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STAT3 signaling in myeloid cells promotes pathogenic myelin-specific T cell differentiation and autoimmune demyelination

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Materials and Methods

Mice: B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J (*LysMcre)* mice and B6.129P2(Cg)-Cx3cr1$^{tm2.1(cre/ERT2)Litt/WganJ}$ (*Cx3cr1-cre^{ERT}*), expressing EYFP and a Cre-ERT2 fusion protein under the control of the endogenous *Cx3cr1* promoter (1), were purchased from the Jackson Laboratory (stock #004781 and #021160, respectively). Conditional Stat3 mutant mice were generated by crossbreeding *Stat3$^{fl/fl}$* with *LysMcre* or *Cx3cr1cre^{ERT}* mice. Cre reporter mice *Rosa26-Ai14* (#007914), congenic C57B6 Cd45.1 (#002014), the 2D2 MOG-specific T cell receptor transgenic TCR^{MOG} mice (#006912) were obtained from the Jackson Laboratory.

Tamoxifen administration: For *Cx3cr1-cre^{ERT}:Stat3$^{fl/fl}$* and littermate controls, tamoxifen (T5648, Sigma-Aldrich) was dissolved in corn oil at 40 mg/ml by incubating while shaking at 37°C for 30 min followed by pulse sonication and given to mice (5-6 weeks) at 2 doses of 8 mg tamoxifen at 48 hr apart via oral gavage.

MOG$_{35-55}$-induced active EAE: Active immunization was carried out as previously described (2) with minor modifications. Both male and female mice were used. Briefly, 20 ml of Incomplete Freund's adjuvant (263910, Difco, Detroit, MI) was mixed with 100 mg heat-killed Mycobacterium tuberculosis H37 RA (231141, Difco) and vortexed for 5 min to make complete Freund's adjuvant (CFA). MOG$_{35-55}$ peptide (AS-60130-10, Anaspec, Fremont, CA) was reconstituted in sterile PBS at 2 mg/ml. CFA was emulsified with MOG$_{35-55}$ at a 1:1 volume ratio by sonication using a Microson ultrasonic cell disrupter followed by vortexing for 45min. After inducing light anesthesia with ketamine/xylazine, mice received two subcutaneous injections of 100 µl MOG$_{35-55}$/CFA into the flanks, followed by i.p. injection of 400 ng pertussis toxin (#180, List Biological Laboratories, Campbell, CA). A second dose of pertussis toxin was administered 48 hrs later. Mice were weighted and clinical symptoms were assessed on a daily basis. Clinical symptoms were scored according to the following criteria: 1, tail paralysis; 2, hind limb paresis; 3, unilateral hind limb paralysis; 4, bilateral hind limb paralysis; 5, moribund or death.

Passive EAE induction by adoptive transfer: Passive EAE was induced as previously described (3) with modifications. Briefly, donor C57BL/6 mice were immunized for MOG$_{35-55}$ using the same procedure as in active EAE induction. Pertussis toxin was not administered. Ten days after immunization, draining LNs were isolated from donor mice and single cell suspension was prepared and cultured at 5.0×10^7 cells in 10 ml complete RPMI 1640 medium containing 10% FBS, 4 mM L-glutamine, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol and 100 IU/ml Penicillin and 100 µg/ml Streptomycin in the presence of 20 µg/ml MOG$_{35-55}$ for 3-4 days. Cells were then collected, washed and resuspended in RPMI at a density of $1.0 - 2.0 \times 10^7$ cells/ml. Cells were aliquoted (5 ml) into several conical tubes. Equal volume of Lympholyte M (CL5031; Cedarlane, Burlington, NC) was layered using a Pasteur pipette to the bottom of each tube. The tubes were then centrifuged at 1,000 × g at RT for 20 min with the brake set to 0. Cells at the interphase were collected, washed twice with sterile PBS, and resuspended at a final density of 1.5×10^7 cells/200ul. Cells ($1.5 – 2.0 \times 10^7$) were injected retro-orbitally into recipient mice that were sublethally irradiated at 400 rad a day before cell transfer. The recipient mice also received 400 ng pertussis toxin on the day of cell transfer and then 48 hrs after. Clinical symptoms were assessed on a daily basis following the same criteria as for active EAE.

Bone marrow chimera: Mouse bone marrow was isolated from donor mice (5-8 weeks) and chimeric mice were generated as described (4) with minor modifications. Recipient mice of age 5-6 weeks were subject to 7Gy lethal dose irradiation and 4 hours later were anesthetized with

isoflurane and received 5.0×10^6 bone marrow cells from donor mice retro-orbitally. Mice were housed in autoclaved cages and supplied with irradiated food and drinking water with 1.25% antibiotics (sulfamethoxazole and trimethoprim; 50383, Hi-Tech Pharmacal, Amityville, NY) for 2 weeks before switched back to regular housing. Six weeks after bone marrow transplantation, active EAE was induced as described above.

Tissue collection: The mice at specified age or EAE stages were deeply anesthetized with ketamine and xylazine. Blood was collected by cardiac puncture and mixed 2:1 with 10 usp/ml heparin solution (H3393, Sigma-Aldrich) for preparation of single cell suspension or placed in empty Eppendorf tubes for preparation of serum. The mice were perfused with 20 ml of PBS. The spleen and draining lymph nodes (inguinal, axillary, and cervical) were removed and placed in Eppendorf tubes with PBS containing 2% FBS on ice. For histological analysis of the CNS, the brain and spine were removed and fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C. For analysis of CNS infiltrating leukocytes, the spinal cord was ejected from the spine with a 10 ml syringe attached to a 20G cannula and filled with ice-cold PBS. For analysis of cytokine mRNA expression in the CNS, the brain and spinal cord were flash frozen and stored at -80°C until use.

Isolation of CNS infiltrating leukocytes: Brains and spinal cords from EAE mice were cut and digested with Accutase (A11105-01; Life Technologies, Carlsbad, CA) for 30 min at 37°C and passed through a 70 μm cell strainer to achieve single cell suspension. Cells were collected by centrifugation, resuspended in sterile PBS containing 35% Percoll (P4937; Sigma-Aldrich) and underlayered with 70% Percoll. After centrifugation at 2,000 × *g* for 20 min at room temperature with the brake set to 0, cells in the interphase between 35% and 70% Percoll were collected, washed and resuspended in complete RPMI 1640 medium or PBS containing 2% FBS for flow cytometry.

Single cell suspensions from spleen, lymph nodes and blood: Spleens and lymph nodes from EAE mice were collected, dissociated with a 3 ml syringe plunger and passed through a 70 μm cell strainer. Cells were centrifuged, resuspended in 3 ml of RBC lysis buffer (R7757, Sigma-Aldrich) and incubated at room temperature for 2 min, and washed in complete RPMI 1640 medium. Blood collected from the mice was mixed with heparin. Erythrocytes were eliminated with RBC lysis buffer. Mononuclear cells were collected by centrifugation and resuspended in PBS containing 2% FBS for flow cytometry analysis. For antigen recall experiments, splenocytes were resuspended in complete RPMI 1640 medium and cultured at 5.0×10^5 cells per well in 96-well U-shaped plates in the absence or presence of 30 μg/ml MOG_{35-55} for 3 d. Cytokine secretion into the supernatant was determined with ELISA.

Immunomagnetic selection of $CD11b^+$ and $CD4^+$ cells and co-cultures: Single cell suspension of splenocytes prepared from mice was resuspended in BD iMAG buffer (552362, BD Biosciences, San Jose, CA) at the density of 2.0×10^7 cells/ml and incubated with 20 μg/ml α-CD11b-biotin (13-0112, eBioscience, San Diego, CA) or 10 μg/ml α-CD4-biotin (13-0042, eBioscience) for 15 min on ice. The cells were then washed twice and resuspended in iMAG buffer at the density of 4.0×10^7 cells/ml. Streptavidin (557812, BD Biosciences) was added (10 μl per 1.0×10^7 cells) and incubated at 8°C for 30 min. The volume was then brought to 2.0×10^7 cells/ml and the tube was placed onto a cell separation magnet (552311; BD Biosciences) and incubated at room temperature for 8 min. The negative portion was removed while the tube was still on the magnet, and the positive portion was resuspended in iMAG buffer at 2.0×10^7 cells/ml. The separation was repeated two more times with 4 min incubation at room temperature

each time. After the last separation, the positive portion was resuspended in complete RPMI 1640 medium.

For myeloid cell and T cell co-cultures, magnetic selected $CD11b^+$ splenocytes (2.0×10^5) from MOG-immunized mice after 9 days and magnetic selected $CD4^+$ splenocytes (1.0×10^5) from naïve 2D2 mice were co-cultured in 48-well plates in the absence or presence of 30 µg/ml MOG_{35-55} for 2-3 d. For myeloid cell monoculture, $CD11b^+$ splenocytes (2.0×10^5) were cultured in 48-well plates and stimulated with 1 ng/ml LPS and 10 ng/ml IFNγ for 24 h. Cells were harvested and stained for flow cytometry analysis.

BMDM cell cultures: The preparation of BMDMs was described previously (4). Briefly, L929 cells were cultured at the density of 5.0 x 10^5 cells in 15 ml complete DMEM medium per 75-cm^2 culture flask. At div 5, the conditioned medium was collected and stored in -20°C until use. Bone marrow cells from wild-type and mutant mice were isolated, seeded at the density of 1.0 x 10^6 cells/ml, and induced for differentiation to monocytes in IMDM medium containing 15% L929-conditioned medium and 10% FBS. The medium was replenished at DIV3. At DIV7, cells were incubated in HBSS containing 5 mM EDTA at 4°C for 40 min and harvested. BMDMs were analyzed by flow cytometry or sub-cultured for in vitro stimulation with IFNγ and LPS as specified in figure legends.

In vitro polarization of T cells: $CD4^+$ cells from splenocytes of naive 2D2 mice were isolated by magnetic selection and cultured with irradiated feeder splenocytes at a ratio of 1:5. The cells were polarized towards Th1 with 2.5 µg/ml α-CD3 (16-0031, eBioscience), 10 ng/ml rmIL-12 (14-8121, eBioscience), and 10 µg/ml α-IL-4 (#16-7041, eBioscience), or polarized towards Th17 with 2.5 µg/ml α-CD3, 5 ng/ml TGFβ1 (7666-MB-005, R&D Systems), 30 ng/ml rmIL-6 (406-ML-025, R&D Systems), and 10 µg/ml α-IFNγ (16-7311, eBioscience) for 3 d in complete RPMI 1640 medium.

Flow cytometry: Cells ($1.0\text{-}5.0 \times 10^5$) were resuspended in 50-100 µl ice cold flow buffer (sterile PBS containing 2% FBS). Nonspecific binding of Fc receptors was blocked with 5 µg/ml α-ms CD16/32 antibody (14-0161, Clone 93, eBioscience) for 10 min on ice. Subsequently, cells were stained with fluorophore-conjugated antibodies at 1/200 dilution in a total volume of 100 µl for 20 min on ice, and then washed twice with 500 ul flow buffer. Antibodies used in this study included α-ms CD11b FITC (11-0112); α-ms Ly6G PE (61-9668); α-ms F4/80 APC, (17-4801); α-ms CD8 FITC (11-0081); α-ms CD19 PE (12-0193); α-ms CD4 APC (17-0041); α-ms CD11c PE (12-0114); α-ms Ly6C APC (17-5932); α-ms/rt CD40 FITC (11-0402); α-ms CD80 PE (12-0801); α-ms CD86 APC (17-0862); α-ms MHC II APC (17-5321); α-ms CD45 PE-Cy5.5 (35-0451); α-ms CD45.1 FITC (11-0453); and α-ms CD45.2 APC (17-0454, all from eBioscience). Flow cytometric data were acquired on a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Color compensation, gating, and data analysis were performed on the proprietary software from the manufacturer. For intracellular cytokine staining, brefeldin-A (00-4506, eBioscience) was added to the cells during the last 5 hr of MOG_{35-55} stimulation. After harvesting, the cells were stained for surface marker CD4, washed, and fixed with fixation buffer (426803, BioLegend) at RT for 20 min. Subsequently the cells were permeabilized by washing twice with the permeabilization buffer (#426803, BioLegend), and then stained with α-IFNγ PerCP-Cyanine5.5 (45-7311), α-IL-17 FITC (11-7177), and α-GM-CSF PE (12-7331, eBioscience) at RT for 20 min.

Western blotting analysis: Brain tissue lysates and western blots were carried out as previously described (5, 6). BMDMs from *Stat3*$^{fl/fl}$ and *LysMcre:Stat3*$^{fl/fl}$ mice were stimulated with 10 ng/ml rmIL-6 (1830-SR, R&D Systems, Minneapolis, MN) for 30 min and then lysed with ice-cold lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% triton-X100, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1.0% PIC (P8340, Sigma-Aldrich), 100 mM Na_3VO_4, and 100 mM PMSF). Antibodies against total STAT3 (9132, 1:1000 dilution) and pSTAT3(Y705) (9145, 1:1000) were from Cell Signaling Technology. Images were acquired with Chemidoc XRS and quantified by densitometry using the Quantity One software (Bio-Rad).

RNA isolation and quantitative RT-PCR: qRT-PCR was used to determine tissue cytokine/chemokine transcripts. Flash frozen spinal cords from EAE mice were thawed, and RNA was extracted with Tri Reagent (T9424, Sigma Aldrich), quantified with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and stored at -80°C until use. RNA samples (500 ng) were treated with 0.1 UI/µl DNase I (#18068, Invitrogen, Carlsbad, CA) for 15 min at room temperature. 2.5 mM EDTA was then added and samples were incubated for 10 min at 56°C to inactivate DNase. Samples were then reverse transcribed to cDNA in a reaction mixture of 5 mM magnesium chloride, 0.25 mM each of dATP, dTTP, dCTP and dGTP, 0.75 U/ml reverse transcriptase (M510F, Promega, Madison, WI), 25 µg/ml random primers (C118A, Promega) and 1 U/ml RNase inhibitor (N251B, Promega). Specific cDNAs were amplified and analyzed using a 7500 Real-Time PCR System (Applied Biosciences, Foster City, CA) in a reaction mixture of 1x SYBR Green PCR Master Mix (#4309155, Applied Biosystems, UK) and 1 µM forward- and reverse- primers (*SI Appendix* Table 2) with the following thermal cycling parameters: 95°C 10 min, [95°C 15 sec, 60°C 1 min] × 40 cycles. All samples were run in duplicate or triplicate, and gene expression was normalized to β-actin and fold expression was calculated using the formula $2^{-\Delta\Delta CT}$.

Multiplex immunoassay: Splenocytes (5×10^5 cells per 96-well) isolated from MOG-immunized *Stat3*$^{fl/fl}$ and *LysMcre:Stat3*$^{fl/fl}$ mice at various clinical stages were re-stimulated with antigen MOG_{35-55} (0-30 µg/ml) for 1-3 days. $CD11b^+$ myeloid mono- and myeloid/2D2 $CD4^+$ T-cell co-cultures were stimulated with LPS or MOG_{35-55} as specified in figure legends. The levels of cytokines/chemokines in the supernatants were determined by either Ready-SET-Go!® ELISA kits or customized ProcartaPlex Mix&Match mouse multiple immunoassay kit (eBioscience) following the manufacturer's instructions. The level of MPO in the supernatant was determined using Mouse Myeloperoxidase DuoSet ELISA kit from R&D Systems (DY3667) following the manufacturer's instructions.

Immunohistochemistry and immunofluorescence microscopy: Brain and vertebral column were isolated from normal and EAE mice and fixed in 4% PFA in PBS at 4°C overnight. The next day, the spinal cord was carefully isolated from the spine using scissors and forceps, and fixed in 4% PFA again for an additional 4 hr. The tissues were then cryoprotected with 30% sucrose in PBS, embedded in Tissue-Tek O.C.T. and cryosectioned into serial 10 µm sections. Myelin was visualized with Oil-Red-O(6). For immunohistochemistry, the slide sections were rinsed in PBS, blocked and permeabilized with PBS containing 5% goat serum and 0.3% Triton X-100 for 1 hr at room temperature, and incubated with primary antibodies overnight in PBS containing 5% goat serum and 0.1% Triton X-100 at 4°C. Antibodies used for staining included: rabbit α-Iba-1 (1:400; #019-19741, Wako, Richmond, VA), rat α-CD68 (1:400; MCA1957, BioRad, Hercules, CA), rabbit α-GFAP (1:100; 18-0063, Invitrogen), Tomato lectin (B-1175, Vector Labs), rat α-ms ICAM-1 (1:100; 14-0542-81, eBioscience), rat α-ms PECAM-1 (1:50; 553370, BD Biosciences), rat α-ms CD3 (1:100, 14-0032, eBioscience), and rat α-ms CD4

(1:100, 14-0042, eBioscience), rabbit α-pSTAT3(Y705) (1:100; #9145, Cell Signaling), ms α-human CD11b (ab63317, Abcam). Tissue sections were washed in PBS three times for 5 min each, and incubated with appropriate secondary antibodies conjugated to Alexa Fluor 488 or 594 (1:1000, Invitrogen) for 1 hr at room temperature in dark. Nuclei were stained with Hoechst 33342. Human MS tissues were also stained with Sudan black as described previously to remove autofluorescence (5). Images were acquired with an Olympus IX71 fluorescence microscope equipped with a DP70 digital camera.

Single cell RNA-seq of myeloid cells and analysis: $CD11b^+$ cells from EAE mice at dpi 9 and naïve $CD4^+$ T cells from normal 2D2 mice were isolated as described above. Myeloid cells in co-cultures were first isolated by FACS. Cells from each treatment group were captured on C1 Single-Cell mRNA Seq IFC. After capture, cells were visualized on a BioTek Cytation 5 and locations of captured cells were noted. Single-cell cDNA samples were prepared on C1 Single-Cell mRNA Seq IFC's following the standard protocol (Fluidigm Inc.). Resulting cDNA samples were quantified and normalized for sequencing library preparation utilizing the Illumina Nextera XT Library Preparation Kit. Libraries were normalized and pooled for sequencing on a NextSeq 500 using a 2x75 Mid-output sequencing kit. Following RNA sequencing of 446 cells, raw reads were trimmed using Trimmomatic (7). Filtered reads were aligned to mouse genome (mm10) using TopHat (8). Gene level FPKM (fragments per kilobase per million reads) values were produced using Cufflinks(8) and an FPKM matrix was generated for all cells. After removing zero count genes across samples, the FPKM matrix was furthered filtered using Monocle (9) to remove cells that did not have at least 100 genes expressed and to remove genes that did not have expression (FPKM > 1.0) in at least 10 cells. The resulting gene set of 1229 genes and 389 cells was used for all downstream analyses. Ingenuity Pathway Analysis (Qiagen) was based on fold change values calculated by applying log2 scale on mean expression of genes in mutant myeloid cells versus WT after coculturing with 2D2 T cells with the antigen.

Statistical analysis: When appropriate, differences between two groups were analyzed with two-tailed Student's t test. Differences between more than two groups were analyzed with multivariate analysis of variance followed by Bonferroni's post hoc test. The cumulative EAE scores of *Stat3*$^{fl/fl}$ and *LysMcre:Stat3*$^{fl/fl}$ mice were analyzed with Wilcoxon rank-sum test. The rate of EAE onset of *Stat3*$^{fl/fl}$ and *LysMcre:Stat3*$^{fl/fl}$ mice was analyzed with Pearson chi-square test and Fisher's exact test. Differences were considered statistically significant if $p < 0.05$. All analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

Table S1. Summary of clinical information on MS and control cases used in this study

Diagnosis	ID	Age/Sex	Clinical Diagnosis/Cause of Death
Control	#C-1	86/F	Clinical diagnosis of MS
	#C-2	74/M	Progressive supranuclear palsy
	#C-3	19/M	Gunshot (abdomen)
	#C-4	15/M	Accidental hanging
	#C-5	31/M	Anemia, pancytopenia
	#C-6	29/M	Motor cycle accident
MS	#MS-1	69/M	MS
	#MS-2	44/M	MS (chronic progressive), aspiration pneumonia, grand mal seizure with status epilepticus
	#MS-3	40/F	MS (progressive, severe, unrelenting for 10 yrs.)
	#MS-4	63/F	MS, sleep apnea, chronic urinary tract infection, respiratory failure, hypertension
	#MS-5	52/F	MS (secondary progressive)
	#MS-6	51/F	MS (secondary progressive)
	#MS-7	75/F	MS, hypertension, kidney stone surgery, Goiter surgery, hypothyroidism, migraine, chronic urinary tract infection
	#MS-8	54/F	MS (relapse-remitting)

Table S2. Primer pairs used for qPCR

Primer Pair Name	Forward 5' → 3'	Reverse 5' → 3'
β-actin	AGACTTCGAGCAGGAGATGG	CAACGTCACACTTCATGATGG
Tnfα	TGTCCCTTTCACTCACTGGC	CATCTTTTGGGGGAGTGCCT
Il1β	CGACAAAATACCTGTGGCCT	CGACAAAATACCTGTGGCCT
Ifnγ	AAGACTGTGATTGCGGGGTT	GCACCAGGTGTCAAGTCTCT
Trem2	GGCTGAGGTCCTGCAGAAAG	GCACCCTCGAAACTCGATGA
Cd206	AAGGCATGCGTTGCACATAC	ATTCTGCTCGATGTTGCCCA
Ccl2	GGCTCAGCCAGATGCAGTTA	GCTGCTGGTGATCCTCTTGT
Ccl3	CCGGAAGATTCCACGCCAAT	GTCTCTTTGGAGTCAGCGCA
Ccl5	GCCAGCTTGGGGATGCCACTC	CAGAGCCTCGGAGCAGCTGAG
Il6	TGGTGACAACCACGGCCTTCC	AGCCTCCGACTTGTGAAGTGGT
Il12p40	CGCAGCAAAGCAAGATGTGT	CGTGTCACAGGTGAGGTTCA

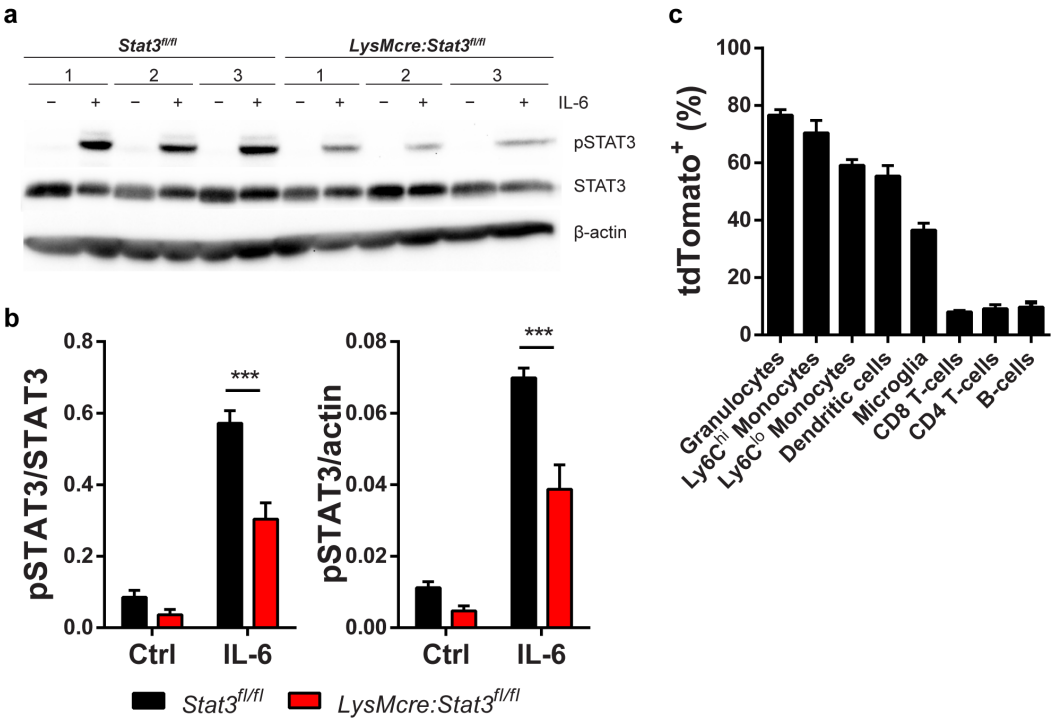


Figure S1. Functional inactivation of STAT3 signaling in cultured *LysMcre:Stat3*$^{fl/fl}$ macrophages and cre recombination efficacy in *LysMcre* reporter mice. a, b, Bone-marrow derived macrophages (BMDM) were prepared from adult *LysMcre:Stat3*$^{fl/fl}$ and *Stat3*$^{fl/fl}$ mice (n = 3 per group) and stimulated *in vitro* with recombinant mIL-6 (10 ng/ml) for 30 min. Cell lysates were subject to Western blot analysis for pSTAT3 (Y705) and total STAT3 (**a**). Relative expression levels of pSTAT3 were quantitated with densitometry (**b**). Data represent mean ± SEM. ***p < 0.005. **c**, Expression of reporter activity in immune cells from the spleen and CNS of *LysMcre:rosa26-Ai14* reporter mice (n = 3) was analyzed with flow cytometry. Granulocytes, CD11b^{+}Gr-1^{+}; Ly6C^{hi} monocytes, CD11b^{+}Ly6C^{hi}; Ly6C^{lo} monocytes, CD11b^{+}Ly6C^{lo}; Dendritic cells, CD11b^{+}CD11c^{+}; CD8 T-cells, CD8^{+}; CD4 T-cells, CD4^{+}; B-cells, CD19^{+}; Microglia, CD11b^{+}CD45^{int}. Data represent Mean ± SEM.

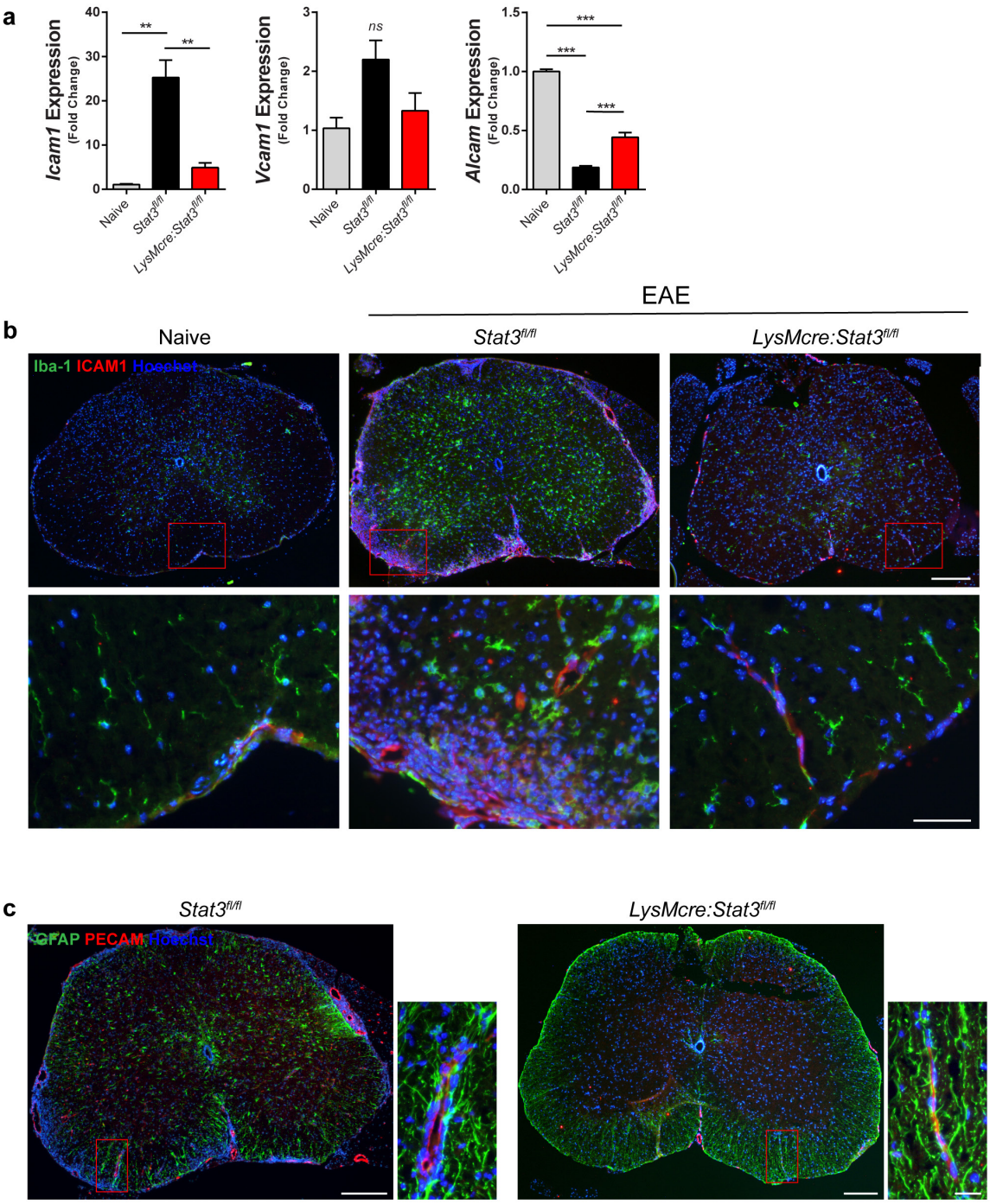


Figure S2. Decreased expression of ICAM-1 cell adhesion molecules during EAE. a, Quantitative RT-PCR analysis of *Icam1, Vcam1* and *Alcam* expression in spinal cords of naïve (n= 3) and MOG_{35-55}-immunized mice at 14 dpi (*Stat3*$^{fl/fl}$, n = 4; *LysMcre:Stat3*$^{fl/fl}$, n = 3). Data represent mean ± SEM. **p < 0.01; ***, p<0.001. **b,** Representative images of spinal cord sections from naïve and EAE mice at 14 dpi immunostained for Iba-1 and ICAM-1. Scale bar, 200 µm. Lower panel are boxed areas at higher magnification. Scale bar, 50 µm. **c,** Representative images of spinal cords of EAE mice (14 dpi) stained for GFAP and PECAM. Scale bar, 200 µm. Lower panels are the magnified view of boxed area showing prominent perivascular infiltrating cells in *Stat3*$^{fl/fl}$ EAE mice. Scale bar, 20 µm.

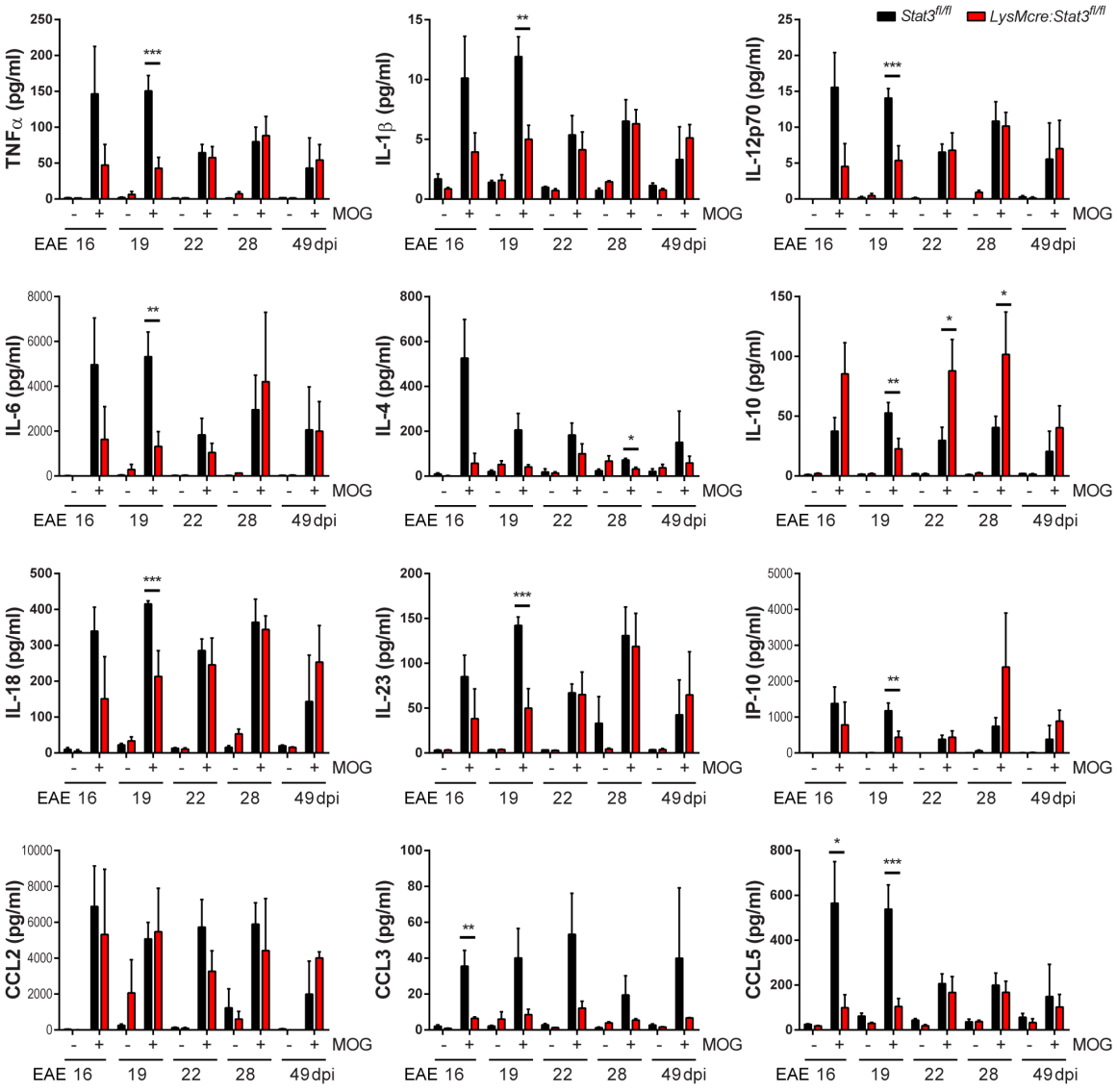


Figure S3. Antigen-specific responses of splenocytes isolated from mice at different time points after MOG_{35-55} immunization. Splenocytes were isolated from mice at specified days post-immunization (dpi) representing EAE onset, peak and chronic stages, and cultured in the absence or presence of 30 µg/ml MOG_{35-55} for 3 days. Cytokine/chemokine secretion into the supernatant was determined using multiplex immunoassay. Mice used for each time point: 16 dpi, *Stat3*$^{fl/fl}$ n = 4, *LysMcre:Stat3*$^{fl/fl}$ n = 3. 19 dpi, *Stat3*$^{fl/fl}$ n = 6; *LysMcre:Stat3*$^{fl/fl}$ n = 4. 22 dpi, *Stat3*$^{fl/fl}$ n = 4, *LysMcre:Stat3*$^{fl/fl}$ n = 4. 28 dpi, *Stat3*$^{fl/fl}$ n = 5, *LysMcre:Stat3*$^{fl/fl}$ n = 2. 49 dpi, *Stat3*$^{fl/fl}$ n = 3, *LysMcre:Stat3*$^{fl/fl}$ n = 2. Data represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.005.

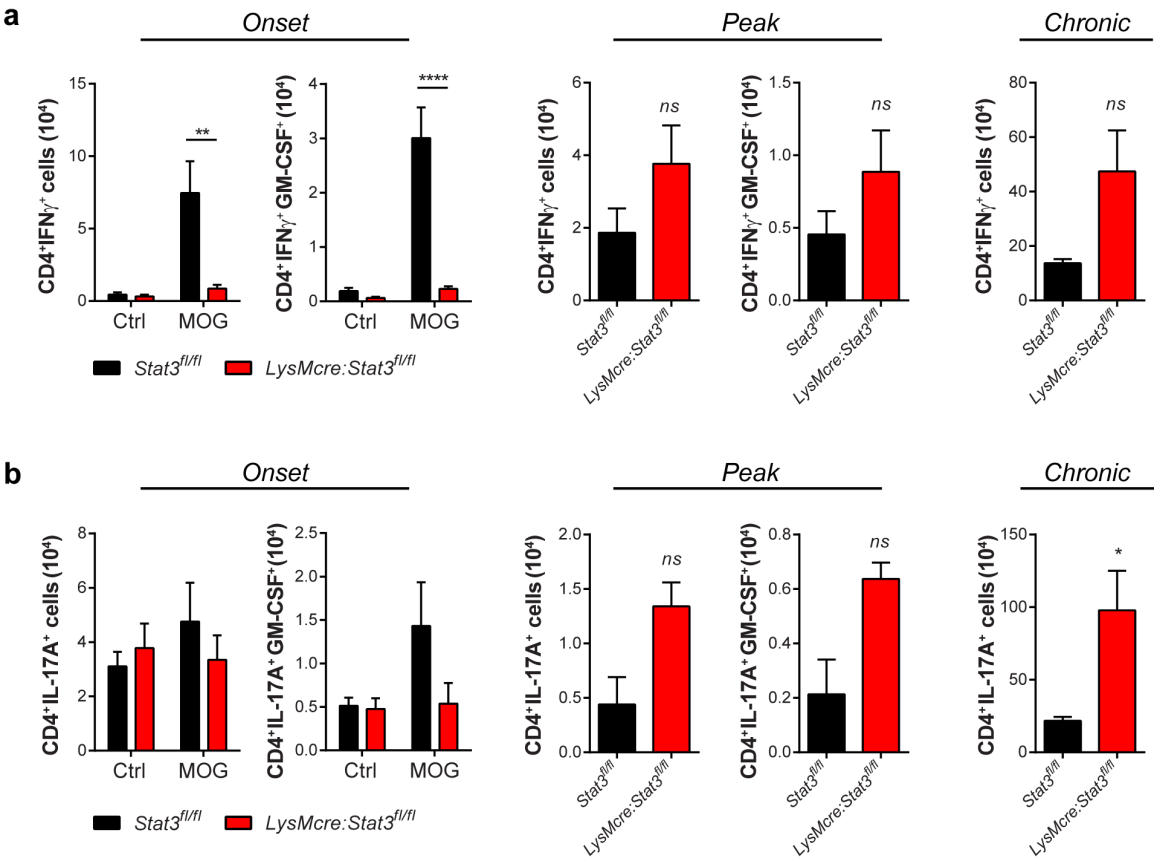


Figure S4. Loss of myeloid *Stat3* suppressed the number of myelin-specific Th1 cells in the secondary lymphoid organ in the EAE model. Active EAE was induced in *Stat3*$^{fl/fl}$ and *LysMcre:Stat3*$^{fl/fl}$ mice. At three different disease stages splenocytes were isolated and exposed to vehicle or MOG_{35-55} (100 µg/ml) in complete RPMI medium for 24 h. Cellular cytokine secretion was blocked with Brefeldin A during the last 5 h incubation. The cells were stained with α-CD4, α-IFNγ, α-IL-17A and α-GM-CSF, and analyzed with flow cytometry. The total number of Th1 (**a**) and Th17 (**b**) cells was determined. EAE onset, *Stat3*$^{fl/fl}$, n = 6; *LysMcre:Stat3*$^{fl/fl}$, n = 4. Peak, *Stat3*$^{fl/fl}$, n = 4; *LysMcre:Stat3*$^{fl/fl}$, n = 4. Chronic, *Stat3*$^{fl/fl}$, n = 3; *LysMcre:Stat3*$^{fl/fl}$, n = 2. Data represent Mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.005.

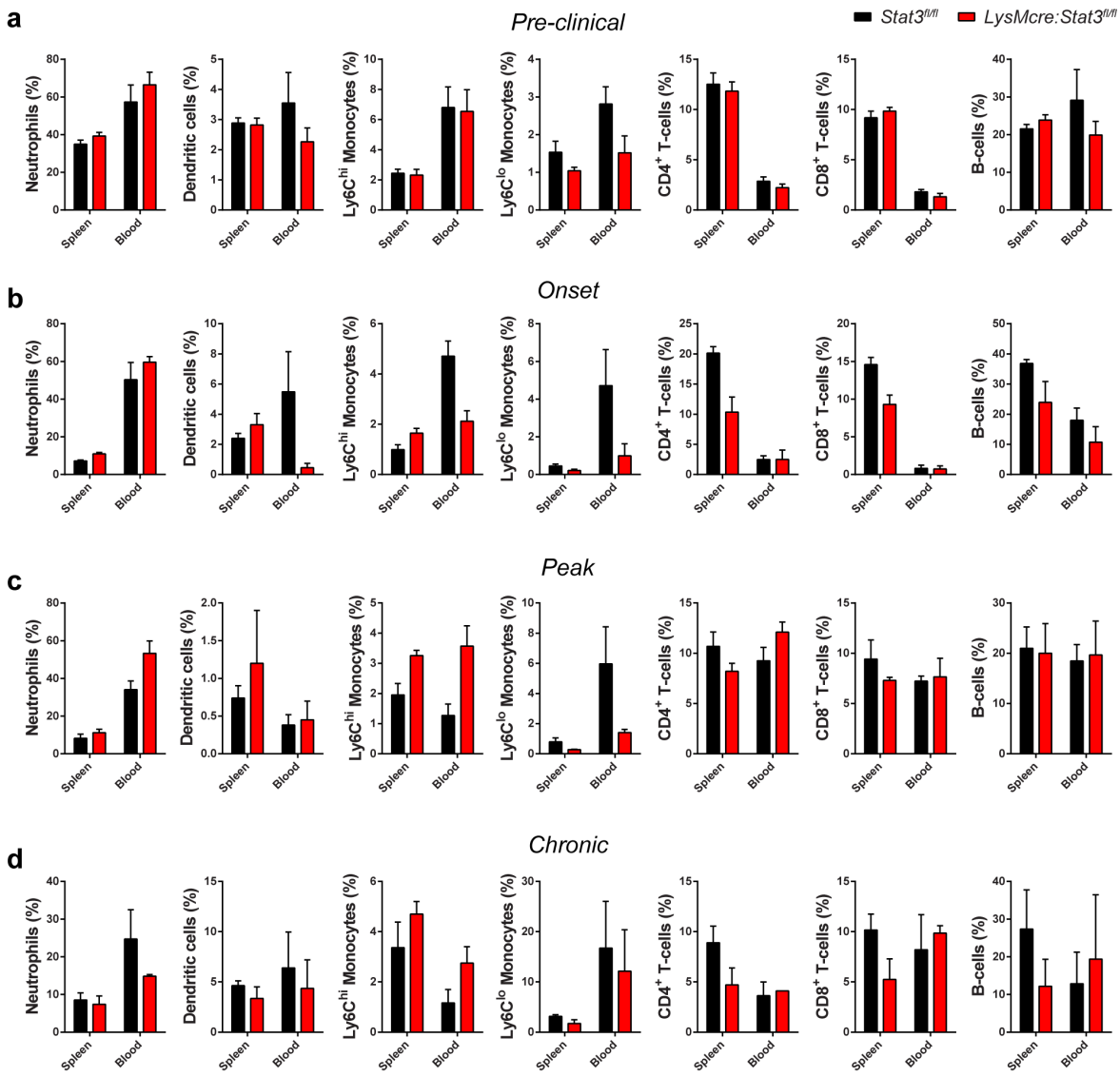


Figure S5. No significant differences in major immune cell populations were observed between *LysMcre:Stat3*$^{fl/fl}$ and *Stat3*$^{fl/fl}$ mice over the course of EAE. Active EAE was induced in *LysMcre:Stat3*$^{fl/fl}$ and *Stat3*$^{fl/fl}$ mice. Splenocytes and blood were collected at four different stages of EAE, stained with antibodies against cell surface markers and immunophenotyped by flow cytometry. Neutrophils, CD11b^{+}Ly6G^{+}; Dendritic cells, CD11b^{+}CD11c^{+}; Ly6C^{hi} monocytes, CD11b^{+}Ly6C^{hi}; Ly6C^{lo} monocytes, CD11b^{+}Ly6C^{lo}; CD4^{+} T-cells, CD4^{+}; CD8^{+} T-cells, CD8^{+}; B-cells, CD19^{+}. **a**, Pre-clinical, *Stat3*$^{fl/fl}$, n = 6; *LysMcre:Stat3*$^{fl/fl}$, n = 5. **b**, Onset, *Stat3*$^{fl/fl}$, n = 6; *LysMcre:Stat3*$^{fl/fl}$, n = 4. **c**, Peak, *Stat3*$^{fl/fl}$, n = 5; *LysMcre:Stat3*$^{fl/fl}$, n = 2. **d**, Chronic, *Stat3*$^{fl/fl}$, n = 3; *LysMcre:Stat3*$^{fl/fl}$, n = 2. Data represent mean ± SEM.

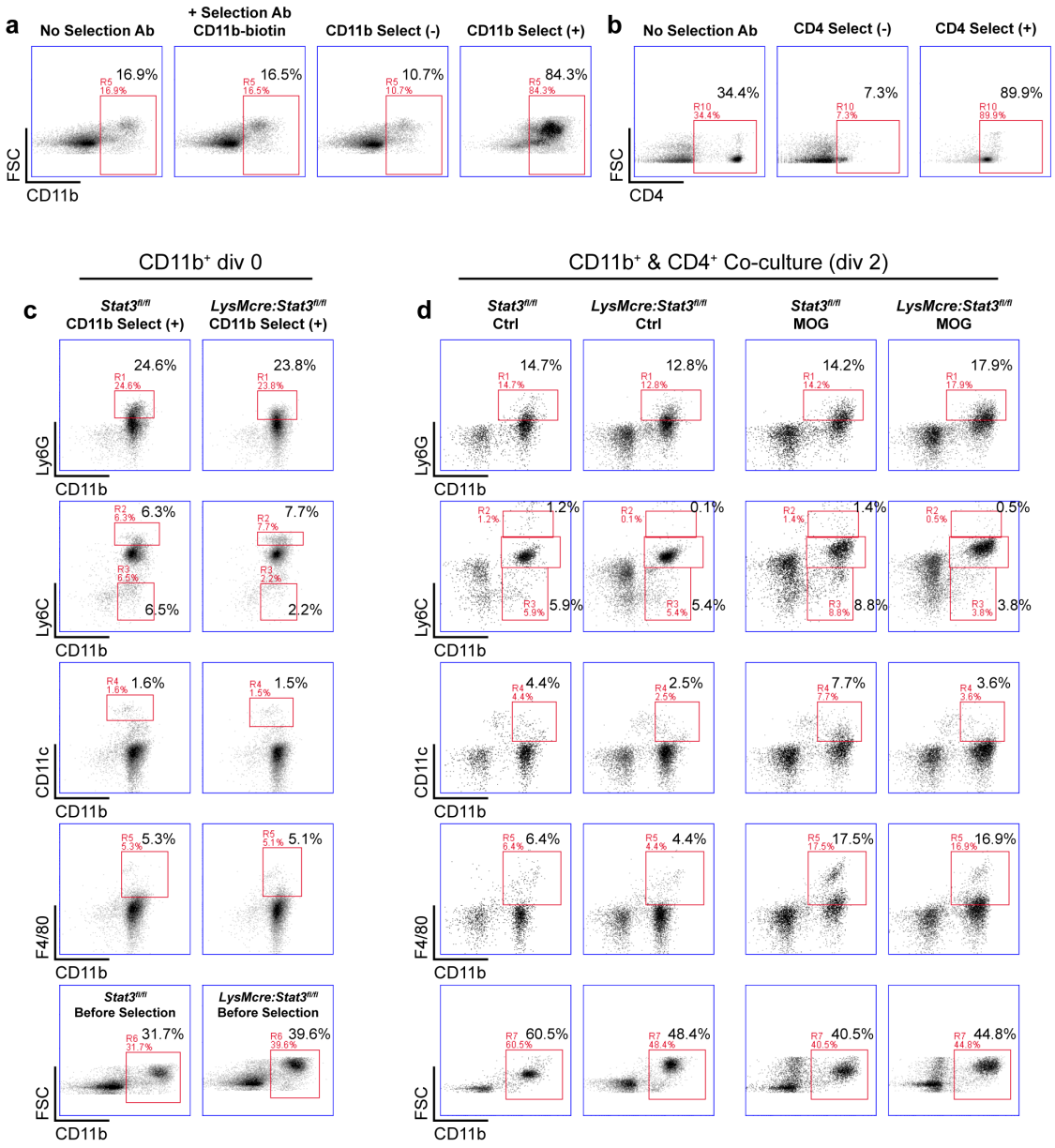


Figure S6. Efficiency and cell profile analysis of immunomagnetic cell selection for the ex vivo co-culture experiments. CD11b^{+} cells from splenocytes of immunized *Stat3$^{fl/fl}$* and *LysMcre:Stat3$^{fl/fl}$* mice at 9 dpi (**a**) and CD4^{+} T-cells from naïve 2D2 mice (**b**) were isolated by positive immunomagnetic cell selection as described in the methods. **a, b,** Efficiency of immunomagnetic bead selection as determined by surface labeling of CD11b (**a**) and CD4 (**b**) of the cells prior to and after beads selection. Positive (+) selected populations were used for mono- or co-culture experiments. **c**, **d**, Flow cytometry analyses of myeloid cell subpopulations in freshly selected CD11b^{+} cells (**c**) or after co-cultured with T cells for 2 days under control and antigen-stimulated conditions (**d**).

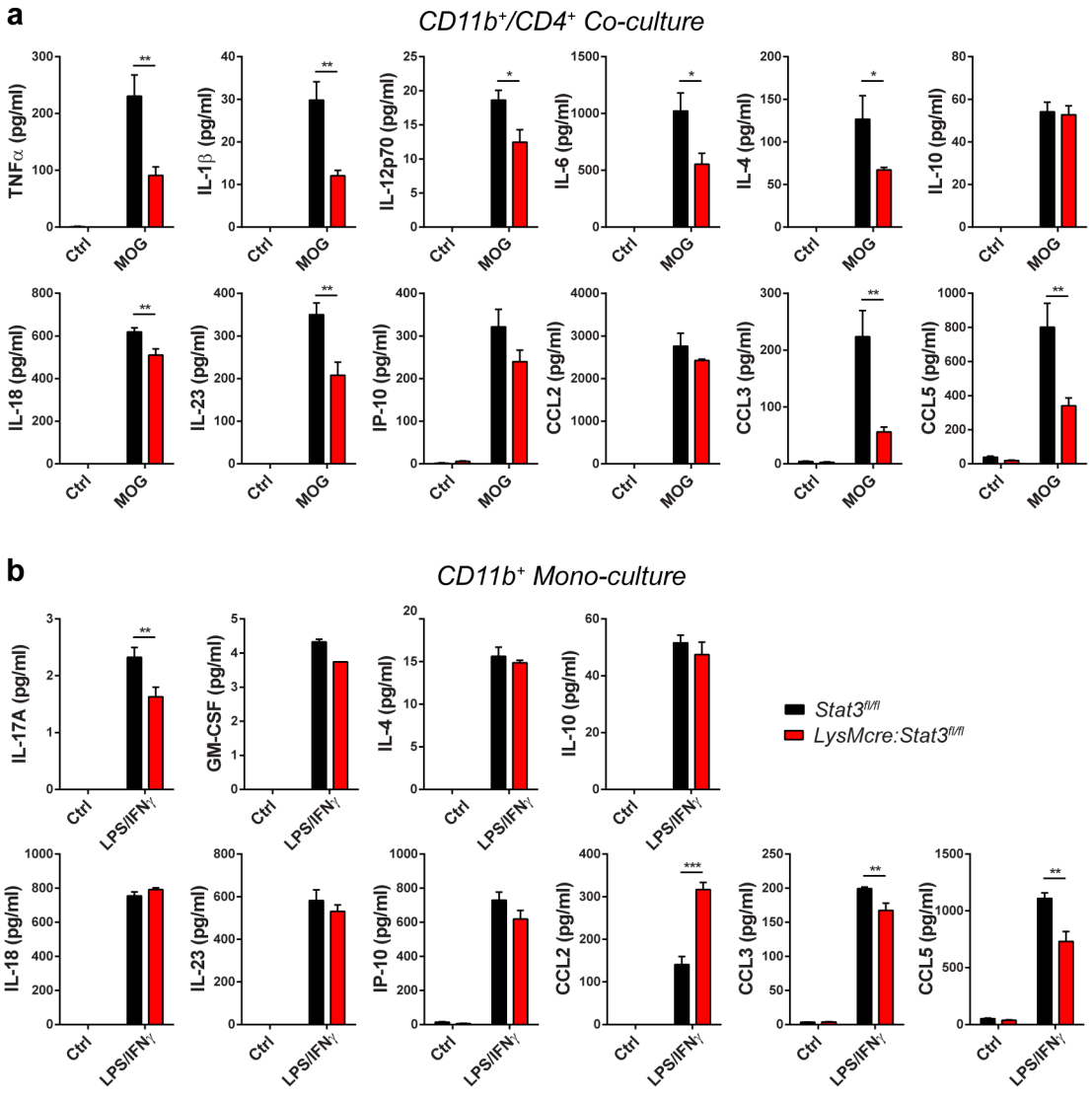


Figure S7. Activation-dependent production of cytokines/chemokines by co-cultured myeloid/T cells and CD11b$^+$ cells. Mono- and co-cultures were prepared as in Fig. S6. Cytokine/chemokine produced by CD11b$^+$/CD4$^+$ co-cultures treated as indicated (**a**) or by CD11b$^+$ mono-cultures (**b**) were analyzed by multiplex immunoassays. Data represent mean ± SEM of a pool of three mice per genotype with technical replicates. *p < 0.05, **p < 0.01, ***p < 0.005.

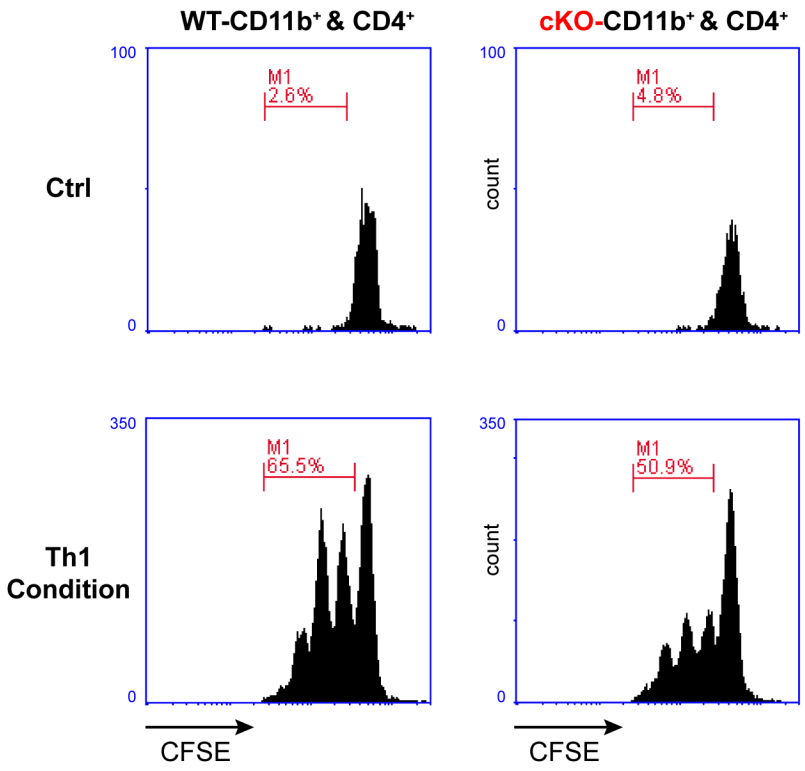


Figure S8. Decreased Th1 cell proliferation in cocultures with *Stat3* mutant myeloid cells. *LysMcre:Stat3*$^{fl/fl}$ and WT littermates were immunized with MOG_{35-55}. At 7 dpi, $CD11b^+$ cells from spleens of immunized mice and $CD4^+$ cells from naïve 2D2 mice were isolated with immunomagnetic separation. $CD4^+$ T cells were pre-labeled with 2.5 µg/ml CFSE, and co-cultured in 1:2 ratio with $CD11b^+$ cells under either control or Th1 polarization condition (2.5 µg/ml α-CD3, 10 ng/ml IL-12, and 10 µg/ml α-IL-4) for 72 hours. Proliferation of $CD4^+$ T cells was analyzed with flow cytometry.

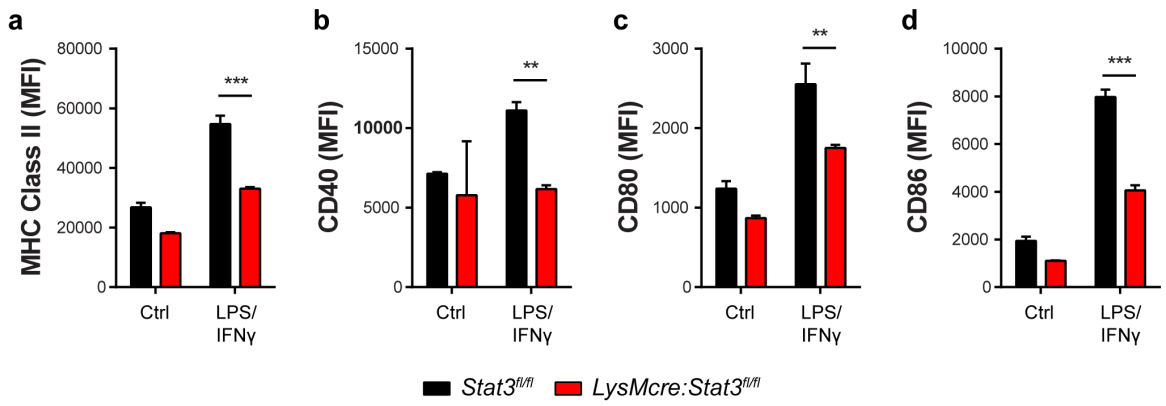


Figure S9. Flow cytometric analysis of antigen-presenting molecules in CD11b$^+$ cells isolated from immunized mice. Adult *LysMcre:Stat3$^{fl/fl}$* mice and WT littermates were immunized with MOG_{35-55} and PT was not administered. At 9-10 dpi, CD11b$^+$ cells from spleens were isolated with immunomagnetic selection. CD11b$^+$ cells (2.0×10^5) were stimulated with 10 ng/ml LPS and 10 ng/ml IFNγ for 24 h, and stained with antibodies against MHC Class II, CD40, CD80, and CD86 and analyzed by flow cytometry. *Stat3$^{fl/fl}$, n = 3*; *LysMcre:Stat3$^{fl/fl}$, n = 3*. Data represent mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.005$.

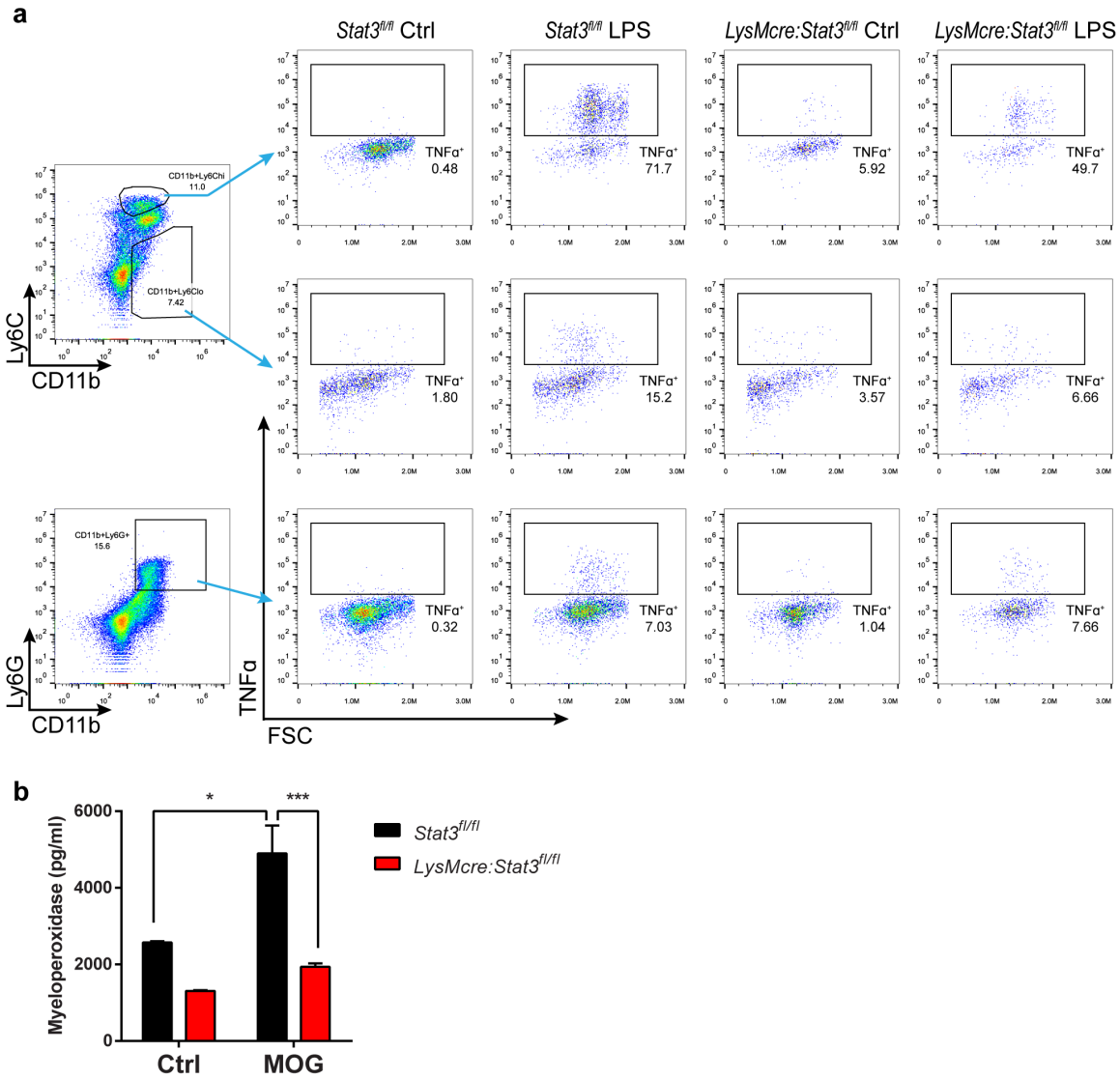


Figure S10. Differential effects of STAT3 inactivation in subsets of myeloid populations. $CD11b^+$ cells from splenocytes of immunized *Stat3*$^{fl/fl}$ and *LysMcre:Stat3*$^{fl/fl}$ mice at 9 dpi and $CD4^+$ cells from naive 2D2 mice were positively immunomagnetic selected and cocultured as described in Fig. 5a. **a**, Ex vivo $CD11b^+$ cells were stimulated with 1 ng/ml LPS for 5 h and stained against CD11b, Ly6C, Ly6G, and intracellular TNFα. **b**, Cocultures were stimulated with vehicle (Ctrl) or 30 µg/ml MOG peptide for 3 days. Secretion of myeloperoxidase in the supernatant was measured by ELISA. Representative data from 3 independent experiments were shown. Data represent mean ± SEM of a pool of three mice per genotype with technical replicates. *$p < 0.05$, ***$p < 0.005$.

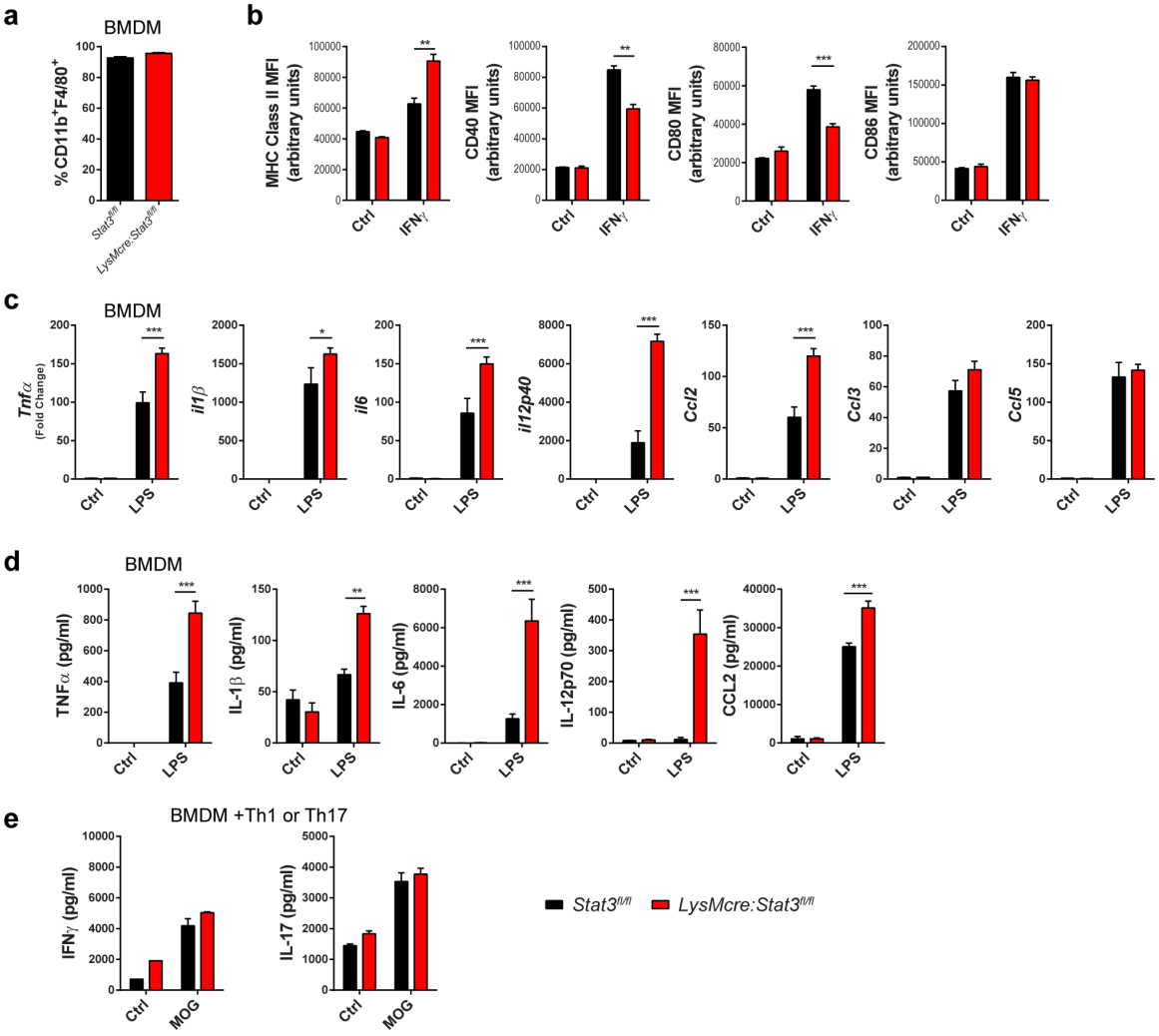


Figure S11. ***Stat3*** **mutant BMDMs exhibited enhanced proinflammatory responses upon immune activation in vitro.** BMDMs were prepraed from bone marrow cells isolated from adult *LysMcre:Stat3*$^{fl/fl}$ or *Stat3*$^{fl/fl}$ mice and cultured in L929 conditioned medium. **a,** The percentage of mature macrophages in BMDM cultures was evaluated by flow cytometry with α-CD11b and α-F4/80 at DIV7. *Stat3*$^{fl/fl}$, n = 3; *LysMcre:Stat3*$^{fl/fl}$, n = 3. **b,** BMDMs were stimulated with 10 ng/ml IFNγ for 24 h, and then stained with α-MHC Class II, α-CD40, α-CD80, and α-CD86 and analyzed with flow cytometry. *Stat3*$^{fl/fl}$, n = 2; *LysMcre:Stat3*$^{fl/fl}$, n = 2. **c,** BMDMs were stimulated with 10 ng/ml LPS for 5 hr. Expression levels of mRNA were evaluated by quantitative RT-PCR. *Stat3*$^{fl/fl}$, n = 3; *LysMcre:Stat3*$^{fl/fl}$, n = 3. **d,** Enhanced proinflammatory cytokine produciton by *Stat3* mutant BMDMs. BMDMs were stimulated with 10 ng/ml LPS for 24 h. Cytokine secretion into the supernatant was measured by ELISA. *Stat3*$^{fl/fl}$, n = 3; *LysMcre:Stat3*$^{fl/fl}$, n = 3. **e,** No differences between wild-type and *Stat3* mutant BMDMs in their capability of promoting antigen-specific production of IFN□ and IL-17A by Th1 and Th17 cells, respectively. BMDMs were co-cultured with in vitro pre-differentiated Th1 or Th17 cells for 1 d (Th1) or 3 d (Th17). Secretion of cytokines was measured by ELISA. *Stat3*$^{fl/fl}$, n = 2; *LysMcre:Stat3*$^{fl/fl}$, n = 2. Data represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.005.

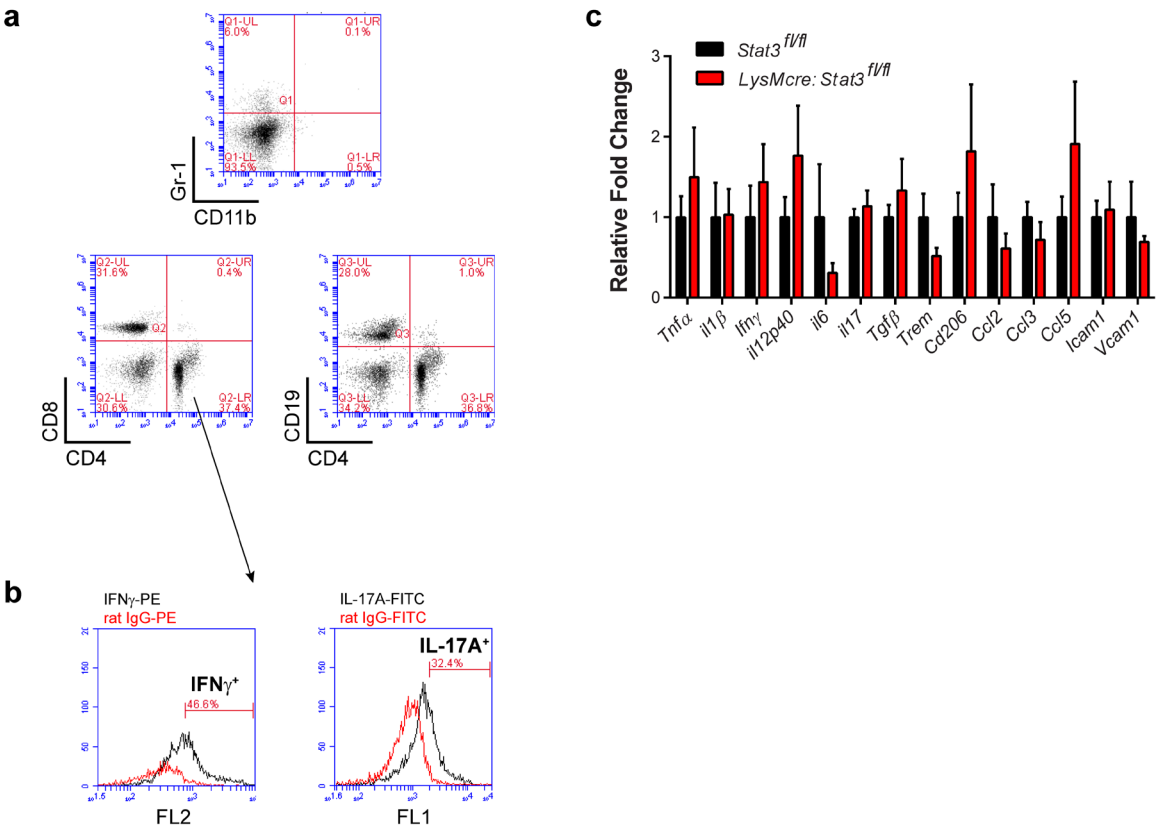
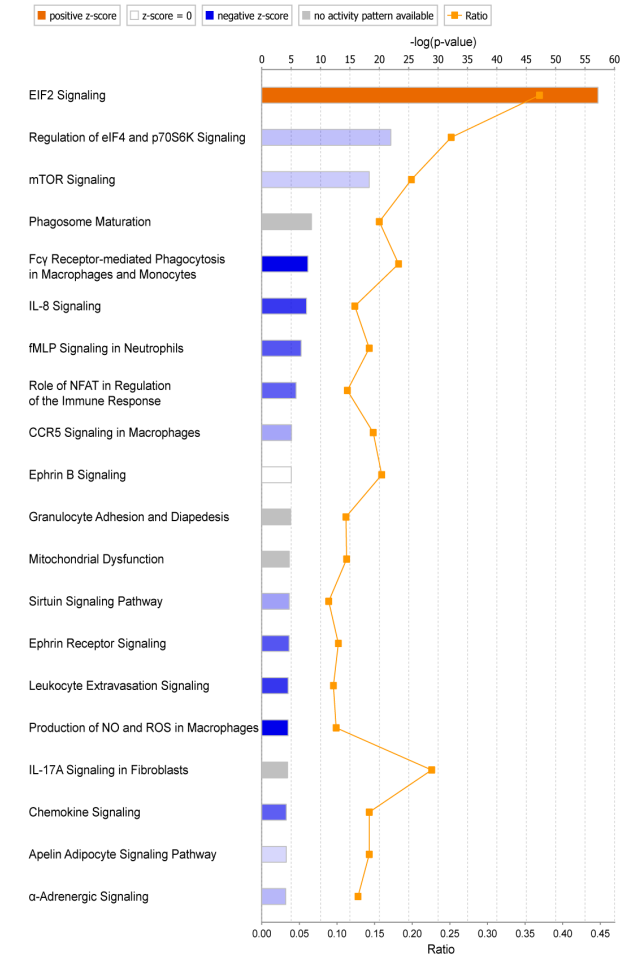


Figure S12. Adoptive transfer EAE donor cells and tissue cytokine mRNA analysis. a, b, Donor C57BL/6 mice were immunized with $MOG_{35.55}$. After 9-10 days, cells from the draining lymph nodes were isolated and cultured in the presence of 20 µg/ml MOG_{35-55} for 3-4 d, and analyzed by flow cytometry. **a,** Immunophenotyping analysis of the donor cells. **b,** Th1 and Th17 populations analyzed by intracellular cytokine staining. Cytokine secretion was blocked with Brefeldin A during the last 5 h of culturing. The percentage of Th1 and Th17 cells was analyzed with flow cytometry using IgG controls as baseline. **c,** Quantitative PCR analysis of cytokine/chemokine mRNA from spinal cords of adoptive transfer EAE mice (*Stat3*$^{fl/fl}$, n = 6; *LysMcre:Stat3*$^{fl/fl}$, n = 5). Data are mean ± SEM of fold expression changes relative to *Stat3*$^{fl/fl}$.

a



b

Top Upstream Regulator

Name	p-value	Predicted activation
RICTOR	2.20×10^{-42}	Inhibited
POLG	4.39×10^{-23}	
DYSF	3.72×10^{-12}	
TP53	1.15×10^{-10}	
NFE2L2	6.27×10^{-08}	

Top Causal Network

Name	p-value	Predicted activation
RICTOR	1.21×10^{-43}	Inhibited
RICTOR	6.46×10^{-43}	Inhibited
PTPN11	2.31×10^{-23}	
RCAN1	3.35×10^{-22}	
TNF	1.07×10^{-21}	

c

Top Molecular and Celluar Function

Name	p-value range
Cellular death and survival	3.62×10^{-04} - 5.17×10^{-26}
Cellular movement	7.19×10^{-04} - 6.63×10^{-27}
Cellular function and maintenance	6.42×10^{-04} - 3.61×10^{-17}
Cell-to-cell signaling and interaction	6.54×10^{-04} - 3.28×10^{-15}
Cell growth and proliferation	5.88×10^{-04} - 1.07×10^{-12}

Figure S13. Pathway analysis of the two most distant groups of *Stat3* wild-type and mutant myeloid cells after activation with MOG-specific 2D2 T cells. a. Predicted canonical pathways dysregulated in the *Stat3* mutant myeloid cells in comparison to wild-type cells after coculturing with 2D2 T cells in the presence of antigen. Ingenuity Pathway analysis (IPA) was based on log2 changes of the mean gene expression levels of myeloid cells isolated from M^{KO}/T +MOG versus M^{WT}/T +MOG groups. The canonical pathways identified ($p<0.0001$) are listed according to their p value and the ratio of list genes found in each pathway over the total number of genes in that pathway (ratio). z-score represents the number of standard deviations by which a sample's expression level for a gene differs from the mean expression level for that gene across all samples. **b, c**, Top upstream regulators, networks, and cellular functions identified by IPA.

References

1. Parkhurst Christopher N*, et al.* (2013) Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. *Cell* 155(7):1596-1609.
2. Stromnes IM & Goverman JM (2006) Active induction of experimental allergic encephalomyelitis. *Nat. Protocols* 1(4):1810-1819.
3. Stromnes IM & Goverman JM (2006) Passive induction of experimental allergic encephalomyelitis. *Nat. Protocols* 1(4):1952-1960.
4. Zhuang G*, et al.* (2012) A Novel Regulator of Macrophage Activation: miR-223 in Obesity-Associated Adipose Tissue Inflammation. *Circulation* 125(23):2892-2903.
5. Kim S, Steelman AJ, Zhang Y, Kinney HC, & Li J (2012) Aberrant Upregulation of Astroglial Ceramide Potentiates Oligodendrocyte Injury. *Brain Pathology* 22:41-57
6. Steelman AJ*, et al.* (2016) Activation of oligodendroglial Stat3 is required for efficient remyelination. *Neurobiology of Disease* 91:336-346.
7. Bolger AM, Lohse M, & Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15):2114-2120.
8. Trapnell C*, et al.* (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* 7(3):562-578.
9. Trapnell C*, et al.* (2014) The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nature Biotechnology* 32:381.