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Review Article

Microglia and ischemic stroke: a double-edged sword

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Abstract: Inflammatory processes have a fundamental role in the pathophysiology of stroke. A key initial event is the rapid activation of resident immune cells, primarily microglia. This cell population is an important target for new therapeutic approaches to limit stroke damage. Activation of microglia is normally held in check by strictly controlled mechanisms involving neuronal-glial communication. Ischemic stroke is a powerful stimulus that disables the endogenous inhibitory signaling and triggers microglial activation. Once activated, microglia exhibit a spectrum of phenotypes, release both pro- and anti-inflammatory mediators, and function to either exacerbate ischemic injury or help repair depending on different molecular signals the microglial receptors receive. Various ligands and receptors have been identified for microglial activation. Experimental tools to detect these inflammatory signals are being increasingly developed in an effort to define the functional roles of microglia. Fine-tuning immunomodulatory interventions based on the heterogeneous profiles of microglia are urgently needed for ischemic stroke.

Keywords: CD45, chimera, inflammatory response, ischemic stroke, macrophage, microglia

Introduction

Inflammation plays a critical role in mediating post-ischemic injury. The activation of microglia, the major resident immune cells in the brain, is a key element in triggering the innate immune response [1]. When ischemia occurs, cessation of cerebral blood flow leads to deprivation of oxygen and glucose to areas supplied by the occluded vessel. Resultantly, vulnerable neurons are subject to death and the endogenous signaling that inhibits inflammatory responses under physiological conditions compromises, leading to microglial activation. Once activated, microglia develop macrophage-like capabilities including phagocytosis, cytokine production, antigen presentation and the release of matrix metalloproteinases (MMPs) that weaken the blood brain barrier (BBB) [2]. As a result, peripheral leukocytes infiltrate into the brain and the normally immune-privileged brain environment is exposed to systemic responses that further exacerbate inflammation and brain damage.

The inflammatory response has dual effects on ischemic injury [3]. Stroke-induced microglial activation causes release of a variety of inflammatory mediators many of which are cytotoxic and/or cytoprotective [4]. Phagocytosis of cellular debris and harmful substances along with the release of anti-inflammatory cytokines by microglia occurs in an effort to restore tissue homeostasis by clearing pathogens or necrotic cells, and consequently attenuate the detrimental effects of inflammation and aid in tissue repair [5, 6]. Because of their critical roles in the immune response to stroke, microglia have become a recent target of interest for many stroke scientists. This review focuses on current findings, providing an update on microglial activation, phenotype identification, and the roles of microglia in the pathophysiology of cerebral ischemia.

Origin and development of microglia

There has been much controversy over the characterization of the cell lineage of microglia. The most significant hypotheses of the development of microglia debate their embryonic neuroectodermal or mesodermal origin. Unlike the ectodermal development of macroglia (astrocytes and oligodendrocytes) and neurons, a

consensus in favor of the mesodermal hematopoietic origin of microglia is currently held [7, 8]. A subset of primitive myeloid precursors, localized in the extra-embryonic yolk sac (YS) at embryonic day 8 (E8) was shown to contribute to the rise of yolk sac microglia that persists into adulthood [9]. This cellular subset of primitive hematopoiesis contributes little to blood leukocyte development, which is largely specific to definitive hematopoiesis of hematopoetic stem cells (HSCs) [9-11]. Microglia precursors originate in the embryonic yolk sac before the emergence of definitive HSCs from the aorto-gonad-mesenephros (AGM). The direct precursors of microglia that travel to the neural tube at E8 are exclusively the $CD45^{-}cKit^{+}$ cells. This subpopulation of erythromyeloid cells eventually begins expressing CX3CR1 and CD45 and travels into the neuroectoderm in a matrix metalloproteinase 8 (MMP 8) and MMP 9 dependent manner to develop into microglia [12, 13]. These precursor cells are seen seeding the brain rudiment by E10 in rodents [9] and have a full microglial morphology beginning at E14 [12].

The transcription factor *Myb* is essential for the development of HSCs [14, 15] and can be found in the AGM during embryogenesis [8, 16]. By using *Myb* knockout (KO)mice, Schulz et al. [15] found that yolk sac-derived $CD45^{+}CX_3CR1^{hi}F4/80^{hi}$ macrophages and microglia still develop in normal numbers and remain independent of *Myb* into adulthood; however, $CD45^{+}CX_3CR1^{+}F4/80^{low}CD11b^{hi}$ monocytic phagocytes continually replaced by bone marrow (BM) in an adult are unable to develop without *Myb*. The transcription factor PU.1, on the other hand, is necessary for myelopoiesis of the YS but dispensable for the development of definitive HSCs [11, 15]. This further suggests two separate myeloid lineages of peripheral macrophages and resident microglia.

Moreover, colony stimulating factor (CSF) and its receptor CSF-1R are necessary for the differentiation of most macrophages/microglia [17]. Recent studies showed that in CSF-1R KO mice, yolk sac-derived microglia do not develop and are deficient throughout life, but HSC-derived monocytes are able to differentiate and circulate without dependence on CSF-1R [8, 9, 18]. Fate mapping analysis of *Flt3*-Cre x $Rosa^{LSL-YFP}$ mice also shows that yolk sac-derived microglia are independent of *Flt3*, a chemokine present on multipotent hematopoetic progenitors in the blood and brain [15]. Taken together, the separation between HSCs and yolk sac derivations is evident and allows insight into microglial ontogeny.

Markers and methods for identification

To date, no single microglial-specific marker has been identified, keeping the cell type indistinguishable from macrophages and various myeloid-derived cells that infiltrate the brain during pathological states [19]. Changes in microglial activation in response to central nervous system (CNS) injury are illustrated by diverse phenotypes and unique expression of cell surface proteins. Microglial activation stages can be detected by characterizations of these changes, but the similarities with other cell types pose a major hurdle for their definitive characterization and detection.

Ionized calcium binding adaptor molecule-1 (Iba-1) expression by immunohistochemistry (IHC)

Iba-1 is amongst the most useful proteins for distinguishing microglia through IHC and immunocytochemistry (ICC) staining [20, 21], especially for studies of cerebral ischemia where the expression of Iba-1 is upregulated [22] (**Figure 1**). However, Iba-1 has also been shown to bind various cells of monocytic lineage [20], and thus its specificity for microglia staining is limited in injured brain tissue where peripheral macrophages may infiltrate. Other markers, such as CD11b, Isolectin (IB4), and F4/80, have also been used for *in vivo* and *in vitro* microglial staining. Although the benefits of IHC include the ability to create a spatiotemporal and morphological profile of microglia within the CNS, it lacks in specificity and may be inconsistent depending on the type of histological preparation.

CD45/CD11b expression by flow cytometry

Flow cytometry provides a sensitive means to detect various markers and create a multiparameter characterization of different cell types. In 1991, Sedgewick et al [23] observed differences in the expression of the hematopoietic cell surface marker CD45 on resident microglia and infiltrating peripheral leukocytes. To date, the most common characterization profile

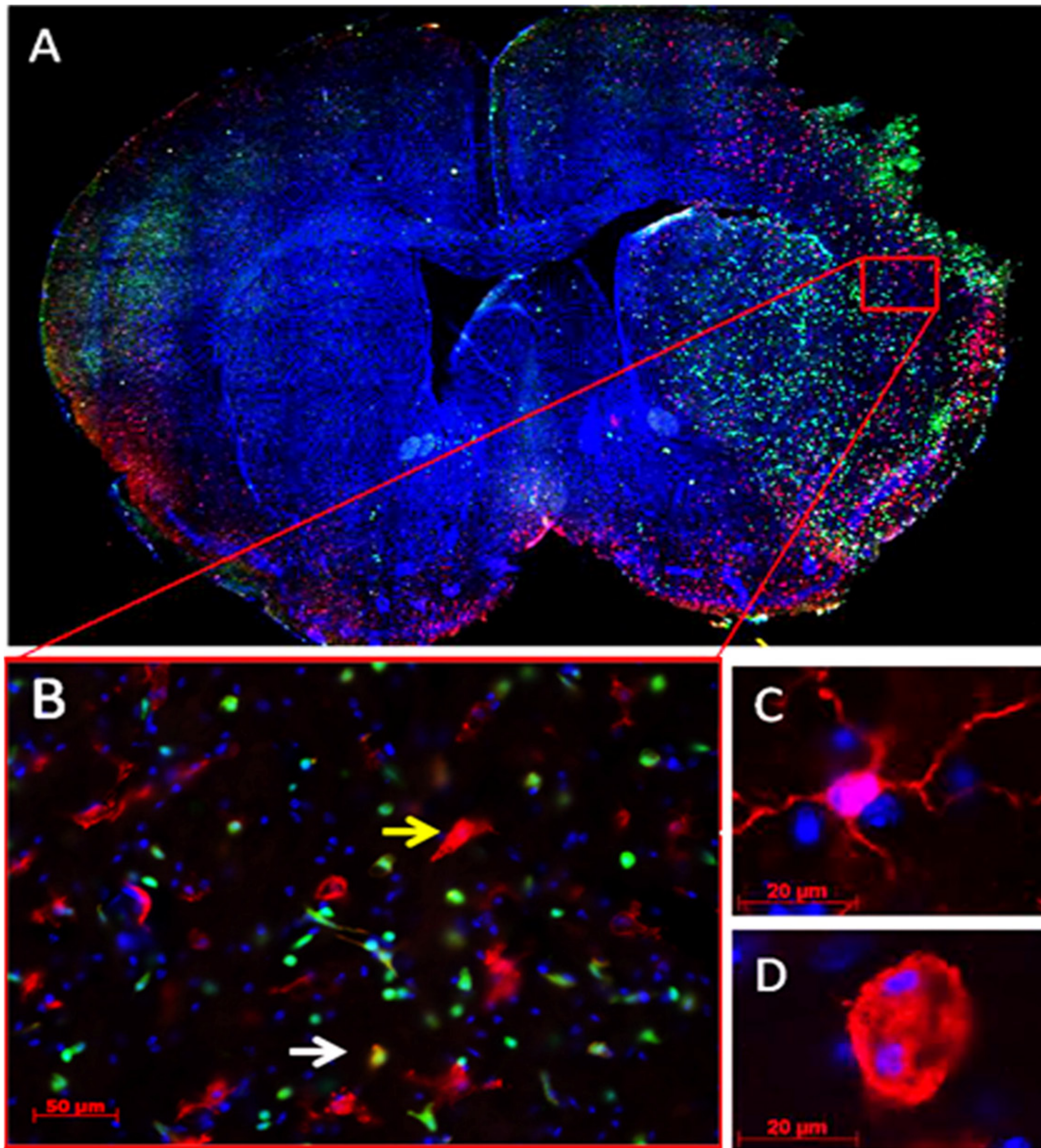

Figure 1. Microglia activation and leukocyte infiltration in the ischemic brain. A. Representative brain slice stained with Iba-1 (red), GFP (green) and DAPI (blue) from a chimeric mouse after stroke (10x magnification). The chimeric mouse model was made by transferring GFP$^+$ bone marrow of a donor mouse to an irradiated WT mouse. Eight weeks after reconstitution, the chimeric mouse was subjected to 90 min MCAO. The mouse was reperfused for 72 hours before sacrifice. GFP$^+$ cells represent bone marrow-derived peripheral leukocyte infiltrates. Green fluorescence is localized to the region of injured tissue in the striatum and cortex. B. 20x magnification of the box area in (A). The yellow arrow indicates a large Iba1$^+$ ameboid microglia that does not colocalize with GFP$^+$ bone marrow-derived cells; the white arrow indicates a cell co-labeled with Iba-1 and GFP. C. Resting microglia morphology with thin, ramified processes in the non-injured brain hemisphere. 63x. D. Activated microglia with large, ameboid cell body in the ischemic cortex. 63x.

derived from flow cytometry sorting still holds that resident microglial cells are CD45^{low}CD11b$^+$, while infiltrating hematogenous myeloid cells are CD45^{high}CD11b$^+$ [24]. Experimental stroke studies with two photon imaging [25] and IHC [26] also demonstrated that infiltrating leukocytes in the ischemic hemisphere showed a higher expression of CD45, whereas microglia

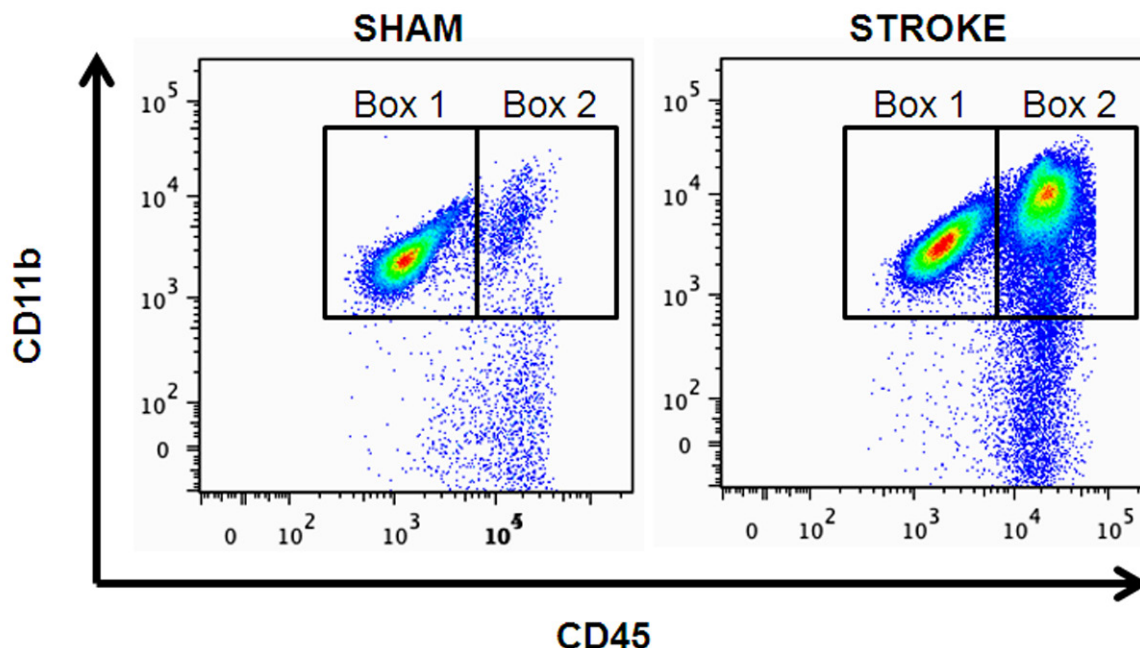

Figure 2. Representative flow cytometry plots of resident microglia and peripheral leukocytes in the mouse brain. In the brains of sham mice (left), $CD45^{low}CD11b^{+}$ (box 1) and $CD45^{high}CD11b^{+}$ (box 2) cells were identified as resident microglia and peripheral leukocytes respectively. 72 hours after 90 min MCAO (right), both $CD45^{low}CD11b^{+}$ and $CD45^{high}CD11b^{+}$ cells were increased in the brain.

had a fainter CD45 immunostaining, which is consistent with our flow cytometry data (**Figure 2**). Although $CD11c^{high}$ and $CD14^{-}$ have sometimes been used to label microglia in flow cytometry, their similarity in expression on other peripheral cell types makes it difficult to justify resident microglial specificity [24, 27].

There are, however, limitations to using variable levels of CD45 expression to distinguish between microglia and other monocyte/macrophage populations. CD45 on microglia can be upregulated in mouse models of EAE in spinal cord towards a CD45 high phenotype [28]. Inflammation in the periphery of humans may also upregulate CD45 in CNS resident microglia with no changes in CD11b expression [29]. The possible instability of this marker's levels during pathological states reiterates the need for a better method of tracking resident microglia and peripheral monocyte/macrophages.

Transgenic mice

A multitude of transgenic mice can also be used to label cell populations by attaching a fluorescent reporter to a myeloid promoter. For example, CD11b-GFP can label all cells of myeloid origin including microglia in the mouse brain [30]. Similarly, Iba-1-EGFP transgenic mice have shown successful fluorescent labeling of CNS microglia/macrophages in tissue samples [31]. More recently, a fluorescent knock-in transgenic mice line created using CX3CR1-GFP and CCR2-RFP has shown to be the most useful to the field to differentiate resident microglia from peripheral monocytes. It is now known that CCR2, though differentially expressed, is specific to the periphery in naïve and EAE mice [32], and unlike CD45, is not upregulated significantly in pathological states. In addition, fractalkine receptor (CX3CR1) is present on microglial progenitors in the yolk sac and, in the CNS, remains specific to microglia into adulthood [13].

Studies using systemic and region-specific depletion of myeloid cells or macrophages can be done using DTR-CD11b transgenic mice. These mice have diphtheria toxin receptors (DTR) linked to the CD11b promoter. Administration of diphtheria toxin will deplete $CD11b^{+}$ (myeloid) cells [30, 33]. Another commonly used technique to selectively ablate $CD11b^{+}$ cells involves treating CD11b-HSV TK transgenic mice with injection of ganciclovir (GCV) [34]. Inducible death of $CD11b^{+}$ cells has

Table 1. Phenotypes of microglia

Phenotype	Identification Markers	References
Resting State	Iba-1	[21, 143]
	CD45^{int} CD11b^{+}	[23, 24]
	F4/80	[46, 144]
	Isolectin (IB4)	[145]
Classical Activation (M1)	MHCII	[28, 45, 146]
	CD16 (FcγR III)	[147, 148]
	CD 32 (FcγR II)	[147]
		[149, 150]
	CD80 (B7-1)	[149, 150]
	CD86 (B7-2)	[151]
	CD40 (TNFR)	
Alternative Activation (M2)	Arg-1	[152]
	CD68 (ED1)	[153]
	Fizz1 (Relmα)	[152, 154]
	Ym-1	[152, 154]
	CD206 (MR)	[155, 156]
	Dectin-1	[155]

MHC, major histocompatibility complex; Arg, arginase; TNFR, tumor necrosis factor receptor; Fizz1, resting-like molecule alpha; MR, mannose receptor.

more recently become a useful technique in the field.

Chimeras

Irradiation chimera models are a popular technique to differentiate microglia from peripheral myeloid cells since there is no exclusive antigenic marker [35]. To generate a BM chimera (**Figure 1**), BM cells of a mouse with ubiquitously expressing fluorescent (GFP^{+}, YFP^{+}, RFP^{+}, etc) protein is extracted and injected into a mouse of interest whose own marrow is first irradiated and eliminated. After weeks to months of reconstitution of the new cell population, it is possible to track the origins of cells in CNS tissue, particularly after neurological diseases, where cells from the periphery that cross the BBB have been labeled with fluorescence. BM chimeras can be performed with transgenic knockout or knockin mice to selectively target cells of the CNS or of the periphery. Unfortunately, the effects of irradiation are confounding and have been implicated in physiological alterations such as weakening of the BBB, potential cell death and activation of microglia [36-40]. Though microglia are mostly radio-resistant, their numbers in the CNS may also be altered in uninjured and ischemic chimeric mice compared to nonchimeric mice in the hippocampus and the cortex [41]. Changes in gene regulation with higher expression of proinflammatory cytokine and CCL2 can also accompany irradiation [38, 42]. These undesireable effects of irradiation pose a caveat to the technique. However, preventive measures can be taken to study the brain using chimeras. For example, targeted, instead of whole body irradiation using lead shielding of mouse heads is now often used in an effort to prevent damage or brain environmental changes due to radiation exposure [36, 43].

Resting microglia

Resting phenotype

Resting microglia in a healthy brain, more representatively known as "surveying microglia", are constantly extending and retracting their thin ramified processes in an effort to inspect the CNS microenvironment [44, 45] (**Table 1**). As the central macrophages of the brain, microglia are implicated in controlling synapse number and remodeling in the developing brain, and function to prevent accumulation of debris in the healthy adult CNS. They are not present uniformly in the adult brain and express differences in location, protein expression, and morphology [46]. Heterogeneity of morphology and location of microglia translates to differences in microglial responses to injuries and in activation states [47]. The majority of microglia are found in the gray matter and express more ramified structure with radial branches than those found in the white matter with longitudinal processes [46]. In the ischemic lesion induced by MCAO modeling, the penumbral area (the border zone of dead and living tissue) showed highly ramified cells (resting), while the ischemic core housed amoeboid bodies with thick ramifications (activated) [25]. To conclude, resting microglia are not "sleeping"; instead they are poised to respond to stimuli resulting from disturbances in the CNS environment by drastically altering their phenotypes and functions.

Inhibitory signaling of microglia

In the healthy brain, microglia express numerous inhibitory proteins, and interact with neurons to form a "brake" on inflammation [48]. The endogenous mechanisms in the form of neuronal-glial interactions may prevent excessive microglial activation in the CNS. Following injury, these interactions may be disrupted due to neuronal cell death and structural tissue damage. Regulating these inhibitions to keep microglia from acquiring a proinflammatory phenotype has been shown to prevent uncontrolled damage in models of Alzheimers Disease (AD), multiple sclerosis (MS), and neurodegeneration [49-53]. Similarly, regulation of these inhibitory proteins may also have beneficial effects on ischemic stroke.

CD200/CD200R1: CD200 is a transmembrane glycoprotein mainly expressed on neurons [54, 55]. The cell-cell contact between CD200 and its receptor (CD200R1), expressed on the surface of all myeloid origin cells, provides microglia with stability to remain in a resting state under normal CNS conditions [56, 57]. Cleavage of this interaction and subsequent microglial activation leads to the upregulation of proinflammatory cytokines and an inflammatory profile [54]. Increased tissue damage was related to a decrease of CD200 level in mouse models of EAE, suggesting a detrimental effect of the activated microglia unbound from CD200/CD200R1 interaction [58]. Aging is also associated with a reduced level of CD200 and long-term potentiation (LTP), as well as an increased microglial activation in the brain; however, treatment of CD200R1 agonist (CD200Fc) can attenuate the LTP deficit and ameliorate microglial activation, even after Lipopolysaccharide (LPS) stimulation [59]. Little is known about the role of CD200/CD200R1 signaling in ischemic stroke, but a primarily descriptive study of CD200 on Iba^+ cells showed a decrease in gene transcripts of CD200 in the ischemic hemisphere [60].

Fractalkine (CX3CL1)/CX3CR1: Much like the immunoglobulin superfamily member CD200R1, fractalkine receptor (CX3CR1) on microglia can bind the soluble and membrane bound forms of CX3CL1, to keep microglia quiescent [61]. However, under injury, neurons significantly decrease CX3CL1 release thereby enabling microglial activation [62]. Loss of this contact has been shown to be neurotoxic in many disease models including Parkinson's disease and ALS by exacerbating neuronal loss [50]. Similarly, LPS stimulation of microglia on $CX3CR1^{(-/-)}$ mice leads to greater IL1-β secretion compared to $CX3CR1^{(+/-)}$ mice [50].

However, the effect of CX3CL1/CX3CR1 signaling in neuroinflammation is controversial as deleterious roles for the CX3CL1/CX3CR1 pair were reported in rodent models of AD and cerebral ischemia [51, 52]. Twenty four hours after a transient MCAO, $CX3CR1^{(gfp/gfp)}$ mice (GFP is inserted into both alleles of the CX3CR1 locus) were noted to have less severe cerebral infarct volumes than WT mice, possibly associated with a coinciding decrease in IL-1β and TNF-α gene transcripts [63], as in the case of fractalkine knockout mice [64]. Similar results were seen in a recent study by Ciprani et al with a pMCAO model in $CX3CL1^{-/-}$ and $CX3CR1^{(gfp/gfp)}$ rodents, both of which showed less severe ischemic damage than WT mice [52]. Furthermore, $CX3CL1^{-/-}$ animals have increased damage after ischemia with exogenous intracerebroventricular CX3CL1 administration. Interestingly, WT rodents show less severe infarct volumes and better functional outcomes with the addition of CX3CL1 in a dose dependent manner [52]. The disparity in the effect of exogenous CX3CL1 between KO and WT animals suggests that CX3CL1 may be protective only when microglia exhibited a normal constitutive CX3CR1-mediated signaling throughout development in the WT brain [52].

SIRPα/CD47: Signal-regulatory protein alpha (SIRPα), expressed on myeloid cells including microglia, binds integrin associated protein CD47 on neurons to activate an intracellular immunotyrosine inhibitory motif [65, 66], keeping microglia silenced and thereby suppressing phagocytosis [67, 68]. Human MS lesions have shown a decrease in CD47 expression [53], though little is known to date about the role of CD47 and SIRPα in ischemia specifically. A reduction of infarct was seen 24 and 72 hours after 90 min MCAO in CD47 knockout mice, potentially due to a decrease in peripheral inflammatory cell infiltration [69]. Wang et al recently also reported a reduction of infarct and improvement of behavior deficit after transient MCAO in SIRPα mutant mice [70].

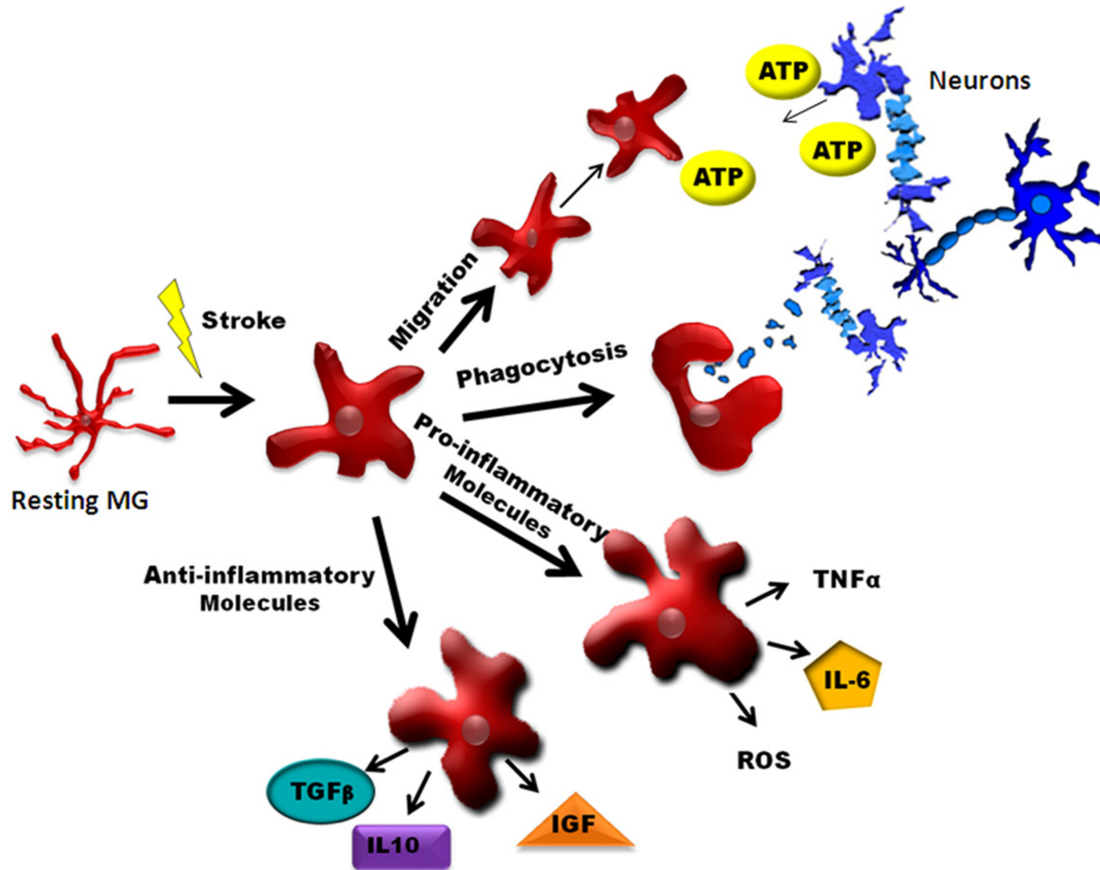

Figure 3. Schematic of Microglia Activation after Ischemic Brain Injury. In the ischemic brain, microglia display prominent changes in morphology associated with various functional states. Activation results in upregulation of transcriptional machinery which serves to increase the production of inflammatory mediators. In response to chemotaxic factors (e.g. ATP, etc.), microglia can migrate to sites of ischemic tissue injury to increase phagocytic uptake of cellular debris and cytotoxic substances. Depending on their activation state, microglia may promote a proinflammatory environment (via M1 activation) or regenerative milieu (via M2 activation). IGF-1: Insulin like growth factor 1; IL-1β: interleukin 1 beta; IL-6: interleukin 6; IL-10: interleukin 10; MG, microglia; ROS: reactive oxidative species; TGFβ: Transforming growth factor beta; TNFα: Tumor necrosis factor alpha.

Triggering receptor expressed on myeloid cells 2 (TREM2): The neuronal-microglial connection of heat shock protein 60 (HSP60)-TREM2 is important for the clearance of apoptotic neurons by microglial phagocytosis [71]. Microglia, via its intracellular adaptor protein DAP12, prevent the release of proinflammatory cytokines and maintain an anti-inflammatory microglial profile [72, 73]. In EAE, mice lacking TREM-2 by antibody blockage suffered worse pathology, but are rescued with greater recovery and tissue repair by injection of myeloid cells expressing TREM-2 [73]. Contrarily, TREM-2 KO mice showed a decrease in amoeboid $Iba1^+$ and $CD68^+$ microglial activation and an overall decreased inflammatory response 7 days post-reperfusion after a 30 minute MCAO model that did not translate into a decreased infarct volume [74]. The inhibitory effect of TREM-2 on microglial activation following stroke is still uncertain at this time.

Activation of microglia

A commonly held assertion is that distinct activation states (based on protein expression signatures) impart defined functional roles of microglia and may account for heterogenic responses to CNS injury. These activation states are generally described in terms of the class of activating signals and selectively induced expression of unique markers. Microglia can change patterns of migration, cell surface protein expression, and functions in response to tissue damage or dysfunction (**Figure 3**). The rapid responses to altered

homeostasis of the brain parenchyma can be visualized in two photon imaging to show immediate (within minutes) increase in the motility of microglial processes towards area of injury in a chemical gradient dependent manner, while the cell bodies remain stationary [44, 45]. *In vivo* data suggest that ATP signaling of P_2Y_{12} receptors on the cell surface is responsible for such extensions and directional guidance [44, 75, 76].

Microglial activation phenotypes: M1 and M2

Changes in microglial phenotype during activation may be analogous to that of peripheral macrophages, as the two cell types are indistinguishable without definitive surface markers for either. Microglial responses to stimuli from a changing brain environment are characterized as either M1, classical activation, or M2, alternative activation (**Table 1**). M1 is a proinflammatory cellular state associated with an increase in protein synthesis of pro-inflammatory mediators (IFNγ, Il-1β, TNFα, Il-6, CXCL10, etc.), ROS and NO production, and proteolytic enzymes (MMP 9, MMP3) that act on the extracellular matrix leading to BBB breakdown [77, 78]. M1 phenotype can lead to increased neuronal death compared to alternatively activated M2 microglia [79]; therefore, there is a growing interest to pharmacologically interfere with the signaling mechanisms that give rise to the classical activation phenotype of microglia. M2 microglia release anti-inflammatory mediators (IL-10, TGF-β, IL-4, IL-13, IGF-1, etc.) [80], leading to enhanced expression of genes associated with inflammation resolution, scavenging, and homeostasis [77, 81-83]. Levels of IL-10, TGF-β and CD206 mRNA increased as early as day 1 after ischemic injury and peaked at 4-6 days. In addition, TGF-β released by microglia promotes an anti-inflammatory profile associated with increased proliferation and neuroprotection in the ischemic brain [84]. This may be therapeutically relevant because TGF-β1 is specifically found in the salvageable peri-infarcted region of the cortex 24 hours after a 60 min MCAO and involved in distinct spatiotemporally regulated inflammatory and neuroprotective processes [85].

During disease progression and in normal ageing, microglial activation phenotypes can switch from M2 to M1 [86]. One recent study suggests that microglia are activated early after MCAO and morph into a reactive M1 phenotype by 7 days [79]. The balance between the M1 and M2 states is dynamic in inflammatory responses and may be offset in chronic disease states such as stroke, representing a novel mechanistic target for therapy [77]. Several proteins have been identified as markers for M1 or M2. MHC II, implicated in antigen presentation as an immune reaction, is upregulated on classically activated microglia, and is commonly used as a marker for M1 [28]. The alternatively activated M2 cells up-regulate presentation of several antigens. Ym-1 (Chitinase 3-like 3), for example, has been found to be associated with the protective, pre-phagocytic state of macrophages after ischemia [25]. Some of $Ym\text{-}1^{+}$ cells co-express CD206 (mannose receptor), which is another marker for M2 activation known to be involved in antigen internalization and processing [25]. CD68 (macrosialin) glycoprotein is another accepted marker for phagocytotic cells and is often used to distinguish the M2 debris clearing state of microglia [25]. Stroke-induced changes in the expression of specific cell surface proteins probably reflect a continuum of the microglial activation spectrum. There is a growing need to identify subpopulations of M1/M2 microglia versus those of peripheral myeloid cells and the relative percentages of each over time to determine the overall functional contribution to stroke injury.

Migration

Microglia, as the first immune responders in the CNS, migrate to areas of injury through detection of chemoattractant gradients to subsequently phagocytize debris in damaged tissue, neutrophils, and apoptotic cells that have the potential to release damaging molecules [44, 87, 88]. CX3CL1 and ATP released from dying neurons can act on microglial receptors to induce chemotaxis. In addition, monocyte chemotactic protein-1 (MCP-1, CCL2) is a chemokine expressed both in the brain and in some peripheral organs that can induce migration of leukocytes and macrophages/microglia to the ischemic area [89, 90]. After MCAO, MCP-1 expression increases at injured region and peaks at 2-3 days [91] when monocytes/macrophages also start to peak in the ischemic brain [92]. Transgenic CCL2 knockout mice show decreased ischemic injury [93], suggesting that the recruitment of monocytes/macrophages to the injured area may be detrimental

and that therapies designed to block the migration of leukocytes may have translational value. Microglia at the border region of the infarct, "penumbral" microglia, may be the major target of therapy given their large numbers, and activated status after stroke [94]. Unlike microglia of the ischemic core, which appear to be dying with increased disease severity, penumbral microglia are presumably living and highly activated [47]. This suggests a regional target of interest to deliver treatments.

Phagocytosis by microglia and macrophages

As the primary phagocytes in the brain, activated microglia phagocytize and uptake damaging components in an effort to reestablish homeostasis after insults [5]. Microglial phagocytosis of neuronal cells begins early even before peripheral macrophages infiltrate into the brain after stroke [95]. These phagocytotic cells were seen interacting with neurons and show neuron engulfment in the ischemic brain. CD68 is a commonly used marker of macrophage phagocytosis and is expressed as early as 6h after pMCAO on ramified $CD11b^+$ cells in the penumbra, and continues to increase later in the hypertrophic ameboid cells of the ischemic core [25]. However, this phagocytosis marker can be expressed on both the resident and infiltrating phagocytes, and therefore may not be exclusive to microglia [25].

Phagocytosis is able to attenuate inflammation but can also be implicated in neuronal damage. A recent study of primary microglial cultures assessed the sequence of events from microglial activation to the phagocytosis of neurons [96]. Production of peroxynitrite during a microglial immune response to TLR4 and TLR2 activation leads neurons to externalize phosphatidylserine on the outer membrane to act as an "eat me" signal to elicit phagocytosis. Interestingly, inhibition of this process at any stage not only leads to the decrease in phagocytosis of the neurons, as would be suspected, but also prevents 90% of neuronal cell death [96]. The potential for microglia to phagocytize viable neurons illustrates the importance of microglial modulation in ischemic stroke [96].

Purinergic receptors

Purinergic receptors ($P2X_7$, etc.) are upregulated on microglia in the periinfarct region of injury [97] and have been shown to interact with ATP that is accumulated extracellularly in the ischemic brain [98], subsequently leading to microglial activation [99]. Activation of these receptors leads to cell death [100] and the prominent release of proinflammatory cytokine IL-1β through caspase-1 pathway [101]. ATP leaked from dying neuronal cells and released by astrocytes [44, 102] can act as a chemoattractant on microglia by interacting with $P2Y_{12}$ receptor [103]; the microglia attracted to sites of ischemic injury can further amplify activation by autocrine signaling of ATP [104]. This positive feedback loop leads to increased proliferation and secretion of IL-1β, TNF-α, and ROS [99], and exacerbates inflammatory responses. Blocking $P2X_7$ receptors was shown to improve behavior deficits in a model of transient global ischemia where decreases in microglial activation and proinflammatory cytokines (TNF-α, IL1-β, and IL-6) were noted [105]. A nonselective P2 blocker, Reactive Blue 2, was shown to reduce infarction in a focal pMCAO model. However, $P2X_7$ receptors are also expressed on neurons and astrocytes. Therefore, the function may not be solely attributed to microglia [106].

Toll like receptor (TLR)

The stroke-induced innate immune response is also associated with the release of various damage associated molecular patterns (DAMPs), which can further activate pattern recognition receptors (PRRs), including members of the TLR family on microglia [107, 108]. TLRs are important in innate immunity in both the central and peripheral systems. TLR4 is the prominent LPS receptor that can lead to activation of LPS-responsive cells, such as monocytes and macrophages, and subsequently causes upregulation of proinflammatory genes via NFκB signaling [109]. The most prominent TLRs on microglia are TLR4 and TLR2, both of which are upregulated after ischemia [108, 110-113]. TLR4 deficient mice have smaller infarct sizes, better neurological scores and decreased downstream NFκB signaling in experimental stroke studies [112, 114]. Recent studies have shown that CNS preconditioning with TLR4 agonist can lead to tolerance, eliciting a diminished proinflammatory response with subsequent less ischemic injury [115, 116]. Although there has been controversy as to the role of TLR2 deficiency in cerebral isch-

emia, recent studies have shown an exacerbation of injury in TLR2 KO mice [108, 111, 117]. Some endogenous ligands of TLRs have recently been identified. Purines and peroxiredoxin (prx) released to the extracellular space from dying cells can bind TLRs on macrophages and produce proinflammatory cytokines IL-23 and IL-17 [118]. Heat shock proteins (HSP) and High mobility group box 1 (HMGB1) are other endogenous ligands for TLRs that result in upregulation of NOS and proinflammatory cytokines (TNF-α, IL-6, and IL-1β) [119, 120].

Functional insights of resident microglia and peripheral macrophages

Microglial proliferation and renewal

There is evidence that activated microglia are proliferative in the ischemic brain. Using carboxyfluorescein diacetate succinimidyl ester (CSFE) to intracellularly label peripheral cells before a 30-60 min MCAO, one study found higher amounts of $BrdU^{+}Iba1^{+}CSFE^{-}$ colabeling microglia after stroke indicating increased proliferation [88]. Interestingly, accumulating data show microglial proliferation is beneficial after ischemia. *In vitro* studies have shown that increased microglial proliferation by stimulation of CSF-1R, which is upregulated in ischemia, is neuroprotective [28, 121]. An in vivo study also showed that defective microglial proliferation was associated with significant increase in the size of ischemic lesion and a 2-fold increase in the number of apoptotic neurons [122].

In addition to microglia, choroid plexus macrophages, perivascular macrophages, and meningeal macrophages inhabit various regions around the CNS [123]. This heterogeneous population of tissue macrophages is continuously replenished by circulating and peripheral monocytes, unlike microglia that are largely thought to be resident in the adult CNS from early development [38, 124, 125]. Theories of another wave of microglial establishment post-embryonic from peripheral monocytic precursers that last into adulthood are part of an ongoing debate [38, 126]. It is uncertain whether these monocytic precursors become integrated into the microglial population or remain bone marrow-derived monocytes. Nevertheless, Varvel et al. [127] suggest yet another mode of microglial replacement apart from proliferation exists in the adult brain. They ablated microglia using intracerebroventricular ganciclovir treatment in Tk^{+}/*Ccr2*$^{+/rfp}$ mice and found abundant levels of $CD45^{high}$ monocytes in neocortical areas of microglial depletion before engraftment. In addition, there were increased levels of *Ccr2 RFP* expression which returned to normal levels after engraftment. These infiltrating cells of monocytic origin are morphologically similar to microglia and functionally active in surveying the microenvironment of the brain, without changes in number over time [127]. It's possible that a population of dying microglia in the ischemic brain could be replenished by peripheral monocyte/macrophages infiltrating into the injured region and downregulating CCR2 and CD45 to morph into microglia. The topic of microglial renewal and proliferation, however, is still controversial.

MMPS

Macrophages, including microglia, are major contributors to the release of MMPs (mainly MMP3 and MMP9) which are responsible for the breakdown of the extracellular matrix and the BBB after ischemia [88, 128]. MMP-3 and MMP-9 knockout mice were shown to suffer less neuronal injury after an ischemic episode [129, 130]. Since MMP-9 can be derived from both brain and peripheral immune cells, its origin after ischemia has been debated. Recently, chimeric studies showed that the increased damage from MMP-9 toxicity in the ischemic brain are attributed to bone marrow cells as opposed to resident microglia [130, 131]. This suggests that infiltrating monocytes/macrophages can also release MMPs and modulate their own entry into the CNS by weakening BBB.

TNF

TNF-α can be released from both microglia and peripheral leukocytes. TNF-α has been largely regarded as neurotoxic [132]; however, chimeric studies have found that TNF-α produced locally by resident brain microglia (but not by peripheral macrophages and leukocytes) exerted neuroprotection in pMCAO model via TNF-p55 signaling [133]. The disparity in implications may be attributed to different signaling mechanisms between the neuroprotective TNF-p55 and neurotoxic TNF-p75 pathways [134].

iNOS

Local accumulation of nitric oxide (NO) is also involved in the inflammatory cascade after cerebral ischemia [135]. Equipped with inducible nitric oxide synthase (iNOS), M1 microglia are partially responsible for the changes in expression level of NO. The role of iNOS in ischemia is inconsistent in literature as both protective and deleterious roles have been reported [136-138]. Pruss et al [139] found that in chimeric iNOS-deficient mice transplanted with WT bone marrow (BM) cells and wild-type mice transplanted with iNOS-deficient BM cells, no difference in cerebral iNOS expression or in infarct volumes can be seen between the chimeric animals after MCAO, suggesting iNOS from microglia and peripheral myeloid cells may not be a significant regulator of ischemic injury.

Peroxiredoxin

The peroxiredoxin family of proteins (Prxs) are intracellular antioxidant enzymes that are needed for cell survival in the brain [140, 141] . However, once released from necrotic brain cells, extracellular Prxs promote neural cell death in ischemia by inducing expression of inflammatory cytokines including IL-23 in macrophages [118]. This study also utilized chimeric mice models and further showed that the infiltrating bone marrow-derived macrophages but not the resident microglia, are responsible for increased ischemic volume and inflammatory response triggered by Prxs release [118]. In addition, they found that neutralization of the released extracellular Prxs with a prx antibody is protective.

Summary

Despite more than one century of research since they were first investigated by Rio Del Hortega [142], "the father of microglia", the precise role of microglia is still shrouded in mystery due to a current lack of tools and fundamental understanding of the heterogeneity of their observed activation spectrum. The nature of the microglial response to stroke is multi-faceted and complicated by the aggregate immune response. The significance of the local and systemic inflammatory response as well as the interplay between the two is widely argued. While many studies suggested that anti-inflammatory agents confer neuroprotection following ischemic brain injury, others pointed to a requirement for pro-inflammatory cytokines and leukocyte activation in orchestrating repair. Enhancing repair may be possible by targeting distinct populations of microglia with special attention to temporal and spatial specific therapeutic intervention in ischemic stroke and other neurological disorders. There is room for improvements of methods for better identification and manipulation of microglia. The roles of microglia in stroke-induced inflammatory responses merit further investigation, in hope that fine-tuning immunomodulatory therapies could be available to avoid the deleterious effects of total immunosuppression and the possible detrimental effects of chronic microglial inhibition.

Conflict of interest

None.

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