Supplementary Methods (eMethods)

Histological examination and analysis of cuprizone-treated mice

All mice were sacrificed on the last day of week 7 and intra-cardially perfused with PBS (3 min 4-5 ml/min) and 4% PFA (4 min 4-5 ml/min) and the brains were removed. The whole forebrains were put in 4% PFA for 48 h at 4°C. After fixation brains were processed for paraffin embedding by dehydration through increasing ethanol series. Automated immunohistochemistry of paraffin sections was performed on 3 µm paraffin sections mounted on SuperFrost+ slides (Thermo Fisher Scientific) and automatically immuno-stained using the Discovery XT technology (Ventana, Roche Diagnostics). Sections were de-paraffinized, re-hydrated, subjected to antigen retrieval by heating with CC1 cell conditioning buffer for 28–68 min according to the antibody, incubated for 1–3 h depending on the antibody at room temperature with the primary antibody diluted in antibody diluent (Ventana), incubated with the respective biotinylated secondary antibody diluted in antibody diluent, reacted with the DAB- Mab kit and counterstained with Hematoxylin II and Bluing reagent (Ventana). Slides were washed with soap in hot tap water and rinsed under cold running tap water to remove the soap, then dehydrated and embedded with Pertex.

For LFB staining, slides were de-paraffinized and rehydrated with 95% ethanol. Slides were then incubated in LFB solution (Solvent Blue 38 (Sigma S3382) in 95% ethanol and 10% acetic acid (Sigma 695092)) overnight at 60 °C, rinsed in 95% ethanol for 1 min, then in distilled water for 2 min and in 0.05% lithium carbonate for 5 s. Subsequently, slides were rinsed in 70% ethanol twice for 10 s, then in distilled water for 2 min. The rinsing was repeated in 0.05% lithium carbonate (Merck 105680) prepared freshly, 70% ethanol and distilled water until there was a sharp contrast between the blue of the white matter (myelin) and the colorless grey- matter. Finally, slides were dehydrated starting with 95% ethanol and mounted in Pertex.

The primary antibody was a rabbit anti-mouse GST-π (MBL 312) 1:500. Secondary detection antibody: Goat anti-rabbit IgG biotinylated (Jackson ImmunoResearch 111–065-144; Jackson ImmunoResearch Europe Ltd, Ely, UK) 1:1000.

Xenopus laevis demyelination model and metronidazole preparation

In this model, myelinating oligodendrocytes selectively express both the GFP reporter and the bacterial nitroreductase (NTR) enzyme, under the control of a portion of mouse *Mbp* regulatory sequence.[1] The NTR enzyme converts the nitro radical of prodrugs, such as metronidazole (MTZ), to a highly cytotoxic hydroxylamine derivative; therefore, introduction of MTZ into the swimming water of transgenic *Mbp:GFP-NTR* tadpoles provokes an oligodendrocyte cell-death.[1,2] Xenopus tadpoles were staged according to Nieuwkoop and Faber.[3] Transgenic tadpoles were treated between stages NF 50 and 55, corresponding to pre-metamorphosis, a stage of ongoing myelination.

MTZ (Fluka) was dissolved in filtered tap water containing 0.1% DMSO (Sigma Aldrich). MTZ was used at a concentration of 10 mM with an exposure length of 10 days. Transgenic or non-transgenic sibling tadpoles were maintained in 600 ml of MTZ solution (maximum 10 tadpoles/600 ml) at 20˚C in complete darkness (MTZ is light-sensitive) and the solution was changed daily throughout the duration of treatment. For regeneration experiments, MTZ-exposed animals were allowed to recover for 3 days in either normal water (control) or water containing siponimod at increasing concentrations in ambient laboratory lighting (12 h light/12 h dark).

Pharmacokinetics study in C57Bl/6J mice

Female, 6-weeks old C57Bl/6J mice (Janvier Labs, Le Genest-Saint-Isle, France) were treated for two weeks with siponimod (BAF312) at 0.3, 1.5 or 3 mg/kg BW, or vehicle (DMSO:H_2O, 1:20), via drinking water or were fed over four weeks with siponimod-loaded pellets at 0.03, 0.1 and 0.2 g/kg of food for the concentration analysis and 0.3, 0.1, 0.03, and 0.01 g/kg of food for a second concentration analysis and the lymphocyte counts. Siponimod-loaded pellets were prepared as previously described.[4] Briefly, dry powdered food (~500 g) for rodents (Provimi Kliba SA, Switzerland) was mixed with siponimod at the final concentrations. The whole mixing procedure was conducted in a fume cupboard as a safety precaution to avoid dust inhalation. Whilst still mixing, 250 ml water was gradually added and final mixture was loaded into a custom-built extruder for compression into a long cylinder of food mixture (~ 1.5 cm in diameter) which was broken by hand in pieces of approximately 2 to 4 cm in length. These food-pellets were left to dry on a metal grid overnight, at 35 °C, in a food-dehydrator (Excalibur®). Drug-free pellets were prepared similarly for controls. All pellets were stored at room temperature and prepared fresh every 2 weeks.

Blood samples were collected (via tail nick) in short intervals and at termination to assess blood siponimod levels by LC/MS/MS (explained below) and lymphocytes counts using Vet ABC™ (scil animal care, Germany) or ADVIA™ hematology analyzers (Siemens Healthineers, Germany).

Results were analyzed by using one-way Anova Holm-Sidak's multiple comparisons test (GraphPad Prism Software Inc., San Diego, California, USA). A value of $p < 0.05$ was considered significant.

Tissues sampling for measurements of siponimod exposures in Xenopus tadpoles

At end of studies, i.e. 3 days after stopping MTZ, tadpoles were euthanized, put on ice and cut in two, separating the head (mainly brain) from the rest of the body. The “Body” parts were weighed (approx. 1 g each), distributed to individual Eppendorf tubes and snap-frozen in liquid N2. Concerning the “Brain” parts, due to their very small size (≤3 mg), they were mixed in one single Eppendorf tube, according to treatment groups, before snap-freezing. All samples were then stored at -80°C until further processing for measuring siponimod levels by liquid chromatography/tandem

mass spectrometry (LC/MS/MS), explained in detail see § “Measurements of siponimod levels in blood/tissues samples of Xenopus tadpoles and mice” below.

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OCT and OMR in mice

The measurements of retinal layers were performed using a Spectralis™ HRA+OCT device (Heidelberg Engineering, Germany) under ambient light conditions. The OCT device was equipped with several adaptions for rodents described elsewhere[5] and the scanning protocol was executed as previously described.[6,7] We report the methodology in line with the APOSTEL recommendations.[8] Automated segmentation was carried out by the Heidelberg Eye Explorer™ software version 1.9.10.0 followed by manual correction of an investigator, blinded for the experimental groups. We calculated the thickness of the inner retinal layers (IRL), consisting of the retinal nerve fibre layer (RNFL), ganglion cell layer (GCL) and inner plexiform layer (IPL) as described elsewhere.[6] High-resolution mode was used; only scans with a quality of at least 20 decibels were included.

The visual function analysis was carried out with a testing chamber and the OptoMotry™ software from CerebralMechanics™, Canada. The mice were positioned on a platform in a virtual cylinder with a moving grid and the head movements (tracking) were evaluated by an investigator blinded on the experimental groups. As a measure for visual acuity, we used the threshold of the highest spatial frequency at 100% contrast. As tracking of the mice is performed exclusively from temporal to nasal direction, left and right eye tracking can be distinguished.

Cuprizone-preparation and randomization of C57Bl/6J mice

Cuprizone (Bis(cyclohexanone) oxaldihydrazone, Sigma-Aldrich, Buchs, Switzerland) was mixed into rodent food pellets (0.2% w/w) by Provimi Kliba AG (Kaiseraugst, Switzerland). Siponimod-loaded pellets (10 mg/kg food) and sham pellets were prepared in-house as previously described.[4] Mice were randomized in 3 study groups (n=7 each), with similar mean baseline bodyweight in each group (20.6 ± 0.3 g; 20.5 ± 0.3 g; 20.8 ± 0.4 g). Mice were regularly monitored via visual inspection and bodyweight. MRI measurements were performed at week 5, 6 and 7 as described below.

Wholemount of Xenopus optic nerve and analysis

Tadpoles were fixed by immersion in 4% paraformaldehyde for 1h at room temperature. Fixed optic nerves were carefully dissected out before being rinsed in PBS (1x) and incubated in methanol-acetone (1:1 v:v) for 10 min at 4 °C, then blocked in 10% normal goat serum (NGS-Thermo-Fisher 50062Z) containing 0.3% Triton X-100® overnight at 4 °C. Optic nerves were incubated in anti-GFP for 3 days at 4 °C in blocking solution (PBS-Triton X-100 0.3%-NGS 1%). Samples were then

rinsed in PBS/0.1% Tween-20 and secondary antibody added overnight at 4 °C in blocking solution. After extensive washes in PBS, optic nerves were mounted on glass slide with an anti-fade mounting medium (Vectashield vibrance, Eurobio).

Z-series using the Olympus FV-1200 upright confocal microscope were performed at 0.3 µm increment and maximum orthogonal projection of images was carried out using Fiji software (NIH, Bethesda, Maryland). Serial images were collected and the number of GFP+ cells counted on the whole optic nerve. We used chicken anti-GFP (1:1000, Aves Lab, USA), and goat anti-chicken Alexa Fluor 488, (1:600, InVitrogen) as secondary antibody.

Microglial cells were stained with *Bandeiraea Simplicifolia isolectin* B4 (IB4) on wholemount optic nerves prepared as above. Samples were incubated for 48h at 4°C with Alexa Fluor 594-conjugated IB4 (1:1000, Invitrogen) in PBT 0,1% supplemented with 1mM calcium under gentle shaking, and then washed in blocking solution before exposures to antibodies, as described above. Counting of $IB4^+$ cells per optic nerve was performed on collected serial images obtained on a Nikon A1R-HD25 confocal microscope.

Electron microscopy of Xenopus tadpole optic nerve

Xenopus larvae were fixed in a mixture of 2% paraformaldehyde, 2% glutaraldehyde, in 0.1 M cacodylate buffer pH 7.4 and 0.002% calcium chloride overnight at 4 °C, washed in 0.1 M cacodylate buffer, and postfixed in 1% osmium tetroxide, 1% potassium ferricyanide in 0.1 M cacodylate buffer. After washing in cacodylate buffer and water, larvae were incubated in 2% uranyl acetate aqueous solution at 4 °C overnight. After rinsing twice in water, larvae were dehydrated in increasing concentrations of ethanol, final dehydration was in 100% acetone (twice 10 min each). Samples were infiltrated with 50% acetone 50% Epon for 2 h, followed by pure Epon for 1 h (EMBed 812, Electron Microscopy Sciences Cat 14120). Blocks were then embedded in a new batch of Epon and heated at 56 °C for 48 h. Semi-thin (0.5 µm thick) and ultra-thin (70 nm thick) sections were cut with an ultramicrotome (UC7, Leica Microsystems). Semi-thin sections were stained with 0.5% toluidine blue in 1% borax buffer and examined under a conventional microscope, objective X63 (Eclipse TiE, Nikon). Ultrathin sections were contrasted with Reynold's lead citrate solution[9] and mounted on 200 mesh copper grids (Electron microscopy science cat# EMS200-Cu).

Wholemounts and histological optic nerve analysis of C57Bl/6J EAEON mice

After 21, 35 or 90 days of EAEON, mice were sacrificed with an overdose of Isofluran (Piramal Critical Care). Ketamine (50 mg/kg, i.p.) was administered for analgesia before cardiac perfusion was performed with cold phosphate-buffered saline (PBS). Eyes were extracted, retinae were isolated and fixed in 4% paraformaldehyde (PFA) for 30 min and wholemounts were stained with anti-Bnr3a (1:200, Santa Cruz Biotechnology, USA).

Optic nerves slices were incubated with antibodies directed against CD3- (1:400, Dako), Iba1- (1:500, Wako chemicals), myelin basic protein (MBP) (1:500, Millipore), Ym1/Chi3l3: Chitinase 3-like-3 (Ym1) (1:100, Stem Cell Technology), Arginase-1 (Arg-1) (Abcam, 1:500), tumor necrosis factor alpha (TNF-α) (1:20, Invitrogen), Olig2 (1:250, Millipore), platelet-derived growth factor receptor A (PDFGRα) (1:250, Neuromics) and glial fibrillary acidic protein (GFAP) (1:1000, Neuromab). Cy5 anti-rat, Cy5 anti-rabbit (1:500, Millipore) and Alexa Fluor™ 488 anti-rabbit (1:500, Invitrogen) were used as secondary antibodies.

The CD3 and MBP staining results were rated by an investigator blinded to the experimental groups by scores described previously.[10]

Fluorescence stained longitudinal optic nerve sections were acquired with a Leica HyD detector attached to a Leica DMi8 confocal microscope (63x objective lens magnification). At least four sections of the optic nerve from one eye of each mouse were analyzed per staining.

Optic nerves were snap frozen in liquid nitrogen and stored at -80°C for qPCR or RNA sequencing (RNAseq) or fixed in 4% PFA over night at 4 °C and dehydrated in sucrose solutions with increasing concentrations. After embedding in O.C.T. compound (Sakura Finetek, The Netherlands), longitudinal sections of 5 µm were cut for immunohistological analysis.

Bulk RNA sequencing of optic nerve from EAEON mice

Total RNA samples used for transcriptome analyses were quantified (Qubit RNA HS Assay, Thermo Fisher Scientific) and quality measured by capillary electrophoresis using the Fragment Analyzer and the 'Total RNA High Sensitivity Assay' (Agilent Technologies, Inc. Santa Clara, USA). All samples in this study showed high quality RNA Quality Numbers (RQN; mean = 8.5). The library preparation was performed according to the manufacturer's protocol using the 'SMART-Seq® Stranded Kit' (Takara Bio Inc. Kusatsu, Shiga, Japan) for Illumina®. Briefly, 10 ng total RNA were used for reverse transcription of total RNA, addition of Illumina adapters with barcodes via PCR, cleavage of ribosomal cDNA and library amplification. Bead purified libraries were normalized and finally sequenced on the NextSeq 550 system (Illumina Inc. San Diego, USA) with a read setup of 1x75 bp. The bcl2fastq tool was used to convert the bcl files to fastq files as well for adapter trimming and demultiplexing.

Statistical analysis on the fastq files was performed with CLC Genomics Workbench (version 20.0.4, QIAGEN, Venlo. NL). The reads of all probes were adapter trimmed (Illumina TruSeq) and quality trimmed (using the default parameters: bases below Q13 were trimmed from the end of the reads, ambiguous nucleotides maximal 2). We performed mapping against the Mus musculus (mm10; GRCm38.86) (March 24, 2017) genome sequence. Multi-group comparisons were made and statistically determined using the Empirical Analysis of DGE (version 1.1, cutoff = 5) after grouping of the samples (n=5 for each group). The Resulting p -values were corrected for multiple testing by FDR. A p-value of ≤0.05 was considered significant.

Iba1 and GFAP staining of the mouse eye cross-sections

At 90 days after immunization, mice were sacrificed with an overdose of Isofluran (Piramal Critical Care). Ketamine (50 mg/kg, i.p.) was administered for analgesia before cardiac perfusion was performed with cold phosphate-buffered saline (PBS). Eyes were isolated and fixated in 4 % PFA over night at 4 °C and dehydrated in ethanol solutions with increasing concentrations. After embedding in paraffin (Paraplast, Leica, Germany), longitudinal sections of 5 µm were cut for immunohistological analysis. Slices of the retinae were incubated with an Iba1 antibody (1:500, Wako chemicals) and Glial fibrillary acidic protein (GFAP) antibody. (1:500, Sigma Aldrich). Cy3 anti-rat (1:500, Millipore) was used as secondary antibody. Fluorescence stained longitudinal optic nerve sections were acquired with a Leica HyD detector attached to a Leica DMi8 confocal microscope (63x objective lens magnification). Microglia (Iba1) and actrocyte (GFAP) number was counted in three eye sections per group in a blinded manner.

Cultivation and treatment of BV2 cells

Microglial BV2 cells were cultivated in DMEM GlutaMAX (Invitrogen Corporation, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS; Lonza, Basel, Switzerland) and 50 U/ml penicillin/streptomycin (Invitrogen, Carlsbad, USA) in T75-flasks at 37°C in a humidified atmosphere of 5% CO2.

For siponimod stimulation experiments, confluent BV2 microglia were washed once with 10 ml PBS, detached by adding 5 ml Trypsin EDTA and a 5 min incubation at 37°C. Afterwards, Trypsin reaction was stopped by adding 10 ml BV2-medium. The cell suspension was collected in a centrifuge tube and spun down at 300 x g for 5 min at 4°C. Supernatant was discarded, the cell pellet resuspended in 5 ml fresh BV2-media and cell viability and numbers were quantified using trypan blue staining.

25.000 cells were seeded into 24-well plates containing 1 ml DMEM glutaMAX (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 2% FCS (Lonza, Basel, Switzerland)), 50 U/ml penicillin/streptomycin (Invitrogen, Carlsbad, USA). Stimulation was performed 24h after cells were seeded with respective Siponimod or DMSO concentrations. Cells were lysed 24h after stimulation using RLT buffer – β-mercaptoethanol mixture (1:100) according to the manufacturer's protocol (RNAeasy Mini Kit, Qiagen, Germany). RNA isolation, reverse transcription, and quantitative real-time PCR was performed as previously described.[11] GAPDH served as endogenous control genes. Primer sequences can be found in Table S1.

Measurements of siponimod levels in blood/tissues samples of Xenopus tadpoles and mice

Xenopus tadpole tissue: Tissues samples from xenopus-tadpoles (brains and bodies) and mouse blood samples were processed for assessing siponimod concentrations using a specific LC/MS/MS

bioanalytical method using an Agilent 1290 Infinity UHPLC system coupled to an Agilent 6490 triple quadrupole mass spectrometer, controlled by a MassHunter Workstation software. Briefly, siponimod was extracted by a two-step protein precipitation using a mixture of acetonitrile/methanol/trichloromethane (40/30/30%). Aliquots of 5 µL of the calibration, Quality Control (QC), recovery control and unknown samples were directly injected on an Agilent Eclipse Plus RRHD C18 2.1x50 mm column, filled with 1.8 µm particles and maintained at 40 °C. Chromatographic separation was performed applying a linear gradient from 20% to 100% B within 1.9 min at a flow rate of 500 µL/min. Solvent A was 0.2% formic acid and solvent B acetonitrile containing 0.2% formic acid. The column flow was guided to the AP-ESI JetStream source of the mass spectrometer run in positive mode (all source parameters were optimized for the siponimod). A structure related compound was used as internal standard. Both siponimod and internal standard were detected as positive ions. For calculation, the siponimod/internal standard ratio of the quantifier ion signals was used applying a polynomial regression with origin included and 1/x weighed regression over 8 calibration points. The regression coefficient was R2 = 0.99990. All calculations and data evaluation were done with MassHunter software and final reporting was done with MS Excel. The accuracy of individually prepared calibration samples between 40 and 80000 pg/sample was better than ±15.0%. The limit of quantification was measured to be 40 pg/sample and the precision, assessed with the help of QC samples was better than 3.1% relative standard deviation. The recovery was found to be 78.9%.

C57Bl/6J mouse blood: To 10 µl of blood samples 20 µl of 1EDTA solution were precipitated with 100 µl acetonitrile and centrifuged at 4 °C and 3000 g for at least 15 min. Approximately 50 µl of supernatant were transferred into a micro¬titer¬ plate and 400 µl of water/acetonitrile (1/1; v/v) was added. An aliquot of each sample was injected into the LC-MS/MS system for analysis as described above.

C57Bl/6J sample preparation brain: After addition of 5 parts of methanol/water (2/8; v/v) to the pre-weighed samples, a homogenization step was performed using a Gentle MACS Dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

To 10 µl of brain homogenate samples 20 µl of a 2blood-EDTA mix were added followed by precipitation with 100 µl acetonitrile and centrifugation at 4 °C and 3000 g for at least 15 min. Approximately 50 µl of supernatant were transferred into a micro-titer¬ plate and 400 µl of water/acetonitrile (1/1; v/v) was added. An aliquot of each sample was injected into the LC-MS/MS system for analysis with a blood calibration curve as described above.

Magnetic resonance imaging (MRI), magnetization transfer ratio (MTR) and T2-weighted signal intensity (T2-WSI)

MRI measurements, with analysis for magnetization transfer ratio (MTR) and T2-weighted signal intensity (T2-WSI), were performed at week-5 (end of the cuprizone-intoxication phase), at week-

6 and week-7 (treatment phase). MTR values at week 5 were used to randomize cuprizone-challenged mice into two groups at week 5, before initiation of vehicle or siponimod treatment. Measurements were performed with a Biospec 70/30 spectrometer (Bruker Medical Systems, Ettlingen, Germany) operating at 7 T. The operational software of the scanner was ParaVision 5.1 (Bruker). Images were acquired from anesthetized, spontaneously breathing animals using a mouse brain circularly polarized coil (Bruker, Model 1P T20063 V3; internal diameter 23 mm) for radiofrequency excitation and detection. Neither cardiac nor respiratory triggering was applied. Following a short period of introduction in a box, animals were maintained in anesthesia with 1.5% isoflurane (Abbott, Cham, Switzerland) in oxygen, administered via a nose cone. During MRI signal acquisitions, animals were placed in prone position in a cradle made of Plexiglas, the body temperature was kept at 37 ± 1 °C using a heating pad, and the respiration was monitored. A T2-weighted, two-dimensional multi slice RARE (Rapid Acquisition with Relaxation Enhancement) sequence[12] was used for determining the anatomical orientation and for evaluating signal intensities. This was followed by a two- dimensional multi slice gradient-recalled FLASH (Fast Low-Angle Shot) acquisition[13] for assessment of MTR. As both sequences had the same anatomical parameters, the choice of the regions-of-interest for evaluations was performed on the RARE images and then transferred to the FLASH images. MRI images were analyzed using the ParaVision software. The parameters of the acquisitions were the following: (a) RARE sequence: effective echo time 80 ms, repetition time 3280 ms, RARE factor 16, 12 averages, field of view 20 × 18 mm, matrix size 213 × 192, pixel size 0.094 × 0.094 mm, slice thickness 0.5 mm, 15 adjacent slices. Hermite pulses of duration/bandwidth 1 ms/5400 Hz and 0.64 ms/5344 Hz were used for radiofrequency excitation and refocusing, respectively. Fat suppression was achieved by a gauss512 pulse of 2.61 ms/1051 Hz duration/bandwidth followed by a 2-ms-long gradient spoiler. The total acquisition time was of 7 min 52.3 s; (b) FLASH sequence: echo time 2.8 ms, repetition time 252.8 ms, 4 averages, field of view 20 × 18 mm, matrix size 213 × 192, pixel size 0.094 × 0.094 mm, slice thickness 0.5 mm, 15 adjacent slices. A hermite pulse of 0.9 ms/ 6000 Hz duration/bandwidth and flip angle 30° was used for radiofrequency excitation. MTR contrast was introduced by a gauss pulse of 15 ms/182.7 Hz duration/bandwidth applied with a radiofrequency peak amplitude of 7.5 µT and an irradiation offset of 2500 Hz. The acquisition was then repeated with the same parameters but without the introduction of the MTR contrast. MTR was then computed using the formula $MTR= (S_0-S_{MTR})/S_0$ where S_0 and S_{MTR} represent respectively the signal intensities in the FLASH acquisitions without and with the introduction of the MTR contrast. The total acquisition time for both data sets was of 6 min 31.6 s.

Signal intensities in T2-weighted images, T2-WSI and MTR were determined in different brain regions, including the Corpus Callosum (CC) at the level of the commissure of fornix. Data acquisition and analysis were performed by an experimenter blinded to the treatment.

Supplementary References

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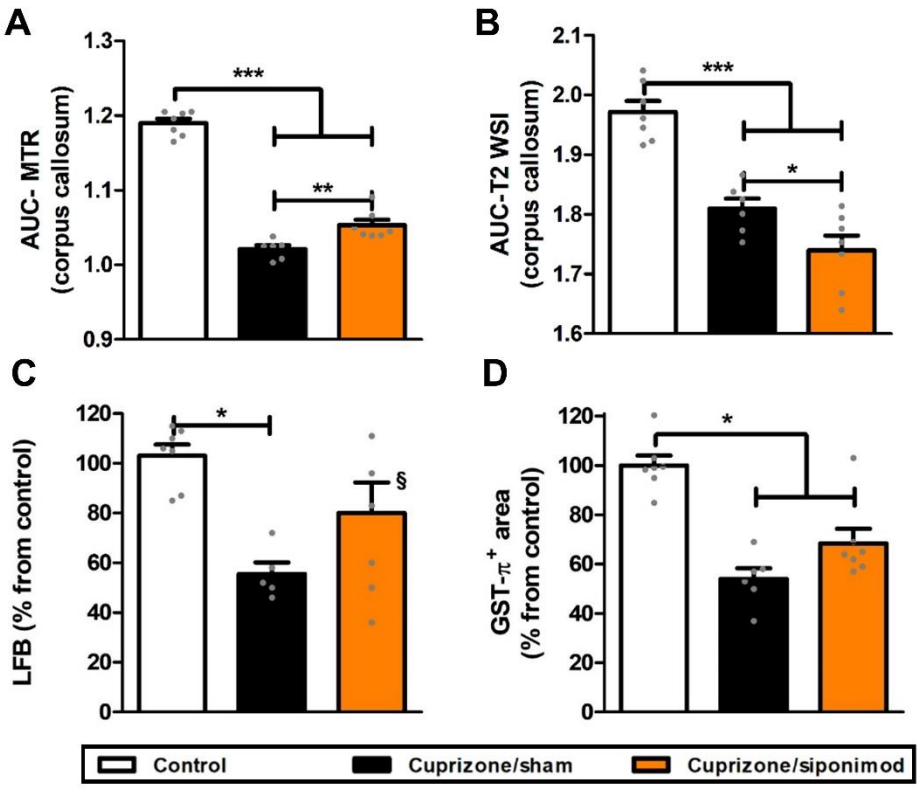
eTable 1. Oligonucleotides used in this study

Gene ID	Gene name	Primer	Sequence (5'-3')
BCAS1	Breast carcinoma-amplified sequence 1	Forward	agaaagctctttaggcacaagg
		Reverse	tggctttgtccgacttgag
CD206	Cluster of differentiation 206	Forward	ccacagcattgaggagtttg
		Reverse	acagctcatcatttggctca
CNPase	2',3'-Cyclic-nucleotide 3'-phosphodiesterase	Forward	cgctggggcagaagaatac
		Reverse	aaggccttgccatacgatct
Galc	Galactocerebrosidase	Forward	ttttgggtgccaagcattat
		Reverse	cggcctttcattccaaatc
Iba1	Ionized calcium-binding adapter molecule 1	Forward	ggatttgcagggaggaaaa
		Reverse	tgggatcatcgaggaattg
MBP	Myelin basic protein	Forward	agccctctgccctctcat
		Reverse	ggtagttctcgtgtgtgagtcct
MD-1	Lymphocyte antigen 86	Forward	attctgaactactcctatccccttt
		Reverse	ggccggcatagtatatctgttct
MOG	Myelin oligodendrocyte glycoprotein	Forward	gaatctccatcggacttttga
		Reverse	ggtccaagaacaggcacaat
NG2	Neuron-glial antigen 2	Forward	cagaggaggtcttggtgaactt
		Reverse	cagaggacatctcgtgctca
PDGFR-α	Platelet-derived growth factor receptor A	Forward	aagacctgggcaagaggaac
		Reverse	gaacctgtctcgatggcact
TMEM 119	Transmembrane Protein 119	Forward	gtcactccatcccagtttcac
		Reverse	ggaccatgttgagctatggaa
Chi3l3/Ym1	Chitinase 3-like-3	Forward	ggtctgaaagacaagaacactgag
		Reverse	gagaccatggcactgaacg
Arg-1	Arginase-1	Forward	cattgtccttaagccgttcc
		Reverse	cagccaacatccccacat
TNF-α	Tumor necrosis factor alpha	Forward	ctgtagcccacgtcgtagc
		Reverse	ttgagatccatgccgttg



eFigure 1. Siponimod therapy promotes remyelination after toxic demyelination by cuprizone.

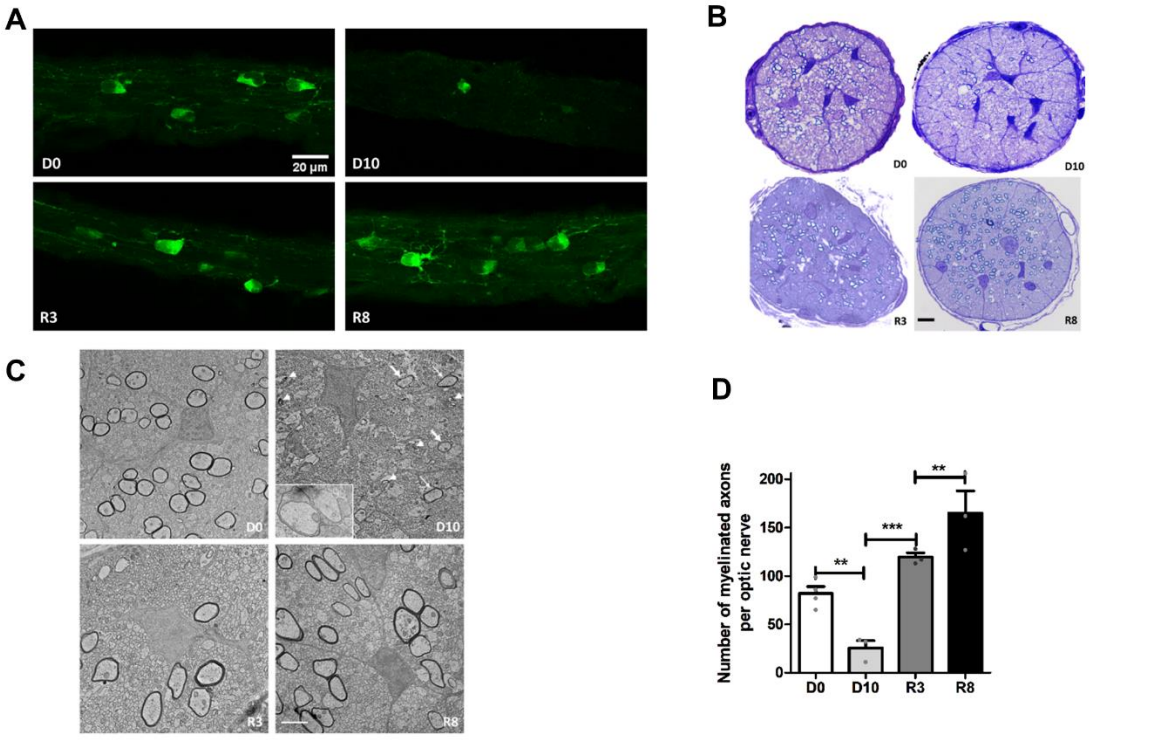
AUC for MTR changes (A) in the CC and AUC for T2-WSI changes (B) in the CC of mice in all study groups. Terminal qIHC for changes vs control in myelin density by LFB staining (C) and in oligodendrocyte numbers, assessed by GSTπ+ cells (D) in the CC (rostral) of siponimod-treated or untreated mice. Changes are expressed as mean ± SEM (n=7 animals per group), grey dots show individual data points. **p<0.01, ***p<0.001, *p<0.05 by ANOVA with Dunnett's post hoc test. §One data points out of axis limits.

Supplementary Results (eAppendix)

Conditional oligodendrocyte ablation and demyelination followed by spontaneous remyelination in the *Mbp:GFP-NTR* Xenopus laevis.

Thanks to the GFP reporter myelin forming oligodendrocytes sending their processes to nearby axons are easily observed in the optic nerve of transgenic *Mbp:GFP-NTR* tadpoles (D0; eFigure 2A). Exposure to metronidazole (MTZ) for 10 days induced a nearly complete ablation of GFP+ oligodendrocytes (D10; eFigure 2A). At the end of the MTZ treatment, animals recovered rapidly as observed already after 3 days (R3; eFigure 2A) and recovery is about complete at 8 days (R8; eFigure 2A). We expected that ablation of GFP+ cells should lead to demyelination. This was indeed the case as shown on transversal semi-thin sections stained with toluidine blue (eFigure 2B, compare D0 and D10) or on ultra-thin sections observed at the electron microscope (EM) (eFigure 2C). Demyelination was quantified on semi-thin sections showing that at the end of MTZ

exposure the number of myelinated axons per optic nerve had decreased from 81.7 ± 7.2 down to 25.6 ± 7.3 (mean ± SEM; n= 3 or 4) (eFigure 2D). On EM of D10 samples, there was a drastic rarefaction of myelinated axons, surrounded with myelin debris as well as some axons undergoing demyelination (inset at D10) (eFigure 2C). As soon as exposure to MTZ was interrupted and tadpoles returned to normal water, spontaneous generation of new GFP+ oligodendrocytes (R3 eFigure 2A) and solid remyelination occurred already after only 3 days (R3; eFigure 2B and C). At R8 myelin recovery was complete and the number of myelinated axons was even double compared to D0 (eFigure 2D). This could be explained by the fact that 18 days occurred between the beginning of the experiment (i.e., D0) and R8, which means that tadpoles went from stage 50 to stage 55, i.e., a developmental stage with a higher number of myelinated axons.

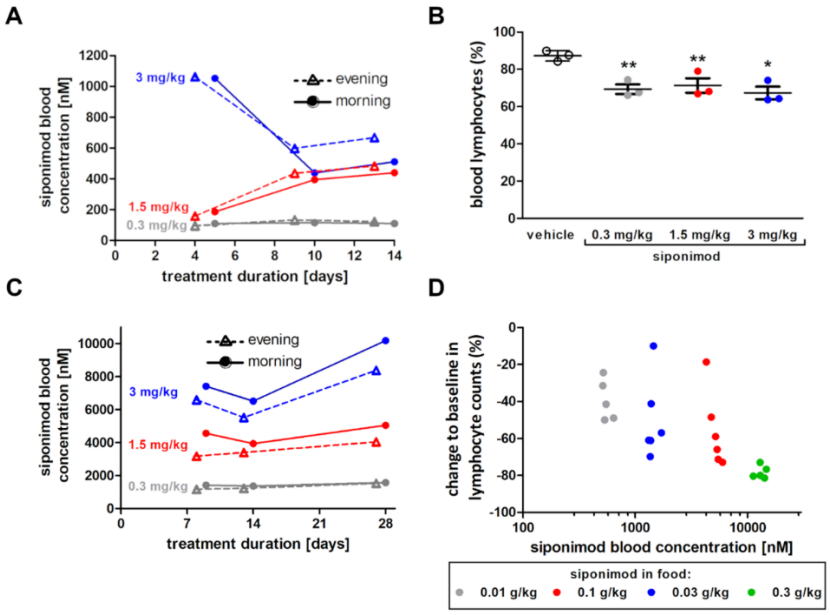


eFigure 2. Confocal and electron microscopic illustration of conditional demyelination and spontaneous remyelination of *Mbp:GFP-NTR* transgenic Xenopus laevis.

(A) *Mbp:GFP-NTR* Xenopus laevis (stage 50 tadpole) before (D0), at the end of metronidazole exposure (D10) and after 3 days (R3) and 8 days (R8) spontaneous recovery, scale bar=20 µm. (B) Semi-thin transversal sections stained with toluidine blue (C) and electron micrographs of transversal ultrathin sections of optic nerve of transgenic *Mbp:GFP-NTR* Xenopus laevis tadpole before (D0), at the end of metronidazole exposure (D10) and after 3 days (R3) and 8 days (R8) of spontaneous recovery, scale bar=2 µm. (D) Quantification of the number of myelinated axons of the semi-thin section between D0 and R8. Data are expressed as mean ± SEM, grey dots show individual data points (n = 3-4 tadpoles per group), with **p<0.01 and ***p<0.001 calculated using an unpaired two-tailed Student's t-test between two groups with a 95% confidence interval.

Pharmacokinetics study in mice shows pellet food is superior over supply via drinking water

We performed a pharmacokinetics study to evaluate the best-suited formulation for oral administration and to assess the bioavailability and the effect of siponimod on the circulating blood lymphocytes. The treatments via drinking water achieved dose-dependent blood concentrations of siponimod, with 0.1, 0.4 and 0.6 µM ranges obtained with 0.3, 1.5 and 3 mg/kg bodyweight (BW), respectively. These results indicate a poor dose-proportionality of siponimod blood levels and a large, time-dependent variability (eFigure 3A). A reduction in blood lymphocytes counts of about 20% was observed in all treatment groups (eFigure 3B). Using siponimod-loaded pellets, dose-dependent blood levels of siponimod were observed together with a dose-related reduction in lymphocyte counts. Pellets loaded at 0.03, 0.1 and 0.2 g/kg of food achieved siponimod blood levels within the 1, 4 and 8 µM ranges with good stability over time and low intra- and inter-individual variability (eFigure 3C). In mice fed with pellets loaded with 0.3, 0.1, 0.03, and 0.01 g/kg of food, circulating lymphocytes were reduced by 81, 56, 50 and 39% respectively (eFigure 3D). We therefore chose pellet-derived siponimod treatment at 0.03 and 0.01 g/kg food for all following experiments.

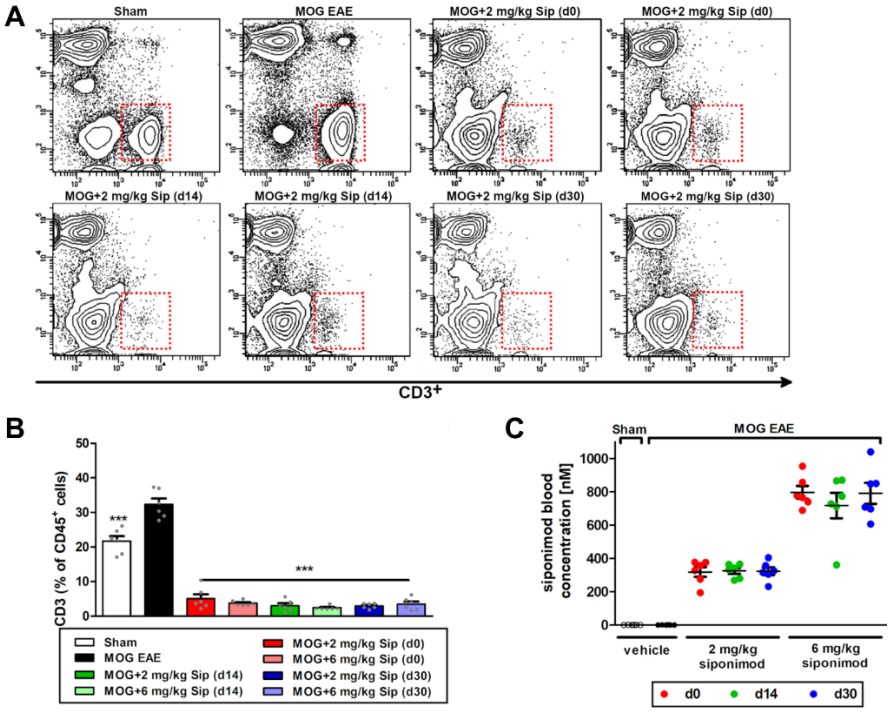


eFigure 3. Longitudinal changes of siponimod blood concentrations and lymphocyte counts in C57Bl/6J mice.

(A) Siponimod blood concentrations over 14 days and (B) lymphocyte counts after 14 days in mice during continuous siponimod treatment via drinking water (n=3 per group, concentrations in mg/kg BW). (C) Siponimod concentration over 28 days and (D) lymphocyte counts in blood after 28 days in mice, fed continuously with siponimod-loaded food pellets (n=5-6 per group, concentrations in g/kg of food). All graphs represent the pooled mean ± SEM with *p<0.05; **p<0.01; ***p<0.001, area under the curve compared by ANOVA with Dunnett's post hoc test for time courses and with *p<0.05, **p<0.01, by ANOVA with Dunnett's post hoc test for scatter plots compared to control untreated mice.

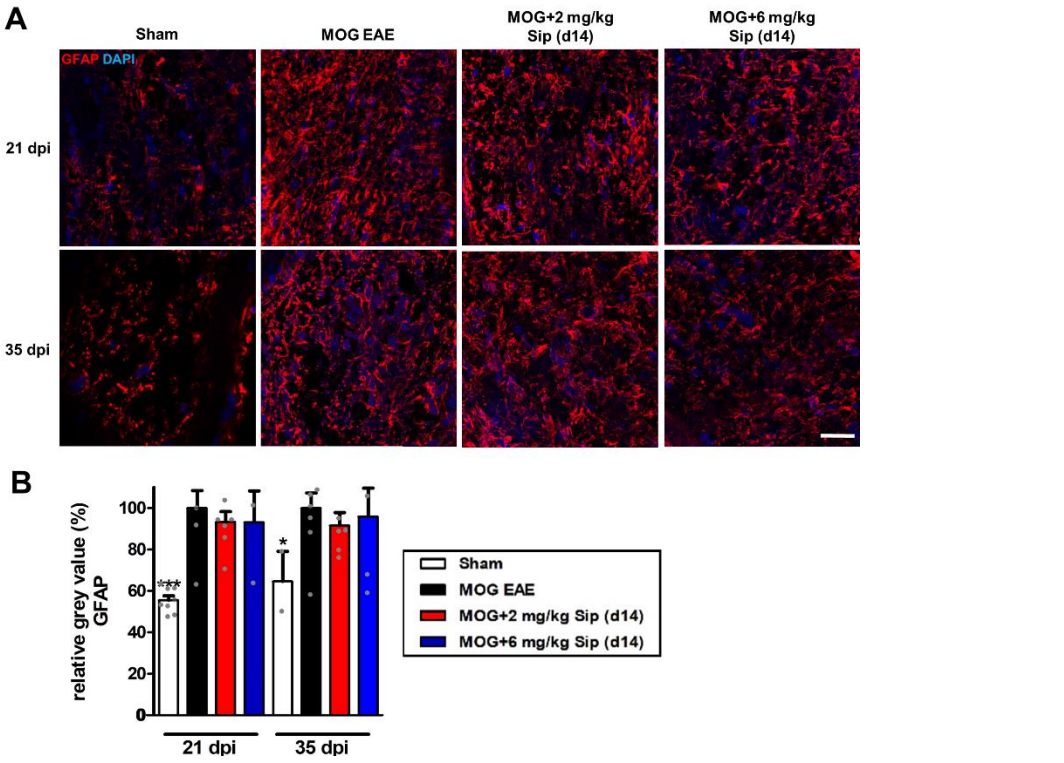
Flow-cytometry analysis of the circulating blood cells and siponimod blood concentrations in EAE animals

As siponimod alters the lymphocyte profile in the periphery by modulation of S1P1/5 receptors, we investigated the effects on the circulating blood cells of EAEON mice. The $CD3^+$ T-cell population, pregated for lymphocytes (by forward- and side scatter) and $CD45^+$ (leukocyte) cells, was increased in MOG EAE mice 90 days after immunization. The siponimod therapy reduced the circulating $CD3^+$ cells by approximately 90% at 2 and 6 mg/kg BW, regardless when the treatment was started (eFigure 4A and B). Of note, the CD45+/$CD3^{low}$ population was decreased in EAEON mice, which was reversed by siponimod therapy, even showing a higher $CD3^{low}$ population compared to sham untreated mice (eFigure 4A). When the blood samples of the animals were analyzed for siponimod, dose dependent concentrations between 300-400 nM and 700-900 nM were detected in mice treated at 2 or 6 mg/kg BW, respectively. No significant difference was measurable between the different treatment starting time points, confirming a robust stability of the substance over the time course (eFigure 4C).



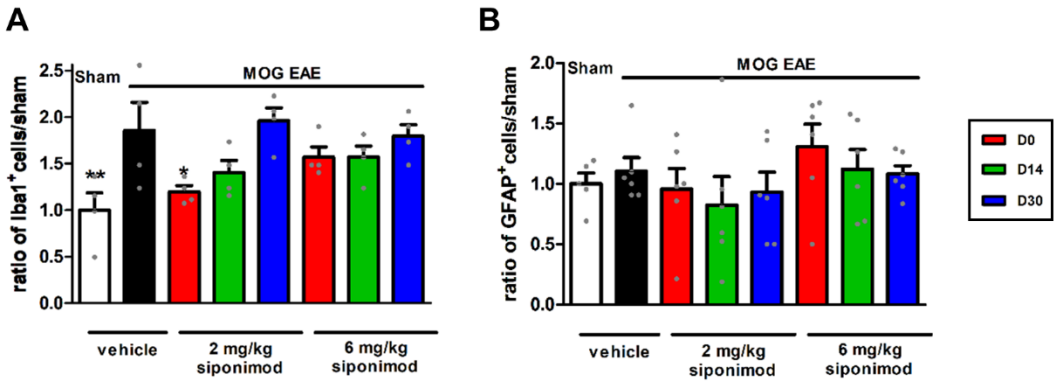
eFigure 4. Siponimod reduces the circulating CD3+ lymphocytes and shows dose dependent blood concentrations.

(A) Leucocytes were isolated 90 days after Sham injection or EAE immunization and directly analyzed. The cell population was gated and percentages of CD45+ (leukocyte) cells were analyzed for CD3+ cells. (B) Quantitative analysis of the flow-cytometry measurement. (C) Siponimod blood concentration 90 days after EAE Immunization. All graphs represent the pooled mean ± SEM, grey dots show individual data points (out of two independent experiments each with n = 6 animals per group) with ***p<0.001, by ANOVA with Dunnett's post hoc test compared to MOG EAE untreated mice.



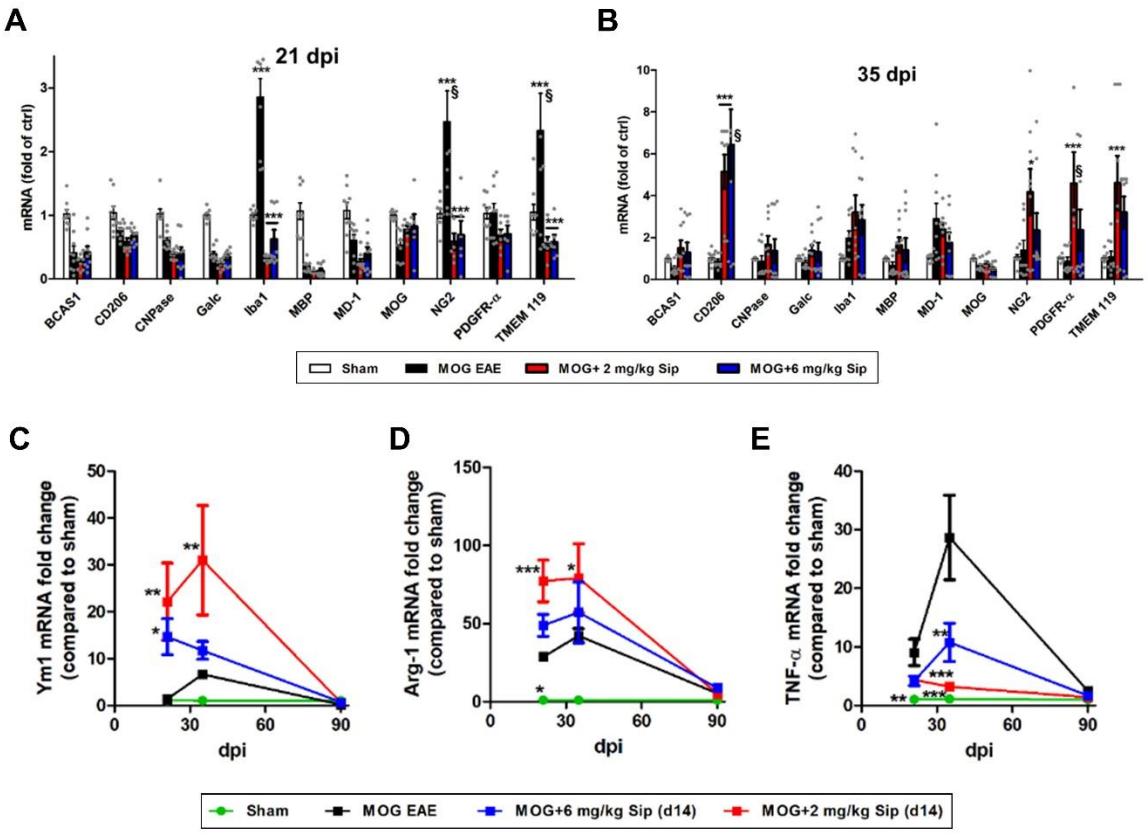
eFigure 5. Histological analysis of GFAP in the optic nerve at 21 and 35 dpi.

Optic nerves were stained for GFAP at 21 and 35 days after MOG_{35-55} immunization, scale bar=50 µm. (B) Quantitative analysis of longitudinal sections of optic nerves of C57Bl/6J mice. One optic nerve per mouse was included for histological examination. Graph represents the pooled mean ± SEM. Grey dots show individual data points (n = 6 animals per group), with *p<0.05, ***p<0.001 by ANOVA with Dunnett's post hoc test compared to untreated MOG EAE.



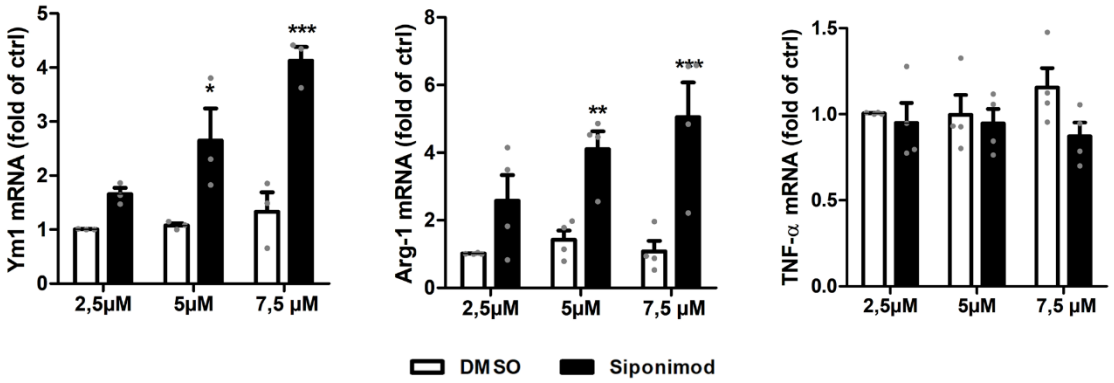
eFigure 6. Histological analysis of retinal cross sections of EAEON mice after siponimod treatment.

(A) Analysis of microglia number (Iba1+) and (B) number of GFAP+ cells (astrocytes and Mueller cells) 90 days after MOG immunization in the retinal cross sections. One eye per mouse was included. All graphs represent the pooled mean ± SEM, (n = 4-6 animals per group) with *p<0.05, **p<0.01 by ANOVA with Dunnett's post hoc test compared to MOG untreated mice.



eFigure 7. qPCR analysis of optic nerve of EAEON mice after siponimod treatment.

Therapy was started at day 14 after immunization and tissue was analyzed at (A) 21 dpi and (B) 35 dpi. qPCR analysis of optic nerve tissue of the microglia related genes Ym1 (C), Arg-1 (D) and TNF-α (E) at 21, 35 and 90 dpi. Graph represent the pooled mean ± SEM. (n=6 out of two separate experiments), with *p<0.05, **p<0.01, ***p<0.001 by area under the curve for time course by ANOVA with Dunnett's post hoc test compared to MOG EAE. §Few data points out of axis limits. BCAS1: Breast carcinoma-amplified sequence 1; CD206: Cluster of differentiation 206; CNPase: 2',3'-Cyclic-nucleotide 3'-phosphodiesterase; Galc: Galactocerebroside C; Iba1: Ionized calcium-binding adapter molecule 1; MBP: Myelin basic protein; MD-1: Lymphocyte antigen 86; MOG: Myelin oligodendrocyte glycoprotein; NG2: Neuron-glial antigen 2, PDGFR-α: platelet-derived growth factor receptor A; TMEM 119: Transmembrane Protein.



eFigure 8. qPCR analysis of BV2 cells after siponimod treatment.

The mRNA levels of Ym1, Arg-1 and TNF-α after siponimod treatment were analyzed. Graphs represent the pooled mean ± SEM. (n=3), with *p<0.05, **p<0.01, ***p<0.001 by two-way ANOVA with Bonferroni post hoc test compared to the DMSO control**.**

eTable 2. Top five downregulated genes of optic nerve linked to immune response from EAEON mice after siponimod therapy.

Feature ID	Description	MGI ID	Fold change		P-value[A]	
Siponimod treatment dose			**2 mg/kg**	**6 mg/kg**	**2 mg/kg**	**6 mg/kg**
Krt1	negative regulation of inflammatory response	96698	-1,532.92	-767.67	3.99E-03	8.81E-03
Muc4	negative regulation of T cell mediated cytotoxicity directed against tumor cell target	2153525	-378.65	-302.62	4.01E-03	8.81E-03
Serpinb3a	protection from natural killer cell mediated cytotoxicity	3573933	-7,252.07	-10,372.70	9.69E-03	9.18E-03
Elf3	inflammatory response	1101781	-51.48	-63.88	2.78E-02	2.57E-02
Pglyrp1	negative regulation of interferon-gamma production	1345092	-8.84	3.75E-02	-6.09	n. s.

[A] P-values (FDR corrected) compared to untreated MOG EAE.