

Additional file 1

Single-cell transcriptomic analysis of the immune cell landscape in the aged mouse brain after ischemic stroke

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Methods

Animals. Animal experiments were approved by the Duke University Medical Center Animal Care and Use Committee. All studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals. Young adult C57Bl/6 mice (3-4 months old) were purchased from The Jackson Laboratory (Maine). Aged C57Bl/6 mice (19-20 months old) were obtained through the NIA Aged Rodent Colonies program. Since this study was not focused on pre-clinical outcome but on the general immune response in aged mice, only male mice were used. The online tool Quickcalcs was used to randomize animals for group assignments. Due to obvious differences between young vs aged mice and sham vs stroke mice, blinding was not feasible.

Stroke surgery. We aimed to establish a transient stroke model that applies a relatively long ischemic period, has a good survival rate, produces readily detectable neurologic deficits, and can be used in aged animals. To this end, we developed a modified transient transcranial middle cerebral artery occlusion (ttMCAO) model, based on our previous permanent ischemic stroke model.¹ Briefly, mice were anesthetized with isoflurane, orally intubated, and mechanically ventilated. Rectal temperature was maintained at $37^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ throughout the procedure. After a small neck midline incision was made, the right common carotid artery (CCA) was isolated and ligated using a 4-0 silk suture. A small transverse incision was then made between the eye and ear. Next, the lowest part of the temporal muscle was cut using a high temperature loop, the tip of the forceps was used to slightly push the temporal muscle aside to reveal the zygomatic arch, and a 3-mm segment of the zygomatic arch was removed. A small window ($1\text{-}2\text{ mm}^2$) was then drilled in the skull above the MCA. The MCA trunk was lifted with an 8-0 needle, and ligated with a 4-0 silk suture proximal to the cortical branch to the rhinal cortex (Fig. 1A) to initiate ischemia. Anesthesia was then terminated, and mice were allowed to recover and extubated. At the end of 6 hours ischemia, mice were anesthetized and intubated again. The sutures around the MCA and CCA were untied and removed. During model development, we used laser speckle contrast imaging (LSCI) to monitor changes in cerebral blood flow (CBF) flux before and during occlusion of the MCA, and after

reperfusion.

Assessment of reperfusion status. To examine reperfusion in our new 6-hour ttMCAO stroke model, we used the following 3 approaches.

Laser speckle contrast imaging (LSCI). LSCI was performed using a full-field laser perfusion imager RFLS (RWD Life Science Co, Kent, Delaware, USA), as described previously.² In addition to stroke surgery, a midline scalp incision was made to expose the skull. The same imaging parameters were used as before.²

Ink perfusion. Brain vessels were visualized using a gelatin-India ink casting method, as described previously.³ Briefly, mice were deeply anesthetized with isoflurane, and transcardially perfused with 15 mL saline, followed by 15 mL 10% formalin. Next, 20 mL of the gelatin-India ink mixture (pre-heated to 40°C) was infused into the mice with the perfusion pressure controlled at 100 mmHg. Immediately, mice were covered with ice for at least 1 hour to allow gelatin solidification. Finally, brains were harvested and immersed in 10% neutral buffered formalin. Blood vessels were imaged with a Leica microscope.

Lectin labeling. Under isoflurane anesthesia, mice were injected with 100 μ L saline solution containing 50 μ g of DyLight- 594 lectin (Vector Laboratories) via the jugular vein. After 15 minutes, mice were transcardially perfused with saline, and then fixed with 4% paraformaldehyde. Mouse brains were collected, immersed in 30% sucrose in PBS for 2 days, and then stored at -80°C. Frozen brain sections (25 μ m thick) were obtained using a Leica cryostat. Images were captured on a Zeiss Axio Imager Z2 motorized fluorescent microscope (Carl Zeiss MicroImaging).

Behavioral tests. The following functional assessments were performed to evaluate functional deficits after stroke, essentially as described previously.^{4, 5}

Open field. Mice were placed in the center of an open field chamber (50 × 50 × 50 cm). Spontaneous locomotor activity was recorded for 10 minutes, and the video data were

analyzed by Topscan 3.0 software (CleverSys).

Tape removal. The tape removal test was used to evaluate sensory-motor impairments after stroke. Before surgery, mice were trained with one trial per day for 4 days. During the test, a small adhesive patch (0.3 cm × 0.4 cm) was applied to each forepaw. Each mouse was then placed in a transparent box, and observed for 2 minutes. The time lapse to contact and remove each adhesive tape was recorded.

Tight rope. Each mouse was placed with its forepaws at the middle of the rope (60 cm long), and the time lapse for the mouse to reach the platform was recorded. Each test had 2 trials, and the average time was calculated.

Neurologic scoring. A 48-point scoring system was used.⁶ This scoring system evaluates general status (spontaneous activity, body symmetry, gait; 0-12), simple motor deficit (forelimb asymmetry, circling, hind-limb placement; 0-14), complex motor deficit (vertical screen climbing, beam walking; 0-8), and sensory deficit (hind limb, trunk, vibrissae, and face touch; 0-14). The final score was the sum of the 4 individual scores, with 0 = no deficit and 48 = maximal deficit.

Flow cytometry analysis. Our standard protocol was used.⁷ Briefly, mice were deeply anesthetized with isoflurane, and transcardially perfused with ice-cold PBS. Brains were rapidly excised, and split into ipsilateral and contralateral hemispheres along the midline. Brain tissues were then cut into small pieces with scissors in ice-cold DMEM medium, followed by enzymatic digestion with Collagenase D (1 mg/mL) and DNase I (0.1 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) for 45 minutes at 37°C. Cells were then filtered through a 70-µm cell strainer, and resuspended in a 70%/37% Percoll gradient (GE Healthcare Life Sciences, Pittsburgh, PA, USA). After centrifugation at 500 x g without braking, the immune cells at the interphase were harvested. After washing with 25 mL PBS, cell pellets were resuspended in 200 µL RPMI 1640 containing 2% fetal calf serum. Cell numbers were counted with a hemocytometer after staining with trypan blue. After incubation with Fc receptor blocking solution for 15 minutes, cells were immunostained

with combinations of different surface antibodies. The antibodies (all from BioLegend) included: CD45-FITC (103108), CD11b-PE/cy7 (101216), Ly6G-APC/cy7 (127624). LIVE/DEAD fixable violet kit (L34963A; Invitrogen) was used to stain dead cells. Flow cytometry data were acquired on FACS Canto (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software.

Single-cell RNA sequencing (scRNA-seq) analysis. The scRNA-seq analysis was performed at Duke Molecular Genomics Core (MGC) following established protocols.

Preparation of single-cell suspension samples. We adopted a published protocol with modifications.⁸ To minimize changes in gene expression during the procedure, each step was performed on ice or at 4°C, whenever possible. Mice were transcardially perfused with ice-cold Hank's balanced salt solution (HBSS), and the brains were quickly excised. The ipsilateral hemisphere of stroke brains and both hemispheres of sham brains were used for sample preparation. Brain tissues were cut into small pieces, and then homogenized with a 2-mL Dounce homogenizer (Kimble) in ice-cold HBSS using the loose and then tight pestles with 15-20 strokes each. Next, cells were filtered through a 70-µm cell strainer, resuspended in a 70%/37% Percoll gradient, and centrifuged to obtain the enriched immune cells at the interphase. The collected interphase cells were washed with 10 mL ice cold HBSS, and resuspended in 200 µL ice cold FACS buffer (0.5% BSA, 1 mM EDTA, in PBS) containing anti-CD45-FITC (103108; BioLegend) and 7-AAD (420404; BioLegend) for 30 minutes at 4°C. Cell samples were then washed in ice cold FACS buffer, centrifuged for 5 minutes at 300 x g, and resuspended in 500 µL ice cold FACS buffer.

Fluorescence-activated cell sorting (FACS). Cells were sorted on a BD FACSAria II with a purity mode. After gating live/dead cells, all CD45⁺ live cells were collected into tubes. FACS-isolated cells were immediately delivered to Duke Molecular Genomics Core.

Library preparation. Cell suspensions were loaded on a 10x Genomics Chromium Controller Single-Cell Instrument (10x Genomics, Pleasanton, CA, USA), and mixed with

reverse transcription (RT) reagents along with gel beads and oil to generate single-cell gel beads in emulsions (GEMs). GEM-RT was performed in an Eppendorf Mastercycler Pro machine using the following cycle: 53°C for 45 minutes, 85°C for 5 minutes; held at 4°C. After RT, GEMs were broken, and the single-strand cDNA was purified with DynaBeads MyOne Silane Beads (Thermo Fisher Scientific). The cDNA was then amplified using the following protocol: 98°C for 3 minutes; cycled 11-13 ×: 98°C for 15 seconds, 67°C for 20 seconds, and 72°C for 1 minute; 72°C for 1 minute; held at 4°C. The amplified cDNA product was purified using the SPRIselect Reagent Kit (0.6 × SPRI) (Beckman Coulter). Indexed sequencing libraries were constructed using the reagents in the Chromium Single-Cell 3' v3.1 Library Kit, following these steps: (1) fragmentation, end repair, and A-tailing; (2) SPRIselect cleanup; (3) adapter ligation; (4) postligation cleanup with SPRIselect; (5) sample index PCR; and (6) PostindexPCR cleanup. The barcoded sequencing libraries were quantified by quantitative PCR (KAPA Biosystems Library Quantification Kit for Illumina platforms). Sequencing libraries were transferred to the Duke Genomic and Computational Biology (GCB) Core, and loaded on a Novaseq 6000 (Illumina, San Diego, CA, USA) for sequencing.

Bioinformatics processing and data analysis. The primary analytical pipeline for the scRNA-seq analysis followed the recommended protocols from 10X Genomics. Briefly, we demultiplexed raw base call (BCL) files generated by Illumina sequencers into FASTQ files, upon which alignment to mm10-3.0.0 Mouse transcriptome, filtering, barcode counting, and UMI counting were performed using 10X's Cell Ranger software version 5.0.0. The secondary statistical analysis was performed using an R package Seurat version 4.0 which performed quality control and subsequent analyses on the feature-barcode matrices produced by Cell Ranger. In Seurat, data were first filtered for minimum gene (200) and cell observance frequency cut-offs (10) for both samples. We then closely examined the data and performed further filtering based a range of metrics (% mitochondrial expression < 20 for both samples, 250 < nFeature > 7500, 500 < nUMI(count) > 50,000 for sham & 250 < nFeature > 8000, 500 < nUMI > 55,000 for stroke) to identify and exclude possible multiplets (ie, instances where more than one cell was present and sequenced in a single emulsified gel bead) followed by normalization and

scaling samples. To further remove doublets/multiplets, DoubletFinder (version 2.0.3) was used in R individually on each sample.⁹ The removal of further technical artifacts was performed using regression methods to reduce noise.

After quality control procedures, we performed linear dimensional reduction calculating principal components using the most variably expressed genes in our dataset. Library size and/or the numbers of genes expressed across subsets of cells may necessitate the restriction of cells upon which the variably-expressed genes are selected for inclusion when calculating principal components.¹⁰ Significant principal components for downstream analyses were determined through methods mirroring those implemented by Macosko et al,¹⁰ and these principal components (64) were carried forward for two main purposes: to perform cell clustering and to enhance visualization.¹¹ Cells were grouped into an optimal number of clusters for de novo cell type discovery using Seurat's FindNeighbors() and FindClusters() functions, graph-based clustering approaches with visualization of cells was achieved through the use of manifold learning technique UMAP (Uniform Manifold Approximation and Projection), which reduces the information captured in the selected significant principal components to 2 dimensions.^{11, 12}

Differential expression of relevant cell marker genes was visualized on UMAP plot to reveal specific individual cell types. Additional downstream analyses included examining the cellular distribution of a priori genes of interest, closer examination of genes associated with cell clusters, and the refined clustering of cells in order to identify further resolution of cell types, in addition to comparing differences between experiments of different states. Combining multiple libraries using the integration strategy described in Stuart and Butler et al¹³ allowed for calculation of differential expression, not only between clusters, but also within clusters across libraries using default parameters in Seurat. This allowed calculation of differential expression within cell type between sham and stroke brains. Enrichment analysis of gene ontology categories was performed using DAVID online tools (<https://david.ncifcrf.gov>). For all volcano plots (Fig. 2D and Fig. 3B), if a p_{adj} equals zero, the value of $-\log_{10}p_{adj}$ is randomly assigned a number between 300-500 in order to be plotted.

Statistical Analysis. The group size for each experiment was based on our previous studies or pilot experiments. Statistical analyses were performed using unpaired Student's *t*-test for 2 group comparisons, and one-way or two-way ANOVA with *post hoc* Holm-Sidak correction for multiple comparisons for more than 2 groups. Neurologic scores were analyzed with the Mann-Whitney *U* test. Data are presented as mean $\pm$ SEM or median. The level of significance was set at $p < 0.05$.

Table S1. List of full gene names.

Gene symbol	Gene name
<i>Aif1</i> (Iba1)	allograft inflammatory factor 1 (induction of brown adipocytes 1)
<i>Apoe</i>	apolipoprotein E
<i>Arg1</i>	arginase 1
<i>Bst2</i>	bone marrow stromal cell antigen 2
<i>C3</i>	complement component 3
<i>Camp</i>	cathelicidin antimicrobial peptide
<i>Ccr2</i>	chemokine (C-C motif) receptor 2
<i>Ccr7</i>	chemokine (C-C motif) receptor 7
<i>Ccr9</i>	chemokine (C-C motif) receptor 9
<i>Cdk1</i>	cyclin-dependent kinase 1
<i>Cd19</i>	CD19 antigen
<i>Cd209a</i>	CD209a antigen
<i>Cd4</i>	CD4 antigen
<i>Cd44</i>	CD44 antigen
<i>Cd74</i>	CD74 antigen
<i>Cd79a</i>	CD79A antigen
<i>Cd8b1</i>	CD8 antigen, beta chain 1
<i>Cd93</i>	CD93 antigen
<i>Cd3e</i>	CD3 antigen, epsilon polypeptide
<i>Cd3g</i>	CD3 antigen, gamma polypeptide
<i>Chil3</i>	Chitinase-like 3
<i>Csf3r</i>	colony stimulating factor 3 receptor
<i>Cx3cr1</i>	chemokine (C-X3-C motif) receptor 1
<i>Cxcr2</i>	chemokine (C-X-C motif) receptor 2
<i>Cybb</i>	cytochrome b-245, beta polypeptide
<i>Fcrls</i>	Fc receptor-like S, scavenger receptor
<i>Flt3</i>	FMS-like tyrosine kinase 3
<i>Fn1</i>	fibronectin 1
<i>Fxyd5</i>	FXDY domain-containing ion transport regulator 5
<i>Gata3</i>	GATA binding protein 3
<i>H2-Aa</i>	histocompatibility 2, class II antigen A, alpha
<i>H2-Ab1</i>	histocompatibility 2, class II antigen A, beta 1
<i>Hexb</i>	hexosaminidase B
<i>Hif1a</i>	hypoxia inducible factor 1, alpha subunit
<i>Hmox1</i>	heme oxygenase 1
<i>Hp</i>	haptoglobin
<i>Ifitm1</i>	interferon induced transmembrane protein 1
<i>Il1b</i>	interleukin 1 beta
<i>Il1rl1</i>	interleukin 1 receptor-like 1
<i>Itgam</i>	integrin alpha M
<i>Itgax</i> (CD11c)	integrin alpha X
<i>Itf</i>	lactotransferrin
<i>Klre1</i>	killer cell lectin-like receptor family E member 1
<i>Lat</i>	linker for activation of T cells

<i>Ly6c2</i>	lymphocyte antigen 6 complex, locus C2
<i>Ly6g</i>	lymphocyte antigen 6 complex, locus G
<i>Ly86</i>	lymphocyte antigen 86
<i>Lyz2</i>	lysozyme 2
<i>mt-Nd1-4</i>	mitochondrially encoded NADH dehydrogenase 1-4
<i>Mki67</i>	antigen identified by monoclonal antibody Ki 67
<i>Mmp9</i>	matrix metalloproteinase 9
<i>Ms4a1</i>	membrane-spanning 4-domains, subfamily A, member 1
<i>Ms4a7</i>	membrane-spanning 4-domains, subfamily A, member 7
<i>Ngp</i>	neutrophilic granule protein
<i>Ncr1</i>	natural cytotoxicity triggering receptor 1
<i>Rps27</i>	ribosomal protein S27
<i>P2ry12</i>	purinergic receptor P2Y, G-protein coupled 12
<i>Pf4</i>	platelet factor 4
<i>Ptprc (CD45)</i>	protein tyrosine phosphatase, receptor type, C
<i>S100a8</i>	S100 calcium binding protein A8 (calgranulin A)
<i>S100a9</i>	S100 calcium binding protein A9 (calgranulin B)
<i>Sall1</i>	spalt like transcription factor 1
<i>Stmn1</i>	stathmin 1
<i>Thbs1</i>	thrombospondin 1
<i>Tmem119</i>	transmembrane protein 119
<i>Tgfb1</i>	transforming growth factor, beta induced
<i>Top2A</i>	topoisomerase (DNA) II alpha
<i>Wfdc17</i>	WAP four-disulfide core domain 17
<i>Xcr1</i>	chemokine (C motif) receptor 1
<i>Ym1(Chil3)</i>	chitinase-like 3

Table S2 (Excel file).

Description: Average expression level of detected genes for each cluster.

Table S3 (Excel file).

Description: This file has 28 worksheet tabs. Each tab contains the top 50 genes enriched in the corresponding cluster. These genes were used to identify cell types.

Table S4 (Excel file).

Description: The list of differentially expressed genes (DEGs) between stroke and sham. The analysis was performed on aggregated data from all clusters. The selection criteria are: $p_{adj} < 0.01$ and fold change ≥ 2 or ≤ -2 .

Table S5 (Excel file).

Description: The list of differentially expressed genes (DEGs) between cluster MG5 and MG1. The selection criteria are: $p_{adj} < 0.01$ and fold change ≥ 2 or ≤ -2 .

Table S6 (Excel file).

Description: The list of differentially expressed genes (DEGs) between cluster Neut1 and Neut2. The selection criteria are: $p_{adj} < 0.01$ and fold change ≥ 2 or ≤ -2 .

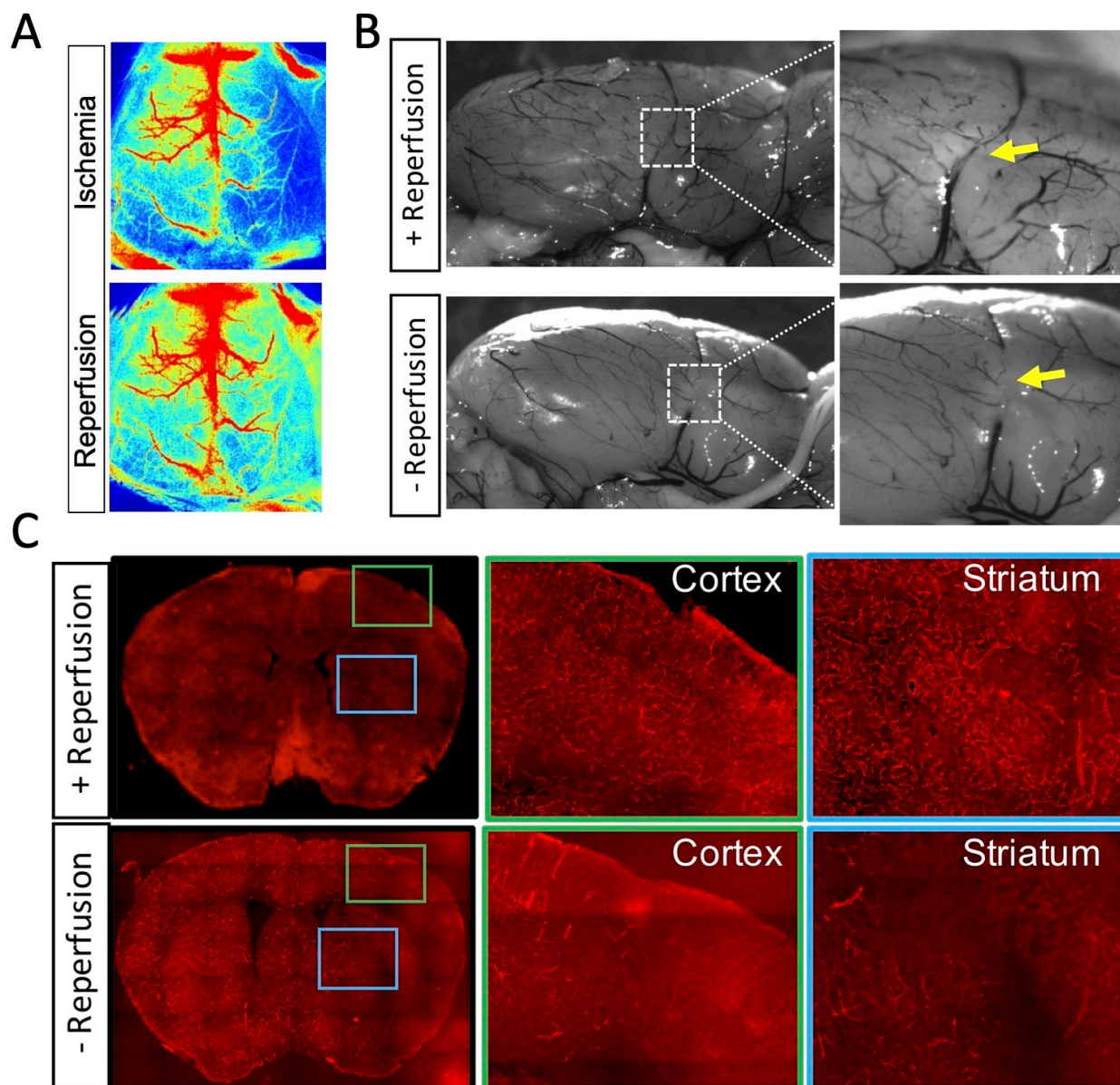


Figure S1. Evaluation of reperfusion after 6 hours ischemia in our ttMCAO model.

A) Laser speckle contrast imaging (LSCI). Mice were subjected to occlusion of middle cerebral artery (MCA) at the site indicated in Fig. 1A. After 5 hours and 40 minutes MCAO, mice were anesthetized again, and the cortex was imaged by LSCI to show cerebral blood flow (CBF) during ischemia (top). Then, at 6 hours ischemia, both sutures from CCA and MCA were removed to initiate perfusion. Ten minutes later, CBF flux was imaged again using LSCI (bottom). **B)** Ink-perfused brain vessels. After 6 hours ttMCAO, mice were subjected to either reperfusion (+) or no (-) reperfusion. The mice were then transcardially

perfused with gelatin-India ink solution under controlled pressure. The right MCA ligated (yellow arrow) in this model was indicated in a representative image. **C)** Lectin labeling. After 6 hours ttMCAO, mice were subjected to either reperfusion (+) or no (-) reperfusion. Then, lectin DyLight 594 was injected through the external jugular vein. After 15 minutes, mice were perfused and fixed. Brains were sectioned, and the perfused brain vessels were visualized under a fluorescent microscope.

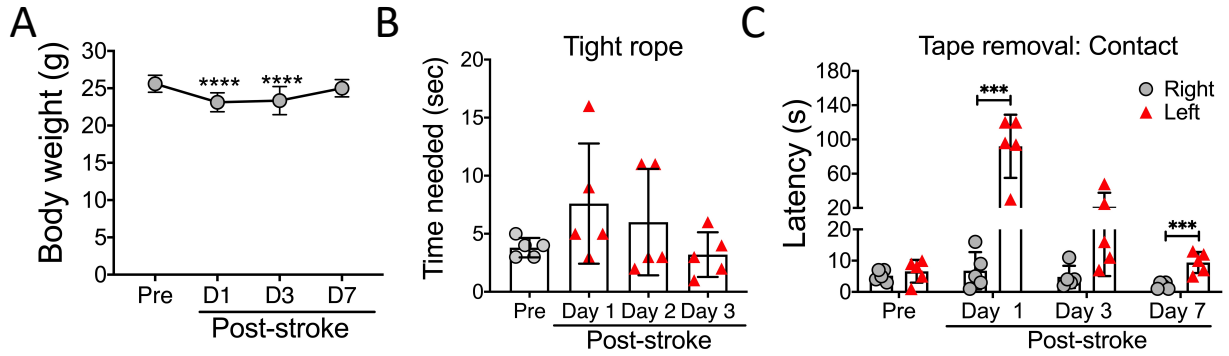


Figure S2. Stroke outcome after 6 hours ttMCAO in young mice. Supplemental information to Figure 1. Young C57Bl/6 mice were subjected to 6 hours ttMCAO. Data before stroke (Pre) are shown for comparisons. **A)** Body weight change in stroke mice. **B)** Tight rope test. **C)** Tape removal test. Data are presented as mean $\pm$ SEM ($n = 5/\text{group}$). *** $p < 0.001$; **** $p < 0.0001$.

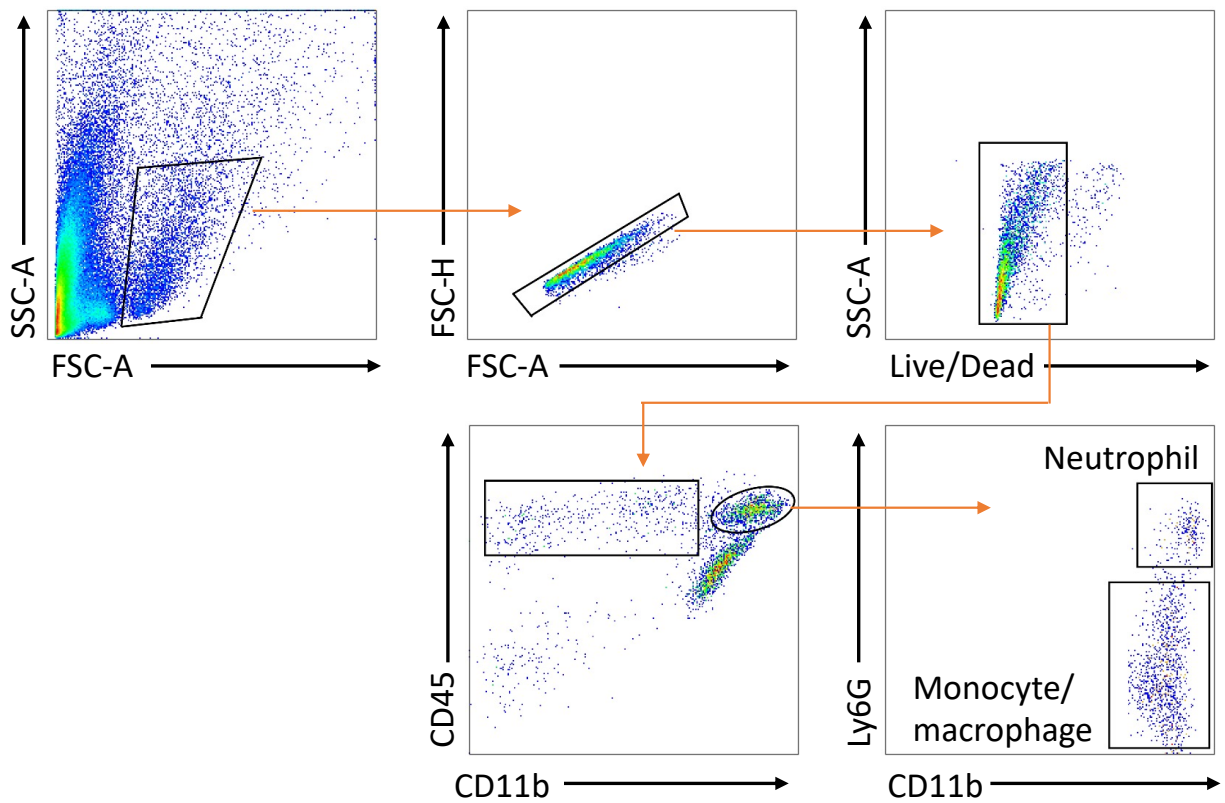


Figure S3. Flow cytometric gating strategy used in this study. Plots depict gating of infiltrated myeloid cells (CD45^{hi}CD11b⁺), neutrophils (CD45^{hi}CD11b⁺Ly6G⁺), and monocytes/macrophages (CD45^{hi}CD11b⁺Ly6G⁻).

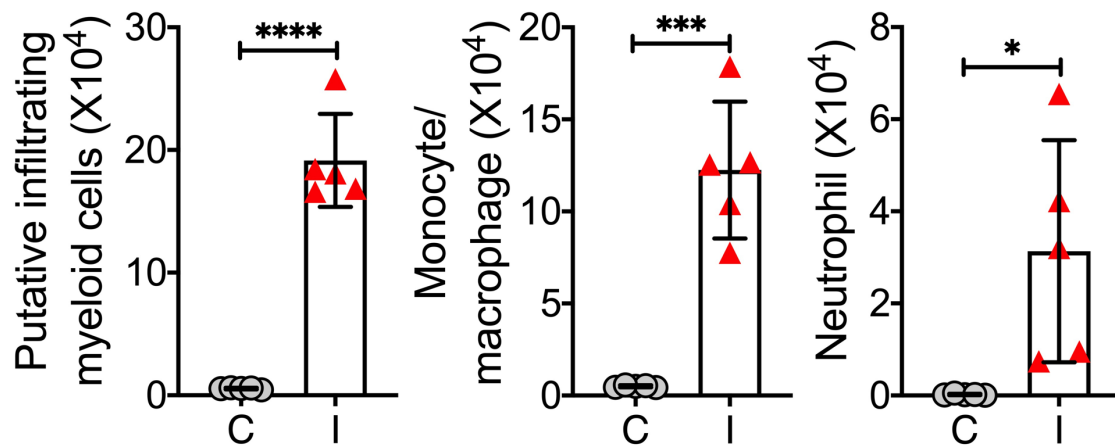


Figure S4. The flow cytometric analysis of immune cell populations in the aged brain after stroke. Aged mice ($n = 5$) were subjected to 6-hour ttMCAO. On day 3 after stroke, the contralateral (C) and ipsilateral (I) hemispheres were collected for flow cytometric analysis. The gating strategy is shown in Figure S3. Data are presented as mean $\pm$ SEM ($n = 5$ /group). * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

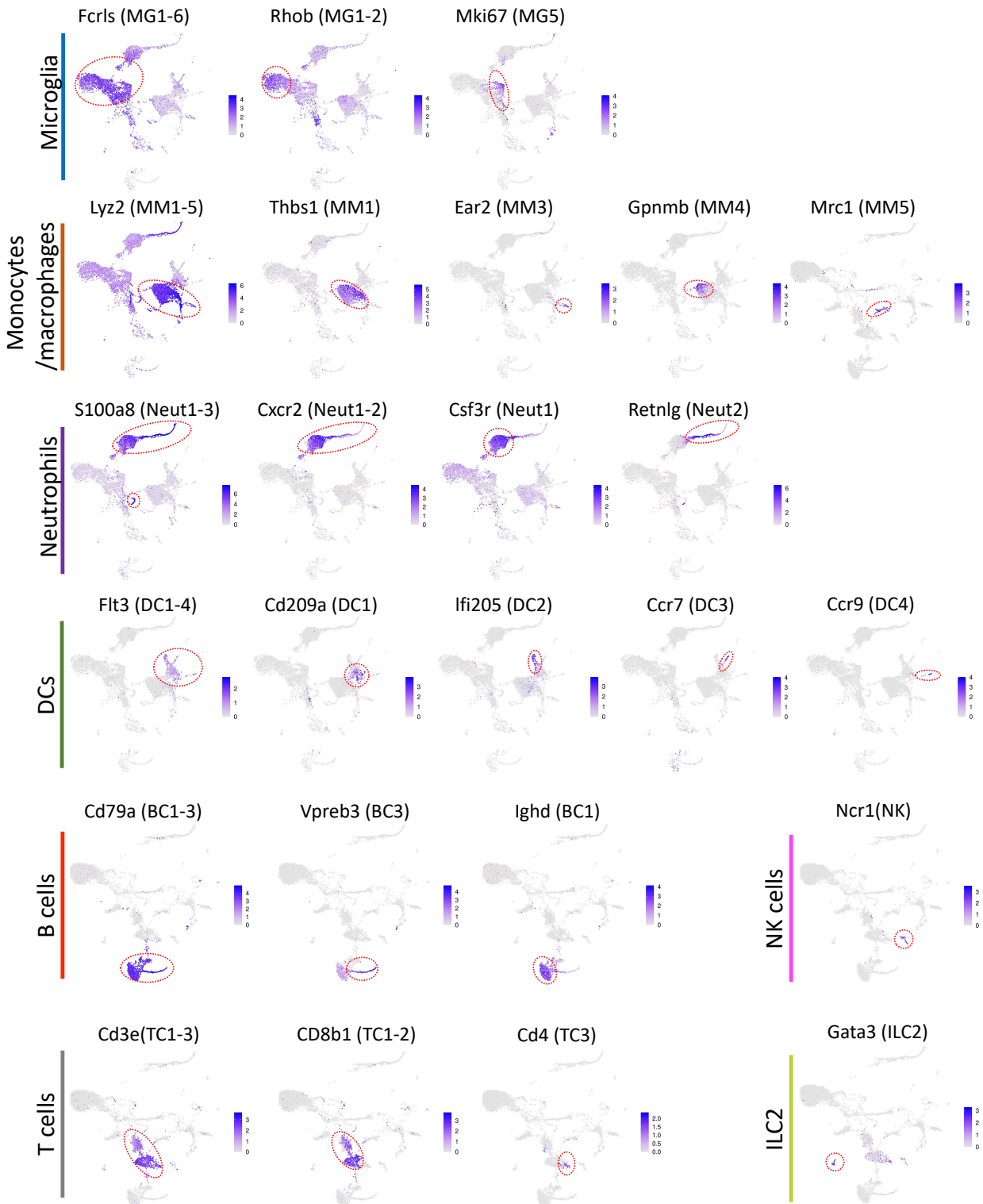


Figure S5. Molecularly distinct subpopulations of immune cells in the post-stroke aged brain. Supplemental information to Figure 2. Aged mice were subjected to 6-hour ttMCAO or sham. Three days later, both hemispheres (sham) or the ipsilesional hemisphere (stroke) were collected for scRNA-seq analysis of all FACS-isolated CD45⁺ cells. Potential molecular markers for immune cell types or individual clusters were identified, and their expression was depicted on UMAP plots. To better visualize different cell populations, we plotted microglial, monocyte/macrophage, neutrophil, and DC populations in the stroke brain, and T-cell, B-cell, NK, and ILC2 populations in the sham brain. The color intensity indicates the expression level of each gene in individual cells. The main cell clusters for each gene are circled in red dotted lines.

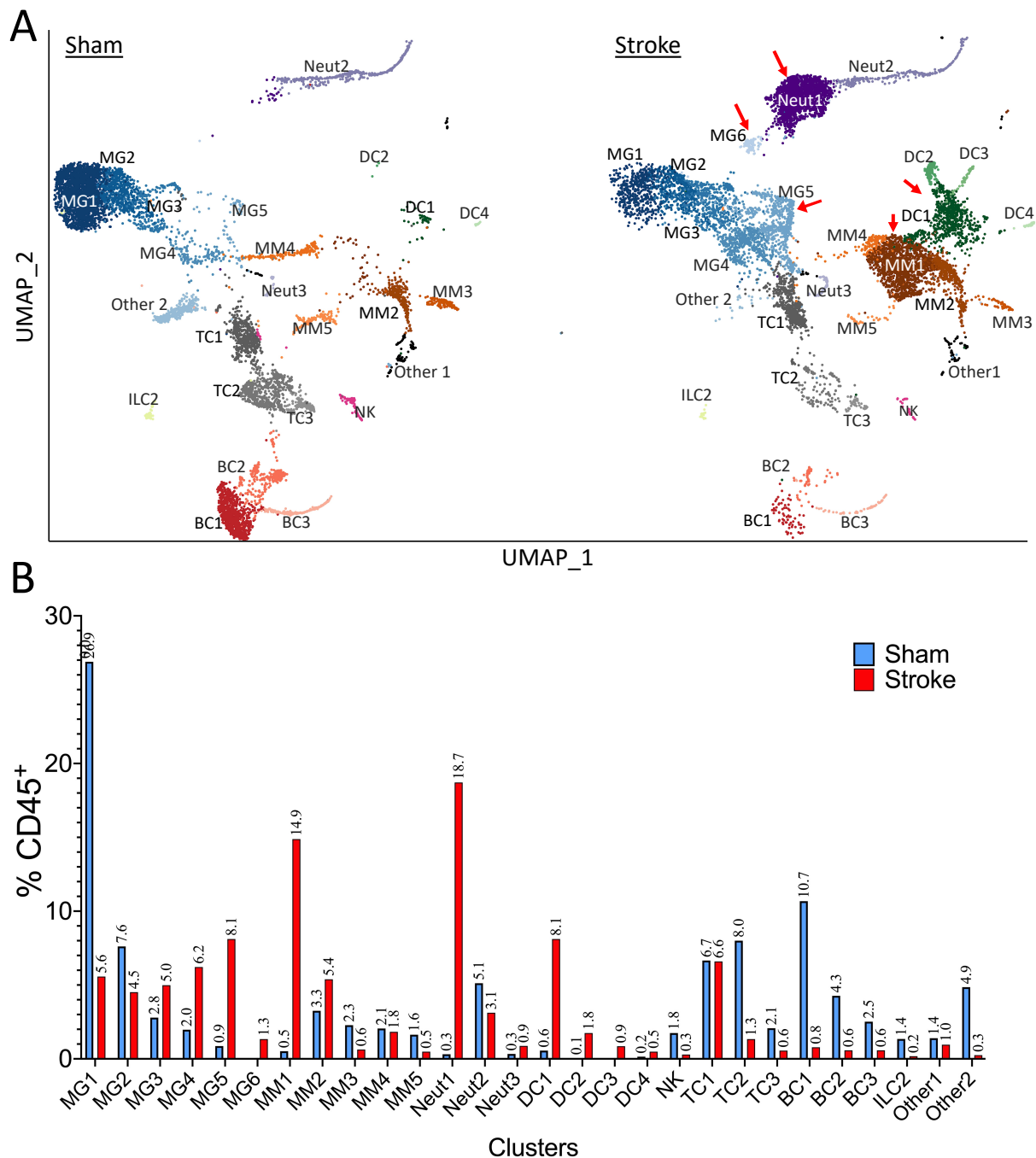
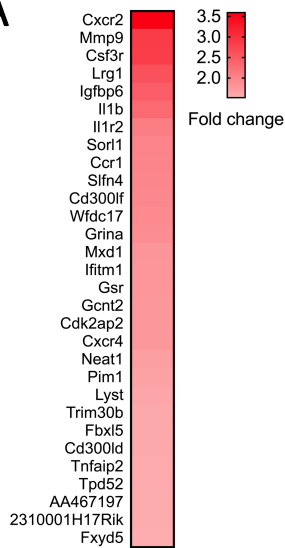
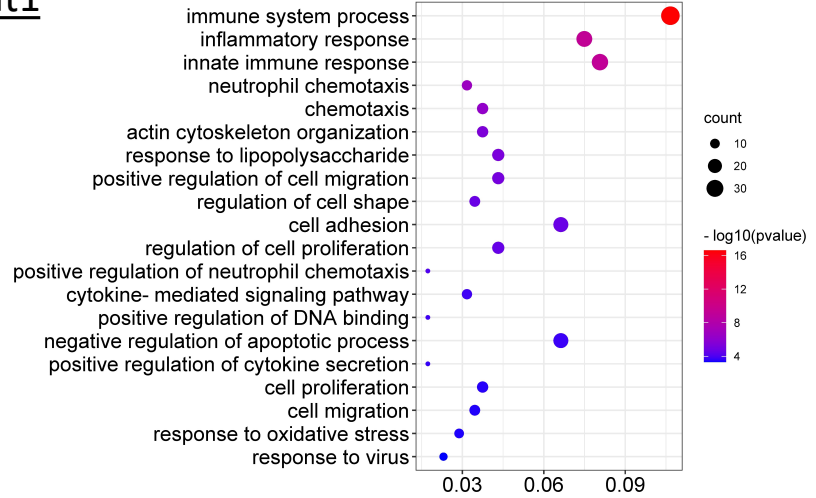


Figure S6. Changes of individual cell clusters in the aged brain on day 3 after stroke vs sham. Supplemental information to Figure 3. **A)** UMAP plots of cells from sham and stroke aged brains. The clusters with a marked increase in cell frequency after stroke (MG5, MG6, MM1, Neut1, and DC1) are indicated by red arrows. **B)** Changes in the frequency of each cell cluster after stroke vs sham.

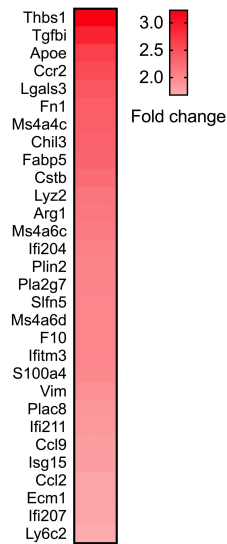
A



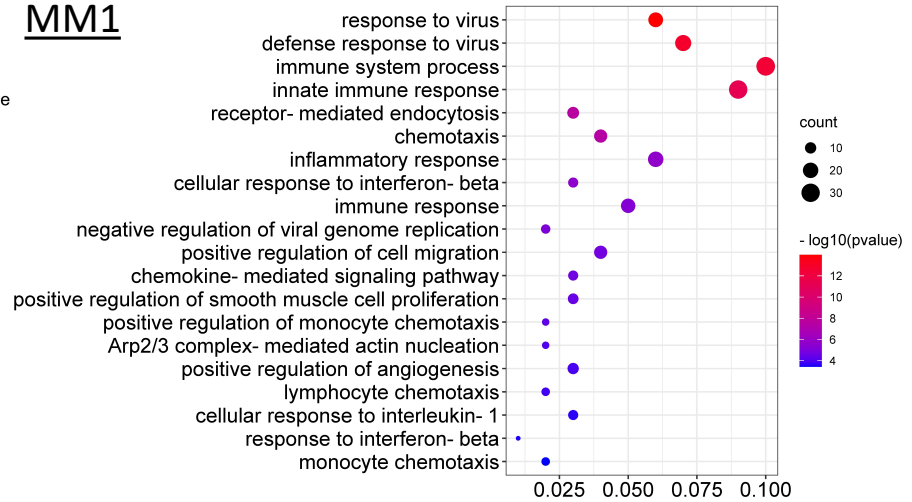
Neut1



B



MM1



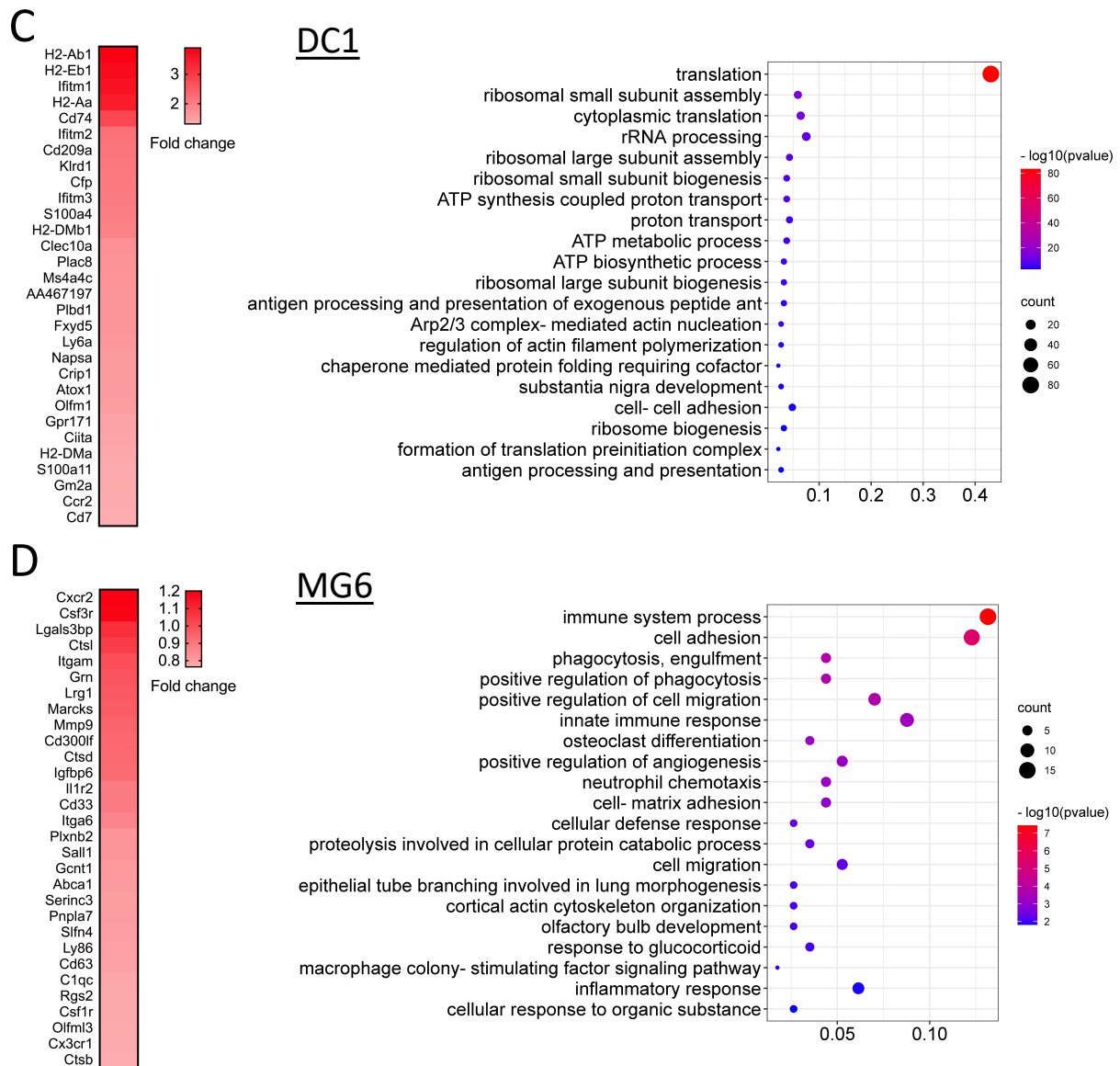


Figure S7. Major clusters with increased percentages after stroke. Four major clusters MM1 (A), Neurt1 (B), DC1 (C), and MG6 (D) are analyzed with DAVID program. Top 30 differentially expressed genes (DEGs) in each cluster are shown on the left. The fold change was calculated by comparing average expression of a gene in each cluster relative to the average expression of this gene in all other clusters combined. Cluster-enriched DEGs with $padj < 0.01$ and fold change ≥ 1.5 were subjected to gene ontology analysis. The top 20 overrepresented terms of biologic process are shown here (right).

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