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Review

Regulation of microglial activation in stroke

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Abstract

When ischemic stroke occurs, oxygen and energy depletion triggers a cascade of events, including inflammatory responses, glutamate excitotoxicity, oxidative stress, and apoptosis that result in a profound brain injury. The inflammatory response contributes to secondary neuronal damage, which exerts a substantial impact on both acute ischemic injury and the chronic recovery of the brain function. Microglia are the resident immune cells in the brain that constantly monitor brain microenvironment under normal conditions. Once ischemia occurs, microglia are activated to produce both detrimental and neuroprotective mediators, and the balance of the two counteracting mediators determines the fate of injured neurons. The activation of microglia is defined as either classic (M1) or alternative (M2): M1 microglia secrete pro-inflammatory cytokines (TNFα, IL-23, IL-1β, IL-12, *etc*) and exacerbate neuronal injury, whereas the M2 phenotype promotes anti-inflammatory responses that are reparative. It has important translational value to regulate M1/M2 microglial activation to minimize the detrimental effects and/or maximize the protective role. Here, we discuss various regulators of microglia/macrophage activation and the interaction between microglia and neurons in the context of ischemic stroke.

Keywords: cerebral ischemia; microglia; macrophage; neuroinflammation; cytokines; microglia/neuron interaction; brain

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Introduction

Ischemic stroke is a leading cause of morbidity and mortality worldwide. Ischemic brain injury is initiated by energy depletion and massive cytoplasmic Na^+/Ca^{2+} accumulation. As a result, a cascade of molecular events occurs, including the production of reactive oxygen species (ROS), the activation of Caspases/Calpain, the inhibition of protein synthesis, mitochondrial dysfunction, cerebral edema formation and cellular DNA fragmentation, which together lead to primary ischemic damage. Almost immediately after the onset of brain ischemia, microglia are activated, and cytokines are extensively produced, which leads to the migration of leukocytes toward the injured brain. The normally immune-privileged brain environment is consequently exposed to systemic responses that further exacerbate the immune responses and cause secondary neuronal damage[1]. The activated microglia are highly plastic cells and are divided into classic (M1) and alternative (M2) activation phenotypes[2, 3], and the polarization of the cells is dependent on the different stages of disease development. Emerging studies have focused on the regulatory mechanisms that underlie the activation of microglia and have aimed to suppress the M1 phenotype and to promote the M2 phenotype to provide neuroprotection. Different molecular pathways and mediators that regulate microglia/macrophage activation after cerebral ischemic injury are reviewed below.

Resting microglia

Microglia, the resident immunocytes in the brain[4], have a myeloid origin[5, 6]. These cells constantly survey their environment with highly motile processes and are thought to be the immediate sensors of the brain pathology[7]. Resting microglia not only constantly extend and retract their thin ramified processes to inspect the microenvironment[8, 9] but also remodel neural circuits by forming synaptic communications with adjacent neurons in healthy brains[10, 11].

During the embryonic stage, there is a major wave of migration of primitive myeloid progenitors that enter into the brain and later develop into resident microglia. Microglia are considered to be long-lived cells that can replenish themselves in the brain[12]; however, the topic of microglial renewal and proliferation remains controversial[13]. There are other macrophage-related populations present in the leptomeninges and in the choroids plexus; the functions of these populations are relatively poorly known, but they were proposed to have monitoring and scavenging functions, given their locations[14].

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Activation of microglia/macrophages

Classic (M1) or alternative (M2) activation has been mostly reported for macrophage responses during peripheral inflammation, and, recently, microglia were found to have a similar activation process upon ischemic insult[15]. M1 macrophages are involved in the acute pro-inflammatory response and possess antigen-presenting capacity. Several signaling pathways have been proposed to contribute to M1 macrophage polarization. Interferon γ (IFNγ) secreted by T helper (Th) 1 cells is instrumental in inducing the M1 phenotype of macrophages[16]. Through Janus kinase (JAK)1/JAK2 signaling, IFNγ activates signal transducer and activator of transcription 1 (STAT1) factor and increases the production of pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin (IL)-23, IL-1β, IL-12, chemotactic factors, reactive oxygen species, and nitrogen monoxide (NO). Another pathway for the induction of M1 activation is triggered by lipopolysaccharide (LPS) or damage-associated molecular pattern (DAMP) stimulation through Toll-like receptor 4 (TLR4)[17], followed by the formation of an "activation complex" that includes myeloid differentiation factor 88 (Myd88), nuclear factor κB (NFκB), p65, p38, and interferon regulatory factor 3 (IRF3)[17, 18]. The complex in turn regulates the secretion of inflammatory mediators [inducible nitric oxide synthase (iNOS), CD16, CD32, *etc*] and cell surface markers [histocompatibility complex (MHC-II), CD86, *etc*] from the polarized cell[17]. The polarization of the M1 phenotype is characterized by the expression of IL-12^{high}, IL-23^{high} and IL-10^{low}[18]. The alternative activation of macrophages (M2) is usually induced by IL-4 or IL-10 and IL-13 and is generally characterized by IL-12^{low}, IL-23^{low}, and IL-10^{high}. To activate macrophages toward M2, IL-4 or IL-13 combines with IL-4Rα or IL-13Rα1, respectively, to activate transcription factors such as STAT6, peroxisome-proliferator-activated receptor γ (PPARγ), Jumonji domain-containing protein 3 (Jmjd3), and IRF4. As a result, cytokines such as IL-10, transforming growth factor β (TGF β), IL-1 receptor agonist, CD302, CD163, and other inflammatory mediators [platelet-derived growth factor (PDGF), Fibronectin 1 and Arginase 1 (Arg1)] are released[18].

In ischemic stroke, sterile inflammation is induced by DAMPs released from injured brain tissue. Endogenous microglia and recruited macrophages are activated and polarize to the M2 phenotype in the acute stage and then gradually switch toward the M1 phenotype, which particularly occurs in peri-infarct regions adjacent to ischemic neurons[15]. *Ex vivo* studies have found that when M1 microglia were added to a cell culture, oxygen glucose deprivation (OGD)-induced neuronal death was exacerbated; by contrast, M2 microglia protected neurons against OGD[15]. It has been demonstrated that microglial polarization also involves NFκB and STAT1 pathway activation, which is seen in inflamed macrophages[19]. Similar to macrophage activation, the production of pro-inflammatory cytokines is essential for microglia to polarize toward the M1 phenotype[20]. It has also been reported that during neuroinflammation, microglia produce IL-10 and TGFβ[21, 22], which leads to anti-inflammatory signaling (M2a) and wound healing (M2c)[22]. During disease progression, the M1 phenotype can switch to M2 or vice versa depending on inflammatory signals[23]. In addition, microglia can self-control their polarization through autocrine and paracrine means, and this response is protective but is downregulated once the damage or pathogen has been addressed. If the self-regulation is interrupted, unregulated, long-term, or chronic inflammation occurs that exacerbates tissue damage[24].

Regulation of microglia/macrophage activation

A plethora of pathways and mediators have been documented to regulate macrophage activation, and many of these pathways and mediators are shared by microglial activation. Most of these signaling pathways overlap with each other to varying degrees, and do not appear to work independently but rather function synergistically to cause an inflammatory maelstrom. Limited studies are available in the literature regarding the regulatory pathways that underlie microglial activation in the context of stroke; therefore, relevant data from other neurodegenerative diseases are also reviewed here.

Toll-like receptors (TLRs)

TLRs are transmembrane proteins that are characterized as pattern-recognition receptors and are the key factors in initiating inflammatory responses. TLRs are expressed on various cells, including macrophages, microglia[25, 26], astrocytes[26–28], Schwann cells[29], and neurons[30]. In the central nervous system (CNS), both pathogen-associated molecular patterns (PAMPs) and DAMPs can activate TLRs to initiate a signaling cascade of immune responses that are regulated by microglia and astrocytes[25, 26, 31]. PAMPs include bacterial DNA and LPS, and DAMPs are known as endogenous ligands that are generated by sterile tissue injury. The cascade mobilizes Toll/IL-1R (TIR) domain-containing adaptor proteins, such as MyD88, NFκB-inducing kinase (NIK), and IκB kinase (IKK), to activate NFκB; subsequently, the activated NFκB translocates into the nucleus to induce the production of pro-inflammatory cytokines such as TNFα, IL-1, and IL-12[32–34]. During spinal cord injury, TLR2 mediates Nox2 gene expression and activates microglia via the NFκB and p38 pathway[35]. It was found that TLR2 activation led to the expression of IL-23 and IL-17 on microglia, which exacerbated inflammatory responses and neuronal damage[36]. TLR2 activation causes either M1 or M2 microglial polarization depending on the type of stimulation and the progression of neuroinflammation. After the administration of Pam2CSK4, a specific TLR2 agonist, microglia robustly increased and were polarized to the M2 phenotype to reduce the secondary degeneration of myelinated fibers in the CNS[37]. TLR3 was found to play vital roles in the JEV-induced microglial response[38]. The TLR3-mediated activation of human microglia can change the profile of immune responses by transmitting Th1 polarizing signals to CD4 T cells. The response leads to IFNγ secretion, Th1 polarization, and the expression of major histocompatibility complex, costimulatory molecules (CD80, CD40, and CD86), and INFα and IL-23 in microglia[39]. TLR4 is mostly expressed by microglia in

the brain[40]; however, both TLR4 and TLR2 expression were increased in cerebral cortical neurons after ischemia/reperfusion injury[30]. A shortage of TLR2 or TLR4 reduces infarct size following focal cerebral ischemic injury[41–43]. The increase in TLR4 expression leads to the activation of the NFκB pathway, the release of the pro-inflammatory cytokines TNFα and IL-6, and the exacerbation of neuronal damage and apoptosis. TLR4 activation also causes the elevated expression of p38, c-Jun N-terminal kinase (JNK) and extracellular regulated protein kinase (ERK)1/2 and iNOS in the ischemic brain, which are responsible for M2 microglial activation[44].

Histamine and substance P

Histamine and substance P are important neuroinflammatory mediators in the CNS that can trigger microglia to become activated and to release pro-inflammatory factors (TNFα and IL-6). The underlying mechanism was proposed to be histamine- or substance P-induced mitochondrial membrane depolarization[45]. Antagonists of histamine or substance P receptors can partially abolish the effect of microglial activation. However, controversy exists because another study reported that in cultured microglia from adult mice, IL-4, an anti-inflammatory mediator, increased the sizes of the histamine-, substance P-, and somatostatin-sensitive cell populations[46], which suggests that histamine and substance P are also involved in anti-inflammatory responses. Taken together, the role of histamine and substance P in microglial activation is still elusive.

Programmed cell death protein 1 (PD1)

PD1 is a transmembrane protein that belongs to the CD28 superfamily. As an inhibitory receptor, PD1 binds to its ligands PDL1 and PDL2 to induce inhibitory signals to maintain the balance between T-cell activation, tolerance, and immune-mediated tissue insult[47, 48]. PD1 also suppresses innate and adaptive immune responses[49]. The PD1/PDL1 signaling pathway plays an important role in the polarization of macrophage activation during spinal cord injury (SCI)[50]. At the onset of SCI, both PD1 and PDL1 expression on microphage/microglia are up-regulated; as SCI progresses, increased PDL1 recruits PD1 from other macrophage/microglia at the site of SCI, providing a negative feedback signal that contributes to an M2 phenotype switch[50]. Other studies have also shown that up-regulated PD1 can suppress M1 polarization and promote M2 polarization by increasing STAT6 phosphorylation[50]. By contrast, down-regulated PD1 signaling may initiate the polarization of macrophages/microglia toward an M1 phenotype by increasing the phosphorylation of STAT1 and NFκB[50].

Secreted protein lipocalin 2 (LCN2)

LCN2 is a member of the lipocalin family that was initially found in neutrophil granules[51]. LCN2 possesses multiple functions to regulate cell death/survival[52], cell migration/invasion[53], cell differentiation[51] and iron delivery[54]. A variety of inflammatory conditions can induce the expression of LCN2 in macrophages[55]. In a mouse model of LPS-induced neuroinflammation, the expression of LCN2 was notably increased in microglia[56]. The secreted LCN2 from microglia in turn stimulated and amplified the M1 polarization of microglia in an autocrine manner. As a result, pro-inflammatory gene expression was increased, including the expression of IL-12, IL-23, iNOS, TNFα, and the chemokine fractalkine (CXCL)10. LCN2 was found to inhibit the phosphorylation of STAT6 and to lead to the suppression of M2 signaling in IL-4-stimulated microglia[56]. It has been suggested that LCN2 is an M1-amplifier in microglia and can cause skewed M1 polarization.

Mitogen-activated protein kinase (MAPK) and AMP-activated protein kinase (AMPK)

The MAPK signaling pathway plays a vital role in microglial activation. Through this signaling pathway, 4-oxobutyric acid (DCPIB), a potent volume-regulated anion channel (VRAC) inhibitor, suppressed the release of glutamate and the excitatory stimulation of neurotoxicity, and it inhibited M1 microglial activation during ischemic stroke injury[57]. Upstream of MAPK activation, the AMPK and Calcium-Calmodulin-Dependent Protein Kinase Kinase (CaMKK) β-dependent signaling pathway also participates in the activation of microglia. This mechanism was identified through experiments showing that microglia treated with hydrogen sulfide (H2S) can polarize to the M2 phenotype"[58]. Another AMPK activator, metformin, also induced the activation of microglia/macrophages to switch toward the M2 phenotype and significantly increased angiogenesis and neurogenesis in the ischemic brain following middle cerebral artery occlusion (MCAO)[58].

MicroRNAs (miRs)

MicroRNAs (miRs) are a family of small (approximately 22 nucleotides) noncoding RNAs. A total of 30%–90% of human genes are regulated by miRs that modulate cell growth, activation, and differentiation[59]. miR124 is expressed in M2-phenotype macrophages, and the over-expression of miR124 down-regulates the expression of M1 genes (MHC II, CD86) and up-regulates M2 markers such as resistin-like α (Fizz1) and Arg1. Exposure to IL-4 and IL-13 increased the expression of miR124 in macrophages[59]. miR424 also plays an important role in neuroprotection after stroke. In a cerebral ischemia/reperfusion study, the over-expression of miR424 reduced infarction volume and brain edema and decreased neuronal apoptosis and microglia polarization by inhibiting ionized calcium binding adaptor molecule-1 (Iba-1) expression and activity after stroke. *In vitro*, miR424 mimics caused G_1 phase cell-cycle arrest and inhibited BV2 microglia activity. miR424 suppresses microglial activation by inhibiting key translational activators of G_1/S[60]. Other investigators found that miR200b was also expressed in microglia, and the expression was down-regulated after traumatic brain injury; knockdown of miR200b in microglia increased JNK activity, pro-inflammatory cytokine secretion, iNOS synthesis and NO production, which resulted in increased neuronal apoptosis. Conversely,

the over-expression of miR200b in microglia suppressed JNK activity, iNOS synthesis, NO production and the migratory potential of activated microglia. miR200b regulates microglial inflammatory processes and neuronal survival by modulating the c-Jun/ MAPK pathway[61].

Notch signaling

Experimental stroke studies found that the activation of Notch induces the M1 polarization of microglia, suppresses M2 activation, and worsens ischemic brain damage[62]. Moreover, the blockade of Notch signaling during microglial polarization could be "memorized" by microglia, which suggests that Notch signaling contributes to post-ischemic inflammation by directly modulating the microglial innate response[63]. The effects of Notch signaling on microglial activation may be pleiotropic; Notch-induced microglial activation also contributes to cell apoptosis in the ischemic brain tissue[64].

IRFs

Emerging data suggest that members of the family of interferon regulatory factors (IRFs) mediate macrophage polarization. In mammals, the IRF family consists of nine members: IRF1, IRF2, IRF3, IRF4, IRF5, IRF6, IRF7, IRF8/ICSBP, and IRF9[65]. The variable domains at the C-terminus determine the functional specificity of each IRF (Table 1).

IRF1

In the peripheral immune system, IRF1 is weakly expressed in resting macrophages; however, the expression of IRF1 is up-regulated in M1 polarized macrophages that are activated by IFNγ-producing NKT cells or Th1 cells. By interacting with MyD88, IRF1 migrates into the nucleus and triggers the expression of TLR-mediated genes (IFNβ, iNOS and IL-12p35)[66]. Moreover, IRF1 prevents other transcription factors (*eg,* IFNγ) from binding to IL-4 and inhibits M2 macrophage activation, which leads to M1 microglial polarization in the ischemic brain[67, 68]. The expression of the IRF1 gene was markedly increased within 12 h of MCAO in mice and reached a peak at d 4 of ischemia[69]. IRF1 expression was also present in intravascular neutrophils that infiltrated the ischemia tissue after MCAO and in neurons at the outer border of the ischemic territory[70]. In an ischemic stroke study with an IRF1$^{-/-}$ mouse model, the infarct volume and neurological deficit scores were reduced compared to the WT cohort, indicating that IRF1 contributes substantially to cerebral ischemic damage[69].

IRF2

IRF2 is a transcriptional repressor with the capacity to inhibit IFNγ expression and IRF1-dependent macrophage activation[71, 72]. IRF2 also suppresses LPS-induced TNFα expression and augments IL-1, IL-6, IL-12, and IFNγ secretion[73]. Sumoylation increases IRF2's ability to inhibit IRF1 transcriptional activity[74]. The expression of TLR3, TLR4, and TLR5 was regulated by IRF2 signaling after LPS stimulation of murine macrophages[75, 76]. Thus far, there are limited data available about the role of IRF2 in microglial activation after

Table 1. The roles of IRFs in macrophage/microglia polarization.

	Primary expression cell	Induction	Pathways involved	Roles in regulating marophages/microgla	References
IRF1	Microglia/ macrophage	IFN-γ	Interact with MyD88.	Regulate TLR-mediated expression of pro-inflammatory genes.	[66–70]
IRF2	Any cells	IFN-γ and virus	Compete with IRF1 for DNA binding sites.	Activator of H4 gene, VCAM-1, and TLR9 gene; augment LPS induced IL-1, IL-6, IL-12, and IFN-γ secretion; repress the transcriptional activation of the IFN-b gene.	[71–76]
IRF3	Ependymal cells and choroid plexus	Molecules mediated by dsRNA and dsDNA	Be the crucial transcription factor in non-MyD88 pathway.	Alter the microglial activation phenotype from M1 to M2; transactivate the IFN-b, CXCL10, CCL5, ISG56, IFIT1 , arginase II and RIG-Ilike eceptors gene.	[77, 78]
IRF4	Bone marrow-derived macrophages/ microglia	Different mitogenic stimuli	Through IL-4 signaling; mediated by the transcription factor signal transducer and activator of Stat6.	Control M2 polarization; regulate MHC-II, Ciita, Cyp1b1, and Il1rn genes.	[79–82]
IRF5	B cells, dendritic cells, macrophages/ microglia	Type I IFN and viral infection	Decrease TLR3-, TLR4-, and TLR9-dependent induction of TNFα and I IFN.	Regulate host immunity against extracellular pathogens, DNA damage-induced apoptosis, death receptor signaling, and classic macrophage polarization.	[83, 84]
IRF7	Microglia/ macrophages	Toll-like receptor 4 signaling	Suppress the activation of STAT1.	Involved in demyelination.	[85]
IRF8	Microglia	Type I IFN	Activate gene expression that transforms microglia into a reactive phenotype.	Increase levels of Iba1, CD206, CD45, CD11b and F4/80 and F4/80; decrease levels of the chemokine receptors CCR2, CCR5 and CX3CR1.	[86–91]

cerebral ischemia.

IRF3

IRF3 is a transcription factor that can be induced by TLR3 or TLR4 activation. A variety of cells express the IRF3 protein, and it was reported that IRF3 could change the microglial phenotype from M1 to M2. In an *in vitro* study with a glial-neuronal co-culture system, transduction with adenovirus-mediated IRF3 suppressed pro-inflammatory gene expression, including the expression of IL-1, TNFα, IL-6, and CXCL1 in microglia. Moreover, IRF3 induced the expression of many anti-inflammatory genes, including IL-1 receptor antagonist, IL-10 and IFNβ[77]. It was found that IRF3 facilitated M2 polarization of microglia and protected neurons from cytokine-induced insult[77]. There was also evidence to indicate that IRF3 suppressed neuroinflammation by regulating miR155, which is highly expressed in lesions of multiple sclerosis and is involved in the induction of pro-inflammatory cytokine genes[78].

IRF4

IRF4 is expressed in macrophages in various organs and tissues[79]. During peripheral immune responses, IRF4 expression is strongly induced in bone marrow-derived macrophages via IL-4 signaling. IRF4 not only regulates the expression of several secondary DNA binding proteins (*eg*, MHC II) that promote M1 macrophage phenotype[80] but also increases the expression of M2 phenotype marker genes such as Arg1, Ym1, and Fizz1. IRF-4$^{-/-}$ macrophages produce pro-inflammatory cytokines, including IL-1β and TNFα, to enhance M1 polarization[81]. In ischemic brains, the expression of IRF4 increases in neurons and microglia in the penumbra after the onset of ischemic stroke in an attempt to rescue neurons from ischemia/reperfusion-induced cell injury[82]. Neuron-specific IRF4 transgenic (IRF4-TG) mice exhibited reduced infarct volumes after MCAO, which was reversed in the ischemic brains of IRF4 knockout mice[82].

IRF5

IRF5 contributes to pro-inflammatory responses[83]. Through TLR signaling, IRF5 binds MyD88 to form homodimers, activates type I interferon, and up-regulates the expression of M1-phenotype related cytokines, including TNFα, IL-6, and IL-12. MyD88 is the key adaptor protein that mediates the regulatory effect of IRF5 on M1 activation; however, IRF4 competes with IRF5 for binding to MyD88 and inhibits the transmission of TLR outside-in signaling to NFκB and to other pro-inflammatory transcription factors[66]. The competition between IRF5 and IRF4 for MyD88 binding causes IRF4 to suppress M1 macrophage polarization[84]. The balance between IRF4 and IRF5 might be a critical determinant in the modulation of macrophage polarization.

IRF7

IRF7 also participates in the regulation of microglial polarization[85]. One study showed that IRF7 expression increased when the phenotype of microglia switched from M2 to M1 after LPS treatment *in vitro*[85]. After the knockdown of IRF7 with siRNA, the phosphorylation of STAT1 was reduced, and the expression of M1 markers (CD86, iNOS) was suppressed[85].

IRF8

IRF8 is an interferon consensus sequence-binding protein and a key IRF for the phenotypic switch of microglial activation in the brain[86]. IRF8 is constitutively expressed in neurons and microglia[87, 88]. The expression of IRF8 was suppressed at the onset of stroke and was further decreased to 26% of the baseline at 72 h of ischemia[88]. However, IRF8 expression was increased in microglia after peripheral nerve injury[89]. IRF8 promoted the M1 activation of microglia/macrophages and exacerbated neuroinflammation[90]. In an EAE disease model, IRF8 activated microglia and induced the production of pro-inflammatory cytokines IL-12 and -23[91].

The transcription factor p53

During HIV-associated neurocognitive disorders (HAND)-induced neuroinflammation, p53 was highly expressed and in turn increased the expression of genes associated with classical macrophage activation, leading to the secretion of pro-inflammatory cytokines in microglia[92]. The phagocytic activity of microglia and the expression of markers for alternative activation were increased in the ischemic brains of p53$^{-/-}$ mice[92]. In a study of Alzheimer's disease, the inhibition of p53 also led to a decrease in microglial apoptosis and prevented microglial neurotoxicity[93]. By contrast, the up-regulation of p53 expression in ischemic brains increased microglial apoptosis and BBB damage, and worsened brain damage[94].

Jumonji domain containing protein 3 (Jmjd3)

Jmjd3 is indispensable for the expression of the M2 phenotype during macrophage/microglia polarization[95, 96]. Jmjd3 can induce microglial polarization toward the M2 phenotype, which ameliorates the inflammatory pathological changes in Parkinson's disease (PD)[95]. In the aforementioned study, the suppression of Jmjd3 inhibited M2 microglia polarization and simultaneously enhanced M1 activation and pro-inflammatory responses, resulting in extensive neuronal loss in the substantia nigra area[95]. Currently, the role of Jmjd3 in microglial activation has only been reported in neurodegenerative diseases; very little data are available from ischemic brains.

Peroxisome-proliferator-activated receptor (PPARγ) pathway

PPARγ aids in the phenotype switch of peripheral macrophages and modulates the secretion of many cytokines[97]. PPARγ activation inhibits inflammation and may have neuroprotective effects. In a mouse model of PD that was induced by treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-probenecid (MPTPp), PPARγ was found to play a pivotal role in microglial activation[98]. After chronic treatment with MPTPp, the expression of pro-inflammatory cytokines (TNFα and IL-1β) gradually increased, whereas the levels

of the anti-inflammatory cytokine CD206 was significantly reduced. However, treatment with a PPARγ agonist (rosiglitazone) decreased the serum levels of TNFα and IL-1β and increased the levels of TGFβ, IL-10 and CD206. In addition, PGC-1-related coactivator (PRC), an important member of the PPARγ coactivator-1 (PGC-1) family, was robustly expressed in M2 microglia and cooperated with the signal transducer and activator of STAT6 to induce M2-related gene expression, *eg,* Arg1, Fizz1, and chitinase 3-like 3 (Chi3l3), *etc*[99]. An experimental stroke study showed after the activation of PPARγ, the expression of M1 markers (CD16, CD32, TNFα, IL-1 and iNOS) was suppressed, and M2 marker expression (CD206, Ym1, TGFβ and IL-10) was elevated[100]. These results suggested that PPARγ stimulates anti-inflammatory responses and suppresses pro-inflammatory responses to tilt microglial polarization toward the M2 phenotype[98].

Granulocyte colony-stimulating factor (G-CSF)

In a mouse model of spinal cord injury, the administration of recombinant human G-CSF (rhG-CSF) boosted the recruitment of microglia to the lesion site 72 h after the injury. Moreover, rhG-CSF inhibited the microglial expression of pro-inflammatory cytokines and promoted the expression of neurotrophic factors[101]. *In vitro* studies also found that G-CSF increased the expression of M2 markers (Arg 1 and CD206) and decreased the expression of M1 markers (CD32) in the culture medium of BV2 microglia exposed to the supernatant from the injured spinal cord. The NFκB signaling pathway was found to play a role in G-CSF-induced polarization of BV2 microglia[101].

Human bone marrow mesenchymal stromal cells (MSCs)

MSCs are well known as rare multipotent cells and are characterized as potent modulators of regeneration and immune responses. Recent studies[102, 103] have shown that MSCs could provoke polarized microglia to switch to an M2 phenotype. In a transient global ischemia study, the administration of hMSCs induced the alternative activation of microglia and/or macrophages[103]. The intracerebroventricular injection of hMSCs in a mouse model of traumatic brain injury up-regulated Ym1, CD206 and Arg-1 mRNA and reduced the expression of CD68 on the membrane surface of $CD11b^+$ microglia. The MSC-mediated M2 phenotype in microglia was correlated with an early and sustained recovery of neurological function and reparative changes of the lesioned microenvironment, which suggests that MSCs can influence the transcription environment to polarize microglia toward the M2 phenotype and resultantly promote neurogenesis and tissue repair[104]. One recent study[102], however, showed that MSCs induced a mixed microglial phenotypes characterized by $Arg1^{high}$, $CD86^{high}$, $CD206^{high}$, $IL\text{-}10^{high}$, prostaglandin(PG) $E2^{high}$, macrophage chemoattractant protein (MCP) 1/CC ligand (CCL)2^{high}, $IL\text{-}1\beta^{moderate}$, $NALP3^{low}$, and $TNF\alpha^{low}$. These MSC-stimulated microglia had high phagocytic activity and antigen presenting ability. The effects of MSCs on microglia polarization warrant further study.

Cross talk between neurons and microglia

In neurological disorders, such as epilepsy, stroke and neuropathic pain, microglia-neuron interactions alter the excitability of the neural network by modulating the neuroinflammatory responses. The substrates of the communication between microglia and neurons include several potential messengers, including cytokines, purines, prostaglandins, and nitric oxide[105]. Emerging data point to the importance of bidirectional interactions between neurons and microglia in the context of ischemic stroke. The underlying mechanisms by which neurons modulate microglial activation include the secretion of factors that influence basal microglial properties, the discharge of neurotransmitters that affect microglial behavior, and the release of purines that direct microglial chemotaxis[106, 107].

CD200 and CD200R

CD200 is a glycoprotein that is mainly expressed by neurons and by microglia when inflammatory responses occur in the brain[108]. Previous studies found that CD200 was prominently expressed in neuronal bodies in cortical layer I and in the hippocampal fissure[109], and the expression started to increase from postnatal d 1, peaked at d 7, and then decreased gradually in adulthood. CD200 interacts with its receptor CD200R, which is expressed by a variety of cells of the myeloid lineage, including microglia. $CD200R^+$ microglia/macrophages were observed at all ages, but the number significantly decreased with increasing age[109]. The interaction of CD200 with CD200R is involved in maintaining microglia in a quiescent state. The decreased expression of CD200 in the brain was associated with the presence of microglial activation accompanied by the increased production of inflammatory cytokines[110]. In CD200-deficient mice, inflammatory cytokines, which are the hallmark of microglial activation, were produced in higher levels in response to lipopolysaccharide than in wild type mice. Moreover, the expression of TLR4 and NFκB in microglia was also increased in CD200-deficient mice, and M1 microglial activation was enhanced[110]. Although neurons constitutively express CD200 in the normal brain, CD200 is also expressed in alternatively activated microglia. It was reported that microglial CD200 interacts with CD200R via an autocrine mechanism to maintain microglia in an alternatively activated state, whereas interactions between neuronal CD200 and microglial CD200R keep microglia quiescent[108]. This finding has been proposed as one of the mechanisms of the anti-inflammatory role of IL-4, as IL-4 can increase CD200 expression in M2 microglia[108]. Similar to CD200, the increased expression of CD200R in activated microglia induced neuroprotection in inflamed brains. The up-regulation of the receptor by CD200 fusion protein can decrease the secretion of IL-1β and IL-6 but can increase the level of IL-10 in activated microglia[111]. The anti-inflammatory role of CD200/CD200R signaling has been demonstrated in mouse models of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE)[112] and in the hippocampus of aged rats[113]. CD200/CD200R signaling

has been increasingly recognized as an important factor in the modulation of neuroinflammation (Figure 1).

Chemokine (C-X3-C motif) ligand

The chemokine fractalkine (CX3CL1) and its receptor CX3CR1 constitute another coupling signal that mediates microglial activation. Fractalkine is expressed constitutively in neurons and can be up-regulated by TNFα and IL-1β, which are released by astrocytes, and CX3CR1 is exclusively expressed in microglia in the CNS[114]. CX3CL1/ CX3CR1 signaling participates in the modulation of the communication between neurons and resident microglia/migrated macrophages in the brain. In addition, fractalkine expression on astrocytes may also be highly relevant to microglial activation via cytokine release caused by astrocyte-microglial cell interactions[114]. A deficiency in CX3CR1 could improve outcomes after ischemic brain injury, including infarct volume, neuronal apoptosis and ROS levels, owing to the withdrawal of macrophage recruitment from the periphery, the inhibition of microglial proliferation and macrophage infiltration, and the facilitation of the M2 phenotype switch[115]. ERK5 is a member of the MAPK family, is highly expressed in activated microglia in a spinal nerve ligation (SNL) mouse model[116] and increases nerve injury-induced heat and mechanical hypersensitivity; pretreatment with a CX3CR1 neutralizing antibody suppressed the expression of ERK5 in the spinal cord 1 and 3 d after SNL and exerted neuroprotection. CX3CL1 infusion significantly induced microglial activation and induced heat and mechanical hypersensitivity[116], which was suppressed by the intrathecal administration of ERK5 antisense oligodeoxynucleotides, which suggests that CX3CL1/CX3CR1 modulates microglial activation through the ERK5 signaling pathway, at least in a spinal injury model[117]. Interestingly, the CX3CL1/CX3CR1 interaction has also been proposed as an endogenous inhibitory signaling pathway that keeps microglia quiescent and confers neuroprotection[13, 117]. In animal models of Parkinson's disease, ALS, and LPS activation, augmenting CX3CR1 signaling protected brains against microglial neurotoxicity, whereas interrupting CX3CL1/CX3CR1 signaling increased neuronal vulnerability[118]. The controversial roles of CX3CL1/CX3CR1 signaling may be ascribed to alterations in signaling pathways in genetically altered animals, opposing roles of this pathway on microglia and CX3CR1^{+} macrophages, different stages of diseases that were examined, or other elements[119].

Triggering receptor expressed on myeloid cells 2 (TREM2)/ DAP12

TREM2 is a member of the innate immune receptor TREM

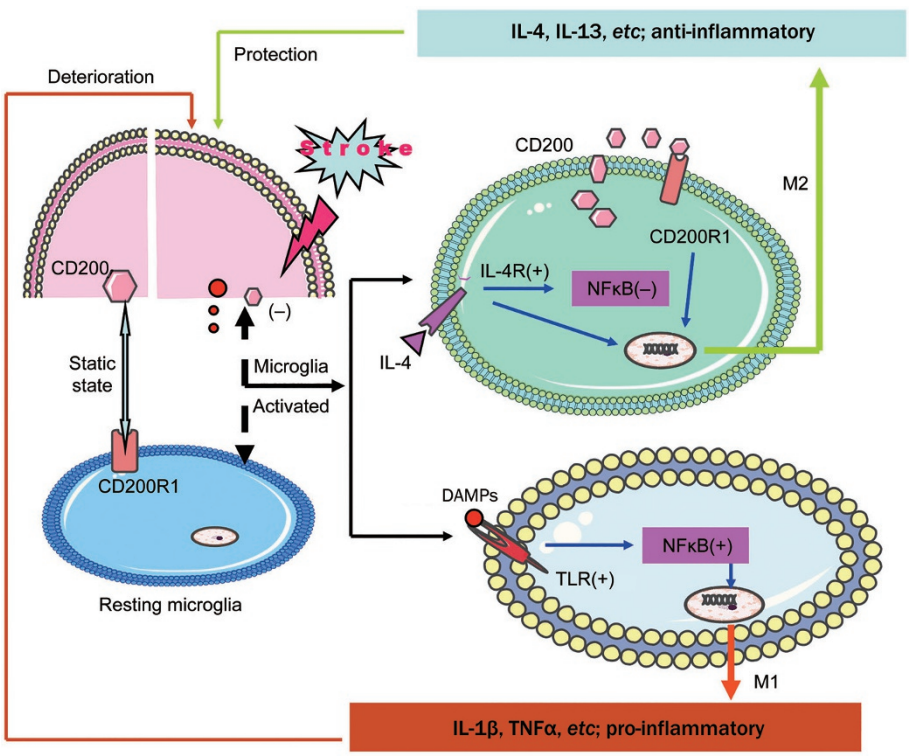

Figure 1. CD200/CD200R1 signaling in microglial activation. CD200 is secreted by neurons and binds to its receptor CD200R1 to maintain microglia in a quiescent state in the healthy brain. When a stroke occurs, CD200 expression by neurons decreases, and the association of CD200/CD200R1 is interrupted, which triggers microglial activation. DAMPs are released by necrotic neurons and bind to TLRs to activate NFκB, and microglia become classically activated (M1); IL-4 activates its receptor IL4R, which leads to NFκB inactivation and microglial M2 polarization. CD200 secreted by microglia also binds to CD200R1 through an autocrine mechanism and augments the M2 activation.

family and is exclusively expressed by microglia in the brain. TREM2 does not have an intrinsic signaling motif and must be bound by its adaptor protein DAP12 to signal intracellularly. TREM has two subtypes named TREM1 and TREM2, and they function differently. TREM1 is an amplifier of the inflammatory response; TREM2, however, inhibits inflammatory responses that are induced by TLR ligands. Upon stimulation with LPS and IFNγ, the expression of TREM2 on microglia is decreased[120–122]. One study showed that TREM2 appeared to sustain immune homeostasis and trigger an anti-inflammatory response in the sub-acute phase following stroke[123]. TREM2 ligand (TREM2-L) is expressed on neurons, and the expression increases when apoptosis occurs; the ligand then serves as an "eat me" signal[122]. TREM2-L on apoptotic neurons will eventually bind to DAP12 to down-regulate the expression of TNFα and iNOS and trigger phagocytic activity in microglia to clear the apoptotic neurons[124]. It was reported that heat shock protein 60 (HSP60) is the only ligand of TREM2 that has been identified on the surface of astrocytes and neuroblastoma cells[125], and another study showed that nucleic acids may also be a potential ligand of TREM2 on ischemic neurons[122]. In an ischemic stroke study using TREM2 knockout mice, subacute inflammation was attenuated at 7 d post-reperfusion, as indicated by a decreased transcription of pro-inflammatory mediators, including the cytokines TNFα, IL1α, and IL1β, the chemokines CCL2 and CCL3, and the receptor CX3CR1, and by reduced invasion of CD3-positive T-cells[123]. It can be concluded that the TREM2/TREM2-L signaling pathway serves as an inducer for M2 microglial activation.

Siglecs

Siglecs belong to a subgroup of the immunoglobulin superfamily that identifies specific arginine residues of sialic acids on the membrane of peripheral immune cells[126, 127]. Siglecs include three subsets: a conserved subgroup (Siglec1, 2, and 4), CD33-related Siglecs (CD33, Siglec5–Siglec11, Siglec14, and Siglec16) and others, which include sialoadhesin, CD22, myelin-associated glycoprotein and Siglec15. As inhibitory receptors, many Siglecs regulate DAMP- and PAMP-mediated inflammation via tyrosine based inhibition motifs (ITIMs) in the cytoplasmic domain of immune cells[128, 129]. CD22 is mainly secreted by B cells as well as by neurons[130]. As an endogenous ligand for the CD45 receptor, CD22 can reduce pro-inflammatory cytokine TNFα production to inhibit microglial activation by combining with the CD45 receptor on the microglial membrane[131]. Most CD33-related Siglecs are predominantly expressed on microglia and macrophages. They can phosphorylate SHP1 and SHP2 to modulate inflammatory responses[129, 132]. When microglial Siglecs interact with polysialylated neuronal cell adhesion molecule, microglial neurotoxicity is attenuated. Siglec11, which is expressed by microglia, can reduce the phagocytic capacity of microglia for apoptotic neurons in a microglia-neuron co-culture system[132]. Accumulating data have indicated that CD33-related Siglecs inhibited microglial expression of pro-inflammatory cytokines to alleviate neuronal injury[133].

Interactions between microglia and other glial cells

Recent studies have found that astrocytes and oligodendrocytes are not simply support cells; in fact, they actively interact with other brain cells under both normal and pathological conditions[134]. During neuroinflammation, these glial cells either directly secret inflammatory mediators or communicate with microglia and/or neurons to modulate inflammatory responses.

In the ischemic brain, astrocytes manifested strong hypertrophic and proliferative changes in the penumbral area, and microglia were characterized as branching cells with thick processes or amoeboid cells with thin processes[135], indicating that both glial cell types were activated after the ischemic insult. Activated microglia have an important role in assisting astrogliosis following brain injury; however, they also delay or reduce subsequent glial scar formation[136]. The interaction between microglia and astrocytes may involve TGF-β signaling. In the acute phase of chronic relapsing experimental autoimmune encephalomyelitis, TGF-β2 expression in astrocytes is downregulated, whereas the secretion of the homologous cytokine TGF-β1 is up-regulated. *In vitro* studies also showed that activated microglia inhibited the secretion of TGF-β2 by astrocytes but induced TGF-β1 expression through soluble factors (IFN-γ and TNF-α). Moreover, TGF-β2 down-regulates the expression of MHC-II and costimulatory/adhesion molecules in microglia to influence antigen-presenting cell functions[137]. Astrocytes and microglia also cooperate in the phagocytosis of ectopic neurons[138]. In a chronic cerebral hypoperfusion rat model generated via 2-vessel occlusion, neurons and astrocytes/microglia interacted to form "ectopic" and apoptotic neurons as well as neuronal debris, and astrocyte processes could infiltrate the cell bodies of ectopic neurons to form "triads" together with activated microglia. The formation of "triads" exacerbates ischemic damage and might be the cause of the burden and severity of neurodegeneration. Evidence of the interplay of astrocytes with microglia and neurons can also be found in *in vitro* studies and other neurodegenerative diseases. In LPS treated co-cultures of astrocytes and microglia, uracil nucleotides (UTPs) that were leaked into the extracellular medium were rapidly converted to UDPs and induced microglial P2Y6 receptors to release NO, which in turn induced astrocyte apoptosis[139]. In Alzheimer's disease, the neuronal overproduction of Aβ stimulates astrocytes to release complement C3, which binds to C3a receptors on neurons and microglia and results in impaired microglial phagocytosis[140].

The main function of the **oligodendrocytes** in the brain is to insulate neuronal axons by wrapping around them to form the myelin sheath structure and to assist in the transmission of electrical signals on the nerve. Under resting conditions, microglia have minimal contact with oligodendrocytes and are not cytotoxic. Once microglia are activated, they produce TNF to kill oligodendrocytes, and the effect can be enhanced by the presence of complements[141]. Studies of MS have found that vascular cell adhesion molecule-1 positive microglia closely apposed or surrounded oligodendrocyte perikarya at

the edges of active lesions that were sites of ongoing demyelination, which suggests an active role for activated microglia to deplete oligodendrocytes[142]. However, other studies suggested that the interaction between activated microglia and oligodendrocytes may be both deleterious and beneficial depending on the developmental stages of the cells; activated microglia were found to be harmful for oligodendrocyte progenitor cells but increased the survival of mature oligodendrocytes[143]. During ischemic stroke, resident oligodendrocytes exhibit resistance to ischemic insults and accumulate at the border of the infarction to participate in tissue repair[144].

Potential therapy targeting microglial responses

To date, there is no specific treatment available to target post-stroke immune responses. All of the promising agents that target microglial activation in the context of stroke have only been used in experimental studies or pre-clinical trials. Although some of them have been applied in the treatment of autoimmune disorders (*eg,* TNF-α antagonists), exploratory research for effective agents that regulate ischemic microglial activation is still in its infancy. The dual function of microglial activation (M1 *vs* M2) after stroke makes it necessary that any therapeutic strategy that targets microglia should be fine-tuned to selectively suppress the pro-inflammatory response and/or promote anti-inflammatory activation.

Experimental studies have found that **metformin**, an AMPK activator, can promote functional recovery and tissue repair by promoting the polarization of microglia toward an M2 phenotype in mouse stroke models[145, 146]. After the administration of metformin, microglia/macrophages are skewed toward an M2 phenotype via the suppression of NF-κB-mediated inflammatory signaling. **Statins**, one class of cholesterol-lowering drugs, have been found to have anti-inflammatory and protective effects in ischemic stroke[147]. Treatment with simvastatin can alter the secretion of cytokines (IL-1β and TNF-α) and brain derived neurotrophic factor from microglia in a cholesterol-dependent manner. Conversely, simvastatin inhibited phagocytosis in microglia in a cholesterol-independent manner[148]. Probucol, another lipid-lowering agent, inhibited microglial release of NO, PGE_2, IL-1β and IL-6 by down-regulating the NF-κB, MAPK and AP-1 signaling pathways in LPS-stimulated primary mouse and BV2 microglia; the *in vivo* pre-administration of probucol reduced microglial production of pro-inflammatory mediators (iNOS, COX-2, IL-1, IL-6) and improved outcomes after MCAO[149]. Treatment with **indomethacin,** one of the non-steroidal anti-inflammatory drugs, remarkably reduced the number of Iba-1 cells and microglia activation but increased neuroblast proliferation 7 d after stroke[150]. **Noggin**, an endogenous antagonist of bone morphogenetic proteins, can protect against ischemic brain injury[151], and this effect may be related to the regulation of M1/M2 microglial activation. Noggin treatment decreased the expression of M1 markers (IL-1β, TNF-α, IL-12, CCL2 and CD86) and increased the expression of M2 markers (IL-1Ra, IL-10, arginase 1, CD206 and Ym1) in activated microglia, which led to an M2-skewed polarization[152]. **Pyrroloquinoline quinone (PQQ)** is a naturally occurring redox cofactor that acts as an essential nutrient and antioxidant. In LPS treated primary microglial cell cultures, pretreatment with PQQ significantly reduced the production of NO and PGE_2 and the expression of pro-inflammatory mediators (iNOS, COX-2, TNF-α, IL-1b, IL-6, MCP-1 and MIP-1a) by inhibiting the nuclear translocation of NF-κB and the phosphorylation of p65[153], an effect that was further verified in an *in vivo* study[153]. It was suggested that PQQ might be a promising therapeutic agent for the alleviation of the progress of neurodegenerative diseases through the modulation of microglial activation[153]. **15d-PGJ2,** a PPARγ agonist, also reduced infarct size and edema formation in stroke and suppressed the release of pro-inflammatory mediators such as TNF-α, IL-1 and CD68 from ischemic microglia after the stroke[154]. **IL-13 immune gene therapy** is a latent therapeutic method with which to modulate microglial activation during neuroinflammation. In a mouse model of multiple sclerosis, lentiviral vector-mediated IL-13 regulated the microglia/macrophage polarization toward an M2 phenotype[155]. Furthermore, IL-13 immune gene therapy can slow the progression of an MS lesion in a pre-existing inflammatory environment[155]. TNF-α has a potent effect on the induction of microglial activation[156]. **TNF-α antagonists** are now clinically available and in use for the treatment of autoimmune diseases but have not been used in any clinical trials for stroke patients. Experimental studies with TNF-α antagonists in stroke patients have resulted in both beneficial and detrimental effects on ischemic injury[157].

Conclusion

In summary, microglial activation plays a vital role in the pathophysiology of neuroinflammation that is induced by various CNS disorders, including ischemic stroke. M1 microglia produce pro-inflammatory mediators and exacerbate the injury of neurons; by contrast, M2 microglia generate anti-inflammatory mediators to alleviate neuronal damage and favor tissue repair. These microglial phenotypes are characterized by different biomarkers, and the balance of the two phenotypes is modulated by multiple pathways. An understanding of the mechanisms that underlie microglial activation might help provide novel therapeutic avenues for the treatment of stroke.

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