

Heterogeneity of Microglia in Ischemic Stroke from the Perspective of Single-Cell RNA Sequencing: Subset Characteristics, Mechanisms and Therapeutic Potential

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Abstract

Ischemic stroke (IS), a predominant cerebrovascular disorder contributing to global disability and mortality, characterizes by a complex, multi-tiered cascade of pathological processes. As the primary innate immune cells within the central nervous system (CNS), microglia exhibit dual functional characteristics following ischemic injury, switching dynamically between pro-inflammatory detrimental phenotypes and anti-inflammatory reparative phenotypes in response to temporal progression, cellular phenotypic transformation, and changes in the local microenvironment. Consequently, therapeutic strategies targeting microglia have garnered considerable research interest, challenging the traditional neuron-centric therapeutic approaches. Microglia display a wide range of phenotypes, and the traditional M1/M2 classification is overly simplistic, failing to capture the full spectrum of their functional diversity. In contrast, single-cell RNA sequencing (scRNA-seq) technology has surpassed the limitations of bulk sequencing, providing a robust tool for elucidating microglial heterogeneity. Recent studies utilizing animal models of stroke have identified several subsets distinct from the conventional M1/M2-like subsets, including but not limited to ischemic stroke-associated microglia (ISAM), *Prdx1*⁺SAM, *Spp1*⁺microglia and SAM-foamy. The mechanisms underlying microglial heterogeneity encompass innate programming influenced by genetic background, dynamic remodeling of epigenetic modifications, metabolic reprogramming in response to extrinsic microenvironmental stress, and intercellular interaction networks. This review systematically examines, from the perspective of scRNA-seq, the biological functions of MG, the heterogeneity observed throughout their CNS life cycle development and regional homeostasis, the characteristics and regulatory mechanisms of heterogeneous microglial subpopulations following ischemic stroke, and potential therapeutic strategies and associated challenges. The aim is to provide a reference for the development of precise therapeutic strategies for IS.

Plain Language Summary

Ischemic stroke causes damage to brain tissue, and microglia play a crucial role in the process of injury repair. Previous studies suggested that these cells have a single function, but the emergence of single cell sequencing technology has enabled us to see that they actually belong to a “large family”. This family consists of various subtypes with different functions, and

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they are distributed in different locations in the brain tissue after stroke and have different marker genes. Some subtypes can clear damaged tissues and protect nerves, while others will aggravate inflammation and expand the damage area. Studying the characteristics and mechanism of action of these subtypes can help us find precise regulatory methods. For example, specifically activating protective subtypes and inhibiting harmful subtypes can provide new treatment options for ischemic stroke. This research has opened a personalized path for stroke treatment and is expected to benefit more patients.

Keywords

single-cell RNA sequencing, ischemic stroke, microglia, cellular heterogeneity, metabolic reprogramming, epigenetic modification

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1. Introduction

Ischemic stroke (IS) represents a significant contributor to disability and mortality within the spectrum of cerebrovascular diseases on a global scale.^{1,2} The pathophysiological progression of ischemic stroke is characterized by a dynamic cascade of events, encompassing multiple interrelated stages. These stages range from initial energy depletion and cellular death to subsequent neuroinflammation and infiltration by peripheral immune cells. Microglia (MG), the resident immune cells of the central nervous system (CNS),³ play a pivotal role in responding rapidly to the stroke-induced injury signals. They engage in the tissue repair process through mechanisms such as phagocytosis, the release of inflammatory mediators, and the regulation of vascular repair.⁴ Given the central role of MG in post-injury tissue repair, there is a growing focus on targeting these cells, which is gradually surpassing the traditional neuro-centric treatment paradigm.

The involvement of MG in the pathological processes following a stroke is highly intricate, encompassing activation, phenotypic transformation, and a multitude of functions, as well as contributing to both neuroinjury and repair mechanisms.^{4,5} Traditional research has often categorized responding MG into pro-inflammatory M1-like and anti-inflammatory M2-like phenotypes; however, this binary classification fails to adequately capture the functional diversity exhibited by MG during tissue repair. Recent advancements in single-cell RNA sequencing (scRNA-seq) technology have transcended the limitations of conventional bulk sequencing methods. This technology enables precise characterization of gene expression at the single-cell level, thereby elucidating the discrete cellular states present under both homeostatic and pathological conditions. Consequently, scRNA-seq provides a robust methodological framework for investigating microglial heterogeneity.^{6,7} This review systematically examines, from the perspective of scRNA-seq, the biological functions of MG, the heterogeneity observed throughout their CNS life cycle development and regional homeostasis, the characteristics and regulatory mechanisms of heterogeneous microglial subsets following ischemic stroke, as well as potential therapeutic strategies and associated challenges. The aim is to provide a reference for the development of precise therapeutic strategies for IS.

2. MG: The Immune Sentinels of the CNS

MG originate from the embryonic yolk sac and migrate into the brain parenchyma. There, exposed to local neural signals, they acquire distinct identity markers and become integral to immune surveillance, neural support, and host defense.^{8,9} MG remain highly dynamic and plastic.¹⁰ Under physiological conditions, they typically exhibit a ramified morphology, continuously interacting with their surroundings and monitoring the CNS to sustain homeostasis.¹⁰ Accordingly, Paolicelli et al refer to these as surveying MG (replacing the former “resting” classification).¹¹ When pathological changes occur in the CNS, microglia respond to damage signals from the microenvironment and undergo phenotypic shifts to perform diverse functions, thus being termed reactive MG (replacing the “activated” designation).¹¹

Microglial responses refer to any physical or biochemical alterations that deviate from the homeostatic state of MG.¹² Pathological cues such as damage-associated molecular patterns (DAMPs) from ischemic injury, drive MG to undergo morphological transformation into amoeboid-like cells with enlarged somata and thickened protrusions—characterized by altered cell size, branching patterns, and morphological complexity—and thereby participate in tissue reparative responses.^{4,13} Early studies categorized reactive MG into pro-inflammatory M1-like and pro-reparative M2-like phenotypes based on their secreted mediators and functional profiles.¹⁴ The M1-like phenotype possesses robust phagocytic capacity and secretes pro-inflammatory mediators to exert pro-inflammatory functions; it can surround and engulf pathogens and aberrant cells, thus contributing to tissue repair.^{5,15,16} However, excessive M1-like activation triggers intense inflammatory responses that impede tissue repair and even exacerbate pathological damage (Figure 1).⁴ Conversely, the reparative M2-like phenotype

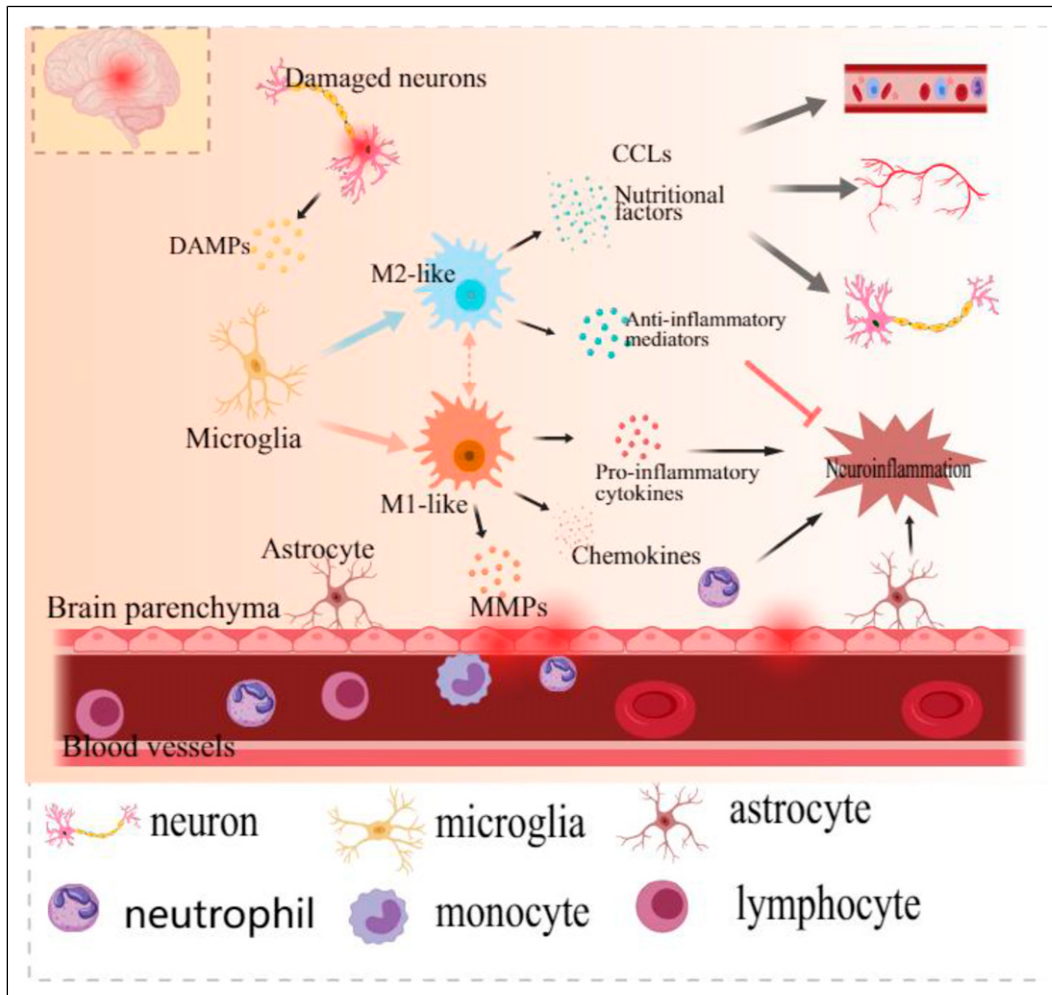


Figure 1. Microglial M1/M2-like polarization responses following ischemic Stroke

exerts anti-inflammatory and neuroprotective effects by secreting anti-inflammatory cytokines and neurotrophic factors, thereby resolving inflammation and promoting recovery. Based on cell surface markers and biological functions, M2-like MG are further subdivided into three subtypes: M2a, M2b, and M2c. M2a MG primarily facilitate cellular regeneration, whereas M2b and M2c subtypes are predominantly involved in phagocytosis and the clearance of tissue debris. The M1/M2 dichotomous classification was initially derived from *in vitro* experimental observations,¹⁷ and has served as a foundational framework for understanding microglial functions.^{11,18} However, this binary model fails to capture the multidimensional, dynamic phenotypic spectrum of MG themselves during *in vivo* pathological responses and also obscures the functional diversity shaped by the spatiotemporal heterogeneity of pathological processes and tissue damage within the CNS.^{11,18} In the ischemic brain microenvironment, microglial phenotypes are highly plastic and heterogeneous, transitioning through intermediate states rather than existing as fixed binaries. Recent investigations have demonstrated that MG exhibit multiple distinct phenotypic states throughout CNS development, aging, and pathological injury¹²; notably, under pathological stress, microglia acquire a diverse array of continuously activated phenotypic states that are not encompassed by the classic M1/M2 paradigm.^{19,20} Accordingly, the existence of microglial subsets with distinct phenotypic characteristics that execute specialized functional roles in different CNS pathological processes has long been a key research focus for neuroscientists.

After ischemic-hypoxic injury, microglia rapidly respond by sensing danger signals such as damage-associated molecular patterns released from dying neurons. Notably, the antagonism between M1-like and M2-like microglia is alleviated to a certain extent during their functional transition, rather than being completely opposed. M1-like microglia primarily secrete pro-inflammatory mediators, cytotoxic substances, and chemokines. Their secretory products are core mediators of neuroinflammation during the acute phase of stroke and amplify inflammatory damage by recruiting peripheral immune cells.

M2-like microglia mainly secrete anti-inflammatory cytokines, neurotrophic and angiogenic factors, and anti-inflammatory chemokines. Some of these factors directly inhibit M1-like polarization, terminate acute inflammation and reduce the antagonism between M1 and M2-like phenotypes. While others promote neuronal survival and angiogenesis through the secretion of trophic factors, thereby facilitating the switch from tissue damage to repair. Created with MedPeer (medpeer.cn).

3. Application of scRNA-Seq in the Study of MG Heterogeneity

High-throughput scRNA-seq allows for the detailed investigation of biological processes that reflect changes and diversity in physiological and pathological states.^{7,21} This technology provides an unbiased characterization of immune cell types, states, and transitions from health to disease, thereby contributing to the construction of a comprehensive transcriptional reference atlas of cells.^{6,7,22,23} Utilizing scRNA-seq, researchers have identified multiple distinct subsets of MG with unique molecular signatures in both animal models and human clinical samples,^{19,20} including but not limited to disease-related MG cells (DAMs) in Alzheimer's disease (AD),²⁴ and inflammatory MG cells (MIMS) in multiple sclerosis²⁵ as well as activated response MG cells (ARMs) and interferon response MG cells (IRMs) in multiple sclerosis (MS).²⁶ These findings reveal the heterogeneity of MG in disease contexts beyond the classical M1/2 dichotomy,²⁷ addressing the limitations inherent in the traditional binary classification framework. Conventional techniques including flow cytometry and immunohistochemistry suffer from inherent limitations, including marker-dependent selection bias, limited throughput, and population-averaged measurements that preclude fine-grained microglial classification and mask single-cell transcriptional diversity.²⁷ By contrast, scRNA-seq technologies circumvent these constraints through single-cell resolution, high-throughput molecular characterization, and data-driven subset identification, facilitating the discovery of disease-specific microglial subsets and multi-dimensional analyses of heterogeneity across transcriptomic, epigenomic, and spatial axes.⁷ Consequently, systematic profiling of microglial subsets and activation states across post-stroke time points, including encompassing their phenotypic attributes, functional contributions, and regulatory mechanisms, to offer potential for precision therapeutic interventions in stroke.

4. Fundamental Heterogeneity of MG: Developmental Heterogeneity Across the Life Cycle

MG are widely distributed throughout the CNS, with their reactivity dynamically adapting to environmental cues.^{28,29} The brain microenvironment is heterogeneous, and factors including neuronal subsets, variations in blood flow and metabolism, and the intensity of external stimuli collectively drive the regional specialization of MG.^{8,30,31} Moreover, diverse functional demands, such as those related to neuronal activity and metabolism, synaptogenesis and myelination, and vascular remodeling, likely necessitate distinct microglial functions.^{29,32} ScRNA-seq not only delineates the abundance, regional distribution, molecular profiles, and functional diversity of MG across CNS regions,³³ but also demonstrates that their regional development follows age-dependent spatiotemporally defined subtypes. Under steady-state conditions, MG exhibit a transcriptional continuum rather than falling into discrete subtypes.^{17,34,35}

Sankowski et al definitively identified the continuous transcriptional states of human MG,³⁵ and their findings revealed that the nine major transcriptional clusters (C1-C9) of MG in healthy adults exhibit higher expression in white matter than in gray matter in a spatial context. Moreover, C3 is enriched in MG from individuals under 30 years of age; the C2, a steady-state subpopulation, predominates in those aged 30 to 50 years; and MG in the C6/C7 from individuals over 50 years of age show high expression of *Spp1* and pro-inflammatory cytokines. Hammond et al identified at least nine microglial subpopulations with distinct transcriptional and spatial features across the murine lifespan, demonstrating that the phenotypic states of these subpopulations undergo temporal changes and that microglial expression patterns display regional heterogeneity throughout life.¹² They further described that MG present diverse phenotypic forms and an uneven spatial distribution in early developmental stages, with the highest level of phenotypic diversity observed in the early postnatal period (embryonic day 14.5 [E14.5] and early postnatal days 4-5 [P4-P5]).¹² In adulthood, this phenotypic diversity diminishes, whereas the characteristic features of homeostatic MG become prominent. Aging and demyelinating injury induce a redistribution of microglial phenotypic states, with a notable increase in inflammation-associated phenotypes. Specific microglial subpopulations predominate in different age groups (Table 1), while gender exerts no significant effect on the normal development of microglial phenotypic diversity.¹² Consistent with these findings, other studies have indicated that MG exhibit greater phenotypic diversity in the early postnatal period; even in distinct brain regions of adult mice, MG display a canonical homeostatic phenotype with highly conserved transcriptomic profiles.³⁶ Similarly, Masuda et al distinctly segregated the transcriptional profiles of embryonic and postnatal microglial clusters in mice: embryonic clusters show high expression of lysosome-related genes, while postnatal clusters are characterized by the high expression of core homeostatic genes. In terms of regional heterogeneity, the proportion of microglial subtypes in the cerebellum remains stable from childhood to adulthood, whereas the distribution of microglial subtypes in the cerebral cortex and hippocampus alters markedly with

Table 1. Developmental Heterogeneity of MG in Normal Lifespan of Mice

Development stage	Major subgroups and marker genes	Trait	Major function	Ref.
Embryonic stage (E14.5)	Proliferative MG: C2a: <i>Mcm6, Pcna, Rrm2</i> C2b: <i>Ube2c, Birc5</i> C2c: <i>Hist1h2bc, Ccnb2</i>	The core subpopulation responsible for the expansion of microglia during embryogenesis is present in extremely low proportions in adulthood.	Rapidly increase the number of microglia to support their distribution in the brain.	Hammond et al. ¹²
	Fabp5 ⁺ MG(C3): <i>Fabp5, Mif, Ldha, Tpi1</i>	Enriched in the embryonic brain, high expression of glycolysis-related genes, showing partial transcriptional overlap with C4.	The glycolytic metabolism is active, supporting cell migration and brain colonization.	
	Ms4a7+MG(C6): <i>Ms4a7, Ccr1, Mrc1, Ms4a6c</i>	Located in the embryonic brain parenchyma. Accounts for approximately 10% of the total microglia population during this period, ≤3% in other periods.	Possesses markers of both macrophages and mature microglia, with overlapping transcriptional features with brain boundary macrophages. May represent precursor or transitional cells.	
Early development (P4/P5)	Axonal tract-associated subgroups (ATM, C4): <i>Spp1, Igf1, Gpnm1, Lgals1/3, Lamp1, CD68</i>	Stage-specific enrichment, with a higher proportion than other stages. Specifically localized to the pre-myelinated axonal tracts, enriched with phagocytosis and lysosome-related genes.	Regulate axonal growth and clear the substance before myelination.	
Adolescence (P30) & Adult (P100)	Steady-state subgroups (C7): <i>Fcrls, Trem2, C1qa</i>	The predominant subgroup in this period. High expression of homeostatic markers. Minimum heterogeneity.	Maintain homeostasis and immune surveillance.	
Old age (P540)	The senescent inflammatory subset (OA2): <i>Ccl4, Ccl3, Il1b, Lgals3, Cst7</i>	Significantly enriched in the brains of aged mice, with 2-4 times higher levels in adulthood, and distributed throughout the entire brain. Shares core markers of the inflammatory subtype following injury.	Secretion of inflammatory factors, participating in age-related brain inflammation. The primary contributor to senile brain inflammation.	
	The aging interferon response subset (OA3): <i>Ifitm3, Rtp4, Oasl2</i>	Significantly enriched, with a quantity 2-4 times that of adulthood and a proportion similar to OA2. Specifically enriched interferon response genes.	Driven by local microenvironment, it lacks distinct brain region specificity and responds to injury signals, indicating neuronal degenerative foci.	

advancing age. Moreover, in the context of neurodegenerative or demyelinating diseases, disease-specific microglial subtypes emerge.³⁴

The heterogeneity of MG during the developmental lifecycle, observed in both humans and mice, collectively indicates that early developmental stages give rise to highly diverse subpopulations adapted to the demands of brain maturation. In adulthood, MG phenotypes converge through stable gene regulatory networks. Nevertheless, the genetic plasticity and reservoir of distinct cellular states established during development form the core foundation for the reactivation and remodeling of MG heterogeneity under pathological conditions, enabling subtype differentiation and functional shifts.^{28,32,34} Microglial heterogeneity is a multifaceted concept encompassing spatiotemporal variation, sex-specific differences, brain-region colonization traits, cellular abundance, morphological phenotypes, and more.^{10,29,32,34} Ultimately, these dimensions

manifest as diverse functional outputs in both physiological and pathological settings. Here, we focus specifically on the regional distribution and heterogeneity of MG during development and further summarize current research on how this heterogeneity is altered following IS injury.

5. Changes and Core Heterogeneous Phenotypes of MG After IS

5.1. Ischemic Stroke-Associated Microglia (ISAM)

The intrinsic developmental heterogeneity of MG not only modulates the modes of CNS immune surveillance and tissue homeostasis maintenance, but also underpins the cellular responses to pathological stimuli.^{9,37} Following IS, DAMPs and other stress signals recruit and activate resident MG in the vicinity, driving them to undergo phenotypic remodeling, functional specialization and accumulation at the lesion site.^{38,39} In the early work using flow cytometry to isolate MG, McDonough et al identified two distinct and functionally heterogeneous microglial populations following ischemia: interferon-responsive MG relying on IFNAR1, and proliferative MG defined by a dedicated cell-cycle transcriptional program.^{40,41} These foundational findings have provided an important reference and guiding direction for subsequent application of single-cell RNA sequencing in deciphering microglial subsets. In Table 2, we have summarized recent literature on studies investigating microglial heterogeneity in animal models using scRNA-seq. Zheng et al mapped the single-cell transcriptional landscape of mouse brains at 24 hours post-ischemic injury, revealing that ischemic insults induce the MG0-dominant microglial subset in control mice to differentiate into five distinct subsets (MG0–MG4), which highlights the central role of MG in orchestrating post-stroke neuroinflammation.⁴² Among these subsets, MG2, MG3 and MG4 are the major subtypes associated with ischemic inflammatory injury. Specifically, MG2 represents a potent pro-inflammatory subset that highly expresses matrix metalloproteinase 12 (MMP12) to disrupt the blood-brain barrier, and also expresses key genes linked to microglial subsets implicated in neurodegenerative diseases. MG3 is primarily associated with the type I interferon signaling pathway, while MG4 functions as a proliferative subset characterized by the expression of core proliferation marker genes.⁴² This observation suggests that adaptive proliferation of MG during the acute phase serves to compensate for their numerical loss after ischemic injury.

In addition to the proliferative *MKI67*⁺ microglial subtype, Zhang et al identified significantly enriched *CH25H*⁺ and *OASL*⁺ clusters within the ischemic lesion sites of transcranial middle cerebral artery occlusion (tMCAO) mice during the acute phase.⁴⁹ These clusters exhibited upregulated expression of stroke-associated genes linked to enhanced phagocytosis and lysosomal activity, leading to their classification as ISAM (Figure 2G). Further analysis revealed distinct spatiotemporal dynamics of *MKI67*⁺ MG: they highly express cell cycle-related genes including *MKI67* and *Top2a*,⁴⁹ undergo a marked expansion in the ischemic penumbra and lateral ventricle by post-stroke day 3, gradually diminish by day 7, and become nearly undetectable by day 21. Lineage-tracing assays demonstrated that *MKI67*⁺ MG can differentiate into the *CH25H*⁺ and *OASL*⁺ clusters. The *OASL*⁺ MG displays a robust pro-inflammatory phenotype, characterized by high expression of type I interferon (IFN) response genes and senescence-associated transcripts. Notably, the proportion of *OASL*⁺ MG exhibits a significant positive correlation with cerebral infarct volume. These cells not only persistently accumulate in the infarct core and peri-infarct regions to sustain a chronic pro-inflammatory microenvironment, but also show increased abundance with advancing age.⁵⁵ Given the marked enrichment of *OASL*⁺ MG in aged mice, and advanced age is a major risk factor for post-stroke neurodegenerative sequelae, this subset may act as a critical mechanistic link between acute ischemic injury and long-term neurodegeneration, thereby elevating the risk of vascular cognitive impairment (VCI), dementia, and other associated disorders. Thus, targeted modulation of the *OASL*⁺ subpopulation represents a promising therapeutic strategy to alleviate acute neuroinflammation and mitigate long-term degenerative risks following the stroke.⁴⁹ In contrast, *CH25H*⁺ MG secrete multiple neurotrophic factors such as *Spp1*, *Gpnmb*, and *Lgals3*, and facilitate post-stroke neurological recovery by augmenting phagocytic activity and tissue repair processes. Depletion of *Ch25h* in MCAO mice resulted in exacerbated neuroinflammation, enlarged infarct volume, and deteriorated neurological outcomes, which confirms the neuroprotective role of this subcluster. In the MCAO/reperfusion (MCAO/R) model, Tang et al reported high *CH25H* expression in ISAM (MG4–7), and further demonstrated that the stress-responsive transcription factor ATF3 directly regulates both *CH25H*, a key enzyme involved in cholesterol metabolism, and *Spp1*, a well-recognized marker of ischemic cerebral injury. Mechanistically, *CH25H* modulates lipid metabolism and inflammatory responses through the production of 25-hydroxycholesterol, whereas *Spp1* promotes immune cell recruitment and tissue repair.⁴⁶ McDonough et al elucidated the glial responses following ischemic brain injury by integrating multi-omics technologies including transcriptomics, translationalomics, spatial transcriptomics, and scRNA-seq. They systematically characterized core microglial subsets after stroke, such as *MKI67*⁺ MG, *OASL*⁺ MG, and *CH25H*⁺ MG. Furthermore, regarding the targeted therapy of cerebrovascular diseases, they summarized next-generation research techniques covering animal models for acute stroke and cerebral small

Table 2. Single-Cell RNA Sequencing for the Study of Microglial Heterogeneity in Animal Models

Animal model	Organization source	Cluster	Main contributions	Ref.
MCAO/R	Left hemisphere 24 hours postoperatively	5	Control group: MG0: Model group: 5 cluster coexist in MG0-4; MG2 (pro-inflammatory type), MG3 (IFN response), and MG4 (proliferative type) are the major clusters, constituting the microglial subsets associated with inflammatory ischemic injury. MG1: no quantitative difference between the two groups, responsible for recruiting monocyte-derived macrophages and peripheral immune cells, and initiating early inflammatory responses.	Zheng et al. ⁴²
MCAO	Postoperative 24-hour half-shade tissue	14	MG: The cells with the highest proportion in the penumbra region, enriched in clusters 3, 4, 6, 8, 9, and 10 in MCAO. The 24h M1/M2 polarization trajectory was obvious. Gadd45b and TGF- β -smad3 may be the key targets to MG.	Guo et al. ⁴³
tMCAO	The damaged hemisphere 1 day postoperatively	12	12 MG features: steady-state-like: C0, 1, 5; DAM-like: C3, 8, 10; M1-like: C2, 10, M2-like: C4, 9. Proliferative type: MG7; stroke-VAM: MG6, 11.	Li et al. ⁴⁴
tMCAO	The ipsilateral and contralateral hemispheres at 24 and 48 hours postoperatively.	2	Induction of transcriptional reprogramming in the total number of MG and intrinsic phenotypes, along with mild heterogeneity in the contralateral homeostatic MG population; identification of SAM with enhanced antioxidant function.	Kim et al. ⁴⁵
MCAO/R	1 day in the cerebral hemisphere	7	Ischemia-related subpopulations (MG4-MG7) undergo metabolic reprogramming with downregulation of SDH gene expression: glycolysis-dependent, upregulation of HIF-1 α and pentose phosphate pathway. The ATF3-CH25H-Spp1 axis inhibition promotes inflammatory damage.	Tang et al. ⁴⁶
tMCAO	Cerebral cell suspension at 2 days and 14 days postoperatively	8	Control: MG1 (maintaining homeostasis) and MG2 (homeostasis monitoring). The acute phase (2 days postoperatively): The major subsets: Mg4 (clearance-repair type), and Mg5 (proliferation type). The subacute phase (14 days postoperatively): The major subsets: Mg3, Mg6, and Mg7 subsets, all of which exhibit inflammation-related phenotypes. The Mg8 subset is present in small quantities throughout the entire phase, with its phenotype and function yet to be verified.	Garcia et al. ⁴⁷
MCAO	Ischemic hemisphere (3 h, 12 h, and 72 h)	4	Major subgroups of MCAO: Cluster 1 and Cluster 3. The spatiotemporal expression of IPAM and ICAM was analyzed.	Li et al. ⁴⁸
tMCAO	Brain tissue in the ischemic half-shade and infarcted areas 3 days postoperatively	7	Control: 98% of the MG0. Model: 7 clusters of MG0-6 were identified, with MG0 accounting for only 2%. Three microglial subsets associated with ischemic stroke (ISAM) were recognized: MKI67+, OASL+, and CH25H+ MG.	Zhang et al. ⁴⁹

(continued)

Table 2. (continued)

Animal model	Organization source	Cluster	Main contributions	Ref.
Transient transcranial MCAO	The elderly brain 3 days postoperatively	6	MG6: a distinct “neutrophil-like” phenotype, which may represent a unique microglial state in the aged brain following stroke.	Li et al. ⁵⁰
dMCAO	Ischemic half-shade after 3 days	8	The MG4 subset: Spp1+ MG with anti-inflammatory properties and unique lipid metabolism reprogramming characteristics.	Li et al. ⁵¹
tMCAO	Postoperative 3, 7, 14,30,90, and 180-day damage to the hemispheres	-	SAM- foamy clusters: extensive reprogramming of cholesterol metabolism, with high expression of lipid metabolism and pro-inflammatory genes, persisting in the chronic phase. SAM- iron: Emerging during the chronic phase (30-90 days), localized to the core injury area. Expressing iron metabolism-related genes such as Fth1 and Ftl1, exhibiting characteristics of abnormal activation of iron metabolism.	Zhao et al. ⁵²
dMCAO	5 days postoperatively	6	Healthy state: microglia in aged mice exhibit enhanced transcriptional signatures of chronic inflammation. Cerebral ischemia: impaired transcriptional activation of key functional gene clusters: immune-inflammatory responses↓, immune cell chemotaxis↓, tissue remodeling↓, intercellular interactions↓, peripheral immune cell infiltration↓.	Shi et al. ⁵³
dMCAO	Hemispheres at 5 days postoperatively and 14 days postoperatively.	-	Healthy state: the MG of aged mice is in a state of chronic inflammatory activation. Cerebral ischemia: impaired or suppressed transcriptional responses in immune inflammation, chemotaxis, tissue remodeling, and cell interactions.	Jiang et al. ⁵⁴

vessel disease, single-cell atlases of vascular cells, and spatial transcriptomics analyses. Interested readers are referred to their original literature for further details.⁵⁶

Most sequencing studies have established that proliferative MG contribute to cell population replenishment in the acute phase after injury.^{44,51} Multicolor fate mapping has further revealed that MG in MCAO mice switch from stochastic proliferation at homeostasis to damage-driven polyclonal expansion. Specifically, multiple brain-resident MG clones of different origins proliferate concurrently, with no single or small subset of clones dominating the expansion process.⁵⁷ This polyclonal process follows a distinct time course: cell numbers rise detectably at 2 days, clone abundance peaks at 2 weeks, clone size reaches a maximum at 4 weeks, and by 12 weeks, clone distribution returns to a random state that is comparable to the contralateral hemisphere.⁵⁷ This sequence demonstrates that MG rapidly mobilize in the acute phase via the formation of numerous coexisting clones to cover the injured area; they then boost local functional involvement through clonal expansion, and gradually restore clonal homeostasis as tissue repairs in the chronic phase, thus reflecting the full spectrum of microglial responses to ischemic injury.⁵⁷ Furthermore, electrophysiological analyses show that cells from the same fluorescently labeled clone share identical electrophysiological phenotypes. In contrast, cells from different fluorescently labeled clones—even when spaced less than 50 μm apart in the same microenvironment, showing significant phenotypic differences.⁵⁷ This suggests that microglial functional heterogeneity is induced by both microenvironmental factors and clonal origin.

5.2. *Spp1*⁺MG and *Prdx1*⁺SAM

Different stroke models and injury severities shape distinct cerebral microenvironments, which further induce the divergence of microglial phenotypes, differentiation outcomes, and associated functions. In the ischemic penumbra of perioperative

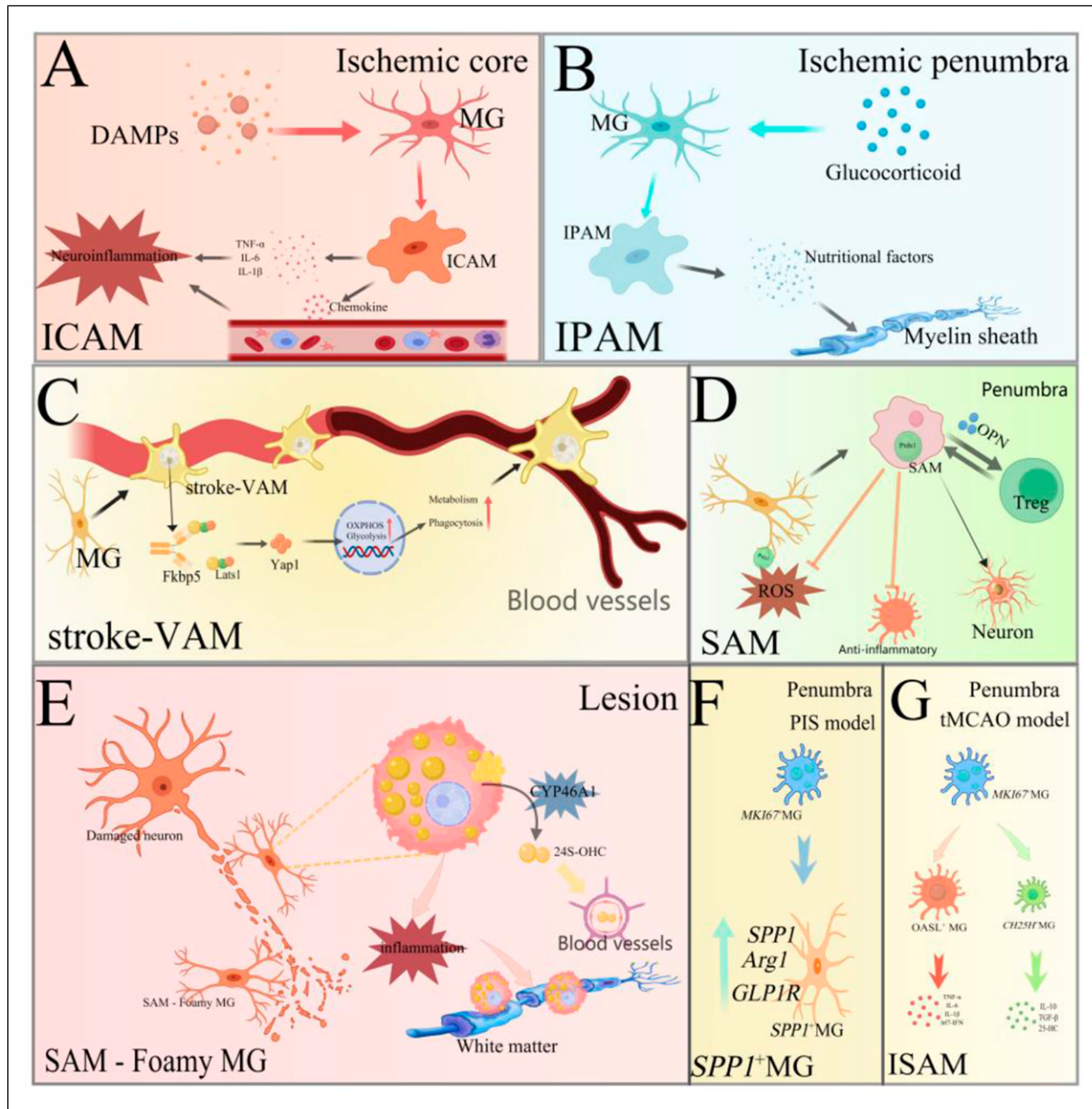


Figure 2. The regulatory mechanism of novel microglial subpopulations distinct from the M1/M2 paradigm. (A) Damage-associated molecular patterns released by injured neurons in the ischemic core drive microglia to polarize toward an ischemic core-associated microglia (ICAM) phenotype. ICAM exhibits high expression of pro-inflammatory cytokines and chemokines, promotes the recruitment of peripheral immune cells, and exacerbates neuroinflammation. (B) Glucocorticoid signaling in the ischemic penumbra induces microglia to differentiate into ischemic penumbra-associated microglia (IPAM), which is characterized by anti-inflammatory metabolic profiles and myelinotrophic properties. (C) Located perivascularly around cerebral vessels and closely interacting with endothelial cells, Stroke-activated vascular-associated microglia (stroke-VAM) enhances glycolysis and oxidative phosphorylation (OXPHOS) via the Fkbp5-Lats1-Yap1 pathway, sustains its hypermetabolic activity and phagocytic function, and ultimately inhibits vascular regeneration. (D) Stroke-associated microglia (SAM) upregulates ROS defense genes and *Spp1* (osteopontin, OPN) in a Prdx1-dependent manner to inhibit oxidative injury, inflammatory injury and interact with Tregs through OPN. (E) Upon phagocytosing cellular debris in the infarct lesion, microglia accumulate cholesterol and undergo cholesterol metabolic reprogramming, giving rise to stroke-associated foamy (SAM-foamy) microglial clusters. These cells maintain persistently high expression of inflammatory genes, which drives chronic neuroinflammation and inhibits white matter recovery. (F) In the mouse model of perioperative ischemic stroke (PIS), Mki67⁺ microglia in the ischemic penumbra are capable of differentiating into *Spp1*⁺ microglia with high expression of Arg1, Spp1, and GLP1R. (G) In the penumbra of tMCAO mice, proliferative MKi67⁺ microglia can differentiate into neuroprotective CH25H⁺ microglia and pro-inflammatory OASL⁺ microglia. These three subsets are collectively referred to as ISAM. (Created with MedPeer (medpeer.cn))

ischemic stroke (PIS) mouse models, the highly enriched *Mki67*⁺MG, despite being characterized as proliferative precursors,⁵¹ displays microenvironment-dependent differentiation patterns. As demonstrated by Li et al, *Mki67*⁺MG can differentiate into *Spp1*⁺MG (Figure 2F), a subpopulation with anti-inflammatory traits and reprogrammed lipid metabolism.⁵¹ *Spp1*⁺MG not only highly expresses the *Spp1* gene and osteopontin (OPN) as well as phagocytosis-related genes, but also specifically expresses GLP1R, thereby directly acting on damaged areas through anti-inflammatory and phagocytic functions. Semaglutide, a clinically used GLP1R agonist for type 2 diabetes, promotes the proliferation of *Spp1*⁺MG via GLP1R activation.

Vascular recanalization to achieve reperfusion in the ischemic penumbra represents the core therapeutic strategy for ischemic stroke. However, abrupt restoration of blood flow can trigger an explosive burst of reactive oxygen species (ROS), thereby inducing severe oxidative stress damage.^{58,59} Endogenous antioxidant defense mechanisms have been proven effective in mitigating the deleterious effects of oxidative stress.⁶⁰ Kim et al conducted comparative analyses of the ipsilateral (IL) and contralateral (CL) cerebral hemispheres in the tMCAO mouse model, and identified a novel MG cluster termed SAM (Figure 2D). Distinct from homeostatic MG, SAM exert potent antioxidant effects in a Peroxiredoxin-1 (Prdx1)-dependent manner.⁴⁵ Specifically, SAM exhibit robust antioxidative capacity by upregulating a battery of ROS-scavenging genes, thus alleviating oxidative stress-induced tissue injury. Moreover, SAM highly express *Spp1* and *Fth1*; the protein product of *Spp1*, OPN, can bind to integrin receptors and Cd63 on microglial surfaces to enhance tissue repair capacity.⁶¹⁻⁶³ Notably, Prdx1 deficiency leads to a specific depletion of the SAM population, which in turn exacerbates cerebral infarction volume. Mechanistically, microglial *Spp1* expression is tightly regulated by the Prdx1-dependent antioxidant pathway. The high level of *Spp1* expression in SAM, coupled with its downregulation in Prdx1-deficient MG, indicates that SAM serve as a critical cellular source of OPN. Given the phenotypic similarity between SAM and *Spp1*⁺MG, we hypothesize that they may share a common cellular origin. Further investigation is warranted to clarify whether they represent independent subpopulations with distinct functional specializations, or alternatively, the same subset at different stages of temporal differentiation.

5.3. Ischemic Core-associated MG (ICAM) and Ischemic Penumbra-Associated MG (IPAM)

Unlike the ischemic core where cells undergo irreversible necrosis, the ischemic penumbra exhibits functional impairment due to inadequate blood supply post-ischemia, with its resident cells retaining limited viability and sustaining reversible damage.^{64,65} The ischemic core and penumbra differ significantly in the extent of injury and metabolic profiles,⁶⁵ and such microenvironmental disparities further drive the phenotypic and functional specialization of MG. Guo et al characterized the cellular heterogeneity and constructed a cell atlas of the cortical penumbra,⁴³ demonstrating that MG constitute the most abundant immune cell population in this region and can be classified into as many as 14 distinct phenotypes. Notably, multiple microglial subclusters are highly enriched within this area. The molecular and functional properties of MG are spatio-temporally regulated.^{18,21} Li et al integrated spatial transcriptomics (ST) with scRNA-seq and identified two spatially distinct stroke-associated microglial subclusters in the ipsilateral hemisphere following MCAO.⁴⁸ According to their spatial localization, these subclusters were designated as ICAM (Figure 2A) and IPAM (Figure 2B). Markers of ICAM are highly concentrated in the ischemic core. Driven by DAMPs and the transcription factor BACH1, ICAM relies on glycolysis for energy production and exerts robust pro-inflammatory and chemotactic activities. Knockdown of BACH1 can downregulate the expression of ICAM-specific markers and pro-inflammatory cytokines, thereby attenuating its pro-damaging effects.⁴⁸ In contrast, IPAM aggregates in the ischemic penumbra, where its differentiation is triggered by glucocorticoids. This subcluster depends on the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) for energy metabolism, displaying anti-inflammatory metabolic features and myelinotrophic properties to exert neuroprotective functions. Administration of glucocorticoids such as dexamethasone (DEX) or corticosterone can upregulate IPAM-specific markers and reduce infarct volume, whereas treatment with the glucocorticoid receptor antagonist RU486 exacerbates ischemic brain damage.⁴⁸

5.4. Stroke-Activated Vascular-Associated MG (Stroke-VAM)

Neovascularization delivers an adequate supply of oxygen and nutrients to facilitate post-injury tissue repair and functional recovery in the chronic phase of IS.⁴⁴ MG can promote neovascularization and consolidate the integrity of the blood-brain barrier (BBB) by relying on their intimate crosstalk with vascular endothelial cells.^{44,66} By analyzing the injured hemisphere of transient MCAO mice, Li et al identified a distinct subset of stroke-VAM (Figure 2C). Gene set enrichment analysis revealed that unlike other clusters with distinct M1, M2, or DAM-like phenotypes, clusters 6 and 11 show robust associations with cerebrovascular functions. These cells are spatially localized around injured blood vessels, and are characterized by low M2-like marker expression, enhanced glycolysis, elevated OXPHOS, increased phagocytic activity, and concomitant mitochondrial fragmentation. The Fkbp5-Lats1-Yap1 signaling pathway potentiates the transcription of glycolysis and

OXPHOS-related genes, sustains the hypermetabolic activity and robust phagocytic capacity of stroke-VAM, and ultimately induces BBB disruption while suppressing neovascularization.⁴⁴ Fkbp5 acts as a pivotal regulator of stroke-VAM, and serum FKBP5 levels in patients are positively correlated with the clinical severity of the disease. Conditional deletion of Fkbp5 in MG suppresses post-stroke VAM activation, which in turn mitigates BBB disruption, neuroinflammation, and cerebral edema, ultimately improving ischemic stroke outcomes. SAFit2, a selective inhibitor targeting the Fkbp5 pathway, improves neurological function, reduces infarct volume, attenuates BBB leakage, and alleviates neuroinflammation in stroke models. Notably, in contrast to classical M2 subset, Fkbp5 deficiency in mice upregulates M2 markers in stroke-VAM; these markers are significantly enriched in pathways governing positive regulation of cell migration, modulation of cell morphology, regulation of apoptotic processes, and angiogenesis. Concomitantly, genes linked to endocytosis, synaptic vesicle endocytosis, chromatin remodeling, and protein trafficking are markedly downregulated. These alterations further underscore the unique heterogeneity of stroke-VAM, differentiating it from conventional microglial subsets.

Building on the acute phase stress response, the subacute phase is centered on clearing residual pathological products and re-establishing homeostasis, accompanied by profound dynamic alterations in immune cells. Through analyzing the dynamics of 8 functional subsets (MG1–8), Garcia et al demonstrated that, unlike the acute phase where MG transition to phenotypes linked to damage response, proliferation, and clearance-repair,⁴⁷ the subset repertoire in the subacute phase shifts toward disease-associated and refined repair-inflammatory phenotypes. Complemented by the proliferative subset (MG5), MG populations are restored.⁴⁷ During this period, MG subsets accumulate in the ischemic core, with marked upregulation of neuroprotective and inflammatory chemotaxis-related genes, while lacking expression of homeostatic or proliferation-associated genes. They synergize with other cell types to co-regulate the local injured microenvironment. Among these subsets: MG3 displays a disease-associated phenotype: while contributing to the clearance of protein fragments and repair of lipid metabolism, it highly expresses neurodegeneration-related genes. Mg6 functions as an inflammatory regulator and mediates residual damage clearance, yet its prolonged activation precipitates chronic inflammation and impairs repair. Mg7 co-expresses protective genes and damage response markers, and facilitates synaptic repair, though its persistent overexpression denotes an exaggerated response.⁴⁷ Residual neuronal debris and abnormal proteins in post-injury ischemic foci can sustain MG in a disease-associated state over the long term, promoting the deposition of pathological proteins, like A β and tau, disrupting synaptic connectivity, and ultimately precipitating cognitive impairment or even dementia. The molecular signatures of these subsets are highly congruent with the pathogenic mechanisms of neurodegenerative diseases such as AD and vascular dementia. Thus, targeting repair subsets (MG3, 6, 7) during the subacute phase to abrogate their transition from repair-competent to pathological phenotypes and halt the chronicization of neurodegenerative pathology holds promise as a novel strategy for treating post-stroke cognitive impairment (PSCI) and preventing neurodegenerative diseases.

5.5. SAM-Foamy and SAM-Iron

Persistent chronic neuroinflammation represents a major obstacle to post-stroke recovery. Microglial responses persist for weeks to months.⁶⁷ By phagocytosing cellular debris at the injured lesion site, MG undergo intracellular accumulation of free cholesterol and cholesterol crystals, which in turn exacerbates their inflammatory responses. In contrast to MG in the acute phase, Zhao et al demonstrated that a distinct population of foamy-MG emerges during the chronic phase (30-180 days post-stroke).⁵² Characterized by prominent lipid loading and sustained high expression of pro-inflammatory genes, these SAM-foamy (Figure 2E) exhibit sex-independent features and are marked by extensive cholesterol metabolic reprogramming.⁵² Owing to their enrichment with abundant lipid droplets and cholesterol crystals, SAM-foamy can continuously activate inflammatory pathways through cholesterol overload, directly impairing myelin sheaths, hindering white matter repair, exacerbating secondary brain damage and compromising neurological functional recovery. CYP46A1 is a brain-specific cholesterol 24-hydroxylase.⁶⁸ It facilitates the conversion of cholesterol into 24S-hydroxycholesterol, a metabolite that can cross the BBB, enter the systemic circulation and be cleared from the CNS. Depleting MG with PLX5622 has been shown to reduce lipid accumulation in these cells. Specifically, microglia-targeted overexpression of *Cyp46a1* or pharmacological activation of CYP46A1 using efavirenz (EFV) can enhance cholesterol metabolism, decrease intracellular lipid droplet accumulation, suppress persistent microglial inflammation and improve myelin integrity.

Beyond cholesterol accumulation, Due to the continuous degradation of necrotic tissue contribute to the release of iron ions, and exogenous transferrin infiltrates the lesion site, prompting infiltrating MG to engage in both active phagocytosis and passive uptake of iron. These processes synergistically enhance intracellular iron accumulation in MG. The study identifies a distinct subset of SAM-iron as well, which is specifically enriched in the core region of chronic lesions. This microglial subset exhibits diminished expression of canonical microglial markers, yet robustly upregulates iron metabolism-associated genes such as *Fth1* and *Fthl1*. Characterized by dysregulated iron metabolism and massive intracellular iron ion accumulation, SAM-iron MG participate in iron ion clearance and homeostasis regulation within the lesion area, while displaying a

moderately pro-inflammatory phenotype. Intracellularly accumulated Fe^{2+} acts as a highly potent catalyst: it triggers the Fenton reaction with hydrogen peroxide (H_2O_2) generated via cellular metabolism, producing highly reactive hydroxyl radicals.^{69,70} These radicals attack cell membranes and unsaturated fatty acids in lipid droplets, initiating lipid peroxidation cascades, promoting cellular ferroptosis, and ultimately impeding neural recovery.⁷¹ However, whether cross-talk occurs between SAM-iron and SAM-foamy to form a vicious cycle of iron accumulation and lipid metabolic dysfunction—one that perpetuates inflammatory damage in the chronic phase, exacerbates poor post-stroke outcomes, and even precipitates neurodegenerative-like pathological changes,^{70,72,73} remains an unresolved question with substantial implications for the development of novel therapeutic targets.

The elderly population represents the primary demographic affected by stroke.¹ Aging can modify the phenotype of MG, and both elderly stroke patients and animal models demonstrate impaired recovery capabilities. Compared to young mice, aged mouse models of stroke exhibit not only enhanced transcriptional signatures of chronic inflammation in MG,⁵³ but also significant suppression in the transcription of key functional gene clusters involved in immune-inflammatory responses, immune cell chemotaxis, tissue remodeling, and intercellular interactions.^{53,54} Lu et al further confirmed that in aged mice following cerebral ischemia, the number of MG-related genes, the breadth of pathway engagement, and the overall activation intensity were markedly reduced, along with significant declines in MG migratory, chemotactic, and intercellular interaction capacities.⁵⁴ These alterations may constitute critical mechanisms underlying the increased susceptibility and poorer functional recovery after cerebral ischemia in aged mice. Although MG dysfunction accompanies aging, experimental depletion of MG using CSF1R inhibitors exacerbates infarct volume post-stroke. This indicates a protective role of MG during the early phase of acute ischemic stroke in aged mice, suggesting they are not merely detrimental inflammatory mediators.⁷⁴ Nevertheless, microglial responses in aged stroke may possess a distinct heterogeneity compared to those in younger subjects. For instance, whether specific functional phenotypes such as *OASL*⁺MG emerge and play a substantive role in aging-related pathological and repair processes, thereby influencing neurological recovery and cognitive outcomes, remains to be fully elucidated.

6. Regulatory Mechanisms of Microglial Heterogeneity

The functional heterogeneity of microglial subpopulations dictates the pathological trajectory of injury. The heterogeneity is orchestrated by convergent regulatory mechanisms, including genetic background and dynamic epigenetic remodeling, metabolic reprogramming (MR) under extrinsic microenvironmental stress and intercellular crosstalk network, that collectively ensure precise, context-dependent responses to injury signals.

6.1. Genetic and Epigenetic Regulation: Intrinsic Determinants of Heterogeneous Subsets

During the early stages of embryonic development, MG migrate from the yolk sac to the brain, undergoing a maturation process that is closely aligned with brain development stages and is meticulously regulated by genetic programs.^{32,75,76} Throughout this developmental process, MG progressively adapt to their specific identity and respond to environmental stimuli. The inherent differentiation and genetic polymorphisms present during development further amplify these genetic distinctions. These fundamental functional characteristics of MG are crucial in determining their diverse differentiation trajectories following IS injury. Epigenetic modifications play a pivotal role as a regulatory nexus, linking genetic background with microenvironmental signals and contributing to normal brain development. Moreover, they are deeply involved in the remodeling of microglial phenotypes and functions during the pathological processes following the stroke.⁷⁷ Although epigenetic modifications do not directly alter the cellular genome, they regulate gene expression through mechanisms such as histone modifications, DNA methylation, and non-coding RNAs, thereby participating in the regulation and reprogramming of cell phenotypes.^{2,77,78} Recent studies have elucidated the pivotal role of the epigenetic modification network in the state transition of MG following stroke and in IS. During temporally dynamic regulation, epigenetic modifications facilitate the stage-specific transformation of microglial subsets. For instance, during the transition from the acute to the subacute phase, acute phase stress triggers an upregulation of DNA methyltransferase (DNMT) activity, leading to the demethylation of promoters of pro-inflammatory genes while maintaining high methylation levels at promoters of repair genes.^{75,79,80} This process promotes a predominant response from the pro-inflammatory subpopulation.^{80,81} In contrast, during the subacute phase, there is a reversal and remodeling through the demethylation of repair gene promoters, which enhances the activity of histone acetyltransferases (HATs) and promotes the transcription of anti-inflammatory repair genes.⁷⁹⁻⁸²

In terms of spatially specific regulation, epigenetic modifications enable MG with identical genetic backgrounds to exhibit differentiated gene expression across various brain regions.^{81,83} This modification heterogeneity becomes more pronounced under the pathological conditions of stroke. For instance, MG within the demyelinated regions of the corpus callosum exhibit

notably elevated expression of genes such as *CD74* and *Spp1*, which is dependent on the activation of the MHC II gene region. In contrast, MG in the ischemic hippocampus are distinguished by elevated expression of *Apoe* and *Cst7*, driven by epigenetic modifications related to the NF- κ B pathway. This variation contributes to the heterogeneity observed in repair efficiency across different brain regions following injury.^{84,85} Furthermore, considering the adaptability of MG, it raises the question of whether epigenetic mechanisms possess a form of memory that governs microglial involvement in specific phenotypes or activation states. Does this epigenetic memory of distinct events account for the differential phenotypic response to stroke injury? This remains an area for future investigation.^{33,86}

6.2. Coupling of MR and Intercellular Crosstalk Under Microenvironmental Stress: Extrinsic Drivers of Microglial Phenotypic Shifts

Metabolic alterations under microenvironmental stress are intricately linked with intercellular communication, driving phenotypic changes in MG.^{85,87} The stress-induced microenvironment and ensuing energy crisis in the brain precipitate specific metabolic transformations within MG. MG exhibit a pronounced reliance on glucose metabolism under both physiological and pathological conditions.⁸⁸ Microenvironmental stress initiates the microglial response and induces metabolic reprogramming, characterized by a comprehensive shift in the metabolic network to meet the energy demands imposed by hypoxia and energy deficiency during injury. MR constitutes an active process through which cells modulate various metabolic pathways to alter their phenotype, balance energy, and fulfill modular requirements.^{89,90} Following IS injury, the metabolic reprogramming of MG in terms of glucose metabolism is evidenced by a reduction in mitochondrial OXPHOS activity, alongside an upregulation of glycolysis and the pentose phosphate pathway (PPP).⁹¹

The MR of MG is predominantly influenced by the HIF-1 α signaling pathway and the upregulation of the pentose phosphate pathway, which directly induces the expression of glycolysis-related genes in MG, thereby promoting an inflammatory phenotype and cytokine production.⁹⁰ Several studies have demonstrated that early inhibition of glycolytic metabolic reprogramming in MG can mitigate their excessive pro-inflammatory responses.^{89,92} The metabolic models based on ICAM and IPAM also demonstrate phenotypic alterations in MG under environmental stress.⁴⁸ MG exhibit heightened activity in lipid and amino acid metabolism, particularly in pathways associated with lipid, cholesterol, and phospholipid metabolism, as well as amino acid biosynthesis following ischemic stroke.⁴⁶ As precursors of CNS neurotransmitters, disruptions in amino acid metabolism, particularly glutamine metabolism, can significantly impact microglial phenotype and function.^{89,93} Lipids function as signaling molecules that activate the phagocytic activity of MG. In response to pathogenic stimuli, MG undergo transformations such as the formation of phagocytic vesicles, necessitating energy acquisition through lipids or the expansion of biological membranes. In their resting state, MG express lipid transport proteins and key genes involved in lipid metabolism.^{89,94,95} Upon stimulation by lipopolysaccharides (LPS), MG upregulate genes associated with fatty acid synthesis and enhance the expression of genes involved in fatty acid uptake and oxidation.⁸⁹ This indicates that the reprogramming of fatty acid metabolism plays a role in the phenotypic alterations of MG.

Furthermore, the affected area comprises a complex network of neurons, glial cells, and infiltrating peripheral immune cells. Following ischemic events, microglial activity is augmented through ligand-receptor interactions, such as *APOE-TREM2* and *Fn1-Itga3/4+Itgb1*, facilitating enhanced interactions with pericytes, endothelial cells, oligodendrocyte precursor cells (OPCs), and other cell types, thereby reconfiguring the communication network.⁴⁶ Infiltrating peripheral immune cells modulate the heterogeneity of the phenotypic regulatory network through direct cell contact or the secretion of signaling molecules. For instance, pro-inflammatory signals from neutrophils can induce M1/M2-like polarization, mediated by inflammatory storms, while regulatory T cells secrete OPN and other cytokines via *CD44* and integrin receptors, contributing to the shaping of phenotypic heterogeneity. The enrichment, signal interactions, and temporal distribution differences in the injured area create a distinct stress microenvironment and metabolic pattern, which drive the phenotypic differentiation of MG. Currently, most studies in this field rely on the binary M1/M2 paradigm. This article provides a concise overview of the core relationship between metabolic reprogramming under microenvironmental stress and the intercellular communication coupling that drives the phenotypic changes in MG.

7. The Therapeutic Potential of Targeting Specific Subsets of MG

MG play a critical role throughout the entire process of post-stroke pathological damage. Currently, numerous basic research studies and clinical trials aim to exert therapeutic effects on IS by either inhibiting the M1-like phenotype of MG or promoting their polarization towards an anti-inflammatory repair phenotype. Given the diverse phenotypes of MG during various pathological stages, it is crucial to focus on facilitating the transformation of MG into beneficial phenotypes, preventing their shift from repair-oriented to pathological types, and enhancing the plasticity of their functions during the stroke disease process to improve the stroke outcomes. Core strategies in cell therapy include

directly supplementing or inducing beneficial repair phenotypes, as well as depleting or inhibiting the pathological types and their transformation. These approaches aim to achieve subtype-specific precise interventions to enhance the efficacy and safety of treatment. We have summarized some characteristics of the novel microglial clusters and their potential cell therapy directions in [Table 3](#).

7.1. Transplantation or Depletion of Specific Subsets of MG

The primary advantage of transplanting beneficial subset cells lies in their ability to directly replenish functional, intact MG with advantageous phenotypes. This approach rapidly facilitates tissue repair and addresses the limitations associated with the insufficient proliferation and diminished function of endogenous repair subsets. Currently identified beneficial microglial phenotypes specifically target mechanisms operative during the post-stroke period, particularly in elderly patients. By elucidating the core target cell phenotype for transplantation, understanding the source of cells, and refining in vitro induction strategies, it becomes possible to directly infuse beneficial MG phenotype. For instance, the in vivo source of *Spp1*⁺MG is the directed differentiation of inherent proliferative cells (*Mki67*⁺MG) within the brain.⁵¹ This differentiation process is regulated by the PIS model, and the functional expression of these cells is highly dependent on their spatial positioning within the ischemic penumbra, which identifies essential parameters for the optimal site and timing of cell transplantation. It is crucial to administer the cells specifically into the ischemic penumbra, selecting a transplantation window during the early critical phase post-stroke to align with the optimal period for injury repair. A primary issue in elderly stroke patients is the diminished functionality of their microglial cells, characterized by a low proportion of *CH25H*⁺ beneficial subsets and a natural predominance of *OASL*⁺ pro-inflammatory subsets. These cells exhibit limited proliferation and differentiation capabilities and are susceptible to compounded inflammatory stress following surgical intervention. Transplanting beneficial subsets can circumvent the inherent proliferation and differentiation deficiencies of aged microglial cells, allowing for the direct deployment of neuroprotective cells within the ischemic region.⁴⁹ Moreover, elderly patients frequently exhibit combined dyslipidemia and metabolic abnormalities, positioning them as a high-risk group for post-stroke cognitive decline. Consequently, elucidating the subgroups and core functions is instrumental in facilitating targeted cell transplantation therapies tailored to various stroke-related complications in the elderly. The accumulation of pathological microglial contributes to microenvironmental damage, which impedes the proliferation and differentiation of endogenous beneficial subgroups and hinders neural recovery. Traditional approaches that broadly deplete microglial indiscriminately eliminate both beneficial and pathological MG. This is particularly problematic for elderly patients, as the overall reduction in MG, coupled with issues such as diminished immunity, can lead to central immune deficits and an increased risk of infection. The heterogeneous phenotype of MG can precisely identify depletion targets, thereby preserving endogenous beneficial MG. For instance, antibody-drug conjugates (ADCs) can be designed by leveraging the defining molecular signatures of distinct pathological microglial subsets: high expression of *APOE* and *Trem2* in SAM-foamy MG, and elevated *Fth1* expression in SAM-iron MG. Such ADCs selectively bind to surface markers of these pathological subclusters via antibody targeting, thereby achieving their specific ablation.⁴⁴

7.2. Therapies Targeting Subtype-specific Genes, Pathways, or Receptors With Agonists/Antagonists

The heterogeneity observed in MG underscores the presence of distinct functional subsets, each characterized by unique transcription factors, signaling pathways, and surface receptor expression profiles. For example, the *Prdx1*-dependent SAM subset, the stroke-VAM subset modulated by the *Fkbp5*-*Lats1*-*Yap1* pathway, and the *Spp1*⁺MG subgroup, which specifically expresses the GLP1R receptor, exemplify this diversity. These molecular entities play a crucial role in regulating the activation, differentiation, and functional maintenance of their respective subtypes, rendering them ideal targets for the precise modulation of microglial subtypes. Consequently, the development of antagonists or agonists targeting specific genes, pathways, or receptors offers a strategy to selectively modulate microglial subtypes while minimizing interference with stable microglial populations and other neural cells, thereby reducing off-target effects. Drug therapy can precisely achieve the inhibition or activation of microglial phenotypes by designing specific agonists or antagonists and combining their expression characteristics. For example, the specific receptor of the *Spp1*⁺MG subgroup expresses GLP1R, and the GLP1R agonist semaglutide promotes the proliferation of this subgroup, upregulates the anti-inflammatory phenotype, and simultaneously improves the expression of tight junction proteins of the blood-brain barrier. For the stroke-VAM characteristic of the pro-damage subgroup, the *Fkbp5*-specific antagonist SAFit2 specifically blocks the activation of the downstream pathway of *Fkbp5*, exerting the inhibitory function of stroke-VAM activation.⁴⁴ Based on the in-depth analysis of its heterogeneity, a temporal combination therapy of antagonists and agonists for subtypes can be constructed to achieve precise regulation of heterogeneity.

Table 3. Characteristics and Potential Functional Implications of Novel Microglial Clusters

Name	Spatio-temporal localization	Marker genes	Functional verification	Potential therapeutic strategies	Ref.
Clearing MG	Day 2 of acute phase, infarct border and ischemic core	<i>Spp1</i> <i>Msr1</i> <i>Lgals3</i> <i>Ccl2</i> <i>Ccl12</i>	-	Transplantation therapy, specific antagonist or agonist therapy: Targeted enhancement of cell number or function helps ameliorate insufficient clearance function in the acute phase. Suitable for elderly patients or those with MG dysfunction	Garcia et al. 47
MKI67+ MG	ischemic penumbra, 3-21 days after surgery	<i>Mki67</i> <i>Top2a</i>	-	Transplantation therapy, specific antagonist or agonist therapy, cytokine therapy: Supplement insufficient number of MG in the acute phase or induce targeted differentiation towards beneficial phenotypes	Zhang et al. 49
OASL+MG	Peri-infarct area	<i>OASL</i> <i>Ifitm3</i> <i>Irf7</i>	Aged mice: proportion of OASL+ MG↑ infarct volume↑	Specific depletion therapy, specific antagonist therapy: Targeted inhibition of activation or proliferation of OASL+ subsets, especially suitable for elderly patients or those with severe inflammatory response	
CH25H+MG	Ischemic penumbra	<i>CH25H</i> <i>Spp1</i> <i>Gpnmb</i>	Ch25h gene knockout mice: infarct volume↑, BBB disruption↑ neurological function↓	Transplantation therapy, specific agonist therapy, cytokine therapy: Targeted activation of these cells or promotion of their differentiation helps facilitate neurological function recovery or supplement the function of protective subsets	
stroke -VAM	Perivascular regions of the ischemic hemisphere	<i>Flt1</i> <i>Slc2a1</i> <i>Tjp1</i> <i>Cldn5</i> <i>Cdh5</i> <i>Hk2</i> <i>Atp5a1</i>	Fkbp5 knockout mice: infarct volume↓, angiogenesis↑, BBB leakage↓. Administration of Fkbp5 inhibitor SAFit2: neurological function↑, infarct volume ↓. Overexpression of Yap1 gene: Reversed the inhibitory effect of Fkbp5 knockdown on the cell phenotype	Specific antagonist therapy: Serum FKBP5 protein concentration in patients can be used as a prognostic stratification marker Targeted inhibition of the Fkbp5-Lats1-Yap1 signaling pathway to regulate the stroke -VAM cell phenotype and improve the BBB function of cerebral blood vessels	Li et al. 44

(continued)

Table 3. (continued)

Name	Spatio-temporal localization	Marker genes	Functional verification	Potential therapeutic strategies	Ref.
SAM	Injured hemisphere	<i>Prdx1</i> <i>Txn1</i> <i>Srx1</i> <i>Mt1/2</i>	Prdx1 gene knockout mice: infarct volume ↑, neurological function↓.	Prdx1+ MG transplantation therapy, specific agonist therapy, cytokine therapy: Increase the number of SAM, or enhance the function of ROS defense gene network, thereby improving anti - inflammatory and cytoprotective functions	Kim et al. 45
IPAM	Appears at 3 hours post - ischemia and persists in the ischemic penumbra	<i>Pik3ip1</i> <i>Cd300lf</i> <i>Gpr65</i> <i>Ms4a6c</i> <i>Cep152</i> <i>Ddit4</i> <i>Zbtb16</i> <i>Abhd15</i>	Glucocorticoid - induced primary MG: expression of IPAM - specific markers↑, activity of the TCA and OXPHOS pathways↑. Intraperitoneal injection of corticosterone in MCAO mice: infarct volume↓ expression of IPAM - specific markers↑, neurological function↑.	Specific agonist therapy, cytokine therapy: Promote the expansion of IPAM number or enhancement of its function, improve the microenvironment of the ischemic penumbra, and limit the expansion of the ischemic core.	Li et al. 48
ICAM	Appears at 3 hours post - ischemia and persists in the ischemic core from 12 to 72 hours	<i>Lgals3</i> <i>Plau</i> <i>Srxn1</i> <i>Ankrd33b</i> , <i>Gas2l3</i> <i>Nes</i> <i>Edn1</i> <i>Fam129b</i>	BACH1 - knockdown MG: expression of ICAM - specific markers↓, asecretion of pro - inflammatory ↓, factorsStimulation of primary microglia with DAMPs/LPS:expression of ICAM - specific marker genes↑, pro - inflammatory factors and chemokines↑.	Specific antagonist therapy, cytokine therapy: Inhibit the activation of ICAM and limit the expansion of the injury area in the ischemic core.	
Spp1+MG	Ischemic penumbra	<i>Spp1</i> <i>Iba-1</i> <i>CX3CR1</i> <i>GLP1R</i> <i>Arg1</i> <i>CD36</i>	Intraperitoneal injection of semaglutide in model mice: proportion of pro - inflammatory cells↓, Cerebral infarct volume↓, BBB leakage↓, motor function↑.	Transplantation therapy, specific agonist therapy, cytokine therapy: Promote the expansion of cell number and exertion of its function, enhance its anti - inflammatory and phagocytic capacity, and improve the expression level of tight junction proteins in the BBB	Li et al. 51

(continued)

Table 3. (continued)

Name	Spatio-temporal localization	Marker genes	Functional verification	Potential therapeutic strategies	Ref.
SAM-foamy	MCAO-injured area, persists for 3-180 days	<i>Apoe</i> <i>Lpl</i> <i>Trem2</i> <i>Nceh1</i> <i>Fabp5</i> <i>Soat1</i> <i>Abca1</i>	MG - specific overexpression of <i>Cyp46a1</i> gene: Increased myelin G ratio↑, lipid droplet area in MG↓, narrowed injury area↓ Treatment with the CYP46A1 activator EFV: neurological function↑, inflammation - related genes↓, injury area↓.	Microglia - specific depletion therapy, specific agonist therapy: Reduce the phagocytosis of lipids in the injured area by microglia and promote the metabolic conversion of cholesterol	Zhao et al. 52
SAM-iron	Injured core area, persists for 30 - 90 days	<i>Fth1</i> <i>Ftl1</i>	-	Specific agonist or inhibitor therapy: Inhibit iron accumulation in these cells, which may alleviate oxidative stress and inflammatory response induced by iron overload; or upregulate the expression level of iron transporters	

7.3. Cytokine Therapy

Given the remarkable perceptiveness and adaptability of MG, along with their subtype-specific response characteristics, the aberrant microenvironment in the brain post-stroke can be modulated through exogenous supplementation or endogenous induction. This modulation can indirectly influence the differentiation and functional phenotype of MG subtypes, thereby minimizing the off-target effects associated with monotherapies and enhancing the overall efficacy and safety of indirect cell therapy. For instance, prior research has elucidated the protective and coordinating functions of OPN. OPN not only facilitates the localization and survival of Spp1⁺MG, contributing to the maintenance of their anti-inflammatory protective phenotype and neuroprotective functions, but also plays a crucial role in sustaining the stability of small acidic molecules. It enhances their antioxidant and anti-apoptotic properties, thereby augmenting the neuroprotective effects of SAM in the context of acute ischemic stroke. In addition to OPN, a variety of cytokines are capable of achieving precise regulation of the microenvironment and functional intervention by specifically targeting the heterogeneous subtypes of MG. These cytokines exhibit distinct effects that are specific to particular subtypes and follow a defined pathological temporal sequence. While previous research has extensively reviewed the regulatory roles of cytokines in the M1/M2-like phenotypes,⁹⁶⁻⁹⁸ the mechanisms underlying the newly identified subtypes remain largely unexplored. [Table 3](#) provides a summary of the partially characterized novel microglial subtypes and potential therapeutic directions; further elaboration is beyond the scope of this discussion. Cytokine therapy has the potential to create a synergistic effect when combined with the aforementioned treatments, thereby addressing the limitations associated with monotherapy.

8. Challenges and Prospects

ScRNA-seq has profoundly delineated the spatiotemporal dynamics, functional diversity, and metabolic complexity of MG, revolutionizing our simplistic dichotomous view of MG. It has revealed that multiple highly specialized subpopulations orchestrate the complex pathological processes of injury response and repair following stroke.^{22,27} While scRNA-seq has greatly advanced research on MG heterogeneity, notable gaps remain in methodology, mechanistic elucidation, and clinical translation. Currently, when capturing transcriptomic profiles of large cell populations, scRNA-seq often sacrifices sequencing depth per cell to balance cell throughput, which leads to the underrepresentation or undetection of functional genes in MG that are regulated by low-abundance factors, potentially resulting in misclassification of cell subtypes. Second, differences in microglial activated phenotypes and functional orientations are tightly linked to their spatial localization and microenvironmental cues. However, tissue dissociation

required for scRNA-seq disrupts the inherent spatial positioning and microenvironmental connections of cells, making it challenging to clarify the triggers of heterogeneity. Furthermore, given the significant difficulties in obtaining healthy or diseased human brain samples and tracking dynamic changes, most current studies on microglial heterogeneity are confined to small clinical cohorts or animal models.^{34,35} Nevertheless, brain tissue injury involves a complex and dynamically continuous pathological process. Most available small clinical samples are derived from patients, which may exhibit substantial interindividual variability and fail to adequately characterize population-level MG phenotypic features. Various discrepancies, including those in animal models, tissue sampling sites, timing, and protocols for dissociation and sequencing, contributing to marked inconsistencies across different research findings. The tissue dissociation process itself damages cellular structures, inducing cell injury and increased debris, which in turn impairs cell viability and the quality of sequencing data. Even though scRNA-seq has successfully identified multiple novel microglial subtypes post-stroke, several critical issues persist: insufficient functional validation of these new subtypes, unclear molecular regulatory mechanisms underlying heterogeneity, and ambiguous specificity of downstream effector pathways for distinct subtypes. Additionally, there are discrepancies in pathophysiological characteristics between animal models and human stroke, and the consistency of subtypes identified in animal models with those in human stroke samples awaits further verification, all of which hinder the clinical translation of heterogeneity research.

Building upon the above considerations, future research should leverage integrated multi-omics technologies to deepen and broaden the analysis of microglial heterogeneity. For instance, spatial sequencing approaches could be employed to precisely map the distribution of distinct microglial subtypes within damaged brain tissue and to clarify their interactions with neighboring cells as well as their modulation by the local microenvironment, thereby addressing limitations inherent to scRNA-seq alone. To tackle core questions regarding functions and mechanisms of specific subtype, integrating data from single-cell transcriptomics, proteomics, and metabolomics will strengthen the identification of specific molecular targets, help screen for its own markers and regulators, and ultimately provide candidate molecules for validation in both in vitro and in vivo models. Furthermore, dynamically tracking spatial redistribution of microglial subtypes across different pathological stages will help elucidate their origins, proliferation and differentiation trajectories, and rules governing functional transitions. This longitudinal approach will clarify how microglial heterogeneity evolves over the course of pathology and identify key regulatory factors at each stage, moving beyond descriptions of a single-time point.

The translational potential of MG heterogeneity resides in the realization of specific subtype interventions, encompassing the development of its targeted drugs, in vitro expansion or genetically modified targeted cell therapies, and the construction of precision therapeutic strategies. As a three-dimensional model that recapitulates the in vivo brain microenvironment, brain organoids can replicate post-stroke pathological features such as ischemia and inflammation, serving as an ideal platform for the in vitro expansion and functional preservation of MG-specific subtypes. Moving forward, priority should be given to leveraging brain organoids as an in vitro validation system to assess key aspects, including the in vitro preparation and expansion of specific subtypes, genetic modification efficacy, functional performance, safety profiles, and individualized validation. This approach will facilitate the optimization of cell therapies and targeted drugs, thereby enabling the precise establishment of a therapeutic regimen focused on microglial reparative subtypes.

MG are innate immune cells residing in the CNS. Upon stroke onset, they rapidly sense alterations in danger signals, undergo phenotypic switching and migrate to the lesion site, contributing to tissue repair and neural function remodeling by clearing damaged debris, suppressing excessive inflammation, and secreting neurotrophic factors.^{5,8,99} Notably, the response of post-stroke MG is not confined to the traditional M1/2 dichotomy; instead, it encompasses a spectrum of diverse phenotypes with pro-inflammatory, antioxidant stress, and angiogenic properties. The scRNA-seq has overcome the averaging limitation of traditional bulk sequencing in analyzing cell populations, emerging as a key tool for identifying novel MG subtypes. Recent studies have uncovered multiple MG subtypes with phenotypes and functions distinct from the classic M1/2 paradigm, such as Prdx1⁺ SAM and stroke-VAM, and delineated the dynamic shifts in their functional phenotypes throughout different pathological stages of stroke. This heterogeneity is tightly associated with epigenetic modifications and the remodeling of the local microenvironment.

While scRNA-seq serves as a pivotal foundational tool for dissecting MG heterogeneity, it is undeniable that this technology harbors inherent limitations, including inadequate sequencing depth, lack of spatial context, and inconsistencies arising from stroke sample variability. Furthermore, the newly identified subtypes in current research still face bottlenecks in functional validation, upstream and downstream pathway elucidation, and dynamic trajectory tracking. Clarifying these core issues represents a critical entry point to link the functions of these subtypes to the repair mechanisms following ischemic injury, facilitate the clinical translation of subtype-specific targeted drugs and cell therapies, enable precise individualized treatment for IS, and ultimately translate basic research findings into tangible improvements in patient outcomes.

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The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Agreement Statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the Corresponding Author is the sole contact for the Editorial process. He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

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