SUPPLEMENTAL MATERIAL

Divergent functions of tissue-resident and blood-derived macrophages in the hemorrhagic brain

Che-Feng Chang, PhD; Brittany A. Goods, PhD; Michael H. Askenase, PhD; Hannah E. Beatty, BS; Artem Osherov, BS; Jonathan Delong, PhD; Matthew D. Hammond, PhD; Jordan Massey, BS; Margaret Landreneau, BA; J. Christopher Love, PhD; Lauren H. Sansing, MD, MS

Graduate Institute of Physiology (C.-F.C.), College of Medicine, National Taiwan University, Taipei, Taiwan; Department of Biological Engineering, Koch Institute for Integrative Cancer Research (B.A.G, J.C.L.) at Massachusetts Institute of Technology, Cambridge; Department of Neurology (C.-F.C., H.E.B., M.H.A., A.O. M.L., J.M., J.D., M.D.H., L.H.S.), Yale University School of Medicine, New Haven.

Table of Contents

SUPPLEMENTAL METHODS

ICH Model

The autologous blood ICH model in male mice was utilized according to our established protocol.[16] In brief, mice were anesthetized throughout the procedure with 1-3% isoflurane in oxygen-enriched air (20%:80%) via nose cone. After sterile midline scalp incision, a burrhole was made in the right side of the skull at 0.0 mm anterior and 2.5 mm lateral to bregma. A total of 25-μL autologous blood was injected to a depth of 3.0 mm below the surface of the dura at a 5-degree angle. The craniotomy was sealed with bone wax, and the scalp was closed with tissue adhesive (3M Vetbond). Rectal temperature was monitored and body temperature maintained at 37.0 ± 0.5°C by an electronic thermostat-controlled warming blanket (FHC Inc.) throughout the experimental and recovery periods. No mice were excluded. Transcriptional data was available from male mice only and therefore all experiments were performed in males.

In Vivo Phagocytosis Assays

Microsphere-based assay

During the autologous blood injection ICH surgery, 4.5 x 10^7 1-μm diameter Fluoresbrite YG carboxylate beads (Polysciences) were mixed with the collected blood prior to injection as above. Animals were euthanized at days 1, 3, and 7 post-ICH by isoflurane overdose and then perfused transcardially with ice-cold PBS prior to brain harvest. 4-mm fresh coronal perihematomal tissue sections were collected based on our established standard procedure.[16] The brain tissues were mechanically and enzymatically digested. Leukocyte-enriched fraction was isolated by 30%/70% Percoll (GE Health) gradient centrifugation and stained with cell surface markers [CD45 (30-F11),

Ly6C (HK1.4), CD11b (M1/70), Ly6G (1A8), CD3e (500A2)]. After fixation in 4% paraformaldehyde and staining with DAPI, the cells were evaluated on an ImageStreamX Mark II (Amnis). The beads containing cells were selected and the number of fluorescent beads in each cell was calculated. The data are presented as beads per cell.

Erythrophagocytosis assay

Erythrocytes were isolated and labeled with the lipophilic cell membrane fluorescent dye PKH-26 (PKH26GL, Sigma) as previously described[1] and injected into the mice brain during autologous blood ICH surgery. Mice were sacrificed at 3 days after PKH-26-labeled erythrocyte injection and the brain samples were prepared as described above. The cells were stained for surface markers CD11b (M1/70), CD45 (30-F11), Ly6G (1A8), Ly6C (HK1.4) and viability dye (Invitrogen). The erythrophagocytosis-positive microglia and MDMs were identified as LIVE/DEAD^{-} CD11b^{+}CD45^{int}PKH-26-erythrocyte^{+} and LIVE/DEAD^{-}CD11b^{+}CD45^{hi}Ly6G^{-}Ly6C^{+}PKH-26-erythrocyte^{+} populations, respectively. The results are presented as the percentage of erythrocyte-positive cells and the mean fluorescence intensity (MFI) of PKH-erythrocyte-positive cells. The experimental gate was set based on the fluorescence-minus-one (FMO) control. Control samples were taken from the naïve brain matching the 4-mm coronal perihematomal region.

Transcriptional Analyses

Previously published longitudinal transcriptional datasets from nanoString analysis (PanCancer Immune Profiling Panel plus 27 custom genes; total 780 genes) were used for microglia and macrophage comparative analysis.[16, 17] Naïve and post-ICH mice at days 1, 3, 7, 10 after surgery were euthanized and perihematomal brain or the corresponding area from naïve mice were

collected. For each replicate, brain samples from three mice were pooled. The brain samples were prepared and stained as described above. After surface and live/dead staining, the microglia ($CD11b^{+}CD45^{int}$) and MDMs ($CD11b^{+}CD45^{hi}Ly6G^{-}CD3e^{-}Ly6C^{+}$) were sorted on a FACSAria directly into 4 µl RLT buffer (QIAGEN) and stored at -80°C until analysis.

Nanostring data were first preprocessed according to the manufacturer's instructions and then further analyzed using custom R (V 3.3.3) scripts as previously described.[16, 17] Briefly, nCounter data were normalized to housekeeping genes and genes with expressed absolute count below 7 (the threshold of detection instructed by the manufacture) at all time points were eliminated. Principal components analysis (PCA) was performed on log transformed data to identify genes driving separation between microglia and macrophages. To identify genes that were significantly different, we compared macrophages to microglia pairwise at each time point. We used Student's t-test and p values were adjusted using the Bonferroni correction to adjust for multiple comparisons. Volcano plots and heat maps were created for each indicated comparison. Enrichment analysis was performed using the Cytoscape (v 3.6.1) plugin GeneMania (v 3.4.1). Gene set enrichment analysis (GSEA) was also performed for each time point.[19] R scripts for the analyses are available at https://github.com/bagoods/Chang_Goods_MacrophagevMicroglia.

Bone Marrow-Derived Macrophages (BMDMs)

Mononuclear cells collected from 6- to 8-week-old C57BL/6 male mice were isolated and differentiated into BMDMs as described previously.[16] In short, adherent stromal cells were depleted by culturing the harvested bone marrow at 37°C overnight in complete α-minimum essential medium: α-MEM (Lonza) supplemented with 10% HI-FBS, 100 U/mL penicillin plus

100 mg/mL streptomycin, and 2 mM L-glutamine (all from Gibco). After RBCs were lysed, remaining non-adherent mononuclear cells were cultured in the complete α-MEM plus 50 ng/mL of recombinant murine M-CSF (R&D Systems) for 7 days, yielding BMDMs.

Primary Microglia Culture

L929-conditioned media

NCTC Clone L929 fibroblast cells (ATCC® CCL-1™) were grown in the DMEM High Glucose media supplemented with 100 U/mL penicillin plus 100 mg/mL streptomycin and 10% HI-FBS (all from Gibco) in 75 cm^2 TC flasks (Corning). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C and allowed to grow to >95% confluence prior to supernatant collection. The supernatants were collected, pooled and passed through a 0.22μm filter (Corning). The fresh filtered supernatants were stored at -20°C. The L929-conditioned media for use in growing mixed-glial cultures was composed of 60% DMEM High Glucose media, 10% HI-FBS, 30% L929 supernatants, and 100 U/mL penicillin plus 100 mg/mL streptomycin.

Microglia isolation and differentiation

The primary microglial cultures were isolated and purified from male mouse brain co-cultures using p0-p2 pups as previously described[20] with slightly modified based on the work from other groups.[20,8,18] Briefly, the cells from brain cortices were processed by Neural tissue dissociation kit (Miltenyi Biotec) according to manufacturer's instructions. The cells were seeded in 75 cm^2 TC flasks pre-coated with poly-D-lysine (100 μg/mL; Sigma) and cultured in the L929-conditioned media for 14 days to obtain mixed-glial culture and to differentiate microglia on the astrocyte bed. The loosely adherent microglia were harvested from the culture medium after slight shaking at 500

rpm for 3-4 hours. Microglia were cultured for another 5 days in 90% DMEM/F12 and 10% HI-FBS, supplemented with 100 U/mL penicillin plus 100 mg/mL streptomycin and 2 mM L-glutamine (all from Gibco), as well as 50 ng/mL TGF-β (Tonbo Biosciences), 10 ng/mL recombinant M-CSF (R&D Systems), and 1.5 μg/mL cholesterol (Avanti Polar Lipids 700000).[6] The cell purity was >95%, measured by flow cytometry.

Real-Time Quantitative Reverse-Transcriptase PCR (RT-qPCR)

Total RNA was isolated from cultured cells using miRNeasy Micro Kit (Qiagen) according to manufacturer's instructions. First strand cDNA was synthesized from 200ng of RNA using SuperScript VILO IV (Thermo Fisher Scientific). An ABI 7500 Fast Real-Time PCR system (Applied Biosystems) was used to carry out RT-qPCR analysis. Primers and probes for genes of interest were obtained from Thermo Fisher Scientific, listed as follows: *Cd74* (Mm00658576_m1), *Tmem119* (Mm00525305_m1), *P2ry12* (Mm01950543_s1), *Sall1* (Mm01950543_s1), *Gfap* (Mm01253033_m1), and *Fcrls* (Mm00472833_m1). *Gapdh* (Mm99999915_g1) was used as the endogenous control, and cycle time values for genes of interest were normalized to *Gapdh* in the same sample after confirming suitability as a housekeeping gene (mean ± SD difference from the mean Ct value of 0.08 ± 0.42 across experiments and conditions). The expression levels of mRNA are presented as fold change versus the control group, as previously described.[16]

Naïve T cell Isolation

Splenocytes were isolated from the spleens of male OT-II mice (B6.Cg Tg(TcraTcrb)425Cbn/J). The spleen was triturated, passed through a 40 μm cell strainer (Fisher Scientific), and washed

with complete RPMI media (Gibco) containing 10% HI-FBS, 2 mM L-glutamine, sodium pyruvate, non-essential amino acids, β-Mercaptoethanol, and 100 U/mL penicillin plus 100 mg/mL streptomycin. The red blood cells were then lysed and $CD4^+$ T-cells were selected for using $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec) according to manufacturer's instructions. $CD4^+$ cells were rested at 37°C for at least 1 hr and resuspended at 2 x 10^6 cells/mL in DPBS (Gibco). The volume was then doubled with 10 µM CellTrace™ CFSE (Thermo Fisher) in DPBS. The cells were stained with 5 µM CFSE for 5 minutes in the dark at room temperature and washed and resuspended in complete RPMI media.

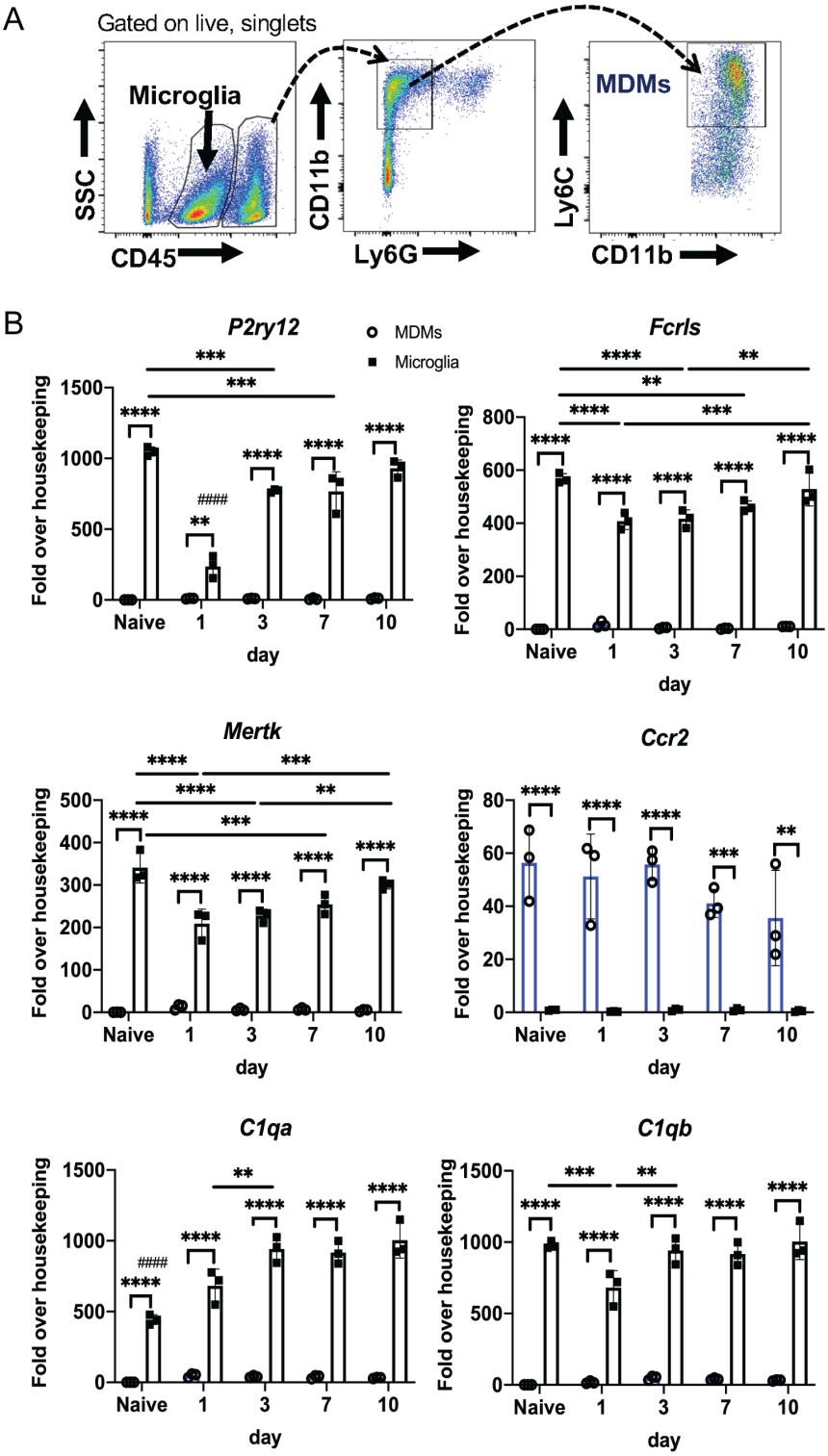
T cell-BMDM and T cell-Primary Microglia Co-cultures

To generate final cultures of differentiated antigen presenting cells (APCs) at similar densities, 7.5 x 10^3 BMDMs and 4 x 10^4 microglia were initially plated in a TC-treated 96-well plate and grown for 7 and 5 days respectively. Prior to co-culture with T cells, APC (BMDMs and microglia) densities were quantified by staining three representative wells for each cell type with 5 µg/ml Hoecsht 3342 (Thermo Fisher) and imaged on a Leica DMi8 microscope, followed by automated counting of fluorescent nuclei with CellProfiler 3.1.5 (Broad Institute), using three representative images from each well. BMDMs and microglia were pre-incubated with or without 50 µg/ml ovalbumin (323-339) (Sigma) ± 10 µg/mL anti-MHCII (BioXCell) at 37°C in 5% CO_2 for 3 hrs. The cells were then irradiated in a XRAD320 cabinet with 34 Gy of radiation and washed with DPBS. CFSE stained OT-II $CD4^+$ T-cells in complete RPMI were added to each well at a ratio of 1:11 APCs to T-cells. An additional dose of 10 µg/mL anti-MHCII was added to any wells pre-incubated with the blocking antibody. These cells were left to co-incubate at 37°C in 5% CO2 for

72 hrs before being harvested and stained for CD4 1:200 (GK15, BioLegend), CD11b 1:500 (M1/70, Tonbo) viability dye 1:2500 (Invitrogen), for 15-20 minutes on ice and analyzed by flow cytometry. Proliferated $CD4^+$ T cells were defined as the $CD4^{hi}CD11b^-CFSE^{lo}$ population.

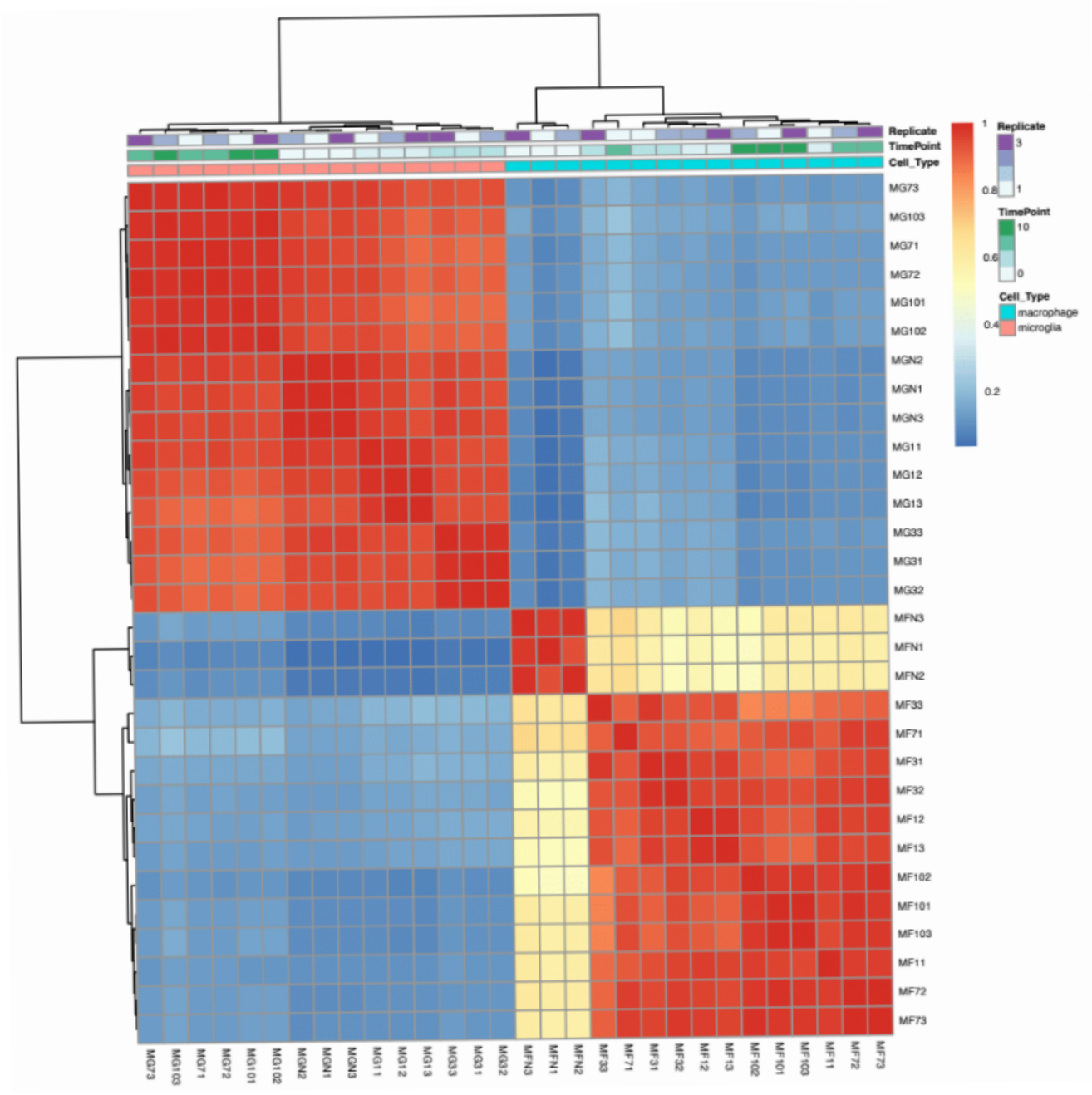
SUPPLEMENTAL FIGURES

Supplemental Figure I



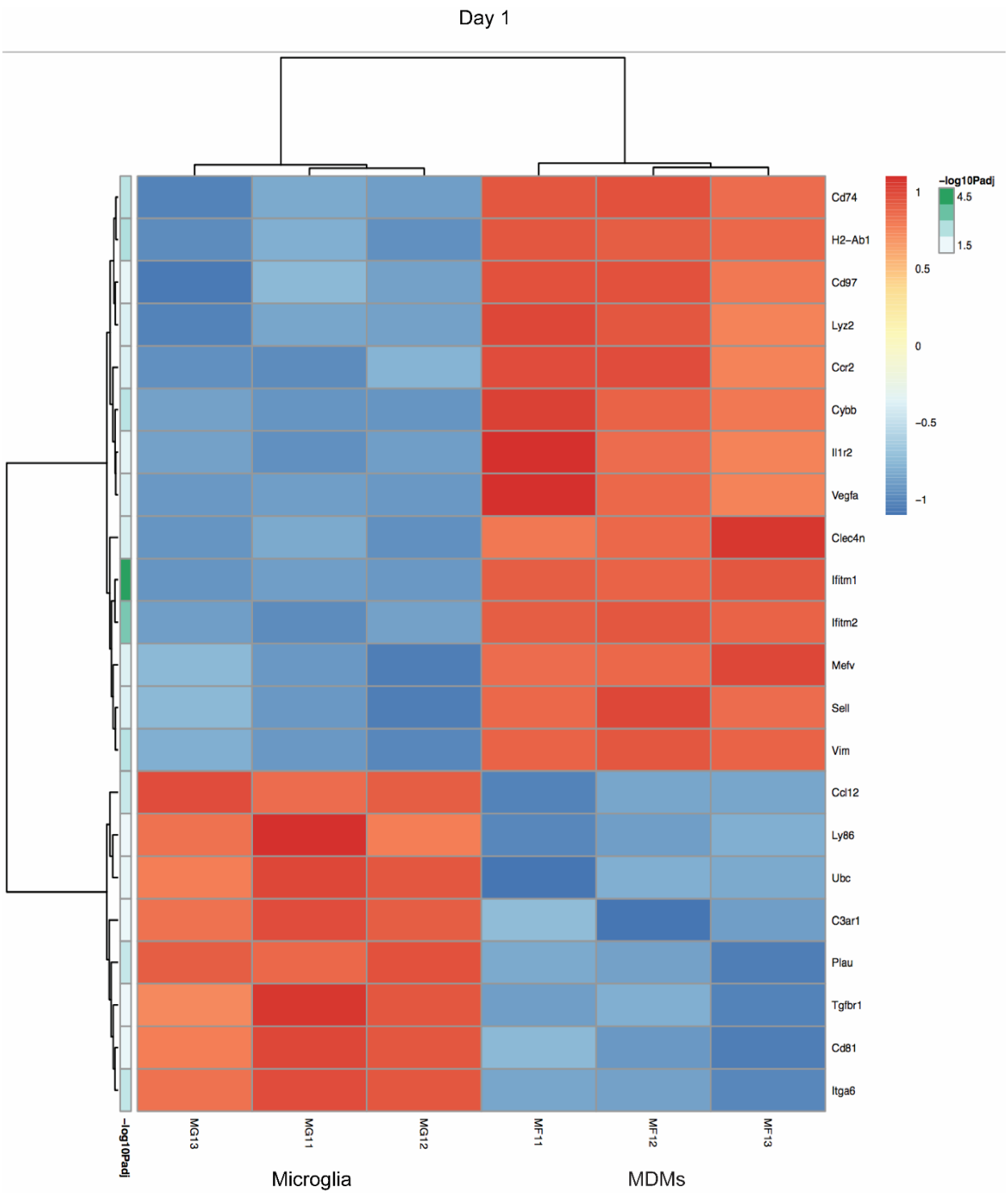
(A) The gating strategy to distinguish microglia and MDMs from ICH brain. Samples were gated on live singlets prior to leukocytes being found by forward and side scatter. Microglia were identified as $CD45^{intermediate}CD11b^{+}$ and MDMs as $CD45^{high}CD11b^{+}Ly6G^{-}Ly6C^{+}$. (B) Gene expression by nanoString confirms high expression of *P2ry12*, *Fcrls*, *Mertk*, *C1qa*, and *C1qb* in microglia and *Ccr2* in macrophages. * $p<0.05$, ** $p<0.01$, *** $p<0.005$, **** $p<0.001$ for each comparison shown, #### $p<0.001$ compared to same population at every other time point, by two-way ANOVA with post-hoc Tukey test. Gene expression is reported as fold over housekeeping to normalize for differing cell counts across time points and cell population.

Supplemental Figure II



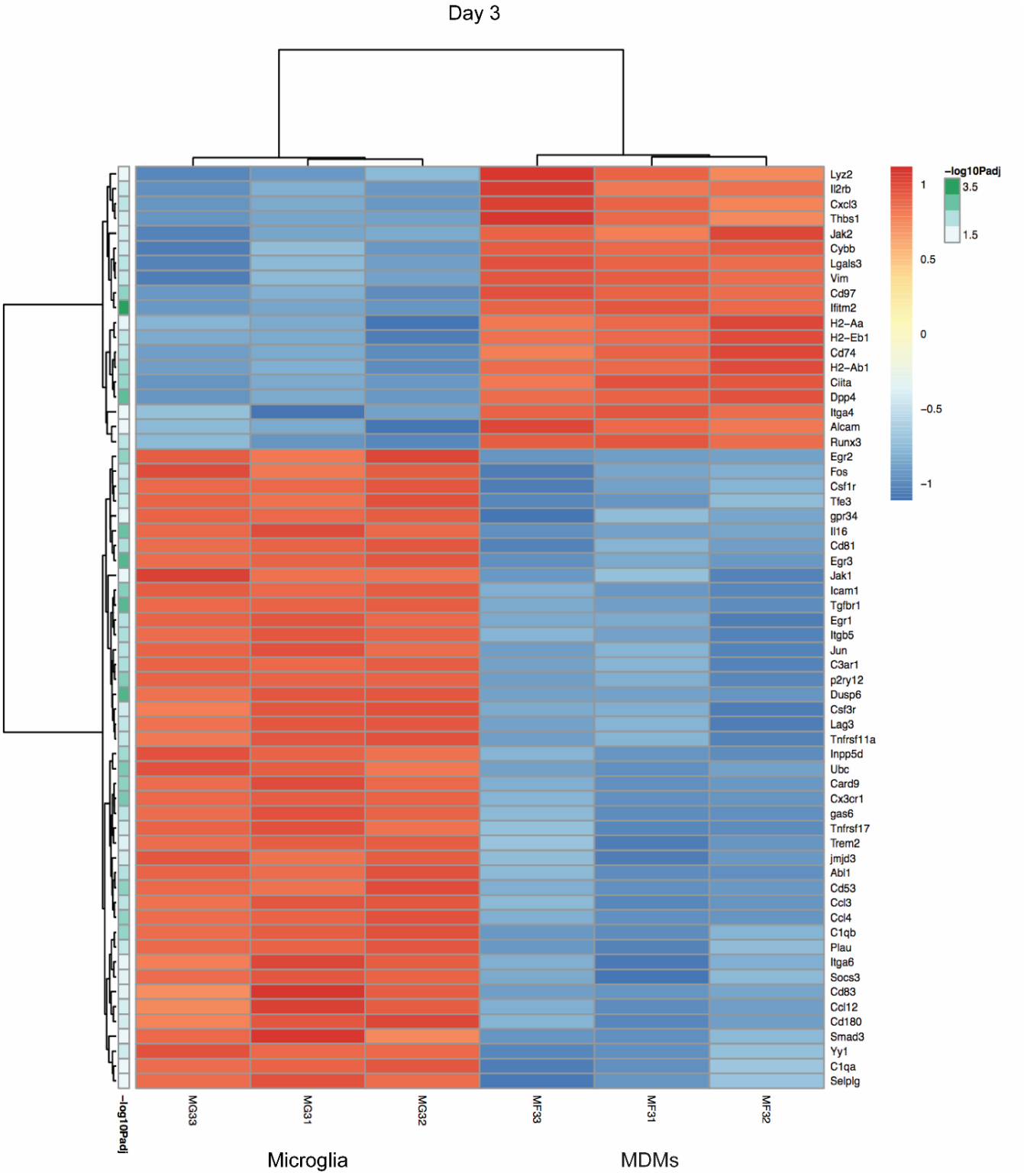
Sample-sample correlation. Pearson correlation values were calculated across all samples pairwise and clustered hierarchically. MF= macrophage, MG= microglia, with the following number indicating time point (days) after ICH or N for naïve, and the final number indicating the biological replicate.

Supplemental Figure III



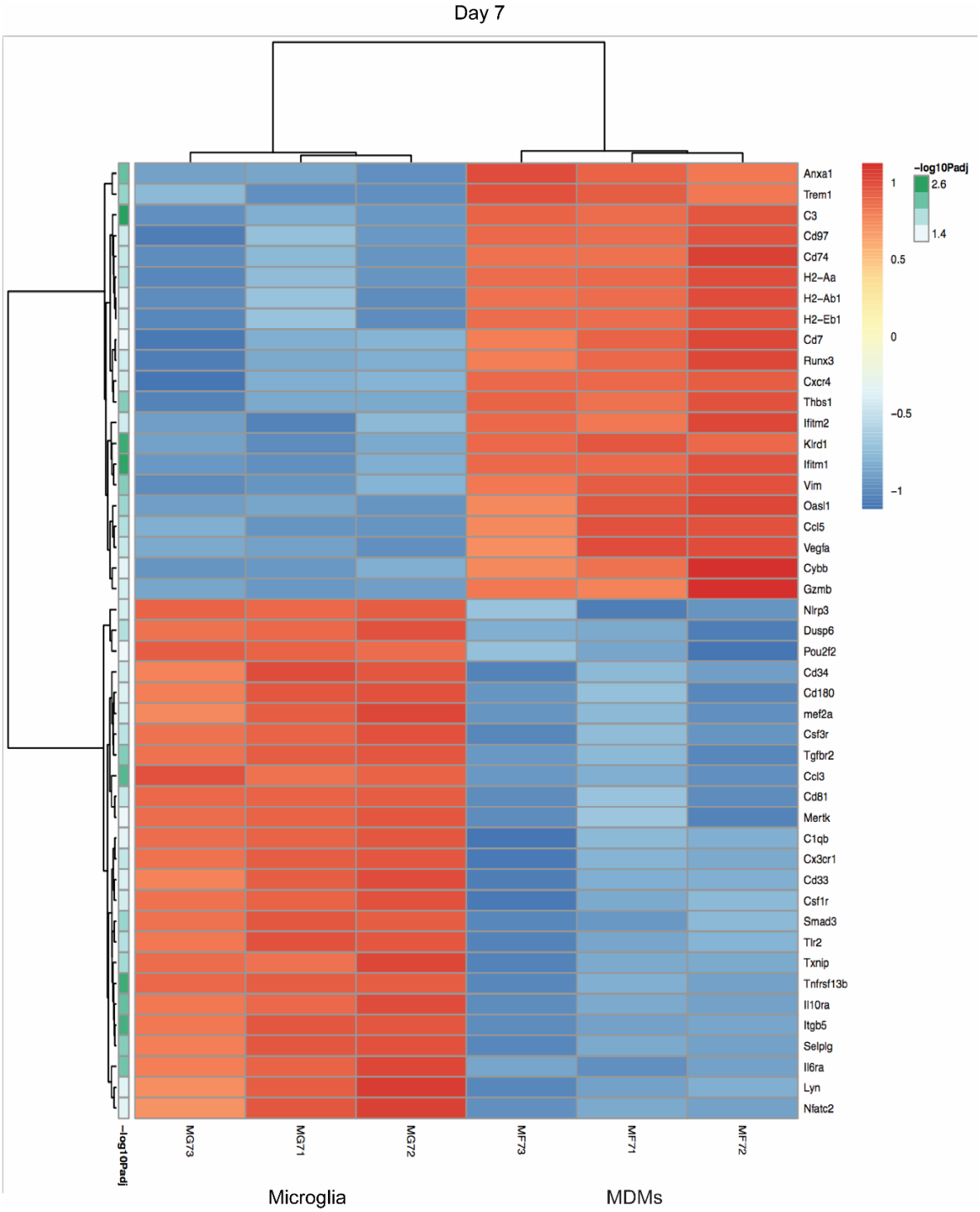
Differential gene expression in microglia and MDMs over time in the day 1 ICH brain

Supplemental Figure IV



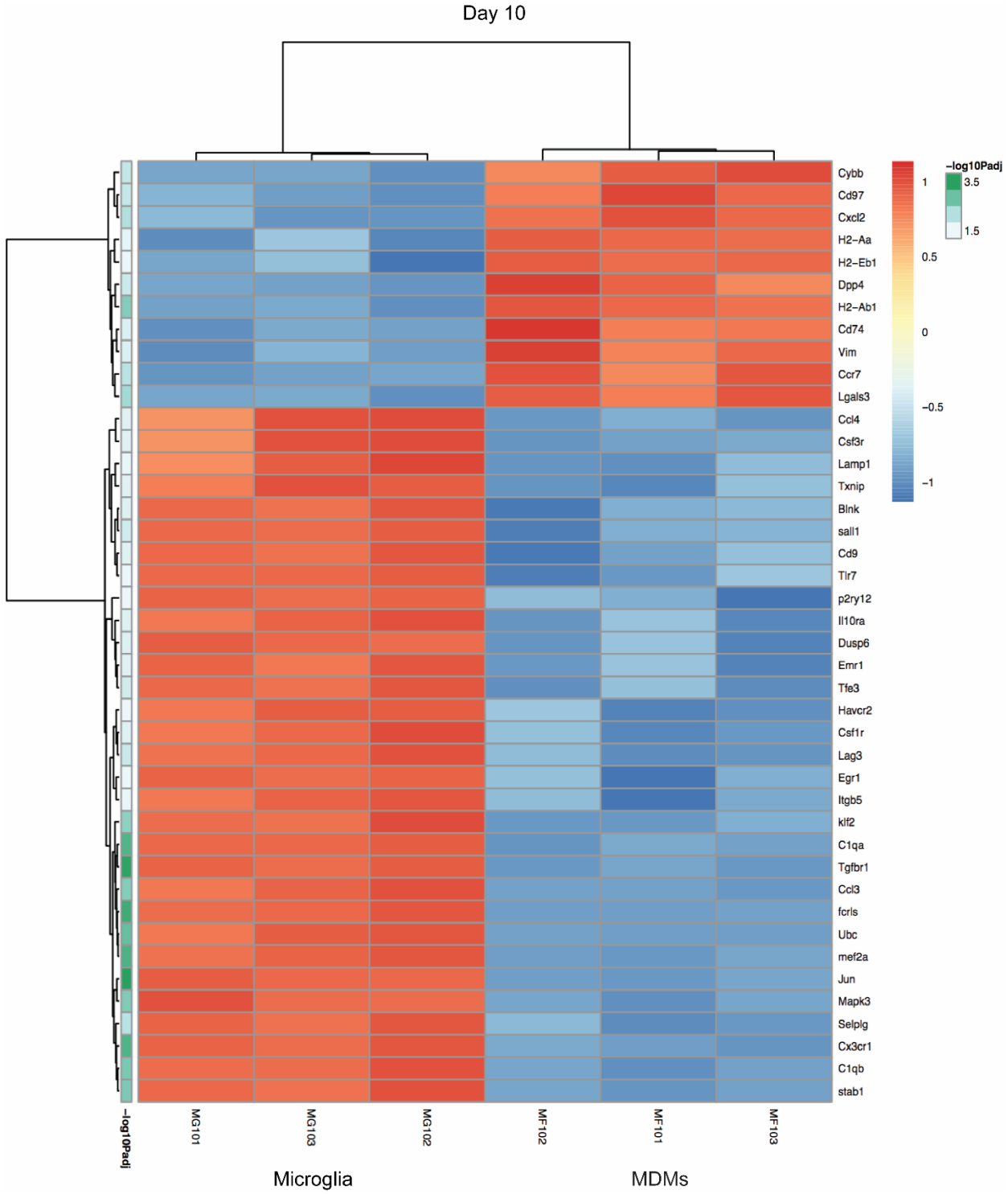
Differential gene expression in microglia and MDMs over time in the day 3 ICH brain

Supplemental Figure V



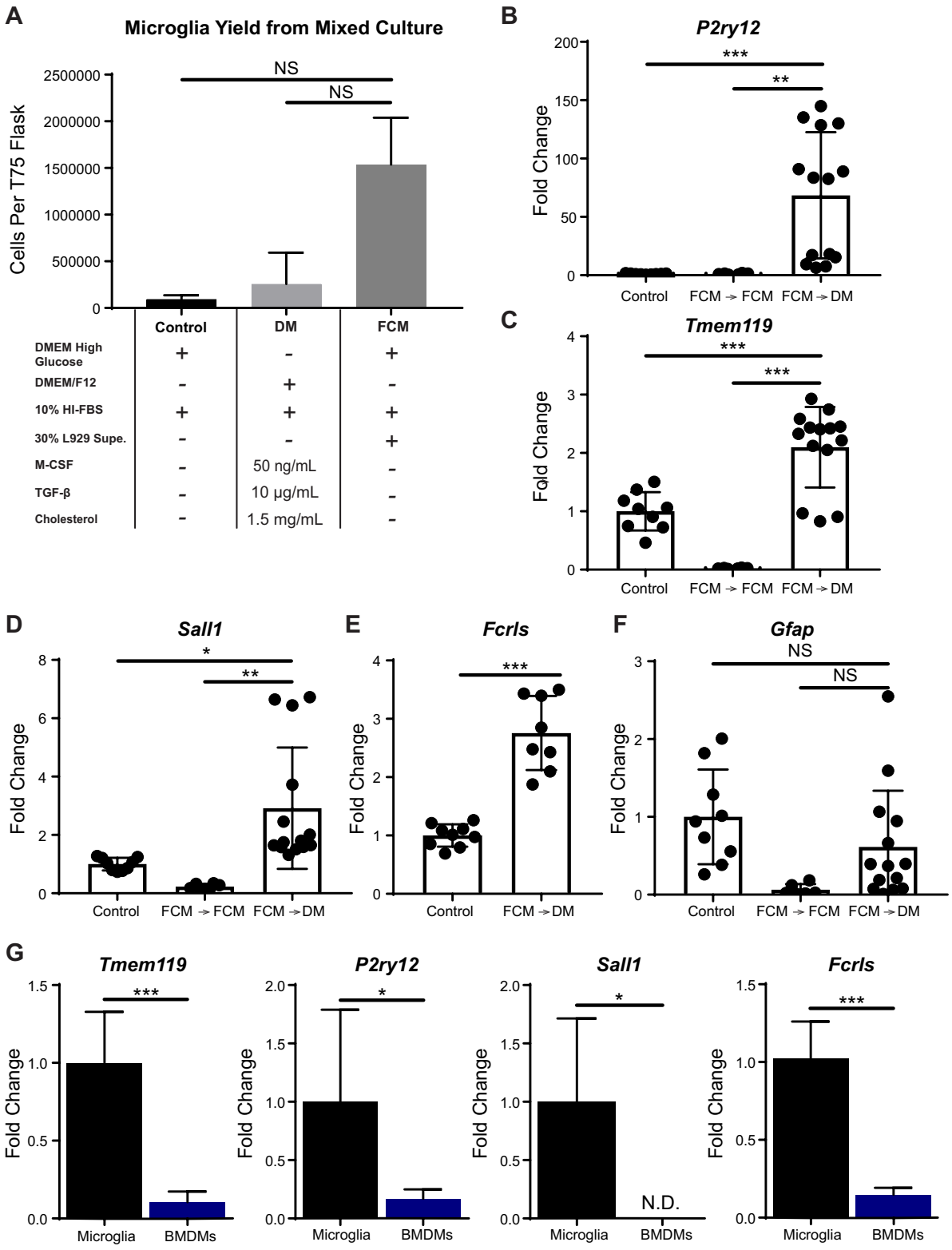
Differential gene expression in microglia and MDMs over time in the day 7 ICH brain

Supplemental Figure VI



Differential gene expression in microglia and MDMs over time in the day 10 ICH brain

Supplemental Figure VII



Qualification of murine microglia derived from primary neonatal tissue. (A) Quantification of progenitor cells collected from 14-17 day mixed glial culture, per T75 culture flask, prior to plating. Media composition of mixed glial cultures corresponding to plotted data is shown. DMEM only n=5, defined media n=3, L929 conditioned n=47. (B-E) Gene expression of microglial markers is significantly improved in cells grown under L929 conditioned mixed glial culture conditions (FCM) followed by differentiation in defined media (DM). ***$p<0.001$ and ** $p<0.01$ versus DMEM-only culture protocol by one-way ANOVA corrected for multiple comparisons. Control n=9, FCM n=6, FCM to DM n=8-14. (F) Gene expression of astrocyte marker *Gfap* not significantly different under different media conditions. Control n=9, FCM n=6, FCM to DM n=14. (G) Expression of microglia markers are much lower in cultured murine bone marrow derived macrophages (BMDMs) in the microglia culture conditions (FCM to DM). Microglia n=8-14, BMDMs n=3-6. *** $p<0.001$ and * $p<0.05$ by Student's *t*-test.

Supplemental Table I. GO functional enrichment for each cell type across time. GO enrichment results for each indicated cell type and time point are shown.

Cell Type	Time	GO id	Description	q-value
Macrophage	Naïve	GO:0009897	external side of plasma membrane	6.88E-13
Macrophage	Naïve	GO:0045087	innate immune response	1.25E-11
Macrophage	Naïve	GO:0050870	positive regulation of T cell activation	6.95E-11
Macrophage	Naïve	GO:0050863	regulation of T cell activation	2.17E-10
Macrophage	Naïve	GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II	1.37E-09
Macrophage	Naïve	GO:0051251	positive regulation of lymphocyte activation	1.37E-09
Macrophage	Naïve	GO:0002443	leukocyte mediated immunity	2.53E-09
Macrophage	Naïve	GO:0002696	positive regulation of leukocyte activation	3.09E-09
Macrophage	Naïve	GO:0042098	T cell proliferation	3.12E-09
Macrophage	Naïve	GO:0002495	antigen processing and presentation of peptide antigen via MHC class II	3.37E-09
Macrophage	Day 1	GO:0002495	antigen processing and presentation of peptide antigen via MHC class II	1.91E-09
Macrophage	Day 1	GO:0002504	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	1.91E-09
Macrophage	Day 1	GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II	1.02E-07
Macrophage	Day 1	GO:0002687	positive regulation of leukocyte migration	1.65E-07
Macrophage	Day 1	GO:0002478	antigen processing and presentation of exogenous peptide antigen	6.18E-07
Macrophage	Day 1	GO:0048002	antigen processing and presentation of peptide antigen	6.91E-07
Macrophage	Day 1	GO:0045087	innate immune response	6.91E-07
Macrophage	Day 1	GO:0002685	regulation of leukocyte migration	6.91E-07
Macrophage	Day 1	GO:0019884	antigen processing and presentation of exogenous antigen	1.35E-06
Macrophage	Day 1	GO:0050900	leukocyte migration	1.46E-06
Macrophage	Day 3	GO:0002504	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	1.78E-11
Macrophage	Day 3	GO:0050867	positive regulation of cell activation	1.78E-11
Macrophage	Day 3	GO:0002696	positive regulation of leukocyte activation	3.19E-10
Macrophage	Day 3	GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II	3.75E-10
Macrophage	Day 3	GO:0002495	antigen processing and presentation of peptide antigen via MHC class II	1.23E-09
Macrophage	Day 3	GO:0051251	positive regulation of lymphocyte activation	2.37E-09
Macrophage	Day 3	GO:0009897	external side of plasma membrane	3.00E-09
Macrophage	Day 3	GO:0002478	antigen processing and presentation of exogenous peptide antigen	3.36E-09
Macrophage	Day 3	GO:0050870	positive regulation of T cell activation	3.47E-09
Macrophage	Day 3	GO:0002449	lymphocyte mediated immunity	1.02E-08
Macrophage	Day 7	GO:0002504	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	3.54E-11
Macrophage	Day 7	GO:0019882	antigen processing and presentation	7.07E-10
Macrophage	Day 7	GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II	7.07E-10
Macrophage	Day 7	GO:0002495	antigen processing and presentation of peptide antigen via MHC class II	2.12E-09
Macrophage	Day 7	GO:0002478	antigen processing and presentation of exogenous peptide antigen	7.39E-09
Macrophage	Day 7	GO:0048002	antigen processing and presentation of peptide antigen	2.72E-08
Macrophage	Day 7	GO:0019884	antigen processing and presentation of exogenous antigen	2.86E-08
Macrophage	Day 7	GO:0002460	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	5.85E-08
Macrophage	Day 7	GO:0042611	MHC protein complex	5.89E-08
Macrophage	Day 7	GO:0002443	leukocyte mediated immunity	1.89E-07
Macrophage	Day 10	GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II	3.39E-10
Macrophage	Day 10	GO:0002495	antigen processing and presentation of peptide antigen via MHC class II	6.97E-10
Macrophage	Day 10	GO:0002504	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	6.97E-10
Macrophage	Day 10	GO:0002478	antigen processing and presentation of exogenous peptide antigen	1.52E-09
Macrophage	Day 10	GO:0019884	antigen processing and presentation of exogenous antigen	6.61E-09
Macrophage	Day 10	GO:0019882	antigen processing and presentation	1.78E-07
Macrophage	Day 10	GO:0048002	antigen processing and presentation of peptide antigen	3.57E-07
Macrophage	Day 10	GO:1990266	neutrophil migration	5.41E-07
Macrophage	Day 10	GO:0030593	neutrophil chemotaxis	5.41E-07
Macrophage	Day 10	GO:0050870	positive regulation of T cell activation	1.24E-06

Microglia continued on next page.

Microglia	Naïve	GO:0051272	positive regulation of cellular component movement	4.30E-13
Microglia	Naïve	GO:0050900	leukocyte migration	4.30E-13
Microglia	Naïve	GO:1902105	regulation of leukocyte differentiation	4.38E-13
Microglia	Naïve	GO:0030335	positive regulation of cell migration	2.06E-12
Microglia	Naïve	GO:2000147	positive regulation of cell motility	2.33E-12
Microglia	Naïve	GO:0030595	leukocyte chemotaxis	4.75E-12
Microglia	Naïve	GO:0060326	cell chemotaxis	9.01E-12
Microglia	Naïve	GO:0071219	cellular response to molecule of bacterial origin	9.01E-12
Microglia	Naïve	GO:0002237	response to molecule of bacterial origin	1.29E-11
Microglia	Naïve	GO:0097529	myeloid leukocyte migration	1.36E-11
Microglia	Day 1	GO:0032103	positive regulation of response to external stimulus	0.001659458
Microglia	Day 1	GO:0009897	external side of plasma membrane	0.007033321
Microglia	Day 1	GO:1901623	regulation of lymphocyte chemotaxis	0.007033321
Microglia	Day 1	GO:0002688	regulation of leukocyte chemotaxis	0.007864568
Microglia	Day 1	GO:0048247	lymphocyte chemotaxis	0.01281474
Microglia	Day 1	GO:0050900	leukocyte migration	0.018936428
Microglia	Day 1	GO:2000401	regulation of lymphocyte migration	0.019370388
Microglia	Day 1	GO:0002685	regulation of leukocyte migration	0.025663873
Microglia	Day 1	GO:0097529	myeloid leukocyte migration	0.025663873
Microglia	Day 1	GO:0050920	regulation of chemotaxis	0.034010915
Microglia	Day 3	GO:0032103	positive regulation of response to external stimulus	4.28E-11
Microglia	Day 3	GO:0050900	leukocyte migration	1.43E-10
Microglia	Day 3	GO:0002573	myeloid leukocyte differentiation	1.78E-10
Microglia	Day 3	GO:0030099	myeloid cell differentiation	3.37E-10
Microglia	Day 3	GO:0060326	cell chemotaxis	3.42E-10
Microglia	Day 3	GO:0045637	regulation of myeloid cell differentiation	1.99E-09
Microglia	Day 3	GO:1902105	regulation of leukocyte differentiation	2.06E-09
Microglia	Day 3	GO:0030316	osteoclast differentiation	2.43E-09
Microglia	Day 3	GO:0030595	leukocyte chemotaxis	6.45E-09
Microglia	Day 3	GO:0002761	regulation of myeloid leukocyte differentiation	1.69E-08
Microglia	Day 7	GO:0009306	protein secretion	9.49E-08
Microglia	Day 7	GO:0050707	regulation of cytokine secretion	1.51E-07
Microglia	Day 7	GO:0050663	cytokine secretion	3.48E-07
Microglia	Day 7	GO:0050715	positive regulation of cytokine secretion	3.63E-07
Microglia	Day 7	GO:0050900	leukocyte migration	1.16E-06
Microglia	Day 7	GO:0050708	regulation of protein secretion	2.41E-06
Microglia	Day 7	GO:0050714	positive regulation of protein secretion	3.92E-06
Microglia	Day 7	GO:0071216	cellular response to biotic stimulus	9.35E-06
Microglia	Day 7	GO:0042113	B cell activation	1.76E-05
Microglia	Day 7	GO:0051222	positive regulation of protein transport	2.00E-05
Microglia	Day 10	GO:0002761	regulation of myeloid leukocyte differentiation	1.22E-06
Microglia	Day 10	GO:0002573	myeloid leukocyte differentiation	1.22E-06
Microglia	Day 10	GO:0032103	positive regulation of response to external stimulus	1.74E-06
Microglia	Day 10	GO:0030099	myeloid cell differentiation	6.15E-06
Microglia	Day 10	GO:0045637	regulation of myeloid cell differentiation	1.08E-05
Microglia	Day 10	GO:0009897	external side of plasma membrane	3.90E-05
Microglia	Day 10	GO:0050900	leukocyte migration	5.59E-05
Microglia	Day 10	GO:0030595	leukocyte chemotaxis	5.59E-05
Microglia	Day 10	GO:1902105	regulation of leukocyte differentiation	6.31E-05
Microglia	Day 10	GO:0001819	positive regulation of cytokine production	6.31E-05

Supplemental Table II. GSEA results. GSEA was performed for each indicated cell type and time point. Only significant results (FDR 1-val < 0.2) are shown.

Supplemental Table 2: GSEA results (FDR q-val < 0.2).

TIME	ENRICHED IN	NAME	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val
Day 0	Macrophage	REACTOME_CELL_SURFACE_INTERACTIONS_AT_THE_VASCULAR_WALL	21	0.6393	1.8426	0.0000	0.0519	0.103
Day 1	Microglia	BIOCARTA_MAPK_PATHWAY	28	-0.4475	-1.7146	0.0230	0.1944	0.172
Day 1	Macrophage	REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	24	0.6543	1.6517	0.0033	0.0689	0.241
Day 1	Macrophage	PID_CD8_TCR_PATHWAY	16	0.6579	1.5441	0.0097	0.0815	0.747
Day 1	Macrophage	REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_AND_A_NON_LYMPHOID_CELL	21	0.6192	1.5530	0.0181	0.0825	0.703
Day 1	Macrophage	REACTOME_CELL_SURFACE_INTERACTIONS_AT_THE_VASCULAR_WALL	21	0.6546	1.6147	0.0057	0.0827	0.4
Day 1	Macrophage	REACTOME_G_ALPHA_I_SIGNALLING_EVENTS	21	0.6341	1.5774	0.0056	0.0847	0.58
Day 1	Macrophage	REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	30	0.5893	1.5558	0.0075	0.0932	0.691
Day 1	Macrophage	REACTOME_GPCR_LIGAND_BINDING	32	0.6020	1.5852	0.0055	0.0954	0.545
Day 1	Macrophage	REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	28	0.6336	1.6646	0.0000	0.1121	0.202
Day 1	Macrophage	KEGG_CHEMOKINE_SIGNALING_PATHWAY	50	0.5324	1.4637	0.0123	0.1792	0.968
Day 1	Macrophage	NABA_MATRISOME	53	0.5089	1.4206	0.0175	0.1897	0.993
Day 1	Macrophage	REACTOME_GPCR_DOWNSTREAM_SIGNALING	29	0.5494	1.4250	0.0410	0.1977	0.993
Day 3	Microglia	BIOCARTA_TOLL_PATHWAY	25	-0.4871	-1.7654	0.0030	0.1140	0.264
Day 3	Microglia	KEGG_COLORECTAL_CANCER	19	-0.5072	-1.7224	0.0136	0.1145	0.351
Day 3	Microglia	BIOCARTA_NTHI_PATHWAY	17	-0.5221	-1.7296	0.0085	0.1274	0.338
Day 3	Microglia	BIOCARTA_P38MAPK_PATHWAY	15	-0.5479	-1.6869	0.0262	0.1277	0.423
Day 3	Microglia	REACTOME_MYD88_MAL_CASCADE_INITIATED_ON_PLASMA_MEMBRANE	37	-0.4410	-1.7684	0.0109	0.1384	0.256
Day 3	Microglia	REACTOME_ACTIVATED_TLR4_SIGNALLING	45	-0.3827	-1.6418	0.0076	0.1539	0.519
Day 3	Microglia	KEGG_WNT_SIGNALING_PATHWAY	17	-0.5474	-1.7866	0.0085	0.1598	0.226
Day 3	Microglia	BIOCARTA_KERATINOCYTE_PATHWAY	24	-0.4339	-1.5795	0.0380	0.1635	0.694
Day 3	Microglia	PID_AP1_PATHWAY	18	-0.4914	-1.5818	0.0413	0.1744	0.689
Day 3	Microglia	KEGG_MAPK_SIGNALING_PATHWAY	41	-0.3907	-1.5926	0.0172	0.1747	0.654
Day 3	Microglia	REACTOME_MAP_KINASE_ACTIVATION_IN_TLR_CASCADE	21	-0.4673	-1.5968	0.0096	0.1876	0.645
Day 3	Macrophage	REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	28	0.5681	1.7963	0.0030	0.0548	0.243
Day 3	Macrophage	REACTOME_G_ALPHA_I_SIGNALLING_EVENTS	21	0.6053	1.8141	0.0046	0.0603	0.182
Day 3	Macrophage	REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	24	0.5947	1.8527	0.0030	0.0754	0.116
Day 3	Macrophage	REACTOME_CELL_SURFACE_INTERACTIONS_AT_THE_VASCULAR_WALL	21	0.5793	1.7253	0.0030	0.0856	0.423
Day 3	Macrophage	REACTOME_GPCR_LIGAND_BINDING	32	0.5056	1.6569	0.0144	0.1265	0.644
Day 3	Macrophage	REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	30	0.4973	1.6188	0.0230	0.1472	0.757
Day 7	Macrophage	REACTOME_G_ALPHA_I_SIGNALLING_EVENTS	21	0.5735	1.7859	0.0062	0.0683	0.289
Day 7	Macrophage	REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	28	0.5534	1.8102	0.0045	0.0776	0.23
Day 7	Macrophage	REACTOME_GPCR_LIGAND_BINDING	32	0.4976	1.6707	0.0097	0.1221	0.625
Day 7	Macrophage	REACTOME_CELL_SURFACE_INTERACTIONS_AT_THE_VASCULAR_WALL	21	0.5609	1.6920	0.0096	0.1293	0.558
Day 7	Macrophage	REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_AND_A_NON_LYMPHOID_CELL	21	0.5335	1.6389	0.0275	0.1349	0.738
Day 7	Macrophage	REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	30	0.4924	1.6009	0.0148	0.1535	0.83
Day 7	Macrophage	REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	24	0.5685	1.8112	0.0030	0.1541	0.228
Day 10	Microglia	BIOCARTA_BCR_PATHWAY	16	-0.4868	-1.6012	0.0382	0.1926	0.714
Day 10	Microglia	REACTOME_TRIF_MEDIATED_TLR3_SIGNALING	34	-0.3744	-1.5714	0.0182	0.1944	0.771
Day 10	Microglia	KEGG_WNT_SIGNALING_PATHWAY	17	-0.4775	-1.5818	0.0331	0.1989	0.76
Day 10	Macrophage	REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	24	0.6273	2.0938	0.0000	0.0069	0.013
Day 10	Macrophage	REACTOME_G_ALPHA_I_SIGNALLING_EVENTS	21	0.6182	2.0076	0.0000	0.0070	0.037
Day 10	Macrophage	REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	28	0.5907	2.0333	0.0000	0.0073	0.026
Day 10	Macrophage	REACTOME_GPCR_LIGAND_BINDING	32	0.5532	1.9484	0.0000	0.0104	0.073
Day 10	Macrophage	REACTOME_CLASS_A1_RHODOPSIN_LIKE_RECEPTORS	30	0.5443	1.9058	0.0061	0.0138	0.119
Day 10	Macrophage	REACTOME_GPCR_DOWNSTREAM_SIGNALING	29	0.5067	1.7706	0.0047	0.0461	0.379
Day 10	Macrophage	KEGG_CHEMOKINE_SIGNALING_PATHWAY	50	0.4486	1.7390	0.0058	0.0513	0.474
Day 10	Macrophage	KEGG_CELL_ADHESION_MOLECULES_CAMS	29	0.4913	1.7120	0.0144	0.0519	0.581
Day 10	Macrophage	REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_AND_A_NON_LYMPHOID_CELL	21	0.5372	1.7222	0.0081	0.0530	0.542
Day 10	Macrophage	REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS	19	0.5194	1.6326	0.0118	0.0739	0.781
Day 10	Macrophage	REACTOME_SIGNALING_BY_GPCR	43	0.4374	1.6416	0.0130	0.0755	0.762
Day 10	Macrophage	NABA_MATRISOME	53	0.4150	1.6433	0.0044	0.0825	0.761
Day 10	Macrophage	NABA_MATRISOME_ASSOCIATED	48	0.3977	1.5354	0.0301	0.1303	0.955
Day 10	Macrophage	REACTOME_CELL_SURFACE_INTERACTIONS_AT_THE_VASCULAR_WALL	21	0.4528	1.4597	0.0769	0.1955	0.993

*** Preclinical Checklist**

Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research. ***This short checklist must be completed, and the answers should be clearly presented in the manuscript.*** *The checklist will be used by reviewers and editors and it will be published. See "Reporting Standard for Preclinical Studies of Stroke Therapy" and "Good Laboratory Practice: Preventing Introduction of Bias at the Bench" for more information.*

This study invovles animal models:
Yes

Experimental groups and study timeline

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study:	Yes
An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated:	Yes
An overall study timeline is provided:	No

Inclusion and exclusion criteria

A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article:	Yes

Randomization

Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided:	Yes
Type and methods of randomization have been described:	N/A
Methods used for allocation concealment have been reported:	N/A

Blinding

Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible:	N/A
Blinding procedures have been described with regard to masking of group assignment during outcome assessment:	N/A

Sample size and power calculations

Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided:	Yes

Data reporting and statistical methods

Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups:	Yes
Baseline data on assessed outcome(s) for all experimental groups have been reported:	N/A
Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms:	N/A
Statistical methods used have been reported:	Yes
Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures:	Yes

Experimental details, ethics, and funding statements

Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described:	Yes
Different sex animals have been used. If not, the reason/justification is provided:	Yes

Statements on approval by ethics boards and ethical conduct of studies have been provided:	Yes
Statements on funding and conflicts of interests have been provided:	Yes

---

Date completed: 11/10/2020 11:28:27
User pid: 49131