Untargeted Plasma Metabolomics Identifies Endogenous Metabolite with Drug-like Properties in Chronic Animal Model of Multiple Sclerosis

Laila M. Poisson1†, 1, Hamid Suhail§§, Jaspreet Singh‡, Indrani Datta†,†, Aleksandar Denic§, Krzysztof Labuzek§, Md Nasrul Hoda§§, Ashray Shankar§, Ashok Kumar**, Mirela Cerghet§, Stanton Elias§, Robert P. Mohney††, Moses Rodriguez§§, Ramandeep Rattan§§, Ashutosh K. Mangalam**, Shailendra Giri§§

From the †Center for Bioinformatics and 1Department of Public Health Sciences, Henry Ford Health System, Detroit, Michigan, the §Department of Neurology, Henry Ford Health System, Detroit, Michigan, the ‡Department of Neurology, Mayo Clinic College of Medicine, Rochester, Minnesota, the §Department of Pharmacology, Medical University of Silesia, Medyków 18, PL 40-752 Katowice, Poland, the §Department of Neurology, Georgia Health Sciences University, Augusta GA 30912, USA, ††Program in Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, Augusta GA, USA, the **Department of Anatomy & Cell Biology, School of Medicine, Wayne State University, Detroit, Michigan, ‡‡Metabolon, Inc Durham, NC 27713, the ‡§Department of Immunology, Mayo Clinic College of Medicine, Rochester, Minnesota, the §§Division of Gynecology Oncology, Department of Women’s Health Services, Henry Ford Health System, Detroit, Michigan, and the §§Department of Pathology, University of Iowa Carver College of Medicine, Iowa City, Iowa

Running title: Resolvin D1 reduces EAE disease progression

1 Both authors contributed equally to this work.

To whom correspondence should be addressed: Shailendra Giri, PhD, Department of Neurology, Research Division, Education & Research Building, Room 4051, Henry Ford Hospital, 2799 W. Grand Blvd, Detroit, MI 48202, USA. Office: (313) 916-7725; Fax: (313) 916-7250; Email: SGiri1@hfhs.org

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ABSTRACT

We performed untargeted metabolomics in plasma of B6 mice with experimental autoimmune encephalitis (EAE) at the chronic phase of the disease in search of an altered metabolic pathway(s). Of 324 metabolites measured, 100 metabolites that mapped to various pathways (mainly lipids) linked to mitochondrial function, inflammation and membrane stability, were observed to be significantly altered between EAE and control (p < 0.05, false discover rate < 0.10). Bioinformatics analysis revealed 6 metabolic pathways being impacted and altered in EAE including alpha linolenic acid and linoleic acid metabolism (PUFA). The metabolites of PUFAs, including omega 3 and omega 6 fatty acids, are commonly decreased in mouse models of multiple sclerosis (MS) and in MS patients. Daily oral administration of resolvin D1, a downstream metabolite of omega 3, decreased disease progression by suppressing autoreactive T cells and inducing a M2 phenotype of monocytes/macrophages and resident brain microglial cells. This study provides a proof of principle for the application of metabolomics to identify an endogenous metabolite(s) possessing drug-like properties, which is assessed for therapy in preclinical mouse models of MS.

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating disease of the CNS affecting more than 2.3 million people worldwide. It is a classic example of chronic disease leading to physical, psychological,
emotional and financial burden for patients and their loved ones. Based on disease course, MS can be classified into 4 categories including relapsing-remitting MS, secondary-progressive MS, primary-progressive MS and progressive-relapsing. Despite various therapeutic options for treatment, there is no cure for MS. Studies suggest that the most destructive changes from MS in the brain occur very early in the disease process and may cause considerable damage even before symptoms appear. MS treatments are more effective during the early course of the disease when symptoms are mild. Therefore, early diagnosis of MS is important in order to initiate treatments. However, no treatments have been able to delay progression of the disease, even though they improve the quality of a patient’s life (1) as a result of fewer exacerbations. Significant effort has been made to identify biomarkers from cerebrospinal fluid to diagnose MS, and this endeavor has proven to be challenging without success (2). The analysis of easily drawn bio-fluids including blood for MS biomarkers has only been minimally investigated but holds significant promise. The pathology of MS is associated with inflammatory response, glial activation and scarring and demyelination that likely result in a systematic change in the circulatory metabolome. Thus, altered plasma metabolites may be a valuable approach as a surrogate for the disease.

Recently, metabolic aberrations have been defined in various disease processes, as either contributing to the disease as potential biomarkers or therapeutic targets (3). Metabolomics is the global exploration of endogenous small molecule metabolites of cellular processes in the biological system, including cell, tissue, organ or organism (4,5). Therefore, metabolic profiling can provide a window to the instantaneous physiological or pathological changes as a complement to transcriptomic and proteomic profiling for the systematic and functional study of living organisms (6,7). Recent reports are implicating the importance of metabolomics in the possible identification of biomarkers in neurological disorders including Alzheimer’s disease (8,9), Parkinson’s disease (10), and in animal models of MS (11-14). We have previously performed a comprehensive analysis of plasma metabolites in the RR preclinical mouse model of MS (SJL-experimental autoimmune encephalitis [EAE])(14) and identified 44 metabolites that distinguished the EAE from control mice. These metabolites were associated with alterations in lipid, amino acid, nucleotide and xenobiotic metabolism (14).

The present study examined plasma from a chronic B6 mouse model of MS to determine the metabolic changes occurring in chronic EAE compared to the control B6 group. Bioinformatics analysis has revealed that metabolism of the PUFA pathway (omega 3 and 6 metabolism) is downregulated in the plasma of EAE, which has been also reported in MS patients (15-19). We therefore examined the therapeutic potential of resolvin D1 (RvD1; 7S, 8R, 175-trihydroxy-docosahexaenoic acid; KH74) for FACS analysis were purchased from Biolegend (San Diego, CA). Anti-CD4 (FITC; M1/70), CD45 (eFluro 450; F11), CD25 (APC; RM4-5), CD25 (PE; PC61), CD80 (Percp/cy5.5; 16-10A1), CD206 (APC; C068C2) and class II (I-A5; FITC; KH74) for FACS analysis were purchased from Biolegend (San Diego, CA). Anti-CD11b (PE-Cy7; RB6-8C5), CD86 (AF700; GL1), IFNγ (PE-CF594; XM1.2) and class II (I-A/I-E; M5/114.15.2) were purchased from BD

**EXPERIMENTAL PROCEDURES**

**Animals** - Female B6 and SJL mice (10-12 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the pathogen-free animal facility of Henry Ford Hospital, Detroit, MI, according to the animal protocols approved by the Animal Care and Use Committee of Henry Ford Hospital.

**Peptide and reagents** - Murine myeloid-oligodendrocyte glycoprotein peptide (MOG35-55) and proteolipid protein peptide (PLP139-151) was synthesized at New England Peptide, Inc. (Gardner, MA). Complete Freund’s adjuvant and mycobacterium tuberculosis lyophilized powder was purchased from DIFCO Laboratories (Detroit, MI). Labeled antibodies to anti-mouse CD4 (FITC; RM4-5), CD25 (PE; PC61), CD80 (Percp/cy5.5; 16-10A1), CD206 (APC; C068C2) and class II (I-A5; FITC; KH74) for FACS analysis were purchased from Biolegend (San Diego, CA). Anti-CD11b (PE-Cy7; M1/70), CD45 (eFluro 450; F11), CD25 (APC-Cy7; PC61.5), IL17a (PerCp-Cy5.5; eBio17B7), IL4 (PE; 11B11) CD4 (APC; RM4-5), FoxP3 (PE-Cy5; FJK-16s) were purchased from eBioscience (San Diego, CA). Anti-Gr1 (APC-Cy7; RB6-8C5), CD86 (AF700; GL1), IFNγ (PE-CF594; XM1.2) and class II (I-A/I-E; M5/114.15.2) were purchased from BD.
Biosciences (San Jose, CA). Anti-mouse scavenger receptor (PE; 214012) was purchased from R&D Systems, Inc. (Minneapolis, MN). ELISA kits for IFNγ, IL17a, IL6, IL23p19 and IL4 were purchased from Biolegend. RvD1 and its ELISA kit were purchased from Cayman (Ann Arbor, MI). The iScript complementary DNA synthesis kit and SYBR Green PCR master mix was purchased from BIO-RAD Laboratories (Hercules, CA).

EAE induction, treatment and recall response - B6 and SJL mice (10-12 week old) were immunized on day 0 by subcutaneous injections in the flank region with a total 200 μl of emulsion containing MOG35-55 peptide (300 μg/mouse) or PLP139-151 (100 μg/mouse), along with killed Mycobacterium tuberculosis H37Ra (400 μg) as described before (21). B6 mice were given pertussis toxin (300 ng/mouse) in the volume of 200 μl in PBS intraperitoneally on day 0 and 2 post immunization. Pertussis toxin was not injected in SJL mice. One set of mice were injected with complete Freund’s adjuvant/PT without peptide named as control. Clinical disease was monitored daily in a blinded fashion by measuring paralysis according to the conventional grading system: 0, no disease; 1, complete loss of tail tone; 2, partial hind limb paralysis (uneven gate of hind limb); 3, complete hind limb paralysis; 4, complete hind and forelimb paralysis; and 5, moribund or dead. On day 45, blood was collected for plasma isolation for metabolomics. Cells isolated from lymph nodes (2 x 10^6/ml) of EAE and control mice were cultured in the presence or absence of peptide (20 μg/ml). Cell proliferation and the production of various cytokines (IFNγ and IL17a) were examined as described before (21). On the same day, blood was drawn from both groups to isolate plasma for metabolomics analysis (in case of B6-EAE). For RvD1 treatment, RvD1 was given orally using a 22 gauge needle with a 1.25 mm ball diameter or intraperitoneally at a dose of 100 ng/mouse in the volume of 100 μl in PBS. In the control EAE group, 100 μl of PBS was given as vehicle.

Histology and pathology of the spinal cord - Following perfusion with Trump's fixative, spinal cords were processed for histology and pathology on day 45 post immunization as described before (14).

Metabolomic Analysis

Metabolite analysis - Metabolomic profiling analysis was performed by Metabolon Inc. (Durham, NC) as previously described (14,22-25).

Sample Preparation for Global Metabolomics - Samples were stored at -80°C until processed. Sample preparation was carried out as described previously (26) at Metabolon, Inc (Durham, NC). Briefly, recovery standards were added prior to the first step in the extraction process for quality control purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills Genogrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: one for analysis by ultra high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; positive ionization), one for analysis by UPLC-MS/MS (negative ionization), one for the UPLC-MS/MS polar platform (negative ionization), one for analysis by gas chromatography-mass spectrometry (GC-MS), and one sample was reserved for backup.

Three types of controls were analyzed in concert with the experimental samples: samples generated from a small portion of each experimental sample of interest served as technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of standards spiked into every analyzed sample allowed instrument performance monitoring. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers (median RSDs were determined to be 4-5%, depending on the matrix tested; n ≥ 30 standards). Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled human plasma or client matrix samples (median RSD = 6-
12%, depending on the matrix tested; n = several hundred metabolites). Experimental samples and controls were randomized across the platform run. Mass Spectrometry Analysis - Non-targeted MS analysis was performed at Metabolon, Inc. Extracts were subjected to either GC-MS or UPLC-MS/MS [1]. The chromatography was standardized and, once the method was validated no further changes were made. As part of Metabolon’s general practice, all columns were purchased from a single manufacturer’s lot at the outset of experiments. All solvents were similarly purchased in bulk from a single manufacturer’s lot in sufficient quantity to complete all related experiments. For each sample, vacuum-dried samples were dissolved in injection solvent containing eight or more injection standards at fixed concentrations, depending on the platform. The internal standards were used both to assure injection and chromatographic consistency. Instruments were tuned and calibrated for mass resolution and mass accuracy daily. The UPLC-MS/MS platform utilized a Waters Acquity UPLC with Waters UPLC BEH C18-2.1x100 mm, 1.7 μm columns and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in acidic or basic LC-compatible solvents, each of which contained 8 or more injection standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic, positive ion-optimized conditions and the other using basic, negative ion-optimized conditions in two independent injections using separate dedicated columns (Waters UPLC BEH C18-2.1x100 mm, 1.7 μm). Extracts reconstituted in acidic conditions were gradient eluted using water and methanol containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM ammonium bicarbonate. A third aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate. The MS analysis alternated between MS and data-dependent MS² scans using dynamic exclusion, and the scan range was from 80-1000 m/z. The samples destined for analysis by GC-MS were dried under vacuum desiccation for a minimum of 18 h prior to being derivatized under dried nitrogen using bistrimethylsilyl trifluoroacetamide. Derivatized samples were separated on a 5% diphenyl / 95% dimethyl polysiloxane fused silica column (20 m x 0.18 mm ID; 0.18 um film thickness) with helium as carrier gas and a temperature ramp from 60° to 340°C in a 17.5 min period. All samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS using electron impact ionization (EI) and operated at unit mass resolving power. The scan range was from 50–750 m/z.

Compound Identification, Quantification, and Data Curation - Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra and curated by visual inspection for quality control using software developed at Metabolon (23,27) Identification of known chemical entities is based on comparison to metabolomic library entries of purified standards. Commercially available purified standard compounds have been acquired and registered into LIMS for distribution to the various UPLC-MS/MS platforms for determination of their detectable characteristics. Additional mass spectral entries have been created for biochemicals that have been identified by virtue of their recurrent nature (both chromatographic and mass spectral) and classical structural analysis but have not been confirmed with purified standards. Peaks were quantified using area-under-the-curve.

Determination of RvD1 plasma levels by ELISA - Mouse plasma (100 μl) from control and EAE (B6 or SJL) was mixed with 9 volume of sodium acetate (0.1 M, pH=5.0) and passed through solid-phase extraction (SeP-Pak; C-18 columns, Water). Samples were eluted with methanol and were
dried under nitrogen. Samples were suspended in 500 μl of buffer (1 M phosphate solution containing 1% BSA, 4 M NaCl, 10 mM EDTA and 0.1% sodium azide) and 50 μl was used for ELISA as per the manufacturer’s protocol (Cayman Chemicals).

Flow cytometry - For surface markers staining, cells were incubated with fluorochrome conjugated antibodies to CD4, CD11b, Gr1, CD206, scavenger receptor, CD45, CD25, FoxP3, CD80, CD86, and class II at recommended dilution antibodies for 30 min at 4°C. To analyze antigen specific T helper (Th)1 and Th17 cells, spleen cells or CNS-infiltrating mononuclear cells were stimulated with 20 μg/ml of PLP135-155 peptide for 18 h, followed by treatment with GolgiPlug for 5 h. Cells were surface stained with monoclonal antibodies against CD4, then cells were washed, fixed and permeabilized with cytofix/cytoperm buffer and intracellular cytokines were stained with antibodies against IFNy and IL17A. Flowcytometric analysis was performed on BD FACSCalibur (BD Biosciences) and results were analyzed using FACSDiva software (BD Biosciences).

Adoptive transfer of PLP specific T cells to SJL mice - SJL mice (8-10 weeks old) were immunized subcutaneously with 100 μg PLP135-155 per mouse in complete Freund’s adjuvant. Post-10 days, we pressed spleen and lymph node cells through a 100 μm mesh into cold PBS to isolate them, then cells were pelleted at 450 g for 10 min at 4°C. Spleen/lymph node cells were resuspended in red blood cells lysis buffer. Post-5 min incubation, cells were washed 3 times with cold PBS and counted with the hemocytometer using 0.4% trypan blue (Sigma-Aldrich, St. Louis, MO). Spleen/lymph node cells were cultured in a 24 well plate (Corning Inc., Corning, NY) at 5 million cells/ml in Roswell Park Memorial Institute medium supplemented with 10% FBS, 1.25% HEPES buffer, 1% sodium pyruvate, 1% penicillin-streptomycin, 1% glutamine, 0.01% 2 mercaptoethanol (Sigma-Aldrich) for 72 h with PLP (20 μg/ml). PLP135-155 primed cells were harvested, washed with cold PBS and counted using trypan blue to check cell viability and injected 10 million cells in the volume of 200 μl intraperitoneally in each recipient SJL mice. Mice received 200 ng of pertussis toxin at day 0 and 2 and were observed daily for disease onset and severity.

Antigen specific response - CD4+ T cells were isolated from PLP135-155 immunized SJL mice using EasySep mouse CD4+ve selection kit (< 92% pure) from Stemcell Technology (Vancouver, Canada). These cells (2 x 10⁶/ml) were cocultured with adherent macrophages from RvD1-treated and untreated groups in the ratio of 1:5 and incubated for 72 h. Cell proliferation was examined using Wst1 reagent (Promega Corp, Madison, WI) and cell supernatant was used for IFNy and IL-17 analysis using ELISA kit (Biolegend).

Coculture studies - Mouse brain microglia cells were cultured from 1-3 day pups and microglia were isolated by shaking flasks of mixed glia at 100 rpm for 1 h at 37°C. Suspended cells are 90% pure for macrophage/microglia cell specific marker (Iba1). Primary microglial cells were treated with RvD1 (100 ng/ml) or vehicle (0.1% ethanol) for 24 h and cells were processed for the expression of arginase 1 and chitinase-3-like-3 (YM1)/2 by quantitative PCR and data was normalized with ribosomal protein L27 (L27) housekeeping gene, whose expression did not change under any of the experimental conditions studied. For coculture study with oligodendrocyte progenitor cells, treated and untreated microglia cells with RvD1 for 24 h were washed and cocultured with rat oligodendrocyte progenitor cells. Post-24 h of coculture, cells were processed for expression of myelin genes including myelin-associated glycoprotein, MOG and myelin basic protein by quantitative PCR and data was normalized to the control gene ribosomal L27 housekeeping gene.

Statistical analysis of metabolites - Metabolites with missing intensity scores, indicating low levels of the metabolite in the sample, were imputed with a small number (half of the minimum value for the study). Principal component analysis was used to detect outlying samples. Partial least squares discriminant analysis was used for assessment of separability of the samples. A z-score plot was drawn with the z-scores based on the mean and standard deviation of the control samples per metabolite. T-tests,
allowing unequal variance, were used to compare changes in mean expression per metabolite between the control and disease groups. An estimate of the false discovery rate (q-value) was calculated to take into account the multiple comparisons that occur in metabolomic studies. A metabolite was considered to be statistically different when p < 0.05 and q < 0.10. Those significant metabolites were included in a heat map using metabolite-level normalized data. Samples were clustered by complete linkage on Pearson’s correlation, rows are ordered by direction of change and then molecule type. Statistical analyses were conducted using log10-transformed data.

Pathway analysis of significantly altered metabolites included the following approaches - Metaboanalyst (http://www.metaboanalyst.ca/) was used to assess 82 murine associated Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (http://www.genome.jp/kegg) against differential metabolites and also for metabolite enrichment. Both overrepresentation analysis using the hypergeometric test and the impact of metabolite changes based on the pathway topology using the relative betweenness centrality measure (28) were considered. Metabolites were mapped to the KEGG pathways using Human Metabolome Database (www.hmdb.ca/) numbers; N = 75 were retained which showed significance compared to control.

Statistical Analysis for EAE Studies - We utilized GraphPad Prism software (GraphPad Software Inc.) for statistical analysis. Student’s t-test were employed to analyze clinical disease score. Statistics for densitometric values comparison for proliferation and cytokine responses were analyzed with one-way multiple-range analysis of variance and Student’s t-test. A value of p < 0.05 was considered significant.

RESULTS

Characterization of the clinical pathological state of chronic-progressive EAE model - To identify the global metabolic changes in the plasma of a chronic preclinical mouse model of MS, distinct from control mice, we employed the approach of untargeted global metabolomics. EAE was induced in B6 mice using MOG35-55 peptide as described previously (21,29). As shown in figure 1A, B6 mice displayed a chronic-progressive clinical course. At the effector/chronic phase of disease (day 45), blood for plasma was collected for metabolomic profiling. Spinal cords were also collected and processed for CNS pathology recording inflammation, demyelination and axonal loss. The lymph nodes were processed for recall response to identify the relationship between altered metabolic changes, clinical pathology and immune response in B6 mice with EAE. Histological analysis of spinal cords showed that the EAE group displayed extensive demyelination, whereas the control group looked completely normal (Fig. 1B). Upon recall response in lymph node cells, we observed the production of pro-inflammatory cytokines (IFNγ and IL17) (Fig. 1C, D). Overall, this set of data characterizes the clinical and pathological state of EAE in B6 mice obtained at day 45 of disease induction.

Cohort of global metabolomics - To characterize the differential plasma metabolite profiles of B6 EAE compared to controls, metabolomics profiling was performed on 3 independent instrument platforms: 1 GC-MS and 2 LC-tandem MS/MS platforms. A total of 324 structurally named biochemicals in plasma were identified and detected across both groups. Metabolite levels were calculated by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries. There was 1.5%-4.3% missing data per sample, which were imputed with the minimum observed value for the experiment. Following log transformation (base 10), Welch’s two-sample t-test was used to identify biochemicals that differed significantly between experimental groups. In plasma, a total of 100 metabolites (30.9% of the 324 metabolites detected) were significantly altered; 13 metabolites were significantly increased and 87 metabolites were significantly decreased in the EAE group compared to the controls (p < 0.05 with false discover rate < 0.10) (Supplemental Table S1). Partial least squares discriminant analysis revealed a clear separation of EAE and control groups based on their metabolite profile (N = 5 per group; Fig. 2A). To discriminate these metabolite alterations, the intensities in the
EAE samples (red dots) were plotted relative to the distribution of the intensities in the control groups (blue dots) (Fig. 2B). Each dot in the Z-score plot represents 1 observation of 1 metabolite (rows) organized by major metabolic pathway. The magnitude is the number of standard deviations above (positive value) or below (negative value) the average value of the control group observation. To visualize the relationship between the 100 altered metabolites, a heat map was drawn with the metabolites arranged on the basis of relative change (up/down compared to control) and then by superpathway (Fig. 2C) and samples ordered by hierarchical clustering. The pie chart (Fig. 2D) enumerates these alterations indicating that the most altered metabolites belonged to the lipid (57%) and amino acid (19%) pathways, followed by the peptide (9%), xenobiotic (5%), carbohydrate (4%), nucleotide (4%) and energy (2%) pathways.

To understand the functional role of these alterations in plasma, the KEGG metabolic library was analyzed using Metaboanalyst (30,31) (Fig. 3A). We assessed both a test for overrepresentation of altered metabolites within a pathway (hypergeometric tests) (32) and the impact of the changed metabolites on the function of the pathway through alterations in critical junction points of the pathway (relative betweenness centrality) (28). Results of each of the 82 mouse pathways of KEGG were simultaneously plotted to show the most significant pathways in terms of hypergeometric test p-values (vertical axis, shades of red) and impact (horizontal axis, circle diameter). The top 6 pathways in plasma by p-value (top 4) or impact (top 2) were indicated including 1) biosynthesis of unsaturated fatty acids (alpha linolenic acid and linoleic acid metabolism), 2) glutamine and glutamate metabolism, 3) fatty acid biosynthesis, 4) pentose pathway, 5) tryptophan metabolism, and 6) arachidonic acid metabolism (Fig. 3B). Alteration in these pathways in plasma during chronic EAE disease suggests that perturbation of certain central metabolites could have an impact on multiple metabolic pathways that are interconnected.

Most altered metabolites were categorized as lipids, suggesting lipid metabolism was heavily perturbed in the chronic model of EAE (Figs. 2D and 4A). A pathway enrichment overview (MetaboAnalyst 3.0) of altered metabolites highlights alpha linolenic acid and linoleic acid metabolism (PUFA) as being significantly enriched in quantitative metabolomics of plasma in EAE vs control mice (Fig. 4B). This pathway was also heavily impacted in the RR-EAE model (14). Moreover, metabolites of PUFAs were found to be significantly lower in the mouse chronic model of EAE (Fig. 4C, Supplemental Table S1) and in MS patients (15-19), suggesting the importance of PUFA in MS. Alpha linolenic acid is the true essential omega-3 fatty acid found in plants. It is similar to the omega-3 fatty acids in fish oil, called eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In the human body, alpha linolenic acid is metabolized into EPA and DHA, which are enzymatically metabolized into eicosanoids, and ultimately to anti-inflammatory mediators, such as resolvins and protectins. Resolvins and protectins are known to have potent anti-inflammatory and pro-resolving actions in inflammatory models (33). We decided to use RvD1, a downstream metabolite of EPA-DHA, to examine its therapeutic potential in the EAE model. RvD1 has been proven to be very potent in treating a number of inflammation associated human diseases, including peritonitis (34), dextran sulfate sodium-induced colitis (35), sepsis (36), adjuvant-induced arthritis (37), asthma (38) and immune complex-induced inflammation (39). There are no reports examining the effect of RvD1 in EAE disease progression.

**RvD1 treatment attenuates the disease progression in EAE model** - We examined the therapeutic potential of RvD1 in EAE. The level of RvD1 in plasma of both EAE mouse models, including RR-EAE and chronic EAE, was found to be significantly reduced during disease compared to the control group in both models (Fig. 5A). Next, we examined whether supplementation of RvD1 in a RR-EAE mouse model could affect the progression of the disease. For this, we induced EAE in SJL mice and started oral administration of RvD1 at a dose of 100 ng/mouse on day 0 of
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immunization. Dose and route of administration of RvD1 was chosen based on a recent study where after oral administration, RvD1 (100 ng/mouse) rapidly accumulated in plasma and significantly reduced leukocyte infiltration in zymosan A-induced acute peritonitis (40). In EAE, RvD1 treatment did not show any effect on disease onset; however, there was a significant reduction in disease severity at peak of the disease course compared to the vehicle treated group (Fig. 5B, Table 1). Vehicle treated groups exhibited 2 courses of relapses, whereas the RvD1 treatment group showed only 1 relapse with a lesser degree of severity (Fig. 5B). We also examined its therapeutic potential in a chronic model of EAE, induced in B6 mice by immunization with MOG35-55 (21,29). Oral administration of RvD1 treatment (100 ng/mouse) provided significant protection in the clinical symptoms in the chronic EAE model when given on day 7 post immunization (Fig. 5C, Table 1). It reduced severity at the onset (0.4 ± 0.27) and the peak (1.1 ± 0.63) of the disease compared to the vehicle treated group (1.4 ± 0.44 and 3.1 ± 0.12, respectively) (Fig. 5C). We also examined the effect of RvD1 given by a different route; i.e., intraperitoneal, and found it equally effective as oral in terms of reducing clinical symptoms in the chronic EAE model when given on day 7 post immunization (Fig. 5C, Table 1). Overall RvD1 treatment significantly suppressed the severity of disease in both models of EAE. Histological examination at day 22 post immunization demonstrated that the RvD1 treated group had reduced CNS infiltration and demyelination as compared with RR-EAE mice treated with vehicle (Fig. 5D).

To evaluate whether RvD1 treatment altered EAE by modulating the autoreactive T cell immune response required for development of the disease, we isolated spleen cells from PLP139-151 immunized groups either treated with or without RvD1 on day 22 post immunization and stimulated them with PLP139-151 (20 μg/ml). Post-72 h of stimulation, the status of pro- and anti-inflammatory cytokines were profiled in the supernatant by ELISA. RvD1-treated groups displayed significantly reduced levels of IFNγ, IL17a, IL23p19 and IL6 with significantly higher levels of IL5 (Fig. 5E). However, no change in the levels of IL4 was detected (data not shown). Consistent with protein level, the mRNA expression levels of IFNγ, IL17, IL23p19 and IL6 were downregulated in the RvD1 treated group compared to EAE (Fig. 5F). We also examined the expression of signature-transcription factors including T-box transcription factor (T-bet) and retinoic acid receptor-related orphan receptor gamma (RORγ) for Th1 and Th17, respectively. We observed that spleen cells isolated from RvD1-treated groups exhibited significantly lower expression of T-bet and RORγ without affecting GATA3 expression, which explained the decreased levels of IFNγ and IL17, with no effect on IL4, when compared to vehicle-treated EAE group (Fig. 5G). PLP139-151-primed CD4 T cells from RvD1-treated mice showed a 70-fold and 3.4-fold reduction in IFNγ and IL17 intracellular staining, respectively, compared to EAE group; however, there was ~1.9-fold induction in regulatory T cells (CD4+CD25+FoxP3+) in the treated group (Fig. 5H).

To examine whether RvD1 treatment could affect the encephalitogenic property of T cells, we immunized SJL mice with PLP139-151 and 1 set of mice was given RvD1 (100 ng/mouse in 200 μl of PBS, daily by gavage), whereas another set received vehicle (1 μl of ethanol in 200 μl of PBS). Post-10 days, we isolated spleen and lymph node cells from both sets of mice and restimulated with PLP139-151 (20 μg/ml). After 96 h of incubation, PLP139-151-primed T cells (107 cells per mouse intraperitoneally) from vehicle and RvD1-treated EAE groups were adoptively transferred into healthy SJL mice. Mice receiving PLP139-151-primed T cells from the RvD1 treated group exhibited a significantly less severe degree of clinical symptoms compared to the adoptive transfer of PLP139-151-primed T cells from the vehicle-treated group (Fig. 6A, Table 1). These results suggest that RvD1 treatment altered the generation of encephalitogenic T cells.

We further examined the Th1, Th17 and Th2 subpopulations through intracellular staining for specific cytokines, including IFNγ, IL17 and IL4 in infiltrated mononuclear cells. As shown in Figure 6B, infiltrated mononuclear cells from RvD1-treated EAE showed twofold reduction of IFNγ and IL17-producing CD4+ve cells compared to
vehicle-treated EAE group, with no change in IL4-producing CD4 cells. These observation were further supported by quantitative PCR of IL17a and IFNγ in spinal cords isolated from RvD1 treated and vehicle EAE groups (Fig. 6C), further suggesting that RvD1 treatment reduced the pro-inflammatory environment in the CNS of treated EAE.

**RvD1 induced M2 type phenotype and inhibited immune response** - RvD1 has been reported to induce macrophage polarization towards an M2-like phenotype (41-45) known to be involved in tissue repair by producing extracellular matrix molecules and anti-inflammatory cytokines (46,47). The importance of the M2 macrophage in MS has been highlighted by recent studies demonstrating that MS approved drugs, including glatiramer acetate and laquinimod reversed EAE by promoting development of M2 activated monocytes (48,49). To examine if RvD1 mediated protection in EAE could be due to polarization of monocytes/macrophages into the M2 type, we assessed the monocyte/macrophage polarization status during EAE disease. We isolated adherent monocytes/macrophages after panning on plastic plates (< 85% CD11b+ F4/80+) and were treated with lipopolysaccharide and IFNγ for 6 h to induce a M1 response and with IL4 for 24 h to induce a M2 response. We observed that adherent monocytes/macrophages from the RvD1 treated EAE group displayed significantly lower levels of M1 markers including inducible NOS and IL6 expression; however, RvD1 treatment significantly induced M2 markers (IL10, arginase 1, resistin-like-a [FIZZ1] and YM1) suggesting that RvD1 treatment altered the monocytes/macrophages phenotype (Fig. 7A). RvD1 treatment also attenuated the expression of co-stimulatory molecules (CD80 and CD86) and major histocompatibility molecule class II molecules on adherent monocytes/macrophages isolated from the treated EAE group compared to the vehicle treated EAE group (Fig. 7B).

We next examined whether monocytes/macrophages isolated from the RvD1-treated mice have the ability to alter the antigen-specific T cell response. For this, PLP139-151-primed CD4 T cells were cocultured with monocytes/macrophages isolated from RvD1-treated and untreated EAE groups in the presence of PLP139-151 (20 μg/ml) in the ratio of 1:5. Coculture of PLP139-151-primed CD4 T cells with monocytes/macrophages isolated from the RvD1-treated EAE group did not show any effect on CD4 T cell proliferation compared to CD4 T cells cocultured with monocytes/macrophages isolated from the vehicle treated EAE group (Fig. 7C). However, production of IL17A and IFNγ was significantly attenuated in the coculture condition when PLP139-151-primed CD4 cells were cultured with monocytes/macrophages isolated from the RvD1-treated EAE group compared to the vehicle-treated EAE group (Fig. 6C). These observations were further supported by intracellular staining of CD4+ IL17A+ and -IFNγ+ cells (Fig. 7D), strongly suggesting that RvD1-treatment regulates the immunomodulatory properties of monocytes/macrophages and alter the antigen-specific immune response of CD4 T cells.

**RvD1 promoted M2 type phenotype in CNS** - Our finding that oral administration of RvD1 protected EAE disease progression by modulating T cell response, monocytes/macrophage polarization and restricted IL17+ve and IFNγ+ve CD4 cells in the CNS compared to the vehicle treated group suggests a less pro-inflammatory environment in the CNS. To capture the overall picture of inflammation, RNA was isolated from spinal cords of control, RvD1- and vehicle-treated RR-EAE groups on day 22 post immunization to detect the levels of inducible NOS, TNFα, IL1β, IL6 (M1 markers) and FIZZ1 (M2 marker). The vehicle-treated EAE group showed significantly (p < 0.001) higher expression of inducible NOS, TNFα, IL1β and IL6, which were attenuated by the RvD1 treatment (Fig. 8A). Moreover, expression of FIZZ1 was significantly downregulated in EAE groups (Fig. 8A), which was restored by RvD1 treatment, suggesting that the RvD1 treatment attenuated the pro-inflammatory environment and induced an M2-repair-mechanism response associated with tissue repair (46,47). Demyelination in EAE is associated with reduced expression of myelin genes due to mature oligodendrocyte dysfunction and inability of oligodendrocyte progenitor cells into develop in mature oligodendrocytes. The expression of myelin genes, including MOG, PLP
and myelin basic protein, were significantly reduced in the spinal cords of the EAE group, which was restored/protected by the RvD1 treatment (Fig. 8A). This data was well supported by the expression of neurotrophic factors like leukemia inhibitory factor and glial cell line-derived neurotrophic factor, which are known to promote myelination (Fig. 8A). RvD1 treatment did not affect the expression of CNTF and PDGFα (data not shown).

To address whether RvD1 treatment altered the phenotype of brain resident microglia and infiltrated monocytes/macrophages during disease, we isolated infiltrated mononuclear cells from RvD1-treated and vehicle-treated EAE groups on day 22 postimmunization. Flow cytometry analysis revealed a 5.8- and 3.1-fold increase in the number of CD206- and scavenger receptor-positive myeloid cells (CD45\(^{\text{high}}\) CD11b\(^+\)), respectively, in the CNS of the RvD1-treated EAE group (Fig. 8B). The RvD1 treated group also showed a 3.3-fold increase in CD204 positive resident microglia (CD45\(^{\text{low}}\) CD11b\(^+\)) (Fig. 8C), suggesting an induction of the M2 phenotype in resident microglia and infiltrated monocytes/macrophages during EAE disease under RvD1 treatment. We also observed an 8.5-fold increase in the number of myeloid derived suppressor cells (MDSCs; CD45\(^{\text{high}}\) CD11b\(^+\) Gr1\(^-\)), which are known to alter immune response in autoimmune diseases, like EAE (50,51), and in cancer (52). Interestingly, MDSCs in RvD1 treated groups displayed the M2 phenotype as they expressed higher levels of CD206 and scavenger receptor compared to the vehicle treated EAE group (Fig. 8D).

Using an in vitro mixed glial cell model, we examined if RvD1 treatment could affect the expression of inflammatory mediator and myelin genes under inflammation. Mixed glial cell culture were treated with RvD1 (100 nM) in the presence or absence of a combination of pro-inflammatory cytokines (TNFα and IFNγ) (20 ng/ml) to create an inflammatory environment. As shown in Figure 8E, cytokines significantly induced inducible NOS expression compared to the untreated cultures. Pretreatment of cells with RvD1 attenuated cytokine- induced inducible NOS gene expression (Fig. 8E). Under a similar experimental condition, we found that cytokine treatment significantly reduced expression of myelin genes (MOG and PLP) and RvD1 treatment restored or protected their expression. These findings further supports the observation that RvD1 mediates attenuation of inflammation and induction of myelin genes in spinal cords of treated EAE groups. We next examined if RvD1-treated polarized microglia could promote oligodendrocyte differentiation in the absence of an inflammatory condition. We treated brain microglia cells with or without RvD1 (100 ng/ml) for 24 h and observed that RvD1 treatment induced the expression of arginase 1 and YM1/2, suggesting M2 polarization of the microglia (Fig. 8F). Treated and untreated microglial were washed and cocultured with OPCs and, after 24 h of coculture, cells were processed for expression of myelin genes. As shown in figure 8Fii, OPCs cocultured with RvD1 polarized microglia significantly induced the expression of myelin genes including myelin-associated glycoprotein, MOG and myelin basic protein, suggesting that RvD1 promotes myelin expression by modulating M2 type microglia under inflammatory and non-inflammatory conditions.

**DISCUSSION**

Thirteen disease-modifying drugs are approved by the U.S. Food and Drug Administration for MS. All of these agents reduce relapse rate, have a beneficial effect on a variety of magnetic resonance imaging measures, and have been reported to delay disability in short-term clinical trials. Most of them are relatively new, and their safety and efficacy in patients has yet to be determined for a longer therapeutic regimen. It is well accepted that treatment of MS is more effective during the early course of the disease with milder symptoms. Recent efforts in search of biomarkers in the cerebrospinal fluid have had limited success. Blood, a more accessible bio-fluid, could be easily drawn for biomarker analysis if such are available for diagnosis of MS at an early stage of the disease. The main goal of this study was to identify potential metabolite(s) and altered pathway(s) in plasma of the chronic mouse model of MS using a global metabolomics approach. We
identified 100 metabolites that were significantly altered between control and EAE animals, and these molecules were classified into lipid, amino acid, peptide, xenobiotic, carbohydrate, nucleotide and energy pathways. The most intriguing part of this study was the observation that most of the changes were found in the lipids. In human MS, being a presumed autoimmune disease, the role of lipids metabolism is understudied. A few key studies have clearly demonstrated roles for specific lipids, including CDP-choline (53), lactosylceramide (54), sulfatide (55) and fatty acids (56,57), in altering disease outcome in animal models of MS, suggesting the importance of lipids in disease pathology. It has yet to be established if the changes in lipids are the cause or the consequences of the disease pathology.

Recently, we reported a comprehensive analysis of plasma metabolites in the RR preclinical mouse model of MS (SJL-EAE)(14) and identified 44 metabolites that distinguished RR-EAE from control mice. These metabolites were drawn from various metabolic pathways that correlated well with the severity of the RR form of EAE disease. The important findings of our plasma metabolomics data in RR-EAE and progressive-chronic EAE models are the distinct signature and the affected pathways. In the RR model, we identified 44 metabolites of which 72% were increased and 28% were decreased. However, in the case of the chronic model, 100 metabolites were significantly altered of which 13% were increased and 87% were reduced. Using KEGG, only 1 pathway was overlapping (i.e., PUFA metabolism) suggesting that other pathways identified either in B6 or SJL animals might be specific to chronic or RR models, respectively. We carefully looked into metabolite signatures of both models and the number of metabolites were similarly altered in both. These are citrulline, alpha-ketoglutarate, trigonelline (N-methylnicotinate) and hippurate. More strikingly, only kynurenate was differentially altered in both models indicating the possible biomarker for distinguishing chronic vs. RR. We have to keep in mind that altered levels of kynurenate in both models may be due to genetic variation in SJL and B6 mice; therefore, this observation needs validation. Moreover, this pathway is altered in neurodegenerative diseases (58,59). While some of the observed metabolite changes in RR and chronic models could easily be developed as biomarkers of the disease, the key to translating metabolomics into therapeutics requires figuring out the central altered metabolic pathway(s).

Although the main emphasis of our metabolomics study was to identify new diagnostic biomarkers of disease, we found that information related to the perturbed pathways enhances the understanding of disease mechanisms, thereby highlighting plausible endogenous metabolite(s) that have drug-like properties. Using KEGG and enrichment pathway analyses, we observed that alpha linolenic acid and linoleic acid PUFA metabolism (omega-3 and -6) were altered in both murine EAE models (RR (14) and chronic [Supplemental Table S1 and Fig. S5]) and the levels of its metabolites were found to be low in MS patients (15-19), suggesting that this pathway could potentially be a therapeutic target for MS. There are 2 major sources of omega 3 fatty acids: alpha linolenic acid, which is found in plants, and EPA and DHA, which are found in fish. In mammals, alpha linolenic acid is converted via elongation and desaturation to EPA, subsequently to DHA, and ultimately to anti-inflammatory mediators, such as resolvins and protectins which are local-acting mediators with both anti-inflammatory and proresolving actions (60-63). An intermediate in the conversion of EPA to DHA is omega-3 docosapentaenoic acid (64). Both omega-3 and omega-6 docosapentaenoic acid and n3 EPA were found to be significantly lower in the plasma of EAE compared to control. A recent study clearly demonstrated that docosapentaenoic acid is converted during -resolution of inflammation in mice and by human leukocytes to n-3 products congenorous to D-series resolvins, protectins and masresins, called specialized pro-resolving mediators (65).

Several epidemiological studies suggest that n3 PUFA supplementation is linked with improved clinical outcomes in MS patients (66-68). Beneficial effects of n-3 PUFA have been shown in EAE by altering the immunomodulatory effect on
dendritic cells (69) and by inducing remyelination and inducing the M2 microglial phenotype in vivo (70). A number of clinical trials have been conducted by supplementing higher doses of EPA and DHA in MS patients (68,71). Although the level of omega 3 (EPA and DHA) could be restored by supplementation in MS patients, most likely there is a failure of production of adequate amounts of resolution-inducing molecules, like resolvins and protectins. This would result in inappropriate inflammation and a delay in the healing/repair process, so neuronal damage continues. This resolution pathway is also found to be dysfunctional in Alzheimer’s disease (72,73). Moreover, omega-3 supplementation or RvD1 prevents cognitive decline in animal models (74), and improves amyloid-β phagocytosis and regulates inflammation in Alzheimer’s patients (75-77).

We decided to use RvD1, a downstream metabolite of EPA-DHA, to examine its therapeutic potential in the EAE model. RvD1 has been reported to have a number of favorable properties including 1) limiting excessive leukocyte infiltration and attenuating the production of pro-inflammatory cytokines (38); 2) inducing macrophage polarization toward an M2-like phenotype (41-45); 3) promoting IL-4-induced alternative activation via STAT6 and PPARy signalling pathways in microglia (78); and 4) being effective at low doses, including 100 ng/mouse (5 micrograms/kg body weight) (40). Oral administration of RvD1 at a dose of 100 ng/mouse was very effective in attenuating EAE disease progression in our model compared to the vehicle group without affecting disease onset. These data support the hypothesis that proresolution and anti-inflammation are distinct processes. Resolution required blocking infiltration of mononuclear cells and clearance of lymphocytes at the site by macrophage-mediated phagocytosis.

Pro-inflammatory microglia and CNS-infiltrating monocytes/macrophages, recruited and activated in response to factors present in the inflamed CNS environment, are thought to contribute to the pathogenesis of MS and other neurodegenerative diseases (79,80). RvD1 has been reported to enhance the macrophage phagocytosis (38,81) and polarized macrophage toward an M2-like phenotype (41-45). These reports are in accordance with our finding of the M2 phenotype in treated mice, both at the periphery as well as in the CNS. RvD1 treatment did not affect the number of infiltrating monocytes/macrophages in the CNS, but did induce the M2 phenotype. Moreover, resident microglia exhibited the M2 phenotype upon RvD1 treatment compared to the vehicle-treated EAE group. Although we have not examined the possibility of clearance of lymphocytes in the CNS, either by induction of apoptosis or inhibition of clonal expansion, we did observe a 33% reduction in CD4 positive cells in the RvD1-treated group in the CNS. Moreover, inhibition of IL17+ve and IFN+ve CD4 cells in the treated group suggests an inhibition of antigen-specific proliferation of CD4 cells. RvD1 also polarized macrophage into M2-like phenotype in number of pathological conditions including adipose tissue inflammation (45), smoke induced lung inflammation (42), smoke-exposed human macrophage (41), obesity-induced steatohepatitis (43), and endotoxic uveitis (44) and provided protection.

Most of the FDA approved MS therapies including interferon beta, glatiramer acetate (GA), dimethyl fumarate, laquinimod and fingolimod are anti-inflammatory in action (82,83). GA and laquinimod have been shown to induce type II monocyte and reverse the EAE disease by modulating T cell response (49,84). Similar action of RvD1 was observed in EAE, however, it yet to be investigated if these drugs modulate the omega 3 metabolism, thereby may be altering the levels of pro-resolving mediators and downstream resolving actions. The dose used for RvD1, 100ng/mouse was effective and potent (5 micrograms/kg body weight) compared to other MS approved therapies used in EAE models (48,49,85,86).

RvD1-mediated protection in EAE involves multiple mechanisms including induction of regulatory T cells, polarization of monocytes/macrophages and microglia into the M2 phenotype, and an increase in myeloid-derived suppressor cells (MDSCs). The role of MDSCs as regulators or inducers of inflammation in EAE is
quite controversial. In general, these cells represent an important class of immunoregulatory cells that can be activated to suppress T cell function (52). We found increased levels of MDSCs (CD11b+ Gr1+) cells in the CNS of the treated group, which correlates well with the decreased CD4+ve immune response. We did not examine the nature of these MDSCs to determine whether they are granulocytic (CD11+Ly6ClowLy6G+) or monocytic (CD11+Ly6C+Ly6G-). Recently it was reported that monocytic and granulocytic MDSCs are potent suppressors of T cells through nitric oxide (87) and by expressing high levels of programmed cell death ligand 1 (51), respectively. However, one study reported that CD11b+Gr1+ cells isolated from mice with EAE inhibited T cell response but induced Th17 cell differentiation (50). We made an interesting observation that MDSCs in the CNS of the treated mice displayed the M2 phenotype (CD206+ and scavenger receptor positive). MDSCs with M2-like phenotype have been identified in cancer with pro-tumoral and immunosuppressive activities (88,89). It remains to be investigated if RvD1 treatment could enhance the M2 phenotype in MDSCs in general.

The current study demonstrates that a metabolomic approach can suggest new therapeutic targets or potential endogenous metabolites which may have drug-like properties that could easily be tested in MS (Fig. 9). RvD1 is one of the candidates that has been reported previously for treating a number of inflammatory diseases(34,35,37-39,70)(34,35,37-39,70). Here we found it to be significantly effective in attenuating clinical symptoms in the preclinical mouse model of MS. This study provided a proof of principle for how a metabolomic approach could lead to the identification of a small molecules that could be used for therapy.

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Author Contributions: SG conceived, designed, performed, analyzed the data, coordinated and wrote the manuscript. LMP and ID analyzed the metabolomics data, wrote and finalized the manuscript. HS, JS, AS and RR performed experiments, contributed in figure preparation and finalized the manuscript. AD and MR performed the pathology and finalized the manuscript. MNH, AK, MC, SE and AKM provided intellectual input, contributed in figure preparation and finalized the manuscript. RPM analyzed the metabolomics data, provided intellectual input, edited and finalized the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES

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The abbreviations used are: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; RvD1, resolvins D1; RR, relapsing-remitting; UPLC-MS/MS, ultrahigh performance liquid chromatography-tandem mass spectrometry; KEGG, Kyoto Encyclopedia of Genes and Genomics; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; FIZZ1, resistin-like-a; YM1, chitinase-3-like-3; MDSC, myeloid derived suppressor cells; MOG, myelin-oligodendrocyte glycoprotein; PLP, proteolipid protein; Th, T helper.

FIGURE LEGENDS

**FIGURE 1.** Characterization of clinical pathological state of EAE in chronic mouse model in B6. (A) EAE was induced in C57B6 mice using MOG35-55 peptide emulsified in CFA and PT was given on day 0 and 2 post immunization. Clinical score was recorded daily (n=10). The control group was given CFA/PT without a peptide. On the day 45, blood was drawn for analysis of isolated plasma. Red arrow indicates the time of sampling. (B) At the end of the experiment, spinal cords were harvested from control (B1 and B2) and diseased mice (B3 and B4) to examine demyelination. Panels B1 and B3 show the whole mid-thoracic spinal cord section, and panels B2 and B4 show closer images from antero-lateral areas of the white matter. A completely normal and healthy axons can be appreciated on panel B2, whereas on B4 an area with clear demyelination is outlined in red. In addition, in areas that surround the demyelinating lesion a patchy demyelination, dysmorphic and collapsed axons can be observed too. (C-D) Recall response in cells isolated from lymph nodes, stimulated with 50 µg of MOG35-55 for 72h. Cell supernatant was used for measuring the levels of IFN- γ and IL17 by ELISA (n=4).

**FIGURE 2.** Metabolomics profiling of plasma distinguishes EAE from the control group. (A) Metabolic profiling reveals clear separation between plasma from EAE (disease; D) and vehicle control mice (sham; S) by partial least squares discrimination analysis. (B) Z-score plot of EAE metabolite intensities (RED, truncated at z-score -15 and +15) against control group metabolites (BLUE, taken as mean). Each dot represents 1 metabolite observation for 1 sample. (C) Heat map of significantly altered metabolites arranged according to direction of change and the super-metabolic pathway they fall under. Yellow represents high and blue represents the low intensity of the metabolite relative to its mean intensity (black). Each of the 5 replicates of plasma from vehicle control (S) and EAE (D) are arranged by hierarchical clustering. (D) Pie chart representing the number of altered metabolites within each superpathway of
metabolism, specifically lipids, amino acids, peptides, xenobiotics, carbohydrates, nucleotides and energy-related.

**FIGURE 3.** MetaboAnalyst analysis of altered metabolites in plasma isolated from EAE compared to vehicle controls. (A) Metaboanalyst analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic library. Both the overrepresentation of altered metabolites within the pathway (hypergeometric test) and the impact of the changed metabolites on the function of the pathway through alterations in critical junction points of the pathway (relative betweenness centrality) were assessed. Results of each of the 82 mouse pathways of KEGG are simultaneously plotted to show the most significant pathways in terms of hypergeometric test p-value (vertical axis as -LN(P), shades of red) and impact (horizontal axis, circle diameter). (B) The top 6 pathways that arise with low p-values (-LN(P) > 15) or with “high” impact (impact > 0.3).

**FIGURE 4.** Visualization of significantly altered lipid pathway changes in EAE. (A) Lipids found significantly downregulated in EAE (p < 0.05) are shown as blue nodes; whereas up-regulated lipids are shown in yellow. Unchanged and undetected lipids are in white and grey nodes, respectively. (B) Metabolite enrichment pathway (MetaboAnalyst 3.0) overview highlights PUFAs being significant enriched in the metabolomic profile of plasma of EAE vs control. (C) Omega 3 and 6 PUFAs are highlighted and key metabolites in the plasma of vehicle control and EAE groups are presented as a bar graph (N = 5). P < 0.05 is considered significant.

**FIGURE 5.** Oral administration of RvD1 attenuates EAE disease progression. (A) Levels of RvD1 in plasma of RR- and chronic EAE by enzyme immunoassay (N = 9-10). (B) SJL mice were immunized with PLP\textsubscript{139-151} on day 0 in complete Freund’s adjuvant. One set of the group were given daily RvD1 from day 0 by gavage and another set was given vehicle in the same manner. Red line indicates the duration of treatment. Clinical score was taken till the end of the study (N = 10). (C) B6 mice were immunized with MOG\textsubscript{35-55} on day 0 in complete Freund’s adjuvant. One set of the group were given daily RvD1 from day 7 by gavage or intraperitoneally (i.p.) and another set was given vehicle in the same manner. Clinical score was measured daily until end of the study (N = 8). Red line indicates the duration of treatment. (D) The photographs of spinal cord sections show inflammation (H&E; 4x magnification) and demyelination (Luxol fast blue-PAS, 10x magnification) in both groups. (E) Spleen cells were harvested and stimulated with PLP\textsubscript{135-155}. Post-96 h, cell supernatants were used for ELISA (N = 4). (F) For quantitative PCR, cells were harvested at 24 h post-stimulation with peptide and expression of target genes were examined using quantitative PCR after normalizing with ribosomal protein L27 (N = 4). At 96 h of peptide stimulation, cells were stimulated with PMA/ionomycin for 4 h in the presence of GolgiPlug. IL17a-, IFNy- and regulatory T cells (CD4+CD25+FoxP3) expressing cells were measured by intracellular staining on a CD4\textsuperscript* gate. ***P < 0.001; **P < 0.01 and *P < 0.05 compared to EAE.

**FIGURE 6.** Oral administration of RvD1 attenuates CNS inflammation. (A) Spleen cells were isolated from RvD1 treated and untreated EAE groups and cultured with PLP\textsubscript{135-155}. Post-72h, PLP\textsubscript{135-155} primed T cells were harvested, washed and 10 million cells were adoptively transferred in the volume of 200 µl via intraperitoneal route in recipient mice (SJL). Mice were given 300 ng of pertussis toxin at day 0 and 2 and were observed every day for disease onset and disease (N = 4). *P < 0.05 compared to adoptive transfer of vehicle treated PLP\textsubscript{135-155}-primed T cells to SJL mice. (B) BILs (brain and spinal cord) were isolated using a Percoll gradient as described in the Methods. The percentage of Th1/Th17/Th2 cells in the CD4 subset was analyzed by FACS through intracellular staining of IFNy and IL17a in BILs isolated from RvD1 treated and untreated EAE groups on day 22 post immunization following restimulation with PLP\textsubscript{135-155} peptide for
FIGURE 7. RvD1 induces M2 type phenotype and inhibits class II and co-stimulatory molecules expression in macrophages. (A, B) Expression of M1, M2-specific genes, class II and co-stimulatory molecules (CD86 and CD60) were examined in adherent macrophages isolated from spleen cells of RvD1 treated and untreated EAE groups after panning on a plastic dish and treated with lipopolysaccharide/IFNγ for 6 h (N = 4). (C) SJL mice were immunized with PLP135-155 in complete Freund’s adjuvant as described before. Post-10 days, CD4 cells were isolated from spleens/lymph nodes and mixed with adherent monocytes/macrophages isolated from RvD1-treated and untreated EAE groups at the ratio of 1:5. After 72 h of incubation, cell proliferation was examined using WST-1 reagent (N = 4). Cell supernatant was processed for IFNγ and IL17 analysis by ELISA (Biolegend) (N = 4). NS; not significant, ***P < 0.001. (D) Cells were stimulated with PMS/ionomycin for 4 h in the presence of GolgiPlug. IL17A- and IFNγ-expressing cells were measured by intracellular staining on a CD4+ gate (N = 2).

FIGURE 8. RvD1 potently attenuates M1 inflammatory markers while increasing the functional M2 marker. (A) On day 22 post immunization, spinal cords were isolated from both groups and the expression of pro-inflammatory M1 cytokines, M2 gene, myelin genes and neurotrophic factors were analyzed and normalized against the L27 housekeeping gene using quantitative PCR (N = 4). (B) Infiltrating mononuclear cells were isolated from RvD1 treated and untreated groups and the cellular M2 phenotype (CD206+ and scavenger receptor positive) was profiled using FACS analysis for activated myeloid cells including monocytes/macrophages (CD11b+CD45hi) and (C) resident microglia (CD11b+CD45int) (N = 4). (D) Infiltrating mononuclear cells were profiled for myeloid derived suppressor cells (MDSCs; CD45hi CD11b+ Gr1+) and their M2 phenotype (CD206+ and scavenger receptor positive) using FACS analysis (N = 4). (E) Rat mixed glia culture were treated with RvD1 (100 nM) for 2 h followed by treatment with pro-inflammatory cytokines combination (TNFα and IFNγ; 20 ng/ml). Post-24h of incubation, RNA was isolated and expression of inducible NOS and myelin genes (MOG and PLP) was normalized with L27 as a housekeeping gene (N = 4). ***p < 0.001 and **p < 0.01 as depicted here.

FIGURE 9. Schematic flow of implication of untargeted metabolomic approach in EAE.
| Groups                  | Treatment           | Number | Incidence | EAE day onset | EAE score at peak | EAE maximum score | EAE cumulative score |
|------------------------|---------------------|--------|-----------|---------------|-------------------|--------------------|----------------------|
| RR-EAE (SJL)           | EAE-vehicle         | 10     | 10        | 15            | 3.1 + 0.12        | 3.1 + 0.12         | 49 + 11.2            |
|                        | RvD1 treated; oral  | 10     | 8         | 16            | 1.1 + 0.63**      | 1.5 + 0.67**       | 13.35 + 14.6         |
| Chronic-EAE (B6)       | EAE-vehicle         | 6      | 6         | 13            | 2.9 + 0.4         | 3.1 + 0.325        | 30.6 + 7.2           |
|                        | RvD1 treated; oral  | 6      | 6         | 13            | 1.2 + 0.41**      | 1.3 + 0.41**       | 11.8 + 5.9           |
| Chronic-EAE            | EAE-vehicle         | 8      | 6         | 14            | 3.1 + 0.12        | 3.2 + 0.3          | 33 + 6.4             |
|                        | RvD1 treated (ip)   | 8      | 6         | 14            | 1.2 + 0.41**      | 1.4 + 0.27**       | 13.3 + 6.7           |
| Adoptive transfer      |                     |        |           |               |                   |                    |                      |
| Chronic-EAE (B6)       | Vehicle             | 4      | 4         | 22            | 1.67 + 0.5        | 1.67 + 0.5         | 70 + 13.1            |
|                        | RvD1 treated        | 4      | 2         | 25            | 0.25 + 0.14**     | 0.5 + 0.28**       | 18 + 5.2             |

** p<0.01 compared to vehicle treated EAE (unpaired t test using GraphPad Prism software).
Resolvin D1 reduces EAE disease progression

Figure 1

A. Clinical score over time showing the progression of disease in CFA/PT/MOG and CFA/PT groups.

B. Histological images of brain sections from different groups.

C. Bar graph showing IFNγ levels with CFA/PT and MOG35-55 treatments.

D. Bar graph showing IL17 levels with CFA/PT and MOG35-55 treatments.
Resolvin D1 reduces EAE disease progression
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Resolvin D1 reduces EAE disease progression
Resolvin D1 reduces EAE disease progression
**Figure 7**

A. M1 marker

- iNOS
- IL6

M2 marker

- IL10
- Arg
- FIZZ
- YM1/2

Normalized value

- IFNγ/LPS
- +RvD1 treatment (100nM)

B. MHC II, CD86, CD80

Normalized value

- IFNγ/LPS
- +RvD1 (100nM)

C. Co-culture PLP primed CD4 with monocytes from EAE and RvD1 treated mice

- Absorbance
- IL17 (pg/20uL)
- IFNγ (pg/20uL)

D. EAE, EAE RvD1

- IL17
- IFNγ

EAE

- IL17: 6.9%
- IFNγ: 2.9%

EAE RvD1

- IL17: 0.7%
- IFNγ: 0.08%
Resolvin D1 reduces EAE disease progression

**Figure 9**

- **Plasma isolation**
  - Untargeted metabolomics
    - LC-MS and GC-MS

- **Data production & curation**

- **Computational analysis**
  - Identification of metabolic pathways
  - Discovery of biomarkers

- **Omega 3- and -6 metabolism**

- **Literature support to identify potential targets**

- **Resolvin D1**
  - Testing in EAE models
    - Th1/Th17
    - M2 monocytes/macrophage
    - Inflammation

- **EAE disease pathology**