Supplementary Materials for

Barcoded Viral Tracing of Single-Cell Interactions in CNS Inflammation

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Materials and Methods

Mice

Experimental animals were kept in the pathogen-free facility at the Hale Building for Transformative Medicine at Brigham and Women's Hospital and all in vivo experiments were carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines. Adult C57BL/6J (#000664) and NOD/ShiLtJ (NOD mice) (#001976) were obtained from the Jackson Laboratory. Postnatal pups were bred in our facility and used between P0-P3 to establish astrocyte and microglia cultures. B6.Cg-Tg(Gfap-cre)73.12Mvs/J hemizygous mice (The Jackson Laboratory, #012886) were crossed to homozygous B6;129P2-Gt(ROSA)26Sortm1(CAG-RABVgp4,-TVA)Arenk/J mice (The Jackson Laboratory, #024708). Tg(CAG-Kaede)15Kgwa mice (*114*) on a C57Bl/6J background were obtained from RIKEN BRC. Male mice were used in experiments at >8 weeks of age. All procedures were reviewed and approved by the Brigham and Women's Hospital IACUC.

Molecular biology

First, a cDNA encoding the mCherry ORF was inserted into the vector, pSADΔG-GFP-F2 (Addgene, #32635), a gift from Edward Callaway (*118*). PCR with mCherry_FWD and mCherry_REV primers (Table S1) on a template plasmid encoding mCherry (Addgene, #80139), a gift from Ellen Rothenberg (*119*) was performed, followed by SbfI and SacII restriction and ligation, which inserted a unique NheI site. A 156 bp dsDNA fragment (Genewiz) was cloned into the NheI/SacII site via XbaI/SacII, which introduced unique NheI/AscI sites flanking a unique PacI site to create pSADΔG-mCherry. Enzymes used in this study were: XbaI (NEB, #R0145S),

SacII (NEB, #R0157S), NheI-HF (NEB, #R3131M), AscI (NEB, #R0558S), SbfI-HF (NEB, #R3642L), DpnI (NEB, #R0176L), and PacI (NEB, #R0547L).

Rabies library barcode generation

Gibson assembly was used to insert barcodes downstream of the mCherry translational stop codon and before the pseudorabies virus polyA transcriptional stop signal (*120*). The barcode VHDBVHDB**AT**VHDBVHDB**AT**VHDBVHDB was synthesized from IDT as a single stranded oligonucleotide with flanking handles to allow for barcode recovery (Table S1). The plasmid pSADΔG-mCherry was linearized with NheI-HF (NEB, #R3131S) and AscI (NEB, #R0558S) for 16 hours at 37C, mixed at a molar ratio of 5:1 barcode:plasmid, and assembled with NEBuilder HiFi DNA Assembly Master Mix (NEB, #E2621L) according to the manufacturer's instructions. Fifty reactions of 20 µL were pooled after assembly, purified using 2.0X Ampure XP magnetic beads (Beckman-Coulter, #A63881), and resuspended so as to concentrate 25-fold. The purified plasmid was electroporated into ElectroMAX Stbl4 Competent Cells (Thermo Fisher, #11635018). Briefly, a 100 µL aliquot of Stbl4 was combined with 4 µL of purified assembled plasmid, divided into 25 µL per cuvette and electroporated (Biorad Gene Pulser II; 1.2 kV, 200 Ohm, 25 µF). A total of 40 electroporations were performed. The cells were recovered at 30°C in Zymo Broth (Zymo Research, #M3015-100) shaken at 225 rpm for 1 hour and plated at 30°C overnight divided across sixteen 625cm^2 agar plates (Thermo Fisher, #240835) with 100 µg/mL ampicillin (Sigma-Aldrich, #A9518-5G). Colonies were scraped into 20 mL LB per plate, shaken at 225 rpm at 30C for 4 hours, and purified using an endotoxin-free plasmid Giga kit (Qiagen, #12391) resulting in 340 µg of plasmid. Test digestions of the assembled plasmid using PacI (NEB, #R0547L) confirmed undetectable levels of linearized plasmid, signifying no carryover of parent plasmid.

Barcode amplification from DNA

Library amplification and sequencing to test for barcode diversity was performed based on similar protocols for genome-wide CRISPR libraries (*121*). PCR reactions were set up using Phusion Flash HF PCR master mix (Thermo Fisher, #F548L) with 0.5 µM each of forward and reverse primers, 3% DMSO, and 4 ng/µl plasmid DNA per reaction. Reactions were thermocycled at 98C for 30s, 18 cycles of [98C for 10s, 62C for 30s, 72C for 60s], followed by 72C for 5 minutes, and 4C forever. A staggered forward primer cocktail, made by combining equimolar concentrations of P5 primers (RAB_P5_s0, RAB_P5_s1, RAB_P5_s2, RAB_P5_s3, RAB_P5_s4, RAB_P5_s6, RAB_P5_s7, and RAB_P5_s8) was used with the RAB_P7_C01 primer to amplify the plasmid (Table S1) .

Rabies virus production

All plasmids used for rabies virus production were prepared using an endotoxin-free plasmid Giga kit (Qiagen, #12391). Pseudotyped G-deficient rabies virus was produced largely as previously described (*115*). Briefly, one day prior to transfection, baby hamster kidney (BHK) cells expressing T7 RNA polymerase, rabies glycoprotein G, and GFP (hereafter: B7GG cells) were seeded into ten 10-cm plates (Thermo Fisher Scientific, #08-772E) at a density of $2.2x10^6$ cells/plate. Cells were grown in DMEM with high glucose, L-glutamine, and sodium pyruvate (Life Technologies, #11995073) supplemented with 10% FBS (Life Technologies, #10438026) (hereafter: B7GG media). The next day, they were transfected with the following helper plasmids: 150 µg pcDNA-SADB19N (Addgene, #32630), 75 µg pcDNA-SADB19P (Addgene, #32631), 75 µg pcDNA-SADB19L (Addgene, #32632), 50 µg pcDNA-SADB19G (Addgene, #32633), all gifts

from Edward Callaway (*118*), and 300 µg RabΔG-mCherry-BC using Lipofectamine 2000 (Thermo Fisher Scientific, #11668019) according to the standard protocol. Cells were transfected at 37C and 5% CO2 for 18 hours. The next day, cells were split into 30 15-cm plates (Fisher Scientific, #08-772-6) and grown at 35C and 3% CO2. The following day, the supernatant was aspirated, and 24 mL fresh media was added to each plate. Thereafter, every 2 days, 10 mL of B7GG media was added to each plate. Then 2 days later, all viral supernatant was harvested, vacuum filtered through 0.45 µm pores (Fisher Scientific, #SCHVU11RE) and frozen. Altogether, 6 batches of viral supernatant were collected. For viral pseudotyping, 10 15-cm plates of BHK cells expressing the rabies virus envelope protein, EnvA, (hereafter BHK-EnvA) were seeded at 60% confluency at 35C and 3% CO2. The next day, unpseudotyped virus was applied to the BHK-EnvA cells for 48 hours. Each dish was washed 10X with 1X PBS to remove unpseudotyped virus. The next day, viral supernatant was aspirated and 24 mL of fresh B7GG media was added. Thereafter, viral supernatant was collected every 2 days. Altogether, 5 pseudotyped viral preparations were collected. To concentrate pseudotyped and unpseudotyped RabΔG-mCherry-BC viruses, 35 mL of the collected supernatant was ultracentrifuged using a Beckman SW28 rotor at 70,000g for 2 hours at 4C. Afterwards, the pellets were resuspended in HBSS, gently vortexed briefly, and were added to 2.5mL of 20% sucrose in HBSS. Virus was centrifuged at 50,000g for 2 hours at 4C using am SW55 rotor. Each viral pellet was resuspended in 100 µL ice cold HBSS, briefly vortexed, and chilled at 4C for >1 hour. Viral titration and pseudotyping specificity were performed using HEK293-TVA cells and HEK293 cells as described (*115*). The B7GG, BHK-EnvA, and HEK293-TVA cell lines were obtained from the GT3 Core Facility of the Salk Institute with funding from NIH-NCI CCSG: P30 014195, an NINDS R24 Core Grant and funding from NEI.

Rabies virus diversity analysis

To analyze rabies virus diversity, five 90% confluent 15-cm plates of HEK293T cells were transduced with rabies virus supernatant in vitro for 24 hours. 7 days post-transduction, RNA was harvested from cells and processed using an RNeasy Maxi kit (Qiagen, #75162). 5 µg RNA was reverse transcribed using 0.2 µL 100 µM Oligo$(dT)_{20}$ primer, 1 µL 10 mM dNTP (Thermo Fisher Scientific, #R0191), 4 µL RT buffer, 0.5 µL RNase inhibitor (Lucigen), 1 µL Maxima H Minus (Thermo Scientific, #EP0752) and filled to 20 µL with nuclease free H2O. RT was performed at 50C for 30 minutes, followed by 85C for 5 minutes, then 4C forever. cDNA was purified using Ampure RNAclean beads at a ratio of 2.0X. PCR primers designed for pSADΔG-mCherry-BC plasmid amplification were then used for barcode amplification and addition of Illumina adapters.

EAE

EAE was induced as previously described (*3, 9, 13*). Specifically, EAE was induced in C57BL/6 or NOD mice with MOG_{35-55} (Genemed Synthesis Inc., #110582) mixed with freshly prepared Complete Freund's Adjuvant (using 20 mL Incomplete Freund's Adjuvant (BD Biosciences, #BD263910) mixed with 100 mg M. Tuberculosis H-37Ra (BD Biosciences, #231141)) at a ratio of 1:1 (v/v at a concentration of 5 mg/mL). Mice received 2 subcutaneous injections of 100 µL each of the MOG/CFA mix. Mice then received a single intraperitoneal injection of pertussis toxin (List Biological Laboratories, #180) at a concentration of 2 ng/µL in 200 µL of PBS. Mice received a second injection of pertussis toxin at the same concentration two days after the initial EAE induction. Mice were monitored and scored daily thereafter. EAE clinical

scores were defined as follows: 0 – no signs, 1 – fully limp tail, 2 – hindlimb weakness, 3 – hindlimb paralysis, 4 – forelimb paralysis, 5 – moribund, as described previously (*3, 5, 6, 12, 13*).

A38 was administered at a dose of 20 mg/kg by i.p. injection twice per day starting at day 16 C57BL/6 mice (disease peak) or 30 days post EAE-induction for NOD mice (start of progressive phase). A38 has desirable physico-chemical properties (molecular weight=355, cLogP=2.4, PSA=57) for a CNS drug, and it was profiled in a number of drug-like property assays. A38 displayed excellent mouse liver microsome stability ($T_{1/2}$ > 5 h, Clint = 4 uL/min/mg protein), and its oxalate salt had very high aqueous solubility (>10 mg/mL at pH 7.4). A preliminary in vivo pharmacokinetic evaluation, using the mono-oxalate salt of A38, was conducted. Following i.p. administration (3 mg/kg in 100% saline solution) to male Sprague-Dawley rats, both plasma and brain concentrations of A38 were determined at 0.25, 0.5, 1, 2, and 8h. In the plasma, the compound reached promising levels (C_{max}=221 ng/mL) with a plasma half-life of $T_{1/2}$=2.25h. Brain levels of A38 were higher (C_{max}=331 ng/mL), and a brain to plasma ratio of 1.5:1 indicated little resistance to brain penetration and that A38 was not a Pgp substrate.

RabΔG viral transduction

Intracranial delivery of RabΔG was performed largely as described previously (*3, 13*). Briefly, mice were anesthetized using 1-3% isoflurane mixed with oxygen. Heads were shaved and cleaned using 70% ethanol and Betadine (Thermo Fisher, #19-027132) followed by a medial incision of the skin to expose the skull. The forebrain was targeted unilaterally using the coordinates: +1.25 (lateral), +1.0 (rostral), -3.0 (ventral) relative to Bregma. Mice were injected with a RabΔG viral dilution in 1 µL capable of seeding 1,000 cells using a 5 µL Hamilton syringe

on a Stereotaxic Alignment System (Kopf, #1900), sutured, and permitted to recover in a separate clean cage. Mice were sacrificed 7.5 days post-injection for RABID-seq experiments.

Isolation of cells from mouse CNS

Cells were isolated by flow cytometry as described (*3, 5, 6, 13*) with modifications. Briefly, mice were perfused with 1X PBS and the transduced CNS region (approx. 27 mm^3) was isolated into 10 mL of enzyme digestion solution consisting of 75 µL Papain suspension (Worthington, #LS003126) diluted in enzyme stock solution (ESS) and equilibrated to 37C, an approach previously deployed for the study of astrocytes (*122*) that is similar to several others used to analyze cells by genomic approaches (*3, 6, 9, 10, 12, 13, 123-126*). Nevertheless, it is important to consider that the use of enzymatic dissociation may alter the transcriptional state of microglia, even in a context of dramatic CNS inflammation such as EAE (*127, 128*). ESS consisted of 10 mL 10X EBSS (Sigma-Aldrich, #E7510), 2.4 mL 30% D(+)-Glucose (Sigma-Aldrich, #G8769), 5.2 mL 1M NaHCO3 (VWR, #AAJ62495-AP), 200 µL 500 mM EDTA (Thermo Fisher Scientific, #15575020), and 168.2 mL ddH2O, filter-sterilized through a 0.22 µm filter. Samples were shaken at 80rpm for 30 minutes at 37C. Enzymatic digestion was stopped by adding 1 mL of 10X hi ovomucoid inhibitor solution and 20 µL 0.4% DNase (Worthington, #LS002007) diluted in 10 mL inhibitor stock solution (ISS). 10X hi ovomucoid inhibitor stock solution contained 300 mg BSA (Sigma-Aldrich, #A8806), 300 mg ovomucoid trypsin inhibitor (Worthington, #LS003086) diluted in 10 mL 1X PBS and filter sterilized using at 0.22 µm filter. ISS contained 50 mL 10X EBSS (Sigma-Aldrich, #E7510), 6 mL 30% D(+)-Glucose (Sigma-Aldrich, #G8769), 13 mL 1M NaHCO3 (VWR, #AAJ62495-AP) diluted in 170.4 mL ddH2O and filter-sterilized through a 0.22 µm filter. Tissue was gently mechanically dissociated using a 5 mL serological pipette and filtered

through at 70 μm cell strainer (Fisher Scientific, #22363548) into a fresh 50 mL conical. Dead cells and myelin were removed from the cell pellet using the Miltenyi MACS isolation kits (Myelin: #130-096-733; Dead cells: #130-090-101). LS columns and the Annexin V binding buffer were used for wash steps and elution. The cell pellet (1/4 to 1/8 mass of whole brain) was resuspended in 150 μL of Myelin removal beads and 150 μL of dead cell removal beads and incubated at room temperature for 15 minutes. Cells were then processed according to the manufacturer's protocol. Eluate was collected and centrifuged at 600g for 5 minutes. Cells were resuspended in of FACS buffer (500 μM EDTA, 1% BSA, 0.9X PBS) on ice until sorting.

Flow cytometry

Cells were filtered through a 35 μm filter prior to sorting (Fisher Scientific, #08-771-23). Cells were sorted on a FACS Aria IIu (BD Biosciences). Gating parameters were established using a WT control during each batch of flow cytometry. For RABID-seq, cells were gated as intact, followed by exclusion of FSC and SSC doublets. Sorting of mCherry+ cells was judged in the PE-Texas Red channel using a yellow-green laser. Cells were sorted at low flow rates (1-2) through a 100 μm nozzle using purity settings. Approximately >90% of all labeled mCherry+ cells were sorted across all mice. For isolation of all other CNS cells, isolated CNS cells were washed with 1X PBS and stained with the following fluorochrome-conjugated antibodies: FITC anti-CD11b (eBioscience, #11-0112-85), APC anti-CD45 (eBioscience, #17-0451-83), BV421 anti-Ly-6C (BioLegend, #128031), PE anti-mouse CD45R/B220 (BD Biosciences, #553089), PE anti-CD140a (eBioscience, #12-1401-81), PE anti-CD105 (eBioscience, #12-1051-82), PE anti-O4 (R&D Systems, #FAB1326P), PE anti-Ly-6G (BioLegend, #127608), and PE anti-mouse TER-119 (BioLegend, #116207). All cells were gated as SSC/FSC singlets and on

$CD105^{lo}CD140a^{lo}CD11b^{lo}Ter119^{lo}O4^{lo}CD19^{lo}$. Microglia were sorted as $CD11b^{+}CD45^{lo}Ly6C1^{lo}$, pro-inflammatory monocytes were sorted as $CD45^{hi}CD11b^{+}Ly6C1^{hi}$, and astrocytes were sorted as $CD11b^{lo}CD45^{lo}Ly6C^{lo}$, as described previously (*3, 6, 9, 12, 13, 126*).

Flow cytometry analysis of transgenic lines

CNS cells in suspension were washed with 0.5% BSA, 2 mM EDTA in 1X PBS and incubated with surface antibodies and a live/dead cell marker on ice. After 30 min, cells were washed with 0.5% BSA, 2mM EDTA in 1X PBS and fixed according to the manufacturer's protocol of an intracellular labelling kit (eBiosciences, 00-5523-00). Surface antibodies used in this study were: BUV661 anti-CD45 (BD Biosciences, #612975, clone: 30-F11, 1:100), BUV737 anti-CD11b (BD Biosciences, #564443, clone: M1/70, 1:100), BV570 anti-Ly6C (Biolegend, #128041, clone: HK1.4, 1:100), BV650 anti-PDGFRA (Biolegend, #740531, clone: APA5, 1:100), and PE-Cy7 anti-CD4 (eBioscience, #25-0041-82, clone: GK1.5, 1:100). Intracellular antibodies were: FITC anti-Rabies Virus Glycoprotein antibody (abcam, # ab193407, 0.025 mg/mL) and BV421 anti-GFAP (Biolegend, #644710, clone: 2E1.E9, 1:200). FACS was performed on a Symphony A5 (BD Biosciences).

Analysis of T cells

For T cell flow cytometry analyses, CNS single-cell suspensions or splenocytes were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, #P1585), 1 μM ionomycin (Sigma-Aldrich, #I9657), GolgiSTOP (BD Biosciences, #554724, 1:1500), and GolgiPLUG (BD Biosciences, #555029, 1:1500) in RPMI 1640 medium (Life Technologies, #11875119) containing 10% FBS (GIBCO, #10438026) and penicillin/streptomycin (GIBCO,

#15140122, 1:100) for 4h at 37C in a 5% CO2 incubator. Following stimulation, T cells were stained with antibodies against surface markers, and thereafter fixed and stained with antibodies against intracellular target proteins using an intracellular antibody labeling kit according to the manufacturer's instructions (eBioscience, #00-5523). Antibodies used were: BV421 anti-CD3 (BioLegend, #100227, 1:100), BV605 anti-CD4 (BioLegend, #100547, 1:50), 405 Aqua LIVE/DEAD cell stain kit (Thermo Fisher Scientific, #L34966, 1:400), FITC anti-IFN-γ (BioLegend, #505806, 1:100), PE anti-IL-17a (eBioscience, #12-7177-81, 1:100), APC anti-IL-10 (BioLegend, #505010, 1:100), PerCP-Cy5.5 anti-FoxP3 (eBioscience, #45-5773-82, 1:100). Samples were acquired on an LSRFortessa (BD Biosciences). Astrocytes were stained with APC anti-EphB3 (Sino Biological, #50581-R001-A-100). For intracellular staining, BV450 anti-S6 (pS235/pS236) (BD Biosciences, #561457) was used.

For recall responses, splenocytes were cultured in complete RPMI medium for 72h at a density of $4*10^5$ cells/well in 96-well plates in the presence of $MOG_{35–55}$ peptide (Genemed Synthesis, #110582). During the final 16h, cells were pulsed with 1 μCi [^{3}H]thymidine (PerkinElmer, #NET027A005MC) followed by collection on glass fiber filters (PerkinElmer, #1450-421) and analysis of incorporated [^{3}H]thymidine in a beta-counter (PerkinElmer, 1450 MicroBeta TriLux). Alternatively, supernatants were collected after 72h of culture for cytokine measurement by enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked immunosorbent assay (ELISA)

Cytokines were measured in murine or human astrocyte supernatants following the manufacturer's instructions using the following kits: mouse TNFα ELISA Ready SET Go (eBioscience, #50-173-31), mouse IL-6 ELISA Ready SET Go (eBioscience, #50-112-8808),

mouse CCL2 ELISA Ready SET Go (eBioscience, #88-7391-86), human IL-6 DuoSet ELISA (R&D Systems, #DY206-05), human TNFα DuoSet ELISA (R&D Systems, #DY210-05), human CCL2/MCP-1 DuoSet ELISA (R&D Systems, #DY279-05). Cytokines in supernatants of MOG re-stimulated splenocytes were measured using the following kits: mouse IFN-γ ELISA set (BD Biosciences, #555138), mouse IL-10 ELISA Set (BD Biosciences, #555252) and mouse IL-17 ELISA Kit (Invitrogen, #BMS6001).

InDrop single cell RNA-sequencing

Cells were resuspended at a concentration of 100,000 cells/mL in PBS with 0.1% BSA, 18% Optiprep and 0.1% Pluronic F-68 (Thermo Fisher, #24040032). Microfluidic cell encapsulation with barcoded beads was performed using the microfluidic device and flow rates previously described (*116*). FACS sorted mCherry+ cells were co-flowed with reverse transcription reagents at equal flow rates. Barcoded beads were obtained from the Harvard Single Cell Core (inDrop v3). Modifications to the molecular biology were made to enable reverse transcription with template switching in drops. A 2X reverse transcription master mix was prepared at a final concentration of 1 mM dNTP (NEB, #N0447S), 50 µM TSO oligonucleotide (Table S2), 0.6% v/v IGEPAL CA-630 (Sigma-Aldrich, #56741), 2X RT Buffer (Thermo Scientific, #EP0751), 1 U/µl NxGen RNase Inhibitor (Lucigen, #30281-2), and 20 U/µl Maxima H minus RT (Thermo Scientific, #EP0751). Drops were collected on ice in batches of 3000 cells, and immediately UV treated for exactly 8 minutes (Analytik Jena Blak-Ray XX-15L UV light source) to release primers. A mineral oil overlay (Sigma-Aldrich, #M5310-1L) was placed on the emulsion and reverse transcription was performed for 60 minutes at 42C in a heat block. Droplets were incubated at 5 minutes at 85°C to inactivate the reverse transcriptase and the mineral oil

overlay was carefully removed with a P1000 pipet. The bottom layer of oil was removed, and the emulsion was broken by the addition of 5X v/v 20% 1H,1H,2H,2H-Perfluoro-1-octanol (Sigma-Aldrich, #370533-25G) in oil (3M, HFE-7500 Novec Engineered fluid). The aqueous phase was transferred onto a spin filter (Corning, #8162) to remove the barcoded beads, purified with 2.0X RNAclean XP (Beckman Coulter, #A63987), and eluted in 20 µL of ddH2O. Whole transcriptome amplification (WTA) was performed using 20 µL of purified cDNA, 0.5 µM inDrops_FWD primer (Table S2), 0.5 µM inDrops_REV primer (Table S2), and 1X KAPA HiFi master mix (KAPA Biosystems #KK2601) in a 50 µL reaction. The PCR program used was 98C for 3 minutes, followed by 15 cycles of: [98C for 15s, 67C for 20s, 72C for 3 min] then 72C for 5 min, followed by 4C forever. After amplification, WTA product was purified with 0.6X AMPure XP beads, diluted, and analyzed using a Bioanalyzer DNA HS assay (Agilent, #50674626). Single cell RNA-seq libraries were prepared using a NEBNext Ultra II FS Kit (NEB, #E7805) using a custom pre-annealed adapter at the concentrations recommended by the manufacturer, in accordance with the concentration of WTA product. The adapter was prepared by mixing 100 µM Ligation_FWD oligonucleotide and 100 µM Ligation_REV oligonucleotide (Table S2) and heating to 95C for 2 minutes, followed by cooling to room temperature for 5 minutes. The annealed adapter was suspended to 1.5 µM, aliquoted, and stored at -20 ℃ until use. DNA was suspended to 50 ng of material and fragmented using the NEB kit, followed by end repair, and adaptor ligation, all according to the manufacturer's protocol. Sequencing libraries were amplified from purified WTA product using 10 µM of inDropV3_P5_r1_S5XX primer (Table S3) and 10µM of inDropV3_P7_r2 primer (Table S3) in NEBNext Q5 PCR master mix. Samples were cycled at 98C for 45s, followed by 14 cycles of: [98C for 20s, 54C for 30s, 72C for 20s], and 72C for 60s, then 4C forever. Samples were purified by 0.8X Ampure XP bead purification and eluted in 21 µL

ddH2O. Libraries were quantified by Bioanalyzer and Kapa Library Quantification Kit (Kapa Biosystems, #KK4824) prior to sequencing on a NextSeq550 or NovaSeqS2.

Rabies barcode recovery from in vivo experiments

To isolate barcodes from cDNA libraries generated from mCherry+ cells, we derived an approach based on the ATAC-seq protocol (*129, 130*). First, 1-25 nM of whole transcriptome amplified cDNA (WTA product) was PCRed using the following: 2.5 µL of 10 µM inDrops_FWD cDNA amplification primer (Table S3), 2.5µL of 10 µM SMART mCherry primer (Table S3), 25 µL of NEBNext High-Fidelity 2X PCR Master Mix (NEB, #M0541L) and molecular grade water to fill a 50 µL reaction under the cycling conditions: 98C for 30 sec, 5 cycles of [98C for 20s, 63C for 30s, 72C for 10 sec], and 4C forever. Following PCR, 5 µL of each sample was analyzed by qPCR in a master mix consisting of 4.41 µL H2O, 0.25 µL 0.5 µM inDrop FWD, 0.25 µL 0.5 µM SMART mCherry, 0.09 µL 100X SYBR Green I (Thermo Fisher, #S7563), and 5 µL NEBNext High-Fidelity 2X PCR Master Mix to determine the relative amount of DNA in the sample; the remainder of the sample was stored on ice. Samples were cycled by qPCR using the following conditions: 98C for 30s and 30 cycles of [98C for 20s, 63C for 30s, 72C for 10s]. The number of cycles (N) required to achieve 1/3 the maximal fluorescence was calculated for each sample and the remaining 45 µL of sample was cycled using the following conditions: 98C for 30s, and N cycles of [98C for 20s, 63C for 30s, 72C for 10s], and 4C forever. Samples were then purified using Ampure XP magnetic beads (Beckman-Coulter, #A63881) using double-sided bead purification according to the manufacturer's protocol. First, large DNA fragments were removed using a 0.7X beads:volume ratio (31.5 µL beads). Supernatant was collected and incubated with 1.0X bead:volume (76.5 µL) purification to eliminate primer dimers. DNA was resuspended in 30

μL of DNase/RNase-free water. Next, DNA samples were PCRed using the following conditions: 23 μL purified PCR product from round 1, 25 μL NEBNext High-Fidelity 2X PCR Master Mix, 1 μL 10 μM inDrop_v3_p7_r2 (Table S3) and 1 μL of 10 μM staggered cocktail, which contained equimolar concentrations of inDropv3_S5XX_R2bc, inDropv3_S5XX_R2bc+1, inDropv3_S5XX_R2bc+2, inDropv3_S5XX_R2bc+3 (Table S3). Indices from N501-N536 were used (Table S4). Samples were cycled using the following conditions: 98C for 30 sec and 10 cycles of [98C for 20s, 55C for 20s, 72C for 10s], followed by 72C for 2 minutes, and 4C forever. Following PCR, samples were purified using a double-sided Ampure XP bead purification (0.7X (35 μL) followed by 1.0X (85 μL) and resuspended in 15 μL of H2O. Samples were then quantified on a 2100 Bioanalzyer (Agilent Technologies). Samples were quantified by qPCR prior to deep sequencing using the Kapa Library Quantification Kit (Kapa Biosystems, #KK4824).

Lentivirus production and transduction

CRISPR/Cas9 lentiviral constructs were generated by modifying the *pLenti-U6-sgScramble-Gfap-Cas9-2A-EGFP-WPRE* lentiviral backbone, described previously (*3, 13*). This backbone contains derivatives of the previously described reagents lentiCRISPR v2 (a gift from Feng Zhang, Addgene plasmid #52961 (*131*)), and lentiCas9-EGFP (a gift from Phil Sharp and Feng Zhang, Addgene plasmid #63592 (*132*)). The *Gfap* promoter is the ABC_1D *gfa2 GFAP* promoter (*133*). The *Itgam* promoter (also known as *Cd11b*) we described previously (*12*). Substitution of sgRNAs was performed through a PCR-based cloning strategy using Phusion Flash HF 2X Master Mix (Thermo Fisher, #F548L). A three-way cloning strategy was developed to substitute sgRNAs using the following primers: U6-PCR-F and U6-PCR-R; cr-RNA-F and cr-RNA-R (Table S5). Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen,

#28104) and digested using DpnI (NEB, #R0176S), BsaI-HF (NEB, #R3535/R3733), AscI (for U6 fragment) (NEB, #R0558), or SbfI-HF (for crRNA fragment) (NEB, #R3642). The pLenti backbone was cut with AscI/SbfI-HF and purified using the QIAquick PCR purification kit. All shRNA-based lentiviral constructs were cloned into either *pLenti-Gfap-EGFP-mir30-shRNA* or *pLenti-Itgam-EGFP-mir30-shRNA*, which have been previously described (*6, 9, 12, 13*). Ligations were performed overnight at 16C using T4 DNA Ligase Kit (NEB, #M0202L). Ligations were transformed into NEB Stable Cells (NEB, #C3040) at 37C, single colonies were picked, and DNA was prepared using QIAprep Spin Miniprep Kit (Qiagen, #27104). Lentiviral plasmids were transfected into HEK293FT cells (Thermo Fisher Scientific, #R70007) according to the ViraPower Lentiviral Packaging Mix protocol (Thermo Fisher Scientific, #K497500) and lentiviruses were packaged with pLP1, pLP2, and pseudotyped with pLP/VSVG. Media was changed the next day, lentivirus was collected 48 hours later and concentrated using Lenti-X Concentrator (Clontech. #631231) overnight at 4C followed by centrifugation according to the manufacturer's protocol and resuspension in 1/100-1/500 of the original volume in 1X PBS. Delivery of lentiviruses via intracerebroventricular (ICV) injection was performed largely as described previously (*3, 13*). Briefly, mice were anesthetized using 1-3% isoflurane mixed with oxygen. Heads were shaved and cleaned using 70% ethanol and Betadine (Thermo Fisher, #19-027132) followed by a medial incision of the skin to expose the skull. The ventricles were targeted bilaterally using the coordinates: +/- 1.0 (lateral), -0.44 (posterior), -2.2 (ventral) relative to Bregma. Mice were injected with approximately 10^7 total IU of lentivirus delivered by two 10 µL injections using a 25 µL Hamilton syringe (Sigma-Aldrich, #20787) on a stereotaxic alignment system (Kopf, #1900), sutured, and permitted to recover in a separate clean cage. Mice were permitted to recover for between 4-7 days before induction of EAE. CRISPR/Cas9 sgRNA sequences were designed using

a combination of the Broad Institute's sgRNA GPP Web Portal (portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design), Synthego (https://design.synthego.com/#/validate), and cross-referenced with activity-optimized sequences contained within the Addgene library #1000000096 (a gift from David Sabatini and Eric Lander) (*134*). sgRNAs used in this study were: sgSema4d, sgPlxnb1, sgPlxnb2, and sgScramble (sequence from Origene, #GE100003) Lentiviral shRNA sequences were designed based on validated MISSION shRNA sequences (Sigma-Aldrich) (Table S6).

Electron microscopy

Electron microscopy was conducted as previously described (*135*). After transcardial perfusion with PBS, spinal cords were fixed in 3% glutaraldehyde solution. The glutaraldehyde solution was prepared as follows: Sørensen buffer, pH=7.45, was used for the dilution of 25% glutaraldehyde solution (Merck-Millipore, #1.042.390.250) and prepared by titration of disodium phosphate solution (17.8g Na_2HPO_4 x $2H_2O$ in 1000 mL ddH_2O, Sigma-Aldrich, #S9763) and monopotassium phosphate solution (4.08g KH_2PO_4 in 300 mL ddH_2O). Next, the tissue was washed in Sørensen buffer and incubated in 1% osmium tetroxide solution (1:1 mix of 2% Osmium (Science Service, #E19172) and 0.365g $K_4Fe(CN)_6$ in 10 mL ddH2O (Merck, #P3289)) for 2h. After the incubation in an ascending alcohol series starting from 30% ethanol to 100% ethanol, the tissue was placed in 100% 1,2-propylenoxide (Sigma-Aldrich, #8.07027.1000) for 30 minutes. Resin was prepared by mixing 30g glycid ether (Serva, #21045.01), 56g 2-dodecenylsuccinic acid (Serva, #20755.01), 16g Renlam (Serva, #13825) and 2 mL phthalic acid dibutylester (Serva, #32805) in a glass stirrer for one hour. Afterwards, 2 mL 2,4,6-tris(dimethylaminomethyl)phenol (Serva, #36975) was added and the solution was mixed for another 5 minutes. The tissue was

incubated in a 2:1 propylenoxide/resin mix for 1 hour, followed by the overnight incubation in a 1:2 mix. After removal of the supernatant and the evaporation of the liquid, 100% resin was added for 4 hours at room temperature. The block was fully polymerized after incubation for 24h in resin at 75C. 70 nm ultra-thin sections were cut with a microtome (Reichert Ultracut S) and Histo Diamant Knives (Diatome). Next, the sections were transferred to a 200 square mesh grid (Science Service, #T200-Cu), treated with 1% uranyl acetate and 3% lead citrate (Leica, #16707235) and analyzed using an electron microscope (Philips, #CM-100).

Analysis of human brain tissue by immunofluorescence

Use of MS patient tissues was approved by the Neuroimmunology Research Laboratory, Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM) under ethical approval number BH07.001. Human brain tissue was obtained from patients with clinical and neuropathological MS diagnosis according to the revised 2010 McDonald's criteria (*136*). Tissue samples were collected from healthy donors and MS patients with full ethical approval (BH07.001) and informed consent as approved by the local ethics committee. Autopsy samples were preserved and lesions classified using Luxol fast blue/H&E staining and Oil Red O staining as described (*3, 13, 137, 138*).

Frozen brain tissue from 4 MS patients and 4 healthy controls was cut into 7 µm thick sections, air dried and fixed in ice-cold acetone for 10 minutes. Sections were delipidised in 70% ethanol for 5 minutes, followed by blocking endogenous avidin/biotin using an avidin-biotin blocking kit (Life Technologies, #004303) and non-specific binding of antibodies was blocked with either 10% goat serum or 10% donkey serum (Sigma-Aldrich). Two primary antibody workflows were used: either rabbit anti-human EPHB3 (Abcam, #ab133742, 1:25) or a

combination of goat anti-human EPHRINB3 (R&D Systems, #AF395, 1:50) and mouse anti-human TMEM119 (Novus Biologicals, #NBP2-76985, 1:200), all of which were incubated in blocking buffer overnight at 4C. The next day slides were washed with 0.05% PBS-Tween and either incubated with goat anti-rabbit biotin (DAKO, #E0432, 1:500) or a mixture of donkey anti-mouse Cy3 (Jackson ImmunoResearch, #715-166-147, 1:200) and donkey anti-goat Alexa Fluor 488 (Jackson ImmunoResearch, #705-546-147, 1:350) for 40 min at room temperature. EPHB3 sections were washed and incubated with streptavidin-Alexa Fluor 488 (Jackson ImmunoResearch, #016-540-084, 1:250) for 40 minutes at room temperature and incubated with mouse anti-human GFAP-Cy3 (Sigma-Aldrich, #C9205, 1:500) for 1hr at RT. After extensive washing, sections were counterstained with DAPI (Sigma-Aldrich, #D9542, 1:500) and mounted in Mowiol containing pro-long gold (Life Technologies, #P36934). As negative controls, primary antibodies were omitted to control for non-specific binding. Images (z-stacks) were acquired using a Leica SP5 confocal microscope with Leica LAS AF software and processed using Fiji and LAS X. All settings were kept the same during acquisition and analysis. EPHB3 intensity was quantified in $GFAP^+$ cells, whereas EPHRINB3 intensity was quantified in $TMEM119^+$ cells.

For SEMA4D/PLEXIN/GFAP/TMEM119 immunostaining of paraffin sections, sections were deparaffinized in 2 washes of Xylenes (#214736, Sigma-Aldrich), followed by 2 washes in 100% EtOH for 5 minutes, 1 wash in 95% EtOH for 5 minutes, 1 wash in 70% EtOH for 5 minutes, 1 wash in 50% EtOH for 5 minutes, and then slides were rinsed with ddH2O. Antigen retrieval was performed by placing slides in boiling Epitope Retrieval Solution (#IW-11000, IHC World) for 20 minutes. Slides were dried, a hydrophobic barrier was made (#H-4000, Vector Laboratories) and sections were washed 3X for 5 minutes with 0.3% Triton X-100 in PBS (PBS-T). Sections were blocked with 5% donkey serum (#D9663, Sigma-Aldrich) in 0.3% PBS-T at RT for 30

minutes. Sections were then incubated with primary antibody diluted in blocking buffer overnight at 4C. Following primary antibody incubation, sections were washed 3X with 0.3% PBS-T and incubated with secondary antibody diluted in blocking buffer for 2 hours at RT. Sections were then washed 3X with 0.3% PBS-T, dried, and coverslips were mounted. Primary antibodies used in this study were: mouse anti-human TMEM119 (Novus Biologicals, #NBP2-76985, 1:200), PLEXINB2-Alexa Fluor 647 (Bioss USA, #BS11215R-A647, 1:50), GFAP-CY3 (Sigma-Aldrich, #C9205, 1:50), rabbit anti-human SEMA4D (Abcam, #ab134128; 1:100). Secondary antibodies used in this study were: Goat anti-mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 405 (Thermo Fisher, #A-31553), Goat anti-rabbit Alexa Fluor 488 IgG (H+L) (Invitrogen, #A11008) both used at 1:500.

Immunostaining

Mice were intracardially perfused with ice cold 1X PBS followed by ice cold 4% PFA. Brains were harvested, post-fixed in 4% PFA overnight at 4C, followed by dehydration in 30% sucrose for 2 days at 4C. Brains were then frozen in OCT (Sakura, #4583) and 30 µm sections were obtained by cryostat on SuperFrost Plus slides (Fisher Scientific, #22-037-246). A hydrophobic barrier was drawn (Vector Laboratories, #H-4000) and sections were washed 3X for 5 minutes with 0.1% Triton X-100 in PBS (PBS-T). Sections were permeabilized with 0.3% PBS-T for 20 minutes, then washed 3X with 0.1% PBS-T. Sections were blocked with 5% donkey serum (Sigma-Aldrich, #D9663) in 0.1% PBS-T at RT for 30 minutes. Sections were then incubated with primary antibodies diluted in blocking buffer overnight at 4C. Following primary antibody incubation, sections were washed 3X with 0.1% PBS-T and incubated with secondary antibodies diluted in blocking buffer for 2 hours at RT. Following secondary incubation, sections

were washed 3X with 0.1% PBS-T, dried, and coverslips were mounted using Fluoromount-G with DAPI (SouthernBiotech, #0100-20). Primary antibodies used in this study were: mouse anti-GFAP (Millipore, 1:500, #MAB360), chicken anti-GFP (Abcam, #ab13970, 1:1000), rabbit anti-mCherry (Abcam, 1:500, ab167453), Armenian hamster anti-PlexinB2 Antibody (Fisher Scientific, 1:100, #5013113), rabbit anti-Iba1 (Abcam, 1:100, ab178846), rabbit anti-Ephrin-B3 (Abcam, 1:100, #ab101699), rabbit anti-Eph Receptor B3 (Abcam, 1:100, #ab133742), rabbit anti-acetyl NF-kB p65 (Lys-310) (Sigma-Aldrich, 1:100, #SAB4502616-100UG), rabbit anti-CD68 (Abcam, 1:100, #ab955), rabbit anti-CLDN5 (Abcam, 1:100, #ab131259), and rabbit anti-Semaphorin 4D/CD100 (Abcam, 1:100, #ab231961). Secondary antibodies used in this study were: Alexa Fluor 647 donkey anti-mouse (Abcam, #ab150107), donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed, Alexa Fluor 568 (Life Technologies, #A10042), Rhodamine Red-X-AffiniPure Fab Fragment Donkey Anti-Rabbit IgG (H+L) (Jackson Immunoresearch, #711-297-003), Rhodamine Red-X-AffiniPure Fab Fragment Donkey Anti-Mouse IgG (H+L) (Jackson Immunoresearch, #715-297-003), Alexa Fluor 488 AffiniPure Fab Fragment Goat Anti-Rabbit IgG (H+L) (Jackson Immunoresearch, #111-547-003), Alexa Fluor 647 AffiniPure Fab Fragment Donkey Anti-Rabbit IgG (H+L) (Jackson Immunoresearch, #711-607-003), Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 405 (Thermo Fisher, #A-31556), Goat Anti-Armenian hamster IgG H&L Alexa Fluor 568 (Abcam, #ab175716), Alexa Fluor 488-AffiniPure Donkey Anti-Rabbit IgG (H+L) (Jackson Immunoresearch, #711-545-152), and Goat anti-Chicken IgY (H+L) Alexa Fluor 488 (Life Technologies, #A11039) all at 1:500 working dilution. Iterative labeling using rabbit primary antibodies was accomplished by incubating with a single primary antibody on Day 1, staining with the anti-rabbit Fab fragment on Day 2, washing 6X with PBS-T, followed by incubation with primary and secondary antibodies as described

above. Paraffin staining of mouse tissue used in the analysis of CLDN5 was performed identical to the protocol specified for humans.

H&E, LFB and silver staining

Transverse sections from lumbar spinal cords of EAE mice at the end of the experiment were fixed in 4% paraformaldehyde, pH=7.4, embedded in paraffin and cut as 10 μm sections. Sections were stained with H&E, Luxol fast blue, or silver nitrate. Images were acquired using a Zeiss Axioskop 2 Plus microscope with AxioCam HRC and AxioVision software and analyzed using ImageJ software (*139*).

RNAscope analysis

Mice were intracardially perfused with ice cold 1X PBS followed by ice cold 4% PFA. Brains were then post-fixed in 4% PFA overnight followed by dehydration in 30% sucrose for 2 days at 4C. Brains from naive or EAE mice were frozen in OCT (Sakura, 4583) and stored at −80C. 30-μm sections were collected in sucrose and stored at −80C. Sections were removed from the freezer and thawed to room temperature and rinsed in reverse osmosis (RO) water. RNAscope protocol was adapted from the Advanced Cell Diagnostics (ACD) user manual using all ACD provided reagents. Endogenous peroxide activity of samples was quenched with H_2O_2 solution (ACD, #322381) free-floating in a 24-well flat bottomed plate for 10 min at room temperature. Samples were then rinsed in RO water, then mounted onto SuperFrost Plus slides (Thermo Fisher Scientific, #22-037-246) in 0.05M phosphate buffer. Slides were dried on a slide warmer for 15 min and dipped in 100% EtOH to ensure tissue adherence to the slides. Target retrieval was performed by submerging slides in Target Retrieval reagent (ACD, #322000) at 90–95C for 5 min

on a hotplate. Samples were rinsed in RO water, dried on the slide dryer, and dipped in 100% EtOH. At this point, a hydrophobic barrier was drawn on the slides around the tissue sections (ACD, ImmEdge Hydrophobic Barrier Pen, #310018). After target retrieval, tissue was further permeabilized using Protease Plus (ACD, #322381) for 30 min in the HybEZ II humidity controlled hybridization oven (ACD, #321710/#321720) at 40C and then washed twice in RO. RNA 4-plex probe hybridization (ACD, *Plxnb2*-C1 #459181, *Il1b*-C2 #316891-C2, *Sema4d*-C3 #498381-C3, *Nos2*-C4 #319131-C4) was performed in the HybEZ oven for 2 h at 40C. Slides were washed twice in Wash Buffer (ACD, #310091) and stored overnight at room temperature in 5X SSC. The next day, slides were washed twice in Wash Buffer. Hereafter, samples were washed twice in Wash Buffer reagent following each subsequent step. RNA probes were next amplified and detected on the slides using the RNAscope Multiplex Fluorescent Detection Kit v2 (ACD, #323110) and RNAscope 4-Plex Ancillary Kit (ACD, #323120) according to the manufacturer's protocol. Specifically, samples were incubated in Amp1, Amp2, and Amp3 sequentially for 30 min, 30 min, and 15 min, respectively at 40C in the HybEZ oven. The channel 1 probe (*Plxnb2*) was developed by first conjugating the HRP using HRP-C1 for 15 min at 40C in the HybEZ oven, then incubating in the Opal520 fluorophore (Akoya Biosciences, #FP1488001KT) diluted to 1:750 in TSA buffer for 30 min at 40C in the HybEZ oven, and finally blocking using the HRP-Blocking reagent for 15 min at 40C in the HybEZ oven. The channel 2 probe (*Il1b*) was developed by conjugating the HRP using HRP-C2 for 15 min at 40C in the HybEZ oven, then incubating in the Opal570 fluorophore (Akoya Biosciences, #FP1487001KT) diluted to 1:750 in TSA buffer (ACD, #322809) for 30 min at 40C in the HybEZ oven, and finally blocking using the HRP-Blocking reagent for 15 min at 40C in the HybEZ oven. The channel 3 probe (*Sema4d*) was developed by conjugating the HRP using HRP-C3 for 15 min at 40C in the HybEZ oven, then incubating in the

Opal620 fluorophore (Akoya Biosciences, #FP1495001KT) diluted to 1:750 in TSA buffer for 30 min at 40C in the HybEZ oven, and finally blocking using the HRP-Blocking reagent for 15 min at 40C in the HybEZ oven. The channel 4 probe (*Nos2*) was developed by conjugating the HRP using HRP-C4 for 15 min at 40C in the HybEZ oven, then incubating in the Opal690 fluorophore (Akoya Biosciences, #FP1497001KT) diluted to 1:300 in TSA buffer for 30 min at 40C in the HybEZ oven, and finally blocking using the HRP-Blocking reagent for 15 min at 40C in the HybEZ oven. Slides were washed a final time in Wash Buffer before proceeding to astrocyte and microglia immunofluorescence. Slides were blocked in SuperBlock (Thermo Fisher Scientific, #37515) overnight at 4C in the dark. The next day, tissue was incubated in primary mouse anti-GFAP (Millipore, #MAB360, 1:500) and primary rabbit anti-P2RY12 (Novus Biologicals, #NBP1-78249, 1:250) for 1 h in the HybEZ oven at 40C. After incubation, slides were washed twice in TBS followed by detection with anti-mouse Alexa Fluor 700 (Thermo Fisher Scientific, #A-21036, 1:300) to detect GFAP and anti-rabbit Alexa Fluor 514 (Invitrogen, #A-31558, 1:300) to detect P2RY12 for 1 h in the HybEZ oven at 40C. Tissues were washed twice in TBS then coverslipped using ProLong Gold (Thermo Fisher Scientific, #P10144) mounting medium. For *mCherry* detection in microglia and astrocytes, the RNAscope protocol was performed as above. The channel 2 probe (*mCherry*, #431201-C2) was conjugated to the Opal570 fluorophore diluted to 1:750 in TSA buffer. GFAP and P2RY12 immunofluorescence was performed as described above. All sections were left to dry overnight in the dark at 4C until imaging. Images were acquired using a Zeiss LSM 710 NLO confocal microscope equipped with a motorized stage, seven lasers and an acquisition camera. Images were quantified using FIJI by first marking cells based on type, then analyzing the presence of genes of interest, and measured as percent of cells.

Primary astrocyte and microglia cultures

Procedures were performed largely as described previously (*3, 12, 13*). Brains of mice aged P0-P3 were dissected into PBS on ice. Brains were centrifuged at 500g for 10 minutes at 4C and resuspended in 0.25% Trypsin-EDTA (Thermo Fisher Scientific, #25200-072) at 37C for 10 minutes. DNase I (Thermo Fisher Scientific, #90083) was then added at a concentration of 1 mg/mL to the solution, and the brains were digested for 10 more minutes at 37C. Trypsin was neutralized by adding DMEM/F12+GlutaMAX (Thermo Fisher Scientific, #10565018) supplemented with 10% FBS (Thermo Fisher Scientific, #10438026) and 1% penicillin/streptomycin (Thermo Fisher Scientific, #15140148), and cells were passed through a 70 μm cell strainer. Cells were centrifuged at 500g for 10 minutes at 4°C, resuspended in DMEM/F12+GlutaMAX with 10% FBS/1% penicillin/streptomycin and cultured in T-75 flasks (Falcon, #353136) pre-coated with Poly-L-lysine (Sigma Aldrich, #P4707) for 1 h at 37C and washed with 1 X PBS. Cells were cultured at 37C in a humidified incubator with 5% CO2, for 7-10 days until confluency was reached. Media was replaced every 2–3 days. Microglia were removed by shaking for 30 minutes at 180 rpm, and the media was changed, then cells were shaken for 2 hours at 220 rpm and the media was changed again. Remaining attached cells were enriched astrocytes. To obtain microglia, media from shaken cells was centrifuged at 500g for 5 min at 4C, pellet was resuspended in 0.5% BSA, 2mM EDTA in 1X PBS, and magnetic sorted using anti-mouse CD11b Microbeads according to the manufacturer's protocol (Miltenyi, #130-049-601).

Primary astrocyte and microglia cytokine stimulation

Cytokine treatment was performed for 18 hours with cytokines diluted in DMEM/F12+GlutaMAX (Life Technologies, #10565042) supplemented with 10% FBS (Life

Technologies, #10438026) and 1% penicillin/streptomycin (Life Technologies, #15140122), unless otherwise indicated. For semaphorin4D studies, the following compounds were used for stimulation: 50 ng/mL TNF (R&D Systems, #410-MT-010) and 100 ng/mL IL-1β (R&D Systems, #401-ML-005). Recombinant mouse semaphorin $4D_{(24-711)}$ extracellular domain (VWR, #75791-390), which possesses agonistic activity (*38-40*) was used to stimulate astrocytes at 1µg/mL. For serum-free culture conditions, serum was replaced with N1 medium supplement (Sigma-Aldrich, #N6530).

For ephrin studies, cell cultures were pre-treated with 10-50 µM A38, 10 µM ZSTK474 (Selleck Chemicals, #S1072), LPS (100 ng/ml Invivogen #tlrl-3pelps) or 100 nM rapamycin (LC Laboratories, #R-5000) for 30 min and then, when indicated, stimulated with 50 ng/mL TNFα (R&D Systems, #410-MT-010) and 100 ng/mL IL-1β (R&D Systems, #401-ML-025). During siRNA knockdown studies, astrocytes were washed with 1X PBS and co-cultured 1:1 with microglia for 18h, after which each cell type was magnetically separated as before and used for downstream analysis. Recombinant human Ephrin-B3 Fc chimera protein (R&D Systems, #7924-EB-050), recombinant mouse Ephrin-B3 Fc chimera protein (R&D Systems, #7655-EB-050), and recombinant mouse EphB3 Fc chimera protein (R&D Systems, #432-B3-200) were incubated overnight at a concentration of 2.5 µg/cm^2 on poly-L-lysine pre-coated plates, then washed with 1X PBS, followed by cell seeding. For serum-free culture conditions, serum was replaced with N1 medium supplement (Sigma-Aldrich, #N6530).

Barcode quantification in co-culture

Gfap$^{TVA/G}$ astrocytes were cultured in vitro and infected with RabΔG-mCherry-BC virus for 3 days. Infected mCherry+ cells were sorted by flow cytometry and cultured 1:10 with EGFP+

astrocytes derived from *CAG*^{Kaede} mice, which ubiquitously express the Kaede GFP variant. Cells were co-cultured for 4 days, followed by sorting of mCherry+ and mCherry+/GFP+ cells to distinguish between early-infected and late-infected cells, respectively, and inDrop scRNA-seq. Captured cell cDNA of each population was amplified according to the inDrop protocol described above. Next, RabΔG barcode abundance was quantified by qPCR using Fast SYBR Green 2X PCR Master Mix (Thermo Fisher, #4385612) according to the manufacturer's protocol. Rabies barcodes were amplified using 500 nM of the SMART mCherry primer and inDrop FWD primer. Barcode abundance was normalized to inDrop-captured cDNA that was amplified using 500 nM of the inDrop FWD and inDrop REV primers using the ΔΔCt method.

Isolation, culture, and stimulation of human primary astrocytes

Human fetal astrocytes were isolated from human CNS tissue (cerebral hemispheres) from fetuses at 17–23 weeks of gestation obtained from the Laboratory of Developmental Biology (Eunice Kennedy Shriver National Institute of Child Health and Human Development, project number: 5R24HD000836) following Canadian Institutes of Health Research–approved guidelines and cultured as previously described (*6, 12, 13*). The sex of the human astrocytes used was unidentified. Astrocyte cultures were obtained by dissociation of the fetal CNS with 0.25% trypsin (Thermo Fisher Scientific, #25200-072) and 50 μg/mL DNase I (Roche, #10104159001) followed by mechanical dissociation. After washing, the cell suspension was plated at a concentration of 3-5*10^6 cells/mL on poly-L-lysine (Sigma-Aldrich, #P4707) pre-coated 75 cm^2 flasks in DMEM supplemented with 10% FCS (#SH3007303, Fisher Scientific) and penicillin/streptomycin. To obtain pure astrocytes, the mixed CNS cell culture (containing astrocytes, microglia, and neurons) was passaged upon confluency, starting at 2 weeks post-isolation, using 0.25% trypsin-EDTA

(Thermo Fisher Scientific, #25200-072). Human fetal astrocytes were passaged 2-4 times and cultures, which corresponds to a time frame of 2 weeks to 3 months post-isolation, and purity (>90%) was determined by immunostaining using rabbit anti-GFAP (Roche, #05269784001, 1:100) followed by goat anti-rabbit Texas Red IgG (Thermo Fisher Scientific, #T-2767, 1:100). The following compounds were used: 10 ng/mL human IL-1β (R&D Systems, #201-LB), A38, and human Ephrin Fc chimera. Stimulation paradigms were performed analogous to mouse studies. For RNA, cell lysate was collected after stimulation and for analysis by ELISA cells were washed and cultured in fresh media, which was assayed 2 days later. For serum-free culture conditions, serum was replaced with N1 medium supplement (Sigma-Aldrich, #N6530).

Generation of astrocyte-conditioned medium (ACM)

To collect ACM, primary mouse astrocytes were pretreated for 1 hour with the inhibitors A38 and C9 and stimulated as stated above. After 24h, cells were extensively washed with 1X PBS, and incubated with fresh astrocyte complete medium for 48h. Supernatants were spun down at 14,000 rpm for 5 minutes and either used fresh or kept at −80C in single use aliquots for downstream assays.

Monocyte migration assay

Monocytes were purified from the spleen of C57BL/6J mice using CD11b Microbeads (Miltenyi, #130-049-601) according to the manufacturer's instructions. 100,000 monocytes were seeded in the upper chamber of a 24-well cell culture transwell, with a 5 μm pore size (Thermo Fisher Scientific, #3421) containing 300 μL of astrocyte-conditioned medium, in the bottom

chamber. After 3h incubation at 37C in 5% CO_2, cells were collected from the lower chamber after shaking and incubation with Accutase solution (Sigma-Aldrich, #A6964) and quantified by FACS.

Neurotoxicity assay

N2A neuronal cells (ATCC, #CCL-131) were plated and pre-activated with 100 ng/mL mouse IFN-γ (R&D Systems, #485-MI-100) for 24h. After extensive washing with 1X PBS, medium was replaced with ACM and 24h later the supernatant was harvested for cytotoxicity evaluation by measuring LDH release with CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, #G1780) according to the manufacturer's protocol. When stated, anti-TNF blocking antibody (Abcam #ab34719) was added to the ACM at a concentration of 25 $\mu g\ ml^{-1}$. Alternatively, TNF (R&D Systems, #410-MT-010) was added to N2A in the stated concentrations.

In vitro knockdown with siRNA

Smart pools of ON-TARGETplus siRNA against *Ephb3* (Dharmacon, #L-043340-00-0005) or non-targeting control (Dharmacon, #D-001810-10-05) were mixed with INTERFERin (Polyplus Transfection, #409) in Opti-MEM (GIBCO, #51985-034) and incubated at room temperature for 10 min, and then added to primary astrocytes in complete medium following the manufacturer's instructions with a working concentration of 1 nM siRNA. After 48 h incubation, media was removed and downstream experiments were performed. Knockdown efficiency was confirmed by qPCR.

Oxygen consumption

Respiration was measured using the XFe24 or XFe96 analyzers (Agilent Technologies) with 70,000 or 30,000 cells per well respectively, starved for 24h and stimulated for 16h with 50 ng/mL TNFα (R&D Systems, #410-MT-010) and 100 ng/mL IL-1β (R&D Systems, #401-ML-025) after 30 min pre-treatment with 50 μM A38 or 100 nM rapamycin. The Seahorse XF Cell Mito Stress Test kit (Agilent Technologies, #103015-100) was used according to the manufacturer's protocol. Oxygen consumption rate (OCR) was then quantified after sequential addition of 2 μM oligomycin, 1 μM FCCP, and 5 μM rotenone/antimycin A. The assay medium (DMEM with 10 mM glucose, 1 mM pyruvate, and 2 mM glutamine) was used during the assay. The OCR rate was normalized to cell number estimated using CyQUANT cell proliferation assay kit (Invitrogen, #C7026).

Mitochondrial ROS measurement

50,000 astrocytes were seeded per well in 96-well black plates and rested for 2 days in complete medium, followed by activation or inhibitor pretreatment for 24h. MitoSOX red (Thermo Fisher Scientific, #M36008) was used to stain for mitochondrial ROS for 10 min and the signal was detected on an Infinite M1000 PRO Microplate Reader (Tecan). The fluorescent signal was normalized to cell number estimated using a CyQUANT cell proliferation assay kit (Invitrogen, #C7026) and compared to the control group.

RNA isolation from cultured astrocytes and microglia

Primary astrocytes were lysed in Buffer RLT (Qiagen) and RNA was isolated from cultured astrocytes using the Qiagen RNeasy Mini kit (Qiagen, #74106) with on-column DNase digestion

(Qiagen, #79254). cDNA was transcribed using the high-capacity cDNA Reverse Transcription Kit (Life Technologies, #4368813). Gene expression was then measured by qPCR using Taqman Fast Universal PCR Master Mix (Life Technologies, #4367846). Taqman probes used in this study are: *Gapdh* (Mm99999915_g1), *Nos2* (Mm00440502_m1), *Il1b* (Mm00434228_m1), *Sema4d* (Mm00443147_m1), *Ephb3* (Mm00802553_m1), *Ccl2* (Mm00441242_m1), *Csf2* (Mm01290062_m1), *Tnf* (Mm00443258_m1), *Il6* (Mm00446190_m1), *CCL2* (Hs00234140_m1), *CSF2* (Hs00929873_m1), *NOS2* (Hs01075529_m1), *TNF* (Hs00174128_m1), *IL6* (Hs00985639_m1), *IL1B* (Hs01555410_m1), and *GAPDH* (Hs02758991_g1). qPCR data were analyzed by the ddCt method by normalizing the expression of each gene for each replicate to *Gapdh* or *Hprt* and then to the control group.

Western blotting

Protein lysates were prepared by lysing astrocytes with 1X lysis buffer (Cell Signaling Technology, #9803S) containing 1X Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, #78441) or alternatively using a cell fractionation kit (Cell Signaling Technology, #9038S), following the manufacturer's instructions. When required, protein content of each sample was normalized to 100 µg/mL after quantification using the micro BCA protein assay kit (Thermo Fisher Scientific, #23235). 1X Laemmli buffer (Boston BioProducts, #BP-111R) was added to protein lysates followed by boiling at 95C for 5 minutes before loading. SDS-PAGE was performed using Bolt 4%–12% Bis-Tris Plus gradient gels (Invitrogen, #NW04125BOX). Western blotting was performed by transferring proteins onto a nitrocellulose membrane (Thermo Scientific, #88018) in 1X Bolt MES SDS Running Buffer (Life Technologies, #B000202). Membranes were blocked in 10% milk (Lab Scientific, #M0841) in TBS-T (Boston

BioProducts, #IBB-180-2L). Primary antibodies were incubated overnight at 4C and secondary antibodies were incubated for 30 min at RT. HRP-conjugated blots were developed using the KwikQuant imaging system and KwikQuant western blot detection kit (Kindle Biosciences, #R1004). Primary antibodies used in this study were: rabbit anti-Phospho-PI3K p85(Tyr458)/p55(Tyr199) (#4228), mouse anti-PI3K p85α (6G10) (#13666), rabbit anti-Phospho-Akt (Ser473) (D9E) (#4060), rabbit anti-Phospho-Akt (Thr308) (D25E6) (#13038S), mouse anti-Akt (40D4) (#2920), rabbit anti-Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) (#4858), rabbit anti-S6 Ribosomal Protein (5G10) (#2217), rabbit anti-P-p65 (Ser536) (#3033) and rabbit anti-GAPDH (#2118), all from Cell Signaling Technology. Secondary antibodies used in this study were: anti-mouse IgG-HRP conjugate (Cell Signaling Technology, #7076S, 1:2000), anti-rabbit IgG-HRP conjugate (Cell Signaling Technology, #7074S, 1:5000).

Radiometric EphB3 kinase activity assay

To study the EphB3 kinase activity, a radioactive filter binding assay using 33P ATP was performed as described (*140*) at the MRU PPU International Centre for Kinase Profiling. Briefly, different concentrations of A38 were combined with a mixture of EphB3 and substrate that was subsequently provided 33P ATP and halted by orthophosphoric acid addition. The mix was harvested onto P81 filter plates and counts were read on a Topcount NXT.

Cytotoxicity and apoptosis assay

Compound cytotoxicity was inferred by quantifying lactate dehydrogenase (LDH) release into cell supernatants after 24h using the CytoTox 96 non-radioactive cytotoxicity assay (Promega, #G1780) following the manufacturer's protocol. Cell apoptosis was evaluated after A38 treatment

by measuring caspase-3/7 activation. Briefly cells were washed with 0.5% BSA, 2 mM EDTA in 1X PBS and incubated with surface antibodies and a live/dead cell marker on ice for 30 min. Afterwards, cells were washed and stained for caspase-3/7 according the manufacturer's protocol (Invitrogen, #C10427).

Bulk RNA-seq

Bulk RNA isolated from flow cytometry sorted cells was used as input with the kit (NEB, #E6420) according to the manufacturer's protocol. Reverse transcription was performed according to the SMART protocol using a template switching oligo. Then, cDNA was amplified and cleaned using Ampure XP beads (Beckman-Coulter, #A63881) and quantified using a Bioanalyzer DNA HS assay (Agilent, #50674626). Libraries were then fragmented, end-repaired, and ligated to Illumina compatible adaptors followed by sample barcoding using NEBNext Multiplex Oligos for Illumina (#E7335S, #E7500S). Samples were selected again using Ampure XP beads and quantified using a Bioanalyzer. Libraries were quantified using a Kapa Library Quantification Kit (Kapa Biosystems, #KK4824) and run on an Illumina NextSeq550. Alternatively, 5 ng of RNA was resuspended 5 µL of nuclease-free H2O and processed using the SMART-seq2 workflow at Broad Technology Labs and the Broad Genomics Platform.

Bulk RNA-seq analysis

Processed RNA-seq data was filtered, removing genes with low read counts. Read counts were normalized using TMM normalization and CPM (counts per million) were calculated to create a matrix of normalized expression values. The fastq files of each RNA-seq data sample were aligned to the *Mus musculus* GRCm38 transcriptome using Kallisto (v0.46.1), and the same

software was used to quantify the alignment results. Differential expression analysis was conducted using DESeq2, and the log2 fold change was adjusted using apeGLM for downstream analysis. Bioinformatic analysis of predicted upstream regulators and differentially regulated pathways was performed using Ingenuity Pathway Analysis (Qiagen). P-values of canonical signaling networks were obtained using the NetworkAnalyst tool (*141*) with differentially expressed genes as input.

Modeling of barcode labeling and network capture

The fraction (*F*) of uniquely labeled cells based on barcode library diversity (*N*) and number of cells seeded (*k*) were predicted using the "birthday problem" equation:

$$F = (1 - \frac{1}{N})^{(k-1)}$$

Curves shown in **Fig. 1E** were then generated based on the fixed library sizes of k=100, 1000, 10000, and 100000. In a separate analysis in **Fig. 1F**, to estimate the network ratio captured (*R*) using inDrop we assumed:

$$T = \frac{S * C}{3000} * t$$

where *T* is the total time required to process all labeled cells in a RABID-seq experiment, given the number of seeded cells (*S*), the number of connections per cell (*C*), an inDrop batch size of 3000 cells, and the time required to process each batch (*t*), which we approximated as 0.5 hours. We assumed 12 hours is the maximum time that could be spent processing all inDrop samples in a day and 0.6 is the maximum fraction of input cells captured by inDrop. Thus, the ratio of the total seeded network (*R*) was estimated using the equations:

$$R(T) = \begin{cases} 0.6, & T \leq 12 \\ \frac{12}{T} * 0.6, & T > 12 \end{cases}$$

R was then calculated for 500, 1000, 2000, 3000, 4000, 5000, 10000, 15000, and 20000 seeded cells and 1-20, 40, 60, 80, 100, 150, and 200 connections per cell.

InDrop scRNA-seq analysis

Filtering. InDrop scRNA-seq data were processed using the InDrop pipeline made public on GitHub (https://github.com/indrops/indrops) (*117*). Reads were trimmed using Trimmomatic (v.0.39) and a customized Python script which removes polyA tails (the script was integrated into the InDrop pipeline). After trimming, reads were excluded if the length of trimmed reads is less than 16 bases or if half of the bases in reads are from one single base. The reads were excluded if the UMI contained an unknown base 'N'. The cell barcodes were filtered by two thresholds: (1) the cell barcode could not contain an N; and (2) the cell barcode must be in the cell barcode whitelist provided by the InDrop pipeline. During the mapping of cell barcodes to the cell barcode whitelist, one base difference was allowed and was corrected based on the cell barcode whitelist.

Alignment and quantification. Sequence alignment was done using Bowtie (*142*) and the reference genome was built from EMSEMBL GRCm38.p6. Quantification was finished using the InDrop pipeline after alignment. During the quantification process, reads that were aligned to more than 10 genes were excluded, and reads that shared one UMI while being mapped to different genes were excluded. After the quantification cells that had less than 500 genes detected were excluded.

Integration of samples, dimensionality reduction and unsupervised clustering of data. Samples were merged and batch effects were corrected using the canonical correlation approach (*143*) built in the R package Seurat. Normalized counts were calculated by SCTransform software (the

percentage of mitochondrial contents in each cell was regressed out during the process to prevent clustering bias owing to high mitochondrial contents cells). Next, 3,000 integration features were selected to integrate different samples and remove technical variability. After integration, the first 100 principal components were calculated and the first 40 are used to obtain the t-SNE plot and find neighborhood relationships between cells. The Louvain clustering algorithm was used to perform the unsupervised clustering of the cells.

Downstream analysis. After clustering, the model-based analysis of single cell transcriptomics (MAST) algorithm (*144*) was used to find differentially expressed genes in each cluster and condition. A graphics-based clustering analysis was conducted and cluster markers were used to identify different cell types with the R package SingleR (*145*). The average number of genes detected per cell was 1,045, the average number of UMIs detected per cell was 2,055, and the average number of reads per cell was 40,967.

Pooling of samples into mice. Cells from each mouse were split into samples of less than 6,000 cells at the time of cell encapsulation in droplets. Samples were processed separately during library prep and sequencing and recombined at the time of data processing. A mouse and sample ID were added to each cell barcode to avoid barcode collisions. A mouse ID was added to each RabΔG barcode to ensure no cross-mouse connections were assigned during network analysis.

Connectome barcode extraction and analysis. Rabies barcodes were recovered from libraries by extracting the 28 base sequence between known flanking handle sequences as follows. If only a 5' handle existed, 28 bases downstream were selected. If only the 3' handles existed, 28 bases

upstream were selected. If both handles were present, the entire internal sequence was extracted and confirmed to be 28 bases long. The structure of the barcode was checked using regex pattern matching and all sequences that did not conform to the designed sequence (VHDBVHDB**AT**VHDBVHDB**AT**VHDBVHDB) were removed from further analysis. Next, barcodes were error corrected at a Levenshtein distance of one using starcode with '--distance 1' (code available at https://github.com/gui11aume/starcode) (*146*). A count matrix of RabΔG barcodes was generated by UMI counting to create a table of RabΔG barcodes, UMI counts, and cell barcodes.

Rarefaction analysis. Rarefaction analysis was performed to ensure that sufficient sequencing depth was obtained in RABID-seq datasets. We randomly sampled 1k, 5k, 10k, 100k, and 500k reads from paired-end .fastq files and applied our pipeline for barcode recovery described above. The number of unique RabΔG barcode sequences was determined and plotted as a function of the number of input reads.

Network analysis. Analysis of RABID-seq datasets was performed in R using the igraph package (version 1.2.5) in R (*147*). Connectome data was used to generate graph objects with vertexes representing cells and edges representing shared rabies barcodes. For each vertex, edges were removed if they contained counts less than half of the maximum count in a given vertex. The strength of the edges was calculated as the average of the UMI counts for each shared rabies barcode. Vertexes were removed if they did not contain corresponding transcriptome information (i.e., if a cell barcode was not found in the scRNA-seq dataset). Vertexes were then assigned metadata corresponding to their cell type based on cell calling done with scRNA-seq data

(described above). Each vertex, which represented an individual cell, contained full transcriptome information (gene name and normalized counts). Summaries of connections by cell type in the form of chord diagrams were performed using the chorddiag package in R. Astrocyte-centered networks were visualized using the edge weights described above and the Fruchterman-Reingold layout. We centered the networks on astrocytes by removing connections between other cell types. This is justified because we genetically targeted *Gfap*-expressing cells with the EnvA-TVA system. Thus, astrocytes were the initial progenitors of virus, and shared RabΔG barcodes between other cells are result of their shared connections to an astrocyte. Analysis of the interactions between cells was performed by generating subnetworks based on the characteristics (cell type, gene expression, inflammation score, etc.) of vertices. First, we found 2 sets of vertices, for example astrocytes expressing *Il10ra* and T cells expressing *Il10*. Next we extracted the edges between these sets. Lastly, we generated subgraphs from edge lists to create subnetworks that contained cells with only the desired properties (astrocytes expressing *Il10ra* connected to T cells expressing *Il10).* We inferred possible ligand receptor interactions using CellPhoneDB (*148*) on cells extracted from subnetworks.

Pathway analysis. GSEA pre-ranked analyses (*149*) were used to generate enrichment of gene sets in subsets of cells extracted from the network analysis. Genes were ranked based on log(FoldChange) differences in gene expression between two cell populations, for example astrocytes connected to microglia in EAE vs. astrocytes connected to microglia in naïve. GSEA analysis used the KEGG/Reactome/Biocarta (c2.cp.all), Gene ontology (c5.cp.all), and Hallmark (h.all) gene sets from Molecular Signatures Database v7.1. Overrepresented transcriptional motifs

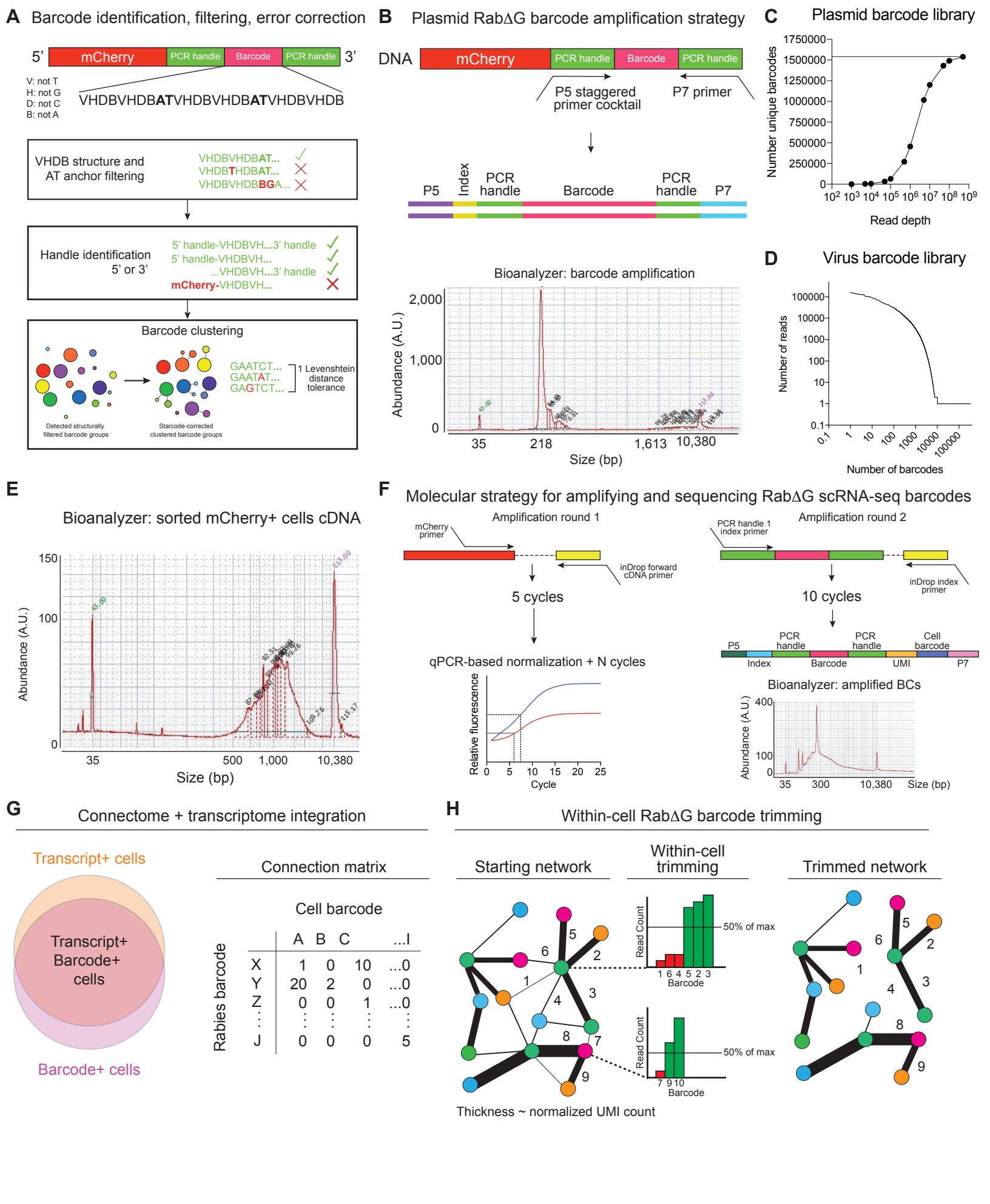
and pathways were found using Ingenuity Pathway Analysis (IPA) software (Qiagen) and ENRICHR (*150, 151*) on lists of differentially expressed genes.

Astrocyte pro-inflammatory score. An inflammation score was calculated for each astrocyte based on its gene expression profile. The inflammation score was determined by first ranking each gene by its scaled counts. The sum of the rank of each gene in gene set M15877 (GO: POSITIVE_REGULATION_OF_INFLAMMATORY_RESPONSE) minus the sum of the rank of each gene in the gene set M13807 (GO: NEGATIVE_REGULATION_OF_INFLAMMATORY_RESPONSE) was calculated. This inflammation score was used to bin astrocytes; those in the bottom 10% and top 90% were extracted from the full network and their adjacent vertices were determined. These subnetworks represented cells connected to astrocytes expressing high pro-inflammatory and low pro-inflammatory transcriptional programs. Differential expression on cells in these subnetworks was performed using Seurat, and the corresponding gene lists were analyzed by GSEA pre-ranked analyses as described above. The same method was used to generate a score for the Ephrin pathway using the gene set M13554 (GO_EPHRIN_RECEPTOR_SIGNALING_PATHWAY).

Quantification and statistical analysis

Statistical analyses were performed with Prism 8 software (GraphPad), using the indicated test and results were considered statistically significant at $p<0.05$. A linear regression slope test was applied for EAE clinical scores in the NOD model only. Multiple independent experiments were performed for all in vitro assays. No statistical methods were used to pre-determine sample sizes. Allocation of samples into groups was randomized. No mice were excluded. All statistical

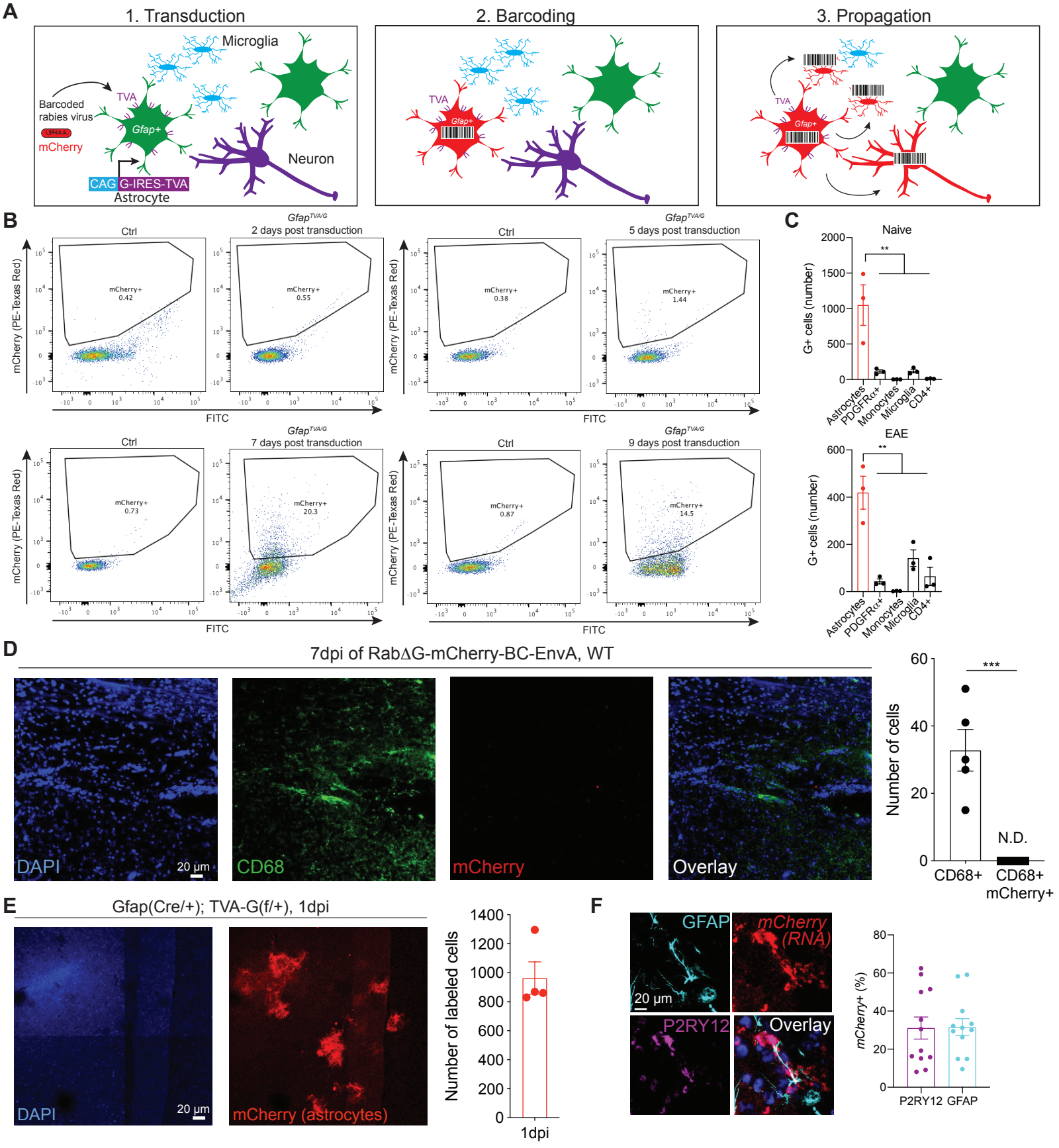
tests, comparisons, and sample sizes are included in the Figures and Figure Legends. All data are shown as mean±SEM.



Supplementary Figure 1

Fig. S1

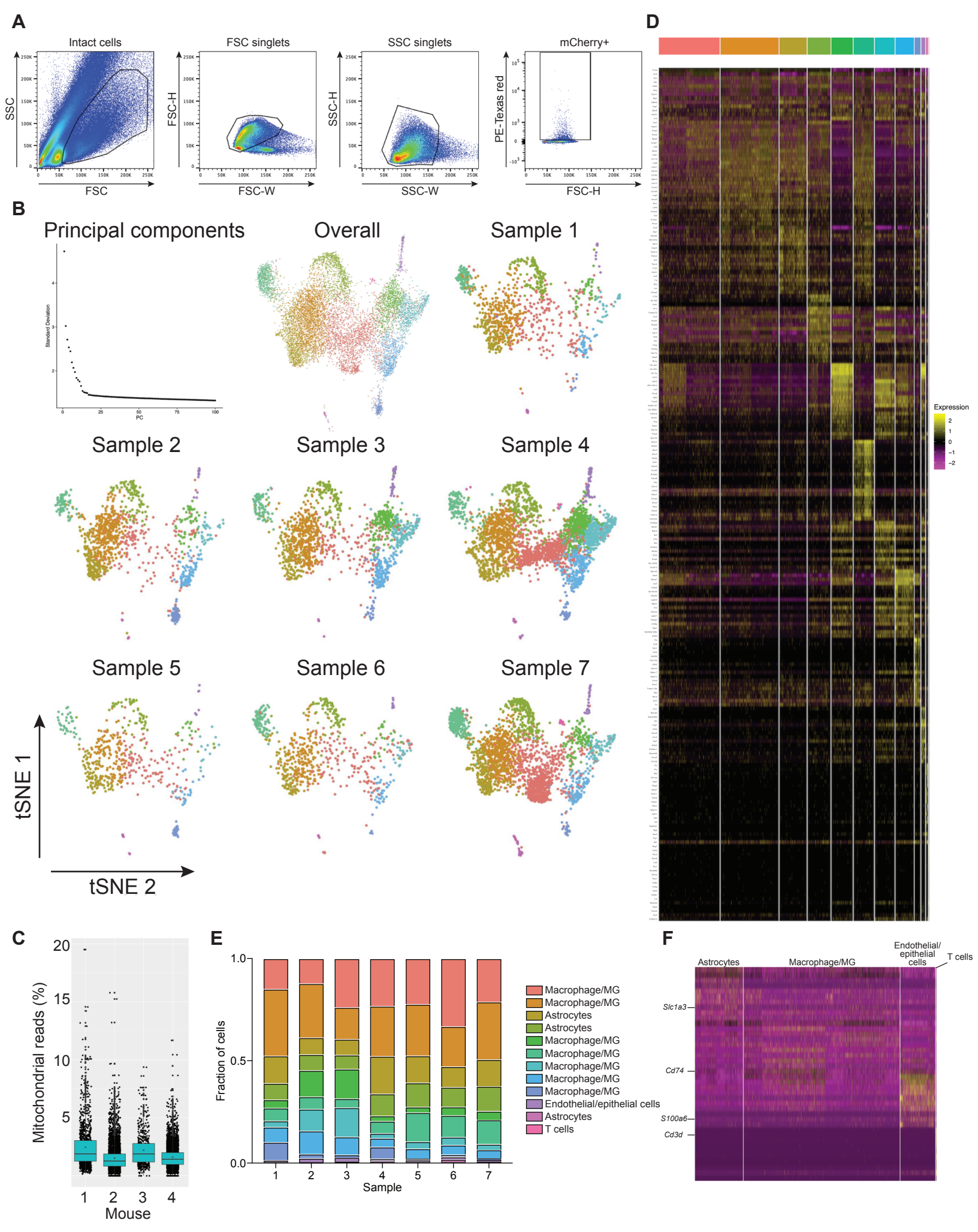
Rabies virus library barcode design, recovery, and analysis pipeline. (**A**) Nucleotide structure of the mCherry barcode. Barcodes were designed using the 28-base sequence VHDBVHDB**AT**VHDBVHDB**AT**VHDBVHDB that contained three variable stretches encoded by the degenerate bases (IUPAC nucleotide code): V is not T; H is not G; D is not C; B is not A; and two AT anchors. Bioinformatic recovery of the barcode from sequencing reads identified the presence of conserved 5' or 3' PCR handles (18 bp each), extracted the intervening 28 bases, filtered sequences with the correct VHDBVHDB structure between AT anchors, and error corrected barcodes using a Levenshtein distance of one. Note: base pair lengths not to scale. (**B**) Top: amplification of barcodes from plasmid libraries used primers that target conserved handles flanking the barcode, generating a sequencing-ready Illumina library. Bottom: Bioanalyzer trace of a representative library. (**C**) Number of unique barcodes recovered after bioinformatic processing versus sub-sampled read depth showed sequencing saturation and an approximate plasmid library barcode diversity of 1.5 million unique sequences. (**D**) After viral packaging and EnvA pseudotyping, the diversity of the viral library was estimated at approximately 10,000-100,000 unique sequences. (**E**) Representative Bioanalyzer trace of whole transcriptome amplified cDNA. (**F**) Molecular strategy for recovering RabΔG barcodes from amplified cDNA. Step one: limited cycle (~5) and qPCR quantification (N cycles) was followed by a semi-nested 10-cycle PCR primed off one of the flanking handles. A representative Bioanalyzer trace from a sequencing-ready library is shown. (**G**) Cells containing both transcriptome and RabΔG barcode information were retained for analysis. (**H**) Networks were generated and trimmed based on UMI counts of shared barcodes to create final networks. Vertices represent cells, edges represent shared rabies barcodes.



Supplementary Figure 2

Fig. S2

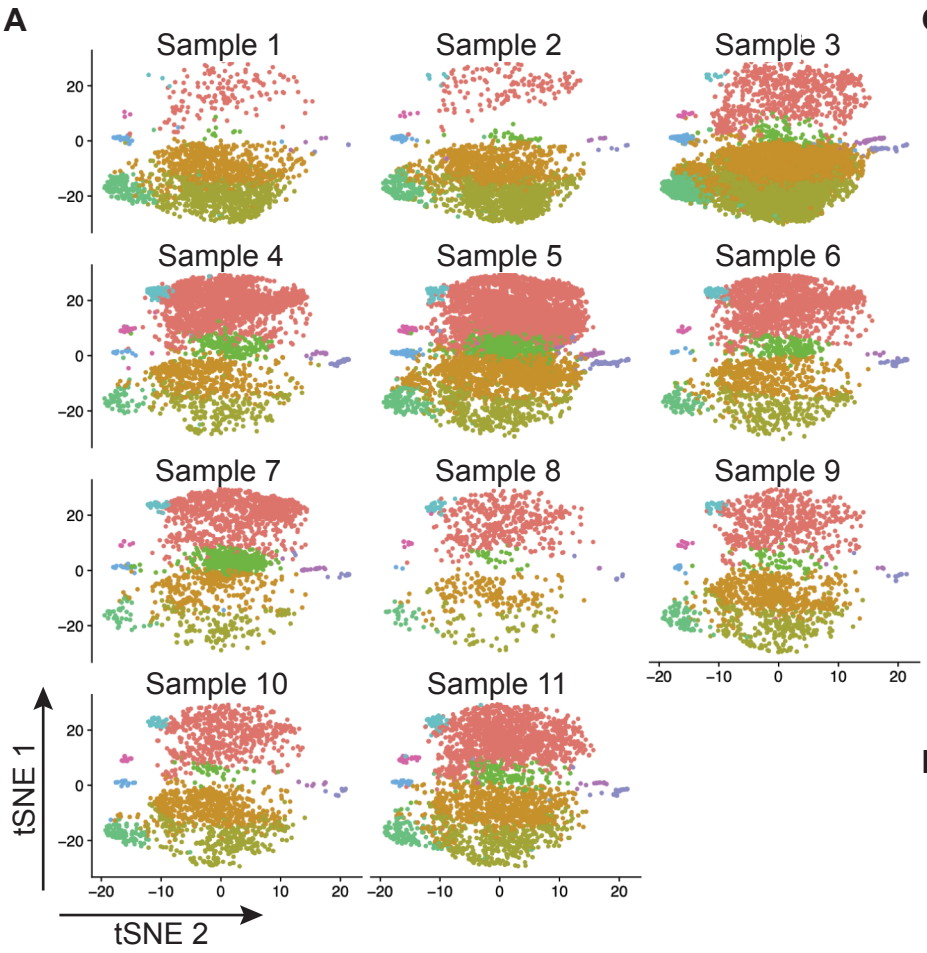
RABID-seq control analyses. (**A**) Schematic of RabΔG barcoding of glia. EnvA pseudotyped rabies virus infects TVA-expressing astrocytes. G-expressing astrocytes produce functional rabies virus that infects neighboring cells but cannot be spread by glycoprotein G-deficient connected cells. (**B**) FACS of mCherry^{+} cells from non-transduced wildtype (WT) and *Gfap*$^{TVA/G}$ mice transduced with EnvA-pseudotyped RabΔG-mCherry-BC virus and analyzed at different times post transduction. **(C)** FACS analysis of rabies glycoprotein G levels in *Gfap*$^{TVA/G}$ mice as a function of cell type staining. n=3 per group. One-way ANOVA, Dunnett post-test. (**D**) Staining of brain tissue from WT mice transduced with EnvA pseudotyped RabΔG-mCherry-BC virus and analyzed at 7 days post-transduction shows negligible background mCherry staining detected in CD68^{+} macrophages. n=5 per group. Unpaired two-tailed t-test. N.D.: not detected. (**E**) Staining of *Gfap*$^{TVA/G}$ mice brain tissue used to titer EnvA pseudotyped RabΔG-mCherry-BC virus to infect 1000 cells at 1 day post-transduction. n=4 mice. **(F)** RNAscope analysis of *mCherry* expression and GFAP or P2RY12 immunostaining. n=12 per group. Data shown as mean±SEM. ***p<0.001.

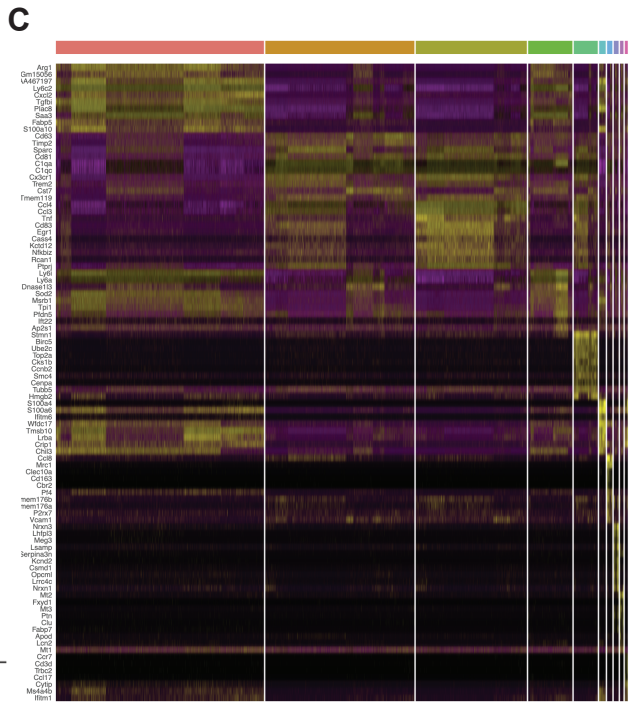


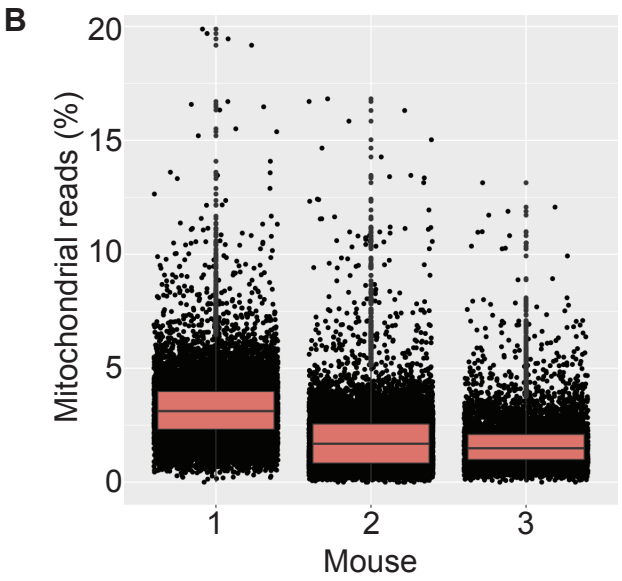
Supplementary Figure 3

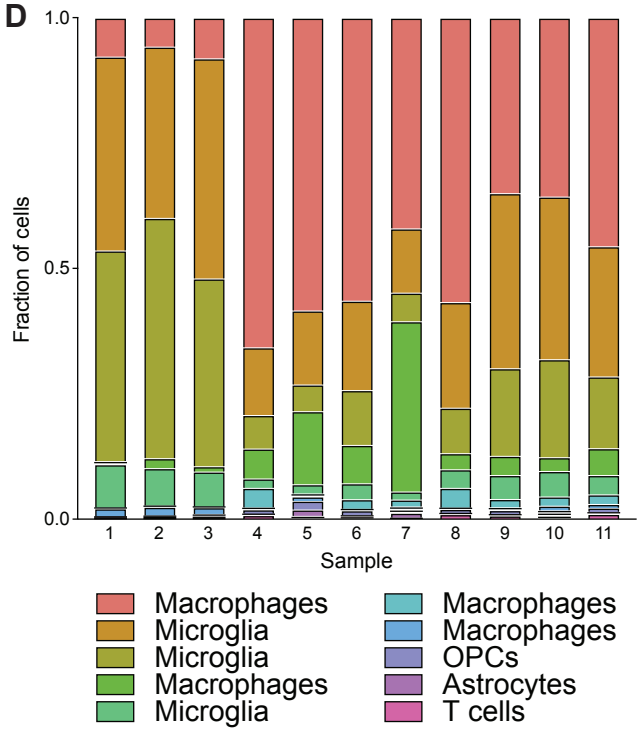
Fig. S3

Control analyses for RABID-seq in naïve mice. (**A**) FACS gating strategy used for the isolation of mCherry^{+} cells. (**B**) Principal components and tSNE plots of naïve samples analyzed in this study. **(C)** Percent mitochondrial reads per cell per mouse. (**D**) Significantly enriched genes by tSNE cluster. (**E**) Distribution of tSNE clusters by sample. (**F**) Significantly enriched genes by cell type.





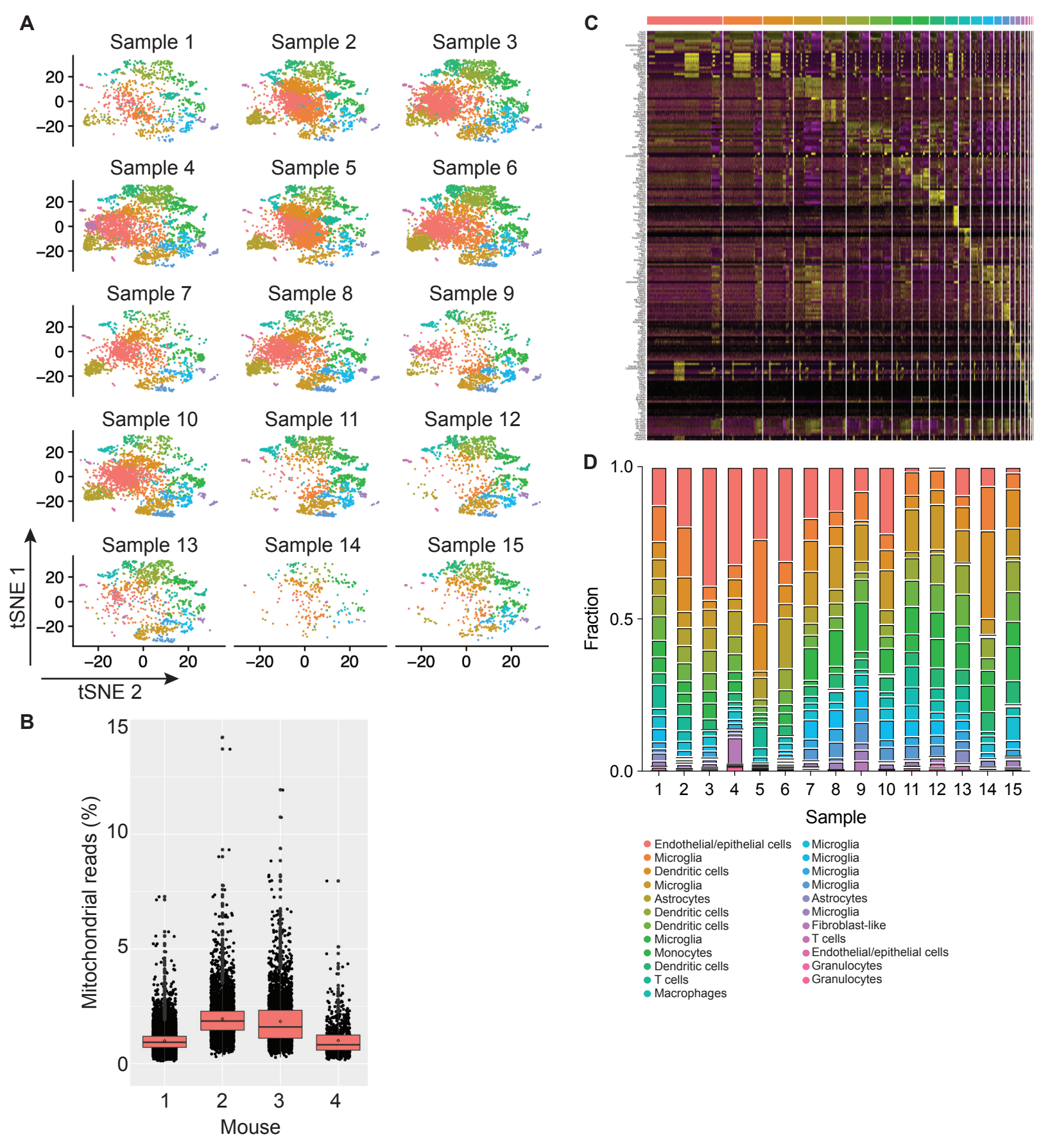




Supplementary Figure 4

Fig. S4

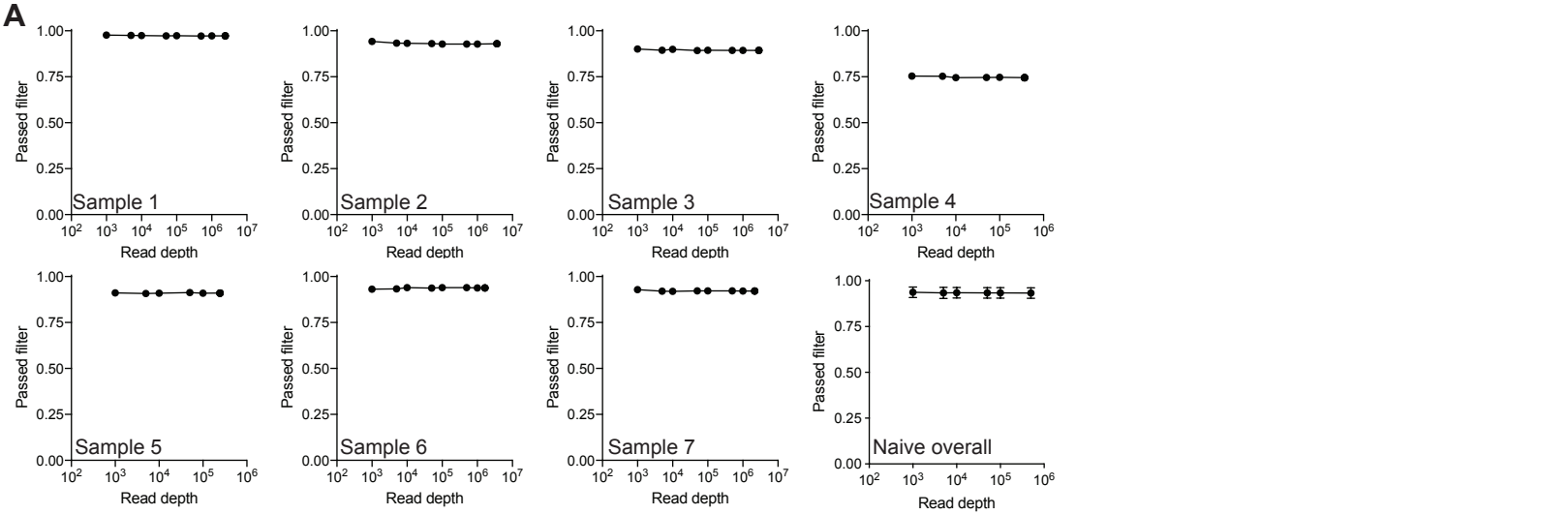
Control analyses for RABID-seq in EAE priming mice. (**A**) tSNE plots by sample. **(B)** Percent mitochondrial reads per cell per mouse. (**C**) Significantly enriched genes by tSNE cluster. (**D**) Distribution of tSNE clusters by sample.

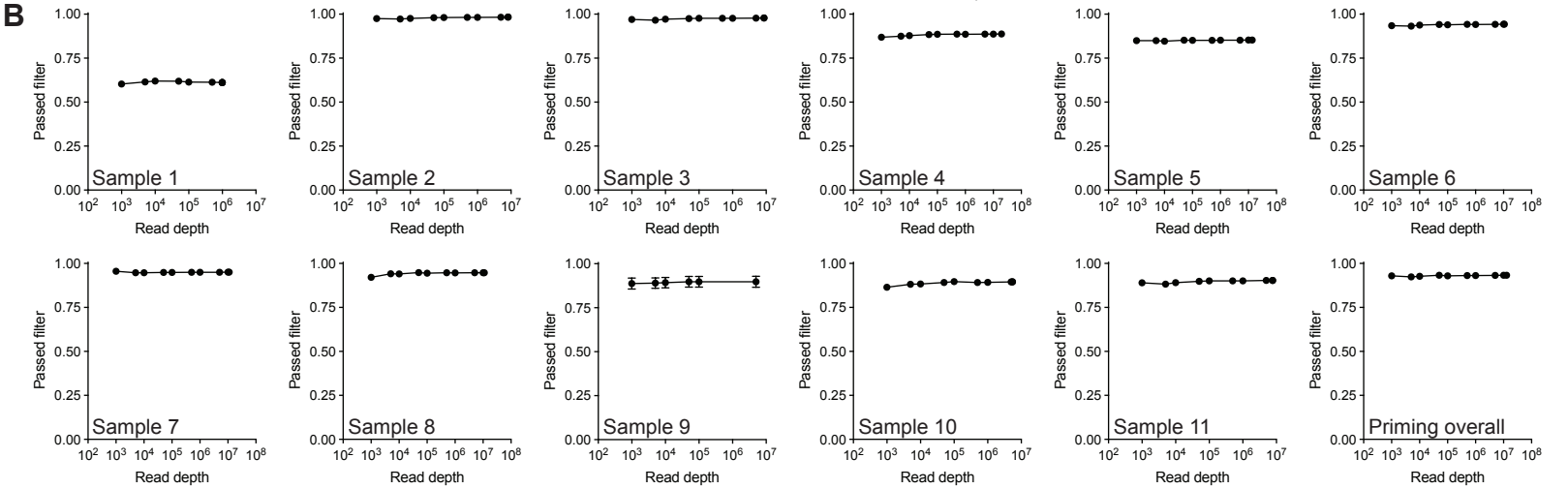


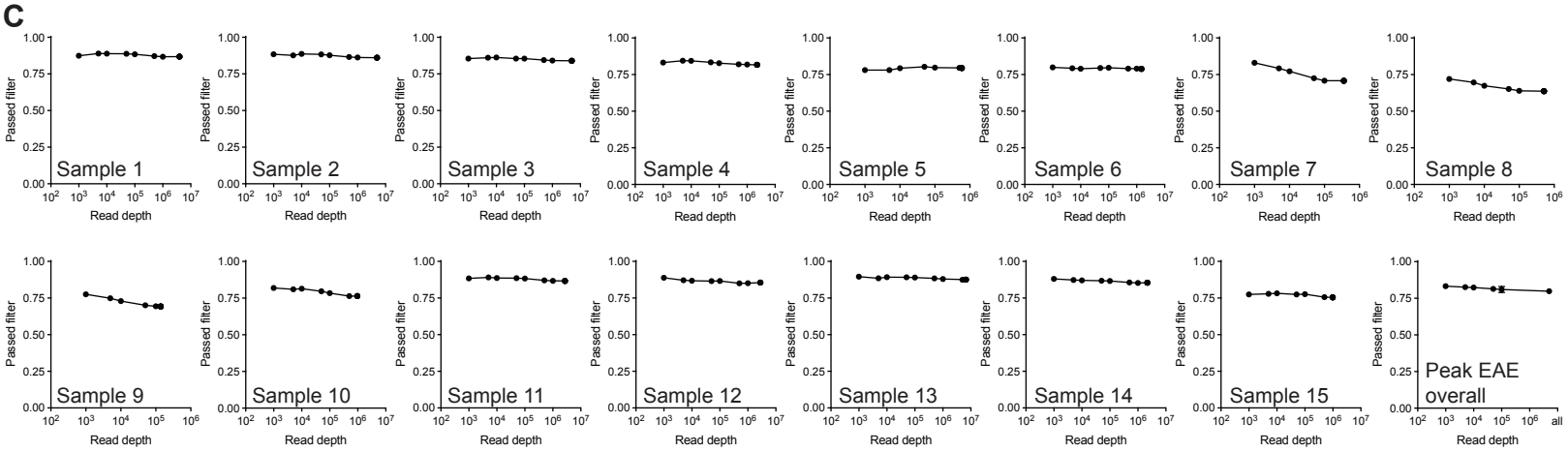
Supplementary Figure 5

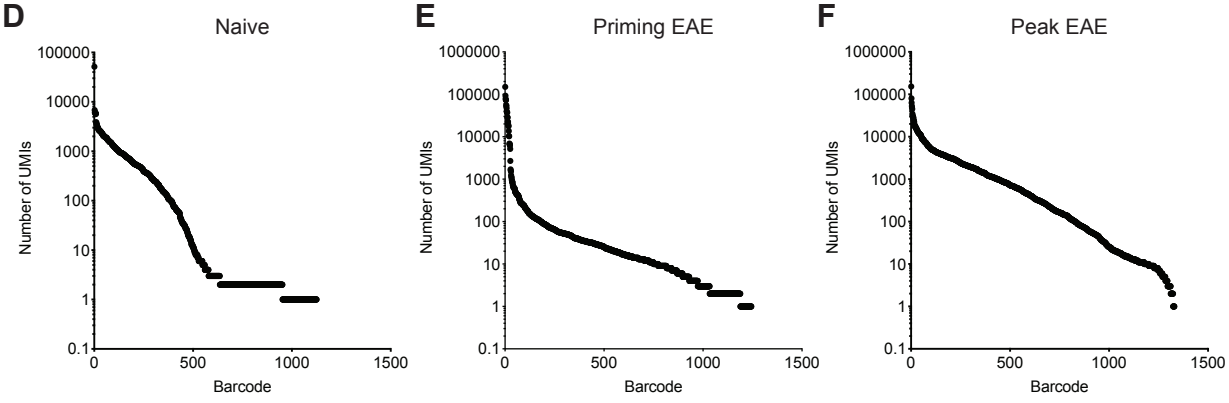
Fig. S5

Control analyses for RABID-seq in peak EAE mice. (**A**) tSNE plots by sample. **(B)** Percent mitochondrial reads per cell per mouse. (**C**) Significantly enriched genes by tSNE cluster. (**D**) Distribution of tSNE clusters by sample.





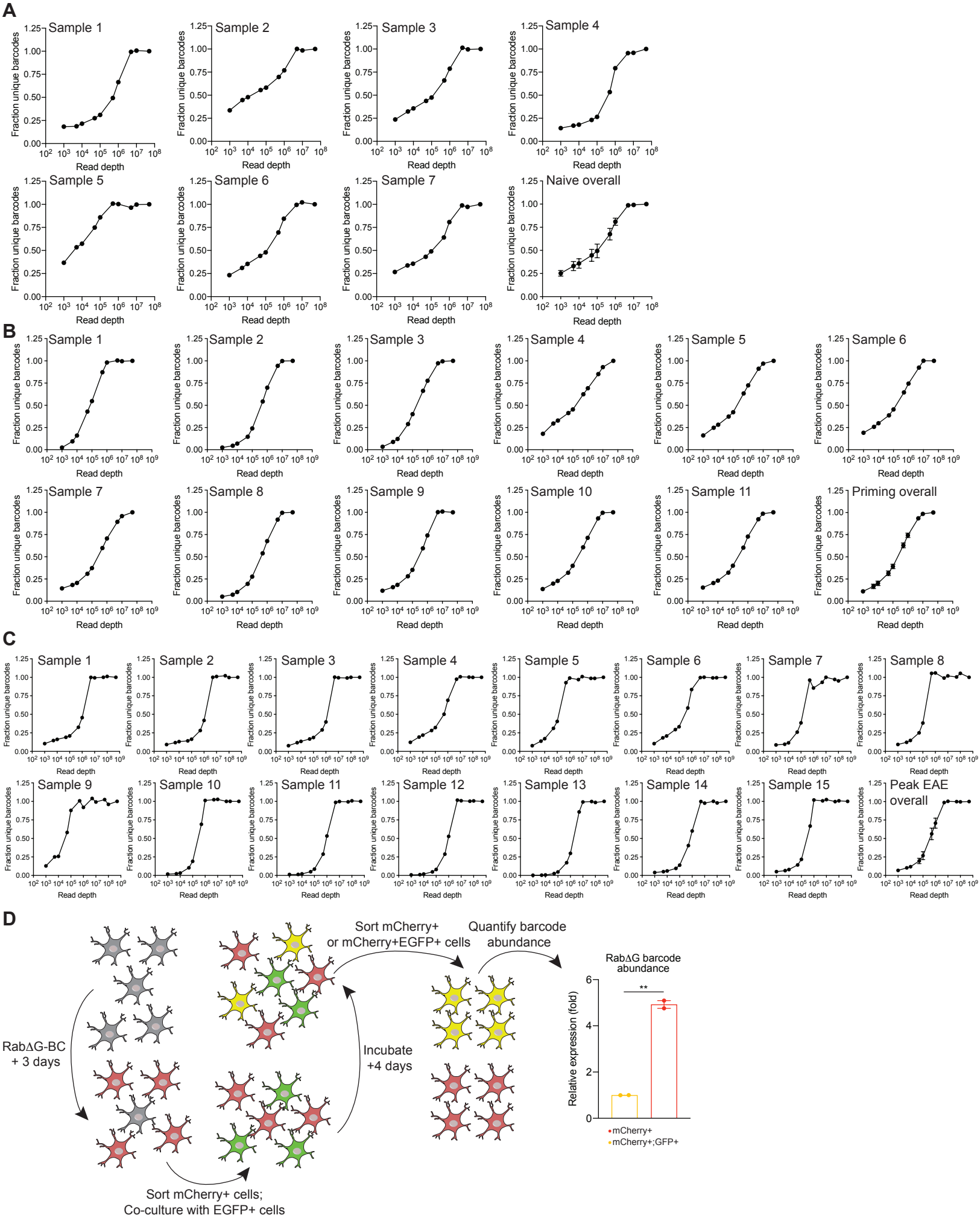




Supplementary Figure 6

Fig. S6

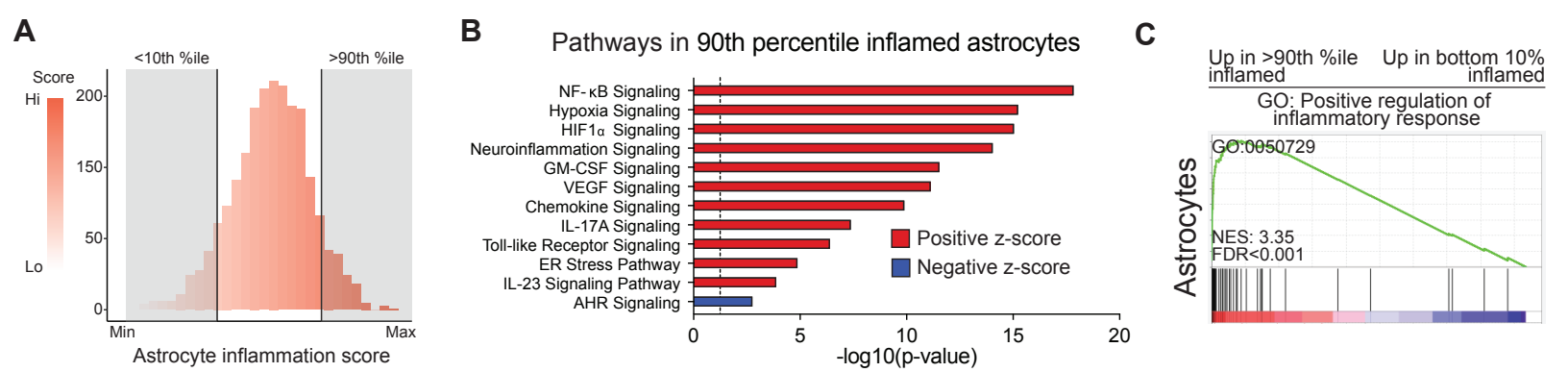
Analysis of barcode quality as a function of read depth. Reads that passed barcode structural filter as a function of sequencing depth. Reads were randomly subsampled to perform this analysis. (**A**) Naïve mice. **(B)** Priming EAE mice. (**C**) Peak EAE mice. **(D-F)** Rank abundance plots of unique barcode abundance per mouse at different EAE stages. The infection points of these curves correspond to roughly 1,000 barcodes.

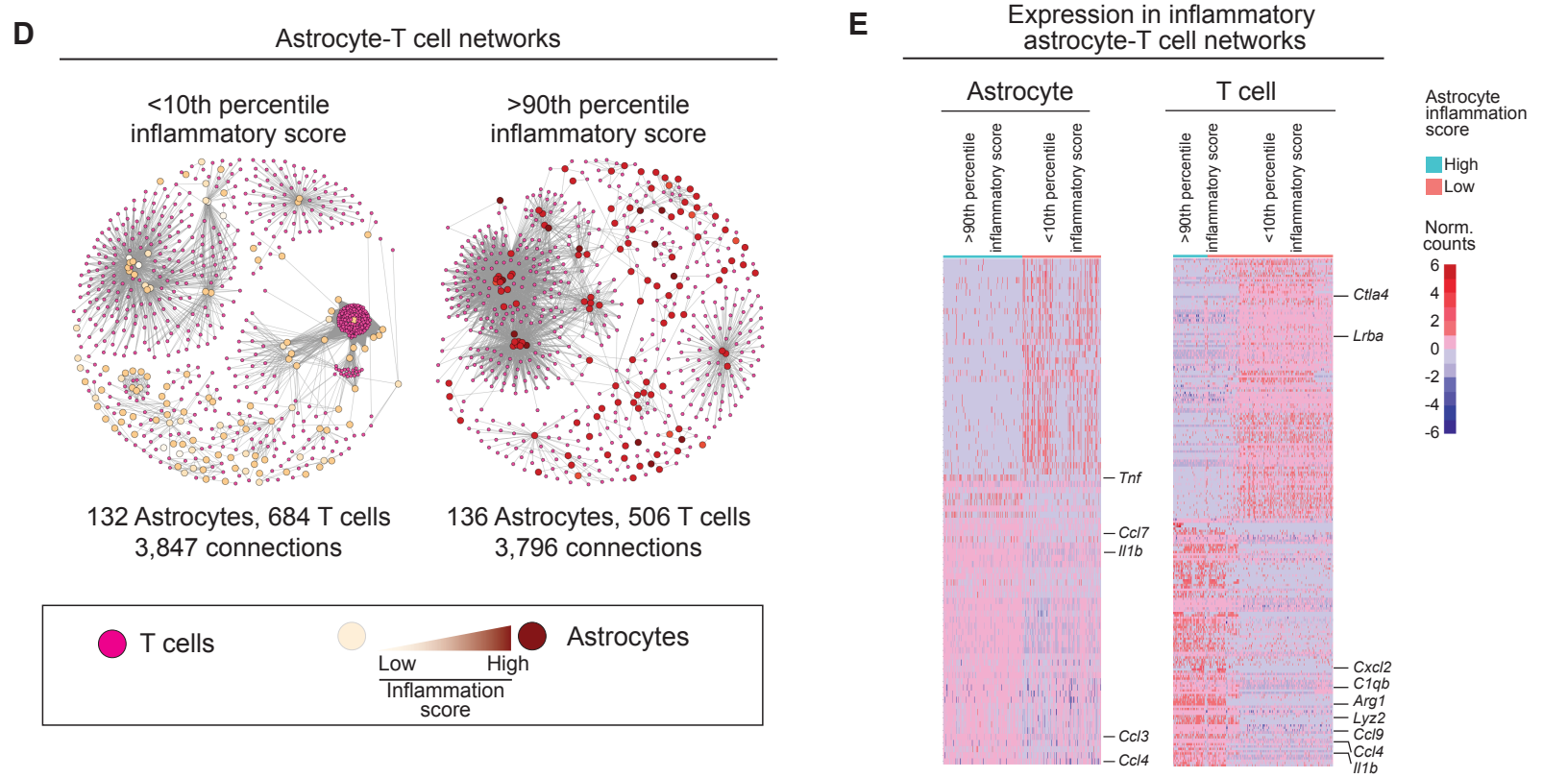


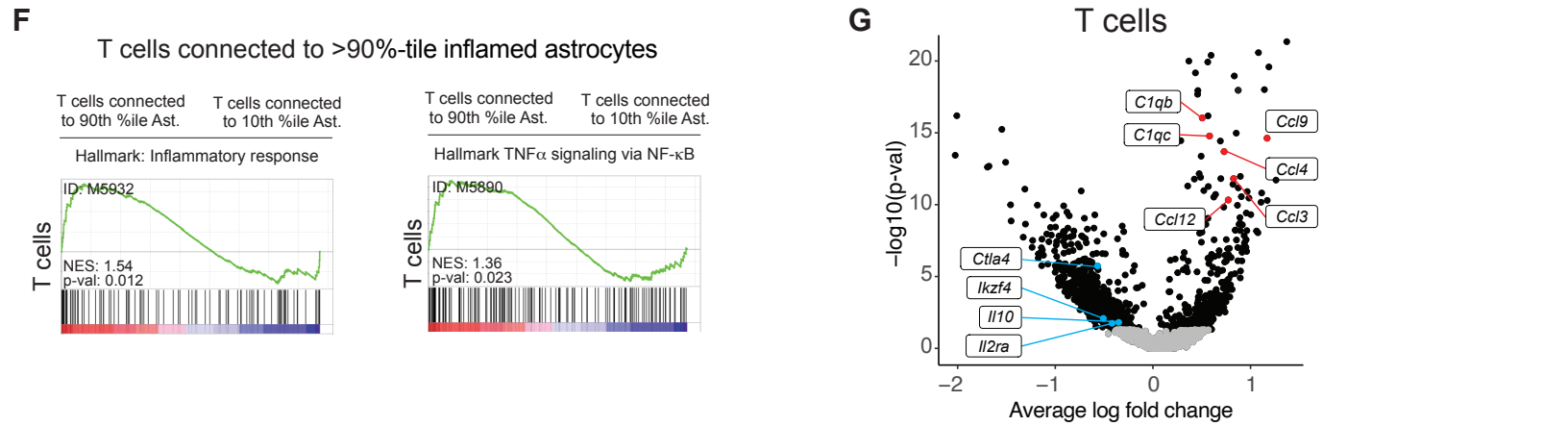
Supplementary Figure 7

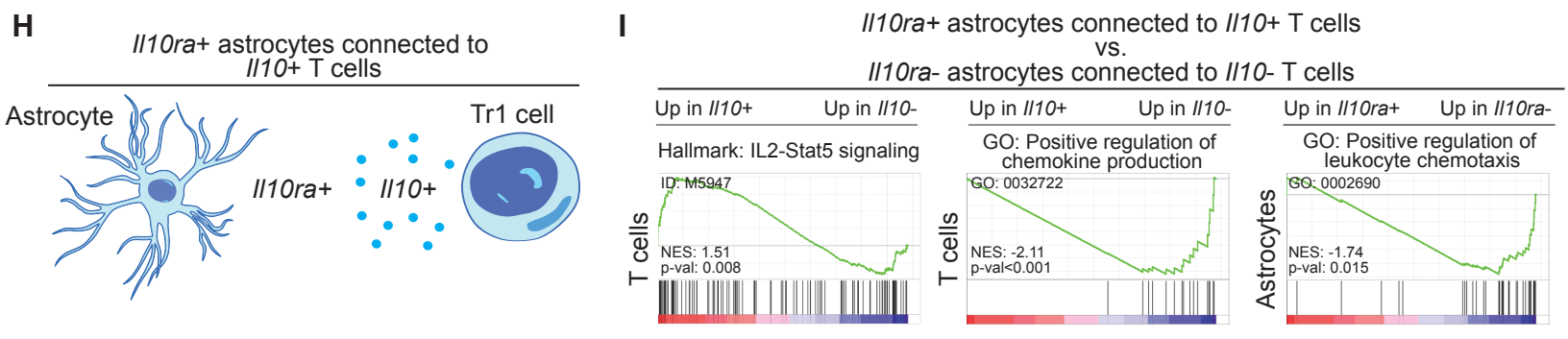
Fig. S7

Control analyses of rabies barcode sequencing. (**A-C**) Analysis of RabΔG barcode sequencing read depth for naïve (A), priming EAE (B), and peak EAE (C) samples. The fraction of reads that passed barcode identification, filtering, and error correction is plotted as a function of sequencing read depth. Reads were randomly subsampled to perform this analysis. (**D**) Schematic (left) and analysis (right) of barcode abundance as a function of time after infection. Experiment performed with n=3 biological replicates pooled, sorted, and captured by inDrop. Graph shows n=2 technical replicates from this experiment. Unpaired two-tailed t-test. Data shown as mean±SEM. **p<0.01.





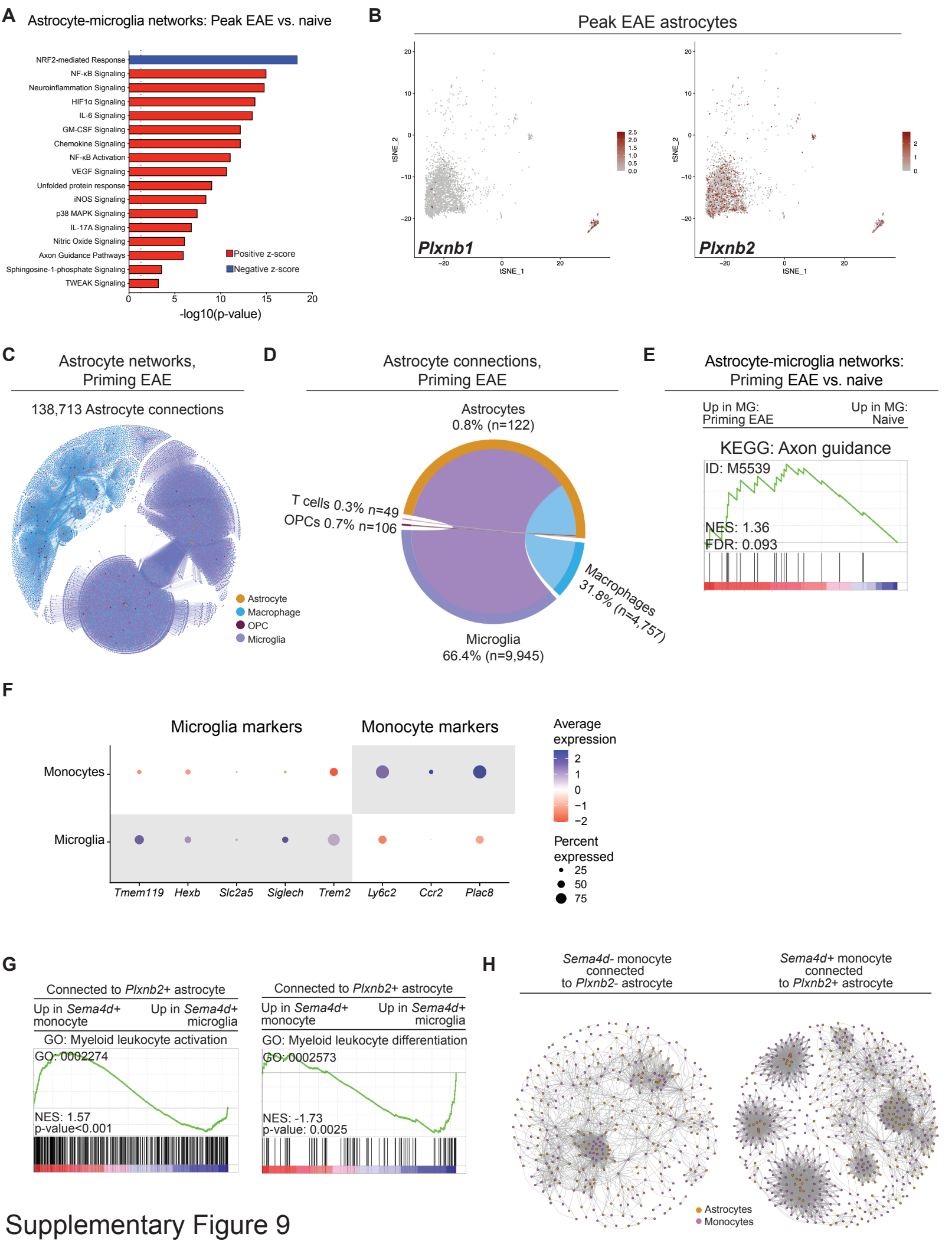




Supplementary Figure 8

Fig. S8

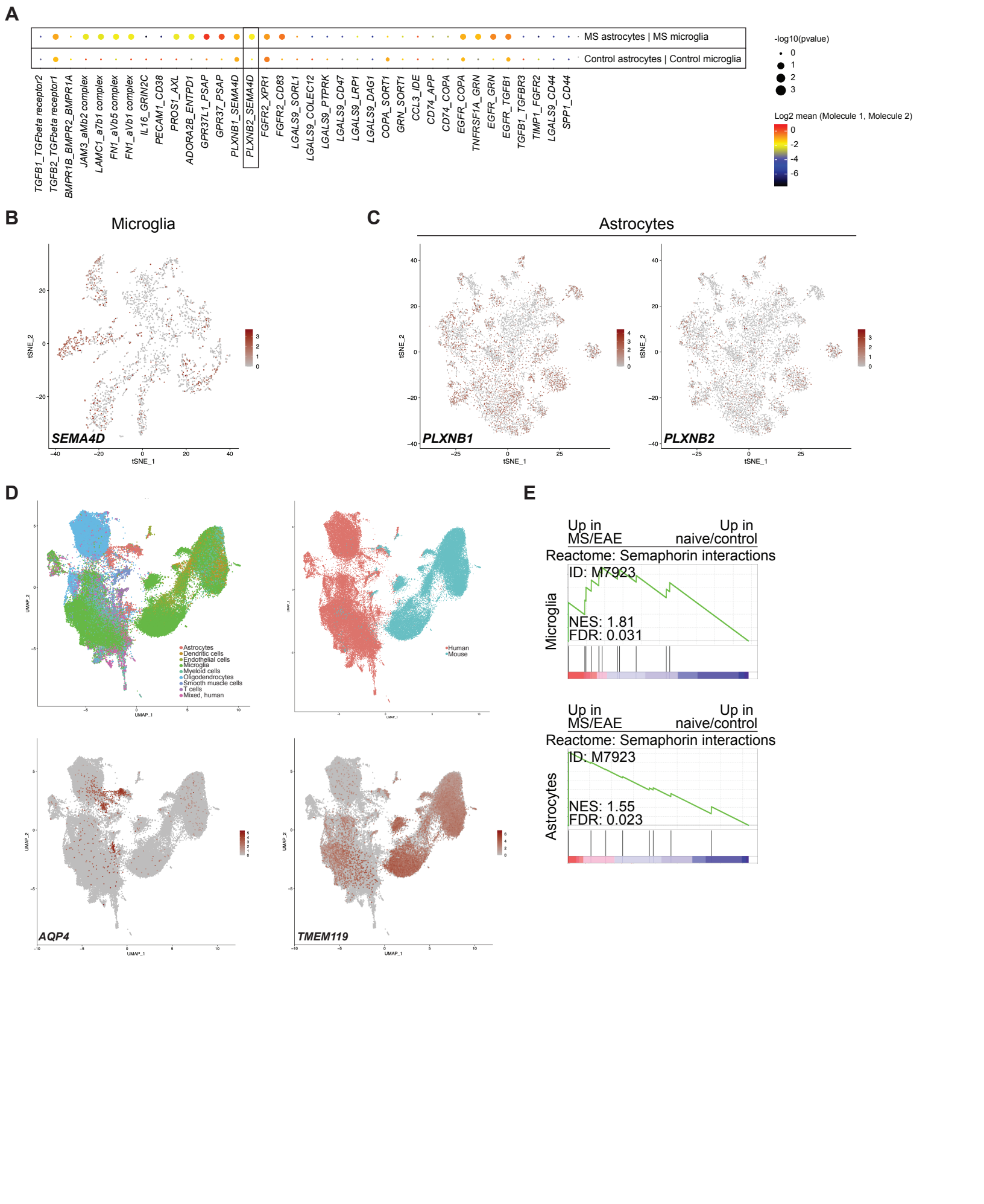
Gene expression analysis of astrocyte-T cell interactions in peak EAE. (A) Distribution of inflammation scores assigned to astrocytes captured by RABID-seq. **(B)** Pathways enriched in the 90^{th} percentile inflamed astrocytes versus the bottom 10^{th} percentile inflamed astrocytes. **(C)** GSEA pre-ranked analysis of scRNA-seq data comparing the $>90^{th}$ percentile inflamed astrocytes to the $<10^{th}$ percentile inflamed astrocytes. **(D)** Subnetworks of astrocytes binned based on their inflammation score ($<10^{th}$ vs. $>90^{th}$ percentile) and interacting with T cells. **(E)** Heatmap of differentially expressed genes (p.adj < 0.05) in astrocyte-T cell subnetworks. Left: $> 90^{th}$ percentile inflamed astrocytes vs. $<10^{th}$ percentile inflamed astrocytes. Right: T cells connected to $>90^{th}$ percentile inflamed astrocytes vs. connected to $<10^{th}$ percentile of inflamed astrocytes. **(F)** GSEA pre-ranked analysis of RABID-seq data from astrocytes connected to T cells vs. astrocytes not connected to T cells. **(G)** Volcano plot of RABID-seq data comparing T cells connected to $>90^{th}$ percentile pro-inflammatory astrocytes to T cells connected to $<10^{th}$ percentile pro-inflammatory astrocytes. Genes with p>0.05 are colored as gray. **(H)** Schematic of *Il10ra*$^{+}$ astrocytes interacting with *Il10*$^{+}$ T cells. **(I)** GSEA pre-ranked analysis of RABID-seq data comparing: *Il10*$^{+}$ T cells connected to *Il10ra*$^{+}$ astrocytes vs. *Il10*$^{-}$ T cells connected to *Il10ra*- astrocytes (left, middle); and *Il10ra*$^{+}$ astrocytes connected to *Il10*$^{+}$ T cells vs. *Il10ra*$^{-}$ astrocytes connected to *Il10*$^{-}$ T cells (right). GO: gene ontology.



Supplementary Figure 9

Fig. S9

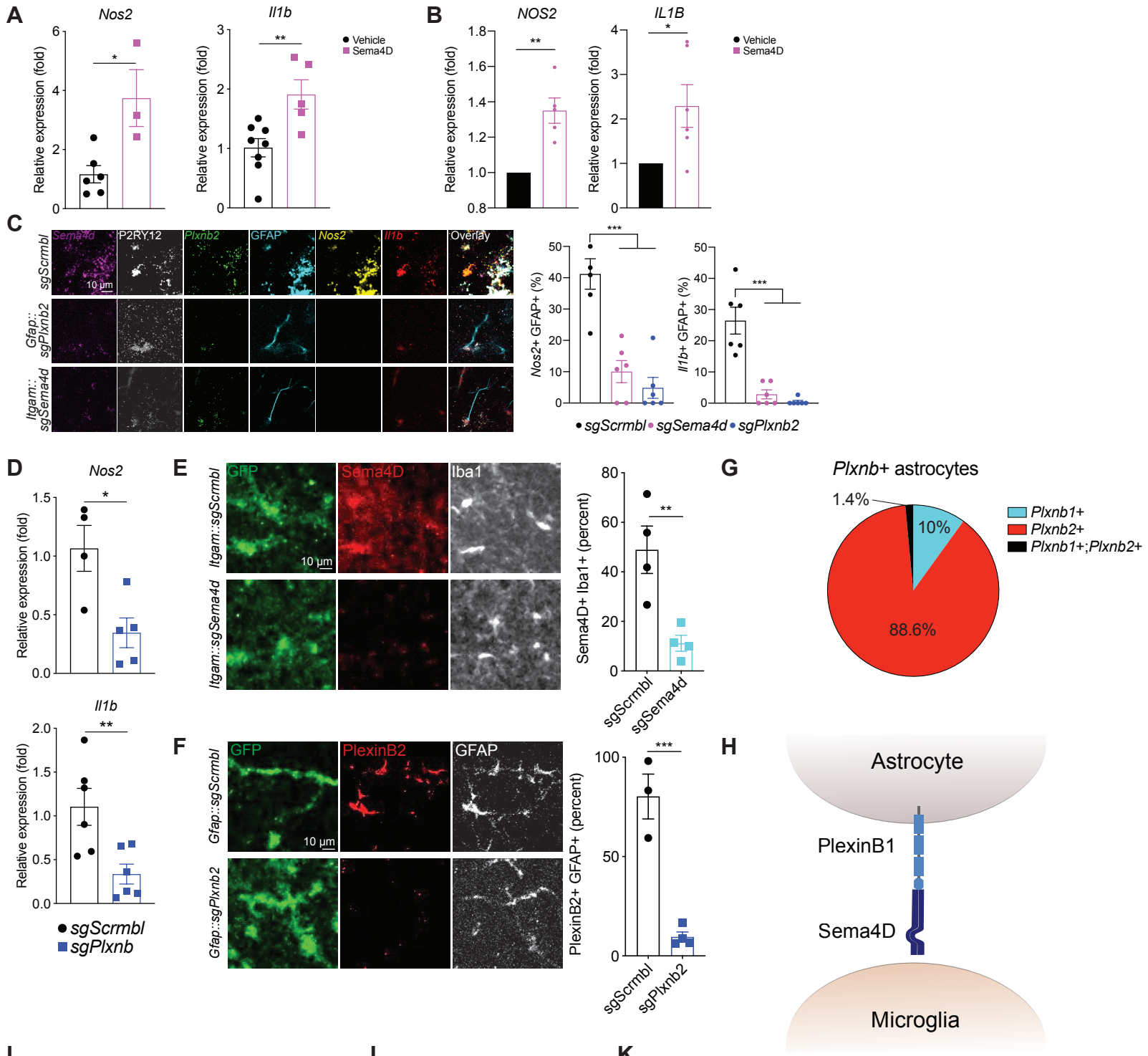
Analysis of Sema4D-PlexinB2 signaling. (**A**) Astrocyte pathways regulated in astrocyte-microglia networks during peak EAE vs. naïve mice. (**B**) tSNE plots of *Plxnb1* and *Plxnb2* expression in peak EAE astrocytes. **(C-D)** Visualization (C) and circus plot (D) of astrocyte connections in the EAE priming phase. **(E)** Analysis of axon guidance pathways in microglia connected to astrocytes in priming phase vs. naïve. **(F)** Comparison of canonical markers expressed by microglia and monocytes captured by RABID-seq. **(G)** Pre-ranked GSEA analysis comparing *Plxnb2*+ astrocytes connected to *Sema4d*+ monocytes or microglia. **(H)** Visualization of *Plxnb2*+ astrocyte networks connected to *Sema4d*+ monocytes.



Supplementary Figure 10

Fig. S10

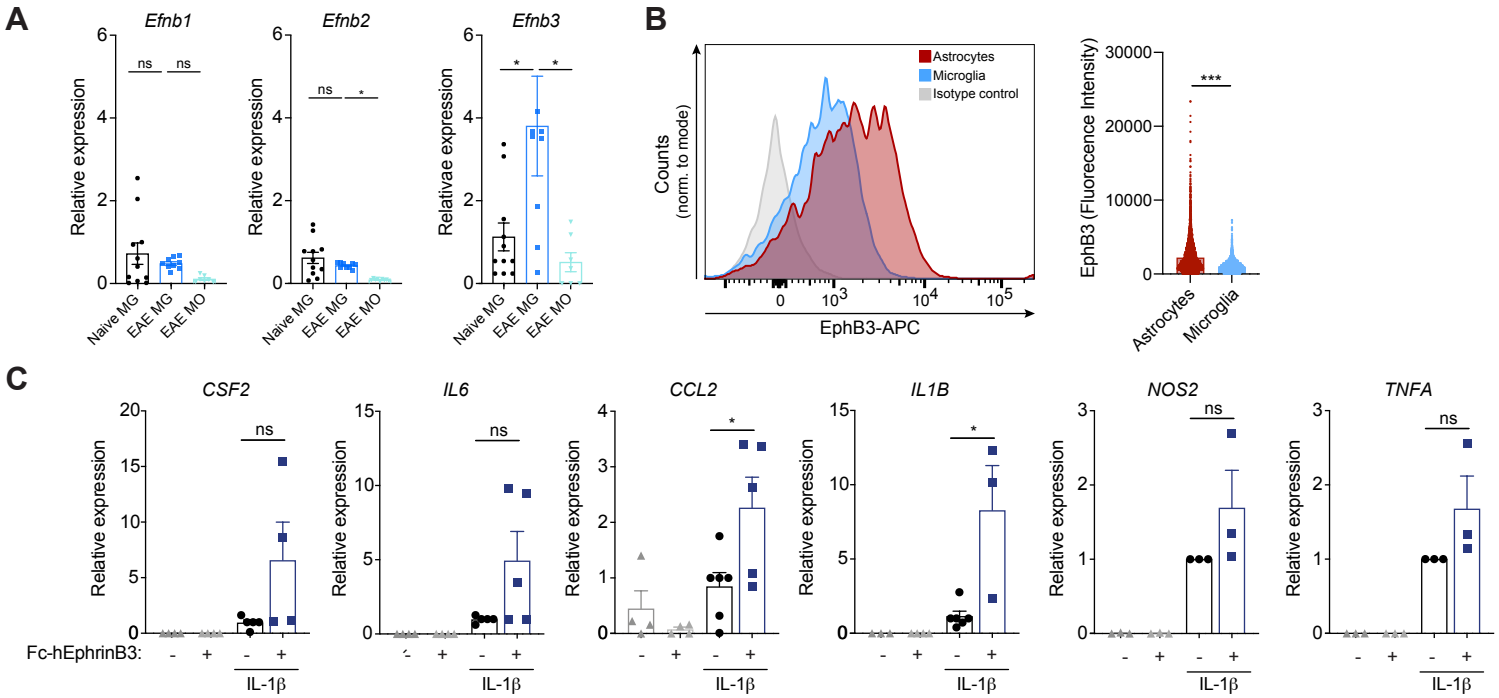
Detection of a Sema4D-PlexinB2 signature in MS. (**A**) CellPhoneDB analysis of astrocytes and microglia isolated from the CNS of MS patients and non-MS controls. Data obtained from the previous published studies in Refs. (*3, 152-154*) and integrated. (**B-C**) Gene expression of *SEMA4D* in microglia (B) and *PLXNB1* and *PLXNB2* in astrocytes (C) overlaid on tSNE subclustering plots. **(D-E)** Merging of previously published human scRNA-seq data from (A) and the RABID-seq data generated in this manuscript. **(D)** tSNE plots showing overlap of mouse and human astrocytes and microglia. **(E)** GSEA analysis showing semaphorin pathway activation in MS/EAE cells compared to healthy control/naïve in humans and mice.



Supplementary Figure 11

Fig. S11

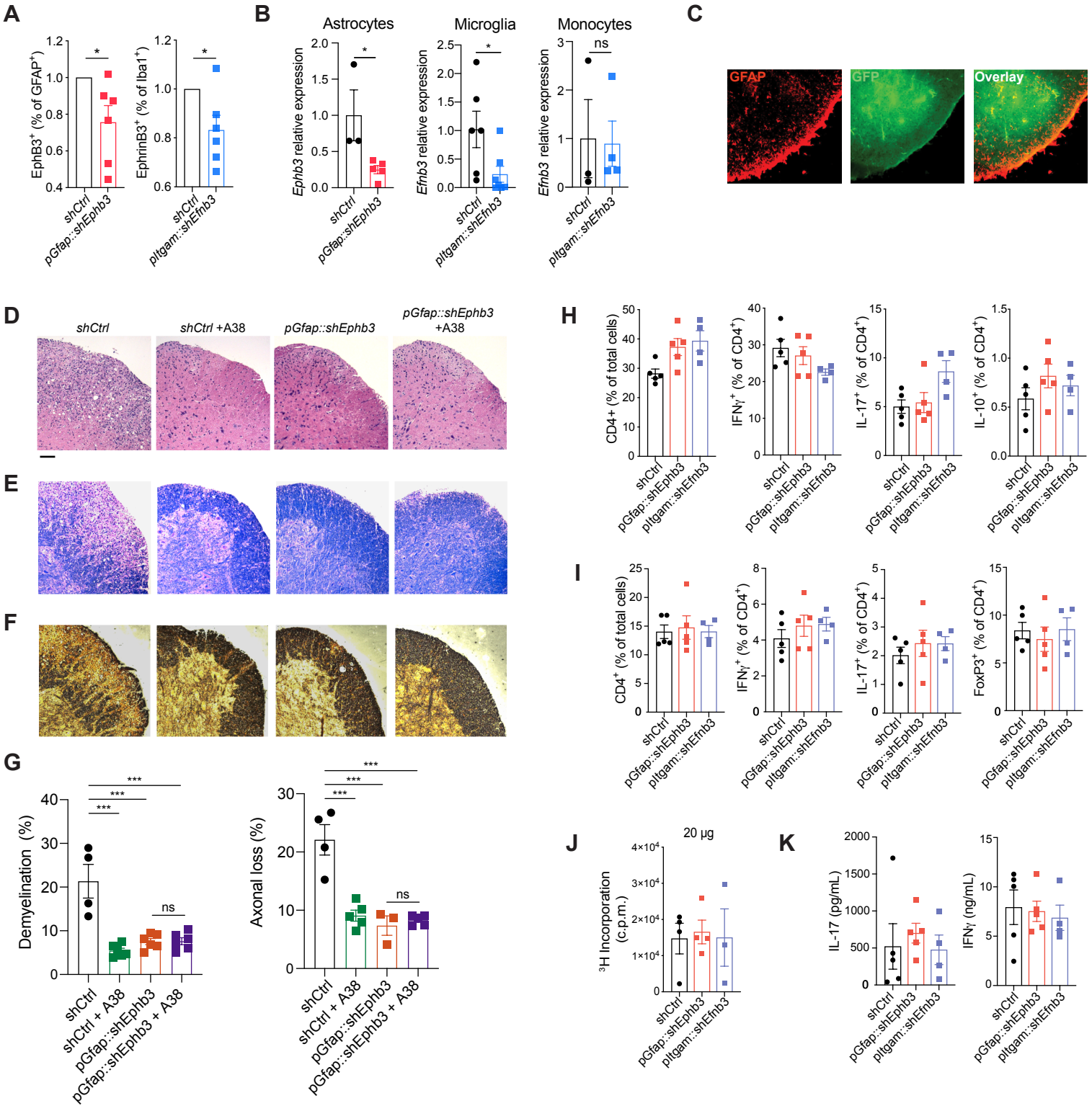
PlexinB signaling in EAE. (A) *Nos2* and *Il1b* expression determined by qPCR in primary mouse astrocytes under serum-free conditions. *Nos2*: n=6 vehicle, n=3 Sema4D; *Il1b*: n=8 vehicle, n=5 Sema4D. Unpaired two-tailed t-test. **(B)** Primary human fetal astrocytes cultured under serum-free conditions and analyzed by qPCR. n=5 *Nos2*, n=6 *Il1b*. One-sample t-test relative to vehicle. **(C)** RNAscope analysis of *Nos2* and *Il1b* expression in astrocytes following microglia *Sema4d* knockdown or astrocyte *Plxnb2* knockdown. n=6 per group. One-way ANOVA, Dunnett post-test. **(D)** *Nos2* and *Il1b* expression determined by qPCR in astrocytes isolated from knockdown mice. *Nos2*: n=4 *sgScrmbl*, n=5 *sgPlxnb*; *Il1b*: n=6 per group. Unpaired two-tailed t-test. **(E)** Validation of Sema4D knockdown in microglia. Left: Levels of GFP (Cas9), Sema4D, and Iba1 determined by immunostaining in *Itgam::sgSema4d* and *Itgam::sgScrmbl* mice. Right: Quantification of Sema4D+ Iba1+ cells, n=3-4 per group. Unpaired two-tailed t-test. **(F)** Validation of PlexinB2 knockdown in astrocytes. Left: Levels of GFP (Cas9), PlexinB2, and GFAP determined by immunostaining in *Gfap::sgPlxnb2* and *Gfap::sgScrmbl* mice. Right: Quantification of PlexinB2+ GFAP+ cells, n=3-4 per group. Unpaired two-tailed t-test. (**G**) Analysis of the distribution of *Plxnb1*- and *Plxnb2*-expressing astrocyte subpopulations during EAE analyzed by RABID-seq. (**H-I**) Schematic (H) and knockdown during peak EAE (I) of *Plxnb1* in astrocytes. n=10 *sgScrmbl*, n=5 *sgPlxnb1*. Two-way repeated measures ANOVA. (**J**) RNA-seq analysis of astrocytes isolated from *Gfap*::*sgScrmbl* vs. *Gfap*::*sgPlxnb1* mice. (**K**) Analysis by IPA of the upstream regulators *Nos2* and *Il1b* in astrocytes isolated from *Gfap*::*sgPlxnb1* vs. *Gfap*::*sgScrmbl* mice. Data shown as mean±SEM. ***p<0.001, **p<0.01, *p<0.05, ns: not significant.



Supplementary Figure 12

Fig. S12

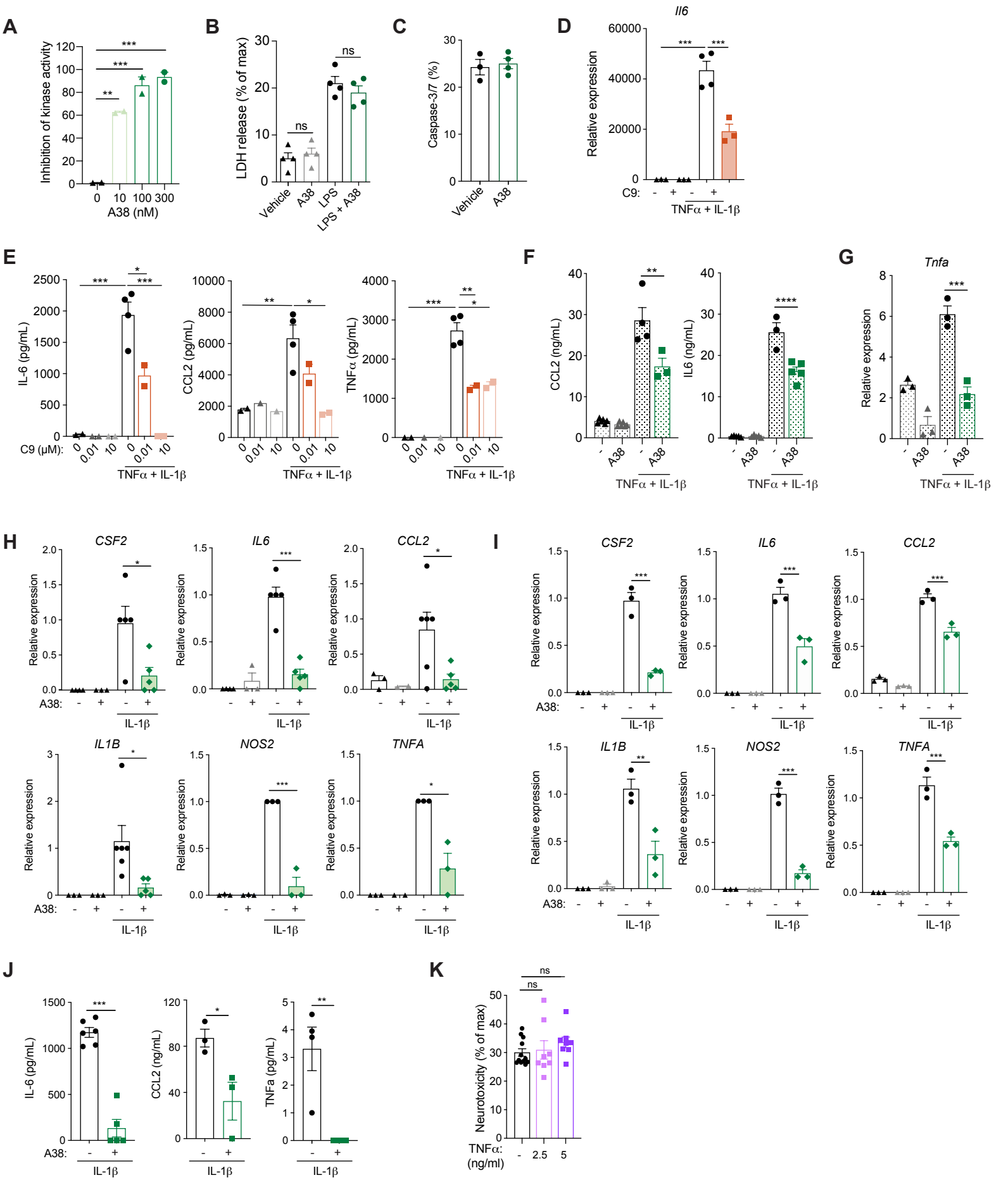
EphrinB3-EphB3 microglia-astrocyte signaling. (A) *Efnb1, Efnb2 and Efnb3* mRNA expression determined by qPCR in microglia (MG) from naïve or EAE mice, and monocytes (MO) from EAE mice. n=11 *Naïve MG*, n=8 *EAE MG*, n=7 *EAE MO*. One-way ANOVA, Tukey post-test. **(B)** EphB3 protein levels determined by flow cytometry in neonatal mouse astrocyte-microglia co-cultures where astrocytes are depicted in red and microglia in blue. Unpaired two-tailed t-test. **(C)** Fetal human astrocytes were stimulated with IL-1β and plate-bound human Ephrin-B3-FcChimera and analyzed for the indicated genes by qPCR. n=4 or 5 (*CSF2, IL6 and CCL2*), n=3 (*IL1B, NOS2 and TNFA*). One-way ANOVA, Tukey or Dunnett post-test. Data representative of 3 independent experiments. Data shown as mean±SEM. ***P<0.001, ***P*<0.01, **P*<0.05.



Supplementary Figure 13

Fig. S13

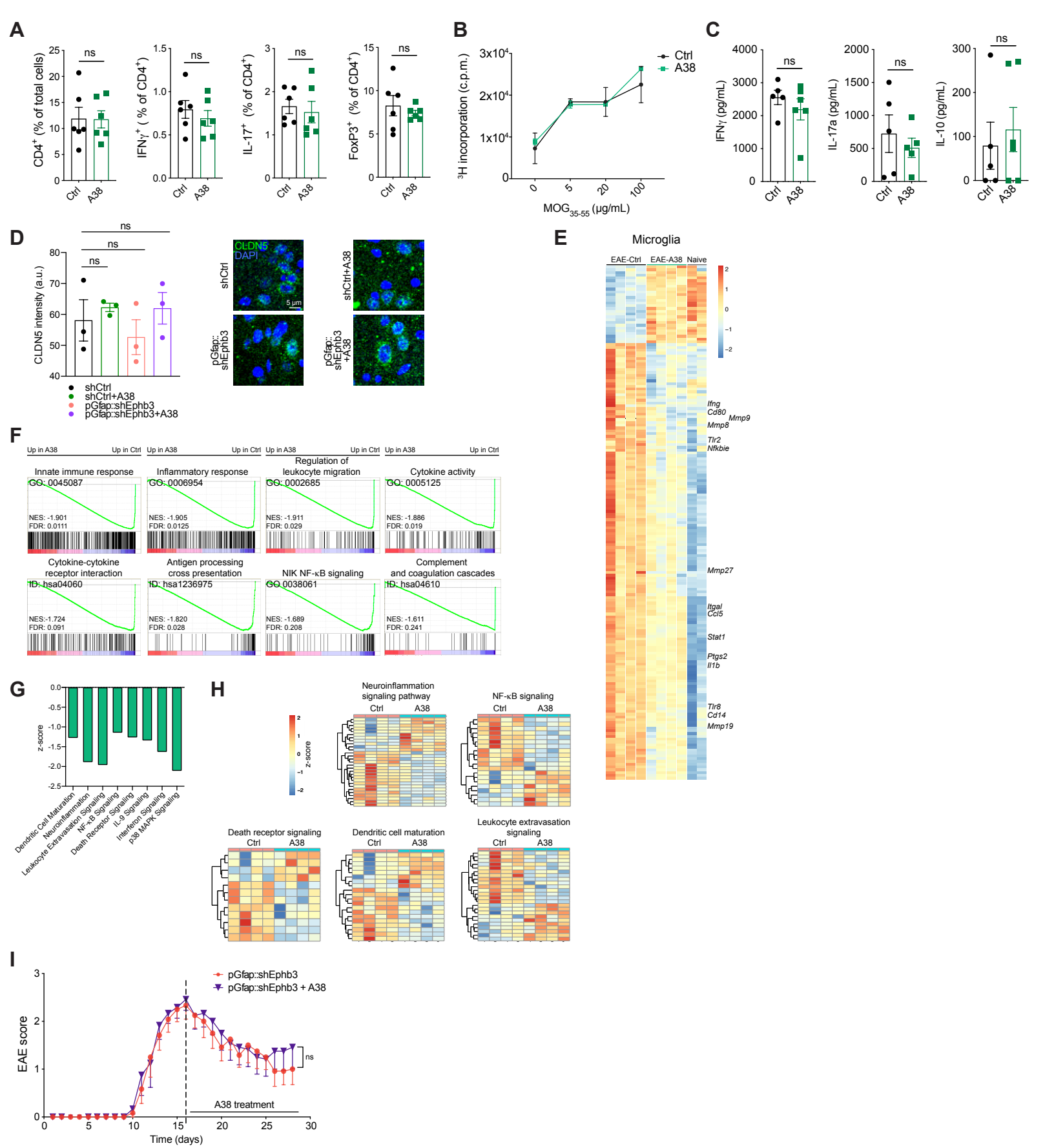
EphB3 expressed in astrocytes contributes to B6 EAE pathogenesis. (A) EphB3 levels in astrocytes (left) and Ephrin-B3 levels in microglia (right) determined by immunofluorescence following transduction with *pGfap::shEphb3*, *pItgam::shEfnb3* or control lentiviral vectors. n=6 per group. One-sample t-test. **(B)** *Ephb3* mRNA expression in astrocytes after transduction in vivo with *pGfap::shEphb3* or control lentiviral vectors, and *Efnb3* expression in microglia and monocytes after in vivo transduction with *pItgam::shEfnb3* or control lentiviral vectors. n=5 per group. Unpaired two-tailed t-test. **(C)** Immunostaining of GFAP and EGFP in the spinal cord of mice transduced with *Gfap*-driven EGFP lentivirus. Representative images from n=5 mice analyzed. **(D-G)** Representative spinal cord sections in EAE C57Bl/6J mice treated with A38 and *pGfap::shEphb3* or control (shCtrl) lentiviral vectors. **(D)** H&E staining. **(E)** Luxol fast blue (LFB) staining for demyelination. **(F)** Silver staining for axonal loss. **(G)** Quantification of demyelination and axonal loss. n=4 *shCtrl,* n=5 *shCtrl+A38,* n=3 *pGfap::shEphb3*, n=5 *pGfap::shEphb3+A38*. One-way ANOVA, Tukey post-test. **(H-K)** $CD4^+$ T cells in EAE C57Bl/6J mice transduced with lentiviral knockdown constructs targeting *Ephb3* in astrocytes (red), *Efnb3* in microglia (blue) or control (black). n=5 *shCtrl* and *pGfap::shEphb3*, n=4 *pItgam::shEfnb3*. T cells in CNS **(H)** and spleen **(I)** analyzed by flow cytometry. **(J)** Splenocyte proliferation assay 28 days after EAE induction. **(K)** IFN-γ and IL-17 production by splenocytes as determined by ELISA. **(L)** EAE development in C57Bl/6J mice treated with lentiviral knockdown constructs targeting *Ephb3* in astrocytes, *Efnb3* in microglia or co-transduction with both viruses. n=5 per group. Two-way repeated measures ANOVA. **(M)** EAE development in C57Bl/6J mice transduced with lentiviral knockdown constructs targeting *Efnb3* in astrocytes. n=5 per group. Data representative of 2 independent experiments. Data shown as mean±SEM. ***P<0.001, **P<0.01, *P<0.05.



Supplementary Figure 14

Fig. S14

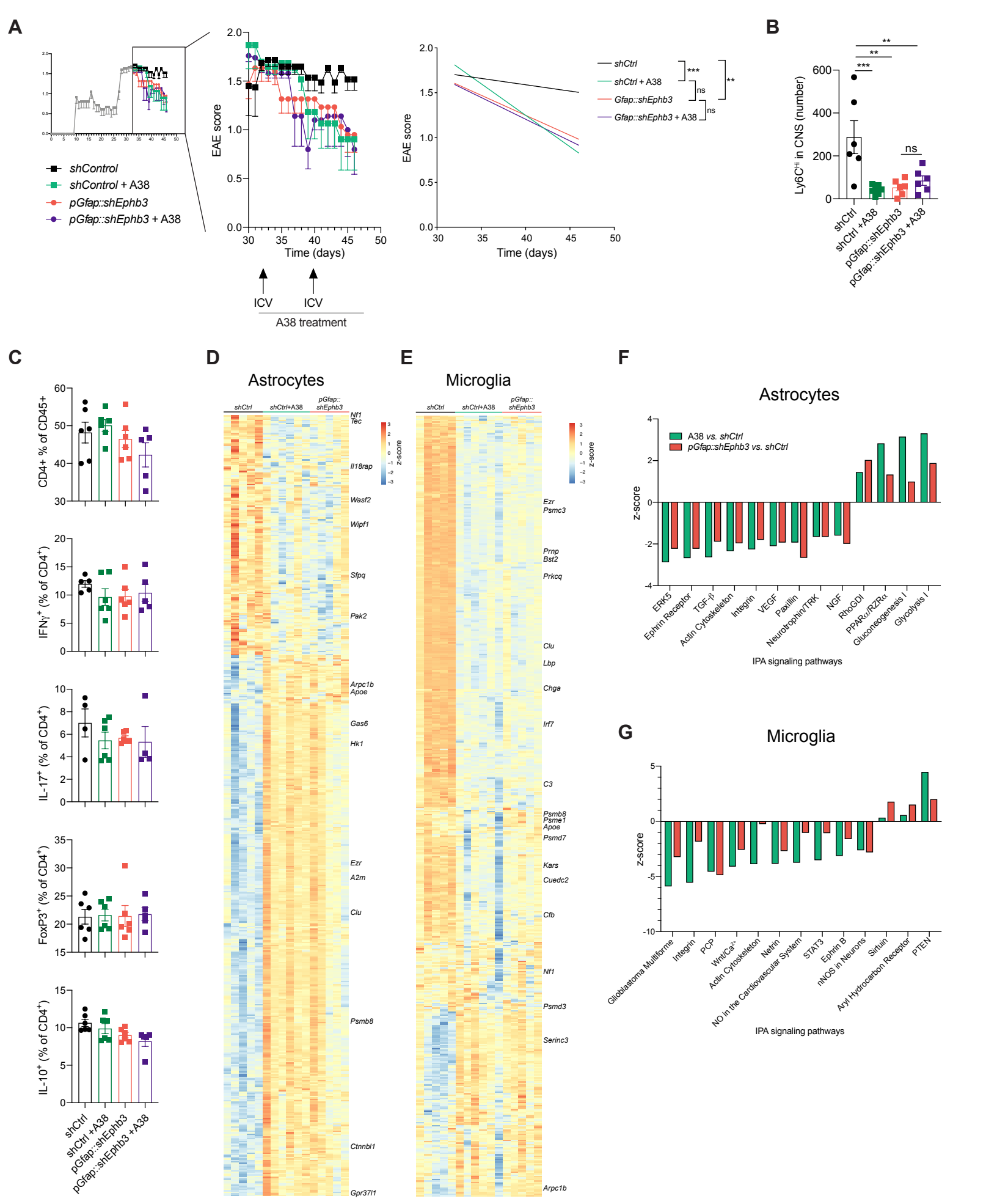
EphB3 signaling promotes pro-inflammatory activities in human astrocytes. (**A**) Inhibition of EphB3 kinase activity by A38 determined using a cell-free radiometric kinase activity assay. n=2. One-way ANOVA, Dunnett post-test. **(B)** Detection of lactate dehydrogenase (LDH) in culture medium. n=4. One-way ANOVA, Tukey post-test. **(C)** Percentage of active caspase-3+ mouse astrocytes. n=3. One-way ANOVA, Tukey post-test. (**D**) *Il6* expression determined by qPCR in neonatal mouse astrocytes stimulated with TNFα and IL-1β in the presence of 10 μM C9. n=3. One-way ANOVA, Sidak post-test. **(E)** IL-6, CCL2 and TNFα concentration measured by ELISA in supernatants of neonatal mouse astrocytes stimulated with TNFα and IL-1β and incubated with the indicated concentrations of C9. n=2. One-way ANOVA, Dunnett post-test. Data representative of 2 independent experiments. **(F)** CCL2 and IL-6 concentration measured by ELISA in supernatants of adult mouse astrocytes cultured in serum-free media and stimulated with TNFα and IL-1β in the presence of A38. n=3. One-way ANOVA, Tukey post-test. **(G)** *Tnfa* expression determined by qPCR in adult mouse astrocytes treated as in (F). n=3. One-way ANOVA, Tukey post-test. (**H-J**) Fetal human astrocytes stimulated with IL-1β in the presence of 10 μM A38. **(H-I)** *CSF2*, *IL6*, *CCL2*, *IL1B*, *NOS2* and *TNFA* expression determined by qPCR in astrocytes cultured with **(H)** n=5 (*CSF2, IL6, CCL2, IL1B*) n=3 (*NOS2, TNFA*) or without **(I)** serum. n=3 per group. One-way ANOVA, Tukey post-test. **(J)** IL-6, CCL2 and TNFα concentration measured by ELISA in human astrocyte supernatants. n=6 (*IL-6*) n=3 (*CCL2* and *TNFa*). Unpaired two-tailed t-test. **(K)** N2A cells were pre-activated with IFNγ and subsequently incubated with the indicated concentrations of TNFα. Cytotoxicity was determined by quantifying lactate dehydrogenase (LDH) release after 24h. n=8. One-way ANOVA, Tukey post-test. Data shown as mean±SEM. ***P<0.001, **P<0.01, *P<0.05.



Supplementary Figure 15

Fig. S15

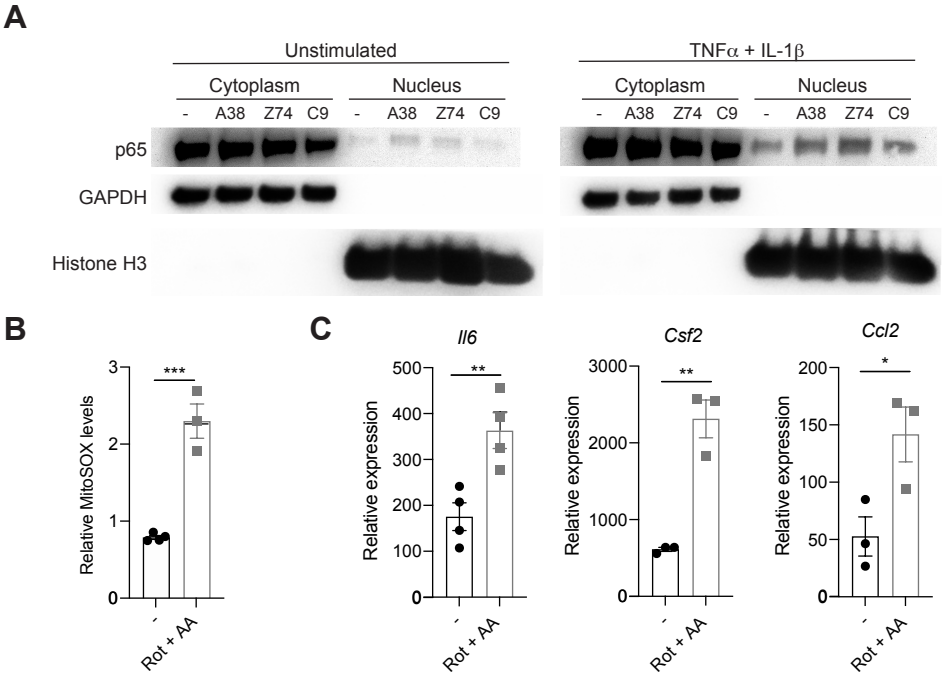
A38 ameliorates EAE. **(A-C)** CD4+ T cells from C57Bl/6J EAE mice treated with A38. **(A)** Analysis of CNS infiltrating T cells by flow cytometry. n=6. Unpaired two-tailed t-test. **(B)** Splenocyte proliferation assay 28 days after EAE induction. n=2. Unpaired two-tailed t-test. Data representative of two independent experiments. **(C)** IFNγ, IL-17 and IL-10 production by splenocytes as determined by ELISA. n=5. Unpaired two-tailed t-test. **(D)** Immunostaining analysis of CLDN5 in EAE mice. n=3 mice per group. Unpaired two-tailed t-test. **(E-H)** RNA-seq analysis of microglia from EAE mice treated with A38 or vehicle. **(E)** Heatmap of differentially expressed genes in microglia. **(F)** Pathways analyzed by gene set enrichment analysis (GSEA). **(G,H)** Ingenuity pathways analysis of genes differentially expressed in microglia from A38 treated mice compared to vehicle. **(I)** EAE development in C57Bl/6J mice treated with A38 and lentiviral knockdown constructs targeting *Ephb3* in astrocytes. n=5 mice per group. Data representative of 2 independent experiments. Data shown as mean±SEM. ***P<0.001, **P<0.01, *P<0.05.



Supplementary Figure 16

Fig. S16

EphB3 in astrocytes contributes to NOD EAE pathogenesis. (A) EAE development in NOD mice treated with A38 or transduced with lentiviral knockdown constructs targeting *Ephb3* in astrocytes during the progressive phase (left); linear regression of progressive phase (right). n=6 mice per group. Data representative of 2 independent experiments. Unpaired t-test performed between regression slopes. **(B)** Monocytes detected in the CNS of NOD mice treated with A38 or lentiviral vectors targeting *Ephb3*. n=6 mice per group. One-way ANOVA, Tukey post-test. **(C)** CNS infiltrating T cells in NOD EAE mice treated with A38 or lentiviral knockdown constructs targeting *Ephb3* in astrocytes. n=6 mice per group. **(D-G)** RNA-seq analysis of astrocytes and microglia isolated from NOD EAE mice treated with A38 or lentiviral knockdown constructs targeting *Ephb3* in astrocytes. **(D)** Heatmap of differentially expressed genes in astrocytes from control, A38 treated and *Ephb3* knockdown mice. **(E)** Heatmap of differentially expressed genes in microglia from control, A38 treated and *Ephb3* knockdown mice. **(F-G)** Ingenuity pathway analysis of differentially expressed genes in glial cells from mice treated with A38 or *Ephb3* knockdown lentivirus. Data shown as mean±SEM. ***P<0.001, **P<0.01 , *P<0.05.



Supplementary Figure 17

Fig. S17

A38 does not suppress NF-κB activation and mitochondrial ROS induces pro-inflammatory genes. (A) Western blot analysis of NF-κB subunit p65 and loading controls GAPDH and histone H3 in subcellular fractions corresponding to cytoplasm and nucleus, respectively, of neonatal mouse astrocytes activated for 30 min with TNFα and IL-1β in the presence of A38, Class I PI3K isoforms inhibitor ZSTK74 (Z74), or C9. **(B)** Mitochondrial ROS abundance measured by MitoSOX staining after overnight treatment in astrocytes with TNFα and IL-1β and rotenone and antimycin A (Rot + AA). n=2 -, n=3 *Rot+AA* per group. Unpaired two-tailed t-test. **(C)** *Il6, Csf2* and *Ccl2* expression determined by qPCR. n=3 per group. Unpaired two-tailed t-test. Data shown as mean±SEM. ***P<0.001, **P<0.01 , *P<0.05.

Table S1

Sequences used to generate and analyze the barcoded pSADΔG-mCherry plasmid.

Primer	Sequence
mCherry-BC Code: mCherry PCR handles **Barcode**	GCCACCATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCA AGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGG CCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAG GGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGC CCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAG GCCTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTC CTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGAC GGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCG AGTTCATCTACAAGGTGAAGCTGCGCGGCACCAACTTCCCCTCCGAC GGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCG AGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCA GAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAG ACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACA ACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACAC CATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGC GGCATGGACGAGCTGTACAAGTAAgctagaGATGTCCACGAGGTCTCTg ctagc**VHDBVHDBATVHDBVHDBATVHDBVHDB**ggcgcgccCGTACGCT GCAGGTCGACccgcggTAGCTTTTCAGTCGAGAAAAAAA
mCherry_FWD	AAACCTGCAGGGCCACCATGGTGAGCAAGGG
mCherry_REV	TTTCCGCGGTTTTTTGCTAGCTTACTTGTACAGCTCGTCCATGC
Rabies barcode	AAGTAAGCTAGAGATGTCCACGAGGTCTCTGCTAGC(V:33333300)(H: 33330033)(D:33003333)(B:00333333)VHDBATVHDBVHDBATVHDBVHD BGGCGCGCCCGTACGCTGCAGGTCGACCCGCGGTAGC
RAB_P7_C01	CAAGCAGAAGACGGCATACGAGATGCACGACCGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCTGTCGACCTGCAGCGTACG
RAB_P5_s0	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGATCTGATGTCCACGAGGTCTCT
RAB_P5_s1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGATCTCGATGTCCACGAGGTCTCT
RAB_P5_s2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGATCTGCGATGTCCACGAGGTCTCT
RAB_P5_s3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGATCTAGCGATGTCCACGAGGTCTCT
RAB_P5_s4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGATCTCAACGATGTCCACGAGGTCTCT
RAB_P5_s6	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGATCTTGCACCGATGTCCACGAGGTCTCT
RAB_P5_s7	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGATCTACGCAACGATGTCCACGAGGTCTCT
RAB_P5_s8	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC TCTTCCGATCTGAAGACCCGATGTCCACGAGGTCTCT

Table S2

Primers used for the SMART-seq based inDrop approach (5’ to 3’).

Primer	**Sequence**
TSO	AAGCAGTGGTATCAACGCAGAGTACATrGrGrG
inDrops_FWD	GGGTGTCGGGTGCAG
inDrops_REV	AAGCAGTGGTATCAACGCAGAGTACAT
Ligation_FWD	CTGTCTCTTATACACATCTGACGCTGCCGACGA
Ligation_REV	AGATGTGTATAAGAGACAGT
PCR_p5_r1_S5XX	AATGATACGGCGACCACCGAGATCTACAC**XXXXXXXX**TCG TCGGCAGCGTC
inDrop_v3_p7_r2	CAAGCAGAAGACGGCATACGAGATGGGTGTCGGGTGCAG
	XXXXXXXX: Indicates S5XX index sequence used.

Table S3

Primers used for RabΔG barcode amplification from WTA product (5' to 3').

Primer	**Sequence**
SMART mCherry primer	TACACCATCGTGGAACAGTACGAAC
inDrop_FWD	GGGTGTCGGGTGCAG
inDrop_v3_p7_r2	CAAGCAGAAGACGGCATACGAGATGGGTGTCGGGTGCAG
inDropv3_S5XX_R2bc	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTC GTCGGCAGCGTCAGATGTGTATAAGAGACAGGATGTCCA CGAGGTCTCT
inDropv3_S5XX_R2bc+1	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTC GTCGGCAGCGTCAGATGTGTATAAGAGACAGaGATGTCC ACGAGGTCTCT
inDropv3_S5XX_R2bc+2	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTC GTCGGCAGCGTCAGATGTGTATAAGAGACAGctGATGTCC ACGAGGTCTCT
inDropv3_S5XX_R2bc+3	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTC GTCGGCAGCGTCAGATGTGTATAAGAGACAGtccGATGTC CACGAGGTCTCT
	XXXXXXXX: Indicates S5XX index sequence used.

Primer conditions for rabies barcode amplification:

Round 1:
- 10 µM FWD
- 10 µM SMART mCherry

Round 2:
- 10 µM inDrop_v3_p7_r2
- 10 µM inDropv3_S5XX_StaggerMix:
  - inDropv3_S5XX_R2bc
  - inDropv3_S5XX_R2bc+1
  - inDropv3_S5XX_R2bc+2
  - inDropv3_S5XX_R2bc+3

Table S4

Index sequences used (5’ to 3’).

Primer name	Index
inDropv3_S501_R2bc	TAGATCGC
inDropv3_S502_R2bc	CTCTCTAT
inDropv3_S503_R2bc	TATCCTCT
inDropv3_S504_R2bc	AGAGTAGA
inDropv3_S505_R2bc	GTAAGGAG
inDropv3_S506_R2bc	ACTGCATA
inDropv3_S507_R2bc	AAGGAGTA
inDropv3_S508_R2bc	CTAAGCCT
inDropv3_S509_R2bc	GGCTACTC
inDropv3_S510_R2bc	CCTCAGAC
inDropv3_S511_R2bc	TCCTTACG
inDropv3_S512_R2bc	ACGCGTGG
inDropv3_S513_R2bc	GGAACTCC
inDropv3_S514_R2bc	TGGCCATG
inDropv3_S515_R2bc	GAGAGATT
inDropv3_S516_R2bc	CGCGGTTA
inDropv3_S517_R2bc	GACCGCCA
inDropv3_S518_R2bc	TAAGATGG
inDropv3_S519_R2bc	ATTGACAT
inDropv3_S520_R2bc	AGCCAACT
inDropv3_S521_R2bc	TACTAGGT
inDropv3_S522_R2bc	TCACGGTT
inDropv3_S523_R2bc	TGTAATGA
inDropv3_S524_R2bc	CACGTCAG
inDropv3_S525_R2bc	CTGAATTC
inDropv3_S526_R2bc	CGTACCGG
inDropv3_S527_R2bc	GATGACGG
inDropv3_S528_R2bc	TATAGACG
inDropv3_S529_R2bc	GTCATTGA
inDropv3_S530_R2bc	GCATCGTT
inDropv3_S531_R2bc	AGGTTGAC
inDropv3_S532_R2bc	TGAAACTG
inDropv3_S533_R2bc	CAATCACA
inDropv3_S534_R2bc	ACATGCAA
inDropv3_S535_R2bc	ATCGCGCC
inDropv3_S536_R2bc	TCGGTTAA

Table S5

Primers used for sgRNA plasmid generation (5’ to 3’)

Primer name	**Index sequence**
U6-PCR-F	AAAGGCGCGCCGAGGGCCTATTT
U6-PCR-R	TTTTTTGGTCTCCCGGTGTTTCGTCCTTTCCAC
cr-RNA-F	AAAAAAGGTCTCTACCG(N20)GTTTTAGAGCTAGAAATAGCAAGTT
cr-RNA-R	GTTCCCTGCAGGAAAAAAGCACCGA
where N20 marks the sgRNA substitution site.	

Table S6

Small RNA sequences used in this study (5’ to 3’).

Small RNA name	**Sequence**
sgSema4d	GCCGAGTAGTTAAAGATGCC
sgPlxnb1	GGGGAAGGCACAGAGCACAG
sgPlxnb2	GGAGGTCACCAGCCCCACGG
sgScramble	GCACTACCAGAGCTAACTCA
shEphb3	CCGGCCATAGCCTATCGGAAGTTTACTCGAGTAAACTTCCGATAGGCTATGGTTTTT
shEfnb3	CCGGCCTGTGTACATTGTGCAGGATCTCGAGATCCTGCACAATGTACACAGGTTTTTG

Data S1 (separate file)

Differential single-cell RABID-seq gene expression between astrocytes with high pro-inflammatory scores (>90th percentile) and low pro-inflammatory scores (<10th percentile) in peak EAE.

Data S2 (separate file)

Differential single-cell RABID-seq gene expression between T cells connected to astrocytes with high pro-inflammatory scores (>90th percentile) and low pro-inflammatory scores (<10th percentile) in peak EAE.

Data S3 (separate file)

Differential single-cell RABID-seq gene expression between astrocytes connected to T cells in peak EAE vs. naïve mice.

Data S4 (separate file)

Differential single-cell RABID-seq gene expression between astrocytes connected to microglia in peak EAE vs. naïve mice.

Data S5 (separate file)

Differential single-cell RABID-seq gene expression between astrocytes connected to astrocytes in peak EAE vs. naïve mice.

Data S6 (separate file)

Normalized astrocyte single-cell RABID-seq gene expression for *Plxnb2*$^{+/-}$ astrocytes connected to *Sema4d*$^{+/-}$ microglia in peak EAE.