reduced pathogenic IL17 producing CD4 cells. IL10 plays a central role in the pathophysiology of MS and other neurodegenerative diseases (Porro et al., 2020), therefore, any molecule (e.g. MaR1) which can increase IL10 production, holds great potential for therapy development. Also, it primarily reduced the infiltration of CD4 T cells within the CNS of EAE mice, suggesting modulation of the inflammatory environment of the CNS by MaR1. It induced metabolic reprogramming in CD4 that might modulate their pathogenic or disease-inducing phenotype as revealed by adoptive transfer data (**Fig. 6B, C**). Further, it also induced metabolic reprogramming in macrophages which rescued their impaired efferocytic activity in EAE. It is pertinent to mention here that MaR1 maintained myelin status by inducing metabolic changes in oligodendrocytes which are myelin-producing cells in CNS, thus implying that neuroprotective effects of MaR1 are partly induced by metabolic reprogramming of disease-associated cell types. RNA seq data further suggests protective role of MaR1 by impacting axonogenesis which could rather reflect improvement of the neurological impairments in EAE. Overall, our results here show that MaR1 salvages EAE-induced transcriptomics changes and helps in regeneration of axons.

Altogether, we provide clear evidence that there is impaired production of MaR1 in EAE and our study is the first to demonstrate that MaR1 exerts its protective actions through IL10 and metabolic reprogramming of disease-associated cell types (CD4, macrophages, and oligodendrocytes). Our data strongly indicates that exogenous administration of MaR1 is effective at activating several protective mechanisms needed for neutralising inflammation in EAE and confers protection against neurological decline and myelin loss. We also observed enhanced behavioural activity through IRAMS in MaR1 treated group compared to EAE which signifies the restoration of neurological impairments. Given the nature of MaR1 as an endogenously produced resolution mediator that can be harnessed to treat autoimmune diseases, particularly MS, our study has provided further evidence to highlight its protective role in MS. Collectively, our findings open a new gateway in targeting resolution mediator MaR1 as a natural and endogenous metabolite, with high potential as a therapeutic agent to treat MS. This overcomes the previous hurdles that have limited the development of lipids as pharmaceutical agents. This will take us closer to a possible treatment strategy by which we can touch the lives of millions of people suffering from MS. However, future studies are critical to determine the protective mechanisms of MaR1 in EAE by which its treatment alters the metabolism and function of innate and adaptive immune response, providing a new fundamental understanding of the regulation of neuroinflammatory processes. Our study will set up the foundation for the development of innovative therapeutic strategies based on endogenous metabolites to resolve inflammation during MS with no side effects and will likely apply to other diseases involving pathogenic activation of the immune system. We envisage

more basic and clinical research on maresin family by providing another dimension to neuroprotective role of MaR1 in EAE through present study. We also expect to discover maresin-related signalling pathways as potential reserve for exploiting endogenous anti-inflammatory mechanisms to limit excessive inflammatory responses in future therapeutic strategies.

Funding This work is in-part supported by research grants from the National Multiple Sclerosis Society (US) (RG-2111–38733), the US National Institutes of Health (NS112727 and AI144004) and Henry Ford Health Internal support (A10270 and A30967) to SG. The funders had no role in the study design, data collection, and interpretation, or the decision to submit the work for publication.

Declarations

Ethics Approval The animal studies performed in this manuscript stand approved by the IACUC committee of Henry Ford Health. The article does not contain any studies with human participants.

Conflict of Interest The authors declare that they have no competing interests.

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Figures

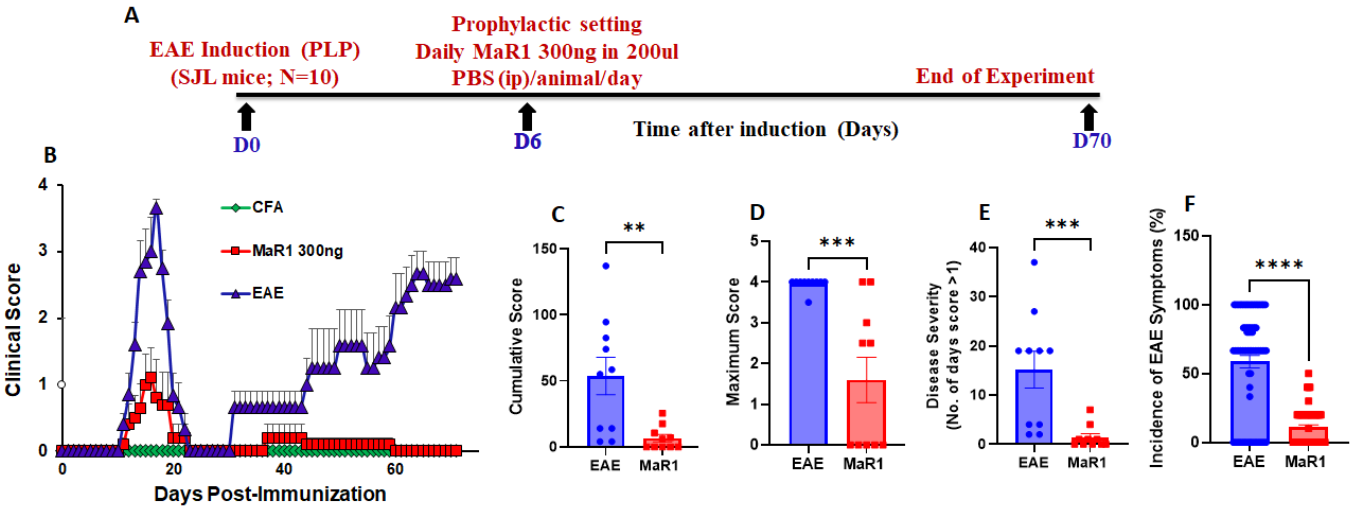


Fig 1. Protective effect of MaR1 treatment on the neurological deficits in RR-EAE mouse model. (**A)** Timeline demonstrating the experimental setup of MaR1 treatment in EAE (SJL) mice after immunization with PLP. (**B)** Clinical score in SJL mice consisting of experimental groups as CFA, EAE, and EAE treated with MaR1 until 70 days after disease induction. EAE mice developed disease symptoms from day 9 after immunization with PLP whereas MaR1 treated mice showed symptoms on day 10 (N=10). (**C)** Cumulative score. (**D)** Maximum score. (**E)** Disease severity score. (**F)** Incidence of clinical symptoms in EAE and MaR1 treated mice. **p<0.01, ***p<0.001, ****p<0.0001 (as determined by Mann-Whitney test) vs vehicle EAE and MaR1 EAE. Data are shown as Mean ± SEM.

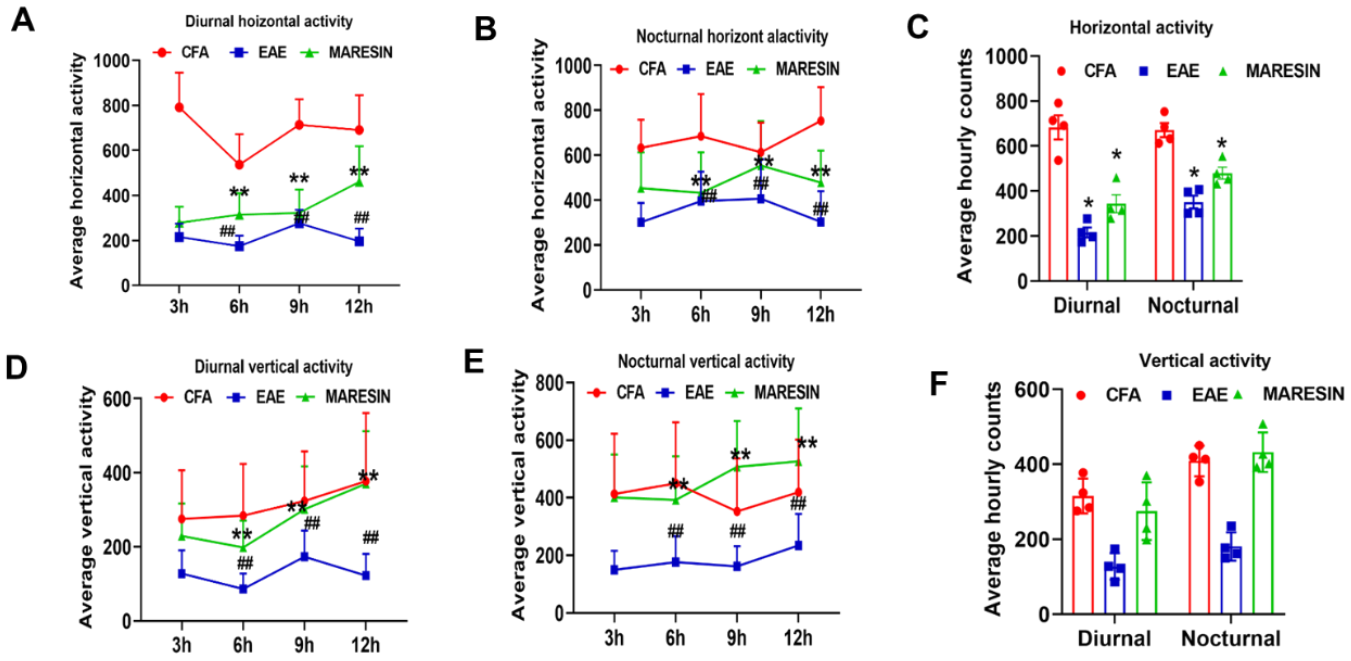


Fig 2. Average spontaneous activity (horizontal & vertical) of CFA, EAE and MaR1 treated mice during the day (diurnal) and night (nocturnal). (A) Average hourly counts of spontaneous day time (diurnal) horizontal activity were measured up to 12h. **(B)** Average hourly night-time horizontal activity (nocturnal) was measured up to 12h after sunset. **(C)** Average of 12h counts of diurnal and nocturnal horizontal, activity of CFA, EAE and MaR1 treated mice. **(D)** Average hourly counts of spontaneous day time (diurnal) vertical activity were measured up to 12h. **(E)** Average hourly night-time (nocturnal) vertical activity was measured up to 12h after sunset. **(F)** Average of 12h counts of diurnal and nocturnal vertical, activity of CFA, EAE and MaR1 treated mice.

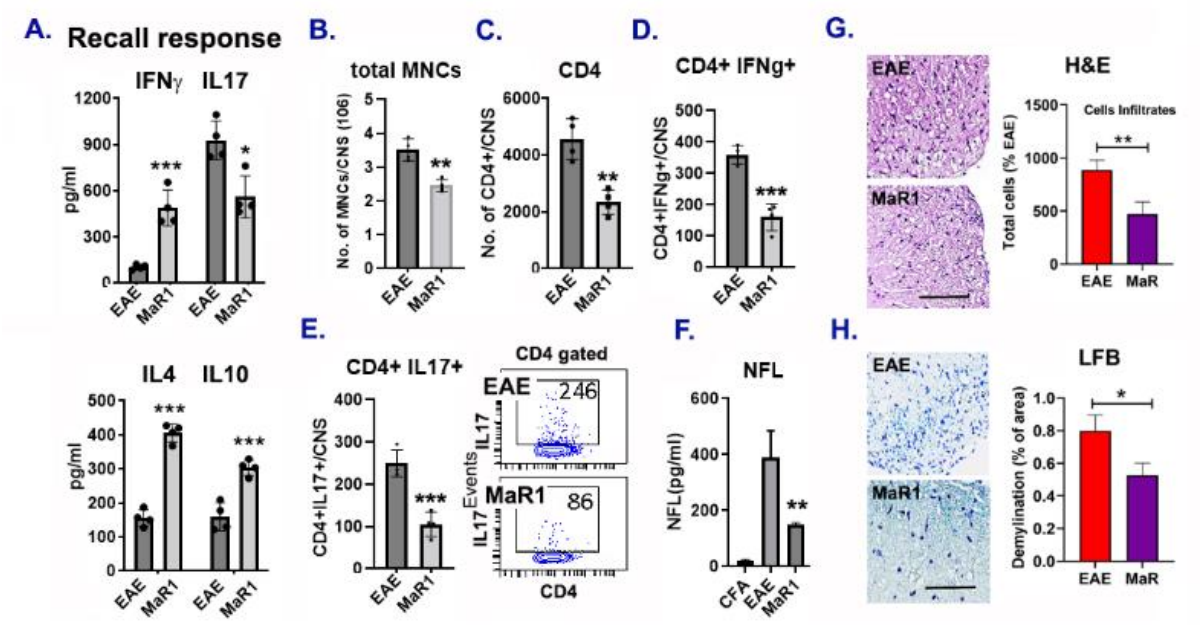


Fig 3. MaR1 reduces peripheral cytokine response and CNS infiltration of IL17 and IFN-G producing CD4 cells. (A) Bar graphs for ELISA and qPCR data showing cytokine response in splenocytes isolated from vehicle-treated EAE mice or MaR1 (N=4). **(B-E)** Bar graphs showing immune cell populations isolated from brain-spinal cord of EAE mice or MaR1 and analysed by flow cytometry (N=4). **(F)** Graph showing the quantification of NFL in plasma samples derived from vehicle-treated EAE mice and MaR1 (N=4). **(G-H)** Representative images showing histopathological changes in the spinal cord tissues in EAE mice with or without MaR1 treatment. Spinal cord sections show inflammatory infiltrates (H&E) **(G)** and demyelination (LFB) **(H).** **p<0.01, ***p<0.001 vs. vehicle EAE. Data are shown as Mean ± SEM.

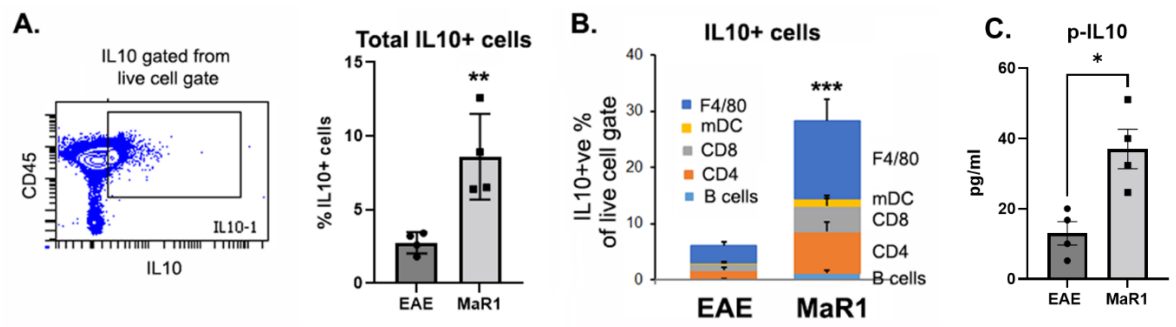


Fig 4. MaR1 treatment induced IL10 expression in all immune cells. (A) Spleen cells from treated and untreated with MaR1 from RR-EAE (day 18) were stimulated with PMA/ionomycin in the presence of GolgiPlug for 4h followed by surface staining for CD4 (CD3+CD4+), CD8 (CD3+CD4+), B cells (CD3-CD19+), mDC (CD11b+CD11c+ClassII+) and macrophage (F4/80+) and intracellular staining for IL10. **(B)** CD45+IL10+ cells were gated from live cells; based on surface marker, profiled all cell type expressing IL10 (N=4). p<0.01**, p<0.001*** compared to EAE. **(C)** Plasma IL10 levels in EAE compared with MaR1 treatment on day 70 using SIMOA (N=4). p<0.05*. Data are shown as Mean ± SEM.

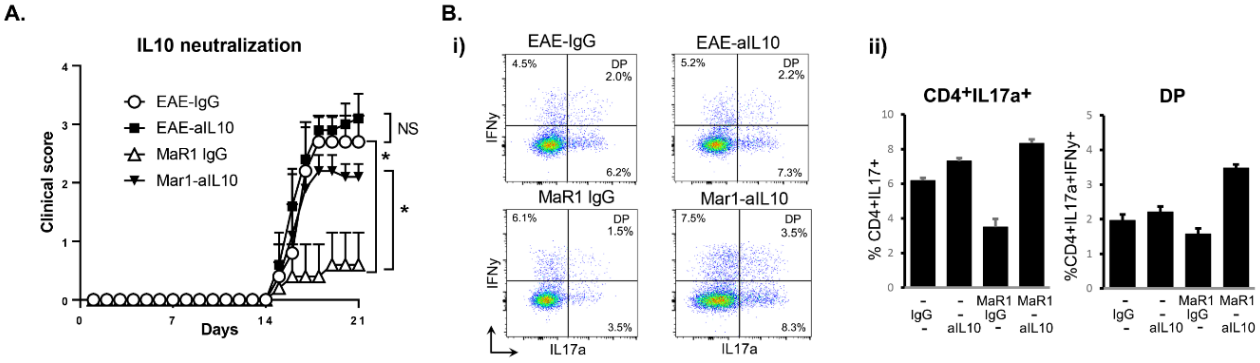


Fig 5. Effect of IL10 neutralization on the protective effect of MaR1 in EAE. (A) Disease severity plot showing abrogation of protective effect of MaR1 by using IL10 neutralizing antibody compared to IgG (N=8)**. (B)** Flow cytometry plots confirming reversal of protective effect **(i)**; % population of IL17 producing CD4 cells **(ii)** (N=5). *p<0.05 vs. vehicle EAE. Data are shown as Mean ± SEM.

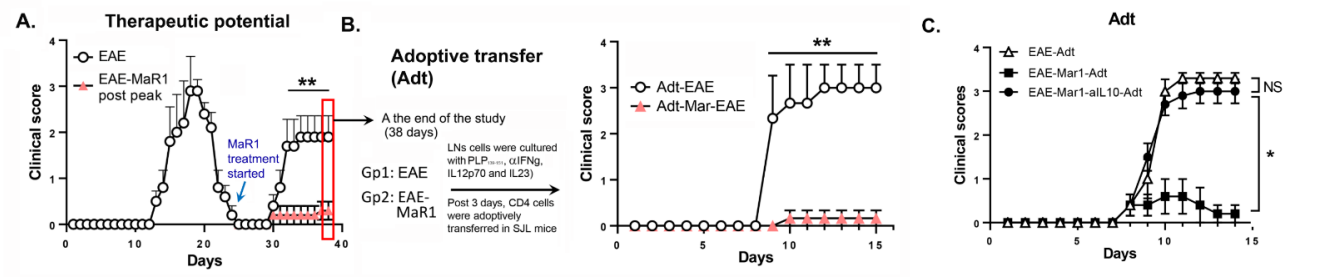


Fig 6. MaR1 treatment impedes encephalitogenic property of CD4 effector cells. (A) EAE was induced in SJL mice and MaR1 treatment was began when mice recovered from disease at day 20 post immunization in randomly divided groups**. (B)** At the end of the study (~38 days), lymph nodes were isolated treated and untreated groups and cultured with $PLP_{139-151}$, anti-IFNy (10ug/ml), IL12p70 and IL23 (10ng/ml). Post three days, CD4 cells were injected in SJL mice (N=5) and monitored for clinical score. Note: Adt-EAE-MaR1 group was not treated with MaR1. P<0.01**. **(C)** Adoptive transfer with CD4 cells derived from IL10 neutralization experimental batch and monitored for clinical score (N = 5). *P<0.05.

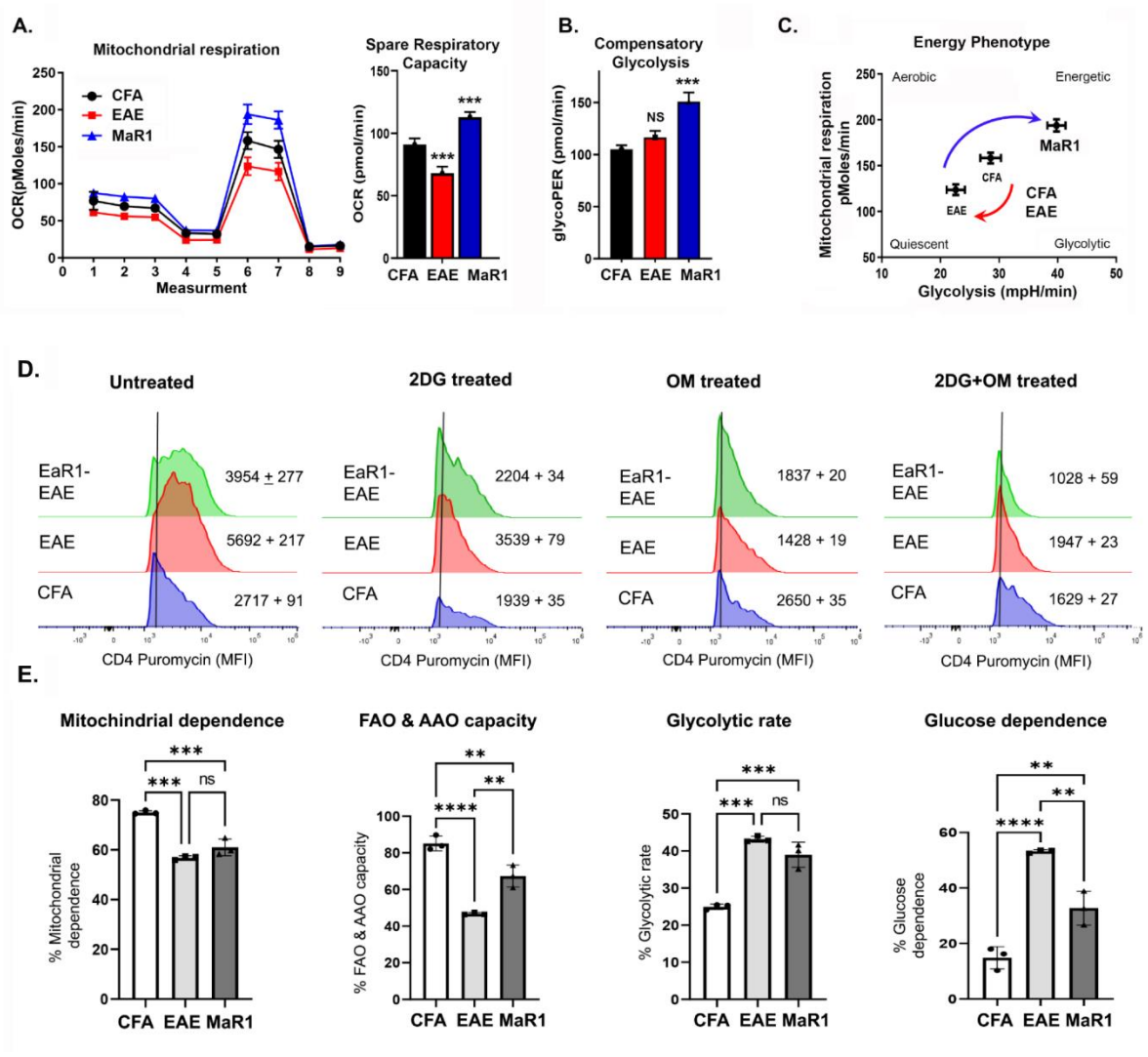


Fig 7. MaR1 induces the metabolic reprograming of CD4 effector cells in EAE. (A) XF mitochondrial Stress Test was performed in isolated CD4+CD25- cells (purity ~95%) isolated from CFA, or mice induced with EAE that were treated or untreated with MaR1 (300 ng/mouse daily) at day 18 to examine mitochondrial respiration. Spare respiratory capacity is presented as a bar graph (n=6). **(B)** Compensatory glycolysis was examined using XF seahorse bioanalyzer and presented as a bar graph (n=6). **(C)** Bioenergetic profile depicts the metabolic state of CD4 cells isolated from mice as in A. **(D-E)** EAE in SJL was treated with MaR1 from day 6 post immunization. At the peak of the disease ~18 days, brain infiltrating cells (BILs) were isolated using percoll gradient from all groups (CFA, EAE and MaR1 treated) and processed for SCENITH. MFI of puromycin across samples treated with different inhibitors for CD4 ($CD45^{+}CD4^{+}$) including deoxyglucose- (DG), oligomycin- (O), or deoxyglucose + oligomycin-treated (DGO) (N=3). Metabolic changes in the infiltrated CD4 in various groups are shown as a bar graph. Data are shown as mean ± SEM (n = 3). NS not significant; ***p < 0.001 compared to control or EAE using student's t test.

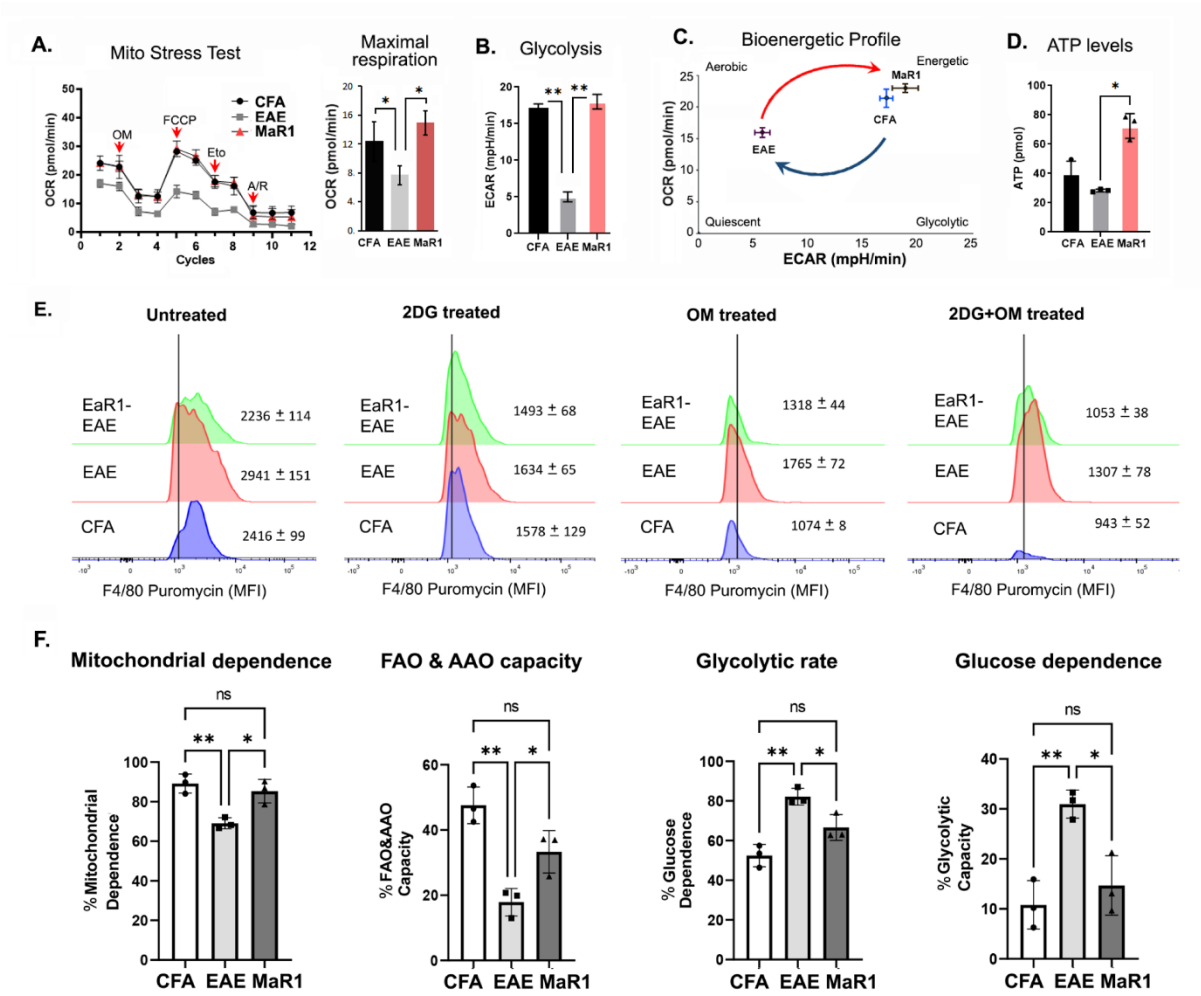
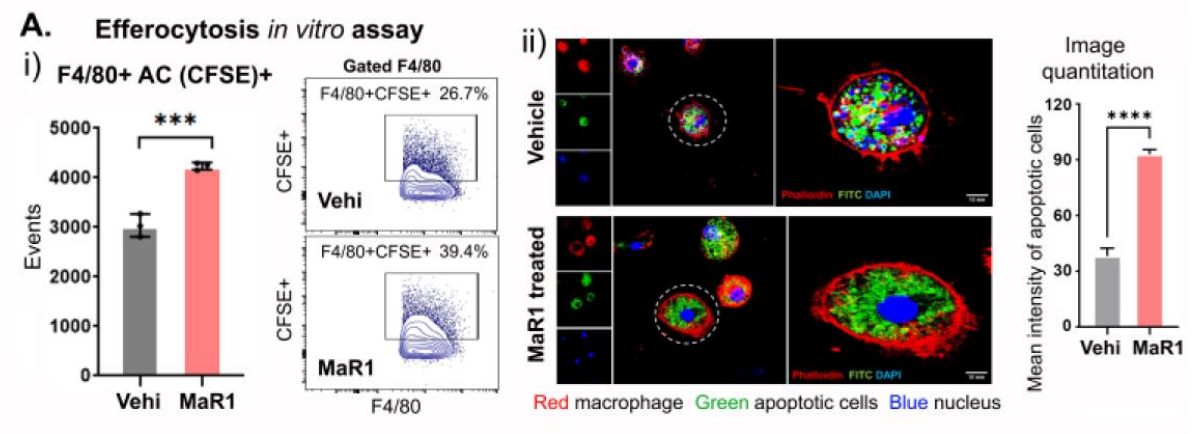


Fig 8. MaR1 induces the metabolic reprograming of macrophages in EAE. (A) XF mitochondrial Stress Test was performed in splenic F4/80+ macrophages (purity ~95%) isolated from CFA, or mice induced with EAE that were treated or untreated with MaR1 (300 ng/mouse daily) at day 18 to examine mitochondrial respiration. Spare respiratory capacity is presented as a bar graph (n=6). **(B)** Compensatory glycolysis was examined using XF seahorse bioanalyzer and presented as a bar graph (n=6). **(C)** Fatty acid oxidation was assayed using seahorse bioanalyzer (N=6). **(D)** Bioenergetic profile depicts the metabolic state of macrophage isolated from mice as in A. **(E)** ATP levels detected in macrophage from various groups in A using ATP assay kit (n = 4). NS not significant; ***p < 0.001 compared to control and EAE using student's t test. **(F)** EAE in SJL was treated with MaR1 from day 6 post immunization. At the peak of the disease ~18 days, brain infiltrating cells (BILs) were isolated using percoll gradient from all groups (CFA, EAE and MaR1 treated) and processed for SCENITH. MFI of puromycin across samples treated with different inhibitors for macrophage ($CD45^+CD11b^+F4/80^+$) including deoxyglucose- (DG), oligomycin- (O), or deoxyglucose + oligomycin-treated (DGO) (N=3). Metabolic changes in the infiltrated $F4/80^+$ macrophage in various groups is shown as a bar graph. Data are shown as mean ± SEM (n = 3). NS not significant; ***p < 0.001 compared to control or EAE using student's t test.



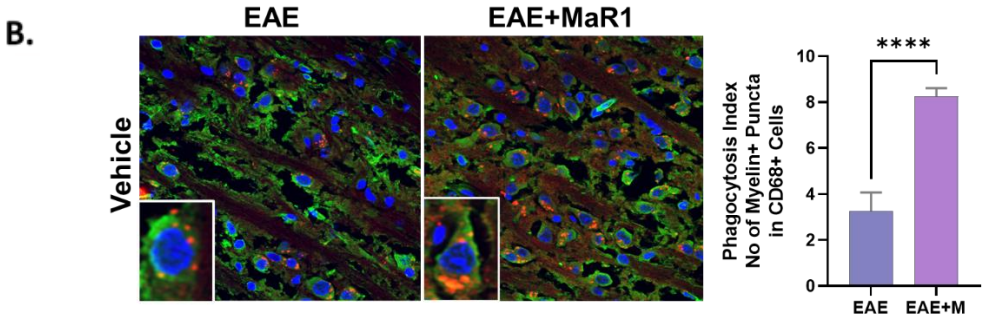


Fig 9. MaR1 induces efferocytosis in macrophage. (Ai) Bone marrow derived macrophages were treated with MaR1 (100nM) or vehicle (0.1% EtOH) for 1h followed by addition of CFSE-labeled 70-75% apoptotic splenic cells devoid of monocytes/macrophage in the ratio of 1:5. Post 18h, cells were washed, stained for F4/80 followed flow to quantitate the number of F4/80 cells engulfed CFSE labeled cells (N=6). Data presented as bar graph and a representative flow plot. **(ii)** Another set was plated in coverslip and in vitro efferocytosis assay was performed as above. Post 18h, cells were washed, fixed and Z-stack images were taken with Olympus FV1000 confocal microscope with 40X objective. 4 consecutive images with the interspace of 1µm were captured. Mean intensity of apoptotic cells were quantified with ImageJ analysis software (Version 1.49; NIH, USA) (N=6). **(B)** Efferocytosis in spinal cord derived from EAE and MaR1 treated groups. ***$p < 0.001$ student's t test.

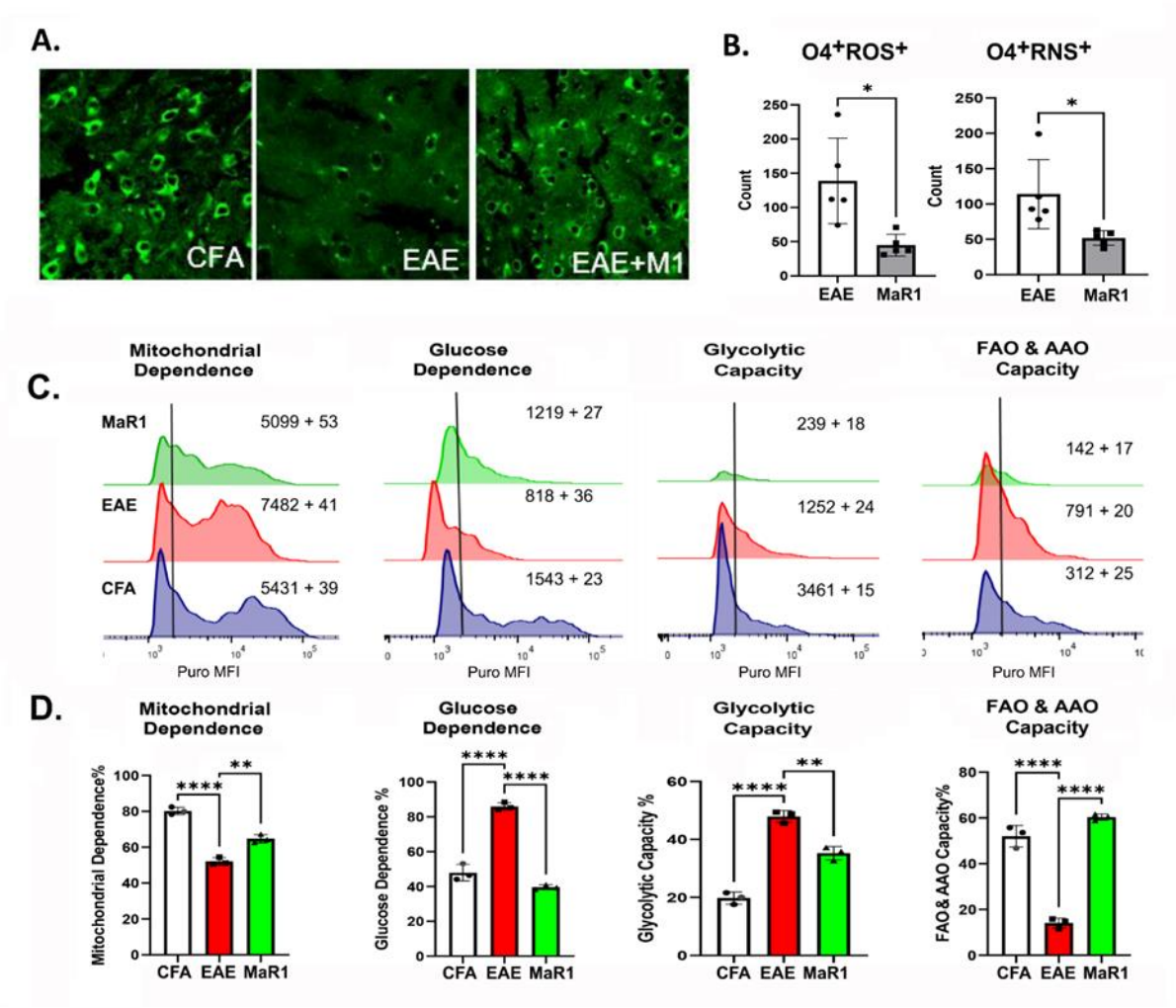


Fig 10. MaR1 protects myelin by controlling O4 oligodendrocytes metabolism. (A) Brain sections from various groups were processed for MBP expression using immunofluorescent and western blot showing expression of MBP with MaR1 treatment. **(B)** At the peak of the disease ~18 days, brain infiltrating cells (BILs) were isolated using percoll gradient from all groups (CFA, EAE and MaR1 treated) and processed for the detection of intracellular Reactive Oxygen and Nitrogen species (ROS and RNS) levels in O4+ oligodendrocytes ($CD45^{-}O4^{+}$) were evaluated using the Cellular ROS/RNS Detection Assay Kit by flow cytometry (N = 5). **(C-D)** Isolated BILs were processed for SCENITH. MFI of puromycin across samples treated with different inhibitors for O4+ oligodendrocytes ($CD45^{-}O4^{-}$) including deoxyglucose- (DG), oligomycin- (O), or deoxyglucose + oligomycin-treated (DGO) (N=3). Metabolic changes in the infiltrated O4+ oligodendrocytes in various groups are shown as a bar graph. Data are shown as mean ± SEM (n = 3). NS not significant; ** < 0.05, **p < 0.01, **** or ***p < 0.001 compared to control or EAE using student's t test.

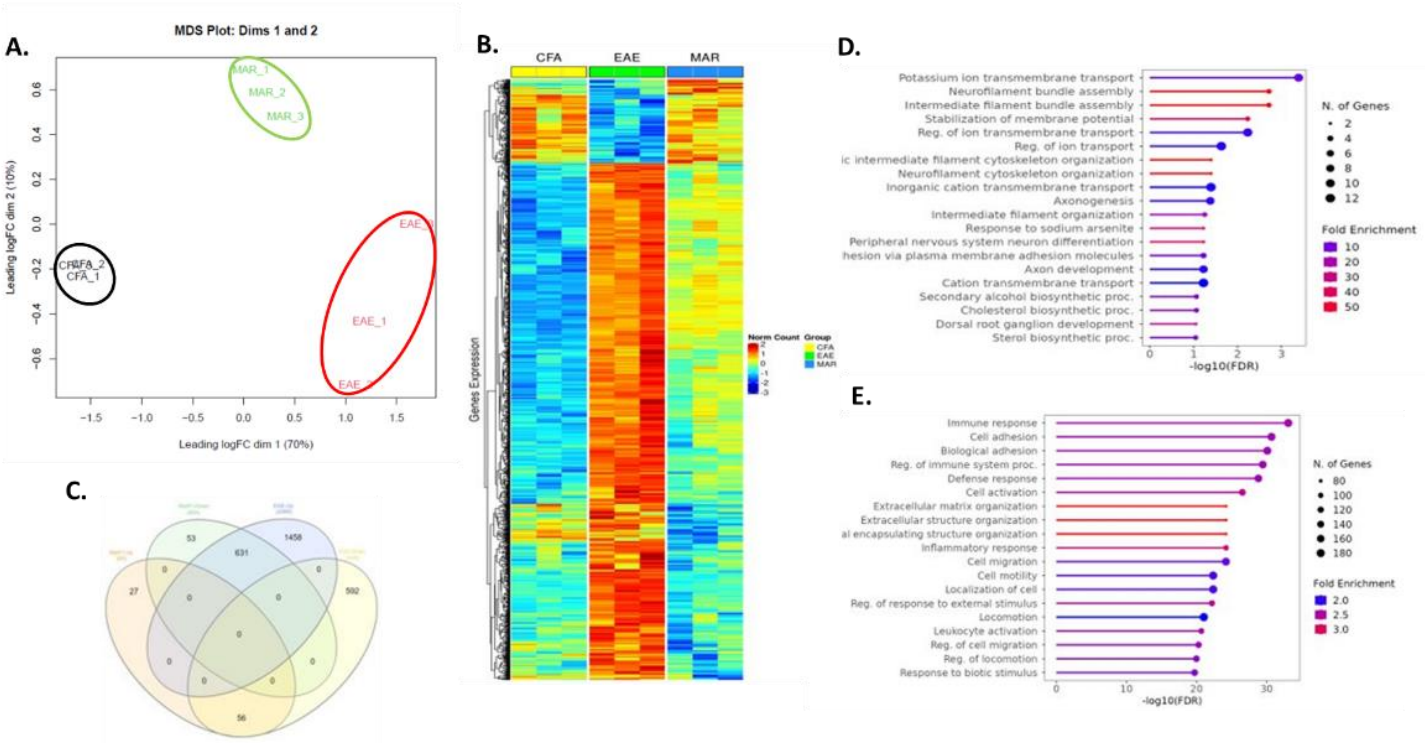


Fig 11. MaR1 rescues EAE-induced spinal cord transcriptomic alterations. **(A)** MDS plot showing segregation of each group- CFA, EAE & MAR. **(B)** Heat map representing significant gene expression changes across the groups. Padj >0.05. (**C)** Venn Diagram representing shared number of up- and downregulated genes in EAE and MAR1. (**D)** Gene ontology (GO) biological processes enrichment of the common genes upregulated with EAE and downregulated by Mar1 treatment. FDR < 0.05. **(E)** Gene ontology (GO) biological processes enrichment of the common genes downregulated with EAE and upregulated by Mar1 treatment. FDR < 0.05.