Review

Cell Heterogeneity Uncovered by Single-Cell RNA Sequencing Offers Potential Therapeutic Targets for Ischemic Stroke

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ABSTRACT: Ischemic stroke is a detrimental neurological disease characterized by an irreversible infarct core surrounded by an ischemic penumbra, a salvageable region of brain tissue. Unique roles of distinct brain cell subpopulations within the neurovascular unit and peripheral immune cells during ischemic stroke remain elusive due to the heterogeneity of cells in the brain. Single-cell RNA sequencing (scRNA-seq) allows for an unbiased determination of cellular heterogeneity at high-resolution and identification of cell markers, thereby unveiling the principal brain clusters within the cell-type-specific gene expression patterns as well as cell-specific subclusters and their functions in different pathways underlying ischemic stroke. In this review, we have summarized the changes in differentiation trajectories of distinct cell types and highlighted the specific pathways and genes in brain cells that are impacted by stroke. This review is expected to inspire new research and provide directions for investigating the potential pathological mechanisms and novel treatment strategies for ischemic stroke at the level of a single cell.

Key words: single-cell RNA sequencing, ischemic stroke, cellular heterogeneity, differentially expressed genes

1. Introduction

Stroke, including ischemic stroke (IS) and hemorrhagic stroke (HS), is the primary cause of death among adults, worldwide, and is characterized by high disability, morbidity, and mortality [1]. Among them, 75–80% of the cases are of IS due to the interruption of cerebral blood flow. It imposes substantial economic and social burdens especially among the elderly [2, 3]. The neurovascular unit (NVU) is comprised of neurons, glial cells, and vascular-associated cells. The current understanding of pathological mechanisms after stroke is based on the multicellular interactions within the NVU and peripheral immune cells [4], both involved in the evolution of damage to the blood-brain barrier (BBB), glial reactions, neuronal cell death, and immune cell infiltration [5-7]. Nevertheless, NVU and peripheral immune cells exhibit highly heterogeneous responses to IS [8], therefore, posing a challenge for evaluating the accurate roles of the specific cell subpopulations during IS [9]. Recently, rapidly evolving high-throughput sequencing technologies including single-cell RNA sequencing (scRNA-seq) have aided the construction of a comprehensive reference map of cell transcriptional states and facilitated further studies on cell heterogeneity in stroke conditions at the level of a single cell [10-15].
scRNA-seq may thus contribute to identifying new potential biomarkers, therapeutic targets, and elucidating the molecular underpinnings underlying the pathological processes in IS [16].

In this review, we have provided an overall introduction to scRNA-seq technology and its promising application in studies related to stroke. Moreover, we have summarized the multiple subpopulations within each cell type and the differentially expressed genes (DEGs) in each of the cellular subpopulations implicated in stroke as evidenced by scRNA-seq. We hope that this review will provide novel insights for further discoveries of novel specific biomarkers and signaling pathways involved in the pathological processes and effective treatment modalities for IS.

2. scRNA-seq of cell transcriptome in stroke

High-throughput scRNA-seq has enabled an unbiased determination of cell heterogeneity at a high resolution and the identification of cellular markers by the assessment of the transcriptomic profiles at the single-cell level [17], thus combining neuroscience with computational biology [18, 19]. scRNA-seq unveils molecular taxonomy and gene regulatory mechanisms of brain cells in neurological disease conditions with unprecedented precision and depth [20-23]. The workflow of scRNA-seq includes tissue dissociation, cell isolation, single-cell partitioning, reverse transcription, library generation, sequencing, and finally the analyses [24]. The general workflow of scRNA-seq is illustrated in Figure 1.

Given that the current treatment strategies for stroke are limited to a narrow treatment window and have failed to address progressive neuronal degeneration and loss of function, scRNA-seq is performed to better understand the mechanisms underlying stroke and provide insights into novel potential therapeutic targets and biomarkers [25]. scRNA-seq provides new insights into the complexity of stroke at the molecular level based on the identification of the new cellular subpopulations and potential disease-specific mechanisms [26]. Additionally, scRNA-seq can be used to compare distinct subpopulations and cell states during stroke which is expected to provide an opportunity to understand stroke mechanisms in detail [26, 27]. Notably, some previous studies elucidate the changes in DEGs, cellular composition with pseudo-differentiation, or pseudo-time tools at cellular resolution through the manipulation of a given gene [11, 13]. The representative DEGs between...
middle cerebral artery occlusion (MCAO) and sham groups, along with the related enriched pathways and processes are presented in Table 1. scRNA-seq can be used to examine the regenerative potential of the brain [12]. Bobadilla et al. have confirmed the presence of dormant neural stem cells (NSCs) that express diverse lineage-specific combinations of genes in IS. Additionally, they have identified that interferon (IFN)-γ signaling affects the transition from the resting to the activated state in stem cells following ischemic injury, thereby providing possible signaling module targets for regeneration post-stroke [12]. However, more studies are needed to translate the results of academic research into clinical practice for investigating novel therapeutic interventions. Moreover, peripheral cells may provide an alternative route to evaluate the mechanisms of a stroke [28]. Understanding the transcriptional landscape in peripheral cells may uncover biomarkers or signatures of neurological diseases. Additionally, blood cells from patients can be easily obtained and are also suitable for the scRNA-seq analysis [29, 30]. Interestingly, whether a subpopulation of peripheral blood can serve as a ‘window’ into the ischemic tissue, remains to be addressed [30-32].

Table 1. Cellular heterogeneity by scRNA-seq in IS.

| Cell types                  | Refs. | Subclusters | DEGs in MACO group                                      | Representative pathways and process                                                                 |
|-----------------------------|-------|-------------|--------------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| Microglia                   | [13]  | 14          | Upregulated: Rcan1, Ccl4, Gadd45b, Cd83, Id2 Downregulated: Cx3cr1, P2ry12, Gpr34, Rgs2, Marcks | IL-17 signaling pathway and toll-like receptor signaling pathway, transcriptional mis-regulation     |
|                            |       |             | Upregulated: Ccl12, Ccl7, Cd72, Lihb4a, Spp1 Downregulated: Hpgd, Selplg, Gpr34, Siglech, P2ry12 | Positive regulation of microglia cell migration, lysosome, apoptosis, neutrophil chemotaxis            |
| Astrocytes                  | [13]  | 7           | Upregulated: Fkbp5, Jund, Cdkn1a, Bos, Cyr61 Downregulated: Hbb-bs, Gm3764, Sox2, Hes5, Phkg1     | Toll-like receptor signaling pathway, estrogen signaling pathway, MAPK signaling pathway, positive regulation of gene expression and metabolic processes |
|                            |       |             | Upregulated: Vim, Ay036118, Glap, Cdkn1a, Ccl4 Downregulated: App2, Itm2a, Ntm, Gria2, Dbp      | Signal transduction respiratory electron transport, cellular responses to stress                       |
| Oligodendrocytes            | [13]  | 9           | Upregulated: Htr1a, Sigk3, Tma16, Phacr3, Cdkn1a Downregulated: Slc1a2, Phyhipl, Sylt2, Marcks, Fabp5 | In response to L-glutamate and acidic amino acid transmembrane transporter activity, neurotransmitter transporter activity and oxygen-containing compounds |
|                            |       |             | Upregulated: Klk6, Phacr3, Gpd1, Tma16, Serpina3n Downregulated: Hs3st1, Omg, Lpar1, 1700047M11Rik, Hebp1 | Regulation of neuron projection and apoptotic process, glial cell differentiation, cytokine-mediated signaling pathway |
| Neurons                     | [13]  | 6           | Upregulated: Ay036118, Ccl4, Gfap, Mt1, Mt2 Downregulated: Cld2a4, Basp1, Stnm2, Nr2f1, Gm17750 | Nervous system development, cell differentiation, neurogenesis                                      |
| Endothelial cells           | [13]  | 5           | Upregulated: Lcn2, Mt2, Mt1, Akap12, Tmem252 Downregulated: Tgfβ2, Slc16a1, Car4, Tfrc, Hmcn1 7 | Cyclic nucleotide metabolism, glutathione metabolism, ROS detoxification and oxidative phosphorylation |
|                            |       |             | Upregulated: Tmem252, Lcn2, Lrg1, Plat, Cthb2a Downregulated: Hm2a2, Ifrit3, Spock2, Cxcl12, Rps27trt | Transport of small molecules, anion transport, regulation of cell death, cellular response to chemical stimulus |
| Pericytes                   | [14]  | 3           | Upregulated: Ccl11, Saa3, Timp1, Il11, Ednrb Downregulated: Tsc22d1, Pltp, Cxcl12, Itm2a, Dbp | Transport of small molecules, response to peptide, regulation of secretion                           |
| Vascular muscle smooth cells | [14]  | 6           | Upregulated: Rasl11a, Sdc4, Cdkn1a, Ccl4, Ifttm1 Downregulated: Myh11, Crmm1, Lbb, Pln, Fbx22 | Relaxation of muscle, calcium ion transmembrane transport, cytokine-mediated signaling pathway, cellular response to chemical stimulus |
| Fibroblast-like cells        | [14]  | 3           | Upregulated: Ccl4, Angpt4 Downregulated: Nme2, Ifi2721a, Dbp | Structural constituent of ribosome, myoblast differentiation                                      |
| Neutrophil                  | [14]  | 4           | Upregulated: Hcar2, Marcksl1, Cxcl2, Ccrf2, Cxcl3 Downregulated: Ly6g, Ngp, Cd177, Camp, Ltf | IL-1 signaling pathway, neutrophil degranulation                                                |
| Lymphocyte                  | [14]  | 6           | Upregulated: Lck, Gzma, Ccl3, Dusp2, Ccl5 Downregulated: Xcl1, Ly6d, Cd74, Lyz2, H2-Aa | Metallopeptase DUBs, leukocyte apoptotic process                                                 |
Taken together, the rapidly evolving scRNA-seq technology provides an unprecedented single-cell-resolution brain map and has led to the accumulation of big data which can be used to further examine aberrations in neurological disorders [33]. Although at present, the knowledge of the brain and neurological disorders including stroke is far from adequate, overwhelming progress is expected in the coming decade with the development of single-cell sequencing technologies.

3. Microglial heterogeneity in IS

Microglial cells, the resident immune cells of the central nervous system (CNS), show an uneven distribution in the main CNS structures, along with a distinct ontogeny [34, 35]. Microglia exhibit regional heterogeneity, with higher densities in the white matter and the substantia nigra [36-38]. Microglia have important physiological functions in the human body [34], including monitoring infections in the brain parenchyma, dysfunction, and damage in motile and ramified processes [39]. Additionally, they rapidly react to the pathological conditions through morphological and functional changes [40], thereby improving the ability of the microglia to adapt and yield a context-specific phenotype [41]. Microglia have two diverse functional phenotypes, namely the M1 pro-inflammatory and M2 pro-regenerative [42, 43]. However, this is an oversimplified classification that points to the M1-M2 dichotomy that represents only two extreme activation states and fails to explain the diversity of different microglial subpopulations in the diseased states of the brain [44].

3.1 Role of microglia in IS

As the major immune cells, microglia undergo morphological and functional changes induced by the interruption of cerebral blood flow and energy supply during the ischemia [45]. At the onset of reperfusion, microglia within the ischemic penumbra are activated and extend their cell protrusions to neighboring vessels, thereby regulating endothelial cell functions [46]. Simultaneously, the adaptable and modifiable features of microglia also contribute to cellular repair and remodeling after stroke [47]. Nevertheless, excessive activation of microglia due to damage-associated molecular patterns after IS result in the production of numerous proinflammatory cytokines, which can damage the BBB and brain cells, as well as influence the neurogenesis [48]. These distinct functions can be attributed to the different subclusters or phenotypes of microglia, including the destructive M1 and neuroprotective M2 types [49]. The lack of high-throughput unbiased approaches to evaluate microglial heterogeneity limits the study of the spatiotemporal distribution of their subpopulations in IS [50]. Nonetheless, the high-resolution scRNA-seq of microglia has provided new insights into the spatial and functional diversity of microglia, thereby expanding the knowledge of different phenotypic changes of microglia in ischemic stroke. In addition, scRNA-seq also contributes to the discovery of potential markers for the identification of different subclusters of microglia in IS [10].

3.2 Microglial heterogeneity in IS by scRNA-seq

In 2020, for the first time, Guo et al. had used scRNA-seq to investigate the cellular heterogeneity and molecular changes in mouse cortex penumbra at the acute stage of IS [13]. They used a model of MCAO and established a sham-operated group [13]. They have analyzed cellular heterogeneity during IS at a single-cell resolution between the MCAO and sham groups [13]. Microglial cells were found highly enriched in the MCAO group as compared to sham group [13], indicating that the microglia had undergone some significant alterations during IS. Another study reports a total of 275 DEGs in the microglia, the first among all the tested cells, between MCAO and sham group [14]. Together, these findings suggest the essential role of microglia in IS.

To better understand the microglial heterogeneity in IS, five unique microglial cell clusters were identified [14]. Microglia0 (MG0) was composed of cells mainly from the sham group. MG1 showed approximately equal percentages in both MCAO and sham groups, while MG2, 3, and 4 clusters were primarily composed of cells from the MCAO group. The highly expressed genes and possible functions of each microglial subcluster are presented in Table 2. MG1 cluster was verified by immunostaining and flow cytometry as CCL2+LGALS3 CXCL10 and CCL2+LGALS3+CXCL10+ [51, 52]. Notably, the MCP family of genes expressed in MG1 reportedly enhances chemotactic migration of peripheral immune cells into the infarcted areas of the brain [53]. The study indicates that the MG2 cluster expresses high levels of matrix metalloproteinases 12 (MMP12), which can damage the BBB after IS [54]. Additionally, ADAM8 is uniquely highly expressed in MG2 and is known to be involved in microglia-mediated neuroprotective effects through modulation of the TNF-R1 shedding [55].

In addition, five distinct transcription factor (TF) subsets were also identified using the single-cell regulatory network inference and clustering (SCENIC) method [56] and a regulatory network in microglia was obtained. In the MCAO group, MG0 and MG1 cells highly expressed cebp, Egr1, and Fos in 3h after ischemic-reperfusion. These TFs are related to the
immediate-early gene family [57]. Some candidate regulons, including Cebb3, Ets2, and Sp3 have been identified in MG2. Ets2 reportedly induces the pro-inflammatory phenotype of endothelial cells and improves the expression of VCAM1, MCP1, and IL6 [58]. Sp3 can regulate NCX1 expression in IS [59]. TFs associated with type I IFN responses like Irf7, Stat1, and Stat2 are enriched in MG3. MG4 highly expresses Nfyb, Myb12, and Ezh2. These indicate that MG4 is involved in cell mitosis, differentiation, and development [60]. Additionally, Dbp, Maf, and Klf2 are candidate TFs in MG0-3 identified in the sham group. However, Spi1 and Hif1a have been identified in MG0-4 from the MCAO group.

Table 2. Microglial heterogeneity by scRNA-seq in IS.

| Subpopulations | Highly expressed genes in IS | Possible functions | Refs. |
|----------------|-----------------------------|-------------------|-------|
| MG0            | Core microglial markers: Siglech, Selplg, TEME119, P2y12, Olfnl3, Gpr34 | Mainly composed cells from the sham group | [14] |
| MG1            | IER3, CCL7, CCL2, CCL12    | Involving in pro-inflammatory reactions | [14] |
| MG2            | MMP12, ADAM8, Fth1, Spp1, Lpl, Lilrb4, Lgals3 | The most inflammatory subcluster in ischemic stroke | [14] |
| MG3            | Cxcl10, Irf7, Ifit3, Isgl5  | Involving in shedding of TNF-R1 in microglia-mediated neuroprotective effects | [14] |
| MG4            | Stmn1, Top2a, Ube2c, Birc5   | Involving in response to virus and interferon-beta | [14] |

Another previous study divided 5108 microglia cells into 14 subpopulations from MCAO and sham groups (Table 1) [13]. Among the DEGs, Gadd45b could activate the TGF-β-smad3 pathway and reduce the infarct volume after IS [61, 62]. Gene set variation analysis (GSVA) was employed to investigate the responses of different microglial subclusters under hypoxic conditions [13]. Some genes that are highly expressed in the MCAO group have been proven subcluster-specific, such as Zbtb16 in subcluster 3; B4galnt1, Plek, and Gadd45b in subcluster 4; Iba1 and P2y12 in subclusters 6 and 8; Csrm1, Gadd45b, and Fkbp5 in subcluster 9, and Eif4ebp1 in subcluster 10 [13]. Inflammation-related pathways consisting of TGF-β, IL-2, and IL-6 and the hypoxia pathway are most enriched in subclusters 3, 4, 9, and 10 [13], indicating that these subclusters underwent an extreme inflammatory response during IS. In contrast, the Kras signaling pathway is enriched in subclusters 6 and 8, and thus, the two subclusters are more likely to survive after IS. Additionally, Arg1 and Ym1, the specific marker genes of M2 microglia [63, 64], are not completely expressed in the early period after a stroke [65], suggesting delayed differentiation of the M2 microglia. In addition, gene enrichment analysis suggests that the DEGs are extremely enriched in the IL-17 signaling pathway (NF-κB, Cebpb), toll-like receptor signaling pathway (Ccl4), and transcriptional misregulation (Cebpb, Id2, Gadd45b) [13].

Thus, the scRNA-seq of microglia indicates that these are prevalent in the early phases of IS and exhibit polarization. Several studies have attempted to regulate microglial activation in IS by inhibiting the M1 phenotype microglial activation, whilst stimulating microglial differentiation into the anti-inflammatory M2 phenotype to enhance restorative processes, axonal remodeling, neurogenesis, and angiogenesis [66, 67]. However, the traditional M1/M2 dichotomy oversimplifies the diversity of microglia due to substantial overlaps between the traditional M1/M2 subset. M1/M2 marker genes are not fully expressed in any of the above-mentioned microglial subclusters [13, 14]. Further application of scRNA-seq to investigate the mechanisms underlying microglial heterogeneity and multi-polarization during IS at single-cell resolution is thus imperative.

4. Heterogeneity in astrocytes in IS

Astrocytes, an abundant cell type in the CNS [68], are critical structural and functional components of the NVU [69]. Astrocytes are responsible for numerous housekeeping functions such as the BBB formation, regulation of cerebral blood flow, and cell communications [70-73]. In addition, they play critical roles in providing nutrition to adjacent neurons as most of the glycogen of the brain is stored in astrocytes [74]. Emerging evidence indicates two subpopulations of astrocytes, namely A1 and A2 [75]. A1 astrocytes, induced by proinflammatory factors like IL-1α and TNF-α, secrete components of the complement cascade. A1 astrocytes specifically express iNOS, regulated by the autocrine LCN-2 [76]. In contrast, protective A2 astrocytes can be induced by transcriptional activity of STAT3 in an effect to produce neurotrophic factors [68, 77].

4.1 Dual roles of astrocytes in IS
Astrocyte response to IS remains unclear. However, undoubtedly, they play important roles in immune responses after IS [78]. On the one hand, astrocyte inflammatory responses may exacerbate ischemic lesions during the acute phase after IS and the glial scar in the peri-infarct area may inhibit neuro-restoration during the late recovery stages [79, 80]. Inflammatory mediators like chemokines and cytokines are released by the BBB upon the destruction of the astrocyte gap junctions in IS [81]. On the other hand, astrocytes also contribute to neuroprotection by releasing neurotrophins and promoting angiogenesis, axonal remodeling, neurogenesis, along with the synaptogenesis [82-84]. Therefore, astrocytes are designated potential therapeutic targets for improving clinical outcomes following a stroke. Inhibition or further improvement in reactivity and function of astrocytes may depend on their location and specific subtypes, as also the time of ischemic lesion. Therefore, more research is required to deepen the understanding of the spatiotemporal dynamics of astrocyte transformation in IS. By employing scRNA-seq, the heterogeneity, distinct gene expression properties, and different transcriptomic patterns of astrocytes in IS can be studied, which could offer novel insights into stroke pathology and unveil potential drug targets [13].

4.2 scRNA-seq of astrocytes in IS

A total of seven subclusters among the 1,083 astrocytes can be detected in cases of IS using scRNA-seq (Table 1)[13]. Among them, subcluster 3 exhibits the greatest diversity in GSVA and pseudo-time trajectories [13]. The kras signaling pathway is enriched in subcluster 3, thereby contributing to neuroprotection and neuro-restoration during the post-acute phase of IS [13]. Additionally, thiamine metabolism and O-glycan biosynthesis functions in subcluster 3 are significantly different from those in other subclusters. Moreover, some specific genes are expressed in subcluster 3, including Cyr61, Sbno2, Socs3, and Klf4 [13]. Previous studies suggest that Socs3 may aggravate neuroinflammatory injury after stroke [85], while Klf4 confers vascular protection against cerebral ischemic injury [86]. SCENIC analysis suggests the overexpression of TFs, BTB and CNC homology 1 (Bach1), and CCHC-type zinc finger protein (Zcchc14), in the MCAO samples and upregulation of Ep300, Rbbp 5, and Mxi1 in the sham group [13].

Table 3. Gene markers and enriched processes in astrocyte (ASC) subclusters.

| Subcluster | Gene markers | Enriched process | Refs. |
|-----------|--------------|------------------|-------|
| ASC1      | Rbp1, C1ql2, Vcan, Pcdh15 | Transcription regulation, synapse function/plasticity, cell proliferation/migration, cell adhesion | [11] |
| ASC2      | Rbp1, Agt, Slc39a12, Gjp6, Sox9, Entpd2 | Transcription regulation, synapse function/plasticity, neurotransmission, gap junction, cell differentiation, metabolism | [11] |
| ASC3      | Rbp1, Txnip, Sox9 | Transcription regulation, immune function, cell differentiation | [11] |
| ASC4      | Nrsn2 | Vesicle transportation | [11] |
| ASC5      | Txnip, Kcnj8 | Immune function, ion modulation/binding | [11] |
| ASC6      | Enpp6, Vcan | Metabolism, cell proliferation/migration | [11] |
| ASC7      | Txnip, Pfn | Immune function, ion modulation/binding | [11] |
| ASC8      | Gjp6, Txnip, Sox9, Entpd2, Enpp6, Plekhh1 | Gap junction, immune function, cell differentiation, metabolism | [11] |
| ASC9      | Vcan, Pcdh15 | Cell proliferation/migration, cell adhesion | [11] |
| ASC10     | Rbp1, Txnip, Sox9, Entpd2, Nme9 | Transcription regulation, immune function, cell differentiation, metabolism, microtubule physiology | [11] |

During the recovery phase of stroke, microglia and astrocytes play important roles in engulfing synapses through MEGF10 and MERTK associated pathways [87, 88], while exhibiting distinct phagocytic characteristics in IS and HS [88]. Recently, it has been reported that microglial-specific knockouts of MEGF10 or MERTK increase the dendritic spines and improve neurobehavioral outcomes in mice models of both IS and HS [11]. However, mice with astrocyte-specific knockout only show increased dendritic spines and improved outcomes in IS but not in HS conditions [11]. The differential analysis of transcriptional profiles using scRNA-seq demonstrates that 135 DEGs in astrocytes are downregulated in HS relative to IS [11]. A total of 10 subclusters from 1380 astrocytes with specific top gene markers representing distinct functional cell identities have been reported [11] (Table 3). The proportion of subcluster3 is about 20% in IS but <2% in HS, indicating that it may be responsible for the various phagocytic features of astrocytes in IS and HS. The analysis of 881 marker genes in subcluster3 demonstrates significant upregulation of processes related to the synapse pruning [11]. In microglia, analysis of scRNA-seq shows similar phagocytic patterns in IS and HS [11]. Collectively, this meaningful study indicates that scRNA-seq may
contribute to the development of more precise therapies for the treatment of stroke in the future.

5. Heterogeneity in oligodendrocytes in IS

Oligodendrocytes (OLs) are found in both grey and white matter in the CNS [89]. OLs produce myelin, a multilamellar lipid structure that wraps around the neuronal axons, thereby accelerating axonal conduction velocity and transferring information across CNS [90]. OLs are derived from the oligodendrocyte progenitor cells (OPCs) [91]. The differentiation of OPCs into myelinating OLs can be promoted by neuronal activity [92], thus altering the internodal length and thickness of myelin. Previous studies also report that OPCs can proliferate and differentiate into myelinating OLs to facilitate the processes of recovery from the CNS injury [93]. Additionally, recent evidence indicates that OLs also possess some novel functions, including immune-related responses, supporting neuronal and axonal energy metabolism, regulating network activity by connecting the neuronal soma, interaction with the vasculature, and participating in processes of learning and memory [94, 95].

5.1 Role of OLs in IS

OLs are vulnerable to ischemic damage and do not have self-renewal capacity. They undergo necroptosis and apoptosis due to the release of toxic glutamate and ATP [96]. However, OLs also produce myelin sheaths on damaged axons during the late recovery phases of IS [97]. Oligodendrogenesis contributes to neuronal recovery and brain repair after stroke, contingent on the OPC proliferation, migration, and differentiation into mature OLs [98]. Previous studies also demonstrate that the number of OPCs increases in the penumbra but decreases in the core lesion after IS [99], suggesting the heterogeneity in OPCs. However, with altered potassium channel permeability, these become hypertrophic or die due to excitotoxicity [100]. Inflammatory cytokines are involved in oligodendrocytic modulation in cases of IS [96]. TNF-α and IFN-γ induce apoptosis in oligodendrocytes and inhibit OPC proliferation and differentiation [101, 102], while IL-4, IL-6, IL-11, and IL17 promote OL differentiation and survival, along with OPC differentiation during IS [103, 104]; additionally, other cells are also involved in neuroinflammation and interact with OLs or OPCs, including peripheral lymphocytes, microglia, and cerebral endothelial cells [105]. However, the heterogeneity of OLs in IS remains unclear and further investigations to evaluate the transcriptomic profiles of OLs at the single-cell level are needed.

6. Neuronal heterogeneity in IS

Cerebral ischemia deprives oxygen and glucose availability in the neuronal core thus inducing neuronal death [106]. In the surrounding penumbra, cells experience partial ischemic conditions and react to detrimental factors of oxidative stress, excitotoxicity, inflammation, and mitochondrial dysfunction; neurons in penumbra may undergo delayed apoptotic cell death, depending on the communication among different cells [107]. Neuronal death is a complicated process, as extensive communication networks are established among the neurons through synaptic transmission. Additionally, there are complex interactions among neurons, glial cells, and blood vessels in the NVU [4, 108, 109]. Failure in the synaptic processes and the NVU results in disconnection, trans-synaptic degeneration, and disruption of the BBB, causing neuronal dysfunction and eventually death [108]. With the expansion in the understanding of electrical synapses, tunneling nanotubes, and extracellular vesicles (EVs), neuronal communication networks have yielded greater complexity [110, 111]. Studies show that synaptic loss may result in neuronal death in the penumbra due to high energy demands [112]. EVs are reportedly involved in processes of neuroinflammation and neurodegeneration and can regulate the behavior of the recipient cells during IS [113]. As neuronal fates in cases of irreversible infarct core and salvageable ischemic penumbra are quite different,
neurons in different areas may express a greater variety of receptor genes. Using scRNA-seq, investigating molecular determinants and lineage relationships of neurons after stroke are expected to yield novel therapeutic targets. A total of 246 neurons were subclustered from the MCAO or sham group into six subclusters in a recent study (Table 1)[13]. The GABAergic neurons showed the highest enrichment in subclusters 1 and 2, while dopamine (DA) neurons were mainly distributed in subcluster 5 [13]. Subcluster 4 was highly enriched in the MCAO group, and the metabolism herein showed elevation in amino acid metabolism, glycolysis, fatty acid metabolism, and gluconeogenesis [13]. However, to date, no study has investigated the heterogeneity and differential gene expressions between neurons in the infarct core and penumbra. Therefore, further investigations should address the aforementioned issue in an effect to provide potential targets for saving neurons in the ischemic penumbra.

7. Heterogeneity in endothelial cells in IS

Endothelial cells (ECs), vulnerable to ischemia, are a critical part of both the BBB and NVU [114]. Cerebral endothelium secretes vasodilators or vasoconstrictors, are involved in the communication network with other brain cells in an effect to regulate cerebral blood flow (CBF) and provide a non-thrombogenic surface [115, 116]. During IS, the main metabolic changes in ECs include the Golgi apparatus, gluconeogenesis and glycolysis, heme metabolism, and porphyrin. Endothelial dysfunction including oxidative stress damage and excess apoptosis induced by IS result in the aggravation of brain edema and neuroinflammation, impairment of endothelium-dependent vasodilation [117-119], reduction in CBF, along with an increased risk of intracerebral hemorrhage after thrombolytic therapy [120, 121]. Mechanistically, IS leads to dysfunction in BBB and hyperpermeability, thereby inducing damage of endothelial tight junctions, allowing water flow across the capillary, increase in transcytosis, and alteration in ion transport [122, 123]. Additionally, leukocyte adhesion molecules, VCAM-1 and ICAM-1, are significantly highly expressed post-stroke, thereby prompting leukocyte extravasation [117, 124]. Previous studies also showed that a possible mechanism underlying EC death post-stroke could be attributed to M1 microglia-derived TNF-α induced endothelial necroptosis and increased breakdown of BBB [125]. Additionally, anti-TNFα treatments, such as administration of infliximab, alleviate endothelial necroptosis and improve outcomes of stroke [126], suggesting a promising therapeutic modality. Nevertheless, the mechanisms underlying endothelial death and functional heterogeneity remain unclear and further investigations are needed to identify potential therapeutic targets for the treatment of injury in specific cell subtypes via scRNA-seq.

Using scRNA-seq, Guo et al. have divided ECs into 6 subclusters [14] (Table 1), and increased EC death induced by ischemia leads to a decrease in the cell number in the MCAO group, while BBB-related subclusters increase accompanied by the upregulation of BBB damage-associated genes, including Pdlim1, Timp1, Upp1, and Adamt54 [14]. The findings also demonstrate that IFN-1 signaling genes, including Ifit3, Isg15, and Usp18, are highly expressed in two EC clusters, capillary (capEC-IFNhi) and arterial (aEC-IFNlo) [14]. Another scRNA-seq study employing GSVA reveals that cluster 3 varies significantly from all clusters and is the most enriched upon myogenesis and epithelial-mesenchymal transition [13]. GO and KEGG analyses show that cluster 3 is primarily oriented in the extracellular matrix, exosomes, and vasculature development, indicating the potential functions in extracellular communication and vascular reconstruction post-stroke [13]. Additionally, ECs in subcluster 3 show upregulated cyclic nucleotide metabolism, glutathione metabolism, ROS detoxification, and oxidative phosphorylation. Taken together, genes encoding inflammatory cytokines like Ccl4, Cd14, Cxcl2, and Spp1 are modulated by ischemia in each EC subcluster [13, 14], and are potential therapeutic targets.

8. Pericytic heterogeneity in IS revealed by scRNA-seq

Pericytes (PCs) are a significant component of the BBB as they maintain its normal physiological functioning [118]. PCs receive, modulate, and process signals to maintain normal homeostasis in the brain. They possess diverse neurovascular functions such as clearance of toxic metabolites, orchestration of BBB permeability, capillary hemodynamics, stem cell activity, and angiogenesis [127-129]. In recent years, accumulating evidence indicates that PCs may play vital roles in the pathogenesis of IS [130].

Due to their versatile functionality, some researchers speculate a heterogeneous cell population of PCs and each subcluster may play its specific role in IS [131]. A recent study has confirmed this hypothesis via scRNA-seq of PCs; 3 PCs subclusters have been identified (Table 1)[14]. PC0 subcluster is involved in the transport of calcium, sodium, and potassium ions, and maintaining normal functioning of the BBB [14]. Highly expressed gene sets in the PC1 subcluster are associated with inflammatory responses such as in cytokine and HIF-1-mediated signaling pathways [14]. The pathway involving syndecans I and muscle contraction associated with the multipotential differentiation capacity of PCs are enriched in the PC2 subcluster, accompanied by the specific...
upregulation of Acta2 in regulating the cerebral blood flow [14]. Although some initial progress has been made in evaluating the functional heterogeneity of various PC subpopulations by scRNA-seq, further studies are needed to develop genetic models and identify novel markers in distinct vessel segments combined with the findings of scRNA-seq and other novel technologies, thus providing novel and precise treatment for targeting specific genes expressed in the different PC subpopulations.

9. Vascular muscle smooth cells in IS

Vascular muscle smooth cells (VSMCs) are the major components of arteries and play a critical role in the modulation of blood pressure and CBF distribution in the CNS. As the main regulators of CBF, VSMCs modulate the relaxation and contraction of cerebral blood vessels [132, 133]. Cerebral ischemic injury may modify VSMC phenotypes, however, the underlying mechanisms remain unclear [133, 134], thus highlighting the importance of further studies for identifying potential therapeutic targets for future pharmacological development.

A recent study reports six VSMC subclusters (Table 1), along with the highly expressed genes associated with the specific markers in each subcluster in IS by scRNA-seq [14], including arteriole SMC subcluster0 (Tagln, Acta2), arterial SMC subclusters 1, 2, and 3 (Eln, Ptgis, and Cnn1), and venous SMC subcluster5 (Art3, Car4) [14]. Notably, the activated SMC subcluster4 is specifically enriched in the processes of regulated exocytosis and neutrophil-mediated immunity, consistent with VSMC lysis and death which is driven by neutrophils in the case of atherosclerosis [14]. Additionally, subcluster5 includes the type I interferon signaling pathway that exhibits a reduced cell proportion in the early stages of stroke. Neutrophils are primarily recruited to the perivascular spaces in the ischemic area and target the NVU after a stroke [14]. The challenges herein can be attributed to the commonly shared numerous molecular markers between FB-like cells and PCs, making it difficult to differentiate them exactly. Further research is needed to identify specific markers in an effect to elucidate the functions of different FB subclusters in stroke.

10. Heterogeneity in fibroblast-like cells in IS

Fibroblast-like cells (FBs) play significant roles in fibrosis, structural support, and injury repair of CNS [135-138]. Emerging evidence shows that FBs migrate to lesion sites post-stroke after the identification of PDGFRβ+ and CD105+ cells without PC markers, NG2 or CD13, but having higher fibronectin expression [139, 140]. However, the functions of FBs in ischemic are debatable. Col1α1+ and fibronectin+ FBs in peri-infarct regions highly express peristin-induced neural stem cell differentiation and proliferation as well as prompt neurorestoration in the recovery phase of IS [141]. However, other studies report that the activation of PDGFRα expressed in FBs increases the cerebrovascular permeability in mice during IS [142, 143]. The dual roles in IS suggest that FBs exhibit functional heterogeneity post-stroke and various subclusters of FBs may possess different biological properties and functions during IS.

A scRNA-seq study reports three subclusters of FBs (Table 1)[14]. FB0 highly expresses genes associated with membrane transporters, including Slc1a3, Slc6a13, Slc7a11, and Slc22a6 as well as collagen fibril organizational genes, such as Col11a1, Col1a2, and Col1a1 [14]. FB1 is specifically enriched in the responses to IFN-β, including increased levels of Gsn, Ifitm2, and Ifitm1, and extracellular matrix organization, through Den, Lum, and Vcam1[14]. FB2 highly expresses genes associated with carboxylic acid transport, such as Slc6a6, Slc16a11, and Slc38a2, and those modulating intercellular pH, namely Slc4a10 and Slc26a2 [14]. Analysis of DEGs among different subclusters between the MCAO and sham groups indicates a subcluster-specific expression pattern for each subcluster [14]. The challenges herein can be attributed to the commonly shared numerous molecular markers between FB-like cells and PCs, making it difficult to differentiate them exactly. Further research is needed to identify specific markers in an effect to elucidate the functions of different FB subclusters in stroke.

11. Neutrophilic heterogeneity in IS

It is well acknowledged that neutrophils are detrimental factors after a stroke and efforts are underway to prevent neutrophils from entering the brain [144]. Neutrophils are reportedly the first immune cells that infiltrate into the ischemic area and provide vigorous and early inflammatory responses after IS [145]. Neutrophils are primarily recruited to the perivascular spaces in the ischemic area and target the NVU after a stroke [146]. In the early stages of stroke, neutrophils are activated and they attach to the endothelium [147] owing to the expression of endothelial adhesion molecules [148], including PSGL-1, MAC-1, CD11a, and ICAM-1. There is an evident increase in neutrophil number within a few hours after stroke [149]. During the acute stages, neutrophils aggravate IS-induced brain damage via several mechanisms [145]. For example, neutrophils physically block the microvascular network, thereby further decreasing CBF and contributing to the damage of adjacent tissues [150, 151]. Moreover, neutrophil count is directly related to the infarct size, and the neutrophil to lymphocyte ratio can be used to predict the risk of hemorrhagic transformation after IS [152, 153].

However, emerging evidence also suggests a possible neuroprotective role of neutrophils in IS [154]. Neutrophils exhibit functional heterogeneity and plasticity. They can transform into the N1 (pro-
inflammatory phenotypes) or N2 (anti-inflammatory phenotypes) neutrophil types [155]. N1 or pro-inflammatory neutrophils induced by IFN-γ possess a short life span and are associated with high cytotoxicity owing to the higher expression of TLR4, CD11b, CD86, and Ly6G [156]. In contrast, N2 or anti-inflammatory neutrophils are long-lived, characterized by the expression of CD206, YM-1, and Arg1 [157]. N2 neutrophils can be induced by TGFβ and produce anti-inflammatory cytokines thereby exerting neuroprotective effects and tissue remodeling [158]. A recent study reports the positive association between N2 neutrophils and the reduction of infarct size after stroke [154]. Therefore, the ratio of N1 to N2 may result in dissimilar clinical outcomes among stroke patients.

Notably, typing method of N1 and N2 oversimplifies the complicated phenotypic diversity of neutrophils [156]. Due to the advances in scRNA-seq, it is now possible to achieve a precise palette of the neutrophil population. Neutrophils are detrimental in the acute phases of IS but become neuroprotective at a later stage, suggesting reprogramming towards anti-inflammatory phenotypes, depending on activation signals and extracellular stimuli. Moreover, Cuartero et al. indicate that PPAR-γ pushes neutrophils toward a protective phenotype [154]. Interleukin-27 (IL-27) can also prompt neutrophilic reprogramming to anti-inflammatory phenotypes. Interestingly, scRNA-seq also demonstrates that bacterial infection may induce reprogramming of the neutrophilic population [159]. However, some studies suggest that neutrophils may be programmed before a stroke-induced insult [160-162], which may explain the distinct outcomes of stroke therapy.

A recent study reports four neutrophil subclusters via scRNA-seq [14]. The highly expressed genes and enriched pathways/processes are detailed in Table 4. Notably, there is a dynamic change in the compositional ratio of different neutrophil subpopulations at various stages of IS [14]. Further investigations are required to examine the relevant signals, the exact cell populations, and specific mechanisms involved in the reprogramming processes of neutrophils during IS by combining scRNA-seq with other new complementary technologies.

### Table 4. Neutrophilic heterogeneity by scRNA-seq in IS.

| Subpopulations | Highly expressed genes in IS | Enriched pathways and process | Refs. |
|----------------|-----------------------------|-----------------------------|-------|
| NEUT0          | Cxcl1, Hcar2, Ptafr, Cd63   | Neutrophil degranulation, neutrophil activation involved in immune response, neutrophil mediated immunity, cellular response to interferon-gamma | [14]  |
| NEUT1          | Irf7, Isg15, Gbp2, Ifitm1   | Type 1 interferon signaling pathway, cellular response to type 1 interferon, interferon-gamma-mediated signaling pathway, cellular response to interferon-gamma | [14]  |
| NEUT2          | Ccr1, Fpr1, Trem1, Ltb4r1, Cxcr2, St6a2H | Cellular response to cytokine stimulus, cytokine-mediated signaling pathway, inflammatory response, negative regulation of insulin receptor signaling and cellular response to insulin stimulus | [14]  |
| NEUT3          | Cebpe, Cd177, Cybb, Camp, Ltf | Granulocyte migration, defense response to fungus, innate immune response in mucosa, positive regulation of vesicle fusion, neutrophil extravasation | [14]  |

### 12. Lymphocytic heterogeneity in IS

#### 12.1 T lymphocytes in IS

T lymphocytes can be subtyped as CD4+ T helper cells (Th) that modulate the functioning of granulocytes and phagocytes, CD8+ cytotoxic T lymphocytes (CTL), and regulatory T cells (Tregs) [163]. T lymphocytes can be activated and thus infiltrate into the ischemic brain and proliferate in the CNS as late as one week after IS [149]. Overall, it is well acknowledged that T lymphocytes amplify neuroinflammation and release cytokotxins and cytokines, thereby contributing to the progression to a secondary stroke [163]. However, emerging evidence shows that subpopulations of T lymphocytes exhibit tremendous heterogeneity in IS [164]. Previous studies suggest that mice deficient in Th1 and Th17 cells exhibit smaller infarct sizes and better recovery [165, 166]. The number of Th1 and Th17 cells increases within the first week after stroke, accompanied by an increase in the proinflammatory cytokines [167], including TNF-α, IL-1, IL-2, IL-12, and IL-17, thereby exacerbating the neurological damage. The mechanism underlying T cell activation in stroke is rather controversial, as some studies demonstrate that T cells are activated in stroke without an adaptive immune mechanism such as co-stimulatory pathways or antigen recognition [168], while others report that antigen-dependent activation of CTL can lead to the expansion of the infarct size [169]. Conversely, Th2 cells are considered to exert neuroprotective effects, along with the secretion of anti-inflammatory cytokines, including IL-13, IL-10, IL-5, and IL-4 in IS.

Growing evidence suggests that regulatory Tregs exhibit neuroprotective effects during IS rather than detrimental effects [170]. The proportion of Tregs, especially CD39+ Tregs, is greatly reduced during IS and...
is accompanied by the decrease in anti-inflammatory cytokines leading to the expansion of infarct sizes [170, 171]. Additionally, Tregs can limit neuroinflammation by inhibiting the MMP9 activity and maintaining the integrity of the BBB [172]. Moreover, MCAO mice with adoptive transfer of Tregs exhibit smaller infarct sizes and better outcomes within one week [173]. Nevertheless, selective depletion of Tregs can prompt neuro-restoration and microvascular dysfunction in an effect to decrease the infarct size [174].

A previous study indicates that mice with Treg depletion experience a rather slow recovery in motor functions, due to axonal myelination disorder in the external capsule and striatum after IS [10]. This suggests that Treg deficiency can impair the WM integrity and retard OL regeneration after IS. Interestingly, scRNA-seq analysis of Foxp3-expressing Tregs from the ischemic brain indicates that Tregs express genes to contact other T cells and microglial populations rather than directly contacting the OLs [10]. In PLX5622-treated mice with microglial depletion, newly matured OLs are not observed despite the adoptive transferring of exogenous Tregs after stroke, which further suggests that Tregs interact with microglia in an effect to affect oligodendrogenesis [10]. Mechanistically, Treg-derived osteopontin activates integrinβ1 receptors on the microglial surface, thereby increasing microglial protective functions, in turn resulting in OL maturation and WM repair post-stroke [10]. Shi et al. have identified a prominent Treg subcluster by scRNA-seq during the recovery stages of IS, thus providing a novel therapeutic method by increasing the effects of Tregs in the ischemic brain.

Collectively, despite the well-known deleterious roles of T lymphocytes in IS, evidence suggests the neuroprotective roles of Th2 and novel Tregs subclusters identified by scRNA-seq. Therefore, targeting the specific subclusters of T lymphocytes prompting neuroprotection is a promising therapeutic approach, however, further investigation is required to identify more optimal targets and the precise subclusters.

### 12.2 B lymphocytes and natural killer cells (NK) in IS

The production of local antibodies in CSF indicates the roles of B lymphocytes during IS [175]. The μMT knockout mice, lacking mature B lymphocytes, exhibit a much larger infarct size and higher mortality post-stroke [176-178]. B lymphocytes produce IL-10 after IS, which can reduce the infiltration of neutrophils, macrophages, and T lymphocytes into the ischemic brain [177]. In 2020, Ortega et al. [179] showed that adoptively-transferred B lymphocytes reduce infarct sizes in the MCAO mice. Moreover, depletion of the B lymphocytes impairs spatial memory, increase anxiety, and delay motor recovery, suggesting that diapedesis of B lymphocytes contributes to functional restoration after stroke. The beneficial effects of B lymphocytes are associated with the inhibition of cytotoxic immune cells. Nevertheless, other studies report the detrimental roles of B lymphocytes in IS. B lymphocyte infiltration into the ischemic area is sustained for at least 12 weeks post-stroke, resulting in cognitive decline, which suggests that B lymphocytes can be used to predict post-stroke dementia [180, 181]. Additionally, post-stroke cognitive decline is attenuated in B lymphocyte-deficient mice. Mechanistically, cognitive deficits induced by stroke may be associated with antibodies produced by B lymphocytes [181, 182]. In clinical trials, patients with post-stroke dementia showed a substantially higher level of B lymphocytes than those without dementia [181]. Therefore, after IS, B lymphocytes infiltrate the brain, and their functions vary at different stages of IS [181]. B lymphocytes secreting IL-10 result in a decrease in the infarct size and inflammatory responses at 48 hours post-stroke. However, B lymphocytes may induce post-stroke dementia during the chronic phases of IS [180, 183]. Furthermore, B lymphocytes also exhibit functional heterogeneity in IS; the B1 (Ly6d, Cd79a) and B2 subclusters (Ramp1, Lmo4) have been identified by scRNA-seq. However, the functions of the two different subclusters need further research in the future [14].

The roles of NK cells during IS are controversial [184]. NK cells can rapidly respond to ischemic insults by increasing cytotoxicity and inflammation, thereby exacerbating brain infarction [185, 186]. Mechanistically, NK cells release IFN-γ to recruit macrophages and dendritic cells, thus attenuating ischemic injury, while IFN-γ can also improve the survival of the infarcted brain by alleviating bacterial infections but not the lesion size [187]. A previous study divided NK cells in IS conditions into NK1 (Klrblc, Ifng) and NK2 types (S100a1, Car2) by scRNA-seq analysis [14]. Previous studies indicate that the expression of TLR4 varies among different NK cell phenotypes and TLR4 increases the secretion of IFN-γ from the NK cells [188, 189]. However, more studies are needed to elucidate the role of TLR4 in different subclusters of NK cells during IS.

### 13. Conclusion and future perspectives

The emergence of scRNA-seq has allowed for targeting a particular cell type and thereby revealing the different phenotypic and molecular changes in cell types of interest during IS [13, 14]. This aids the detection of intercellular contact, cellular heterogeneity, and new treatments in stroke. Several cell types and subpopulations accompanied by the cell-type-specific gene expression patterns have been identified in IS penumbra, including...
glial cells, neurons, brain vasculature-associated cells, and peripheral immune cells. After describing the transcriptional landscape and “therapeutic time window” in stroke by scRNA-seq [13, 14], future studies should combine scRNA-seq and mechanistic examination to investigate the pathological process during the stroke for identifying new potential therapeutic targets.

Due to the morphological complexity of cells in the CNS, many transcripts may be lost during sample preparation for scRNA-seq [190, 191]. Nuclear transcripts account for 20%–50% RNA in the cells, including unspliced and immature RNA molecules [192]. New methods such as single-nuclei RNA sequencing (snRNA-seq) are less influenced by technical artifacts during isolation unlike scRNA-seq [193, 194]. These reveal the localization of transcripts and provide different information for intronic versus exonic readers [195]; snRNA-seq may provide a deeper understanding of the pathological mechanisms during the stroke. scRNA-seq and snRNA-seq limit the spatial context, however, spatial connectivity and context between cells are significant to cell function [196, 197]. Single-cell spatial transcriptomics can combine the morphology of normal tissues, infarct core, and ischemic penumbra with sequencing data to yield more accurate information [198, 199] (Fig. 2).

Figure 2. Single-cell RNA sequencing in stroke research. The advancements in scRNA-seq technologies present an unprecedented single-cell-resolution map of the brain. scRNA-seq is useful for identifying novel cell populations in stroke conditions and analyzing the cellular heterogeneity in a novel cell population. Additionally, scRNA-seq can reveal organ- or tissue-specific characteristics, cell lineage trajectories, as well as cell-to-cell communication states. Furthermore, single-nuclei RNA sequencing and single-cell spatial transcriptomics may provide a deeper understanding of the pathological mechanism during a stroke. Taken together, scRNA-seq contributes to the identification of potential new biomarkers, therapeutic targets, and the molecular underpinnings underlying pathological processes in stroke.

Future studies combining scRNA-seq, snRNA-seq, single-cell spatial transcriptomics, and complimentary in-depth mechanistic investigations in stroke research will bring forth exciting findings in pathological structure-function changes, which may provide more effective treatment strategies for stroke therapy.

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Conflict of interest

The authors declare no competing interests.

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