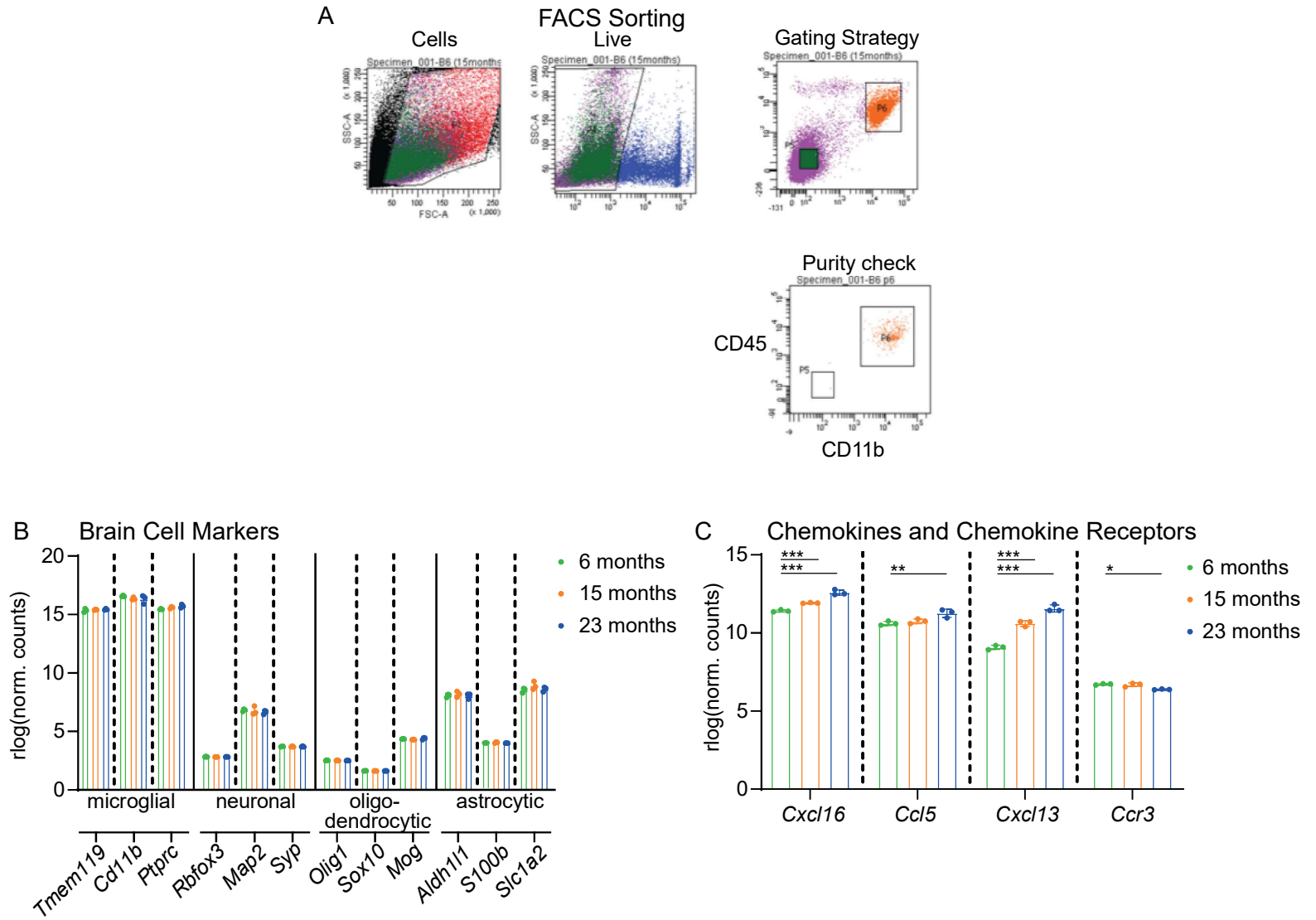
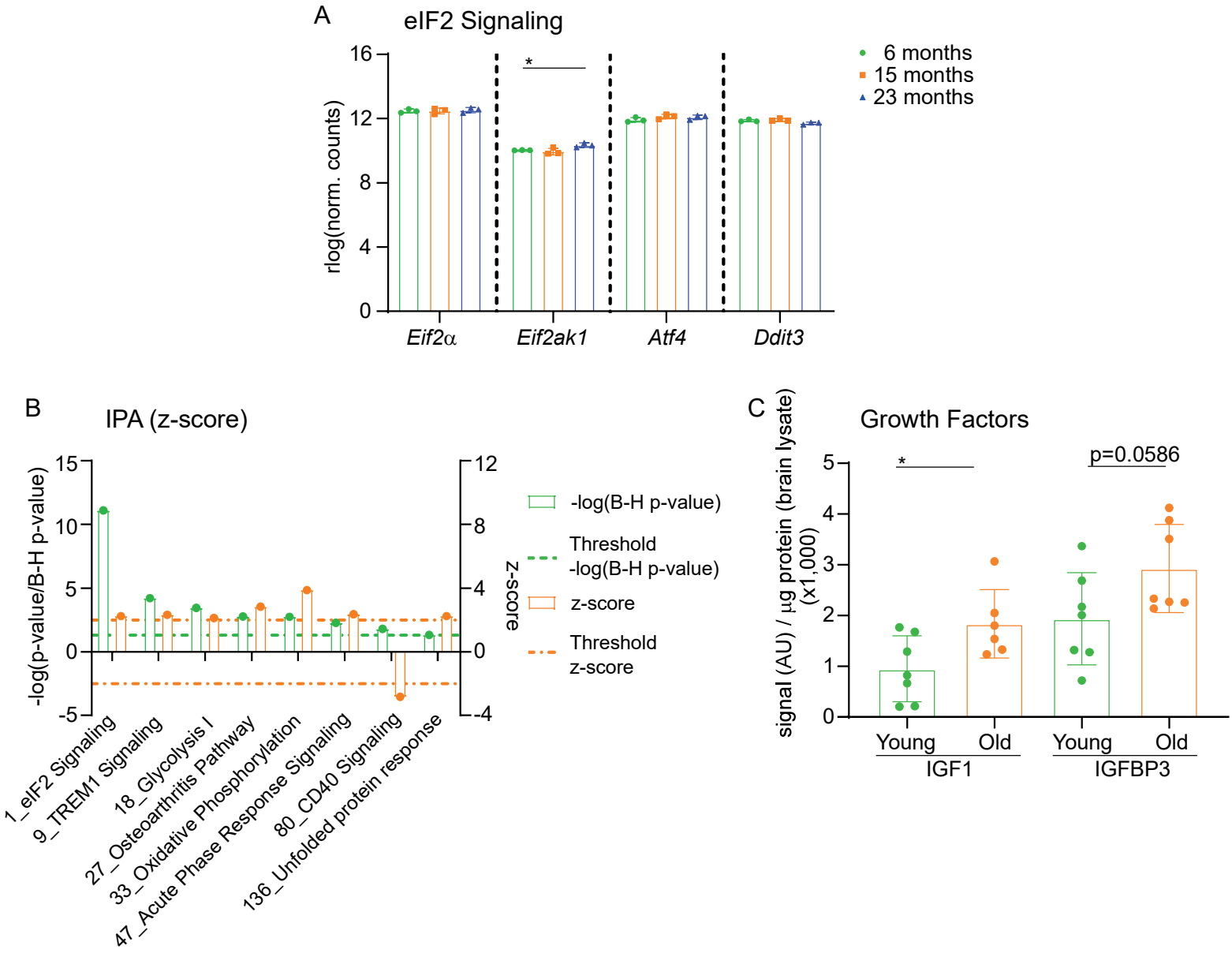
Supplemental Figure 1



Supplemental Figure 1. Microglia isolation strategy and additional RNA sequencing results.

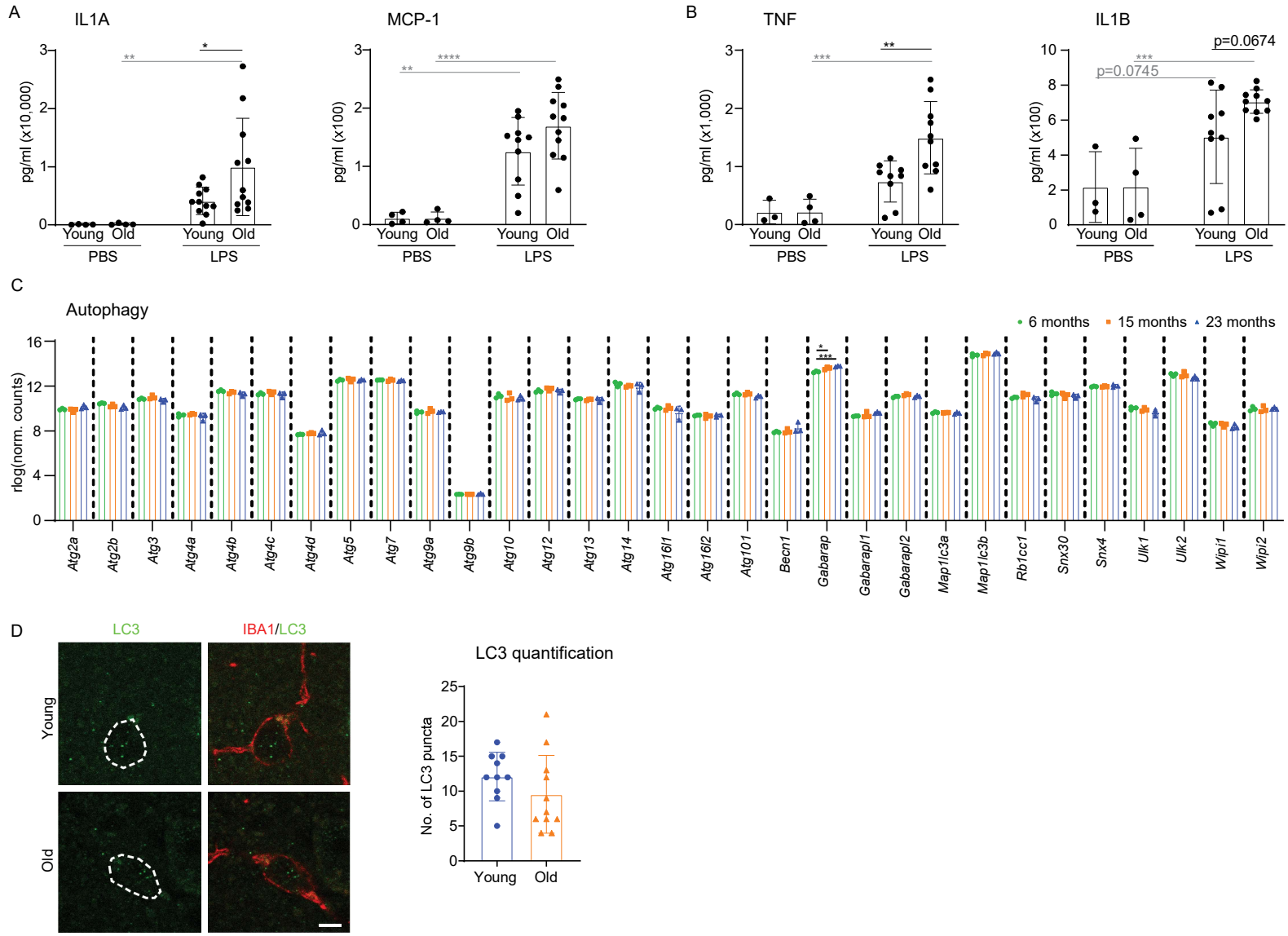
A) Gating strategy for microglia cells isolated by FACS sorting, based on expression of CD11b and CD45, including an example of a purity check run after the sort, n=3. **B**) rlog normalized count expression values for genes specific for microglia, neurons, oligodendrocytes and astrocytes, n=3. **C**) Chemokines and chemokine receptors found differentially expressed between young and aged microglia by DESeq2 analysis of RNA sequencing results, data shown as rlog normalized counts, n=3. **D**) Validation by qPCR of representative genes differentially expressed in microglia from aged mice compared to younger counterparts. Fold change is shown relative to *Hprt* expression. Microglia preparations were obtained from a new cohort of aging mice, n=3-6.(*: p-value <0.05, **: p-value <0.01, ***: p-value <0.001; DESeq2 results for **C**, one-way ANOVA for **D**).

Supplemental Figure 2



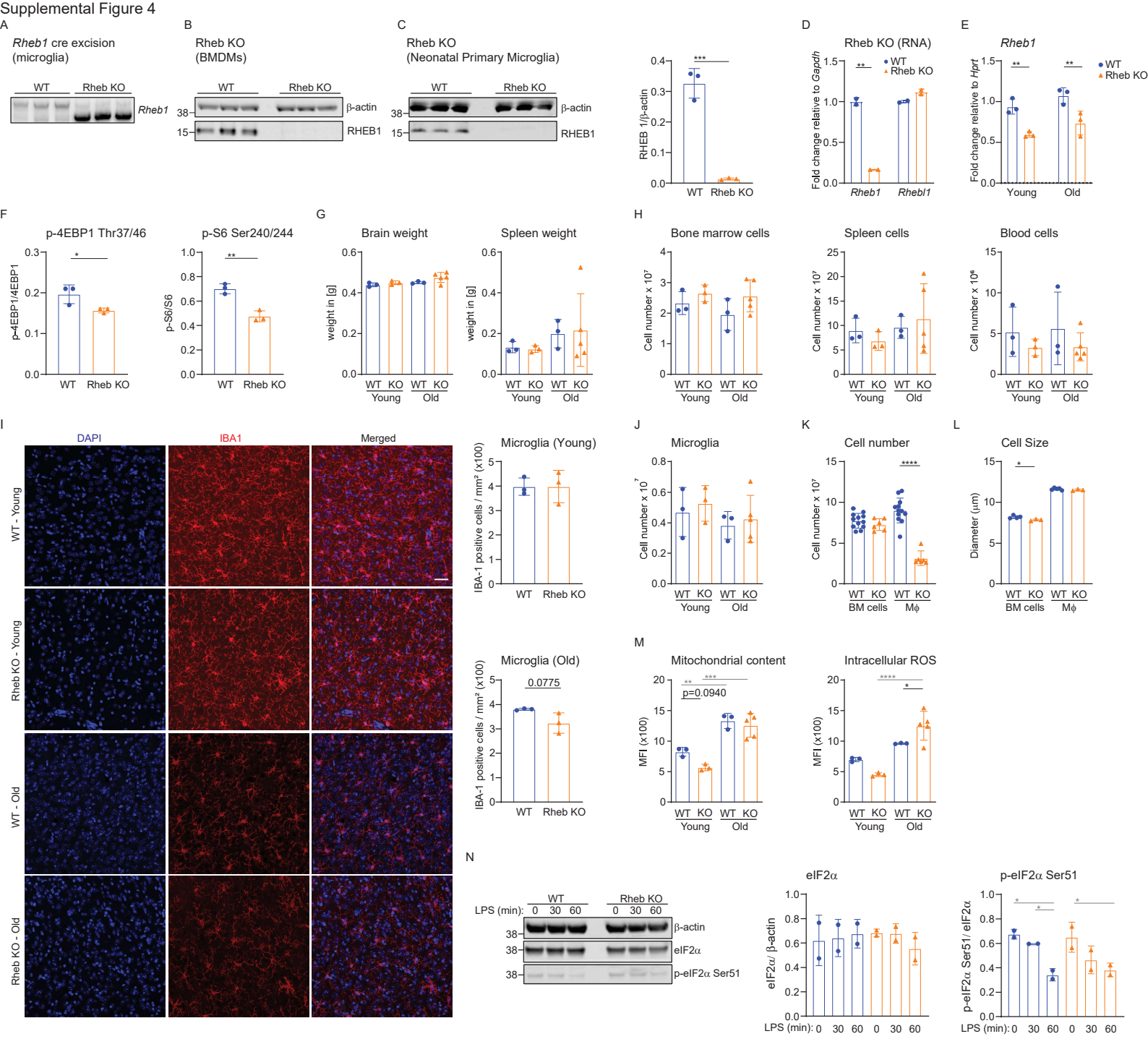
Supplemental Figure 2. Dysregulated IPA pathways and validation of RNA sequencing results. **A**) RNAseq data (rlog normalized counts) of genes involved in eIF2 signaling. *Eif2ak1* encodes for the kinase regulating eIF2 activity, which phosphorylates its Ser51 residue. The transcription factor *Atf4* is downstream of eIF2 phosphorylation, whereas the transcription factor CHOP (coded by *Ddit3*) is an ATF4 target gene. Neither were found upregulated with age. Data are shown as rlog normalized counts, n=3. **B**) Pathways significantly dysregulated with age detected by IPA, reaching the threshold for differential expression (green) and threshold for Z-score (orange), which indicates IPA can predict whether the pathway is up or downregulated. **C**) Protein levels of growth factors detected in protein lysates of young and aged brains, measured using a sandwich-based chemiluminescence assay, n=6-7. (*: p-value <0.05; DESeq2 results for **A**, Student's T-test for **C**).

Supplemental Figure 3



Supplemental Figure 3. Microglia and circulating cytokines upregulated in aging and the role of autophagy in aging microglia.

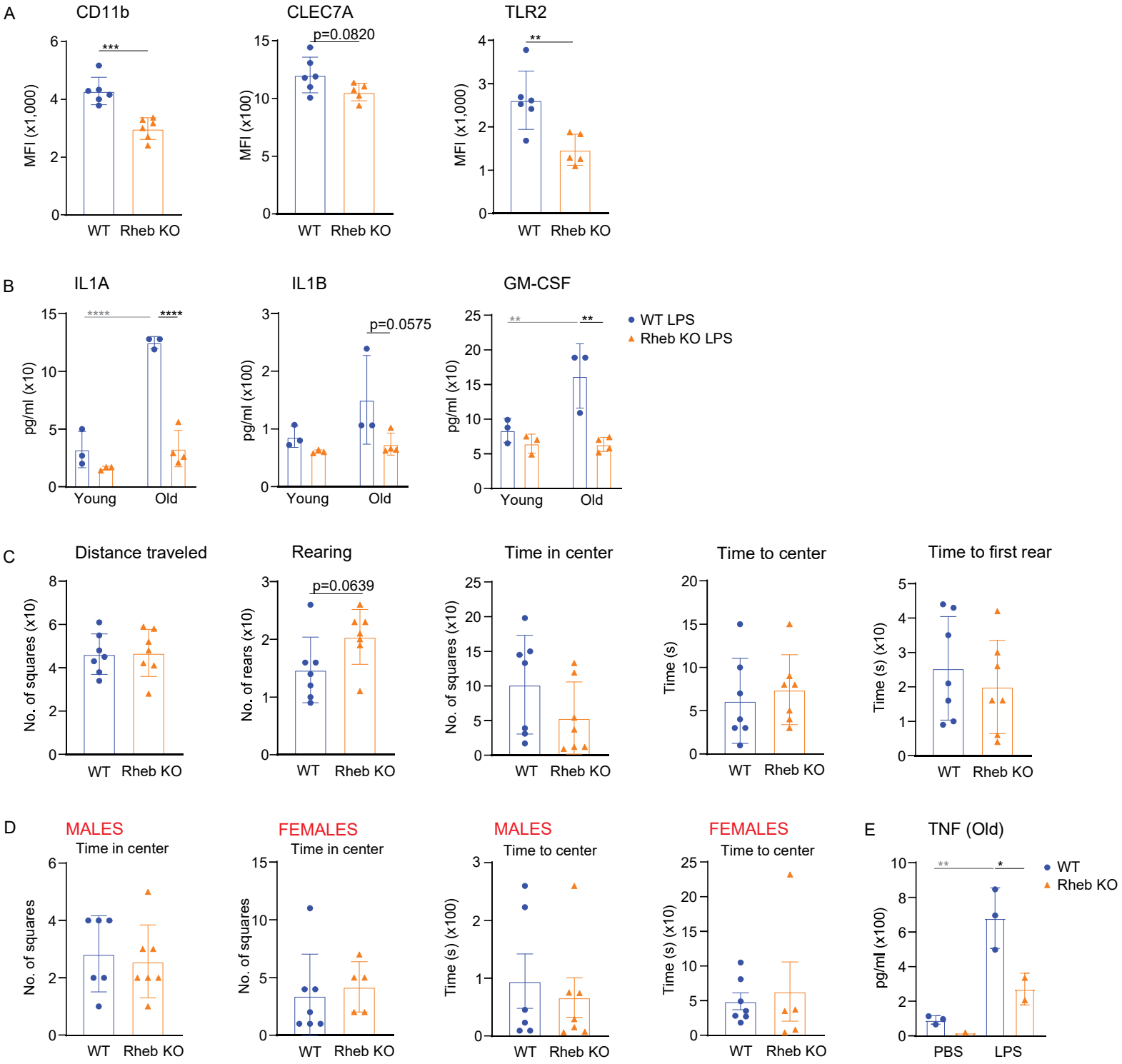
A) Protein levels of microglia cytokines, assessed in a microglia protein lysate from young and aged mice treated with 5 mg/kg LPS for 4 h or PBS as control, measured using the Legendplex assay system. **B**) Plasma cytokine levels from 3-month and 16-month old mice treated with 5 mg/kg LPS or PBS as control and sacrificed 4 h later. Cytokines were measured using a Legendplex assay, n=3-10. **C**) Expression levels of all autophagy related genes in microglia isolated from 6-, 15- and 23-month old mice, assessed by RNA sequencing. No autophagy gene was differentially expressed with age except for *Gabarap*. Data shown were analyzed by DESeq2 analysis of RNA sequencing results and presented as rlog normalized counts, n=3. **D**) LC3 staining (green) in IBA1+ microglia (red), as detected by immunofluorescence staining of young and aged brain sections. Scale bar= 2 µm. (*: p-value <0.05, **: p-value <0.01, ***: p-value <0.001, ****: p-value <0.0001; DESeq2 results for **B**, Student's T-test for **D**, two-way ANOVA for **A** and **B**).



Supplemental Figure 4. Characterization of *Rheb1*$^{f/f}$; *Csf1r*^{Cre} (Rheb KO) mice

A) PCR on genomic DNA of neonatal microglia, using primers to detect Cre-excised *Rheb1*, n=3 mice per genotype. **B**) RHEB1 immunoblot of WT and Rheb KO BMDMs, n=3 mice. **C**) RHEB1 immunoblot of neonatal microglia. Right panel, quantification of RHEB1 protein expression, n=3 mice. **D**) qRT-PCR of *Rheb1* and *Rhebl1* in primary neonatal microglia, n=2 technical replicates. **E**) *Rheb1* qPCR in microglia isolated from young and aged mice, n=3 mice. **F**) Flow cytometry analysis of phospho-4EBP1 Thr 37/46 vs. total 4EBP1 and phospho-S6 Ser240/44 vs. total S6, carried out in microglia isolated from WT and Rheb KO animals, n=3 mice per genotype. **G**) Brain and spleen weight of young and old WT and Rheb KO mice, n=3-5 mice per genotype. **H**) Bone marrow, spleen, and blood cell numbers of young and old WT and Rheb KO mice. Blood cells were counted post red-cell lysis. **I**) Immunofluorescence staining of IBA1+ microglia cells (red) in the cortex of WT and Rheb KO brains of young and old mice and relative quantification, n=3. Scale bar= 25 μm, 40x objective. **J**) Number of microglia cells isolated from young and old WT and Rheb KO mice, n=3-5. **K**) Number of bone marrow (BM) cells and derived BMDMs (MΦ) from WT (n=12) and Rheb KO (n=6) mice. **L**) Cell size of bone marrow (BM) cells and BMDMs (MΦ) from WT (n=5) and Rheb KO mice (n=3). **M**) Mitochondrial content (MitoTracker staining) and intracellular ROS (CellROX staining), measured by flow cytometry of primary microglia isolated from WT and Rheb KO animals, n=3-5 mice. **N**) Western blot of eIF2α and phospho-eIF2α Ser51 in WT and Rheb KO BMDMs. Right panels, densitometry analysis of eIF2α and phospho-eIF2α, n=2 mice. (*: p-value <0.05, **: p-value <0.01, ***: p-value <0.001, ****: p-value <0.0001; Student's T-Test for **C**, **D**, **F**, **I**, **K** and **L**, two-way ANOVA for **E**, **G**, **H**, **J**, **M** and **N**).

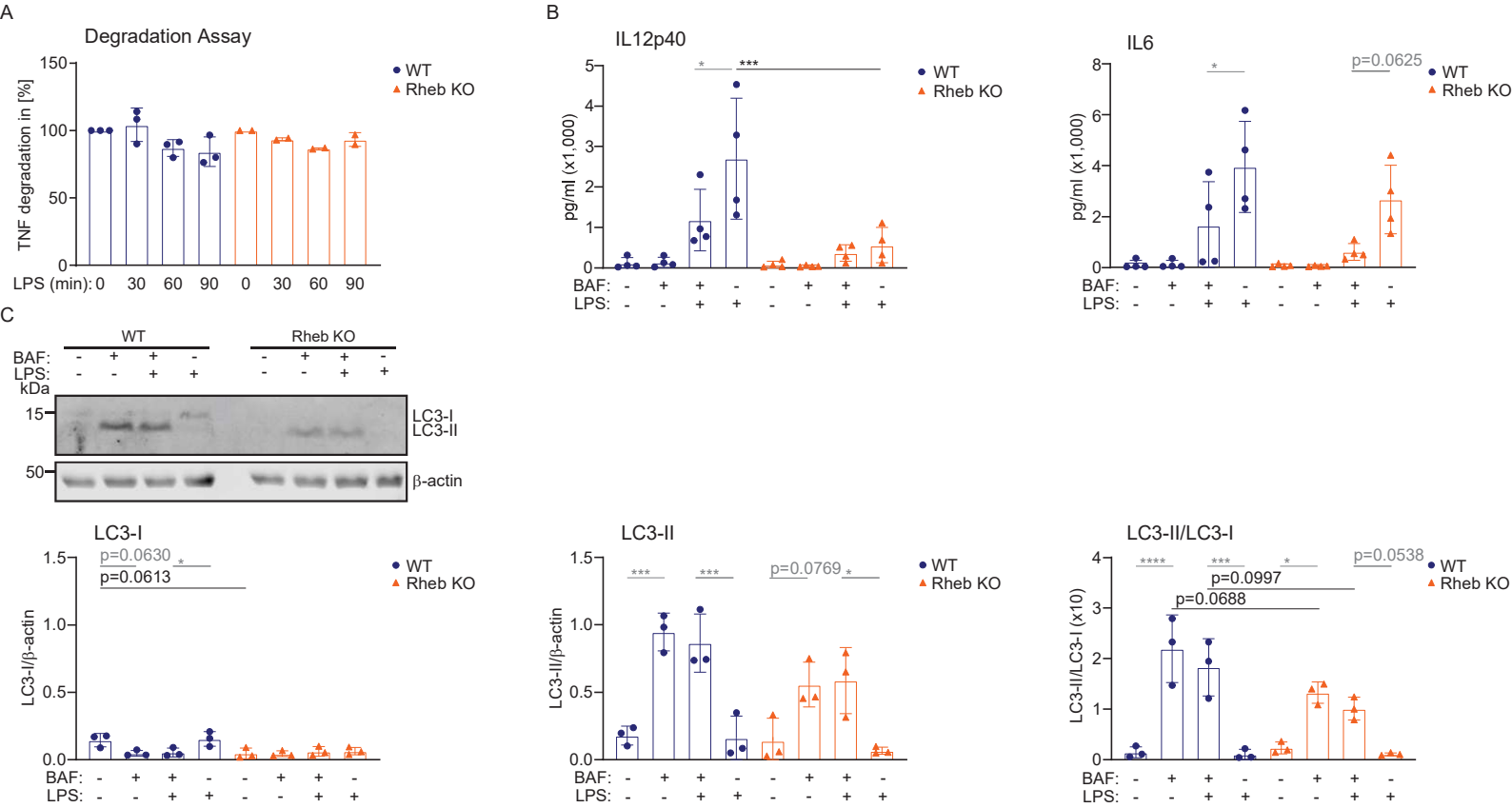
Supplemental Figure 5



Supplemental Figure 5. Young and aged Rheb KO mice show reduced microglia and systemic inflammation after LPS challenge.

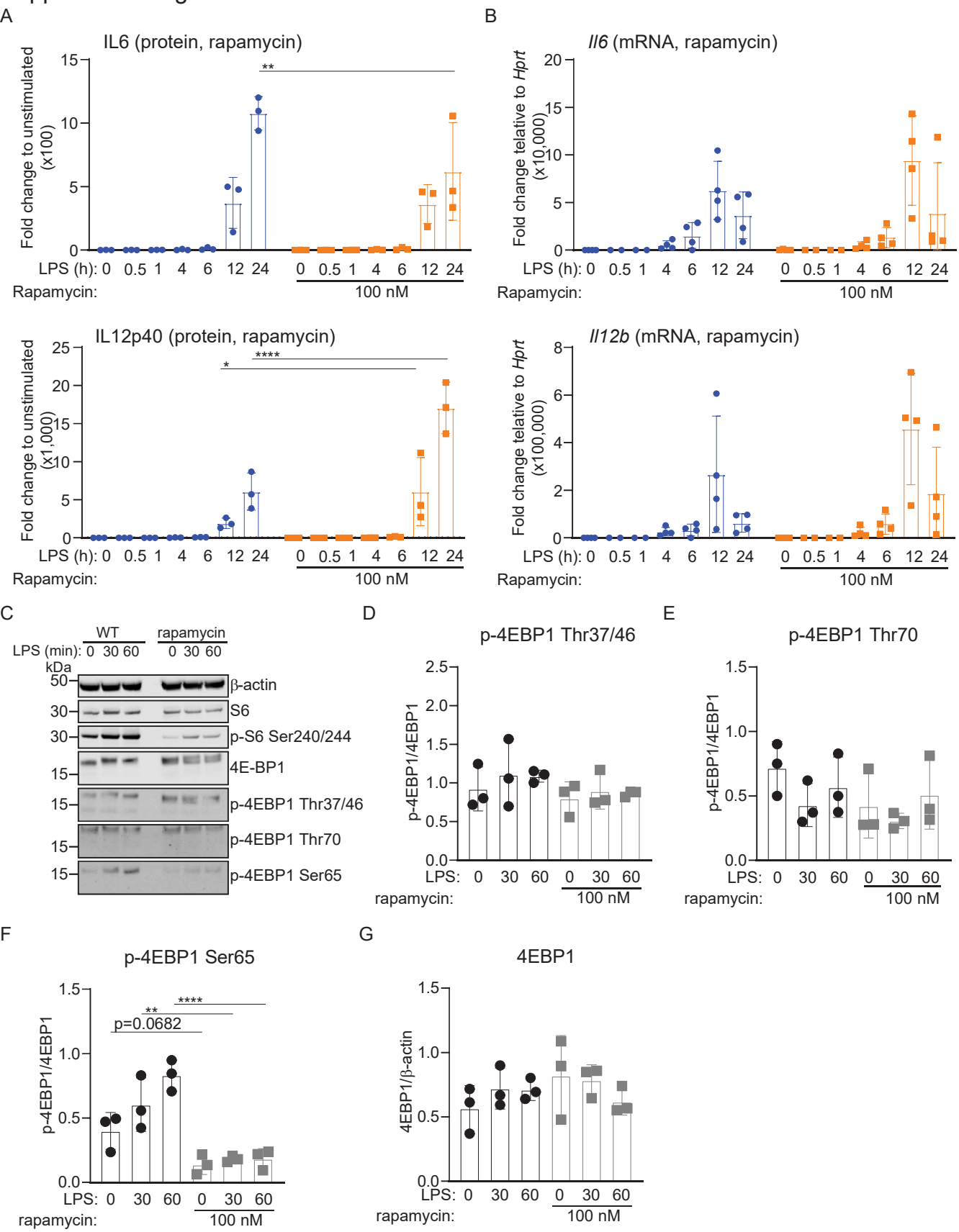
A) Microglia activation markers as assessed by flow cytometry of microglia from aged (15-20 months) WT (n=6) and Rheb KO mice (n=5), 4 h after intraperitoneal injection of 5 mg/kg LPS. Data are shown as median fluorescence intensity (MFI). **B**) Plasma cytokine levels of LPS-challenged young (2-6 months) and aged (15-20 months) WT and Rheb KO mice. Cytokine levels were assessed with a Legendplex assay, n=3. **C**) Baseline analysis of sickness behavior parameters in WT vs. Rheb KO mice as measured by the open-field test. Mice were placed into a 15-square gridded rat cage for a 5-minute period. Each test was recorded and later analyzed blind. The overall locomotor activity of each mouse was assessed by measuring the number of squares it passed through (distance traveled) and the number of times it reared with both paws (rearing) during the test. Time spent in the 3 central squares (Time in center), elapsed time before the mouse reached one of the 3 central squares (Time to center) and elapsed time before the mouse reared for the first time (Time to first rear) were measured as indicators of stress or anxiety behavior. **D**) Time spent in the 3 central squares (Time in center) and elapsed time before mice reached these squares (Time to center) in male and female WT and Rheb KO mice 6 h post intraperitoneal LPS injection (0.33 mg/kg), n=5-7. **E**) TNF levels as measured by ELISA from culture supernatants of acute brain slices from aged WT and Rheb KO mice (20 month), after 6 h culture with 2 µg/ml LPS. (*: p-value <0.05, **: p-value <0.01, ***: p-value <0.001, ****: p-value <0.0001; Student's T-Test for **A**, **C** and **D**, two-way ANOVA for **B** and **E**).

Supplemental Figure 6



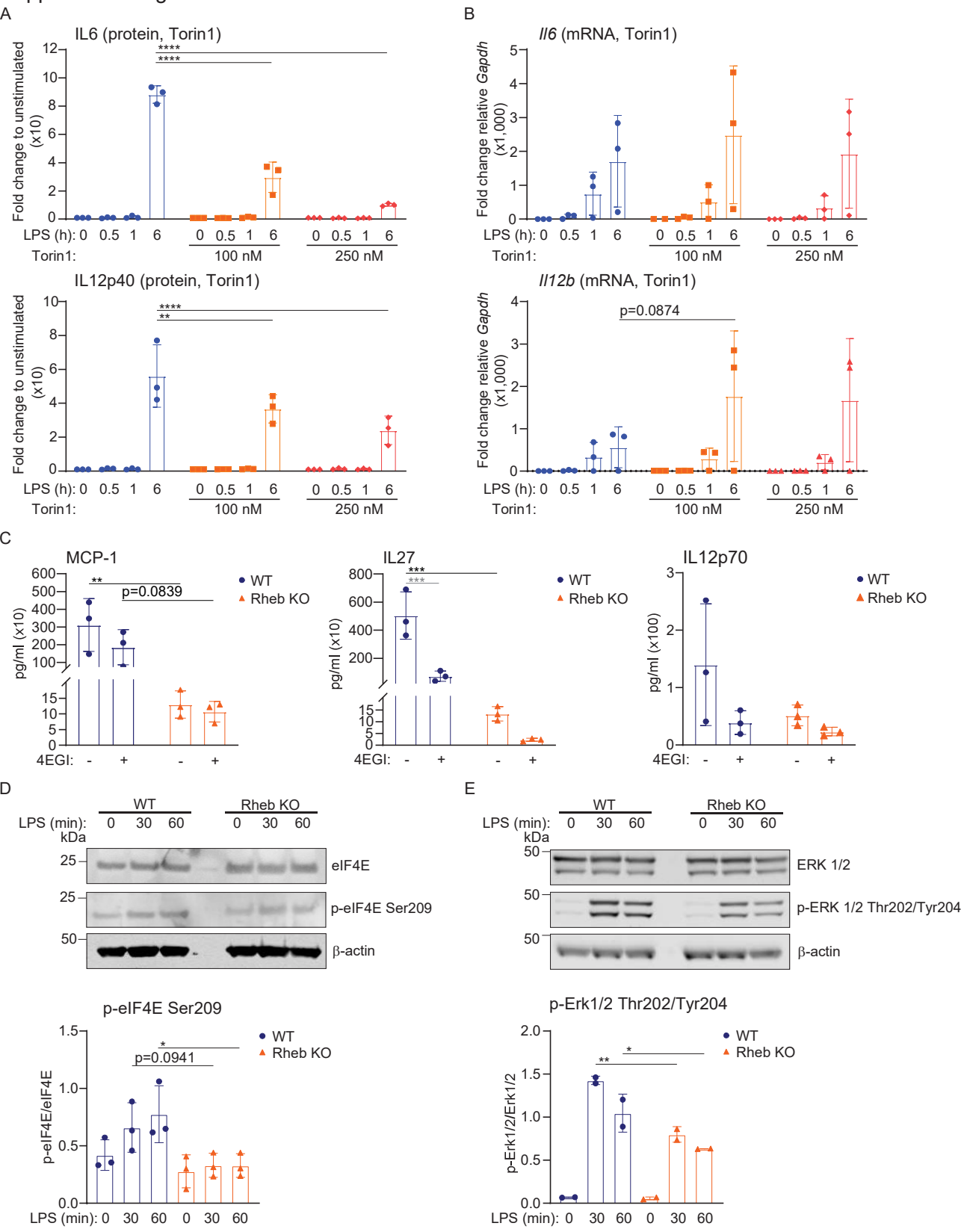
Supplemental Figure 6. Autophagy is not upregulated in Rheb KO cells, nor plays a role in cytokine degradation. **A**) ELISA TNF levels detected in WT and Rheb KO BMDMs, stimulated with 200 ng/ml LPS for 90 min prior to the addition of 10 µg/ml cycloheximide for the indicated time points, in order to assess TNF protein degradation. Values are expressed as percentage of vehicle-treated cells (=100%). **B**) IL12p40 and IL6 measured by ELISA of culture supernatant from WT and Rheb KO BMDMs stimulated with 100 ng/ml LPS for 4 h and treated with 100 nM Bafilomycin A1 or DMSO control, for the last 3 h, n=4 mice in 4 independent experiments. **C**) Western blot of LC3 in WT and Rheb KO BMDMs. Lysates were obtained from the same experiments described above. Graphs below, densitometry analysis of LC3-I, LC3-II and relative ratio, n=3. (*: p-value <0.05, **: p-value <0.01, ***: p-value <0.001, ****: p-value <0.0001; two-way ANOVA for **A**, three-way ANOVA for **B** and **C**).

Supplemental Figure 7



Supplemental Figure 7. Cytokine production and 4EBP1 phosphorylation after rapamycin treatment. **A**) ELISA of culture supernatants of WT BMDMs pre-treated with 100 nM rapamycin for 90 min and stimulated with 100 ng/ml LPS for indicated time points, n=3 BMDMs from 3 mice. **B**) qRT-PCR of cytokine mRNAs from rapamycin-treated WT BMDMs, as described in (**A**), n=4. Data are shown as fold change relative to *Hprt* expression. **C**) Western blot of WT BMDMs pre-treated with 100 nM rapamycin for 90 min and then stimulated with 100 ng/ml LPS for the indicated time points. **D-G**) Densitometry analysis of **D**) Phospho-4EBP1 Thr37/46, **E**) Phospho-4EBP1 Thr70, **F**) Phospho-4EBP1 Ser65 and **G**) 4EBP1, n=3.(*: p-value <0.05, **: p-value <0.01, ****: p-value <0.0001; two-way ANOVA).

Supplemental Figure 8



Supplemental Figure 8. Effect of Torin1 on signaling and cytokine production, differences in WT and Rheb KO response to 4EGI-1 translation inhibitor and differences in cell signaling.

A) ELISA of culture supernatants of WT BMDMs pretreated with either 100 nM or 250 nM Torin1 for 30 min and stimulated with 100 ng/ml LPS for indicated time points, n=3 in 3 independent experiments. Data are reported as fold change to unstimulated (LPS time 0). **B**) qRT-PCR of cytokine mRNAs from Torin1-treated WT BMDMs, as described in (**A**). Data are shown as fold change relative to *Gapdh* expression, n=3 collected in 3 independent experiments. **C**) MCP-1, IL27 and IL12p70 measured by Legendplex of culture supernatants from WT and Rheb KO neonatal primary microglia. Cells were pre-treated for 90 min with 25 μM 4EGI-1 inhibitor or media as control and then stimulated with 200 ng/ml LPS for 3 h, n= 3 mice. **D**) Western blot of eIF4E and phospho-eIF4E Ser209 from WT and Rheb KO BMDMs stimulated with 100 ng/ml LPS. Bottom panel, densitometry analysis of phospho-eIF4E Ser209, n=3 mice. **E**) Western Blot of ERK1/2 and phospho-ERK1/2 Thr202/Tyr204 from WT and Rheb KO BMDMs stimulated with 100 ng/ml LPS. Bottom panel, densitometry analysis of p-Erk1/2 Thr202/Tyr204, n=3 mice. (*: p-value <0.05, **: p-value <0.01, ***: p-value <0.001, ****: p-value <0.0001; two-way ANOVA).

SUPPLEMENTAL METHODS

Primary neonatal microglia. Primary microglia were obtained from P0-P3 day old pups. First pups were decapitated and heads were kept in ice-cold HBSS. The skin, skull and then the meninges were removed using a dissection microscope. Brains were placed in 1 ml of DMEM containing 20% FBS and 1% pen/strep (mgMEDIA) and manually disrupted using a P1,000 pipette tip. Following manual disruption, brain lysates were passed through a 70 μM cell strainer in order to remove any remaining meninges and to obtain a single cell suspension. Cells were spun down at 700 x g for 5 min and then resuspended in 10 ml of mgMEDIA. Each brain was placed in a T25 cm^2 flask that had been previously coated with poly-D-lysine for at least 1 h, in order to allow the formation of an adequate feeder layer of astrocytes. The following day, 5 ml of the media was removed and replaced with fresh mgMEDIA supplemented with 10% L929 supernatant. Cells were then supplemented in a similar way every 2 d until they were harvested by gentle shaking 7-10 d later for further re-plating and analysis. For 4EGI-1 experiments, cells were pre-treated for 90 min with 25 μM 4EGI-1 (Merck, 324517) and then stimulated with 200 ng/ml LPS. Preparation of L929 supernatant: L929 cell line was a kind gift from Michael T. Heneka group, cells were cultured as described (1) for 7 d before harvesting the supernatant.

Bone marrow derived macrophage differentiation (BMDMs). Cells were obtained from murine bone marrow by flushing the femurs and tibia with HBSS (Cat. No. 14025092, Gibco). Cells were then strained through a 70 μm cell strainer (Cat. No. 352350, Falcon), centrifuged at 300 x g for 10 min at RT and counted by Cell Counter CASY (OLS OMNI Life Science). About 14 x 10^6 cells were seeded in 20 ml of DMEM (cat. No D5671, Sigma), supplemented with GlutamaX (Cat. No. 35050-061, Gibco), 20% Fetal Bovine Serum (Cat. No. P30-193306, PAN-Biotech), 1% penicillin/streptomycin (Cat. No. 15140-122, Sigma) and containing either 20% L929 supernatant (preparation: see below) or 20 ng/ml M-CSF (eBioscience) into a 150 mm Petri dish (Cat. No. 351058, Falcon).

Cells were incubated at 37°C / 5% CO2 for 6 d. On day 4, half of medium was gently removed and new complete medium was added. On day 6, BMDMs were carefully washed twice with PBS and incubated in Cell Dissociation Buffer (Cat. No. 13151014, Gibco) for 10 min at 37°C / 5% CO2. Cells were re-plated at different concentrations in complete medium as described above, supplemented with 10% FBS without L929 supernatant or M-CSF for subsequent experiments and allowed to adhere at 37°C / 5% CO_2 overnight. The following day, cells were treated and/or stimulated as described. For pharmacological treatments, cells were pre-treated for 90 min with either 100 nM Rapamycin (Sigma, 553210) or 18 μM of SN50 (Merck, 481480) or for 30 min with either 100 nM or 250 nM Torin1 (Tocris, 4247). Cells were then stimulated with 100 ng/ml LPS from *E. coli* 0111:B4 (Cat. No. L2630**,** EC Number 297-473-0) or medium control for indicated time points in 6-well plates. For autophagy experiments, cells were first stimulated with 100 ng/ml LPS for 4 h and then treated with 100 nM Bafilomycin A1 (Sigma, B1793) for 3 h. For degradation experiments, BMDMs were treated with 200 ng/ml LPS for 90 min, then 10 μg/ml cycloheximide (Sigma, C4859) was added for the indicated time points.

RT-PCR. For RNA extraction from BMDMs, the RNAeasy Mini Kit (Qiagen) or the NucleoSpin RNA Mini Kit (Macherey-Nagel) were used as recommended by the manufacturers. RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Cat. No. 4368814). qPCR was carried out using either the Biorad iTaq master mix and FAM labeled primers or using Thermo Scientific Fast SYBR Green Master Mix and Sigma unlabeled primers (Supplemental Table 6). Reactions were run with corresponding standard programs using the Step One Plus Real Time PCR Machine (Applied Biosystems). Target gene computed tomography (CT) values were normalized to CT values obtained for the housekeeping genes *Hprt* or *Gapdh* using the delta CT method (2).

Flow cytometry staining. For surface staining, 2 x 10^6 cells were transferred to a v-bottom 96-well plate and spun down at 300 x g for 5 min. Cells were then incubated with a combination of surface antibodies (CD11b – Clone M1/70, CD45 – Clone 30-F11, TLR2 – Clone 6C2, CLEC7A – bg1fpj, CD11c – Clone N418, eBioscience/Biolegend) diluted in FACS buffer (PBS + 2.5% BSA and 2 mM EDTA) and incubated in the dark for 20 min at 4 ° C. Cells were then washed twice with FACS buffer and then fixed with 4% paraformaldehyde (PFA) for 10 min on ice. Samples were then analyzed within 24 h on the LSR-fortessa or stained for intracellular markers with the following protocol; cells were washed twice in 0.1% saponin (Sigma Aldrich), before being incubated overnight under gentle agitation with either SPP1 antibody or the following anti-phospho antibodies (all 1:50, Cell Signaling), anti-phospho S6 242/244 (Cat. No. 5018), anti-phospho 4EBP1 Thr37/46 -Alexa 488 (Cat. No. 2846), anti-total S6-Alexa 647 (Cat. No. 5548) or anti-total 4EPB1-PE (Cat. No. 34470). Then cells were washed once more in 0.1% saponin and then PBS, prior to being analyzed on an LSR-Fortessa (BD Biosciences). For measurement of mitochondrial content or intracellular ROS, microglia were first purified from the whole brain as described above and 2 x 10^5 cells were then allowed to adjust to room temperature for 20 min.

Cells were then washed once in warm HBSS and stained with either 5 μM CellRox (C10422, Thermofisher) or 200 nM of MitoTracker (M22426, Invitrogen) for 30 min at 37°C. Cells were then washed twice in warm FACs buffer before being analyzed immediately on the LSR-Fortessa.

Preparation of protein lysates. $1.5x10^6$ Microglia or $2.0x10^6$ BMDMs per ml, cultured in 6-well plates, were lysed in 10 ml Ripa buffer (Sigma, Cat. No. R0278) supplemented with 1 tablet of Complete Mini, EDTA-Free Protease Inhibitor (Roche, Cat. No. 12371700), Phosphatase Inhibitor Cocktail 2 (1:100, Sigma, Cat. No. P5726) and Phosphatase Inhibitor Cocktail 3 (1:100, Cat. No. Sigma, P0044). Briefly, media was removed and cells were washed twice with PBS. After 70 μl lysis buffer was added, cells were scraped vigorously, incubated on ice for 45 min and then centrifuged at 9,391 x *g* for 10 min at 4°C. The supernatant was then removed, snap-frozen and stored at -80°C. For Western blot analysis, protein quantification was carried out using the Bicinchoninic Acid assay (BCA).

Western blot. Samples were supplemented with β-mercaptoethanol (Cat. No. M3148, Sigma-Aldrich**)** and 4x NuPAGE LDS Sample Buffer (Cat. No NP0007, Thermo Fisher Scientific), heated at 95°C for 5 min and loaded on either 4-12% or 12% NuPAGE Bis-Tris gel (Cat. No. NP0335BOX and NP0341BOX, Invitrogen) or 12% or 15% Bis-Tris hand-cast gels (Protein size marker: Cat. No. 928-60000, LI-COR). Protein gels were run for 30 min at 80 V and after that at 120 V until the loading dye reached the desired height. Proteins were transferred overnight at 30 V at 4°C to PVDF membranes (Cat. No. IPFL00010, Merk Millipore) and blocked in Intercept® (TBS) Blocking Buffer (Cat. No. 927-60001, LI-COR) for 1 h at room temperature. Blots were incubated overnight at 4°C with antibodies recognizing the following targets: RHEB1 (1:500, Cat. No. 13879), phospho-4EBP1 Thr37/46 (1:500, Cat. No 2855), phospho-4EBP1 Ser65 (1:200, Cat. No 9451), phospho-4EBP1 Thr70 (1:200, Cat. No, 9455), 4EBP1 (1:500, Cat. No 9644), eIF4E (1:200, Cat. No. 2067), phospho-eIF4E Ser209 (1:200,

Cat. No 9741), phospho-S6 ribosomal protein Ser240/244 (1:3,000, Cat. No. 5364), S6 ribosomal protein (1:1,000, Cat. No. 2217), phospho-p44/42 MAPK T202/Y204 (ERK1/2) (1:500, Cat. No. 4370), p44/42 MAPK (ERK1/2) (1:500, Cat. No. 9102), phospho-eIF2a Ser51 (1:250, Cat. No 3398), eIF2a (1:250, Cat. No. 5324), eIF4GI (1:250, Cat. No. 8701), mTOR (1:250, Cat. No. 2983), phospho-mTOR Ser2481 (1:250, Cat. No 2974) – all from Cell Signaling Technology. Anti-β-actin (1:5,000, Cat. No A1978) and anti-LC3 (1:250, Cat. No L8918) were from Sigma-Aldrich. Membranes were washed 3 times in TBS-0.1% Tween® 20 for 5 min at room temperature and incubated with secondary antibodies (LI-COR, Cat. No 926-32213 and 926-68022) diluted 1:5,000 in blocking buffer for 1 h at room temperature. Image acquisition was performed with a LI-COR ODYSSEY. Sometimes membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies, diluted 1:10,000 in blocking buffer – for 1 h at room temperature. Then proteins were visualized using the ECL Western Blot Detection System from Amersham (Cat. No. RPN2106) and the Chemidoc system from Biorad.

Densitometry analysis of Western blot. Densitometry analysis of bands was carried out using the software ImageJ (Java 1.8.0_172 64-bit) and the Gel Analysis tool, measuring the area under the peak of each band. Values were then normalized to the respective loading control, β-actin detected in the same membrane.

Cytokine measurements. Cytokine levels in serum or in microglia protein lysates were determined using the Legendplex mouse inflammation 13-plex panel, (Biolegend, Cat. No. 740446); a bead-based assay that allows simultaneous measurement of analytes based on cell size using flow cytometry. Specifically, the cytokines measured were CCL2 (MCP-1), GM-CSF, IFN-β, IFN-γ, IL-1α, IL-1β, IL-6, IL-10, IL-12 (p70), IL-17A, IL-23, IL-27 and TNF-α.

The assay was carried out in a 96-well plate following manufacturers' instructions. For TNF, IL-6, IL-12p40 measurements on BMDMs or microglia culture supernatants, the BD OptEIA ELISA kits were used (BD Pharmingen, Cat. No. 558534, 555240 and 555165) and the assay was carried out according to manufacturers' guidelines.

Growth factor array assay. Brain growth factors were detected using the growth factor array C3 assay (RayBio, AAM-GF-3-2), a sandwich-based chemiluminescence assay that detects 30 growth factors simultaneously. Specifically, the growth factors measured were FGF-7, IGF-1, IGFBP-5, PDGF-AA, VEGF-A, beta-NGF, GCSF, IGF-1R, IGFBP-6, PDGF-BB, VEGF-D, bFGF, GM-CSF, IGF-2, IL-2, PLGF-2, VEGFR1, EGF, HGF, IGFBP-2, IL-7, SCF, VEGFR2, EGFR, HGFR, IGFBP-3, M-CSF, TGF beta 1 and VEGFR3. The assay was carried out according to manufacturer's guidelines using a total of 200 μg of proteins for each sample measured. Protein lysates were prepared using the Gentle Macs dissociation system from Miltenyi Biotec and tissue lysis buffer containing 20 mM Tris, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA and 1% Triton® X-100.

Sickness behavior. Wild-type or *Rheb1*$^{f/f}$; *Csf1r*^{Cre} mice were given an intraperitoneal injection of either LPS (0.33 mg/kg) or PBS before being assessed in an open-field test 6 h post injection. Mice were brought into the testing room 30 min prior to the test, so that they could acclimatize. The test was conducted in a 15-square gridded box. Each mouse was placed in the left corner of the gridded box at the start of the test and was recorded for a total of 5 min. The test was performed so that the analyzer was blind to the genotype and treatment group of each mouse. Analysis was conducted by counting the number of times the mouse passed through each square during the 5 min test (distance traveled). All four paws of the mouse were required to pass within each square to be counted. The number of times the mouse reared (both paws) was counted as another measure of locomotion. Anxiety or stress-like behavior was also assessed by measuring the number of times the mouse passed

through any of the 3 central squares (time in center) or the first time the mouse passed through one of these central squares (time to center).

Preparation of acute brain slices. Brains were cut into 300 µm coronal sections using a vibratome (VT1200S, Leica), using cold carboxygenated artificial cerebrospinal fluid (ACSF), which contained 1.25 mM NH_2PO_4, 87 mM NaCl, 2.5 mM KCl, 7 mM $MgCl_2 \bullet 6H_2O$, 0.5 mM $CaCl_2 \bullet 6H_2O$, 25 mM glucose, 25 mM $NaHCO_3$, 50 mM sucrose. Sections were allowed to equilibrate in ACSF for 30 min and then pre-treated with either inhibitors or media as control for 90 min. For the inhibitor ribavirin (Merck, 55580), sections were pre-treated for 2 h with 400 µM ribavirin and then incubated with 2 µg/ml LPS for 6 h. Both pre-treatments and LPS stimulations were carried out at 37°C, 95% CO_2, 5% O_2.

Histological staining: Mice were narcotized with ketamine/xylazine and then transcardially perfused with 0.9% (w/v) saline followed by 10% (v/v) formalin neutral buffer solution (Sigma, Cat. No. HT501128-4). Dissected brain samples were fixed overnight with 10% formalin neutral buffer solution, and then preserved in sucrose 30% (w/v). Sagittal brain sections were cut into 30 µm-thick sections on a VT1200S vibratome (Leica) and free-floating immunostaining was performed. In brain tissue from aged mice, auto-fluorescence was quenched by incubating the tissue in $CuSO_4$ for 2 h at room temperature. Sagittal brain sections were blocked in 2.5-5% (w/v) BSA, 0.5% (v/v) Triton® X-100, and 5-10% (v/v) donkey serum in PBS for 1 h at room temperature. Primary antibodies, goat anti-IBA1 (1:500, Abcam, Cat. No. ab5076); rabbit anti-LC3 A/B (1:100, Cell Signaling, Cat. No. 4108S); and/or rabbit anti-TNF, (1:200, Cell Signaling, Cat. No. 11948) were mixed in 2.5% (w/v) BSA, 0.1% (v/v) Triton® X-100 in PBS and incubated overnight at 4°C. Sections were washed 3 times for 15 min with PBS and primary antibody was detected by incubating the brain slices with appropriate secondary donkey

anti-goat, Alexa Fluor594-conjugated (Thermo Fisher Scientific, Cat. No. A110034; A11058) and the nuclear dye Dapi (1:10000, 4',6-diamidino-2-phenylindole; Thermo Fisher Scientific) in 2.5% (w/v) BSA in PBS for 3 h at room temperature. After washing the sections 3 times for 15 min in PBS, brain slides were cover-slipped with Fluoromount medium (Sigma, Cat. No. F4680) and visualized with a LSM700 confocal laser-scanning microscope (Carl Zeiss) and analyzed using Fiji software. For acute brain slides, sections were fixed with 4% PFA overnight prior to being incubated in blocking/permeabilizing solution (2.5% BSA in PBS with 1% Triton® X-100 and 5% donkey serum for 1 hour at room temperature). Primary antibodies goat anti-IBA-1, (Cat. No. ab5076, Abcam) and/or rabbit anti-TNF, (Cat. No. 11948 Cell Signaling) diluted in blocking buffer, were applied overnight at 4°C. Sections were washed 3 times for 15 minutes in PBS-T and incubated with secondary antibodies (donkey anti-goat Alexa Fluor 594, Invitrogen, Cat. No. A-11058 – donkey anti-rabbit Alexa Fluor 488, Invitrogen, Cat. No. A-11058) in blocking buffer overnight at 4°C. After washing once with PBS-T for 15 minutes, nuclei were stained with 4,6-diamidino-2-phenylindole (Dapi; 1:10,000) for 10 minutes at room temperature. Following 3 washing steps, sections were mounted with with ProLong™ Gold Antifade Mountant (Cat. No. P36930, Thermo Fisher Scientific). Images were acquired using a LSM700 confocal microscope (Zeiss) and analyzed using Fiji software.

Immunofluorescence staining of cells. BMDMs or microglia were seeded at 0.2 x 10^6 cells on coverslips in 24-well plates and allowed to adhere at 37°C / 5% CO_2 overnight. The following day, cells were treated and/or stimulated as described. After aspirating the medium, cells were first washed in PBS for 5 min and then fixed with 10% (v/v) formalin neutral buffer solution (Sigma, Cat. No. HT501128-4) for 15 min on shaker. After washing 3 times in PBS-T (0.5% Tween®-X) for 5 min, cells were incubated in blocking buffer (2.5% BSA / PBS, 5% Donkey Serum) for 1 h on shaker. Primary antibodies, Rabbit Anti-NFκB p65 (1:250, Cat. No

8242, Cell Signaling Technology), Rabbit Anti-phospho-4EBP1 Thr37/46 (1:200, Cat. No 2855) or Rat Anti-F4/80 (1:50 ab6682, Abcam) were mixed in blocking buffer and incubated for 1 h at room temperature. Cells were washed 3 times in PBS-T for 5 min and incubated with appropriate secondary antibodies Alexa Fluor488- or Alexa Fluor594-conjugated for 1 h at room temperature. After washes, nuclear dye Dapi (1:10,000, Thermo Fisher Scientific) was incubated in PBS for 5 min. Coverslips were mounted on slides with ProLong™ Gold Antifade Mountant (Cat. No. P36930, Thermo Fisher Scientific). Images were acquired by LSM700 confocal laser-scanning microscope (Carl Zeiss) and analyzed using Fiji software.

References

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