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Immune-neurovascular Interactions in Experimental Perinatal and Childhood Arterial Ischemic Stroke

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Abstract

Emerging clinical and preclinical data have demonstrated that the pathophysiology of arterial ischemic stroke (AIS) in the adult, in neonates and children share similar mechanisms that regulate brain damage, but also have distinct molecular signatures and involved cellular pathways due to the maturational stage of the central nervous system and of the immune system at the time of the insult. In this review we discuss similarities and differences identified thus far in rodent models at two different diseases—neonatal (perinatal) and childhood AIS. In particular, we review acquired knowledge of the role of resident and peripheral immune populations in modulating outcomes in models of perinatal and childhood AIS and the most recent and relevant findings in relation to the immune-neurovascular crosstalk and how the influence of inflammatory mediators is dependent on specific brain maturation stages. Finally, we discuss the current state of treatments geared towards age-appropriate therapies that signal via the immune-neurovascular interaction and consider sex differences in order to achieve successful translation.

A brief summary

We review the role of maturational stage of the CNS and of the immune system at the time of arterial ischemic stroke (AIS) on the pathophysiological mechanisms of perinatal and childhood AIS.

Keywords

Neonatal stroke; pediatric stroke; inflammation; leukocytes; microglia; blood-brain barrier

Introduction

The developmental stage of the brain at the onset of arterial ischemic stroke (AIS) plays key role in injury [1,2]. Perinatal AIS (PAIS), the most common form of stroke in newborn infants, occurs in one per 2000–4000 live births [2]. Compared to PAIS, the incidence of

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Authors' contributions

CM, DF and ZSV searched the literature and wrote and revised the manuscript.

childhood AIS (CAIS) is lower but the rate of recurrence is strikingly high[3]. The causes of these diseases are distinct in many respects, with emerging literature pointing to the state of neurovascular development and maturation-dependent immune-neurovascular interactions as critical factors in triggering and modulating stroke in the developing brain. Inflammation, which is a hallmark of ischemic brain injury in infants [1]and children[4], affects brain repair and brain connectivity later in life. We will discuss evidence of maturation-dependent distinctions in immune-neurovascular interactions in humans and in rodent models in relation to the pathophysiology of PAIS and CAIS and identify what needs to be learned to better understand the diseases and how to treat them.

Epidemiology of PAIS and CAIS

The incidence of PAIS is high and accounts for ≈80% of perinatal strokes [2]. By comparison, the incidence of perinatal hemorrhagic stroke is ~6.2 per 100 000 live births [5]. Another type of perinatal ischemic brain injury, hypoxic-ischemic encephalopathy (HIE), occurs in 1 to 8 per 1000 live births in developed countries. PAIS produces significant morbidity and severe long-term neurological and cognitive deficits including cerebral palsy and neurodevelopmental disabilities. The likelihood of recurrence after PAIS is low except in those with congenital heart disease [6]. As of now, therapeutic hypothermia (TH) is the only approved treatment that offers neuroprotection and reduces long-term disability caused by HIE, but the benefits are limited and applicability of TH to PAIS has not been established.

The incidence of CAIS is lower compared to PAIS, one to two in 100 000 children[2], but the rate of recurrence is strikingly high [3]. Cerebral arteriopathies are reported in up to 80% of children with AIS, and are strongly predictive of stroke recurrence [6,7]. Focal cerebral arteriopathy of childhood is a leading cause of CAIS, likely via post-infectious inflammatory mechanisms [8], but precise mechanisms are not sufficiently understood. Advanced MRI measures demonstrated vascular inflammatory arteriopathy that causes arterial stenosis and links inflammation and arteriopathy to stroke recurrence [7]. Therapeutic approaches have been considered, including endovascular recanalization in CAIS, but no effective therapies exist.

Infection as a Risk Factor for PAIS and CAIS

Epidemiologic studies reported several common maternal and fetal/neonatal risk factors for PAIS. Chorioamnionitis in the mother is associated with a 3-fold increased risk of stroke in newborn, and maternal intrapartum fever, a clinical feature of chorioamnionitis, is independently associated with a 10-fold increased risk of PAIS [9]. Interaction of released inflammatory mediators with the coagulation cascade leads to increased hypercoagulability via platelet activation, injury to vascular endothelium and impaired fibrinolysis [10].

In children who had infection within a month before stroke, a retrospective population study revealed 3.1-fold increase of CAIS cases [11]. A prospective study reported a robust 6.5-fold increase in risk of CAIS following infection in the prior week [12]. Upper respiratory tract infection, chickenpox (varicella zoster virus, VZV), and other viral infection types are found as contributors, with viruses acting in part through the vasculature [13]. Together, these findings suggest that while the etiology of PAIS and CAIS are different, infection

in infants and children plays the priming role in predisposing to stroke through distinct maturation-dependent mechanisms. A better understanding of how inflammation leads to PAIS and CAIS or affects injury evolution and function of regions that are not directly affected by injury could lead to new strategies to prevent and treat these diseases.

Neurovascular interfaces and the immune system during physiological brain development

The neural parenchyma is separated from peripheral immune cells by the barrier systems [14], including the blood-brain barrier (BBB), and the barriers between the blood and the cerebrospinal fluid (CSF) at the choroid plexus (CP), i.e., blood-cerebrospinal fluid barrier (BCSFB), and the leptomeningeal barrier. All barriers form during fetal life and continue to evolve during postnatal brain maturation [15–17]. Individual components of the BBB mature largely in an unsynchronized fashion. Tight junction (TJ) proteins, such as occludin and claudin-5, are detected in the fetal brain mid gestation [18], whereas astrocytes and pericytes appear later [15]. Microglial cells appear in the brain early [19], before vascular sprouting begins, and contribute to vascular formation and function and proper brain development [16].

The immune system is relatively immature at birth [20]. Newborns have an overall higher white blood cell count than adults, which is primarily due to a sharp postnatal increase in neutrophils and, to a lesser extent, monocytes and lymphocytes. Despite the higher numbers, peripheral myeloid cell responses are blunted, such as lower chemotaxis and adhesion to endothelial cells [21] and undeveloped innate immunity [22]. Microglia contribute to vasculogenesis and shaping of neuronal circuits during development.

Rodent models of PAIS and CAIS

The age that approximates human brain at term is species-dependent (reviewed in [23,24]). In rodents, most brain development occurs after birth, like in humans, but developmental growth of individual brain regions is distinct in rodents and humans, thus making it difficult to adhere to a single postnatal day as a comprehensive representation of brain development in humans. Based on cross-comparisons of gross neuroanatomy, the timing of neurogenesis, synaptogenesis, gliogenesis, and myelination, rodent brain at postnatal day 1 (P1)–P5 is thought to correspond to 23–32 weeks of gestation in humans and, thus, suitable for studies of preterm brain injury, whereas the rodent brain at P7–P10 corresponds to 36–40 weeks of gestation in humans, thus, suitable for studying brain injury close to at term. Brain myelination in rodents is completed between P17-P25, an age considered to represent childhood in human (reviewed in [25]).

The first established model of ischemia-related pathology in the neonatal rodent brain, a hypoxia-ischemia (H-I) model in P7 rats and P9 mice, involves unilateral/bilateral ligation of the common carotid artery followed by systemic hypoxia of variable length and oxygen deprivation [26]. This model is more representative of HIE rather than focal stroke.

Models of focal ischemic injury without a hypoxic exposure were developed to examine the pathophysiology of PAIS by using middle cerebral artery occlusion (MCAO), the most common type of ischemic stroke in at term infants. Both transient MCAO (tMCAO) [27] and permanent MCAO in conjunction with transient occlusion of left common carotid artery [28] were characterized in P7 rats, tMCAO models developed in P10 rats [29] and P9-P10 mice [30].

MRI enabled demarcation of ischemic injury with a definable ischemic core and penumbra [31] and allowed studies of injury evolution over time [32].

CAIS has different etiologies, risk factors and presentation compared to either neonates or adults [2]. To understand the pathophysiology of CAIS, models of tMCAO [33–35] and endothelin-1 injection [36] were developed in juvenile (P17-P25) rodents. Intracortical IL-1β injection or brain trauma models in juvenile rats were also utilized to examine mechanisms of leukocyte-neurovascular interactions in juvenile rats [37,38]. Administration of a viral mimetic/TLR3 agonist Poly-IC in P18 mice was shown to induce cerebral arteriopathy [39], which commonly precedes CAIS. The latter model, when applied in conjunction with a CAIS model, should enable studies of how viral infection affects stroke evolution and outcome in CAIS.

Pathophysiology and early mechanisms of ischemic injury in the maturing brain

Consequences of CBF disruption in neonatal rodents subjected to H-I or MCAO, including rapid disruption of ATP production and energy metabolism, activation of excitotoxic and oxidative pathways, which cumulatively lead to neuronal death through different mechanisms, have been extensively reviewed [1,40].

The brain barrier interfaces in acute PAIS

The breakdown/leakage of the BBB is comprehensively characterized after adult stroke (reviewed in [41]). In neonatal rats and mice the BBB is not as permeable 2–24 hours after tMCAO as after a similar insult in the adult, as is evident from much lower leakage of Evans Blue (i.e., albumin) and 70kDa dextran, and is consistent with low neutrophil infiltration [42]. Endothelial transcriptome analysis showed markedly different gene “signatures” between adults and neonates post-stroke [42]. Altering blood-to-brain neutrophil chemoattractant gradient drives neutrophil infiltration into post-stroke neonatal brain, triggers BBB leakage and enhances injury [42], demonstrating a link between maturation status of the neurovascular interface and neutrophils. It is not well understood whether the higher resistance of the neonatal BBB to stroke is a cause or a consequence of reduced leukocyte infiltration. Of note, in the H-I model, BBB leakage to sucrose is notable but transient and mainly confines to the hippocampus [43].

At the BCSFB, the CP serves as an entry route to the parenchyma for circulating immune cells under both physiological and pathological states [44], including trafficking of inflammatory cells of myeloid lineage to the damaged area in models of CNS injuries and stroke [45]. tMCAO in P9 mice induces robust accumulation of myeloid cells at the CP ipsilateral to injury, including neutrophils as well as inflammatory and beneficial monocytes [46]. RNA sequencing analyses in the isolated CP demonstrated marked unilateral changes in gene expression in the CP ipsilateral to tMCAO, including several clusters of genes involved in inflammatory, metabolic and extracellular matrix signaling [47]. Flow cytometry in the isolated CP, in turn, revealed stepwise CD36-mediated recruitment of myeloid cells into the CP ipsilateral to tMCAO early after reperfusion, with a predominant increase first in inflammatory monocyte subsets and neutrophils followed by patrolling monocytes [47]. tMCAO markedly increased cytokine levels in the CSF in neonatal mice [46], likely

contributing to parenchyma injury without reaching the injured penumbra. In neonatal mice, the patterns of leukocyte migration are strikingly different following administration of TLR2 or TLR4 ligands [48].

The meninges serve as an important site of immune cell expansion and reactivity in the developing brain and during early phases of immune response after preterm brain injury [17]. Mice lacking macrophages, but with conserved brain microglia, or mice bearing macrophage-specific deletion of Stat1 or Ifnar, exhibit extensive viral spread into the CNS, demonstrating non-overlapping functions with microglia and protect against viral infection [49]. Using single-cell RNA sequencing, we have generated the first comprehensive transcriptional atlas of neonatal mouse meningeal leukocytes under normal conditions and after H-I and identified novel meningeal microglia-like cell populations that may participate in white matter development [17]. Increased granulopoiesis early after H-I suggests that the meninges are an important site of immune cell expansion that contributes to the initiation of inflammatory cascades after neonatal brain injury [50]. The role of meningeal macrophages in PAIS is yet to be studied.

The brain barrier interfaces after acute CAIS

BBB permeability differs in a rodent CAIS model compared to both PAIS and AIS models [35,42]. The use of a novel in vivo technique to examine vascular structural-functional responses to CAIS [51] revealed marked loss of vasculature in the ipsilateral hemisphere, ranging from partial to an almost complete loss of microvessels and the overall reduced average vessel length and number of junctions [35]. Dysfunctional CX3CR1-CCR2 signaling is also shown to reduce extravascular albumin leakage, accumulation of $Ly6G^+$ neutrophils and acute injury [35]. Taken together, these data demonstrate that the structural-functional neurovascular response differs between PAIS and CAIS models, and microglia-monocyte-neutrophil interactions serve as injury modifiers, as demonstrated in Figure 1.

BBB disruption is also much higher in juvenile than in newborn or adult rats following intrecerebral IL-1β administration [37]. Distinctions are likely due to evolving myelination and leukocyte maturation during postnatal development. Furthermore, in the mouse brain, cortical vessel branching reaches a plateau between P15 and P25, which could affect the extent in collateral circulation and stroke severity [52]. Systemic immune activation in juveniles can also promote pro-coagulant effects and local inflammation and induce fragility of cerebral arteries, yielding the juvenile brain susceptible to subsequent stroke [53].

NEUROINFLAMMATION

Phenotypic and functional diversity of microglial cells

Microglial cells are the primary immune cells in the brain under physiological conditions and represent 12–15% of the CNS cellular component. For decades microglial cells were viewed as purely toxic in stroke due to release of oxidants and inflammatory molecules, whereas in reality both microglial cells and monocyte-derived macrophages account for the observed effects. Several discoveries and novel fate-mapping techniques have helped distinguish activated microglia and brain-infiltrating monocytes under neuroinflammatory

conditions and redefine microglial states under physiological conditions and after brain injury (reviewed in [54]). As examples, the origin of microglial cells, yolk-sac-derived, is distinct from that for monocyte-derived macrophages, there are several microglia-specific surface markers such as P2RY12, TMEM119, FCRLS, and intracellular markers such as SALL1, and microglia are shown to self-renew without any contribution from bone-marrow-derived cells and long-lived within the CNS parenchyma. The microglial pool is now recognized as heterogeneous, plastic, and exhibiting region-specific phenotypes dependent on the local signals from the CNS microenvironment and from peripheral cues [55]. A switch from “homeostatic” phenotypes under physiological conditions to disease associated microglia (DAM) phenotypes following sustained activation, phenotypes that hamper neuronal damage upon broken homeostasis and play detrimental role in injury, is also recognized [54].

Age has a key influence on temporal diversity of microglial homeostatic states over lifespan; the heterogeneity of microglia declines with brain maturation [56]. Microglia are the source of trophic support to neurons and endothelial cells. Disrupted growth factor production in microglia interrupts cortical layer formation [57] and synaptic refinement during the neonatal to adolescent period [58]. Thus, microglia in the healthy brain exhibit a spectrum of distinct functional states and depends on brain maturation (reviewed in [59,60]).

Microglia interact with other CNS cell types. Microglia-neuronal interactions are bi-directional and are regulated by a number of intracellular checkpoints and receptor-mediated mechanisms [61]. Microglia shape the brain by eliminating synapses during early postnatal development [59] as well as promoting neuronal homeostasis and survival [57]. The microglial-astrocyte and microglial-oligodendrocyte interactions are also bi-directional and contribute to postnatal development and to brain injury via CX3CR1-dependent mechanisms [62]. Microglial maturation is regulated by other immune cells [63]. Border-Associated Macrophages (BAMs), another type of resident macrophages that populate the CP, meninges and perivascular spaces, express both genes found in resident microglia and bone-marrow derived cells and can repopulate perivascular spaces after ischemia. Specific transcriptome differences in BAMs at the meninges were identified in the developing brain [17].

Microglial cells as injury modulators in PAIS and CAIS

In the neonatal brain, microglial cells undergo morphologic transformation after both H-I and focal arterial stroke. Activated microglia/macrophages were viewed as mediators of H-I and excitotoxic injury in earlier studies, before advances in discriminating microglial and monocyte-derived macrophages, it remains unknown which cell type is actually harmful. In the acute injury phase following tMCAO in P7 rats or P9 mice, we demonstrated that pharmacologic depletion of microglia by intracerebral injection of liposome encapsulated clodronate before tMCAO disrupts BBB integrity and exacerbates injury [64]. A number of mechanisms can account for protection. 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, we showed that limited engulfment/removal of neuronal debris in pups with genetically deleting the scavenger receptor CD36, which contributes to several phagocytic steps, increases residual cleaved caspase-3,

enhances inflammation and worsens injury [30]. Another mechanism of microglia-mediated protection in PAIS is through stabilizing BBB integrity. The majority of microglia interact with vessels via extended network of processes under physiological conditions, but the spatial physical microglia-vessel contacts are altered when microglia acquire activated phenotypes. We demonstrated that microglial depletion or abolishing TGFβ1 signaling in activated microglia trigger BBB leakages and induce hemorrhages following neonatal tMCAO [64]. Several fold higher expression of TLR2 expression in microglia in neonatal compared to adult brain [65,66] may also play an important role in maturation-dependent AIS pathophysiology, as outlined in Figure 1B. In adult stroke models microglia set a critical line of defense against spreading depolarization [67] and damaging capacity of neutrophils [68], but these aspects of microglial function have not been studied in PAIS models. It is also essentially unknown whether a particular microglial subpopulation provides endogenous cerebrovascular protection. For example, in chronic phases after stroke in adult and ageing mice repopulation of the microglial pool after microglia deletion before tMCAO drives brain repair [69].

Bacterial infection is known to exacerbate H-I brain injury in the neonate. Recent transcriptome network analysis uncovered the underlying mechanisms by linking perinatal Staphylococcus epidermidis infection to microglia reprogramming in the immature hippocampus, including increased NOD-receptor signaling and inflammasome activation, that leads to leukocyte infiltration to the brain and disruption of the BBB [70].

Leukocytes

Monocytes: Monocytes can signal from the blood or after differentiating into macrophages once they reach injured brain. One caveat in the stroke field has been difficulty in differentiating the relative role of monocytes and microglia when these cell types yield similar antigenic expression. These cell types use many same pathways for activation, but their contribution to stroke is not interchangeable, as is evident from distinct and even opposite roles of microglia and monocytes in stroke [71]. Both human and mouse classical monocytes express high levels of CCR2 and low levels of CX3CR1 while non-classical monocytes of both species express high CX3CR1 and low levels of CCR2. The relative presence of these two key receptors is the basis of the migration and homing mechanism of monocytes into areas of inflammation. In adult stroke models, peripheral monocytes contribute to both damage and healing by polarizing monocytes into a pro- or anti-inflammatory phenotypes [72]. Nonetheless, targeting monocytes following adult stroke has had varying success.

Monocytes and microglia have essential non-redundant functions in brain development [73]. Compared to adult monocytes, fetal monocytes display limited levels of antigen recognition and phagocytic capabilities [74], but exhibit more proliferative potential [75]. After H-I, multiple peaks of monocyte accumulation and an array of related pro- and anti-inflammatory effects ultimately determine long term hippocampal damage and spatial learning deficits [76].

In a CAIS model, $CCR2^{+}$ monocytes infiltrate after acute injury and mice deficient in CCR2 signaling are protected [35]. In patients with CAIS, dis-coordination between neutrophils and

monocytes correlates with endothelial repair response genes [77]. Together, these data suggest a bidirectional role in monocyte-endothelial signaling in CAIS.

Neutrophils

While neutrophils are vital for immunity against invading microorganisms and inflammation, there is ample evidence of the damaging role of neutrophils early after stroke, including blocking the vessels and reducing CBF. In humans, neutrophil accumulation is rapid, the majority of genes induced within 24h after AIS are in neutrophils [78], and neurological outcome is worse in patients with more severe neutrophil accumulation. Neutrophils can reach the perivascular spaces after crossing the endothelial cell layer and basal lamina of post-capillary venules or migrating from the leptomeninges following pial vessel [68]. Despite attenuation of BBB disruption and reduced infarct size in experimental ischemia-reperfusion models by neutralizing neutrophil actions, therapies targeting neutrophil egress have been largely ineffective. More recent studies demonstrated the damaging role of neutrophil elastase (NE) activation and assembly of neutrophil extracellular traps (NETosis) in stroke that is mediated by neutrophil and platelet TLR4 signaling [79] and HMGB1 [80]. “Re-shaping” neutrophil phenotypes from toxic “N1” to an anti-inflammatory “N2” phenotype is shown protective in stroke [81,82]. A novel TLR4-Binding DNA Aptamer is shown protective in several adult stroke models [83], acting via TLR4-neutrophil axis.

In neonatal rats we observed loosely attached but not transmigrated neutrophils 1–24h after tMCAO and demonstrated that the lack of neutrophil recruitment is likely not due to their intrinsic inability to transmigrate to injured neonatal brain, but due to the developmental status of neurovaculature and high circulating chemoattractant levels after tMCAO [42].

In juvenile mice subjected to controlled cortical impact, genetic deletion or pharmacologic inhibition of NE provides short-term protection [84] but lack of NE does not limit leukocyte numbers, suggesting NE role in changing neutrophil phenotypes post-trauma. In the CAIS model, genetic disabling CX3CR1 and CCR2 receptors significantly reduces neutrophil infiltration [35], pointing to regulation of neutrophil activation and migration by the cells of the monocyte lineage. Viral mimetic/TLR3 agonist Poly-IC also triggers cerebral arteriopathy in juvenile brain via NE activation and NETosis [39], suggesting that disruption of this pathway in neutrophils can potentially alleviate arteriopathy-prompted stroke in children. Overall, these findings highlight a potential key role of neutrophils in influencing CAIS.

Other immune cells

T cells mediate microvascular dysfunction in post-ischemic adult brain by producing multiple inflammatory factors that degrade extracellular matrix (ECM) and damage endothelial cells [85]. In neonates, lymphocytes infiltrate the brain for months after H-I injury, suggesting their long-term contribution to the inflammatory response [86]. While pharmacological depletion of T cells after neonatal H-I exacerbates brain injury and increases infiltration of innate immune cells into the brain parenchyma, deficiency of both B and T cells using Rag−/− mice reduces white matter injury [87]. Interestingly, infiltration of

regulatory T-cells (T-regs) into injured brain provides endogenous neuroprotection in female mice while induces secondary neurodegerenation due to vascular injury after neonatal H-I in male mice [88]. As of now, the role of lymphocytes in PAIS and CAIS remains undefined. Spleen-associated NK cells are shown to contribute to H-I injury in neonates [89]. Activated mast cells are reported to be among the first responders in both H-I and PAIS models by undergoing degranulation, histamine release, leading to neuronal death [90].

Toll-like receptors (TLR)

Multiple TLR mediate inflammation and brain injury in stroke. While TLR4 seems to be purely injurious in both experimental [91] and human stroke [92], the role of TLR2 is more complex. Data in TLR2 knockout mice show both its injurious and beneficial role by supporting neurogenesis. The effects in part depend on the type(s) of heterodimers that TLR2 forms [93], recruitment of various individual intracellular TLR adaptor molecules [94] and significant down-regulation of the endogenous Nrf2 anti-oxidant system.

The involvement of TLR2 is a differentiating factor between adults and neonates after stroke. Longitudinal bioluminescence imaging in adult mice bearing the dual *luc/gfp* reporter under the TLR2 promoter showed that the vast majority of cells expressing TLR2 are microglia and that ~ 20-fold increase in TLR2 expression occurs 24h following tMCAO [65]. In healthy neonatal brain TLR2 expression is 20–30-fold higher than in adult TLR2-*luc/gfp* mice, with microglia as the predominant source of TLR2, but no significant TLR2 upregulation occurs in the parenchyma or the CP of neonates after tMCAO [66]. By contrast, TLR2 expression is increased in the neonatal brain following intra-cortical IL1a or intra-peritoneal LPS administration [66]. Following H-I, TLR2 or TLR4 deficiency reduces TNF-α, iNOS, and COX2 production, limits brain infarct volume and improves neurological outcome [95]. TLR4-mediated effects are stimulus-specific, as LPS and TLR2 involve distinct brain chemokine responses to trigger leukocyte infiltration into neonatal brain and barrier leakage [96]. Collectively, these data demonstrate age and context-dependent responses in the magnitude of leukocyte accumulation in the CP and the magnitude and the timing of cytokine accumulation.

LIPID SIGNALING

Lipid composition in cellular membranes plays an important role in maintaining the structural integrity of cells and in regulating cellular signaling. After AIS an assembly of receptors within the plasma lipid layers and signaling via various bioactive lipids serve as major modulators of injury. We limit discussion to the lipid signaling pathways to the ones studied in PAIS models.

Sphingosine-1-phosphate (S1P) signaling mediates many physiological processes. An imbalance of sphingolipids “rheostat” serves as a prerequisite to developing pathological states, including stroke [97]. S1PR1 and S1PR3 regulate T- and B-cell trafficking, whereas S1PR2 mediates vascular inflammation, disrupts cerebrovascular integrity and limits angiogenesis in stroke [98]. [98]Global S1PR2 deletion enhances neuronal survival and attenuates both short-term and longer-term behavior deficits in a mouse PAIS model [99]. In neonatal rats, administration of a S1PR1,3–5 agonist FTY720 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 [100] as well as suppresses LPS-triggered autophagy in primary microglia from S1P ablated mice. Therefore, S1PR2 can be a potential new target for mitigating PAIS.

The scavenger receptor CD36 is central to multiple biological functions in endothelial cells, microglia and monocytes, including uptake of long chain fatty acids and oxLDL, phagocytosis of apoptotic debris and cell chemotaxis. It serves as a “master switch” in assembling lipid signaling and triggering inflammatory pathways and ROS production under injurious conditions via multiple ligands and partnering with multiple receptors in the lipid fraction, including TLR2/4/6 [93]. CD36 is injurious after acute adult stroke but protective in acute perinatal stroke by phagocytosis apoptotic debris [30,101]. Such a rather opposing maturation-dependent response in part depends on availability of ligands in neonatal vs. adult brains as well as the recruitment of both neutrophils and monocytes at the level of the CP, influencing the metabolic and ECM signaling [47]. Further studies should define long-term effects of CD36-mediated effects in the injured developing brain.

Extracellular vesicles (EVs), another aspect of lipid signaling under physiological and injurious scenarios, is important in heterocellular communication between different cell types in the brain (reviewed in [102]). EVs— microvesicles and exosomes—are secreted by all cells and, based on their cell origin and biogenesis, may have different composition, functions and subtypes, depending on cellular “cargo” they carry, including proteins, lipids, nucleic acids, including miRNA [102]. Exosomes from mesenchymal stem cells (MSC) increase neurovascular remodeling, improve neurological, behavioral and cognitive outcomes during recovery, and facilitate endogenous rewiring of neuronal circuitry in multiple brain injury models [103] but can be detrimental in other conditions like tau pathology.

EVs released by microglia are thought to mirror the dynamic nature of their donor cells, exerting important and versatile functions in the CNS [102]. Microvesicles derived from microglia are shown injurious in multiple sclerosis, both in humans and in an animal model [102], but beneficial by modulating neural stem cell proliferation in the neonatal SVZ [104]. We demonstrated that microvesicles and exosomes isolated from $CD11b^{+}$-microglia/macrophages that are pulled down from ischemic-reperfused and contralateral cortex of neonatal mice 24h after tMCAO, carry distinct “cargo”, and that uptake of EVs is significantly higher by microglial cells isolated from ischemic-reperfused regions [105]. Silencing or inhibition of nSMase2, an enzyme involved in EVs release, impacts exosomal secretion and injury after neonatal stroke [105]. Administration of exosomes isolated from MSCs at the time of tMCAO in neonatal mice leads to their selective accumulation in injured regions and protections [106].

One of Omega-3 fatty acids, docosahexaenoic acid (DHA), which accounts for over 50% of all n3-Polyunsaturated Fatty Acids (n3-PUFA) in the CNS cell membranes, mediates fluidity, permeability, and elasticity of cell membranes in the brain. Pharmacologic n3-PUFA

supplementation protects the adult brain against stroke [107] by inducing anti-inflammatory and anti-oxidative effects, enhancing angiogenesis, neurogenesis and oligodendrogenesis. In human infants, Omega-3 fatty acids and lipid derivatives/metabolites play an important role in normal postnatal brain development [108]. Increase in n3/n6 fatty acid ratio or enriched dairy fat matrix diet protects immature brains from cognitive deficits induced by immune challenges [109]. n3-PUFA deficiency during the perinatal period alters microglial and neuronal plasticity-associated genes and functional ability of microglia to prune synapses in the developing brain [110]. We showed that n3-PUFA enriched diet during gestation and postnatal markedly changes lipid composition in brains of neonatal mice under physiological conditions and, interestingly, while it does not affect brain cytokine/chemokine levels under physiological conditions, n3-PUFA enriched diet mutes neuroinflammation and protects neonatal brain from stroke [111]. While the beneficial effects of lipid composition in protecting from acute injury in PAIS model are clear, the relative interplay of immune cells with dietary modifications during development and injury is not well understood.

Effects of sex

Sex differences in histological outcomes in H-I and PAIS are not necessarily apparent, but mechanisms of neuronal death are sex-dependent [112] and responses to anti-inflammatory therapies after perinatal brain injury can be sex-dependent. Microglia are sexually dimorphic [113]. Peak of postnatal phagocytosis occurs earlier but transiently in female microglia [114] but in a more sustained manner in male microglia, potentially influencing social circuitry and behavior in juvenile rats [115]. Abnormalities in microglia morphology/function during early development contributes to neurodevelopmental disorders such as autism, psychiatric diseases and depression (reviewed in [116]). Significantly higher microglial density in males than in females in several brain regions may provide broader access of communications with other cells [117] or, more specifically, via distinct miRNA signatures in male and female mice [118]. In a neonatal pMCAO model, gene expression of pro-inflammatory markers Cox-2 and TNFα is higher in male than in female microglia, pointing to sex differences in the inflammatory injury component [119]. Therefore, sex-dependent immune responses should be considered while identifying therapeutic targets.

Therapies – can we protect post-ischemic immature brain by modulating the immune responses?

Finding therapeutic strategies for AIS with sustained benefits has been difficult, with therapeutic options essentially limited to tPA. Most recently the *Stroke* Pre-Clinical Assessment Network (*SPAN*) has embraced heterogeneity of preclinical AIS rodent studies while examining multiple therapeutic agents in randomized, placebo-controlled, blinded studies [120], including drugs with known immunomodulatory properties. One potentially significant direction in the AIS therapeutic field is the demonstration that a novel agent, a TLR4-Binding DNA Aptamer, is protective in several adult stroke models [83] and in a phase Ib/IIa, double-blind, randomized, placebo-controlled acute stroke trial in AIS patients [121].

In infants, TH is the only approved treatment for HIE that offers neuroprotection and reduces long-term disability [122], but its beneficial role is limited. Pharmacological strategies geared towards promoting neurogenesis after PAIS have been tested. For example,

erythropoietin (EPO) administration is shown to preserve the ECM and attenuate BBB disruption after cerebral ischemia by limiting oxidative injury, activation of various inflammatory signaling mechanisms as well as by skewing the microglial phenotype, leading to protection and enhanced angiogenesis (reviewed in [123]). In the PAIS model in P10 rats, administration of multiple EPO doses significantly improves behavior outcomes, preserves hemispheric brain volume long-term and increases neurogenesis and oligodendrogliosis [124]. However, clinical trials of rhEPO for acute PAIS were not successful [125]. With the premise that limiting oxidative and inflammatory injury by each EPO and hypothermia may further enhance beneficial effects, a combination of the two paradigms, EPO and hypothermia, was tested in the H-I model but no further benefits by EPO supplementation observed [126]. A recently completed clinical trial in at-term neonates with HIE treated with HT and multi-dose EPO administration did not improve beneficial effects of hypothermia [127] but led to serious adverse events [128]. Benefits of EPO for PAIS are to be seen.

Cell-based therapies have provided encouraging results by preventing perinatal brain injuries or enhancing repair in experimental settings (reviewed in [129]). Administration of umbilical cord blood cells (UCBCs), for instance, inhibits microglial activation following neonatal H-I [130], attenuates reactive gliosis, reduces infiltration of leukocytes into the brain and supports BBB function [131]. MSCs are shown to promote regeneration and reduce gliosis when administered intranasally days after H-I [132] and provide white matter protection and improve long-term functional outcomes in P10 rats after tMCAO [133]. Observations in the neonatal H-I model that transplanted MSCs are short-lived and become undetectable soon after administration [134] point to a changing local microenvironment rather than their continued presence that supports myelination. Importantly, safety and feasibility of intranasal administration of MSCs was recently shown in the first human PAIS study [135]. In a mouse PAIS model MSCs-derived exosomes recapitulate beneficial effects of MSCs [106]. Exosomes migrate specifically to ischemic-reperfused regions, are taken up by a subpopulation of endothelial cells and by activated microglia/macrophages, where they reside for lengthy time periods [106]. The role of EVs for myelination and long-term recovery after neonatal tMCAO are unknown. Cell modulation via EVs could thus represent a novel potentially powerful therapeutic alternative to cell-based treatments, but efficacy and safety of EVs subtypes are yet to be demonstrated in clinical trials. Other strategies are being considered for cerebral palsy and perinatal stroke, such as approaches aimed at enhanced repair explore occupational therapies, and their potential synergy with non-invasive neurostimulation [136].

In children, the ongoing VIPS II (Vascular effects of Infection in Pediatric Stroke) study aims to identify the array of pathogens that may lead to CAIS, and two clinical trials of corticosteroids for focal cerebral arteriopathy—the PASTA trial (Paediatric Aspirin Steroids Arteriopathy trial) in Europe and the FOCAS trial (Focal Cerebral Arteriopathy Steroid trial) in North America—are the first pediatric stroke prevention trials outside of the setting of sickle cell disease (reviewed in [4]). Mechanical thrombectomy has revolutionized care in adult stroke [137]. In children, retrospective case thrombectomy series suggested possible benefit [138], however risks of cerebral hemorrhage and reperfusion injury raise concerns on whether children can safely benefit from endovascular thrombectomy.

Finally, preconditioning, a phenomenon that consists of induction of sublethal stress (i.e., hypoxia, ischemia) or drug administration before the main ischemic event, as well as post-conditioning, meant to induce resistance to a subsequent potentially lethal ischemic insult, have been demonstrated to be safe andare being actively studied in adult animal stroke models and in small stroke clinical trials and in neonatal cerebral ischemia (reviewed in [139,140]). Such therapeutic approaches may also prove beneficial in children.

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Availability of data and materials

Not applicable; review article.

Non-standard Abbreviations and Acronyms

CAIS	Childhood arterial ischemic stroke
CP	choroid plexus
CSF	cerebrospinal fluid
ECM	extracellular matrix
EPO	erythropoietin
EVs	extracellular vesicles
HIE	hypoxic-ischemic encephalopathy
MCAO	middle cerebral artery occlusion
MSCs	mesenchymal stem cells
NE	neutrophil elastase
NETosis	neutrophil extracellular traps
P9	postnatal day 9
PAIS	perinatal arterial ischemic stroke
TH	therapeutic hypothermia
TLR	Toll-like receptors
tMCAO	transient MCAO

T-regs	regulatory T-cells
S1P	Sphingosine-1-phosphate

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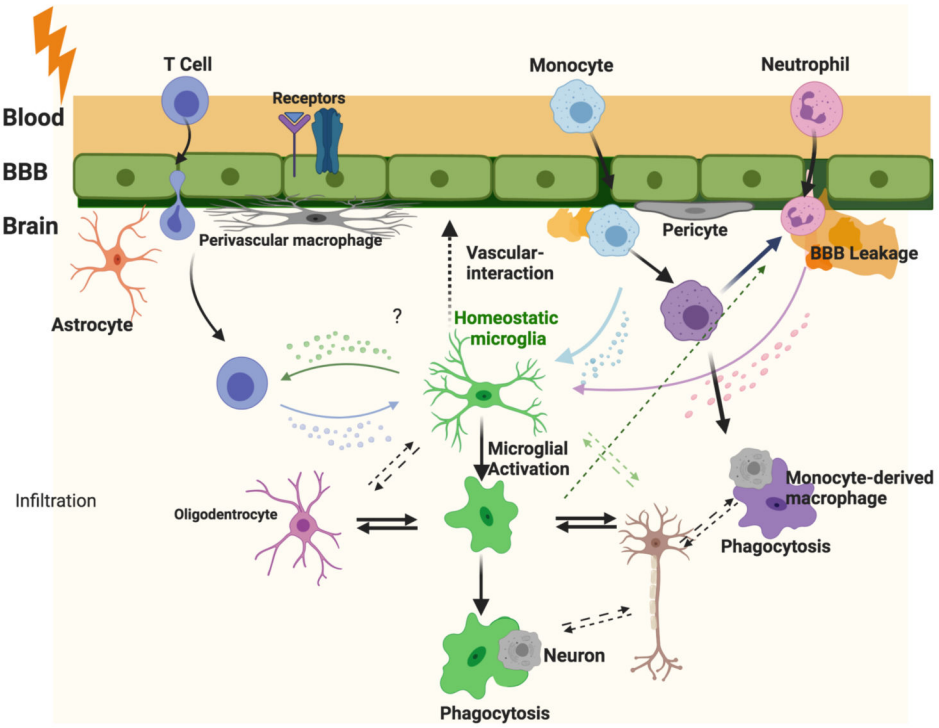
A



B

	PAIS	CAIS
BBB leakage	Low	Increased
TJ protein expression	Relatively preserved	*Not defined*
ECM integrity	Disrupted	*Not defined*
CP leakage to large molecules	Permeable	Permeable
Neutrophil infiltration via the BBB	Low	Increased
Monocyte infiltration via the BBB	Low	Increased
Neutrophil/Monocyte infiltration via the CP	Increased	*Not defined*
	Low	Increased
Toll-like receptors 2/3/4	Constitutive&Inducible	Inducible
CD36 effects	Protective	*Not defined*
Microglial activation	Rapid	Distinct pattern
NfkB activation	Modest	*Not defined*
Cytokine accumulation	Rapid & transient	Rapid, distinct

Figure 1.

Schematic representation of the immune and vascular responses after acute ischemia-reperfusion (**A**) and the relative magnitude (**B**) in PAIS and CAIS rodent models. Figure created with BioRender.com.