

Original Article

Single-Cell RNA Sequencing Characterizes Microglial Phenotypic Heterogeneity Across Different Stroke Subtypes

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ABSTRACT: Microglia-mediated neuroinflammation is a key contributor to secondary brain injury across diverse cerebrovascular pathologies, yet the precise transcriptomic diversity and temporal evolution of microglial states across different stroke subtypes remain to be fully elucidated. We utilized single-cell RNA sequencing to profile the temporal transcriptomic landscape of the immune microenvironment in mouse models of ischemic stroke (IS), hemorrhagic stroke (HS), and chronic cerebral hypoperfusion (CCH) at 3, 7 and 14 days post-injury. Our data showed a “temporal mismatch” in immunological trajectories between acute and chronic insults. Specifically, IS and HS triggered an immediate, burst-like microglial activation as early as 3 days, the CCH model exhibited a significant latency window, maintaining a predominantly homeostatic profile until a profound transcriptomic transition toward active phenotypes occurred at 14 days. Notably, we identified a prominent S100A8⁺/CHIL3⁺ inflammatory cluster enriched during the hyperacute phase of HS, which may represent an early inflammatory signature associated with intraparenchymal hemorrhage. Interestingly, despite these early divergent responses, the 14-day microglial landscapes across the three models showed a putative shared chronic activation pattern characterized by SPP1⁺ DAM-like signatures and MHC-II-associated antigen-presentation features. Furthermore, we identified a pre-existing stress-responsive signature (Hspa1a⁺/Hspa1b⁺) in steady-state white matter microglia. These findings provide a high-resolution roadmap of the divergent and convergent immune signatures across cerebrovascular disease spectra, offering hypothesis-generating insights for the development of stage-specific and etiology-tailored immunotherapeutic interventions. Overall, our pooled-library scRNA-seq analysis provides a descriptive and hypothesis-generating framework for stroke-associated microglial and supported by selected tissue-level validation.

Keywords: Epigenesis, cerebrovascular diseases, single-cell RNA sequencing, microglial heterogeneity

INTRODUCTION

Cerebrovascular disease is the third leading cause of death and estimated to affect more than 143 million people each year in the world [1, 2]. Stroke is by far the most important and life-threatening clinical manifestation of

cerebrovascular disorders [3-5]. Ischemic stroke (IS) and hemorrhagic stroke (HS) are the two main types of strokes, and chronic cerebral hypoperfusion (CCH) represents a critical yet distinct cerebrovascular pathology that drives progressive cognitive decline [6-8]. Despite their high prevalence, there is still no effective treatment

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for cerebrovascular disease, largely due to our incomplete understanding of the divergent pathophysiological mechanisms underlying these distinct stroke subtypes.

The microglia-mediated immune response following stroke, incorporating both innate signaling and coordination with adaptive components, acts as a double-edged sword that governs neuronal survival and long-term functional recovery [9-11]. Microglia were simplified into pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes several years ago; however, emerging evidence suggests that these categories fail to capture the multidimensional transcriptional states observed *in vivo* [12-14]. While microglia are established as central orchestrators of the neuroinflammatory microenvironment, their specific functional states across different stroke etiologies remain highly controversial [15-17]. For instance, the rapid transformation of microglia from a homeostatic state to a reactive profile is not uniform but spatio-temporally regulated by the disruption of blood-brain barrier, oxidative stress, and systemic immune infiltration [18-20]. A primary obstacle to developing precision immunotherapies lies in the profound cellular heterogeneity and the complex, context-dependent interactions within the brain.

Recent advancements in single-cell RNA sequencing (scRNA-seq) have enabled high-resolution investigations into the microglia landscape in response to stroke [21-23]. For instance, Zheng et al. utilized scRNA-seq to map the acute cellular changes 24 hours after ischemic stroke, identifying distinct clusters of activated microglia but without focusing on their long-term diversity [24]. Other studies, such as those by Shi et al., identified specific lipid-associated microglia (LAMs) or disease-associated microglia (DAM) in the context of ischemic injury [25]. Bai et al. have defined a “stroke-associated microglia” phenotype, characterized by the dysregulation of PANoptosis regulators (MCL1, TNFRSF1A, and STAT3), and altered expression of SPP1, MIF, FN1, and TNF [26]. Moreover, our group pointed out an interferon responsive microglia after stroke, which inhibited the differentiation of neural stem cells (NSCs) and aggravated neural injury [27]. However, whether these populations are conserved across other stroke subtypes remains unknown. The transcriptional diversity and functional specialization of microglia across distinct stroke subtypes, especially comparing acute injury with chronic hypoperfusion remain poorly understood, representing a critical knowledge gap in the field.

In the present study, we employed scRNA-seq to systematically characterize the microglial heterogeneity across three distinct cerebrovascular diseases: ischemic stroke, hemorrhagic stroke, and chronic cerebral hypoperfusion. Notably, our analysis showed a distinct heat shock protein (HSP)-associated microglial state

intrinsically linked to the white matter of the corpus callosum, alongside a specific inflammatory state (S100A8⁺/CHIL3⁺) that emerges exclusively at day 3 following hemorrhagic stroke. Our findings provide a time-resolved comparative framework for describing how microglial responses are differentially associated with both the pathological insult and the anatomical niche.

MATERIALS AND METHODS

Animals

Animal experiments were approved by the Institutional Animal Care and Use Committees of Shanghai Jiao Tong University and conducted in accordance with ARRIVE 2.0 guidelines. Male C57BL/6 mice (8 weeks old) were housed in specific pathogen-free facilities under controlled conditions (22 ± 2°C, 55 ± 5% humidity, 12/12 h light/dark cycle) using autoclaved individually ventilated caging systems. CX3CR1^{GFP/WT} mice were gifts from Dr. Donglei Sun at Shanghai Jiao Tong University, Shanghai, China.

Establishment of a mouse model of transient middle cerebral artery occlusion (tMCAO)

We performed tMCAO as previously described [28]. Briefly, mice were anesthetized with 4% isoflurane and maintained at 1.5% isoflurane in a 30% O₂/70% NO mixture by anesthesia machine (RWD Life Science Co., Shenzhen, China). The internal carotid artery and external carotid artery were separated. A 6-0 nylon suture coated with silica gel was carefully inserted through the external carotid artery and advanced into the middle cerebral artery through the internal carotid artery. The efficacy of the occlusion was determined by measuring the decrease in surface cerebral blood flow (CBF) to 20% of baseline CBF using laser Doppler flowmetry (Moor Instruments, Devon, UK). After a period of 1.5 hours of occlusion, reperfusion was initiated by withdrawing the suture. After reperfusion, the blood flow was returned by 80%. Mice were sacrificed at day 3, 7, and 14 for scRNA-seq. Ischemic group consisted of 6 mice, pooling was performed at the tissue level before enzymatic dissociation.

Establishment of a mouse model of intracerebral hemorrhage

After sterilizing the scalp with 75% alcohol, an incision was made to expose the skull. A 29-gauge needle loaded with collagenase IV (Sigma-Aldrich, MO) in PBS was inserted into the right striatum (coordinates: mediolateral, 2.0 mm from bregma; anteroposterior, 0.2 mm;

dorsoventral, 3.0 mm) [29]. Using a micro-infusion pump (WPI, Sarasota, FL, USA), 0.075 U of collagenase IV in 4 μ L PBS was injected over 10 min. The needle was left in place for an additional 5 min to prevent any reflux. Scalp was sutured after needle withdrawal and put on a heating pad (RWD Life Science Co.) for recovery. Mice were sacrificed on days 3, 7, and 14 for scRNA-seq. The hemorrhage group consisted of six mice, and pooling was performed at the tissue level before enzymatic dissociation.

Establishment of a mouse model of bilateral common carotid artery stenosis (BCAS)

To induce BCAS, mice were anesthetized with 1.5% isoflurane. Through a midline incision, the common carotid artery was carefully exposed and isolated from the vagus nerve and surrounding tissues. After gently lifting the carotid artery, the steel spring microcoil (0.18-mm internal diameter) was twined by rotating around the common carotid artery and placed below the carotid bifurcation. Animals were returned to the normal cage to recover with free access to food and water. Mice were sacrificed and isolated the corpus callosum (CC) on day 3, 7, and 14 for scRNA-seq. BCAS group consisted of 8 mice, pooling was performed at the tissue level before enzymatic dissociation.

Single cell preparation

Brain tissues from the regions of interest were collected in a sterile petri dish containing PBS and minced into small pieces using scissors and forceps (Fig. S1A). To ensure comparability across different tissue types, all brain tissues were processed using the same dissociation workflow, including mechanical mincing, enzymatic digestion, debris removal, red blood cell lysis, and single-cell suspension preparation. For each disease model at each designated time point, a single independent library was generated. To ensure a representative sample and minimize individual biological variation, brain tissues from 6-8 mice per condition were pooled and minced together using scissors prior to the tissue dissociation process. The minced tissue was then transferred to a gentleMACS C Tube (Miltenyi Biotec, Bergisch Gladbach, Germany) containing a digestion cocktail of 1900 μ L Buffer X, 50 μ L Enzyme P, 20 μ L Buffer Y, and 10 μ L Enzyme A. Tissue dissociation was performed using the gentleMACS Dissociator (Miltenyi Biotec) with the pre-set program 37C_NTDK_1. Following dissociation, cell suspension was filtered through a 70 μ m MACS SmartStrainer (Miltenyi Biotec) into a 50 mL tube and washed with 10 mL of HBSS. The suspension was centrifuged at $300 \times g$ for 10 min at room temperature

(RT). To remove myelin and cellular debris, the cell pellet was resuspended in 3100 μ L of cold buffer and mixed with 900 μ L of Debris Removal Solution (Miltenyi Biotec). The mixture was carefully overlaid with 4 mL of cold buffer and centrifuged at $3000 \times g$ for 10 min at 4 °C. The bottom phase containing the cells was collected, diluted to 15 mL with cold buffer, and centrifuged at $1000 \times g$ for 10 min at 4 °C. To eliminate potential blood contamination, the pellet was treated with 1 mL of $1 \times$ Red Blood Cell Lysis Solution (Miltenyi Biotec) for 10 min at RT. After a final centrifugation at $300 \times g$ for 5 min, the supernatant was completely aspirated. The single-cell suspension was then processed for library construction using the Chromium Single Cell 3' library with Chromium Single Cell 3' v3 reagent (10x Genomics) according to the manufacturer's protocol. Approximately 8,000 cells per sample were loaded into the Chromium Controller. Sequencing was performed on the Illumina NovaSeq 6000 platform [30]. For each disease model at each time point, one independent scRNA-seq library was generated. For the ischemic stroke and hemorrhagic stroke models, each library was generated from brain tissues pooled from 6 mice. For the BCAS model, each library was generated from brain tissues pooled from 8 mice.

scRNA-seq data analysis

The raw sequencing data were processed using the Cell Ranger pipeline (v5.0.0, 10x Genomics) to perform demultiplexing, alignment, and cellular barcode/UMI counting. Downstream analysis was conducted using the Seurat R package. To ensure high-quality data, we filtered out low-quality cells and potential multiplets based on the following criteria: (1) fewer than 200 detected genes; (2) fewer than 1,000 UMIs; (3) $\log_{10}$ (Genes Per UMI) score < 0.7 ; (4) $> 10\%$ of total counts mapping to mitochondrial genes; and (5) $> 5\%$ of counts mapping to hemoglobin genes. Additionally, the DoubletFinder package was employed to identify and remove potential doublets. Following these QC steps, the remaining high-quality cells (specifically focusing on the microglia population) were retained for further analysis, no additional ambient RNA correction was performed beyond the aforementioned quality-control filtering steps.

Library size normalization was performed using the `NormalizeData` function with the `LogNormalize` method, where gene expression counts for each cell were normalized by total expression, multiplied by a scaling factor of 10,000, and log-transformed. Highly variable genes (HVGs) were identified using the `FindVariableFeatures` function. Principal component analysis (PCA) was then performed for linear dimensionality reduction using `RunPCA`. Graph-based

clustering was executed using the FindNeighbors and FindClusters functions. For visualization, the clusters were projected onto a two-dimensional space using UMAP. Cell-type identity was assigned by comparing cluster-specific marker genes, identified via the FindAllMarkers function, with known canonical markers in the literature. Differentially expressed genes (DEGs) were identified using the FindMarkers function (interfaced with the presto package for computational efficiency). Positive markers for each cluster were defined by $|\log_2(\text{fold change})| \geq 0.1$, the expression ratio of the cell population ≥ 0.25 , and the p-value ≤ 0.05 . For more stringent downstream DEG analysis, a threshold of P value < 0.05 and $|\log_2(\text{fold change})| > 0.58$ was applied. Finally, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed based on the hypergeometric distribution to elucidate the functional profiles of the identified DEGs.

For each condition/time point, one pooled scRNA-seq library was generated. Although pooling allowed us to profile cellular heterogeneity across conditions, the design did not include independent biological replicate scRNA-seq libraries for each condition/time point. Therefore, downstream differential expression analysis, cell-cluster proportion comparisons, pathway enrichment, and pseudotime analyses were considered exploratory and hypothesis-generating. The lack of independent replicate libraries precluded replicate-aware pseudobulk analysis.

Pseudo-time analysis

We applied the Single-Cell Trajectories analysis utilizing Monocle3 (<http://coletrapnell-lab.github.io/monocle-release>) with DDR-Tree and default parameters. Before Monocle analysis, we selected marker genes from the Seurat clustering results and raw expression counts of the cell-passed filtering. Based on the pseudo-time analysis, branch expression analysis modeling (BEAM Analysis) was applied for branch fate determined gene analysis.

Immunostaining

Brain slices were rinsed sequentially in PBS, permeabilized with 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO), and blocked with 2% BSA (Meilunbio, Dalian, China). Samples were incubated overnight at 4°C with primary antibodies of Iba1 (1:200, Novusbio, Centennial, CO), HSP70 (1:200, Abcam, Cambridge, UK), S100a8 (1:200, Abcam), and Ym1 (1:200, Abcam). After washing, slices were incubated with Alexa Fluor-conjugated secondary antibodies (1:500, Thermo Fisher) for 1 h and mounted with Antifade Mounting Medium containing DAPI (Beyotime, Shanghai, China). Images

were acquired using a confocal microscope (Leica, Wetzlar, Germany).

Immunostaining images were captured at 20× magnification using a confocal microscope (Leica, Wetzlar, Germany). Quantification was performed using samples from 3–6 mice per group, with 3–4 brain sections analyzed per mouse and 3 regions of interest examined per section (Fig. S1B). Adult male C57BL/6J mice weighing 22–25g were randomly assigned to different experimental groups. Mice that died as a result of surgical procedures were excluded from the analysis. Image quantification and statistical analyses were performed in a double-blinded manner. Immunostaining validation was performed using an independent cohort of animals, distinct from those used for single-cell RNA sequencing. Regarding lesion-area criteria, the lesion-associated areas were defined according to the brain regions affected in each model: the striatum was analyzed in both the IS and HS models, whereas the corpus callosum was analyzed in the BCAS model. The regions of interest were selected consistently with these lesion-area criteria across comparable anatomical levels and experimental groups.

Statistical Analysis

For all non-scRNA-seq experiments, data are presented as the mean $\pm$ standard deviation (SD) for normally distributed variables and as the median with interquartile range (IQR) for non-normally distributed variables. Normality was assessed using the Shapiro–Wilk test. For comparisons between two groups, unpaired two-tailed Student's t-tests or Mann–Whitney U tests were used, as appropriate. For comparisons among multiple groups, one-way or two-way ANOVA followed by Tukey's post hoc test, or Kruskal–Wallis test followed by Dunn's post hoc test, was applied as appropriate. Statistical analyses were performed using GraphPad Prism version 9.0, and $p < 0.05$ was considered statistically significant. The Immunostaining validation statistical unit was the individual mouse.

For single-cell RNA sequencing analysis, each experimental condition/time point was represented by one pooled scRNA-seq library. Therefore, independent biological replicate libraries were not available for each condition/time point. Differential expression analysis was performed at the individual-cell level using the statistical test implemented in the Seurat single-cell RNA sequencing analysis pipeline. In these analyses, individual cells were treated as the statistical unit. Because cells from the same pooled library do not represent independent biological replicates, these differential expression results should be interpreted as exploratory and hypothesis-generating, with a potential risk of pseudoreplication. Similarly, cluster proportion comparisons, pathway

enrichment analyses based on differentially expressed genes, and pseudotime analyses were performed to describe cell-state changes and generate mechanistic hypotheses, rather than to provide replicate-aware inferential statistical conclusions. Replicate-aware

pseudobulk differential expression analysis was not performed because the current experimental design included only one pooled scRNA-seq library per condition/time point. This limitation was considered when interpreting the single-cell RNA sequencing results.

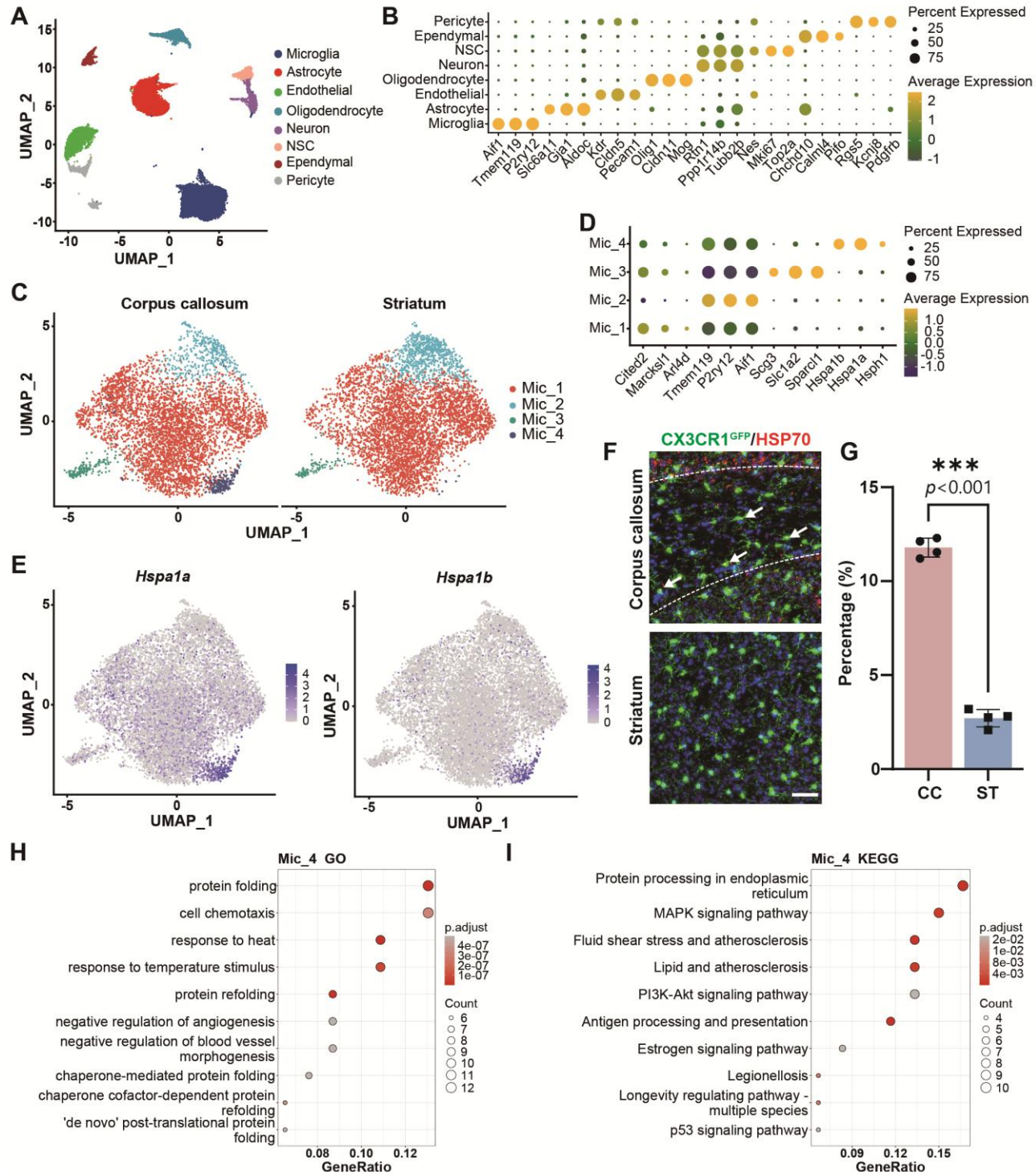


Figure 1. Single-cell transcriptomic atlas and regional microglial heterogeneity in the healthy mouse brain. (A) UMAP visualization of major cell types identified in the mouse brain, including microglia, astrocytes, endothelial cells, oligodendrocytes, neurons, NSCs, ependymal cells, and pericytes. (B) Dot plot showed the expression of canonical marker genes across the identified cell

populations. The dot size represents the percentage of cells expressing the targeted gene, and color scale represents the average expression level. (C) Split UMAP visualization of microglial sub-clusters (Mic_1 to Mic_4) comparing CC and the striatum. (D) Dot plot illustrating the top differentially expressed genes (DEGs) across the 4 microglial sub-clusters. (E) Feature plots showed the localized expression of stress-response genes *Hspa1a* and *Hspa1b* within the microglial clusters. (F-G) Representative immunostaining images showed the co-localization of CX3CR1^{GFP} (green) and HSP70 (red) in CC and striatum. Arrows indicate HSP70⁺ microglia in the CC. Scale bar=50 μ m. (H) GO enrichment analysis of Mic_4-specific DEGs. (I) KEGG enrichment analysis of Mic_4-specific DEGs. Data are presented as mean $\pm$ SEM. *** p <0.001. Statistical significance was determined by t-test in G. scRNA-seq data in this figure are based on one pooled library per condition/time point and should be interpreted descriptively. DEG/pathway analyses are exploratory.

RESULTS

Cellular Heterogeneity and Regional Microglial Diversity in Healthy Mouse Brain

To establish a comprehensive baseline of the cellular composition across different brain regions, we performed scRNA-seq on CC and the striatum from healthy adult mice. To obtain sufficient cell numbers, sequencing libraries were generated from pooled tissue samples for each region (n=9), representing a single biological replicate per condition. After rigorous quality control and batch-effect correction using Seurat, a total of 23,366 high-quality cells were recovered, with a median of 1,837 genes and 3,959 unique molecular identifiers (UMIs) per cell. Unsupervised clustering and Uniform Manifold Approximation and Projection (UMAP) visualization identified 8 major cell lineages based on their canonical markers: microglia (*Aif1*, *Tmem119*, *P2ry12*), astrocytes (*Slc6a11*, *Gja1*, *Aldoc*), endothelial cells (*Kdr*, *Cldn5*, *Pecam1*), oligodendrocytes (*Olig1*, *Cldn11*, *Mog*), neurons (*Rtn1*, *Ppp1r14b*, *Tubb2b*), NSCs (*Nes*, *Mki67*, *Top2a*), ependymal cells (*Chchd10*, *Calml4*, *Pifo*), and pericytes (*Rgs5*, *Kcnj8*, *Pdgfrb*) in both CC and striatum (Fig. 1A-B). To further explore the potential regional heterogeneity of microglia, we performed high-resolution sub-clustering analysis and clustered microglia into four putative transcriptional states, designated Mic_1 to Mic_4 (Fig. 1C). While the majority of microglia in both regions exhibited a homeostatic signature defined by high expression of *Tmem119* and *P2ry12* (primarily within Mic_1 and Mic_2), our descriptive comparison of cluster proportions suggested an enrichment of a stress-responsive sub-cluster (Mic_4) specifically within CC compared to the striatum (Fig. 1D). Differential expression analysis (DEA) showed upregulation of heat shock proteins (HSPs) including *Hspa1a*, *Hspa1b*, and *Hsp1b* in Mic_4 (Fig. 1D-E). This regional divergence was further supported via immunostaining in CX3CR1^{GFP} mice, which showed more abundant HSP70⁺ microglia in CC than in the striatum (Fig. 1F). Consistently, quantitative analysis showed a significantly increased proportion of HSP70⁺ microglia in CC compared with the striatum, supporting the preferential enrichment of a stress-responsive microglial phenotype in white matter

under physiological conditions (Fig. 1G). To further elucidate the functional landscape of the observed DEGs, GO and KEGG enrichment analyses were conducted. As illustrated in Fig. 1H, the GO terms were associated with proteostasis and environmental stress responses, including protein folding, cell chemotaxis, and responses to thermal stimuli. Complementary KEGG analysis demonstrated that these genes are integral to pathways such as protein processing in the endoplasmic reticulum, MAPK signaling, and PI3K-Akt signaling, suggesting a robust involvement in cellular stress management and signal transduction within the Mic_4 cluster (Fig. 1I). Together, these findings provide a descriptive framework of regional microglial heterogeneity in the healthy brain, indicating that white matter microglia harbor a more pronounced stress-responsive molecular signature at baseline than their gray matter counterparts, a factor that may influence their subsequent responses to acute or chronic insults.

Temporal Dynamics and Functional Specification of Microglia State Following IS and HS

To elucidate the longitudinal evolution of the immune microenvironment, we performed scRNA-seq on the ischemic striatum at 3, 7 and 14 days post-ischemia. To ensure sufficient cell yields, tissues from n=6 mice per condition were pooled prior to cell dissociation to generate sequencing libraries for each time point, representing a single biological replicate per condition. While this design provides a high-resolution descriptive map of single-cell landscapes at a lower cost, we emphasize that these findings are exploratory, robust inference would require further validation across additional biological replicates. Clustering analysis identified 12 distinct cell populations, including resident microglia, various neurovascular components (endothelial cells, oligodendrocytes, astrocytes, pericytes), and a robust infiltration of peripheral immune cells, such as macrophages, monocytes, neutrophils, and T cells (Fig. 2A-B). Focused sub-clustering of the microglial compartment showed 5 transcriptionally divergent states: Homeostatic (Stable), Interferon-responsive (IFN), Proliferative (PRO), DAM and Intermediate (DAM-low) (Fig. 2C). The acute phase of IS

(3 days post injury, dpi) was characterized by a dramatic contraction of the homeostatic pool, accompanied by a massive expansion of the PRO cluster, which was defined

by the high expression of cell-cycle drivers such as TOP2A and MKI67 (Fig. 2D-E).

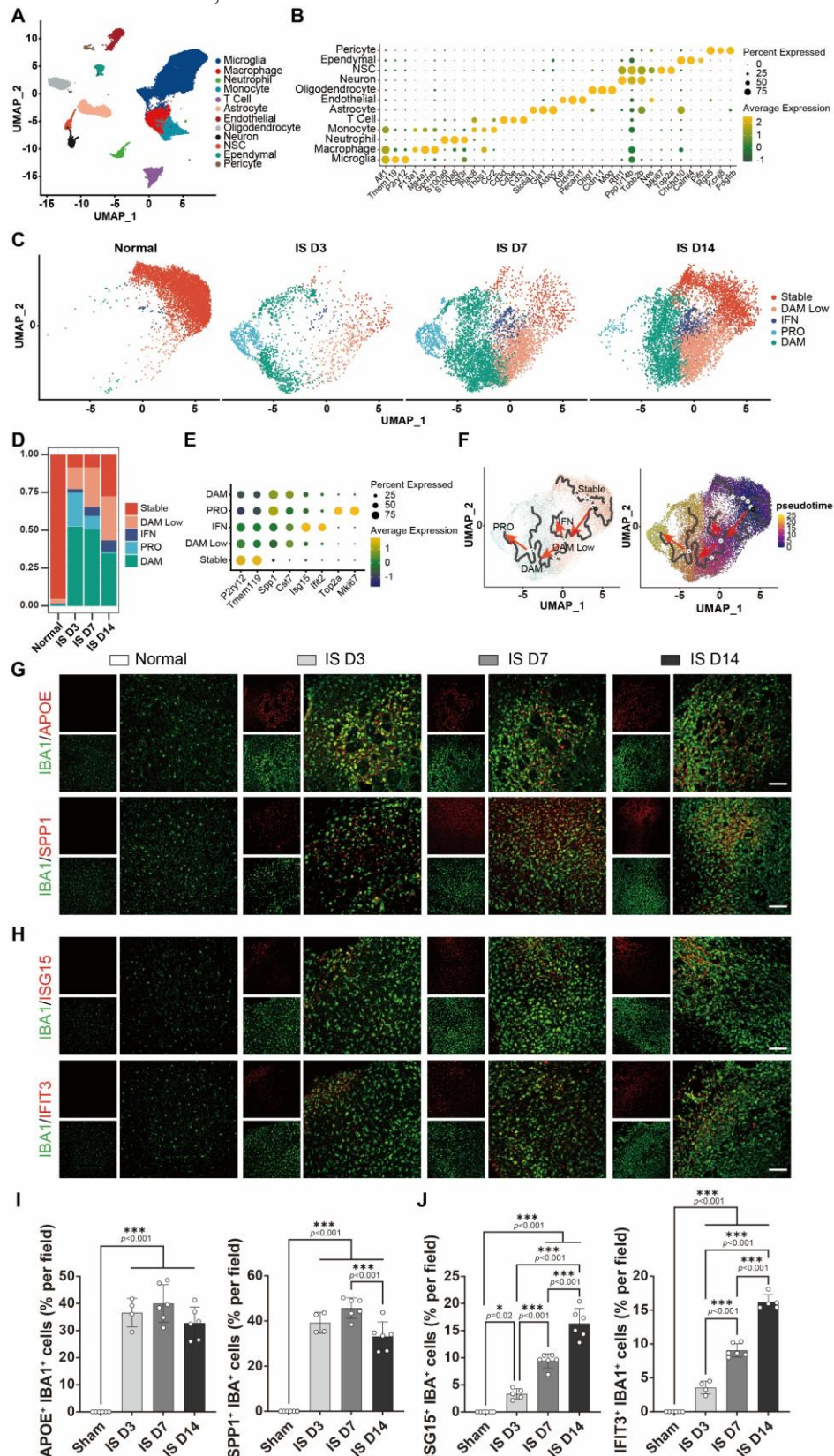


Figure 2. Spatiotemporal transcriptomic profiling and putative transcriptional trajectory of microglia in IS brain. (A) UMAP visualization showed the global cellular landscape of the brain post-ischemia, integrating samples from Normal and IS groups across multiple time points (Day 3, Day 7, and Day 14). (B) Dot plot illustrated the expression of canonical marker genes for the identification of major cell lineages defined in (A). (C) Split UMAP visualization showed the redistribution of microglial sub-clusters at specific stages (Normal, IS D3, IS D7, and IS D14). Five major states were identified: Stable (Homeostatic), DAM (Disease-associated microglia), DAM Low (Intermediate DAM state), IFN (Interferon-responsive), and PRO (Proliferative). (D) Stacked bar plot showed the dynamic compositional changes of microglial states. (E) Dot plot displayed the expression of signature genes across microglial sub-clusters: P2RY12/TMEM119 (Stable), SPP1/CST7 (DAM), ISG15/IFIT2 (IFN), and TOP2A/MKI67 (PRO). (F) Lineage trajectory of microglia reconstructed by Monocle pseudotime analysis. The trajectory (left) originates from the homeostatic state and inferred cell-state relationship. The pseudotime map (right) illustrates the temporal progression from the homeostatic state (dark purple) to the advanced reactive states (yellow). (G) Representative immunofluorescence images showed the co-localization of IBA1⁺ microglia with APOE or SPP1 in normal and IS brains at Day 3, Day 7, and Day 14 after ischemic stroke. Scale bar = 50 μ m. (H) Representative immunofluorescence images showed the co-localization of IBA1⁺ microglia with ISG15 or IFIT3 in normal and IS brains at Day 3, Day 7, and Day 14 after ischemic stroke. Scale bar = 50 μ m. (I) Quantification of the percentages of APOE⁺ or SPP1⁺ cells among IBA1⁺ microglia across Normal and IS groups at different post-ischemic time points. (J) Quantification of the percentages of ISG15⁺ or IFIT3⁺ cells among IBA1⁺ microglia across Normal and IS groups at different post-ischemic time points. Data in (I, J) are presented as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparisons test. * p <0.05, *** p <0.001. scRNA-seq data in this figure are based on one pooled library per condition/time point and should be interpreted descriptively. Stacked bar plots indicate descriptive cell-cluster proportions, DEG/pathway analyses are exploratory, and Monocle pseudotime analysis represents inferred putative transcriptional relationships rather than definitive lineage trajectories.

Simultaneously, we observed the emergence of the DAM-low state, likely representing a transitional gateway toward advanced activation. By 7 dpi, microglial heterogeneity reached its zenith, marked by a significant influx of the IFN-responsive and DAM-low clusters (Fig. 2D-E). During the chronic phase (14 dpi), although homeostatic markers exhibited a partial resurgence, a substantial proportion of microglia remained sequestered in a terminal DAM state, characterized by the persistent expression of SPP1 and CST7 (Fig. 2D-E). Given the absence of independent sequencing replicates, these proportional shifts are presented as descriptive observations. To infer potential transcriptional relationships among these states, pseudotime trajectory analysis via Monocle3 was employed. Through pseudotime ordering, we constructed these trajectories as hypothesis-generating models of potential cell-state transitions. The model may reflect a putative evolutionary continuum where homeostatic microglia initially branch through either an intermediate “DAM-low” or an “IFN” state before converging toward the highly reactive PRO and DAM-like phenotypes (Fig. 2F). To validate these scRNA-seq-defined microglial states at the tissue level, we performed immunofluorescence co-staining of IBA1 with representative DAM-associated markers, APOE and SPP1, and IFN-responsive markers, ISG15 and IFIT3, across the IS time course (Fig. 2G-J). In the normal striatum, IBA1⁺ microglia showed minimal expression of APOE, SPP1, ISG15, and IFIT3. Following ischemic injury, APOE and SPP1 signals were markedly induced within IBA1⁺ cells, with prominent co-localization observed from 3 dpi and sustained expression at 7 and 14 dpi (Fig. 2G, I). We next examined IFN-responsive

microglial activation by staining for ISG15 and IFIT3. Both IFN-related markers were minimally detected in normal striatum but were progressively induced in IBA1⁺ microglia following ischemia (Fig. 2H). In contrast to the DAM-associated markers, the proportions of ISG15⁺IBA1⁺ and IFIT3⁺IBA1⁺ cells increased in a time-dependent manner from 3 dpi to 14 dpi, reaching the highest levels during the chronic phase (Fig. 2J). These immunofluorescence data corroborate the scRNA-seq findings and demonstrate that ischemic injury is accompanied by sustained microglial diversification, characterized by persistent DAM-associated activation and a progressive IFN-responsive program within the injured striatum. GO and KEGG were then conducted to characterize the distinct transcriptional profiles of the DAM and IFN clusters. The DAM cluster exhibited a strong metabolic signature, with GO terms significantly enriched in oxidative phosphorylation, cellular respiration, and energy derivation via oxidation of organic compounds (Fig. S2A). KEGG analysis further underscored its association with neurodegenerative conditions, including Alzheimer's, Parkinson's, and Huntington's diseases (Fig. S2B). Conversely, the IFN cluster displayed a robust antiviral and immunological profile. GO enrichment showed a high concentration of genes involved in the regulation of innate immune responses and defense responses to viruses and symbionts (Fig. S2C). These findings were corroborated by KEGG results, where top-enriched pathways included various viral infections (e.g., Herpes simplex virus 1 and Influenza A) and key inflammatory mediators like the NOD-like receptor signaling pathway (Fig. S2D). Taken together, these descriptive findings provide a time-

resolved framework for understanding the microglial response after IS, may reflect a transition from early-stage

proliferation and interferon-mediated signaling to late-stage functional specialization.

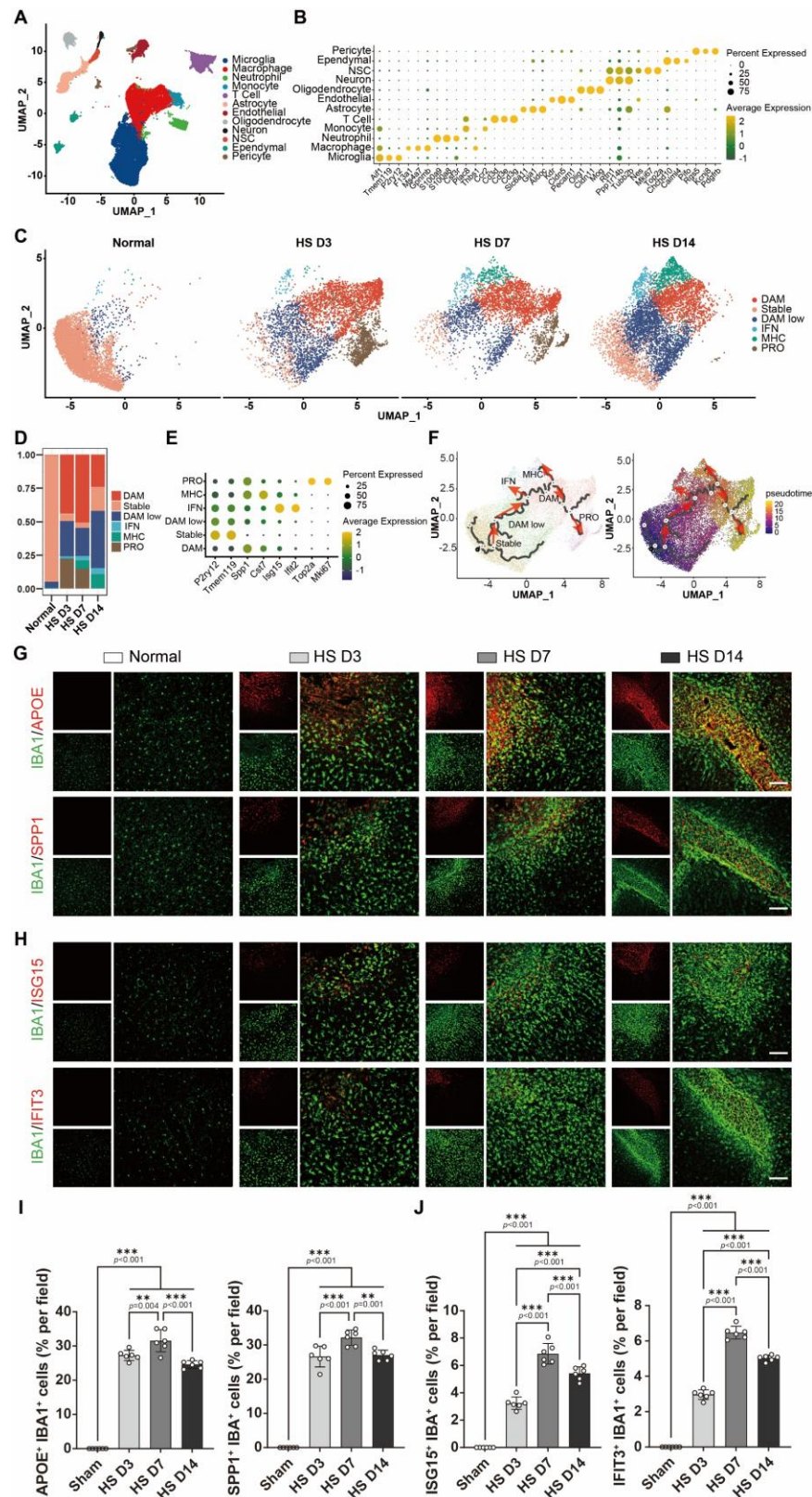


Figure 3. Spatiotemporal transcriptomic profiling and putative transcriptional trajectory of microglia in HS brain. (A) UMAP plot displayed the global cellular landscape of the brain following HS, integrating data from Normal and HS groups at Day 3, Day 7, and Day 14. (B) Dot plot showed the expression of signature marker genes used to identify each major cell population in (A). (C) Split UMAP visualizations of microglial sub-clusters across different times. Six distinct states were identified: Stable, DAM, DAM low, IFN, MHC, and PRO. (D) Stacked bar chart illustrated the dramatic shift in microglial composition following HS. (E) Dot plot showed specific gene expression profiles for each state. (F) Pseudotime trajectory analysis of microglia in the HS model using Monocle. (G) Representative immunofluorescence images showing the co-localization of IBA1⁺ microglia with APOE or SPP1 in Normal and HS brains at Day 3, Day 7, and Day 14 after HS. Scale bars = 50 μ m. (H) Representative immunofluorescence images showing the co-localization of IBA1⁺ microglia with ISG15 or IFIT3 in Normal and HS brains at Day 3, Day 7, and Day 14 after HS. Scale bars = 50 μ m. (I) Quantification of the percentages of APOE⁺ or SPP1⁺ cells among IBA1⁺ microglia across Normal and HS groups at different post-hemorrhage time points. (J) Quantification of the percentages of ISG15⁺ or IFIT3⁺ cells among IBA1⁺ microglia across Normal and HS groups at different post-hemorrhage time points. Data in (I, J) are presented as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparisons test. ** $p < 0.01$, *** $p < 0.001$. scRNA-seq data in this figure are based on one pooled library per condition/time point and should be interpreted descriptively. Stacked bar plots indicate descriptive cell-cluster proportions, DEG/pathway analyses are exploratory, and Monocle pseudotime analysis represents inferred putative transcriptional relationships rather than definitive lineage trajectories.

In HS mouse model, we identified 12 major cell clusters across Normal and HS brains at 3, 7, and 14 dpi, encompassing both CNS-resident populations and infiltrating myeloid and lymphoid lineages (Fig. 3A-B). To ensure sufficient cell yields, tissues from $n=6$ mice per condition were pooled prior to cell dissociation to generate sequencing libraries for each time point, representing a single biological replicate per condition. High-resolution sub-clustering of the microglial compartment showed six transcriptionally specialized states including Stable, DAM Low, IFN, PRO, DAM, and major histocompatibility complex class II-enriched (MHC) (Fig. 3C). While microglia in the normal group remained predominantly in the stable state, HS triggered a rapid and robust expansion of the PRO, IFN, and DAM subclusters by 3 dpi (Fig. 3C-D). Analysis of gene expression profiles further delineated the functional specialization of these subsets. The PRO cluster, driven by cell-cycle regulators *Top2a* and *Mki67*, peaked early at 3 dpi, representing a hyperacute proliferative burst (Fig. 3D-E). By 7 dpi, the microglial landscape shifted toward a DAM-like signature, characterized by the upregulation of *Spp1* and *Cst7* (Fig. 3D-E). Notably, the MHC cluster became increasingly prevalent by 14 dpi, suggesting a late-stage shift toward antigen presentation (Fig. 3D-E). Furthermore, the IFN subcluster exhibited a unique progressive expansion from 3 to 14 dpi, signaling a protracted interferon-related inflammatory milieu in the late subacute phase of HS (Fig. 3D-E). To explore potential transcriptional relationships among these states, pseudotime trajectory analysis via Monocle3 was employed. We constructed these trajectories as hypothesis-generating models of potential cell-state transitions, originating from the Stable cluster. One trajectory progressed through an intermediate DAM-low state before reaching a terminal DAM phenotype. A secondary developmental branch gave rise to the PRO,

IFN, and MHC subclusters, reflecting a multifaceted programming of proliferation, sustained inflammation, and antigen-presenting capacity (Fig. 3F).

To further validate the temporal dynamics of disease-associated microglial markers at the tissue level, we performed immunofluorescence co-staining for IBA1 with representative markers across the HS time course (Fig. 3G-J). In the normal brain, IBA1⁺ microglia showed minimal expression of APOE, SPP1, ISG15, and IFIT3. Following HS, APOE and SPP1 signals were markedly induced within IBA1⁺ microglia as early as 3 dpi, became more prominent at 7 dpi, and remained detectable at 14 dpi, although the signal appeared partially reduced relative to the 7 dpi peak (Fig. 3G, I). These findings are consistent with the expansion of DAM-like microglial states observed in the scRNA-seq dataset. We next examined IFN-responsive microglial activation by assessing ISG15 and IFIT3 expressions. Both IFN-related markers were barely detectable in Normal brains but were robustly induced in IBA1⁺ microglia after HS (Fig. 3H, J). Compared with the DAM-associated markers, ISG15 and IFIT3 displayed a sustained late-phase activation pattern, with increased co-localization in IBA1⁺ cells from 3 dpi to 7 dpi and persistent expression at 14 dpi. Quantification of marker-positive IBA1⁺ cells confirmed the dynamic induction of DAM- and IFN-associated microglial programs after HS (Fig. 3J). Together, these immunofluorescence data support the scRNA-seq results and demonstrate that HS induces sustained microglial diversification, characterized by DAM-like activation, prolonged IFN-responsive signaling, and late-stage immune remodeling in the injured brain.

GO and KEGG databases revealed divergent transcriptional programs for the DAM and IFN populations. DAM cluster demonstrated a robust signature for aerobic metabolism and mitochondrial function, with top GO terms significantly enriched in

oxidative phosphorylation, cellular respiration, and the generation of precursor metabolites and energy (Fig. S3A). KEGG analysis further underscored this metabolic shift, identifying strong associations with oxidative phosphorylation and multiple neurodegenerative conditions, including Alzheimer's, Parkinson's, and Huntington's diseases (Fig. S3B). IFN cluster was defined by a strong immunological and antiviral profile. Key GO terms were heavily concentrated in the regulation of

innate immune responses, defense responses to viruses, and cellular responses to IFN- β (Fig. S3C). KEGG pathway mapping corroborated this immune-centric identity, with high enrichment scores for various viral infectious diseases and critical inflammatory signaling cascades, such as the NOD-like receptor and NF-kappa B signaling pathways (Fig. S3D).

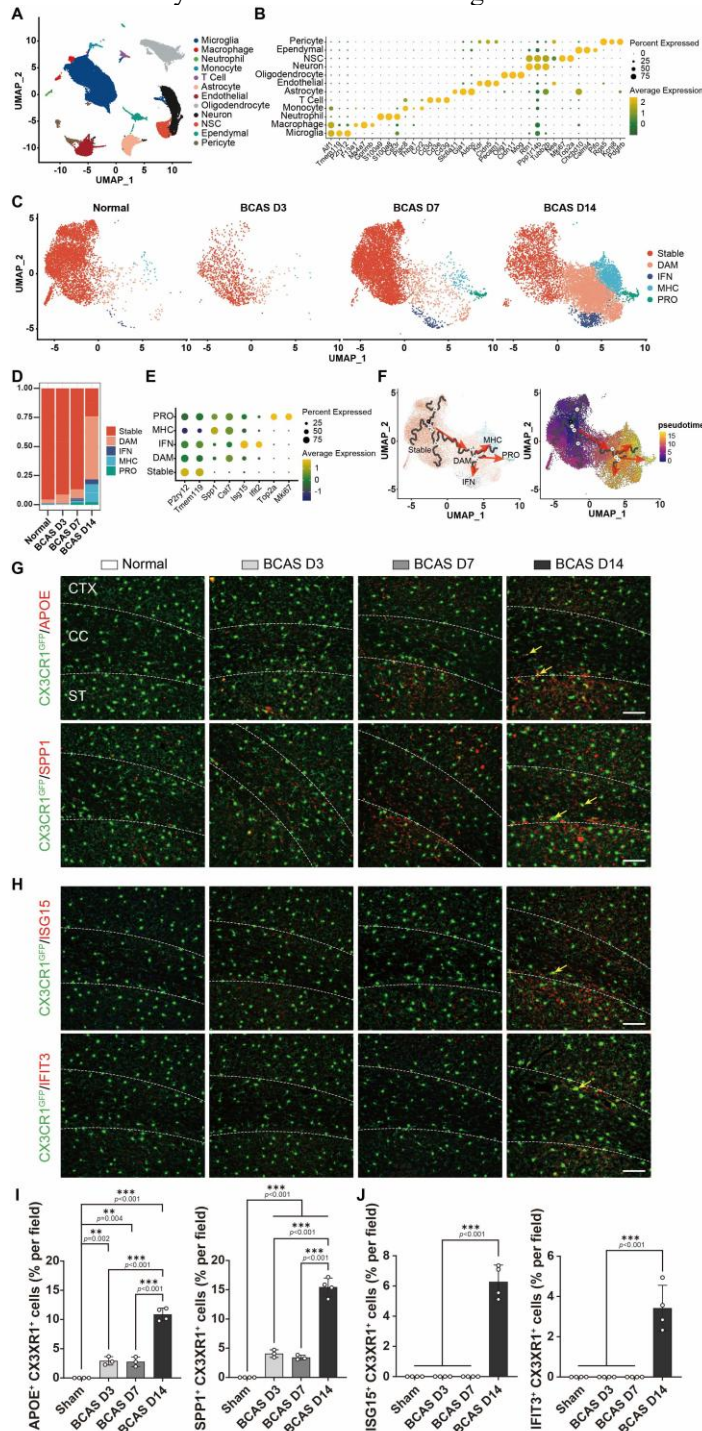


Figure 4. Temporal transcriptomic profiling and putative transcriptional trajectory of microglia in BCAS model. (A) UMAP visualization of total brain cells integrated from Normal and BCAS groups at Day 3, Day 7, and Day 14. (B) Dot plot showed the expression of signature marker genes used to identify each major cell population in (A). (C) Split UMAP plots showed the sub-clustering and distribution of microglial states. (D) Stacked bar plot illustrated the temporal shifts in the proportions of microglial sub-populations. (E) Dot plot showed signature gene expression for each microglial state. (F) Monocle pseudotime trajectory analysis of microglia. (G) Representative immunofluorescence images showing the co-localization of CX3CR1^{GFP} microglia with APOE or SPP1 in Normal and BCAS brains at Day 3, Day 7, and Day 14 after BCAS. Scale bar = 50 μ m. Arrows indicate APOE⁺ or SPP1⁺ microglia. (H) Representative immunofluorescence images showing the co-localization of CX3CR1^{GFP} microglia with ISG15 or IFIT3 in Normal and BCAS brains at Day 3, Day 7, and Day 14 after BCAS. Scale bars = 50 μ m. Arrows indicate ISG15⁺ or IFIT3⁺ microglia. (I) Quantification of the percentages of APOE⁺ or SPP1⁺ cells among CX3CR1^{GFP} microglia across Normal and BCAS groups at different post-CCH time points. (J) Quantification of the percentages of ISG15⁺ or IFIT3⁺ cells among CX3CR1^{GFP} microglia across Normal and BCAS groups at different post-CCH time points. Data in (I, J) are presented as mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparisons test. ** p < 0.01, *** p < 0.001. scRNA-seq data in this figure are based on one pooled library per condition/time point and should be interpreted descriptively. Stacked bar plots indicate descriptive cell-cluster proportions, DEG/pathway analyses are exploratory, and Monocle pseudotime analysis represents inferred putative transcriptional relationships rather than definitive lineage trajectories.

These data provide a descriptive result that HS induces a complex biphasic immune response: transitioning from rapid proliferative programming in the hyperacute phase to a sustained phase of DAM-driven remodeling and MHC-mediated immune surveillance, highlighting stage-specific signatures that may serve as distinct targets for HS-specific immunomodulation.

Delayed Immunological Transition and Progressive Microglial Activation in CCH

To compare the immune dynamics of acute stroke with those of chronic cerebrovascular disease, we employed BCAS model to induce CCH [31]. To ensure sufficient cell yields, tissues from $n=8$ mice per condition were pooled prior to cell dissociation to generate sequencing libraries for each time point, representing a single biological replicate per condition. We identified 12 distinct cell clusters within CC at 3, 7 and 14 days post-surgery (Fig. 4A-B). In contrast to IS and HS, microglia under chronic hypoperfusion exhibited a delayed and progressive phenotypic shift. Sub-clustering of the microglial population showed five transcriptionally divergent states: Stable, DAM, IFN, PRO, and MHC (Fig. 4C). A hallmark of the CCH model was the prolonged maintenance of the homeostatic pool; stable microglia remained the predominant population at 3 and 7 days (Fig. 4C-E). It was not until 14 days post-BCAS that the stable population contracted to its minimum proportion. Correspondingly, the DAM cluster remained relatively quiescent during the first week, reaching its peak only at 14 days (Fig. 4C-E). Furthermore, the proportions of IFN, PRO, and MHC clusters exhibited a steady, time-dependent escalation, all culminating in their maximum levels by day 14 (Fig. 4C-E). To explore potential transcriptional relationships among these states, pseudotime trajectory analysis via Monocle3 was employed. Through pseudotime ordering, we constructed these trajectories as hypothesis-generating models of potential cell-state transitions during BCAS (Fig. 4F). The model may reflect a putative evolutionary continuum characterized by the primary conversion of homeostatic microglia into the DAM state as an initial response. This was followed by a secondary divergence, where DAM cells appear to branch into PRO, MHC, or IFN states, thereby potentially fueling downstream proliferation, antigen presentation, and interferon-mediated inflammatory signaling (Fig. 4F).

To validate the microglial state transitions inferred from the single-cell transcriptomic analysis, we next performed immunofluorescence staining in CX3CR1^{GFP} mice across the BCAS time course. Consistent with the scRNA-seq data, DAM-associated markers APOE and SPP1 were barely detectable in the corpus callosum under

normal conditions and remained relatively low at day 3, but progressively increased after BCAS, with the most pronounced accumulation observed at day 14 (Fig. 4G). Notably, APOE⁺ and SPP1⁺ signals were spatially enriched within the white matter region and frequently overlapped with CX3CR1^{GFP} microglia, supporting the emergence of a DAM-like microglial phenotype during chronic hypoperfusion. Quantitative analysis confirmed a time-dependent increase in APOE⁺ and SPP1⁺ microglia, both reaching their highest levels on day 14 (Fig. 4I).

We further examined the IFN-responsive microglial by staining ISG15 and IFIT3. In agreement with the delayed expansion of the IFN cluster identified by scRNA-seq, ISG15 and IFIT3 expression was minimal in normal and early BCAS brain but became clearly elevated at day 14, particularly within the corpus callosum (Fig. 4H). Co-localization with CX3CR1^{GFP} cells indicated that these interferon-stimulated genes were predominantly associated with microglia/macrophage-lineage cells in the hypoperfused white matter. Quantification showed a marked increase in ISG15⁺ and IFIT3⁺ microglia at day 14 compared with earlier time points (Fig. 4J). Together, these histological validations corroborate the single-cell findings and demonstrate that chronic hypoperfusion induces a delayed but robust microglial activation program characterized by progressive DAM-like and IFN-responsive phenotypic remodeling in the corpus callosum.

Functional enrichment via GO and KEGG was utilized to delineate the transcriptional identities of the DAM and IFN clusters. DAM cluster was characterized by a robust metabolic and translational signature, with GO terms predominantly enriched in cytoplasmic translation, aerobic respiration, and ribosome biogenesis (Fig. S4A). KEGG analysis corroborated this, identifying significant enrichment in oxidative phosphorylation and pathways associated with Alzheimer's and Parkinson's diseases (Fig. S4B). IFN cluster was defined by a strong immunological profile. Top GO terms included the positive regulation of response to biotic stimulus, innate immune response, and antiviral defense mechanisms (Fig. S4C). KEGG pathway mapping further confirmed this immune-centric role, with high enrichment scores for Coronavirus disease, Herpes simplex virus 1 infection, and ribosomal components (Fig. S4D), suggesting a coordinated response to pathogen-associated stressors within this specific cell population.

Divergent Early Microglial Landscapes and Stroke Subtype-Specific Inflammatory Programs at 3 Dpi

To capture the nascent cellular responses following cerebrovascular insult, we performed a comparative analysis across IS, HS, and BCAS models at 3 dpi. While

the global cellular composition remained consistent across 12 clusters (Fig. 5A-B), the microglial compartment exhibited immediate and profound transcriptomic shifts that sharply distinguished IS and HS

from CCH (Fig. 5C). At this early stage, the BCAS group was defined by a high prevalence of homeostatic microglia (Mic_1: *P2ry12*, *Tmem119*) (Fig. 5D-E).

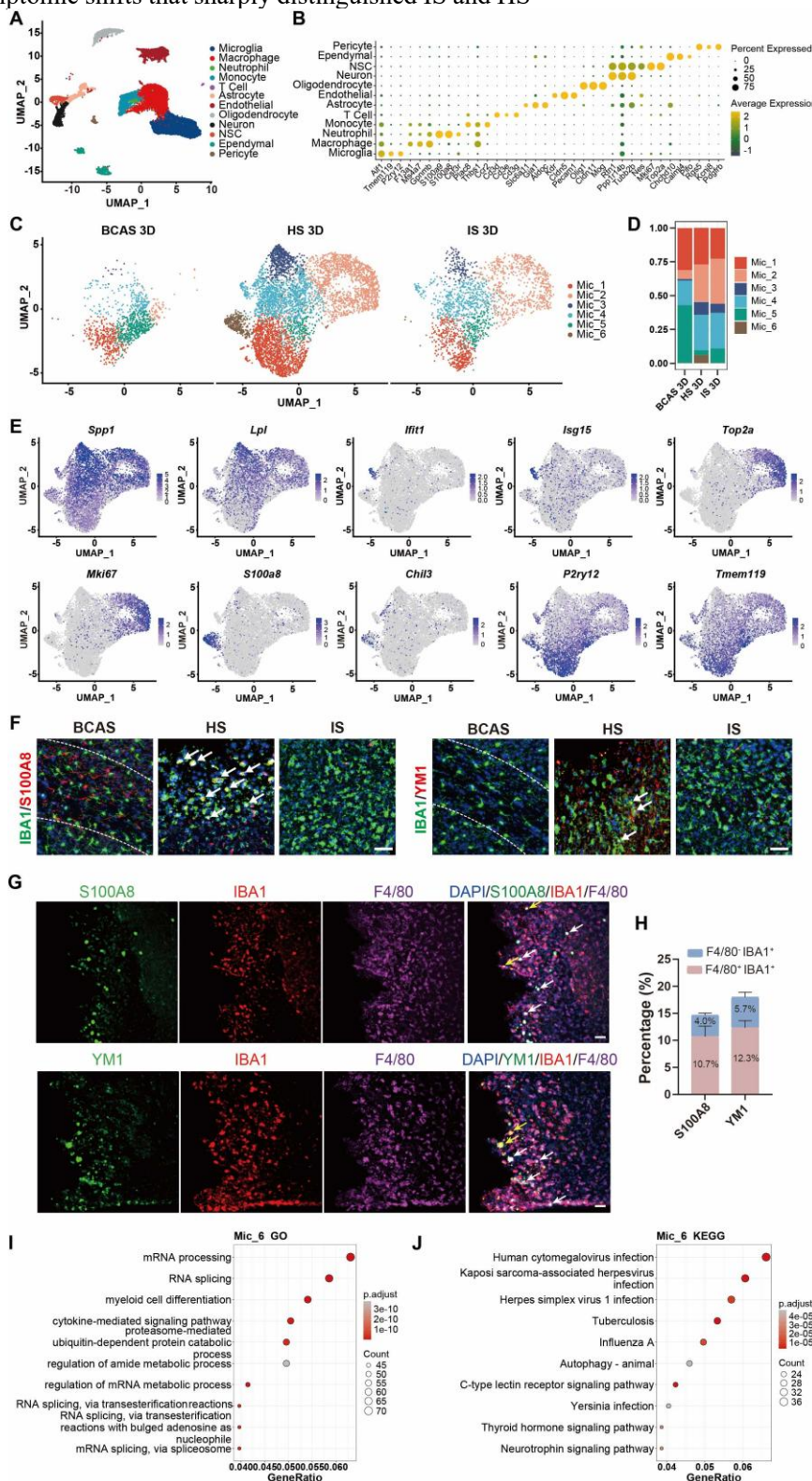


Figure 5. Rapid divergence of immune landscapes and microglial activation states at 3 dpi. (A) UMAP visualization of the integrated single-cell dataset at day 3, identifying 12 major cell populations across BCAS, HS, and IS models. (B) Dot plot showed the expression of canonical marker genes used for cell-type annotation. (C) Split UMAP plots showed the sub-clustering and distribution of microglial states. (D) Stacked bar plot compared the proportions of microglial sub-clusters among the three models. (E) Feature plots showed the expression of key functional markers across microglial clusters. (F) Representative immunostaining images showed the co-localization of IBA1 with S100A8 and YM1 (CHIL3) across the three models to validate subtype-specific protein expression. Scale bar=50 μ m. (G) Representative high-magnification immunofluorescence images showed the co-localization of S100A8 or YM1 with IBA1⁺F4/80⁺ myeloid. Nuclei were counterstained with DAPI. Scale bars = 50 μ m. White arrows indicate IBA1⁺F4/80⁺ myeloid cells and yellow arrows indicate IBA1⁺F4/80⁻ microglia. (H) Quantification of the percentages of S100A8⁺ or YM1⁺ cells among IBA1⁺ cells across the three models at 3 dpi. Data are presented as mean $\pm$ SEM. (I) GO enrichment analysis of Mic_6-specific DEGs. (J) KEGG enrichment analysis of Mic_6-specific DEGs. scRNA-seq data in this figure are based on one pooled library per condition/time point and should be interpreted descriptively. Stacked bar plots indicate descriptive cell-cluster proportions, DEG/pathway analyses are exploratory.

In contrast, both HS and IS models displayed a precipitous contraction of the homeostatic pool, replaced by a massive expansion of reactive phenotypes. A signature feature of acute injury was the early peak of the proliferative cluster (Mic_2: Top2a, Mki67), indicating a robust in-situ microglial expansion immediately following the insult (Fig. 5D-E). Furthermore, the DAM cluster (Mic_3: SPP1, LPL) and IFN-responsive cluster (Mic_4: IFIT1, ISG15) were already well-established in the HS and IS groups by 3 dpi. Notably, we identified an HS-enriched acute inflammatory myeloid/microglia-associated cluster (Mic_6), characterized by a neutrophil-associated transcriptional signature, including high expression of S100A8 and CHIL3. Given the hemorrhagic nature of this model and the absence of computational ambient RNA correction, we interpreted this cluster as an HS-associated inflammatory cell state rather than as definitive evidence of a purely cell-intrinsic microglial program. This cluster was characterized by a neutrophil-like transcriptional signature, including the high expression of S100A8 and CHIL3 (encoding YM1) (Fig. 5D-E). We further validated this finding via multi-color immunofluorescence, confirming that IBA1⁺/S100A8⁺ and IBA1⁺/YM1⁺ microglia were predominantly localized within the HS lesion core, while remaining virtually absent in the IS and BCAS groups (Fig. 5F). To further define the cellular identity of this Mic_6-like population and determine whether it retained a core microglial identity distinct from infiltrating myeloid populations, we performed triple immunostaining for S100A8 or YM1 together with IBA1 and F4/80. Both S100A8 and YM1 signals were concentrated within the hemorrhagic lesion area and showed clear co-localization with IBA1⁺F4/80⁺ or IBA1⁺F4/80⁻ cells, supporting the presence of an HS-related inflammatory microglial population that retained microglia-associated features (Fig. 5G). Given that IBA1 and F4/80 do not definitively distinguish resident microglia from infiltrating peripheral macrophages under hemorrhagic conditions and considering the strong association of S100A8 with peripheral inflammatory myeloid programs, we interpret this population as a

hemorrhage-enriched inflammatory myeloid/microglia-like state. Definitive lineage attribution will require future fate-mapping or lineage-tracing approaches. Quantitative analysis further showed that S100A8⁺ and YM1⁺ myeloid populations accounted for approximately 14.7% and 18.0% of lesion-associated cells, respectively, with S100A8-associated fractions of 10.7% and 4.0%, and YM1-associated fractions of 12.3% and 5.7% (Fig. 5H).

GO and KEGG enrichment analyses were performed for the Mic_6 cluster. The GO terms were predominantly enriched in RNA metabolism and protein degradation pathways, including mRNA processing, RNA splicing, and ubiquitin-dependent protein catabolic processes, suggesting a high level of post-transcriptional regulation within this population (Fig. 5I). KEGG analysis demonstrated that these genes are significantly involved in viral infectious pathways and critical signal transduction pathways such as C-type lectin receptor and Neurotrophin signaling (Fig. 5J). These findings underscore that acute stroke subtypes initiate a high-intensity inflammatory and proliferative program as early as day 3, whereas the BCAS model maintains a relatively stable microglial equilibrium during this initial phase.

Divergent Neuroinflammatory Peak and Temporal Dynamics at 7 Days Post-Stroke

Global UMAP clustering at 7 dpi consistently resolved 12 major cell lineages across all models (Fig. 6A-B). At this subacute stage, the temporal divergence between acute and chronic models became more pronounced (Fig. 6C). Similar to the 3-dpi phase, microglia in the BCAS group remained predominantly in a homeostatic state (Mic_1), whereas the IS and HS groups had successfully transitioned from a stable profile to a diverse active landscape (Fig. 6C). A defining feature of the acute stroke models at 7 dpi was the substantial expansion of the IFN-responsive cluster (Mic_6), characterized by the robust expression of Ifit1 and Isg15 (Fig. 6D-E). This suggests that interferon signaling is a dominant orchestrator of the subacute immune response in acute injury but remains

largely dormant in CCH at this time point. Furthermore, the MHC-II-high cluster (Mic_4), defined by H2-Ab1 and Cd74, was significantly more abundant in both IS and HS brains (Fig. 6D-E). This enrichment indicates an early and vigorous initiation of antigen presentation and adaptive immune recruitment following acute insult, processes that were conspicuously absent in the BCAS model.

Additionally, we identified a proliferative cluster (Mic_5) (Top2a, Mki67) and a DAM-like cluster (Mic_3) (Spp1, Lpl) across all stroke models (Fig. 6D-E). Notably, the proportions of these active states were markedly higher in the acute stroke groups, with the co-existence of proliferation and DAM markers suggesting a sustained expansion of the disease-associated pool (Fig. 6D-E).

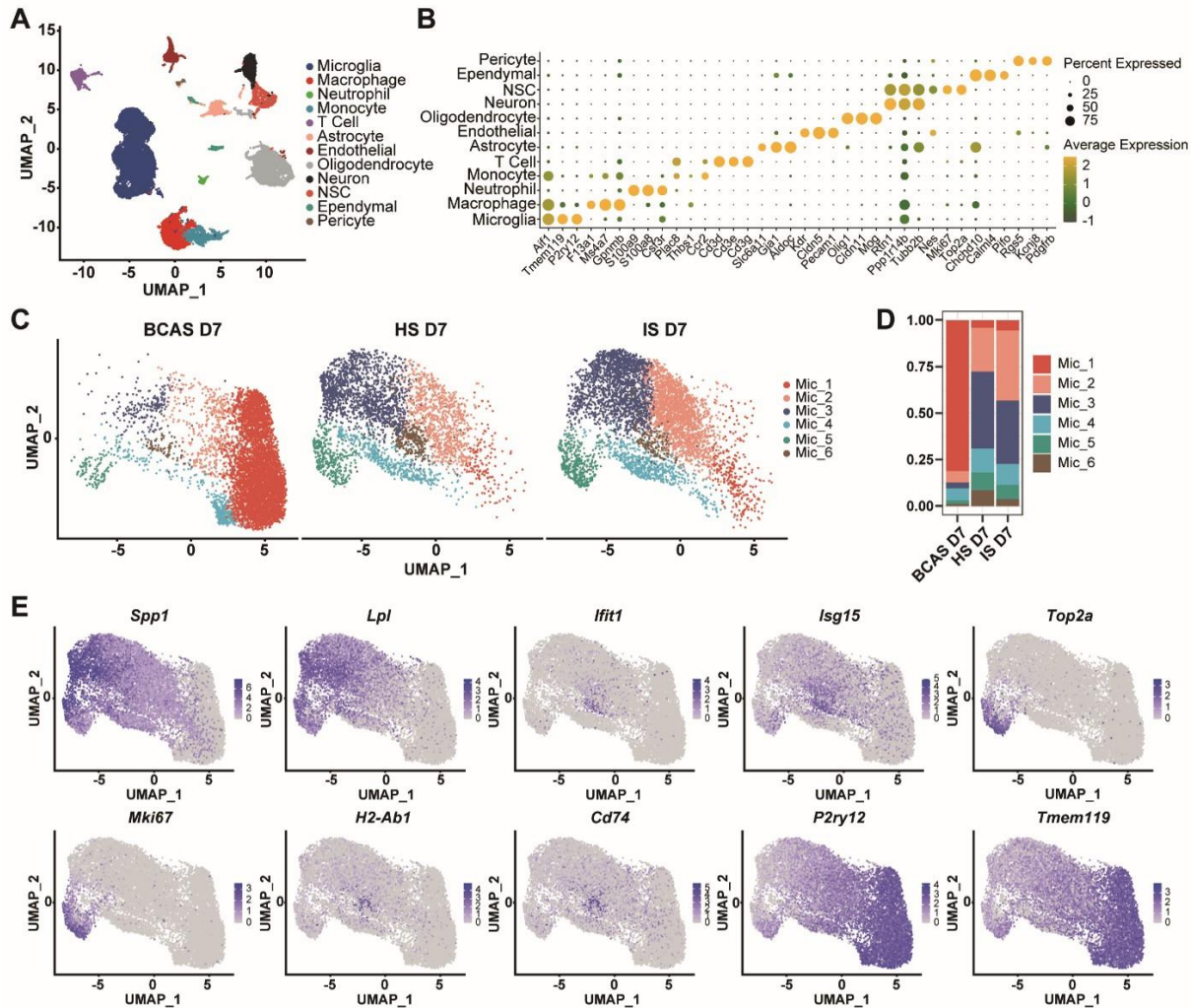


Figure 6. Single-cell transcriptomic landscape and microglia heterogeneity across different stroke subtypes at 7 dpi. (A) UMAP visualization of the integrated single-cell dataset at day 7, identifying 12 major cell populations across BCAS, HS, and IS models. (B) Dot plot showed the expression of canonical marker genes for each major cell cluster. (C) Split UMAP plots showed the sub-clustering and distribution of microglial states. (D) Stacked bar plot compared the proportions of microglial sub-clusters among the three models. (E) Feature plots illustrated the spatial expression of representative marker genes within the microglial clusters. scRNA-seq data in this figure are based on one pooled library per condition/time point and should be interpreted descriptively. Stacked bar plots indicate descriptive cell-cluster proportions.

Shared Late-Phase Microglial Activation Patterns Across IS, HS and BCAS at 14 Days Post-Injury

To characterize the late-phase immune environment, we analyzed the cellular landscapes of BCAS, HS, and IS models at 14 dpi. Global UMAP visualization consistently identified 12 major cell populations across all groups (Fig. 7A-B). At this chronic stage, the microglial landscapes

across the three models displayed partially overlapping activation features, including DAM-like, IFN-responsive, and MHC-associated programs (Fig. 7C-E). However, because each condition/time point was represented by a pooled scRNA-seq library, these similarities were interpreted as an apparent shared late-phase activation pattern rather than definitive convergence into a single chronic microglial state. In a departure from the divergent

responses observed at 3 and 7 dpi, the BCAS model at 14 dpi had undergone a profound transcriptomic transition, with a concomitant expansion of reactive sub-clusters that rendered its profile highly comparable to the acute IS and HS models. High-resolution sub-clustering revealed that the homeostatic pool (Mic_2 and Mic_3), characterized by *P2ry12* and *Tmem119*, remained the dominant population across all three models, suggesting a partial re-establishment of the steady state (Fig. 7D-E). However, significant reactive compartments persisted: the DAM-like cluster (Mic_1) (*SPP1*, *LPL*), the IFN-responsive cluster (Mic_4) (*IFIT1*, *ISG15*), and the MHC-II-high cluster (Mic_5) (*H2-AB1*, *CD74*) were present in relatively similar proportions across all models (Fig. 7D-E). This shared signature indicates a sustained

inflammatory milieu and ongoing antigen presentation activity regardless of the initial insult etiology. Interestingly, the proliferative cluster (Mic_6) (*TOP2A*, *MKI67*) remained detectable primarily in the BCAS and IS groups, suggesting active in-situ remodeling in these models even at 14 dpi (Fig. 7D-E). Collectively, these findings suggest that although the temporal dynamics of neuroinflammation differ substantially during the acute and subacute phases, the three models may develop partially overlapping late-phase microglial activation programs by 14 dpi. This apparent shared profile, characterized by DAM-associated markers, interferon-responsive genes, and MHC-related immune-surveillance features, suggests a putative chronic activation pattern across diverse cerebrovascular disease models.

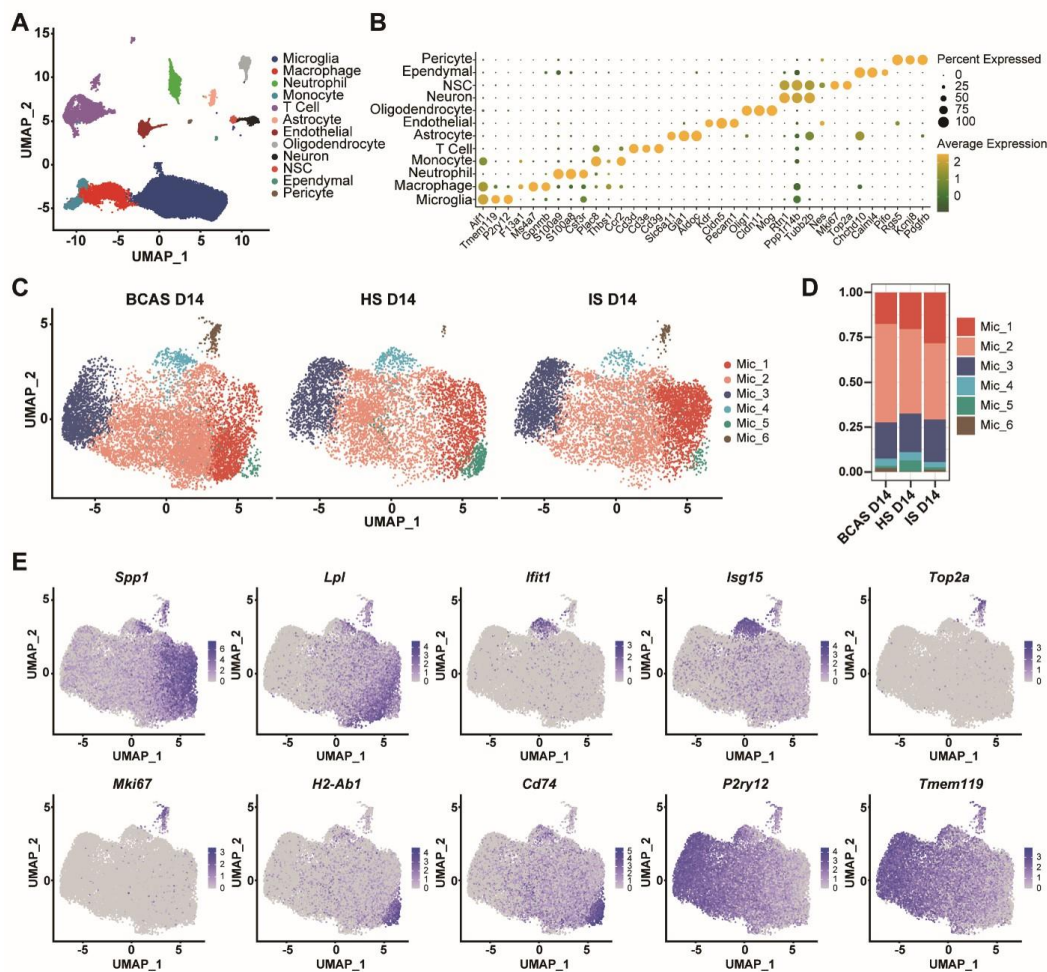


Figure 7. Single-cell transcriptomic landscape and microglia heterogeneity across different stroke subtypes at 14 dpi. (A) UMAP visualization of the integrated single-cell dataset at day 14, identifying 12 major cell populations across BCAS, HS, and IS models. (B) Dot plot illustrated the expression of canonical marker genes used to identify the specific cell lineages shown in (A). (C) Split UMAP plots displayed the distribution and clustering of microglial sub-populations. (D) Stacked bar plot showed the relative proportions of the 6 microglial sub-clusters. (E) Feature plots showed the expression levels of key signature genes across the microglial clusters. scRNA-seq data in this figure are based on one pooled library per condition/time point and should be interpreted descriptively. Stacked bar plots indicate descriptive cell-cluster proportions.

DISCUSSION

In this study, we employed high-resolution scRNA-seq to systematically decode the immune microenvironment across three distinct cerebrovascular disease models. By integrating longitudinal datasets from IS, HS, and CCH, we have observed a complex temporal atlas of microglial activation. Our findings highlight a shift from highly divergent, model-specific immune responses in the acute phase toward an apparent overlap in selected microglial activation programs at the chronic stage. This observation provides a hypothesis-generating framework for understanding shared and distinct immune features across cerebrovascular disorders, while we acknowledge that the pooled-library design precludes definitive conclusions about transcriptomic convergence.

A key observation in the healthy mouse brain was the inherent heterogeneity of microglia between CC and striatum (Fig. 1). The specific enrichment of a stress-responsive sub-cluster (Mic_4) characterized by HSPs (HSPA1A, HSPH1) in the healthy CC suggests that white matter microglia exist in a “primed” or “pre-activated” state. This regional predisposition may be an evolutionary adaptation to the high metabolic demands and lipid-rich environment of myelinated tracts [32, 33]. However, this lower threshold for stress response might also explain the heightened sensitivity of white matter to chronic hypoperfusion (BCAS), where a cumulative insult eventually triggers a profound inflammatory surge.

The most striking distinction among the three models lies in their temporal immunological trajectories (Fig. 2-4). In acute models (IS and HS), we observed a “burst-like” activation pattern as early as 3 dpi, characterized by a precipitous decline in homeostatic microglia and a massive expansion of PRO and IFN clusters, which is consistent with previous reports [34]. In contrast, the CCH model exhibited a significant latency window, maintaining a predominantly homeostatic profile through the first week post-injury. Importantly, these divergent trajectories should not be attributed solely to stroke subtype. Instead, they likely reflect a combination of factors, including differences in injury mechanism (ischemic vs hemorrhagic vs chronic hypoperfusion), temporal kinetics of tissue damage, and regional anatomical context (striatum in IS/HS versus corpus callosum in BCAS), as well as intrinsic differences between gray matter and white matter microenvironments and baseline stress-responsive microglial states. Collectively, acute stroke likely triggers an immediate “emergency response” driven by abrupt blood–brain barrier disruption and necrotic cell death, whereas chronic hypoperfusion induces a slower and cumulative metabolic stress response.

Our cross-model comparison at 3 dpi identified a highly specific microglial state (Mic_6: S100A8+/CHIL3+) exclusive to the HS model (Fig. 5). This neutrophil-like microglial phenotype, which we validated via immunofluorescence, likely reflects the unique pathological pressure of intracerebral hemorrhage, such as the sudden exposure to heme and iron-mediated oxidative stress [35–38]. The sustained expansion of the IFN subcluster and the late-stage emergence of the MHC-II cluster in HS further underscore a more complex and prolonged immune surveillance program compared to IS. Unlike IS, where ischemic core forms without direct blood extravasation, HS is characterized by the rapid release of blood components, including erythrocytes, hemoglobin, and heme, into the brain parenchyma [39, 40]. These blood-derived products serve as potent damage-associated molecular patterns (DAMPs) that can be recognized by pattern recognition receptors such as Toll-like receptors (TLRs) and scavenger receptors [41, 42]. Heme, in particular, has been shown to induce oxidative stress and promote antigen presentation pathways in microglia, potentially driving the upregulation of MHC class II molecules [43]. Furthermore, erythrocyte-derived factors may facilitate the recruitment and activation of peripheral immune cells, creating an inflammatory milieu that sustains MHC-related microglial activation [44, 45]. Thus, the appearance of the MHC subcluster in HS likely reflects a distinct microglial state shaped by the complex inflammatory environment associated with intracerebral hemorrhage.

A technical consideration specific to the HS dataset is the potential influence of blood-derived ambient RNA on single-cell transcriptomic profiles. Hemorrhagic lesions contain erythrocyte products, hemoglobin transcripts, neutrophil-associated RNAs, and inflammatory myeloid cells, all of which may contribute extracellular RNA during tissue dissociation and droplet-based library preparation. In the present analysis, hemoglobin-rich cells were removed using QC filtering, and potential doublets were excluded using DoubletFinder; however, no additional computational ambient RNA correction, such as SoupX or DecontX, was performed. Therefore, residual ambient RNA contamination may have inflated the apparent expression of blood- or neutrophil-associated genes in HS-enriched inflammatory clusters, particularly the S100A8+/YM1+ Mic_6 cluster identified at 3 dpi.

Nevertheless, several observations support the biological relevance of this HS-enriched inflammatory population. First, the cluster was detected in the hemorrhagic condition at the early post-injury stage, consistent with the expected exposure of local myeloid cells to blood products and acute inflammatory cues. Second, tissue-level immunofluorescence confirmed

S100A8 and YM1 signals within lesion-associated IBA1+ cells, including IBA1+F4/80+ and IBA1+F4/80–myeloid/microglia-lineage cells. These findings suggest that the HS-enriched inflammatory signature is unlikely to be explained solely by ambient RNA. We therefore interpret Mic_6 as a hemorrhage-associated inflammatory myeloid/microglia-like state shaped by both cell-intrinsic activation and the blood-rich lesion microenvironment, while acknowledging that future studies using ambient RNA correction, spatial transcriptomics, and independent biological replicate libraries will be required to precisely separate true cellular expression from ambient blood-derived signals.

Despite the initial divergence, all three models displayed an apparent similarity in selected reactive microglial features by 14 dpi (Fig. 7). Rather than establishing definitive immune convergence, these data suggest a putative shared chronic activation pattern involving DAM-like markers, interferon-responsive signaling, and antigen-presentation-related programs. The DAM phenotype, characterized by high expression of SPP1 and CST7, originally described in Alzheimer's disease [46], was commonly observed across all three models. Notably, in both IS and HS, the DAM subcluster was enriched at day 3 and 7, with only a modest reduction at day 14, suggests that DAM-like microglia in stroke may play dual roles in both lipid metabolism/debris clearance and the modulation of chronic neurodegeneration [17, 47]. BCAS model exhibited a markedly delayed microglial response. The DAM subcluster did not become apparent until day 14, indicating that the prolonged, low-grade ischemic stress in BCAS requires a substantially longer latency to drive microglial activation compared with acute stroke models. Nevertheless, it is plausible that extending the time course of analysis, such as to day 21 or beyond, would reveal further accumulation of DAM or other activated microglial states, potentially recapitulating features observed in the acute models.

There are several limitations of our study. First, although the sequencing samples were pooled from multiple mouse brain tissues, they represented only a single biological replicate per condition, limiting the reproducibility of our findings. Second, only male mice were used in this study. This design was intended, in part, to reduce experimental variability associated with cyclic fluctuations of ovarian hormones, particularly estrogen and progesterone, during the 4–5-day estrous cycle. However, we recognize that the exclusive use of male mice represents an important limitation and restricts the generalizability of our conclusions. Biological sex is a critical modifier of ischemic stroke pathology. Previous studies have shown that young female rodents often exhibit smaller infarct volumes and improved neurological outcomes compared with males after

experimental ischemia, whereas this relative protection can be influenced by age, ovariectomy, and gonadal hormone status [48, 49]. In addition, male and female brains may engage distinct ischemic injury pathways, including differential involvement of caspase-dependent and PARP-1/AIF-related cell-death mechanisms [50, 51]. Sex-dependent differences have also been reported in microglial activation after stroke. Male mice may display stronger pro-inflammatory microglial/macrophage responses, whereas female-derived microglia can show greater sensitivity to IL-4/IL-10-mediated immunoregulation and M2-associated responses [52–55].

Furthermore, post-stroke neuroinflammatory responses, including inflammatory gene expression, cytokine production, splenic and peripheral immune activation, T-cell responses, leukocyte infiltration, and gut–immune interactions, may differ between males and females [53, 56, 57]. Therefore, the present findings should be interpreted as male-specific and should not be assumed to generalize to female animals or to both sexes. Future studies including both male and female mice are required to determine whether the microglial subclusters and temporal response programs identified here are conserved, attenuated, or reversed in female animals. Third, our analysis was confined to CC and striatum of healthy mice, without including comparisons with other brain regions such as the cortex and hippocampus. Another limitation is that we focused solely on the heterogeneous changes in microglia across different models and time points, without exploring alterations in other cell types, such as astrocytes, neurons, or infiltrating peripheral immune cells. We did not investigate the intercellular interactions among different cell populations under different diseases. Finally, our current experimental design does not fully decouple the influence of the regional niche from that of the pathological model. Specifically, we cannot definitively determine whether the observed differences between chronic hypoperfusion (BCAS) and acute stroke models are inherently driven by the stress-responsive, HSP-enriched microglial subpopulation found in the white matter. To rigorously distinguish these effects, future investigations should involve a cross-regional comparison, such as analyzing the corpus callosum in acute stroke models and the striatum in chronic hypoperfusion models. Our study provides a high-resolution roadmap for “Temporal Precision Medicine” in stroke. The identified stage-specific and model-specific sub-clusters suggest that the optimal therapeutic window for acute stroke is significantly earlier (within 3–7 days) than that for chronic hypoperfusion. Future interventions should consider not only the type of cerebrovascular insult but also the specific temporal trajectory of the microglial response. While our transcriptomic data provide deep insights into

cellular states, future studies combining spatial transcriptomics and lineage tracing will be essential to fully elucidate the spatial niches and long-term fates of these reactive microglial populations.

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Author contributions

JW, BL, and YT designed the project and revised the manuscript. YG did the bioinformatic analysis, TX and CL conducted the animal experiments and prepared the manuscript draft. SD, LG, QL, and JY contributed to animal model establishment. All authors read and approved the final manuscript.

Competing interests

The authors declare no conflict of interest.

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2026.0803.

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