

SUPPLEMENTARY INFORMATION

Repair-Associated Macrophages Increase after Early-phase Microglia Attenuation to Promote Ischemic Stroke Recovery

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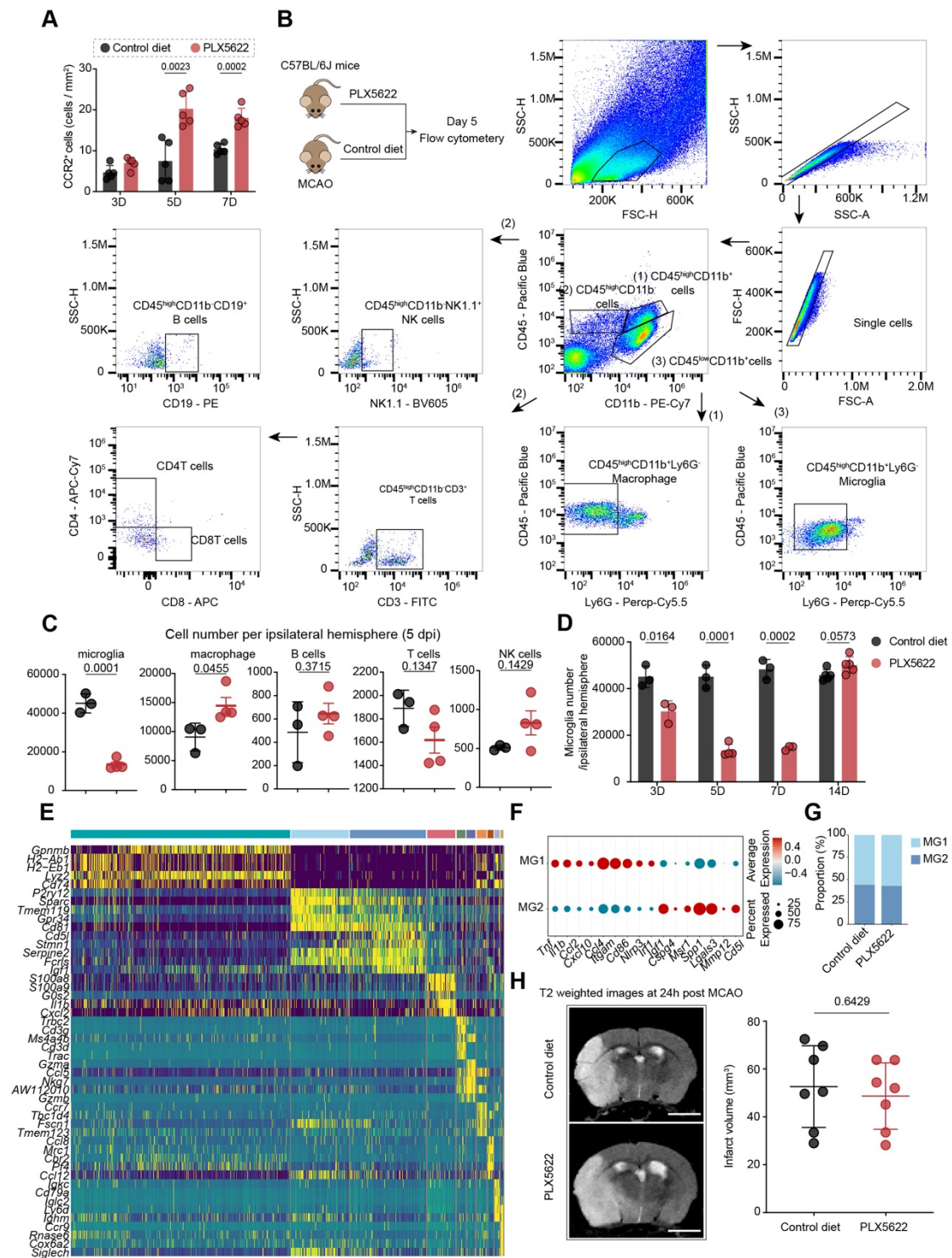
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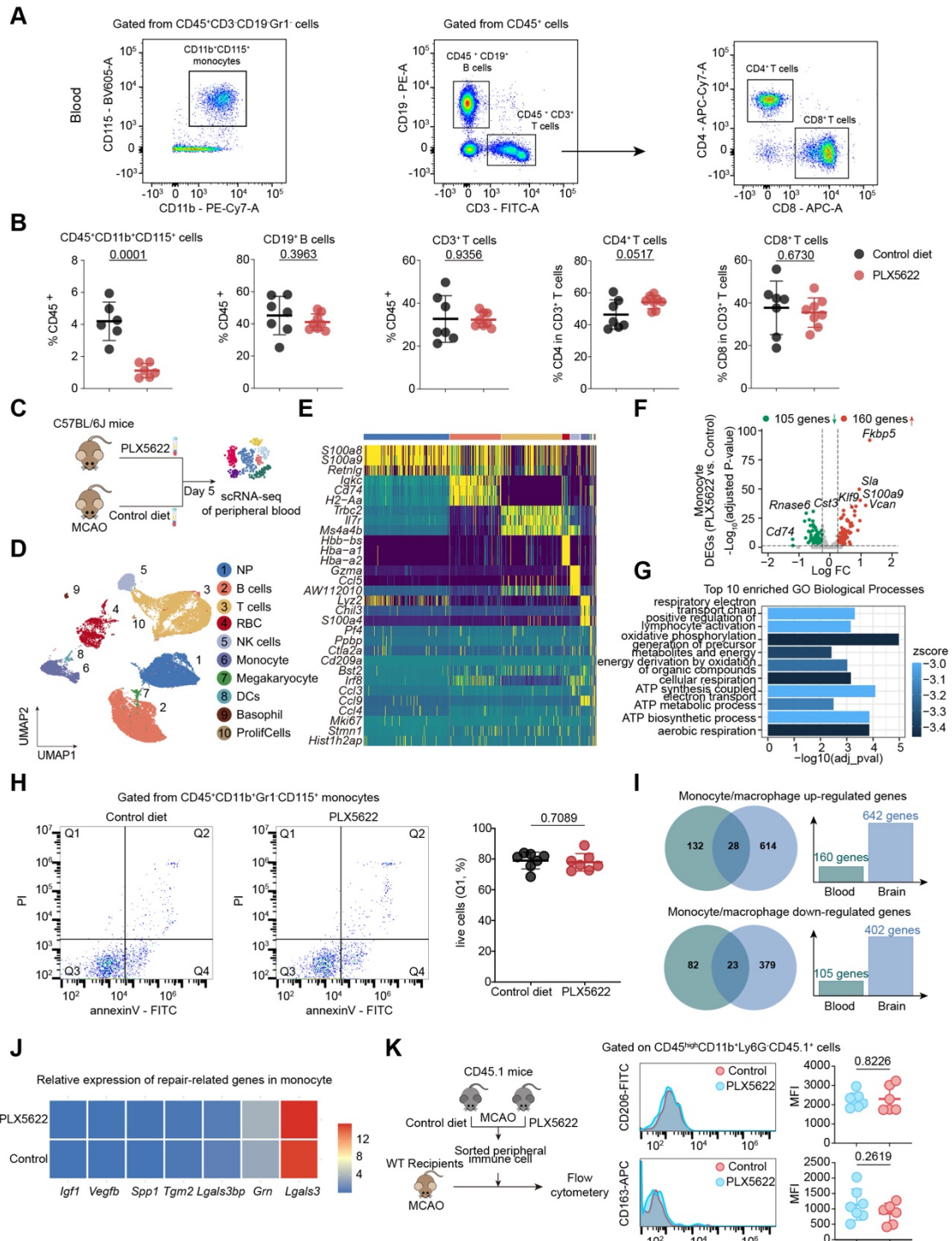
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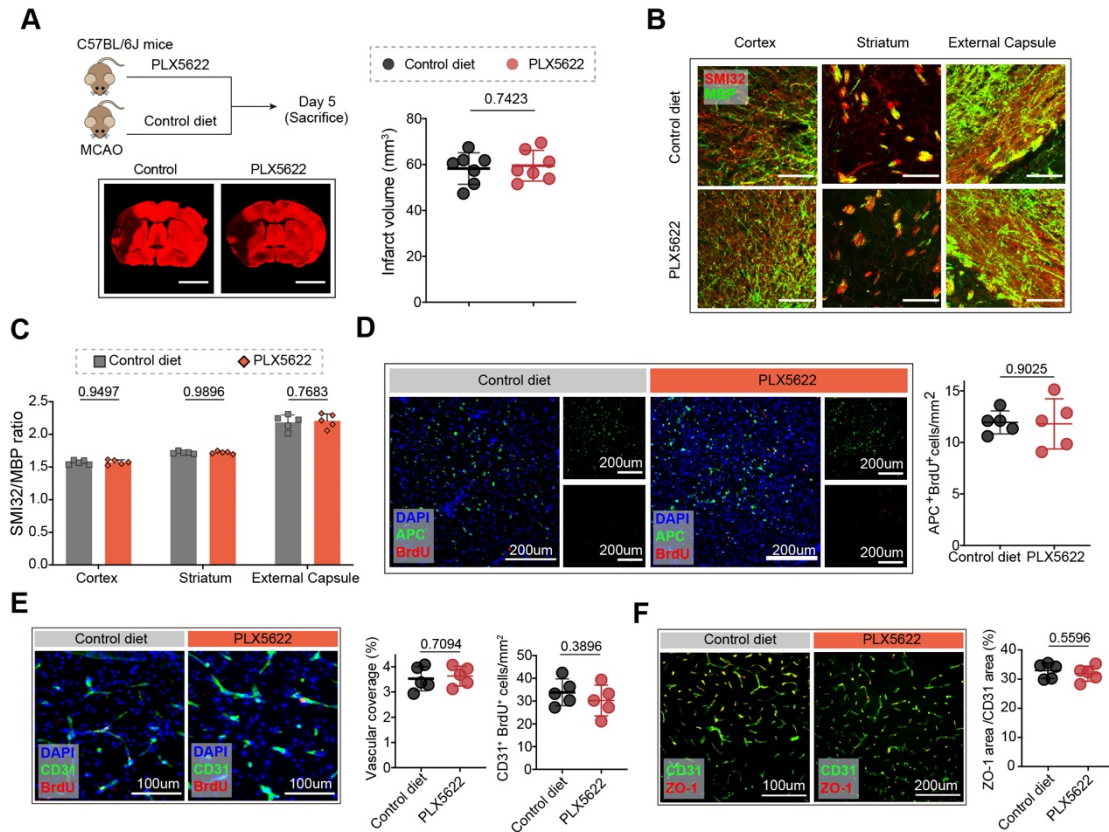
Supplementary Figure1. Immune cell changes induced by early-phase CSF1R inhibition after MCAO. **A** Dot plots showing the CCR2⁺ cells in the ipsilateral brain hemisphere at several time points (3, 5, and 7 days) following ischemic stroke. n=5 per group. Student's t test, two-sided. **B** Flow cytometry was performed to quantify the numbers of various immune cells in the brain. Gating strategy: microglia (CD45^{int}CD11b⁺Ly6G⁻), macrophages (CD45^{high}CD11b⁺Ly6G⁺), NK cells (NK1.1⁺), B cells (CD19⁺), CD4 T cells (CD3⁺CD4⁺) and CD8 T cells (CD3⁺CD8⁺). **C** Dot plots showing the absolute numbers of various immune cells

in the ipsilateral hemisphere at 5 dpi. n=3 for control group, n=4 for PLX5622 group. Student's t test, two-sided. **D** Dot plots showing the absolute numbers of microglia in the ipsilateral hemisphere at several time points (3, 5, 7, and 14 days) following ischemic stroke. n=3 for 3D, 7D, and 5D control diet group, n=4 for 5D PLX5622 group, n=5 for 14D. **E** Heatmap showing the expression levels of top five marker genes in each cell type. The most significantly upregulated genes are shown in yellow and downregulated genes are shown in Tibetan blue. **F** Dot plot illustrating the expression of selected genes in microglia subclusters at 5 dpi. **G** Stacked bar plot showing proportions of microglia subcluster in the mouse brain of each group. **H** Left panel: T2-weighted magnetic resonance imaging showing the infarct (high signal) in mouse brains 24 hours after MCAO induction. Scale bars, 2.5mm. Right panel: Brain lesion volume was assessed on T2-weighted images. n = 7 per group. (**A, C, D and H**): Student's t test, two-sided. Data in **A, C, D and H** are presented as mean $\pm$ SD. Source data are provided as a Source Data file.



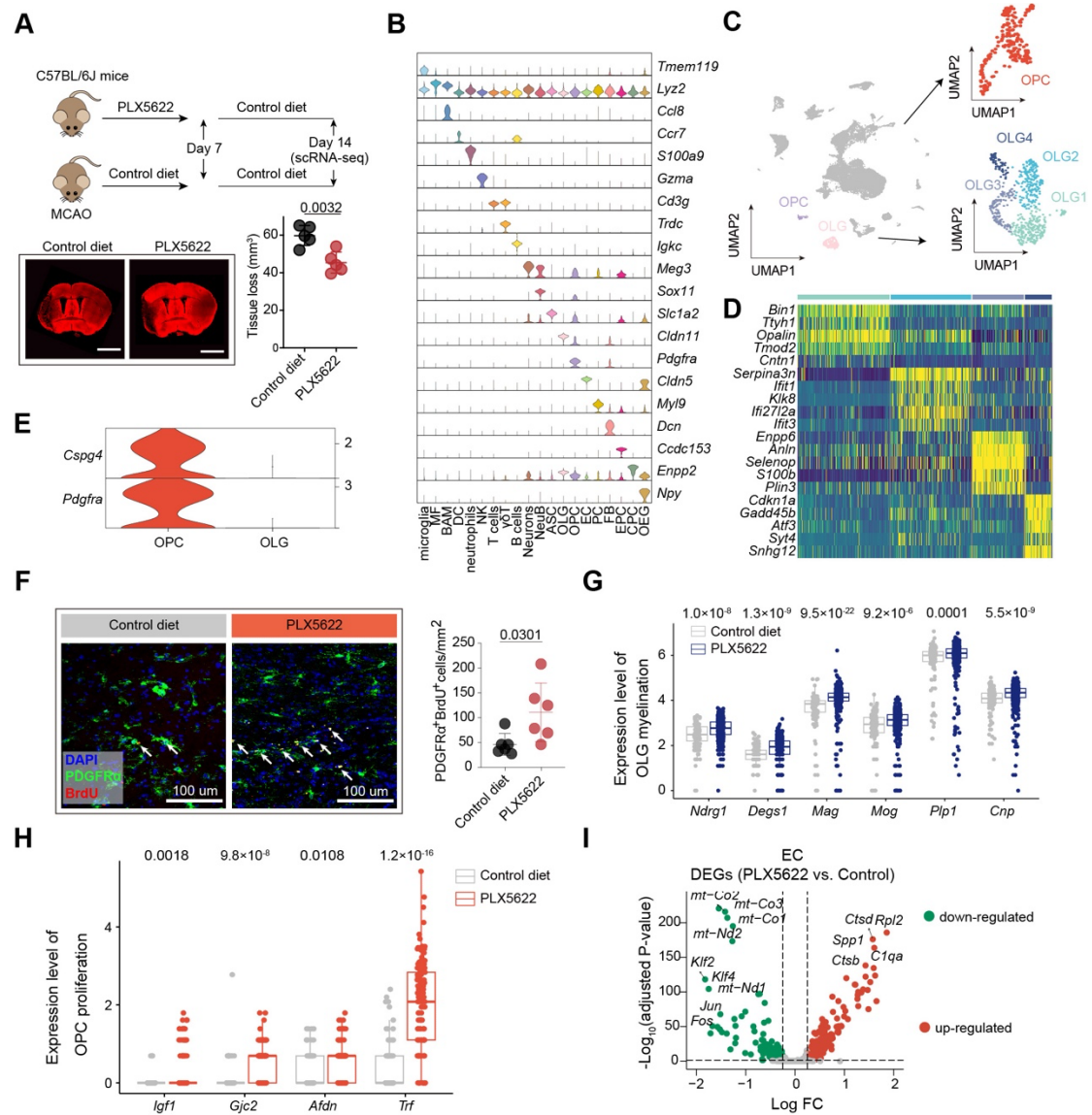
Supplementary Figure2. PLX5622 reduced the number of peripheral monocytes without altering their reparative function. **A** Flow cytometry was performed to quantify the numbers of various immune cells in the blood. **B** Dot plots showing the proportion of various immune cells among CD45⁺ cells in the blood at 5 dpi. n=6 for monocytes, n=7 for control diet group and n=8 for PLX5622 group of B cells, T cells, CD4⁺ T cells and CD8⁺ T cells. **C** Experimental scheme showing scRNA-seq of the peripheral blood from mice at 5dpi. n = 2 biological replicates for each group. **D** UMAP projection plot showing ten distinct cell types revealed by unsupervised clustering analysis in **C**. **E** Heatmap showing the expression levels of top five

marker genes in each cell type. **F** Volcano plot showing 160 upregulated genes and 105 downregulated genes in monocytes from the PLX5622 group compared to the control diet group. **G** Top 10 enriched GO terms of monocytes in the PLX5622 group compared to control diet group. The enriched GO terms were listed in **Supplementary Data 2**. **H** Flow cytometry was performed to assess cell survival of monocytes using Annexin V/PI staining. n=7 for each group. **I** Venn diagrams showing unique/shared DEGs from brain and blood at 5 dpi. **J** Relative expression of repair-related genes in monocyte at 5 dpi with control diet or PLX5622 group. **K** Experiment scheme of CD45.1 immune cells adoptive transfer (left). Mean Fluorescence Intensity (MFI) plots of CD206 and CD163 of brain-infiltrated CD45.1⁺ macrophages (right). n = 7 for control diet group, n=6 for PLX5622 group. (**B, H and K**): Student's t test, two-sided. Data in **B, H and K** are presented as mean $\pm$ SD. Abbreviations: NP neutrophils, RBC red blood cell, NK natural killer cells, DCs dendritic cells, ProlifCells proliferating cells. Source data are provided as a Source Data file.



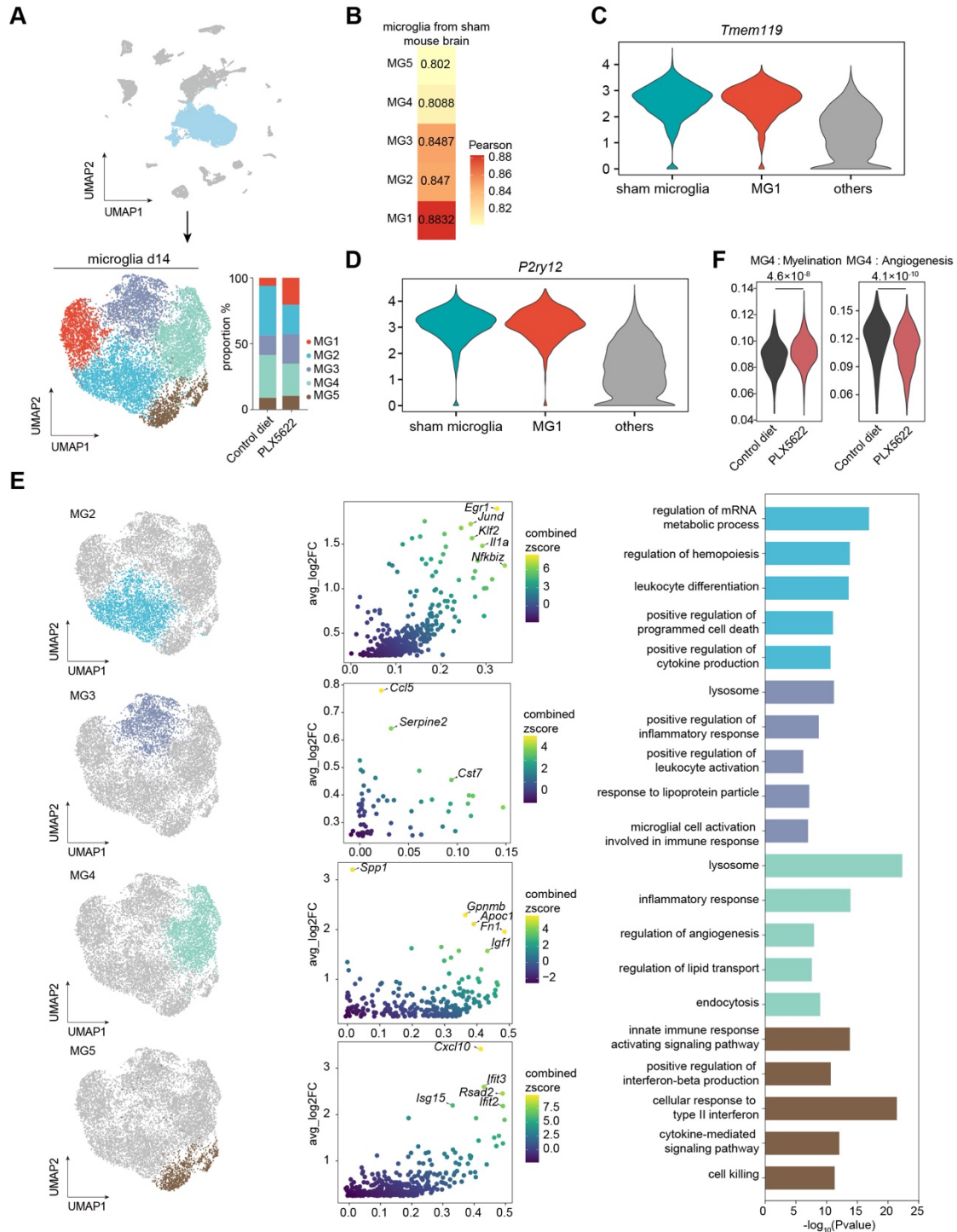
Supplementary Figure3. Ischemic brain injury was comparable between groups at 5 dpi.

A Experimental scheme showing infarct volume evaluation at 5 dpi in the PLX5622 group and the control diet group. $n = 7$ per group. Scale bars, 2.5mm. **B-C** Representative images of SMI32 and MBP dual immunostaining and expression ratio at the cortex, striatum and external capsule at 5 dpi. $n = 5$ per group. Scale bars, 100 μ m. **D** Representative images for APC (green) and BrdU (red) double immunostaining in the peri-infarct areas in brains at 5 dpi (left). Quantification of APC⁺ BrdU⁺ cells in the peri-infarct areas(right). $n = 5$ per group. **E** Representative images for CD31 (green) and BrdU (red) double immunostaining in the peri-infarct areas in brains at 5 dpi (left). Quantification of vascular coverage and CD31⁺ BrdU⁺ cells in the peri-infarct areas (right). $n = 5$ per group. **F** Representative images for CD31 (green) and ZO-1 (red) double immunostaining in the peri-infarct areas in brains at 5 dpi (left). Quantification of ZO-1 area / CD31 area in the peri-infarct areas (right). $n = 5$ per group. (**A**, **C**, **D**, **E** and **F**): Student's t test, two-sided. Data in **A**, **C**, **D**, **E** and **F** are presented as mean $\pm$ SD. Source data are provided as a Source Data file.

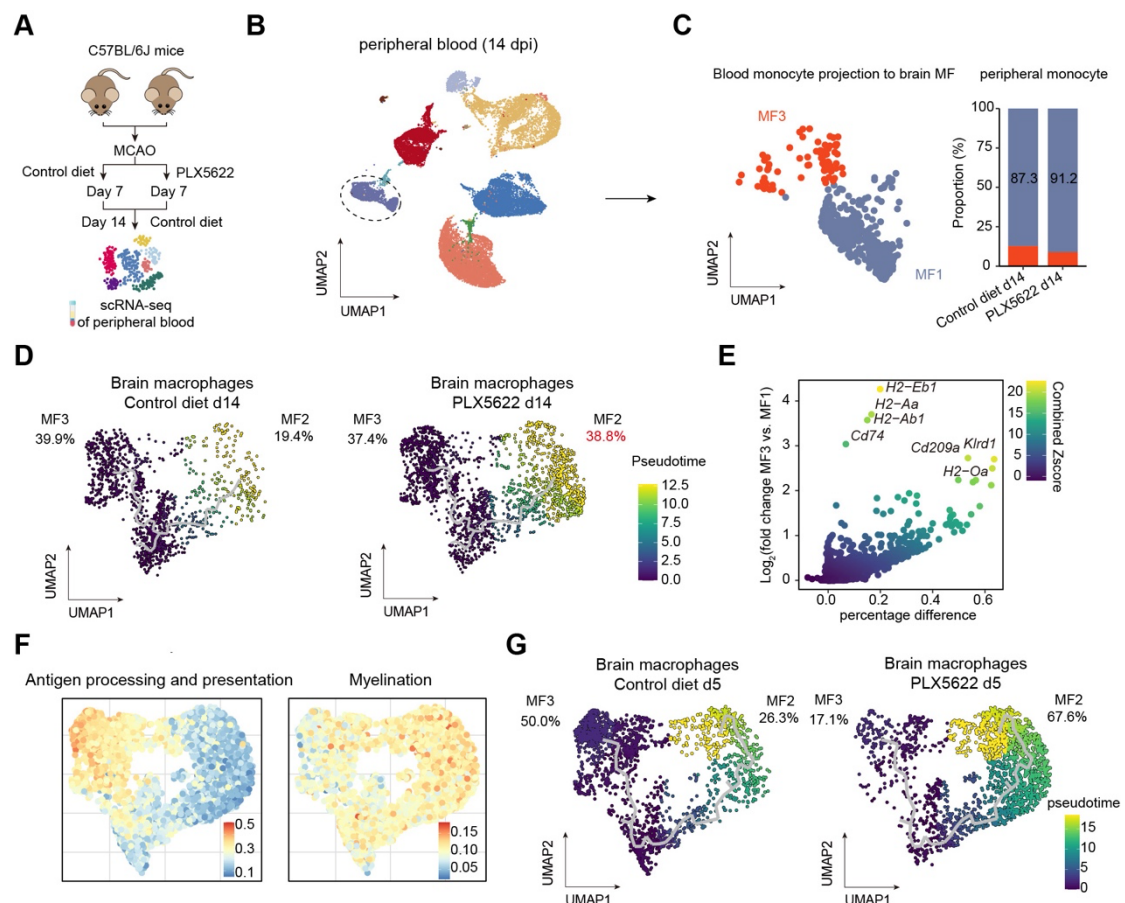


Supplementary Figure 4. Changes of oligodendrocyte lineage cells and endothelial cells in mice with early-phase CSF1R inhibition after MCAO. **A** The experimental scheme for scRNA-seq. $n = 2$ biological replicates for each group (up). Representative images of tissue loss of control diet or PLX5622 group and quantification of tissue loss (down). $n = 5$ per group. Scale bars, 2.5mm. **B** Violin plot illustrating the selected marker genes of each cluster of the whole mouse brains at 14 dpi. **C** UMAP projection plot showing cell clusters harvested from the ipsilateral brain hemisphere at 14 dpi (left). OPC and OLG were extracted for sub-clustering analysis (right). **D** Heatmap showing the expression levels of top five marker genes in each cell type of OLGs. The most significantly upregulated genes are shown in yellow and downregulated genes are shown in Tibetan blue. **E** Violin plots showing the expression of marker genes for OPC. **F** Representative images of PDGFR α ⁺BrdU⁺ cells in the peri-infarct areas (left) and quantification of the number of PDGFR α ⁺ BrdU⁺ cells (right) at 14 dpi. $n = 6$ per group. **G-H** Box plot displaying the upregulation of genes related to myelination in OLGs (G) and OPCs (H). $n = 118$ OLGs for control group and $n = 466$ OLGs for PLX5622 group, n

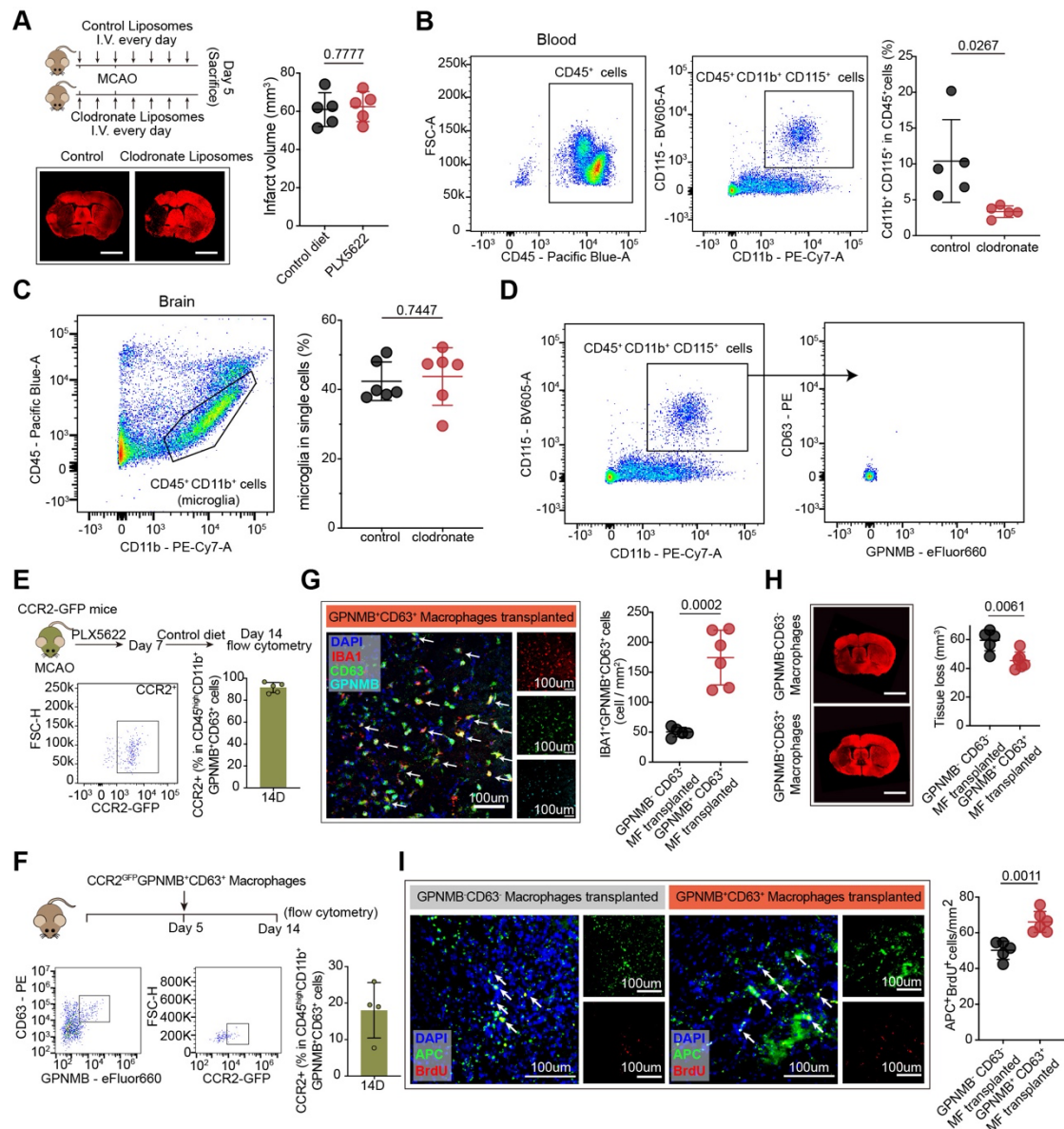
= 68 OPCs for control group and n = 147 OPCs for PLX5622 group, from two biological replicates. Wilcoxon rank sum test, two-sided. Box plot represents the first quartile, median, and third quartile with whiskers extending to 1.5 times the interquartile range. **I** Volcano plot showing upregulated genes and downregulated genes of endothelial cells between PLX5622 group and control diet group. (**A and F**): Student's t test, two-sided. Data in **A and F** are presented as mean $\pm$ SD. Source data are provided as a Source Data file.



Supplementary Figure5. Single-cell transcriptomic analysis of microglia at 14 dpi. **A** microglia were extracted for sub-clustering analysis. Stacked bar plot showing proportions of cells from each group. **B** Pearson correlation coefficients of microglia subsets with microglia from the sham mouse brain. **C-D** Violin plots showing the expression of marker genes (*Tmem119*, *P2ry12*) for resting microglia. **E** UMAP visualization of microglia from different cell cluster (left). Scatter plot illustrating upregulated DEGs of this cell cluster compared to the others, with the percentage difference along the x-axis and $\log_2(\text{fold change})$ along the y-axis. The combined z-score of percentage difference and $\log_2(\text{fold change})$ was color-coded (middle). Functional enrichment analysis was performed on the DEGs of this cell cluster, with the $-\log_{10}(\text{Pvalue})$ along the x-axis. **F** Violin plot depicting myelination and angiogenesis related function of MG4 at 14 dpi, measured by AUCell score. n = 309 microglia for control group and n = 705 microglia for PLX5622 group, from two biological replicates. Wilcoxon rank sum test, two-sided.

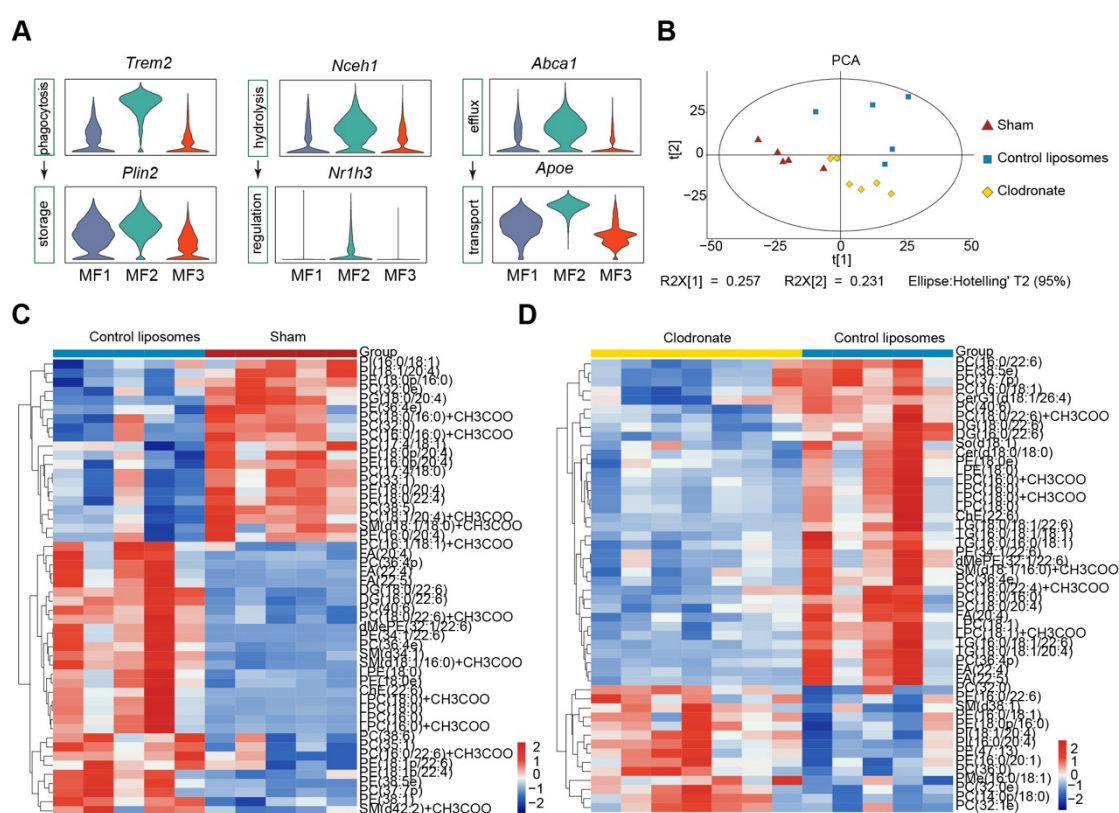


Supplementary Figure6. Assessment of blood monocytes and brain macrophages in PLX5622 group and control diet group. **A** Experimental scheme showing scRNA-seq of the peripheral blood from mice in the PLX5622 group and the control diet group at 14dpi. n = 2 biological replicates for each group. **B** UMAP projection plot showing ten distinct cell types revealed by unsupervised clustering analysis corresponding to A. **C** Blood monocytes projection into the reduced dimensional space of brain macrophages (left) and the proportion of each cluster (right). **D** Monocle pseudotime trajectory analysis of macrophages at 14 dpi revealed different activation states, with a predicted shift from MF1 (lower) to MF2 (upper right) or MF3 (upper left) based on acquired cellular trajectory. **E** Scatter plot illustrating upregulated DEGs of MF3, with the percentage difference along the x-axis and $\log_2$ (fold change) along the y-axis. The combined z-score of percentage difference and $\log_2$ (fold change) was color-coded. **F** UMAP plot depicting the functions of macrophages from 5 dpi based on the AUCell scores of functional gene sets listed in **Supplementary Data 1**. **G** Monocle pseudotime trajectory analysis of macrophages at d5 revealed different activation states, with a predicted shift from MF1 (lower) to MF2 (upper right) or MF3 (upper left) based on acquired cellular trajectory.

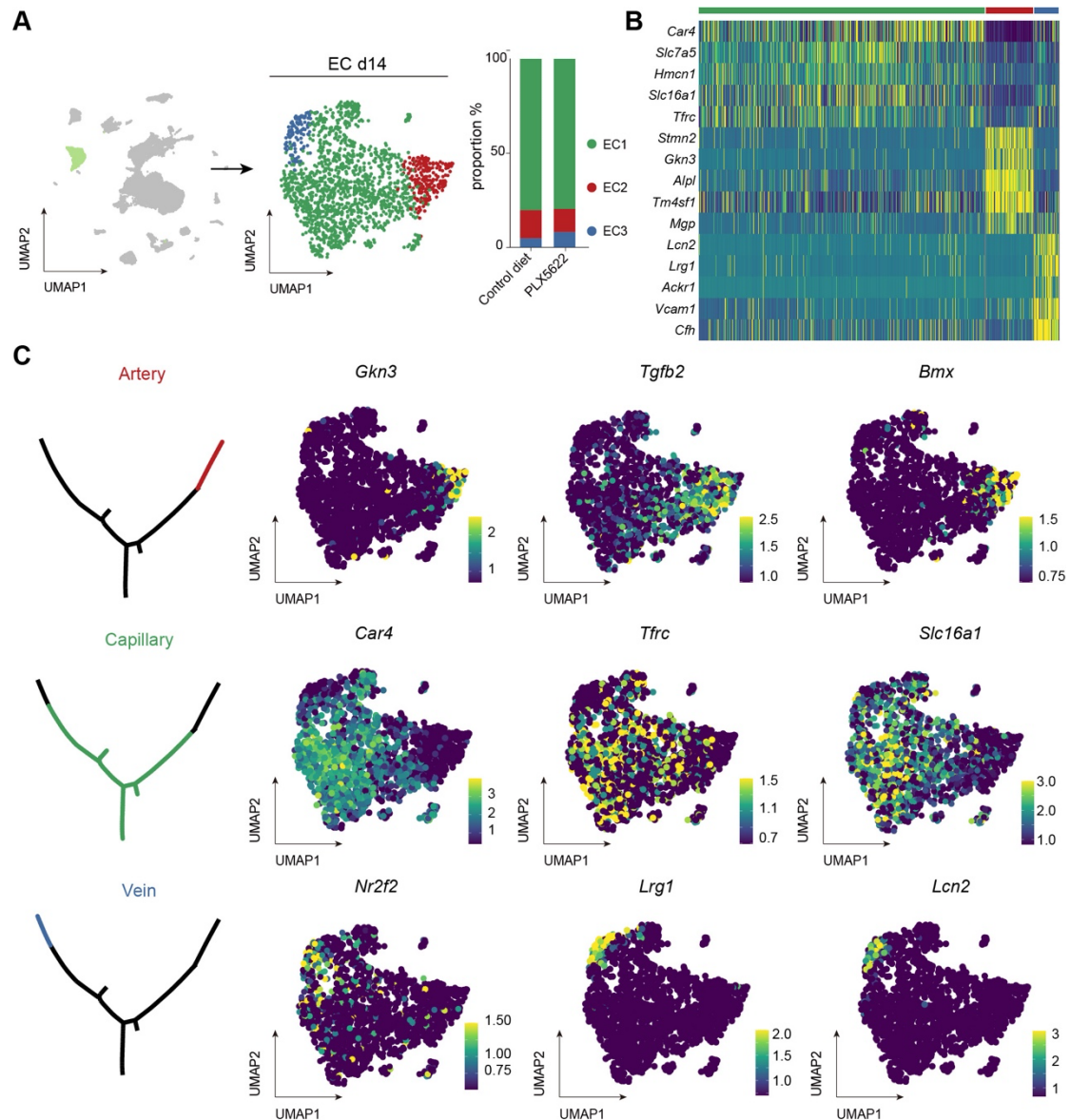


Supplementary Figure 7. Clodronate liposomes efficiently deplete monocyte in blood and RAMf transplantation promotes brain repair. **A** Experimental scheme illustrating the administration of clodronate liposomes in C57BL/6J mice (left) and assessment of infarct volume at 5 dpi. n = 5 per group. Scale bars, 2.5mm. **B** Flow cytometry was performed to quantify the numbers of monocytes in PBMC. Gating strategy: monocytes are defined as CD45⁺ CD11b⁺ CD115⁺ cells (left). Quantitative comparison of the numbers of CD45⁺ CD11b⁺ CD115⁺ cells in the PBMC (right). n = 5 per group. **C** Brain microglia proportion remains unchanged after clodronate treatment. n = 6 per group. **D** Representative flow plots showing GPNMB⁺CD63⁺ macrophages were scarce in peripheral blood at 5 dpi. **E** Experimental scheme illustrating the PLX5622 diet of CCR2^{GFP} mice after MCAO (left). Gating strategy of CD45^{high} CD11b⁺ CD63⁺ GPNMB⁺ cells in ischemic brain and quantification of the CCR2-positive ratio (right). **F** Experimental scheme illustrating the cell transplantation of CCR2^{GFP} CD63⁺ GPNMB⁺ cells (left). Gating strategy of CD45^{high} CD11b⁺ CD63⁺ GPNMB⁺ cells in ischemic brain at 14 dpi and quantification of the CCR2-positive ratio (right). **G** Representative images

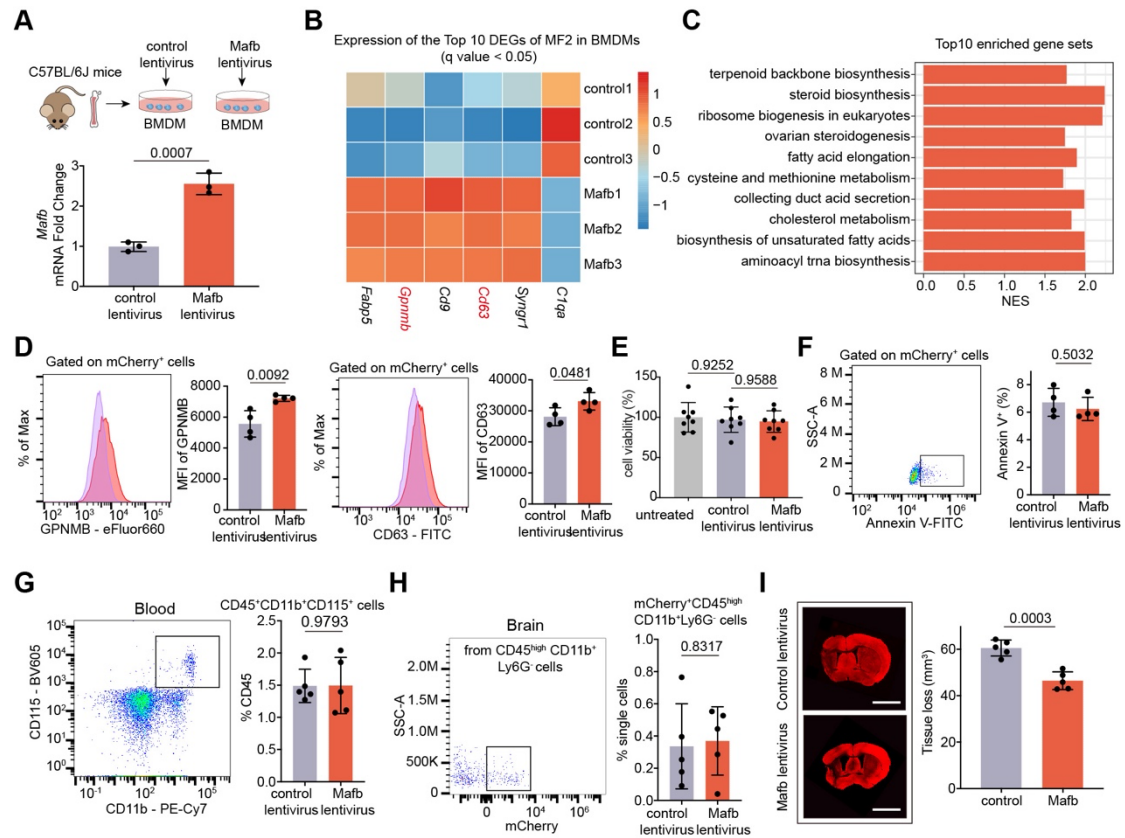
for IBA1 (red), CD63 (green) and GPNMB (cyan) immunostaining in the mouse brains after MCAO. Quantification of IBA1⁺GPNMB⁺CD63⁺ cells (right). n = 5 for GPNMB⁺CD63⁺ macrophages transplantation group, n= 6 for RAMf transplantation group. **H** Assessment of tissue loss at 14 dpi in MCAO mice transplanted with either GPNMB⁺CD63⁺ macrophages or GPNMB⁺CD63⁺ macrophages. n = 5 for GPNMB⁺CD63⁺ macrophages transplantation group, n= 6 for RAMf transplantation group. Scale bars, 2.5mm. **I** Representative images of BrdU and APC immunostaining (left) and BrdU⁺APC⁺ cell number quantification (right) at 14 dpi in MCAO mice transplanted with either GPNMB⁺CD63⁺ macrophages or GPNMB⁺CD63⁺ macrophages. n = 5 for GPNMB⁺CD63⁺ macrophages transplantation group, n= 6 for RAMf transplantation group. (A, B, C, G, H and I): Student's t test, two-sided. Data in A, B, C, E, F, G, H and I are presented as mean ± SD. Source data are provided as a Source Data file.



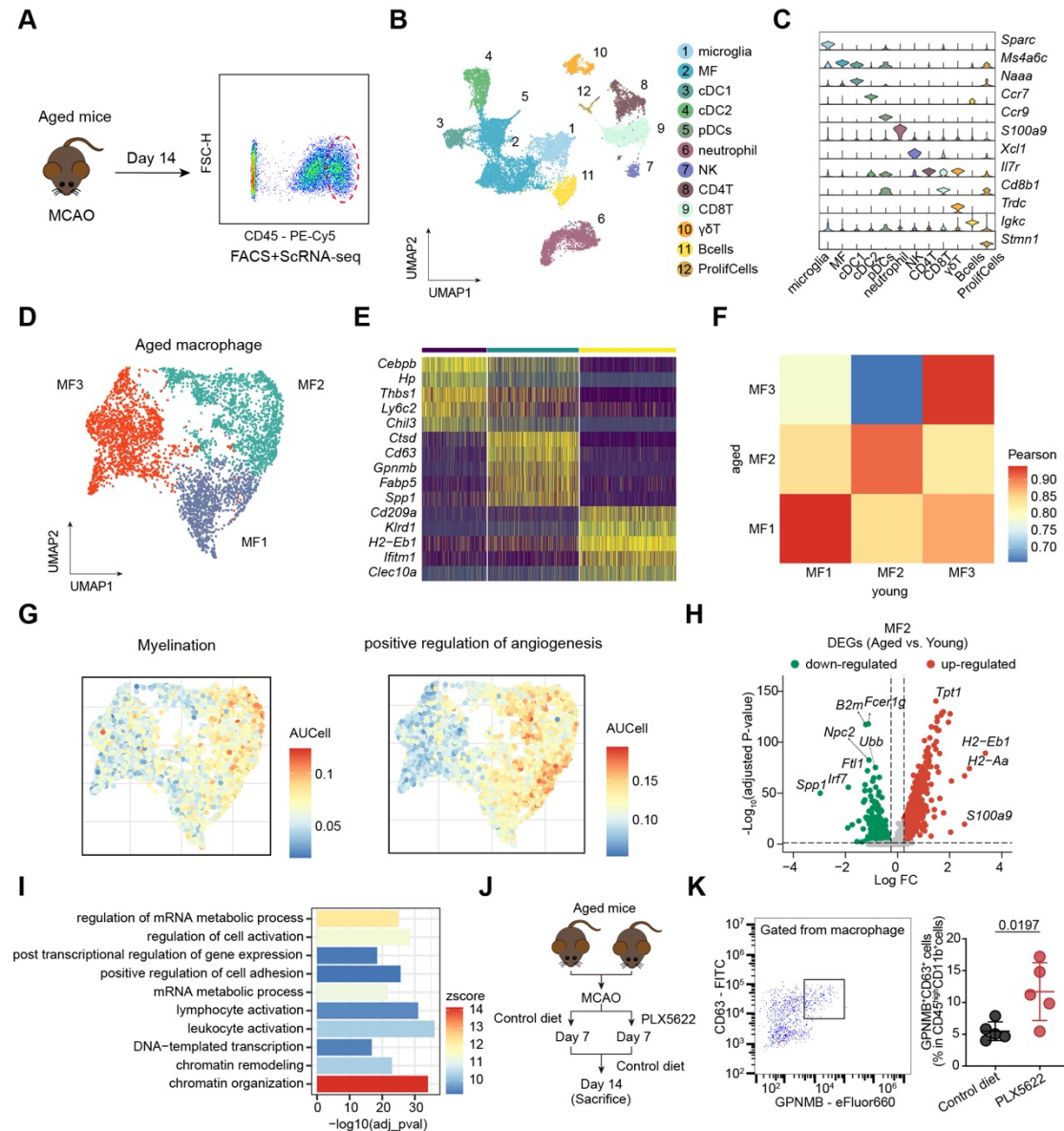
Supplementary Figure8. Lipid profiles of ischemic mouse brain. **A** Violin plots showing expression of genes involved in myelin debris phagocytosis(*Trem2*), lipid storage (*Plin2*), hydrolysis (*Nceh1*), regulation (*Nr1h3*), efflux (*Abca1*) and transport (*Apoe*) in each of three distinct clusters. **B** Principal component analysis (PCA) plot illustrating a tendency towards separation among the three groups. Different color and shape representing distinct sample groups, and the oval area represents the 95% confidence interval. **C** Heatmap showing the changes in lipid metabolites between control and sham group. **D** Heatmap showing the changes in lipid metabolites between clodronate and control group.



Supplementary Figure9. Single-cell transcriptomic analysis of endothelial cells at 14 dpi. **A** Sub-clustering analysis for extracted endothelial cells(left). Stacked bar plot showing proportions of endothelial cells from each group (right). **B** Heatmap showing the expression levels of top five marker genes in each cell type of ECs. The most significantly upregulated genes are shown in yellow and downregulated genes are shown in Tibetan blue. **C** Feature plots showing the expression of known arterial (*Gkn3* and *Stmn2*), capillary (*Car4* and *Trfc*) and venous (*Lrg1* and *Lcn2*) markers in endothelial cells.



Supplementary Figure 10. Mafb-Overexpression promotes BMDMs to acquire RAMf-like phenotype. **A** Experimental scheme of quantitative PCR analysis of *Mafb* expression levels in Mafb lentivirus treatment group and control lentivirus group. *n* = 3 per group. **B** Relative expression of specific genes associated with the RAMf phenotype. **C** Top 10 enriched GSEA terms of BMDMs transfected with Mafb-lentivirus compared to that with control lentivirus. **D** Representative flow plots of MFI levels and quantification of MFI of GPNMB and CD63 measured by flow cytometry. *n* = 4 per group. **E** Cell viability assessed by MTT assays for untreated group, lentivirus treatment group and controls. Data were normalized to the untreated group. *n* = 8 per group. One-way ANOVA and Tukey. **F** Annexin V expression levels measured by flow cytometry in lentivirus treatment group and controls. *n* = 4 per group. **G** Levels of monocytes (CD45⁺ CD11b⁺ CD115⁺) in the peripheral blood after Mafb-overexpression BMDMs transfer or control-BMDMs transfer. *n* = 5 for per group. **H** Levels of macrophages (mCherry⁺ CD45^{high} CD11b⁺ Ly6G⁻) in the brain after Mafb-overexpression BMDMs transfer or control-BMDMs transfer. *n* = 5 for per group. **I** Assessment of tissue loss at 14 dpi in MCAO mice transplanted with either Mafb-overexpressing macrophages or controls. *n* = 5 per group. Scale bars, 2.5mm. (A, D, F, G, H and I): Student's *t* test, two-sided. Data in A, D, E, F, G, H and I are presented as mean $\pm$ SD. Source data are provided as a Source Data file.



Supplementary Figure11. RAMf presents in aged mice and exert tissue reparative functions. A-B ScRNA-seq on enriched CD45^{high} infiltrating immune cells isolated from the brains of aged ischemic mice (20 months old) at 14 dpi. n = 2 biological replicates. C Violin plot illustrating the selected marker genes of each cluster of aged mice. D-E Sub-clustering analysis and heatmap of top 5 genes expression in each cluster for extracted macrophages. F Pearson correlation between three subsets of macrophages from young and aged mice. G UMAP plot depicting the myelination-related and angiogenesis-related functions of macrophages based on the AUCell scores. H Volcano plot showing differentially expressed genes in MF2 between aged and young mice. I Enriched functional terms of the up-regulated genes in MF2 between aged and young mice. J Experimental scheme showing flow cytometry of the ischemic brain from aged MCAO mice (20-22 months old) at 14dpi. K Proportion of RAMf cells in the CD45^{high} CD11b⁺ cells at 14 dpi in aged MCAO mice. n=5 per group. Student's t test, two-sided. Data are presented as mean $\pm$ SD. Abbreviations: MF macrophages,

cDCs classical dendritic cells, NK natural killer cells, $\gamma\delta$ T gamma delta T cells, ProlifCells proliferating cells. Source data are provided as a Source Data file.