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Supplementary Information for

Siponimod ameliorates metabolic oligodendrocyte injury via the sphingosine-1 phosphate receptor 5

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Supplementary Material and Methods

Experimental groups.

Control-group (***Ctrl; group a***); animals were provided a diet of standard rodent chow for the entire duration of the study.

Cuprizone combined with experimental autoimmune encephalomyelitits (EAE)/Vehicle-group (***Cup/EAE-Veh; group b***); animals were intoxicated with cuprizone (0.25%) for three weeks and treated with vehicle solution (1% carboxymethy cellulose sodium; 200 µl) daily *via* oral gavage during the cuprizone intoxication period. At the beginning of week six, the mice were immunized with $MOG_{35–55}$ peptide and pertussis toxin (PTX) as published previously (1, 2).

Cuprizone combined with EAE/Siponimod-group (***Cup/EAE-Sipo; group c***); animals were intoxicated with cuprizone for three weeks and treated with Siponimod solution daily *via* oral gavage during the cuprizone intoxication period. At the beginning of week six, the mice were immunized with $MOG_{35–55}$ peptide and pertussis-toxin (PTX).

3 weeks Cuprizone/Vehicle-group + 2 weeks normal chow (***Cup3wks-Veh + 2wks; group d***); animals were intoxicated with cuprizone for three weeks and treated with Siponimod solution daily *via* oral gavage during the cuprizone intoxication period. After week 3, the animals were provided normal chow for another 2 weeks to allow autonomous lesion progression as published previously (3).

3 weeks Cuprizone/Siponimod-group + 2 weeks normal chow (***Cup3wks-Sipo + 2wks; group e***); animals were intoxicated with cuprizone for three weeks and treated with Siponimod solution daily *via* oral gavage during the cuprizone intoxication period. After week 3, the animals were provided normal chow for another 2 weeks to allow autonomous lesion progression.

1 week Cuprizone/Vehicle-group (***Cup1wk-Veh; group f***); animals were intoxicated with cuprizone for one week and treated with vehicle solution daily via oral gavage during the cuprizone intoxication period.

1 week Cuprizone/Siponimod-group (***Cup1wk-Sipo; group g***); animals were intoxicated with cuprizone for one week and treated with Siponimod solution daily via oral gavage during the cuprizone intoxication period.

3 weeks Cuprizone/Vehicle-group (***Cup3wks-Veh; group h***); animals were intoxicated with cuprizone for three weeks and treated with vehicle solution daily via oral gavage during the cuprizone intoxication period.

3 week Cuprizone/Siponimod-group (***Cup3wks-Sipo; group i***); animals were intoxicated with cuprizone for three weeks and treated with Siponimod solution daily *via* oral gavage during the cuprizone intoxication period.

Cuprizone intoxication and EAE induction

To induce demyelination within the forebrain, mice were intoxicated with the copper chelator cuprizone (bis(cyclohexanone) oxaldihydrazone; Sigma-Aldrich, US) as previously published by our group (4). In brief, 0.25 g cuprizone was weighed using precision scales and mechanically mixed with 100 g ground standard rodent chow (0.25%) using a commercially available kitchen machine (Kult X, WMF Group, Geislingen an der Steige, Germany). The chow was mixed at low speed and manual agitation of the entire machine for 1 min and was provided within the cage in two separate plastic Petri dishes. The cuprizone-ground chow mix was prepared freshly every day. Active EAE induction was realized by immunization with an emulsion of Myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) dissolved in complete Freund's adjuvant (CFA), followed by intraperitoneal injections of PTX in phosphate- buffered saline (PBS), first on the day of immunization and then again the following day. For the immunization, a ready-to-use kit provided by Hooke Laboratories was used (US, order numb EK-2110). Scoring of disease severity was performed daily as follows: A score of 0.5 was assigned if the tip of the tail was limb when the mouse was picked up by the base of the tail; a score of 1 was assigned if the entire tail dropped over the finger of the observer when the mouse was picked up by the base of the tail; a score of 1.5 was assigned when the mouse was dropped on a wire rack, and at least one hind leg fell through consistently; a score of 2 was assigned when the legs of the mice were not spread apart but held close together when the mouse was picked up by the base of the tail, or when the mice exhibited a clearly apparent wobbly gait; a score of 2.5 was assigned when the tail was limp and both hind legs had movement, but were dragging at the feet, or one hind leg showed complete

paralysis, whereas the other one still showed movement; a score of 3 was assigned when the tail was limp and the mice showed complete paralysis of hind legs; a score of 3.5 was given if the mouse was unable to raise itself when placed on its side; a score of 4 was assigned if the tail was limp and the mice showed complete hind leg and partial front leg paralysis, and the mouse was minimally moving around the cage but appeared alert; a score of 4.5 was given if the mouse showed no movement around the cage and appeared not alert.

Histology and immunohistochemistry

For the histological studies, the sections were deparaffinized, rehydrated, and if necessary, antigens were unmasked by cooking in either Tris/EDTA (pH 9.0) or Citrate (pH 6.0) buffer. After washing in PBS, unspecific binding sites were blocked by incubating the slides in blocking solution (serum of the species in which the secondary antibody was produced) for 1 h. Then, the sections were incubated overnight (at 4 °C) with the primary antibodies diluted in blocking solution. The next day, the slides were incubated in a 0.3% hydrogen peroxide PBS-solution for 30 min. After repetitive washing in PBS, the slides were incubated with biotinylated secondary antibodies for 1 h and then with peroxidase-coupled avidin-biotin complex (ABC kit; Vector Laboratories, Peterborough, UK). Finally, the sections were treated with 3,3′-diaminobenzidine (DAKO, Hamburg, Germany) as a peroxidase substrate. Appropriate negative controls (omission of primary antibodies or incubation with isotype control antibodies) were performed in parallel to ensure specificity of the staining.

Histological evaluations

All histological analyses were performed with coronal sections using a Leica DM6 B microscope equipped with the Leica DMC 6200 camera.

To evaluate demyelination in LFB/PAS stains, a nonparametric grading approach was performed by two independent and blinded evaluators. In region 265, the midline of the CC was defined as the ROI and the slides were scored on a scale from 1 (complete demyelination) to 4 (fully myelinated).

In H&E stained sections, the number and location of perivascular infiltrates within the entire forebrain were analyzed by two blinded observers and the results were averaged.

To analyze cell/spheroid densities in immunohistochemically processed sections, the area of the midline of the CC was manually outlined using ImageJ (NIH, Bethesda, MD, US), and positive cells/spheroids were counted by at least two evaluators blinded to the treatment groups. The results were averaged and are shown as cells or spheroids/mm^2.

Since defining individual cells in the fully demyelinated corpus callosum was sometimes challenging, in some cases staining intensities were quantified by densitometrical analyses. Therefore, binary-converted pictures were evaluated within the midline of the CC using ImageJ. A value of 100% represents a maximum and 0% a minimum of staining intensity. Results are shown as staining intensity in [%] Area of the entire ROI.

The extent of inflammatory demyelination in spinal cord sections was quantified by measuring an inflammatory-demyelination index, which is defined as the area covered by inflammatory demyelination in relation to the entire white matter area of each slide. This index was measured in two slides per animal by two different blinded observers, and the values were averaged.

PET/CT

Anesthesia was initially administered with 5% isoflurane (AbbVie, US) vaporized in oxygen gas and maintained during the scanning procedure with 1.5–3%. Body temperature was held constant at 38 °C via a heating pad and respiration rate of the animals was monitored throughout the PET/CT measurement procedure. Each mouse was placed in head-prone position centered in the field of view of a commercially available preclinical PET/CT scanner (Inveon®, Siemens Healthcare,

Knoxville, US). [^{18}F]-GE180 TSPO-PET (10.6 ± 2.1 MBq; injected via the tail vein) with an emission window of 60–90 min post injection (p.i.) was used to measure cerebral glial activity. All analyses were performed using the software package PMOD (V3.5, PMOD technologies, Switzerland). To delineate the target regions, an implemented MRI-based mouse brain atlas from Mirrione and colleagues (5) was supplemented with predefined VOI (volume of interest) of the target regions (the medial corpus callosum [2.2 mm^3] and the lateral corpus callosum [2.9 mm^3]). Animal-specific CT datasets were co-registered with the Mirrione Atlas MRI dataset, the corresponding transformation matrix was saved, and in a final step, PET data were transformed using the saved transformation matrix. The TSPO-PET values (given in kBq/cc), extracted from both target VOI, were normalized by the previously validated myocardium correction method (6).

Mixed glia cell cultures

Primary astrocyte cultures were prepared from the brains of P1–P3 C57BL/6 mice as previously described with some modifications (7). Briefly, the cerebral cortex including the white matter tract corpus callosum was dissected, the meninges carefully removed and dispersed in Dulbecco's phosphate-buffered saline containing 1% trypsin and 0.02% EDTA, filtered through a 50-µm nylon mesh, and centrifuged at 400×g for 5 min. Cells were resuspended in Dulbecco's modified Eagle's medium (Invitrogen, Germany) supplemented with 1% penicillin/streptomycin (Pen/Strep; Gibco Life Technologies, Germany), and 10% fetal bovine serum (FBS; Gibco Life Technologies, Germany). Finally, cells were seeded in 75 cm^2 plastic cell culture flasks coated with poly-L-lysine (PLL, MW 70,000–150,000; Sigma-Aldrich, US). Mixed glia cell cultures were grown at 37°C in a humidified incubator with 5% CO_2. The medium was replaced every third day. Upon reaching confluency, the cells were trypsinized (Trypsin/EDTA 0.05%, Gibco Life Technologies, Germany) and replated at a lower density. This procedure was repeated twice. Mixed glia cell cultures were used for experiments after the third replating and before reaching confluency. Rigorous shaking was avoided to maintain the microglia cells within the mixed glia cell culture. Before starting treatments, cultures were kept under serum-free conditions for individual times which are given at the respective material and method sections.

Flow cytometry analysis

To analyze the relative proportion of astrocytes and microglia in our mixed glia cell cultures, we performed flow cytometry analyses. To this end, confluent cultures were detached from the flasks and after centrifugation, the cell pellet was resuspended in 1.5 mL Dulbecco's Modified Eagle's Limiting Medium (DMEM; Thermo Scientific, US). Cells from up to four samples from different 75cm^2-flasks were pooled to reach sufficient cell numbers. The resulting suspension was pelleted by centrifugation and then cells were stained with the live/dead marker Zombie Green (BioLegend, US, order numb 423111) for 30 min. Afterwards, cells were pelleted, washed once in 1000µL fluorescence-activated cell sorting (FACS) buffer (MiltenyiBiotec, Germany) and resuspended in 100µL FACS buffer. Samples were incubated with 10 µL fetal calf serum (FCS; Hyclone, US) and 2 µL True Stain Fc Block (BioLegend, US) for 15 min. Samples were then stained with anti-ACSA2-PE and anti-CD11b-APC for 20 min. All samples were pelleted, washed with 1 mL FACS buffer, resuspended in 250 µL FACS buffer and analyzed at the same flow rate until 10000 events had been registered to allow comparisons among all samples using a CytoFLEX flow cytometer (Beckman Coulter, US). All centrifugation-steps were performed at 400 × g and 4 °C for 5 min. Data were analyzed using a FACS-CytExpert software (Version: 2.1.0.92, Beckman Coulter, US). Details about the used antibodies are given in SI table 1.

Immunocytochemistry (ICC)

Immunofluorescence stains of the cultured cells were performed to analyze S1PR1 and S1PR5 expression. To this end, cells were seeded on coverslips in a 24-well-plate, washed, fixed in a 3.7% paraformaldehyde/PBS solution for 10 min at 4°C, permeabilized with a 0.1% TritonX-100 solution for 3 min at 4°C and then incubated with blocking solution containing 10% normal donkey serum for 90 min, washed in PBS and incubated with either anti-S1PR1 or anti-S1PR5 antibodies and anti-GFAP antibodies overnight at 4°C. After washing in PBS the next day, cells were incubated

with donkey anti-rabbit IgG Alexa 488 and donkey anti-goat IgG Alexa 594 antibodies for 90 min at room temperature. The applied concentrations and suppliers of the antibodies are given in SI table 1**.** Coverslips were mounted using a DAPI-containing medium to visualize the cell nuclei (Sigma-Aldrich, US, order numb F6057). Stained and processed cells were documented using an epifluorescence microscope (Leica DM6 B) and the software Leica Application Suite X (version 3.7.0.20979, 2019, Germany). Acquisition of confocal images was performed on a Zeiss LSM900 using Zen-Blue (version 3.1, Carl Zeiss Microscopy GmbH, Germany). Image processing was performed using NIH Image J software.

Protein analyses

For western blotting experiments, the cells were lysed in ice-cold RIPA-Buffer containing protease-inhibitor cocktail tablets (Complete, EDTA-free; Roche, Switzerland), beta-glycerolphosphat (20mM) and sodium-orthovanadate (4mM). After incubation on ice for 5 min, sonication for 15 min and centrifugation at 14.000 × g at 4°C for 15 min, the supernatant was recovered. The protein concentration compared to an internal BSA-Standard was determined by BCA-Assay (Thermo Scientific, US) for each sample. 7.5µg of each sample were denatured in Laemmli buffer followed by separation in a 4-15% SDS-Page (Bio-Rad, US) and transfer to a polyvinylidene difluoride (PVDF)-membrane (Bio-Rad, US). Tris-buffered saline with 0.1% Tween-20 (TBS-T) was used as washing-buffer. Membranes were blocked in a 5%-powdered milk-TBS solution and then incubated with primary antibodies over-night at 4°C. After rinsing four times in TBS-T, the membranes were incubated with HRP-conjugated secondary antibodies diluted in a 5%-powdered milk-TBS solution for 1h at 4°C. Membranes were rinsed another four times in TBS-T followed by detection with ECL-chemoluminescence solution (Abcam, UK) and visualization on a Proxima-2850 (Isogene Lifescience, the Netherlands) using the AQ-4-Software (version 1.29, Isogen Lifescience, the Netherlands). Details of antibodies used for western blotting are given in SI table 1.

Realtime reverse transcription-PCR

The primer sequences and individual annealing temperatures are given in SI table 1. Relative quantifications of gene-expression were performed for each sample using an internal standard-curve generated by pooling cDNA from all samples. 18S expression levels were used as internal reference. Gel electrophoresis and melting curves of the PCR products were routinely performed to determine the specificity of the PCR reactions. To exclude contamination of the reagents with either RNA or DNA, appropriate negative controls were performed (i.e.; omission of RNA or cDNA; melting curve analyzes, gel electrophoresis of the PCR products).

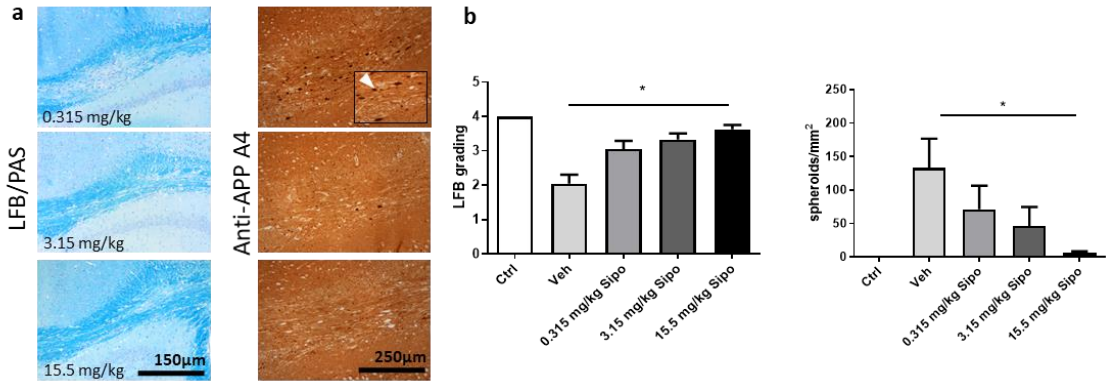

Fig. S1. Siponimod ameliorates cuprizone-induced pathologies in a dose dependent manner

(a) LFB/PAS and anti-APP A4 stains of the medial CC from cuprizone-intoxicated mice treated with different Siponimod doses (0.315 mg/kg, 3.125 mg/kg, 15.5 mg/kg; n=5 per experimental group). Arrowhead indicates an APP^+ spheroid. (b) Evaluation of the extent of demyelination (LFB/PAS) and acute axonal injury (anti-APP A4). Differences were determined using ANOVA followed by Kruskal-Wallis multiple comparisons test (vehicle versus all other groups). Data are shown as Mean ± SEM.

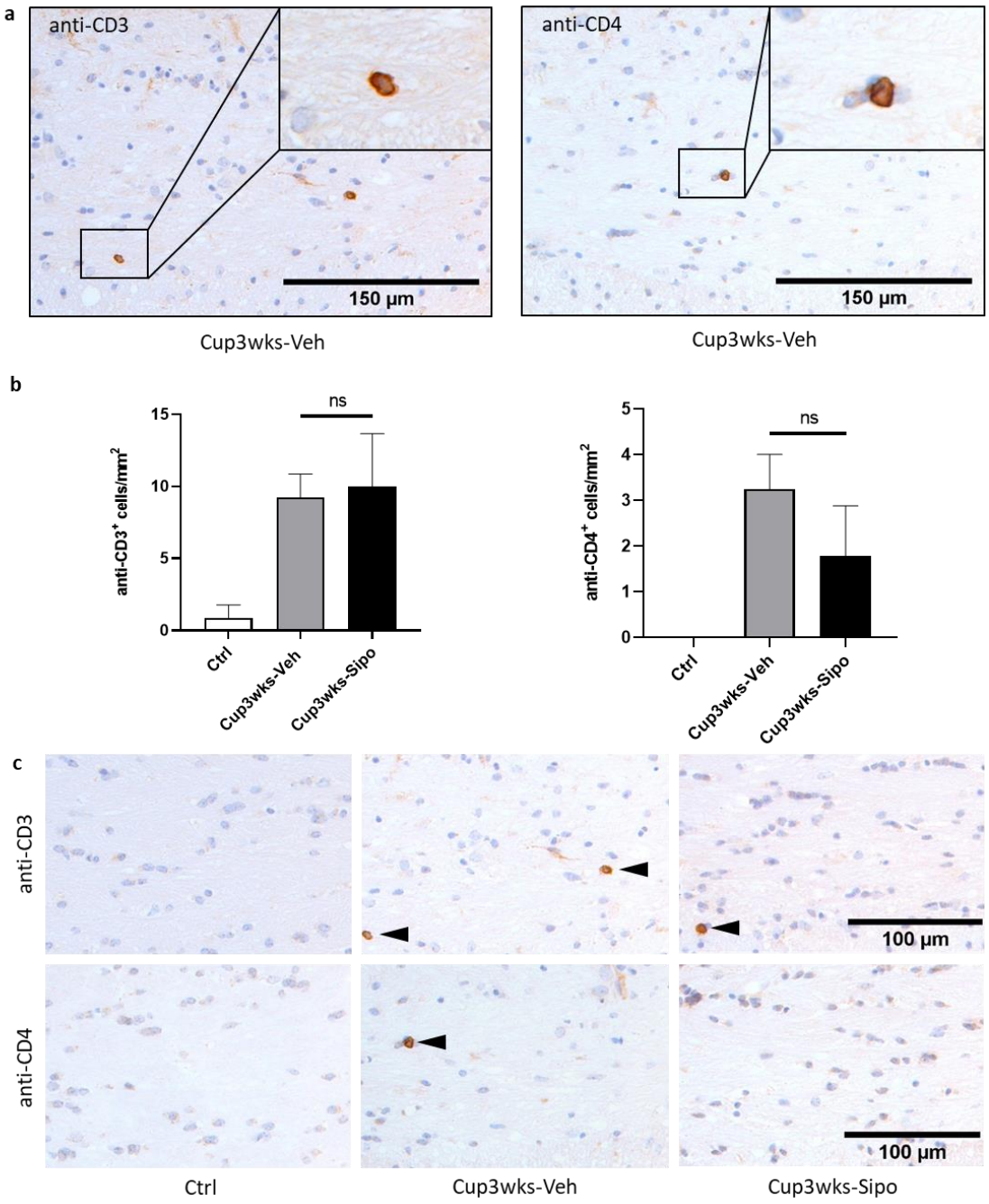

Fig. S2. Siponimod does not reduce T-lymphocyte recruitment in the cuprizone model

(a) Representative anti-CD3 and anti-CD4 stains in Cup3wks-Veh mice. (b) Evaluation of anti-CD3$^+$ and anti-CD4$^+$ cell numbers. Differences were determined using Mann-Whitney tests as indicated. (c) Representative anti-CD3 and anti-CD4 stains of the medial CC from control (n=4), Cup3wks-Veh (n=4) and Cup3wks-Sipo (n=4) mice. Data are shown as Mean ± SEM.

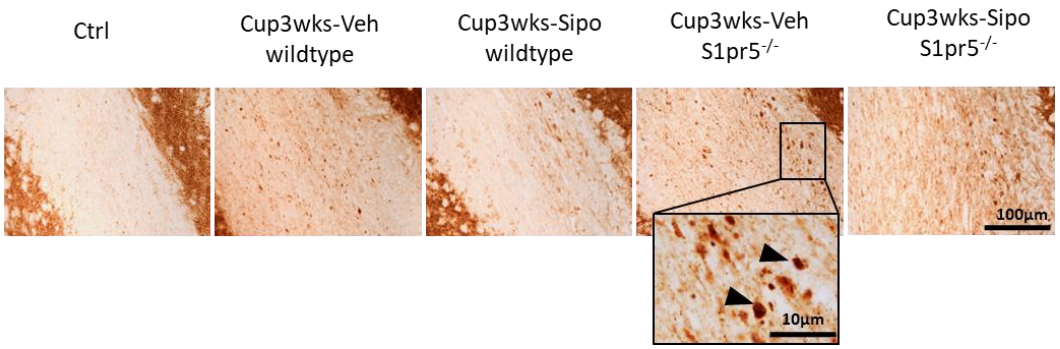

Fig. S3. Siponimod ameliorates acute-axonal injury in the cuprizone model

Representative anti- vesicular glutamate transporter 1 (vGlut1) stains of the medial CC from control (n=4), Cup3wks-Veh-wildtype (n=4), Cup3wks-Sipo-wildtype (n=4), Cup3wks-Veh-S1pr5-/- (n=5) and Cup3wks-Sipo-S1pr5-/- (n=5) mice.

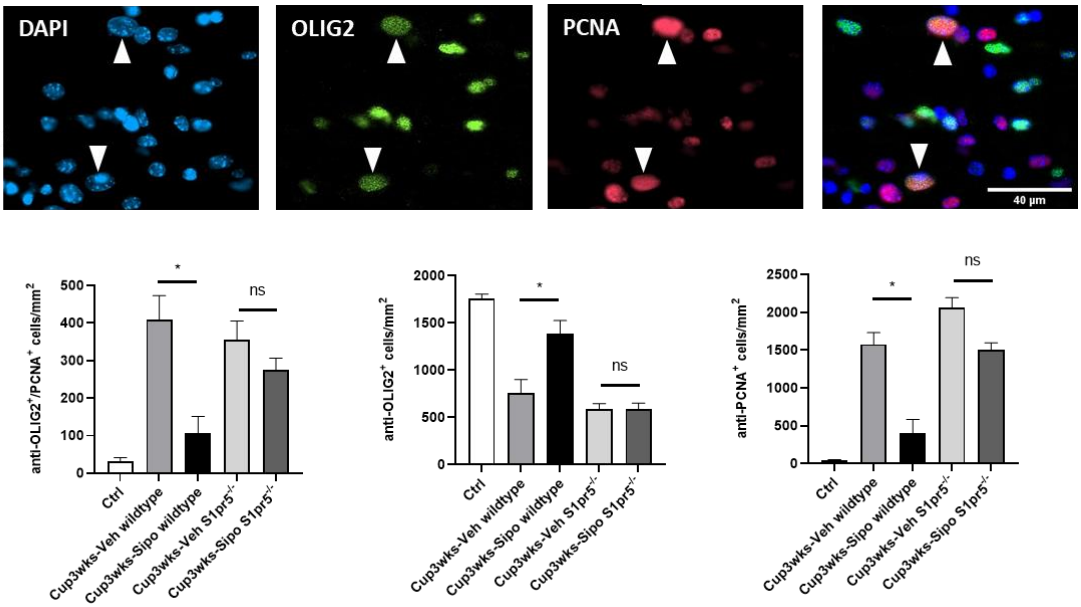

Fig. S4. Siponimod ameliorates the cuprizone-induced proliferation of oligodendrocyte progenitor cells in a S1PR5-dependant manner

(a) Representative anti-OLIG2/PCNA stains of the medial CC from control (n=4), Cup3wks-Veh-wildtype (n=4), Cup3wks-Sipo-wildtype (n=4), Cup3wks-Veh-S1pr5$^{-/-}$ (n=5) and Cup3wks-Sipo-S1pr5$^{-/-}$ (n=5) mice. Arrowheads highlight double-positive cells. (b) Evaluation of proliferating oligodendrocyte progenitor cells (anti-OLIG2^{+}/anti-PCNA^{+}), oligodendrocytes (anti-OLIG2^{+}) and proliferating cells (anti-PCNA^{+}). Differences were determined using Mann-Whitney tests as indicated. Data are shown as Mean ± SEM.

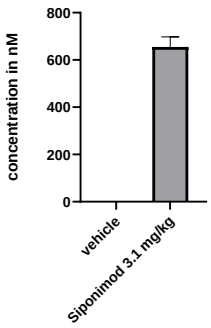

Fig. S5. Siponimod levels in isolated brain tissues

Quantification of Siponimod levels in isolates brain tissues from vehicle- (n=8) and Siponimod-treated mice (3.1mg/kg; n=6). Measurements were performed ~12 h post-last treatment.

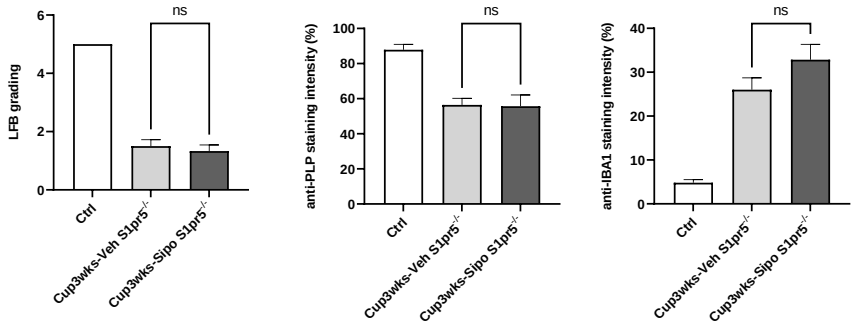

Fig. S6. Protective Siponimod effects are absent in S1pr5$^{-/-}$ mice

Quantification of LFB/PAS, anti-PLP and anti-IBA1 stains of the medial CC from control (n=4), Cup3wks-Veh-S1pr5$^{-/-}$ (n=6) and Cup3wks-Sipo-S1pr5$^{-/-}$ (n=6) mice. Statistical analyzes were performed by Mann-Whitney tests as indicated. Data are shown as Mean ± SEM.

SI Table 1: List of primary antibodies, secondary antibodies and primers used in this study

List of Primary Antibodies used in this study

Antigen	Host\Clone	Dilution	Purchase number	RRID	Supplier	Antigen retrieval method
PLP	Mouse monoclonal	1:5000	MCA839G	AB_2237198	Bio-rad , US	-
IBA1	Rabbit polyclonal	1:5000	019-19741	AB_839504	WAKO, US	Tris/EDTA
APP A4	Mouse monoclonal	1:5000	MAB348	AB_94882	Millipore, US	Tris/EDTA
GFAP	Mouse monoclonal	1:500	G-A-5	AB_2314539	Sigma-Aldrich, US	Tris/EDTA
vGLUT1	Mouse monoclonal	1:2000	ab134283		Abcam, UK	Tris/EDTA
OLIG2	Rabbit polyclonal	1:4000 1:1000 *(Fluorescence)*	AB9610	AB_570666	Millipore, US	Tris/EDTA
ATF3	Rabbit monoclonal	1:400	ab254268	AB_2910214	Abcam, UK	Tris/EDTA
APC /CC1	Mouse monoclonal	1:100 *(Fluorescence)*	OP80	AB_2057371	Millipore, US	Tris/EDTA
CD3	Rat monoclonal	1:250	ab11089	AB_2889189	Abcam, UK	Tris/EDTA
CD4	Rabbit monoclonal	1:750	ab183685	AB_2686917	Abcam, UK	Tris/EDTA
S1PR1	Rabbit polyclonal	1:100 ICC	ab77076	AB_1523525	Abcam, UK	-
S1PR5	Rabbit polyclonal	1:100 ICC	ab140932		Abcam, UK	-
GFAP	Goat polyclonal	1:200 ICC	SAB-2500462	AB_10603437	Sigma-Aldrich, US	-
Total-Akt	Rabbit polyclonal	1:1000 *Western Blot*	4691S	AB_915783	CellSignaling, US	-
P-Akt (Ser473)	Rabbit polyclonal	1:1000 *Western Blot*	9271S	AB_329825	CellSignaling, US	-
ACSA-2	Rat monoclonal PE-coupled	FACS	130-123-284	AB_2811488	Miltenyi Biotec, Germany	-
CD-11b	Rat monoclonal APC-coupled	FACS	ab25482	AB_470574	Abcam, UK	-

List of secondary antibodies used in this study

Antigen	Host\Clone	Dilution	Purchase number	RRID	Supplier
Goat anti-rabbit IgG	Goat Polyclonal	1:200	BA-1000	AB_2313606	Vector Laboratories, Burlingame, US
Goat anti-mouse IgG	Goat Polyclonal	1:200	BA-9200	AB_2336171	Vector Laboratories, Burlingame, US
Donkey anti-rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Donkey Polyclonal	1:200	A-21202	AB_141607	Thermo Fisher Scientific, US
Donkey anti-mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	Donkey Polyclonal	1:200	A21203	AB_141633	Thermo Fisher Scientific, US
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 488	Donkey	1:250	A21207		Thermo Fisher Scientific, US
Donkey anti-Goat IgG (H+L) Alexa Fluor 594	Donkey Polyclonal	1:250	Ab150132		Abcam, UK
Goat anti-rabbit IgG HRP-conjugated	Goat polyclonal	1:2000 (WB)	SA00001-2	AB_2722564	Proteintech, Germany

List of primers used in this study

Target-gen	Sequence 5´-> 3´	Expected product size	Annealing -temp.
18S	*Sense:* CGGCTACCACATCCAAGGAA *Anti-sense:* GCTGGAATTACCGCGGCT	187bp	64°C
S1pr1	*Sense:* ATGGTGTCCACTAGCATCCC *Anti-sense: CGATGTTCAACTTGCCTGTGTAG*	112bp	63°C
S1pr5	*Sense:* CCAACAGCTTGCAGCGATCCCC *Anti-sense:* GGTTGCTACTCCAGGACTGCCG	167bp	68°C
Ccl-2	*Sense:* TAAAAACCTGGATCGGAACCAAA *Anti-sense:* GCATTAGCTTCAGATTTACGGGT	120bp	65°C
Cxcl-10	*Sense:* CCAAGTGCTGCCGTCATTTTC *Anti-sense:* GGCTCGCAGGGATGATTTCAA	157bp	66°C

Dataset S1 (separate file). List of genes differentially expressed in control *versus* cuprizone/vehicle groups. Up-regulated genes are highlighted in red, downregulated genes are highlighted in green.

Dataset S2 (separate file). List of genes differentially expressed in cuprizone/vehicle *versus* cuprizone/Siponimod groups. Genes higher expressed in Siponimod-treated groups are highlighted in green, genes lower expressed in Siponimod-treated groups are highlighted in red.

Dataset S3 (separate file). Cytokine concentrations in the supernatant of mixed glia cell cultures stimulated with either LPS, TNFα or IFNγ and treated with either vehicle-solution or Siponimod.

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