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Microglia-Leukocyte Axis in Cerebral Ischemia and Inflammation in the Developing Brain

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Abstract

Development of the CNS is reliant on the proper function of numerous intricately orchestrated mechanisms that mature independently, including constant communication between the CNS and the peripheral immune system. This review summarizes experimental knowledge of how cerebral ischemia in infants and children alters physiological communication between leukocytes, brain immune cells, microglia, and the neurovascular unit (NVU)—the "microglia-leukocyte axis"—and contributes to acute and long-term brain injury. We outline physiologic development of CNS barriers in relation to microglial and leukocyte maturation and the plethora of mechanisms by which microglia and peripheral leukocytes communicate during postnatal period, including receptor-mediated and intracellular inflammatory signaling, lipids, soluble factors and extracellular vesicles. We focus on the "microglia-leukocyte axis" in rodent models of most common ischemic brain diseases in the at-term infants, hypoxic-ischemic encephalopathy (HIE) and focal arterial stroke and discuss commonalities and distinctions of immune-neurovascular mechanisms in neonatal and childhood stroke compared to stroke in adults. Given that hypoxic and ischemic brain damage involve Toll-like receptor (TLR) activation, we discuss the modulatory role of viral and bacterial TLR2/3/4-mediated infection in HIE, perinatal and childhood stroke. Furthermore, we provide perspective of the dynamics and contribution of the axis in cerebral ischemia depending on the CNS maturational stage at the time of insult, and modulation independently and in consort by individual axis components and in a sexually dependent ways. Improved understanding on how to modify crosstalk between microglia and leukocytes will aid in developing age-appropriate therapies for infants and children who suffered cerebral ischemia.

Keywords

stroke; neonatal stroke; neuroinflammation; immune cells; monocytes; neutrophils; T cells

Introduction

Immune sensing between the blood and the CNS plays an essential role in brain homeostasis and function. Immune-CNS interactions are rather hierarchal and multifactorial. For instance, the type of stressor, injury or disease, age at the time of stress, and genetic

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background, all serve as major determinants of brain pathology. Considering the emerged concepts that many brain injury-induced inflammatory mechanisms differ between infants, children and adults [1, 2], in this review, we will focus on how development of the CNS and immune system, which occur at different paces, modulate brain injury induced by ischemia-related insults and/or infectious stimuli in the immature brain.

The vision of microglial cells has broadened enormously during the last decade. Several types of important advances have been made in understanding microglial complexity, diversity and ample roles of microglia as "gate-keepers" of homeostasis in healthy brain. In neurodegenerative diseases and stroke, while previous attention was given predominantly to the purely toxic role of microglial cells, with advancement of tools that can differentiate microglia from blood-derived monocytes/macrophages, beneficial roles of at least of a subpopulation of microglia have become apparent. In the immature brain, recent data have identified unique ability of microglia to function in age- and context-dependent manner to regulate brain homeostasis and orchestrate postnatal brain connectivity.

Peripheral leukocytes are sparse in the brain under physiological conditions but become major players in triggering or modulating acute or long-term injury. In adults, depending on the nature of injury, the types of cells involved and the timing after insult, peripheral immune cells can either be detrimental by furthering the injury or beneficial by resolving inflammation [3]. Recent work has suggested that the magnitude and specific leukocyte-mediated mechanisms can be different in newborns, children and adults. Yet, information continues to be sparse on how evolving leukocyte maturation affects injury in the developing brain in relation to the strength and relative input of the individual CNS barriers. In this review, we will discuss preclinical findings from small animal models of stroke, hypoxia-ischemia (H-I) and infection to describe how the immune cells of the CNS and periphery form a "microglia-leukocyte axis" to mature and co-dependently interact after insult in both age- and context-dependent manners.

The CNS barriers as age-dependent interfaces in glia-leukocyte communications

Multiple barriers shield the CNS from harmful molecules and pathogens from the blood and provide homeostatic stability, transport nutrients, and eliminate brain waste products. Under physiological conditions, the brain is under constant immune surveillance by both blood-born immune cells in leptomeningeal, perivascular and choroid plexus (CP) spaces by macrophages and by resident microglia within the parenchyma. The neural parenchyma is protected from peripheral immune cells by the barrier systems [4] including the blood-brain barrier (BBB), which consists of cerebral endothelial cells intertwined with pericytes and surrounded by the glial limitans, and the more permissive barriers barrier between the blood and the cerebrospinal fluid (CSF) at the CP, and the leptomenial barrier between dura mater and subarachnoid space that separates fenestrated dural vessels from the subarachnoid CSF.

The meninges and the outer brain barrier form during early fetal life [5, 6], with completion of the sub-pial end feet layer by 11th post conception week in humans and the presence of tight junction proteins such as claudin-11 in the arachnoid blood-CSF barrier by embryonic

day (E)18 in mice [7, 8]. The CP epithelium develops from the neural tube early in fetal life in close association with the development of the surrounding vasculature [9]. The BBB, in turn, is formed by mid gestation and BBB permeability is tightly restricted from unregulated peripheral exposure due to the emergence of tight junctions by E15. Specific transcription factors are temporally activated to facilitate transcellular and paracellular infiltration of peripheral components to the developing brain [10, 11]. For example, tight junction protein ZO-1 starts to be expressed at E15 in cerebral vessels and differentiates on endothelial tight junctions by E19 in the mouse [12]. In addition, non-barrier cells contribute to the development and function of the BBB. As examples, vasculogenesis, vascular sprouting and BBB development is modulated by embryonic microglial cells prior to astrocytic ensheathment around the vessels [13–16]. In the neonatal brain, higher percentage of microglia interact with vasculature in areas lacking astrocyte endfeet coverage, and the high motility of microglia along the vessel cease when astrocyte endfeet more fully ensheath vessels after development the brain. The timing of this microglia-vascular interaction has been recently identified to be regulated by CX3CR1 [17].

Microglia and other CNS cells also provide trophic support to neurons and endothelial cells, notably via their production of growth factors including BDNF, IGF-1/2 and TGFβ. Disrupted growth factor production in microglia interrupts cortical layer formation [18] and synaptic refinement during the neonatal to adolescent period [19, 20]. Overall, the heterogenous barriers of the brain contribute to orchestrating proper brain development.

Microglia-leukocyte communications as CNS barrier regulators in injured immature brain

Systemic infections inflame the vasculature and alter BBB integrity, which, in turn, leads to increased traffic of peripheral immune cells. For example, Toll-like receptor 3 (TLR3) ligand Poly-IC can either directly disrupt endothelial tight junctions or facilitate permeability of the BBB by affecting astrocytes that form the glial limitans [21]. Several studies also suggest that bacterial infection in the newborn gives rise to meningitis but also involves opening of the BBB, at least partly due to the microbial-induced systemic inflammation [22, 23]. Several findings point to the existence of immune-vascular “memory” over several days to months, as shown following multiple intraperitoneal injections of LPS during P0–P8 [24] and increased BBB permeability to sucrose in adult brains following early postnatal LPS treatment [25].

Following experimental stroke, systemic inflammation alters the kinetics of tight junctions disruption [26], but the extent of disruption and the pattern of BBB disintegration induced by transient middle cerebral artery occlusion (tMCAO) in adult and neonatal rodents differ greatly [27]. Microglia-derived TGFβ signaling in particular attenuates leukocyte-mediated BBB damage, while inhibition of TGFβ receptor in microglia disrupts the BBB and promotes hemorrhage formation in the acute phase of neonatal stroke [28]. Other growth factors serve to protect the BBB following ischemic brain injury in neonates. For instance, BDNF protects the neonatal brain from H-I injury by attenuating caspase-3 activation and via the ERK pathway [29, 30] while VEGF strengthens vasculature to limit neuronal damage after neonatal stroke [31].

Phenotypic and functional diversity of microglial cells during embryonic and postnatal physiologic brain development

Microglial cells feed the embryonic neuro-epithelium through trophic factor release and actively shape neuronal circuits in the developing CNS [32]. Over time, they adopt relative quiescence and ramified morphology during postnatal period, as reported in both human and rodent brains. Many features of microglia in early physiological brain development have been comprehensively reviewed elsewhere [33–36]. Therefore, we focus on aspects most applicable to homeostasis and response to brain injury in the immature brain. In human fetus, the presence of amoeboid $CD68^+$ microglia is reported as early as 13 weeks of gestation, with the highest number observed between 13–18 gestational weeks in the germinal matrix [37], white matter [38] and in proximity to growing axons [39]. Single-cell gene expression and bulk chromatin profiles of microglia during 9–18 gestational weeks showed that microglia are heterogeneous at all studied ages and that microglia progress toward a more mature, immune-sensing competent phenotype, which can render the fetal human CNS vulnerable to environmental stressors [40].

The origin for microglia is distinct from monocytes [41, 42] with the local microglial pool in the brain established ahead of mid-gestation. Microglial progenitors originate from the embryonic yolk-sac before BBB development and increased pericyte and astrocyte coverage [15, 43–45]. Microglial deficiency, disrupted PU.1, VEGF or SDF-1 signaling in microglia/macrophages, lead to distorted vasculature and embryonic lethality [46]. Consistent with different origins and partly distinct signaling in these cell subtypes, monocytes are unable to substitute for the microglial roles in vasculogenesis in the developing brain [14]. Integrin and chemokine signaling, such as αVβ8 and transforming growth factor beta (TGFβ) signaling, contribute to embryonic angiogenesis, BBB formation and vascular branching/sprouting to prevent germinal matrix hemorrhagic damage [47]. Secretion of trophic factors and phagocytosis by microglia regulate stem cell pools and embryonic neurogenesis as well in both macaques and rats [48]; depletion of microglia *in utero* by clodronate administration increased the neural precursor cell pool while microglial activation decreased the neural precursor cells in the cortex. The functional implications of modifying microglia to influence neural precursor populations is still not completely understood.

Another key function of microglia during development is to organize brain circuits and regulate brain circuit connectivity through phagocytosis of apoptotic cells and weak neuronal synapses during the postnatal period [19, 49–52]. Direct microglial contacts with axon terminals and dendritic spines are revealed by electron microscopy, with almost all microglial processes containing synaptic elements in the adolescent mouse cerebral cortex [53]. Microglia interact functionally with excitatory synapses [54] by dynamically contacting axon terminals and dendritic spines *in vivo* [55] as well as by shaping presynaptic properties at developing glutamatergic synapses [56].

Microglial properties rapidly evolve and contribute to somatosensory barrel field formation in layer IV [57] and trophic support to layer V neurons for survival early postnatal [18]. Microglia also ensure proper positioning of interneurons in the forebrain [32] and contribute to synaptic refinement [19, 20]. CX3CR1 deficiency delays maturation of postsynaptic glutamate

receptors which normally occurs in P6–P8 mice [57], and transiently reduces microglial number in the hippocampus, leading to higher dendritic spine density and less mature physiological responses and long-term depression [49, 50]. Disruption of microglia-specific C3/CR3 complement signaling leads to sustained deficits of synaptic connectivity during adolescence and adulthood [19]. Accumulation of synaptic debris following pharmacological depletion of microglia further confirms microglia as the dominant phagocytosing cell type during development [58] as well as after neonatal stroke [28].

A wave of recent studies has strengthened the concept of microglial heterogeneity. A new fate mapping system revealed context-dependent random or clonal expansion of microglia [59]. Putative microglial subtypes were summarized with unique genomic, spatial, morphological, and functional specializations, such as satellite microglia, KSPG-microglia, Hox8b-microglia, CD11c-microglia, microglia supporting neurogenesis, as well as “dark” microglia typically viewed as disease-associated microglia [60, 61]. The heterogeneity of microglia was shown to decline with brain maturation [62] and acquire a more uniform homeostatic phenotype with transcriptomic similarity.

Postnatal microglial maturation is also regulated by other types of immune cells. $CD4^+$ T cells, which enter the brain around birth, are required for microglia to mature and acquire proper synaptic pruning function. $CD4^+$ T cell deficiency traps microglia in a fetal-like transcriptional state leading to behavioral abnormalities [63]. Conditional prenatal microglial dicer ablation, in turn, leads to spontaneous microglia activation and affects DNA repair and genome integrity, a response distinct from that in adult brains [64]. Additionally, transcriptional single-cell sorting of microglia identified disease-associated subtypes that are shared between transgenic Alzheimer’s disease mouse lines and proliferative microglial subsets in neonatal developing white matter [65].

Role of microglia following neonatal focal arterial stroke and H-I

Neuroinflammation is a major contributor to stroke injury in adults [66] and in neonates [1]. Several rodent models of infection and stroke have helped reveal the mechanisms of neuroinflammatory responses to injury in the developing brain, as we previously reviewed [67]. Historically, in adult stroke, microglia were viewed as injurious due to production of inflammatory mediators, reactive oxidant species (ROS) and other toxic molecules [66]. However, more recently, diversity of the microglia has been demonstrated, with a subpopulation of beneficial microglial phenotypes that support neuronal homeostasis and neurogenesis and microglia were reported to contribute to long-term maintenance of neurogenic niches through the phagocytosis secretome within the hippocampus [68]. In conjunction with advanced tools developed to discriminate microglia and monocytes, beneficial effects of microglia in preserving neuronal network activity and in limiting injury after stroke were also shown [69–71]. For instance, P2RY12 signaling appeared to mediate protective microglial phenotypes [71]. Furthermore, the importance of timing after stroke as a factor influencing microglial effects is being increasingly acknowledged, yet the usefulness of classifying microglia into M1/M2A/2B phenotypes is often considered as an oversimplification due to their dynamic phenotypic capabilities during various *in vivo* situations.

The patterns of microglial activation are also context-dependent [72] and age-dependent [73–77]. In the neonatal brain, microglial cells undergo morphologic transformation after both H-I and focal arterial stroke. Activated microglia/macrophages were viewed as contributors to H-I injury [78–82] and excitotoxic injury [83]. Contrary to the notion of microglial toxicity in the neonatal brain following H-I, in acute neonatal stroke, we demonstrated that pharmacologic depletion of microglia before tMCAO in P7 rats or P9 mice exacerbate injury [28]. Consistent with the notion that phagocytosis is particularly important in post-ischemic neonatal brain due to the high level of apoptotic neuronal death compared to adult post-ischemic brain [84]. We showed that limited engulfment/removal of neuronal debris in pups with genetic deletion of the scavenger receptor, CD36, a receptor that contributes to several phagocytic steps, increases residual cleaved caspase-3, enhances inflammation and worsens injury [76, 85].

Microglial cells also provide neurovascular protection after neonatal stroke [28]. The majority of microglia interact with vessels extended processes under physiological conditions, but the spatial physical microglia-vessel contacts are altered when microglia acquires activated phenotypes (Figure 1A–D). In ischemic-reperfused neonatal brains, microglial depletion or abolished microglia-derived TGFβ1 signaling triggers BBB leakages and induces hemorrhages following tMCAO [28]. Altogether, these data suggest an array of beneficial microglial effects after ischemia-reperfusion in neonatal rodents, as we demonstrate in the schematic in Figure 2. It is not clear whether the divergent results relate to different models of neonatal brain injury (i.p., the presence of systemic hypoxia in H-I model) or to time-resolved differing microglial phenotypes that participate in an acute and chronic injury. It is also essentially unknown whether a particular microglial subpopulation provides endogenous cerebrovascular protection. Addressing these topics is critical to truly dissect the capabilities of neonatal versus adult microglia during health and disease.

Peripheral leukocytes as modulators of neuroinflammation and injury in neonatal brain

While microglia are able to actively respond and rapidly modify local inflammation after stroke, DAMPs released by neurons and vascular cells compromised by hypoxia or disrupted CBF initiate chemical cues for peripheral cells to respond to injury. This can result in both acute and long-term neurological effects [86]. Here, we describe how each peripheral immune cell type contributes to brain injury and interacts with microglia.

Neutrophils

As large secretory phagocytes of the innate immune system that carry cytoplasmic granules and secrete vesicles, neutrophils contribute to intercellular responses. In adult cerebral ischemia models in several species, neutrophils rapidly accumulate at injury sites and contribute to reducing CBF, causing a “no-reflow” phenomenon [87], and disrupt vascular integrity by producing myeloperoxidase (MPO), matrix metallopeptidases (MMPs) [88], cytokines and ROS [89, 90]. Anti-adhesion strategies, neutralizing anti-CD18 antibodies and neutropenia were effective in preserving CBF and reversing the opening of the BBB after transient ischemia [87, 91, 92]. Pharmacological inhibition or genetic depletion of proteolytic enzymes in leukocytes such as neutrophil elastase (NE), cathepsin G or MMP-9 also reduced

ischemic injury [93–96]. Neutrophil extracellular traps (NETosis) contribute to injury and limit brain repair after stroke as well [94, 96 97, 98]. It is still being debated whether neutrophils actually transmigrate, as long believed (summarized in [99]), or predominantly signal from intravascular and/or the luminal surfaces and perivascular spaces after stroke, as shown by two-photon imaging [100, 101].

In neonatal stroke, neutrophil chemoattractant protein CINC-1/KC rapidly and markedly rises first in peripheral blood and, within hours, in the brain in parallel to decline in peripheral levels [102] but neutrophils do not accumulate in ischemic-reperfused neonatal brain regions as prominently as in adults [27]. In contrast, neutrophil accumulation is rapid and profound in response to intra-cerebral CINC-1 injection, demonstrating ability of neutrophils to migrate in response to direct chemokine gradient and suggesting that lack of coordination between endothelial activation and neutrophil activation, rather than the state of neutrophil maturation, halts neutrophil infiltration after neonatal stroke [27]. Low neutrophil infiltration can in turn contribute to the more preserved BBB after acute stroke in neonates. Despite limited neutrophil numbers in post-ischemic neonatal brain, there is evidence that neutrophil depletion before H-I is protective, as evident from reduced brain swelling, decreased cerebral atrophy, and higher levels of adenine nucleotides [103], but beneficial effects are lost if neutropenia is executed after H-I. Consistent with such a notion, administration of an anti-neutrophil serum reduces MPO accumulation and brain swelling hours after H-I in the neonatal rat brain [104] and neutropenia induced by pre-treatment with a specific anti-Ly6G antibody attenuates the increase of mRNAs involved in neutrophil-recruiting. Additionally, anti-Ly6G administration at 4h post-LPS/H-I fails to prevent the influx of neutrophils and brain damage [105].

Comparative studies between newborn, juvenile (P17-P25) and adult rodents subjected to various excitotoxic and inflammatory conditions further extended the concept of a critical role of maturational stage in leukocyte-NVU interactions. These models include intracortical IL-1β injection [106, 107], brain trauma [108–110] and stroke [111–113]. Comparisons of neutrophil accumulation within 24 hours after tMCAO in P7-P9 [27] and P21 rodents [113] depicted more profound neutrophil accumulation in juvenile brains compared to neonatal brains.

A number of studies have shown that neutrophil signaling and infiltration in stroke are modified by both microglia and monocytes. Two-photon intravital microscopy of adult brain demonstrated that microglia engulf infiltrating neutrophils within 24 hours after photothrombotic stroke [114]. Microglial depletion by feeding a diet containing a CSF1R antagonist, in turn, promotes neutrophil invasion and increases infarct volume after tMCAO [115]. A link between microglial and neutrophil function in injured neonatal brain following H-I was established by genetic deletion of a transcription factor for leukocytes development, interferon regulatory factor 4 (IRF4), that led to attenuated neutrophil infiltration but increased release of inflammatory cytokines from microglia [116]. Deficiency of monocyte CCR2 signaling, in turn, limits neutrophil activation in neonatal stroke [77]. Echoing the overall same pattern in neonatal stroke, disrupted CCR2 and CX3CR1 signaling attenuated neutrophil accumulation and acute injury in a model of childhood stroke [113]. While the underlying signaling mechanisms of interaction need to be better understood, the notion

of dynamic monocyte-neutrophil interactions as modulator of injury in immature brain is growing.

Monocytes

Monocytes can signal while circulating in the blood and after differentiating into macrophages once they reach their target tissue. Under physiological conditions, monocytes are relatively sparse in the developing and adult brain. While monocytes and microglia use many same pathways for activation, the monocyte-mediated responses after stroke are not interchangeable by microglia, as in some instances, the distinct and even opposite roles of microglia and monocytes in stroke are reported [117]. However, one long-standing caveat in the stroke field has been difficulty in differentiating the relative role of monocytes and microglia. Recent novel fate-mapping techniques have helped overcome this limitation, especially under neuroinflammatory conditions when activation of microglia and brain-infiltrating monocytes can yield similar antigenic expression [59, 118–120] or monocytes reportedly can even transition into microglia both genetically and phenotypically after neonatal stroke [121].

In adult stroke models, peripheral monocytes were shown to contribute to both damage and healing by polarizing monocytes into a pro- or anti-inflammatory phenotypes and associated release of inflammatory or anti-inflammatory cytokines [86, 122–124]. Nonetheless, targeting monocytes following adult stroke has had varying success [125, 126].

Compared to adult monocytes, fetal monocytes display limited levels of antigen recognition and phagocytic capabilities [127], but exhibit more proliferative potential [128]. As they mature, monocytes begin expressing $Ly6C^+$ [129]. Essential non-redundant functions of monocytes and microglia in brain development are under investigation [120, 130].

Early after neonatal stroke, as with the case with neutrophils, the extent of peripheral monocyte infiltration is lower than in adults, likely due to continuing NVU development postnatally [2]. Yet several studies have shown a critical role for monocytes in neonatal brain injury. For example, an array of pro- and anti-inflammatory related genes in monocytes and macrophages depended on timing and context in H-I and neonatal stroke [131, 132]. The use of neonatal Lys-EGFP-ki mice, in turn, helped demonstrate multiple peaks of monocyte accumulation in the neonatal brain after H-I [133] and the lack of CCR2, receptor mediates early recruitment of toxic monocytes to damage neonatal brain [134], appeared to exacerbate long term hippocampal damage and spatial learning deficits after H-I [135].

In a childhood arterial stroke model in P21 mice, we demonstrated the presence of $CCR2^+$ monocytes after acute injury and protection in mice deficient in CCR2 signaling [113]. In patients with childhood arterial ischemic stroke, dis-coordination between neutrophils and monocytes correlated with endothelial repair response genes [136]. Together, these data suggest a bidirectional role in monocyte-endothelial signaling in childhood stroke.

Lymphocytes

Lymphocyte responses in adult stroke are well established and several laboratories have parsed out the role of lymphocyte specific cytokines in modulating stroke injury [86, 122, 123].

The three major types of lymphocytes—natural killer (NK) cells, B cells, and T cells—act differently in stroke via the innate immune system (NK cells), which does not require antigenic stimulation to exert an inflammatory response, and adaptive immune system via B cells and T cells that require antigen stimulation to develop an effector or memory phenotype.

In adult mice, $CD11c^+$ cells accumulate by the border of the infarct region early after tMCAO and gradually enter the ischemic core [137]. While lymphocyte-deficient adult mice exhibit reduced lesion sizes and stroke related neuroinflammation [138], lymphocytes and dendritic cells can have varying effects depending on the cytokine signals and which phenotype of lymphocytes they activate. For example, T cell specific cytokines, including IL-17 [139–141], and IL-21 [142], are detrimental in stroke whereas a subpopulation of lymphocytes can produce IL-10 (which is beneficial after stroke) via various mechanisms including regulatory T cells (Tregs) [143, 144], $CD8^+$ Tregs [145], B cells [146], and MOG-specific $CD4^+$ T cells [147]. Dendritic cells, in turn, promote damaging Th17-mediated immunity via IL-23 production [140], whereas plasmacytoid dendritic cells protect the brain from stroke by priming Tregs [148]. Moreover, B cells can induce delayed cognitive impairment by producing toxic antibodies [149].

While the number of studies targeting lymphocyte function in neonatal rodents is relatively limited, splenectomy in rats before H-I reduced the numbers of NK cells and, to a lesser degree, T cells in the brain, resulting in smaller infarct volumes and decreased behavioral deficits. Furthermore, siRNA mediated NK cell deletion modestly limits the damaging effects of H-I. White matter loss is attenuated following H-I in Rag1ko neonatal mice, which are deficient of both T cells and B cells [150]. At he same time, blocking lymphocyte trafficking to the neonatal brain after H-I with FTY720, a S1P/S1PR1 inhibitor which affects T cell infiltration [151] and activation/polarization [152], led to varying results after H-I of different severity [153, 154], reduced $CD4^+$ T cell number and exacerbated brain injury after mild H-I [154] whereas in the more severe injury in a rat LPS/H-I model, FTY720 depleted $CD4^+$ T cells and prevented injury [153], suggesting that the magnitude of insult could determine whether lymphocyte migration is protective or detrimental following ischemia-related injury.

Lymphocyte-microglial interaction can mediate stroke pathology. For instance, $CD4^+$ T cells require microglial inflammatory chemokines, such as CXCL10, to infiltrate, increase white matter injury and induce depressive-like behavior following TBI [155]. Lack of a negative regulator of NF-κB signaling (A20) in microglia, in turn, enhances infiltration of $CD8^+$ T cells to the brain [156]. Microglial chemokines attract dendritic cells to the brain in adult stroke, and infiltrated dendritic cells are able to induce proliferation of T cells [157].

Evaluation of the long-term dynamics of antigen presenting cells (APCs) and lymphocytes in the brain showed a prolonged peak of $CD11c^+CD86^+$ APCs and a spike in $CD11c^+$MHC class II^+ dendritic cells between two weeks and 3 months after H-I. Correspondingly, there is a spike in $CD4^+$ and $CD8^+$ lymphocytes at the same time points with a strong response of $CD25^+$ lymphocytes and $CD69^+$ lymphocytes at 3 months [158].

Therefore, modulatory effects of lymphocytes may depend on injury severity in injured neonatal brains as well as on the stage of development based on potential therapeutic target in preterm infants [159].

Receptors mediating microglial-leukocyte interactions in injured neonatal brain

Microglial cells exert their functions—homeostatic, protective or toxic—via an array of receptors [160]. Hyperactivation of and/or imbalance in microglial receptor expression alter their immune function in the brain and communication with the peripheral environment following cerebral ischemia.

Toll-like receptors

TLR2 and TLR4 activate microglia, mediate inflammatory cytokine production and cause brain damage after stroke. Injurious effects are mediated by recruiting individual intracellular TLR adaptor molecules [161, 162] and significant down-regulation of the endogenous Nrf2 anti-oxidant system, events that occur in conjunction with leukocyte trafficking via the CP and oxidative mechanisms at the blood–CSF barrier interface [163].

In stroke, the pattern of involvement of TLR2 is a differentiating factor between adults and neonates. While microglial cells are the predominant source of TLR2 in both adult and neonatal mice, an approximate 20-fold increase in TLR2 expression occurs 24 hours following tMCAO in adult luc/GFP-TLR2 mice [73], whereas in neonates, when TLR2 expression in the brain is highest, no significant upregulation occurs in the parenchyma or the CP 24–72 hours after tMCAO in P9 luc/GFP-TLR2 mice [74]. Comparative analysis of leukocyte trafficking following tMCAO and of TLR2 ligand administration showed context-dependent responses in the magnitude of leukocyte accumulation in the CP and the magnitude and the timing of cytokine accumulation [77].

Deficiency of TLR2 or TLR4 reduces TNF-α, iNOS, and COX2 production and contributes to reduced brain infarct volume and improved neurological outcome following H-I [164]. Yet, TLR4 stimulation by LPS and TLR2 activation by PAM provoke distinct brain chemokine responses, which lead to increased barrier permeability, particularly of the blood-CSF barrier [163]. These results indicate specific TLR-mediated mechanisms of CNS inflammation and leukocyte infiltration into the neonatal brain. Moreover, TLR ligand administration increases traffic of immune cells in the CP of neonatal mice [77]. Further understanding of the timing and context-dependent response of TLR activations is key.

The scavenger receptor CD36

CD36 is central to multiple biological functions in endothelial cells, microglia and monocytes, phagocytosis of apoptotic debris and cell chemotaxis [165, 166]. It serves as “master switch” in assembling inflammatory pathways, including ROS production via multiple ligands and partnering with multiple receptors in the lipid membrane fraction, including TLR2, TLR4 and TLR6. CD36 exerts opposite effects in acute stroke—exacerbates injury in adult [75] but reduces stroke injury incidence in neonates [76]. Given

that stroke studies were performed in global CD36 knockouts, the specific role of microglial cells is yet to be better understood, but significantly reduced phagocytosis of apoptotic debris in post-ischemic brains of injured neonatal CD36ko mice concomitant with enhanced necrotic cell death and rise of inflammatory cytokines suggest direct beneficial effects of microglia in acute neonatal stroke [76]. However, as an anti -angiogenic factor, CD36 may have divergent effects on long-term injury after neonatal stroke. The influence of CD36 on microglia versus peripheral leukocytes and their infiltration into the ischemic neonatal brain is yet to be understood.

Purinergic receptors

P2RY12, a Gi/o-coupled purinergic receptor with high affinity to ADP, is abundantly and exclusively expressed in microglia in the brain and plays homeostatic roles. Based on *in vivo* 2-photon imaging, P2RY12 elicits rapid chemotactic responses of microglial processes and shields injured sites with increased ATP/ADP [167–170] whereas in mice lacking P2RY12 microglia are unable to polarize, migrate, or extend processes toward nucleotides [167]. After stroke, microglia preserve neuronal network activity and limit injury in part via P2RY12 [69–71], whereas global ischemia leads to mortality in P2RY12ko mice [171] and P2RY12 inhibition extends BBB disruption after laser injury by disabling microglia-vessel interactions [172].

P2RY12 expression rapidly increases between P4 and P21 [20] but information on the role of P2RY12 in brain of injured neonates is more than sparse. We recently demonstrated that essentially all Iba1^{+}TMEM119^{+}CX3CR1^{+} microglia express P2RY12 in uninjured P9 mice hours after tMCAO suggesting limited entry of peripheral cells early after injury. [77] (Figure 1D) but P2RY12 expression is gradually lost within 8–24h after tMCAO.

Lipid signaling

Bioactive sphingolipids and their synthetizing/metabolizing enzymes mediate many physiological processes. S1P is a major regulator of vascular and immune systems by acting on its G-protein coupled receptors S1PR1–5 [173]. They also act as major regulators of T- and B-cell trafficking and as angiogenic receptors. In the brain, S1PR2 plays a key role in mediating vascular inflammation [174], whereas both S1PR1 and S1PR2 are highly expressed in microglia [153].

LPS, which has been extensively used as the *in vitro* and *in vivo* model to examine effects on S1PRs in microglia by using respective S1PR1,3–5 agonist FTY720 and S1PR2 inhibitor JTE013, landed mixed results. Some studies show that FTY720 treatment of cultured microglia attenuates LPS-induced production of inflammatory mediators, including CD16 and iNOS, increases production of neurotrophic factors and expression of anti-inflammatory markers Arg-1 and CD206 [175]. However, in primary microglia from P1-P3 mice and primary microglia from 17–23 gestation week human brains no significant effect on mRNA expression of inflammatory cytokines, neurotoxic mediators or migration are reported. [176].

In neonatal rats, FTY720 administration shortly after H-I is protective only in a combined model of LPS followed by H-I, but not following H-I alone, indicating that FTY720 affects the inflammatory injury component. JTE013, in turn, attenuates phosphorylation

of MAP kinases following LPS stimulation of BV2 cells and in post-ischemic brains [177] as well as suppresses LPS-triggered autophagy in primary microglia from S1P ablated mice [178]. Although regulation of lipid signaling in microglia and leukocytes can impact stroke outcome, studies are sparse.

Dietary lipid components as modulators of the inflammatory response and injury

Docosahexaenoic acid (DHA) accounts for over 50% of all n3-Polyunsaturated Fatty Acids (n3-PUFA, i.e., Omega-3 fatty acids and derivatives/metabolites) in the CNS cell membranes [179, 180]. The specific molecular properties of this highly unsaturated fatty acid are responsible for fluidity, permeability, and elasticity of cell membranes [181]. n3-PUFA protects against stroke [182, 183] and brain trauma [184, 185] by inducing anti-inflammatory and anti-oxidative effects [186], enhancing angiogenesis, revascularization [187], neurogenesis [188], and oligodendrogenesis/white matter integrity, [189], and improving functional outcomes [190], even when treatment is delayed, suggesting a larger therapeutic window for potential intervention [185].

In human infants, Omega-3 fatty acids and lipid derivatives/metabolites play an important role in normal postnatal brain development [191]. Increase in n3/n6 fatty acid ratio [192] or enriched dairy fat matrix diet [193, 194] protect immature brains from cognitive deficits induced by immune challenges. Additionally, nutritional n3-PUFA deficiency during the perinatal period alters microglial and neuronal plasticity-associated genes [195], possibly due to the influence of Omega-3 fatty acids on the functional ability of microglia to prune synapses in the developing brain [196]. In neonatal H-I mice, gestation-to-postnatal n3-PUFA supplementation was determined to be beneficial by inducing anti-inflammatory effects [197], including activation of the Akt pathway, formation of phosphatidylserine [198], attenuation of MMP-9 and vasogenic edema, and reduction in the permeability of small molecules and IgG [199]. While the beneficial effects of dietary modulators to support functionality of the neonatal brain and protect from injury are clear, the relative interplay of immune cells with dietary modifications during development and injury need to be better understood, particularly in children, where research is sparse. It will be key to parse out the influence of dietary lipids on immune cell recruitment to the ischemic brain and the overall neuroinflammatory milieu.

Intra-to-extracellular signaling as interconnector of the microglial-leukocyte axis in injured neonatal brain

Mitochondrial signaling

The number of links between mitochondrial function and leukocyte and microglia in stroke is growing. Release of mitochondria into extracellular spaces in a stroke model was shown to serve as a “help-me” signal to attract peripheral leukocytes to propagate neuroinflammation or expedite recovery [200]. In patients, a significant association was found between low mtDNA content and ischemic stroke [201], demonstrating oxidative stress as means of the CNS to communicate with the periphery through mitochondrial

DNA. Microglia can protect neuronal function through purinergic junctions [71]. Fragmented mitochondria released from microglia, however, can trigger astrocytic response and propagate leukocyte-mediated inflammatory neurodegeneration [202], in part via NLRP3 inflammasome activation [203]. Recent findings implicate perturbations in mitochondrial dynamics (fission, fusion), mitophagy and biogenesis in neonatal brain injury [204]. Several survival kinases contribute to preserve intracellular homeostasis and the inner mitochondrial membrane. Activation of protein kinase Cε (PKCε), phosphoinositide 3 kinase (PI3K)-AKT and glycogen synthase kinase-3β (GSK3β) reduce brain vulnerability to ischemia in adults [205] and neonates [206, 207] and attenuate ischemia-induced triggers of cell death machinery [208], in part by influencing the recognition of compromised neurons by activated microglia and peripheral macrophages.

Cytokines

Cytokines have important physiological and endocrine functions in the brain but when produced in excess, they can trigger microglia-mediated cytotoxic effects. Repeated systemic administration of interleukin(IL)-1β in P1–P5 mice results in a transient increase in microglial density and long-term myelination deficits, which are accompanied by cognitive defects [209]. As we have already discussed, systemic activation of innate immune TLRs during gestation and postnatal period activates microglia, skews the microglial phenotypes toward pro-inflammatory, recruits leukocytes and adversely affects brain development [210, 211]. The patterns of the inflammatory response, cytokine induction and leukocyte infiltration into the immature brain are also stimulus-dependent upon TLR2 and TLR4 stimulation [212] and depend on TLRs communication with other receptors, such as CD36 receptor. Following tMCAO in neonatal wild type mice, CD36ko and Galectin-3ko mice exhibit a pattern of early cytokine response distinct from that in adult stroke. Interestingly, while the levels of some cytokines, such as TNFα and IL-1β, which are rapidly increased in adult stroke model, remain unaffected or only modestly increased in ischemic-reperfused regions of neonates [76, 213]. In addition, dysfunction of CX3CR1 and CCR2 receptors in neonatal mice also drives stimulus-specific cytokine accumulation [77].

While it is clear that microglia respond to systemic infectious stimuli and sterile inflammation produced by stroke, in part by producing an array of cytokines, a caveat exists regarding the sparse data on the precise cellular sources for cytokine release in injured neonatal brain. Filing this gap in knowledge is particularly important given a long-standing notion that activated microglia release inflammatory cytokines. The strongest piece of evidence of microglia-mediated endogenous protective effects in neonatal stroke is an observation that pharmacologic depletion of microglia increases production of IL-1β, TNFα and other toxic cytokines to activate nearby and surrounding endothelium in post-ischemic neonatal brains [85]. This may at least in part be due to the essential function of microglia to serve as a regulatory checkpoint for limiting toxic peripheral immune cell responses. These data reveal a delicate and maturation-dependent balance between microglia and peripheral leukocytes and how they modulate brain environment.

Extracellular vesicles (EVs)

It has traditionally been thought that cells transfer information either via direct cell-cell communication or via molecules released from cells. More recently, a novel mechanism of cell-cell communication has been discovered via release and uptake of extracellular vesicles (EVs), microvesicles and exosomes. EVs carry a cargo of nucleic acids and proteins enclosed in a phospholipid bilayer and have garnered much interest in recent times for their therapeutic potential. While the understanding of small EVs, exosomes, as disease modifiers is constantly evolving, they have been shown to play a critical role in neurodegenerative diseases [214–216]. Microglia were shown to modulate neural stem cell (NSC) proliferation in the neonatal SVZ via release of EVs [217]. Another example is mesenchymal stem cell (MSC)-derived exosomes that have been shown to increase neurovascular remodeling, improve neurological, behavioral and cognitive outcomes during recovery, and facilitate endogenous rewiring of neuronal circuitry in multiple brain injury models [218]. Exosomes were identified as the only constituents of the MSCs secretome that successfully recapitulate the beneficial effects of the parent cell therapy [218].

In neonates, MSCs were shown to enhance functional outcomes in H-I and stroke models [219, 220], raising an exciting possibility to develop MSC-exosomes as alternative to cell-based therapies and their potential risks. The fact that MSCs survival sharply declines to <1% [221] suggested that MSCs act primarily by reshaping the brain microenvironment in injured neonates to permit remodelling and improvement of neurological function [222]. These data pointed to microglial cells as potential important mediator of beneficial effects but a better understanding of how interaction of MSC-exosomes with activated microglia promotes recovery of injured neonatal brain.

Microglial interactions with other CNS cell types

Microglia-neuronal interactions are bi-directional in the developing and adult brain. As we have already eluded, microglia shape the brain by eliminating synapses during early postnatal development [33] as well as promoting neuronal homeostasis and survival [18]. Microglial maturation, in turn, is regulated by other immune cells [63]. Microglial cells play principle role in rapidly promoting spine turnover after synaptic photodamage [223] and protecting neurons from stroke-induced brain damage [71]. Microglia-neuron interactions are regulated by a number of intracellular checkpoints and receptor-mediated mechannisms [224].

The microglial-astrocyte and microglial-oligodendrocyte interactions are also bi-directional (Figure 3) and contribute to postnatal development and to brain injury [225, 226]. Microglia interact with cells of the oligodendrocyte lineage, including oligodendrocyte precursor cells (OPCs), pre-myelinating oligodendrocytes, mature oligodendrocytes, and myelin sheath associated cells (reviewed in [227] with particular focus on interaction in the developing brain [228, 229]). Engulfment of OPCs by microglia peaks at P7 in the mouse corpus callosum and is blocked by deletion of CX3CR1, resulting in an increased number of OPCs and reduced myelin thickness [230]. Real time observation of microglia–myelin interactions during development in zebrafish showed that microglia selectively eliminate myelin sheaths in a neuronal activity-regulated manner [228]. Co-culturing astrocytes with microglia was shown to mute the effects of LPS on TNF-α mRNA expression and upregulated expression of an

anti-inflammatory cytokine IL-10 [231]. Astrocyte-derived IL-15, in turn, promotes microglial expression of TNFα and IL-1β and aggravates brain injury after intracerebral hemorrhage in mice [232].

Moreover, the three-way communication between microglia, astrocytes and oligodendrocytes have been elucidated in the context of excitotoxic damage in the immature injured brain [233]. The crosstalk between CNS cells is further affected by circulating and infiltrating peripheral cells in a context- and maturation-dependent manner, in part via cytokine signaling [234].

Microglial sexual dimorphism in healthy and diseased immature brain

By now, many studies revealed that microglia are sexually dimorphic [235–238] and that microglial sexual dimorphism plays key role in various psychiatric diseases (reviewed in [239]). Phagocytosis occurs earlier but transiently in female microglia [240] but in a more sustained manner in male microglia, particularly in the medial amygdala, potentially influencing social circuitry and behavior in juvenile rats [241]. Abnormalities in microglia morphology/function during early development contributes to neurodevelopmental disorders such as autism, schizophrenia, and depression [49, 242] as well as Rettt syndrome [243] in a sex-dependent manner. For example, children with ASD have increased IL-9 in peripheral blood mononuclear cells [244] and peripheral cytokine profiles at birth have been associated with severity of ASD in childhood [245].

While the links between sex-dependent signaling between peripheral leukocytes and microglia are postulated, the mechanisms in relation to triggering/propagating these diseases are far from understood. Some of the mechanisms can be related to significantly higher microglial density in males than in females among several brain regions [235, 236], providing broader access of communications with other cells. More specifically, this can happen via distinct miRNA signatures in male and female mice [246], differences in transcriptional activation, as postulated in mice with dysfunction of Hoxb8-lineage microglia [247], or via receptor-mediated effects, as was shown in a murine model of ASD in CX3CR1 knockouts [248]. In a neonatal pMCAO model, higher gene expression of pro-inflammatory M1 markers Cox-2 and TNFα in male than in female microglia together with much higher $Iba1^+$ numbers in the male ischemic somatosensory cortex [249] pointed to sex differences in the inflammatory injury component. Therefore, the intrinsic sex-specific differences in the immune responses, local and peripheral, point to the need to take into account sex-dependent pathologies in developing mice.

Therapeutic considerations and future outlook

Essentially all therapies tested in stroke clinical trials failed, including immune-targeted therapies [250]. Shortcomings in pre-clinical trials and in selecting patients for clinical trials have contributed to this rather gloomy scenario. For immunomodulatory therapies, an additional challenge is stroke-induced immune depression and vulnerability to infections [251]. Better translation from pre-clinical models to clinical trials, in conjunction with

advancing our knowledge of stroke biomarkers and utilizing modern technological platforms to selectively target individual cell types give hope for effective therapeutic interventions.

As of now, for infants after HIE, there is “light at the end of the tunnel”— therapeutic hypothermia—which reduces the risk of long-term neurological disabilities [252, 253]. The caveat is that beneficial effects of hypothermia are limited to modest-to-moderate cases only. There is solid evidence of immune-, excitotoxic- and oxidative stress-mediated injury mechanisms after HIE [254–256], yet nuances of cooling and rewarming play an important role in the outcomes [257]. Literature has also emerged that in children, predominantly in boys, viral infections such as the flu and varicella zoster virus (VZV) can induce vascular changes via triggering immune responses, leading to stroke in children [258, 259]. Yet the molecular signature of immune responses and how it contributes to childhood stroke remains largely unknown.

Focusing on drug effects on individual cell types, rather than on effects of cell-cell interactions has been another hurdle. Moreover, evaluating how therapies affect the balance of the microglia-leukocyte axis is vital. Therefore, to look forward, in this review we focused on what is known, insufficiently known or revisited in the field of neonatal brain injury, relying upon information on immune therapies in the context of particular mechanisms and how they influence the neuroinflammatory network, rather than focusing on individual therapies. While we put mechanistic context in comparing adult injured brain to immature injured brain to obtain a better perspective, we do recognize that mechanistic knowledge in the adult stroke field is more elaborate than in the neonatal stroke field. We hope that recent progress in identifying maturation specific stroke-related mechanisms will ignite a wave of research toward developing therapies for neonatal and childhood disease. Advances in the identification of critically important cross-talks between inflammation and CNS barriers function under physiological and ischemic conditions, such as those related to the microbiome [139] and the glymphatic system [260], can further sprout progress toward these goals.

Whether therapies directly target immune cells or involve them indirectly, via crosstalk with other cell types, it is essential to account for the diverse range of pleiotropic functions within the microglia-leukocyte axis particularly. Notwithstanding, it is critical to account for how the developmental status of the brain and immune system integrate at the time of injury and without compromising long-term essential physiological functions. Thus far, recent preclinical progress to unveil mechanisms of neuroinflammatory signaling has been promising and hopefully a trend towards identifying maturation-specific treatments will lead to even better outcomes for injured infants and children.

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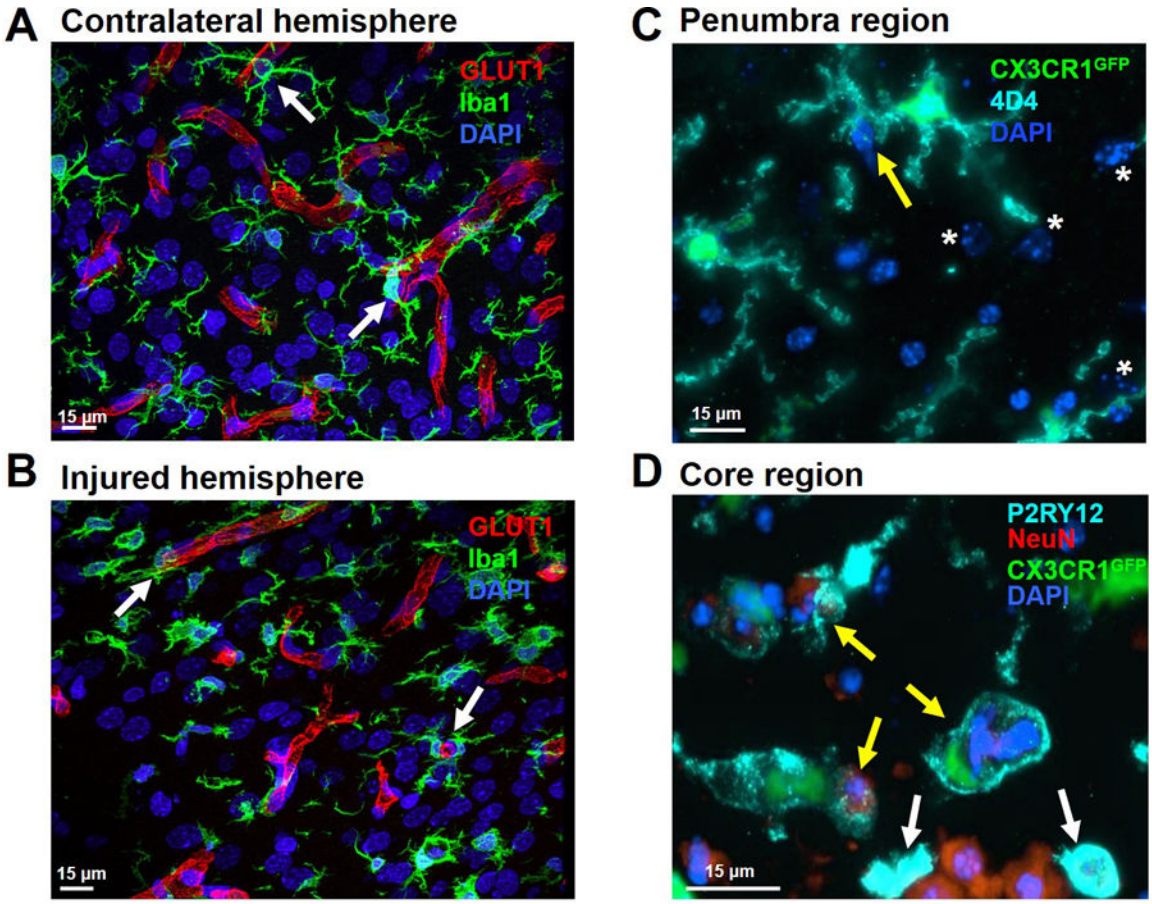

Figure 1.

A-B. Representative immunofluorescence images on Iba1^{+} microglia in proximity to the vessels in the contralateral (A) and ischemic-reperfused cortex (B) at 24 hours after tMCAO. Iba1^{+} microglia in proximity to the vessels (white arrows) **C.** 4D4^{+}/CX3CR1^{+} cells 8h after tMCAO. Damaged DAPI^{+} nuclei (asterisks) in proximity to 4D4^{+}/CX3CR1^{+} microglia (yellow arrow) in the forming penumbra. **D**. P2RY12^{+}/CX3CR1^{+} microglia either engulf (yellow arrows) or are adjacent to dying neurons (white arrows).

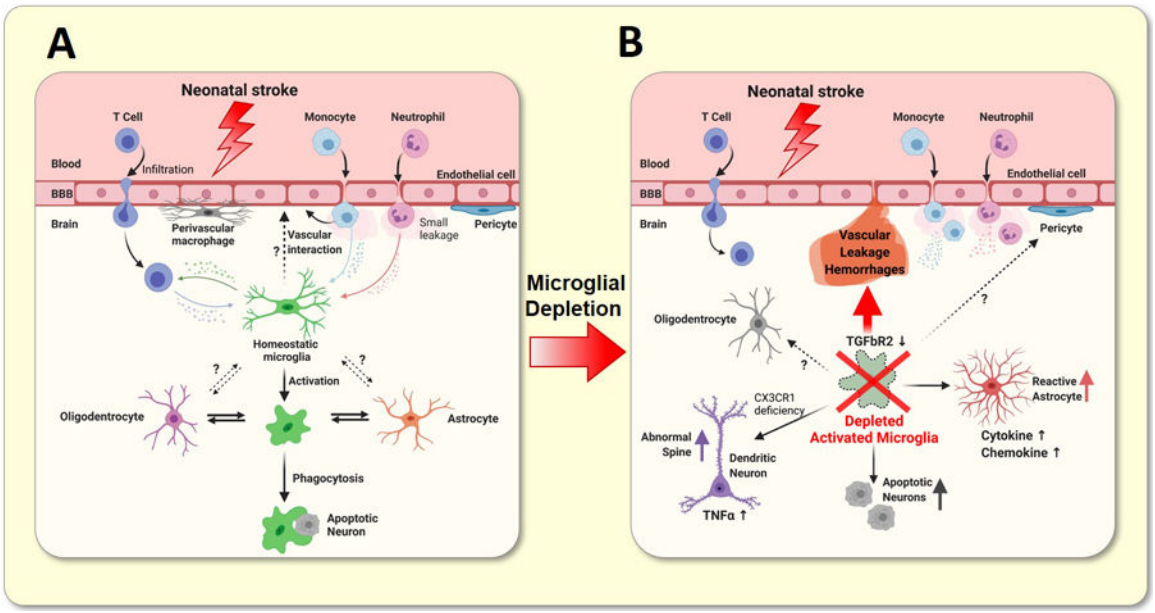

Figure 2.

Schematic representation of the immune and vascular responses fowling acute neonatal stroke in two scenarios, when activated microglia are present (A) or pharmacologically depleted in the brain (B). [Diagrams created using BioRender.com]

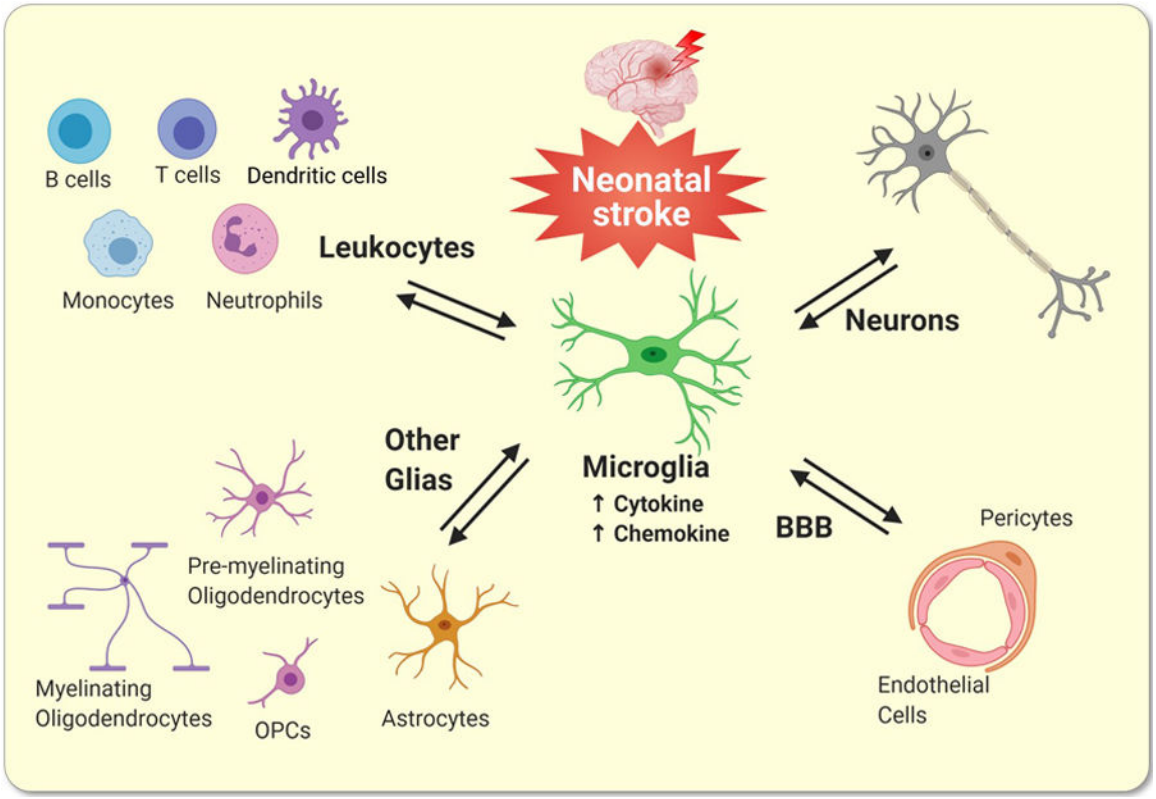

Figure 3.

Schematic representation of bidirectional interaction between microglia and other CNS cells. Infiltrated leukocytes including neutrophils, monocytes, dendritic cells, T cells and B cells interact with microglia in neonatal stroke. [Diagrams created using BioRender.com]