Inflammatory responses in hypoxic ischemic encephalopathy

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Immune cells

Microglia/macrophages

Microglia are a major glial component of the CNS and provide immuno-surveillance in the brain[17]. Resting microglia in a healthy brain, known as surveying microglia, are constantly extending and retracting their thin ramified processes to inspect the CNS microenvironment[18, 19]. When an ischemic event occurs, microglia are activated and develop...
macrophage-like abilities including phagocytosis, the production of inflammatory and anti-inflammatory cytokines, antigen presentation and the release of matrix metalloproteinases (MMPs), which lead to blood-brain barrier (BBB) breakdown\textsuperscript{[20]}. As a result, peripheral leukocytes infiltrate the brain, and the normally immune-privileged brain environment is exposed to systemic responses that further exacerbate inflammation and brain damage (Figure 1). The innate immune response is characterized by the classical activation (M1) of microglia and the subsequent production of specific cytokines, chemokines and reactive intermediates, followed by resolution and alternative activation (M2) that leads to anti-inflammatory signaling (M2a), the clearance of reactive oxygen (ROS) and nitrogen (RNS) species (M2b), and wound healing (M2c)\textsuperscript{[21]}. During disease progression, microglial activation phenotypes switch from M1 to M2 or vice versa depending on inflammatory signaling\textsuperscript{[22]}. The M1 phenotype of microglia can lead to increased neuronal death compared to the alternately activated M2 microglial phenotype\textsuperscript{[23]}; therefore, there is a growing interest in controlling the classical activation phenotype of microglia.

![Figure 1. Schematic diagram of inflammatory responses in ischemic stroke.](image)

ASTROCYTES

Both astrocytes and microglia are activated within minutes after injury by pro-inflammatory mediators, cytokines, and ROS that are secreted by injured neurons and glial cells\textsuperscript{[42]}. The activation of astrocytes has both detrimental and beneficial roles in brain ischemia. Astrocyte support of neurons after a stroke can be achieved by several mechanisms, including the release of glutathione and superoxide dismutase (SOD)\textsuperscript{[43–45]}, enhanced extra-synaptic glutamate uptake\textsuperscript{[46–48]}, and the maintenance of ion gradients, such as that for potassium\textsuperscript{[49,50]}. However, activated astrocytes can also produce pro-inflammatory cytokines, including IL-6, TNF-α, IL-1α, and β and interferon γ\textsuperscript{[42,51,52]}. Rapid increases in the levels of these cytokines exacerbate an ischemic injury by directly inducing the apoptosis of neuronal cells\textsuperscript{[53]}, increasing toxic NO levels and inhibiting neurogenesis\textsuperscript{[54]}. Apart from cytokines, reactive astrocytes also secrete chemokines after ischemia, which results in the attraction of immune cells to the ischemic site and worsening of the brain injury\textsuperscript{[55,56]}

In the brains of human neonates, astrocytes do not readily become reactive and responsive to injury signals until 20 to 23 weeks of gestation\textsuperscript{[57]}. Experimental studies regarding astrocytic responses to HIE or systemic LPS stimulation performed in fetuses from various species, e.g., lamb\textsuperscript{[58–60]} baboon\textsuperscript{[61]}, and kitten\textsuperscript{[62]}, found astrocyte hypertrophy and hyperplasia. These studies concluded that astrocytes generally are resistant to damage during the neonatal period and that the astrocytes adjacent to regions of necrosis are ready to proliferate. Similar to the findings in adult ischemic models, astrocytes in P7 rat neonates are rarely observed within the ischemic core but
are abundant in the penumbra area 24 h after HIE\textsuperscript{[37]}. One unique role of neonatal astrocytes in HIE-induced inflammatory responses is that, in addition to the self-release of cytokines and chemokines, reactive astrocytes in neonatal brains have the ability to up-regulate the expression of inflammatory mediators in neuroblasts and angioblasts, which are chemotactic for bone marrow-derived immune cells\textsuperscript{[63]}. 

**Neutrophils**

During ischemia, neutrophils can exacerbate brain injury through multiple mechanisms, including ROS production\textsuperscript{[35]}, decreased microvascular flow resulting from capillary plugging by neutrophils\textsuperscript{[64]}, the enhanced release of cytotoxic agents into the vasculature and brain parenchyma\textsuperscript{[65, 66]}, and MMP-9 secretion\textsuperscript{[67]}. The accumulation of neutrophils in ischemic brain tissue occurs as early as 4 h to 6 h after the onset of ischemia in adult animals\textsuperscript{[65, 66-70]} and lasts to 48 h post insult, during the period while the brain injury is evolving\textsuperscript{[71-73]}. In contrast to the exacerbated neutrophil infiltration observed in adults, neonates have a diminished ability to mount a neutrophil response to ischemia. Neonatal neutrophils show reduced extravasation from blood vessels\textsuperscript{[74, 75]}. A previous study has shown that neutrophils did not transmigrate into the brains of P7 rats following HI injury within 42 h and were almost exclusively intravascular at all time periods examined\textsuperscript{[80]}. Similarly, it has been reported that neutrophils were most often found within vessels and only transiently invaded brain tissue in the infarct region after induction by HI in P7 rats\textsuperscript{[35]}. These studies indicated that neutrophils do not accumulate in ischemic brain parenchyma in neonatal rodents to the extent that they do in adults. Interestingly, this concept translated well into the neutrophil protection achieved with anti-neutrophil strategies; treatment with neutrophil inhibitory factor initiated after HI insult was neuroprotective in adult animals\textsuperscript{[77-79]} but was less efficacious in neonatal rats. Beneficial effects were only observed when neutropenia was induced before the HI insult\textsuperscript{[80]}, making this a less clinically relevant target for treating neonatal injury.

**Lymphocytes**

Generally, lymphocytes are thought to play a negative role in acute ischemic brain pathogenesis. Yilmaz et al\textsuperscript{[81]} reported that Rag1\textsuperscript{-/-} mice, deficient in both T cells and B cells, had significantly smaller infarcts and neurologic damage compared to WT mice when subjected to middle cerebral artery occlusion (MCAO). In the same study, Rag1\textsuperscript{-/-} mice reconstituted with splenocytes from WT mice were no longer protected from stroke, suggesting that the peripheral lymphocytic response plays an important role in mediating post-stroke injury. Infiltration of T cells and B cells into the ischemic brain can be observed as early as a few hours\textsuperscript{[82, 83]} and lasts days after injury in adult rodents\textsuperscript{[84, 85]}. However, in neonates the infiltration of these cells following HIE and focal stroke may be less profound\textsuperscript{[85, 86]} or only briefly present in the parenchyma\textsuperscript{[87]}. The minimal involvement of lymphocytes in ischemia-induced inflammatory responses in the neonatal brain may reflect the immaturity of lymphoid progenitor cells. Recent clinical studies showed that peripheral blood mononuclear cells of newborns are relatively undifferentiated and have a very low expression level of surface markers\textsuperscript{[88]}. There are few studies investigating the role of lymphocytes in HIE. It is likely that a lymphocytic response is involved in the more chronic immunoinflammatory activation following HIE; the Hagberg group\textsuperscript{[53]} found that CD4 lymphocytes invaded the infarct region quite late after injury (7 d after HIE) and persisted in damaged areas for 14 d to 35 d. Whether this lymphocytic response enhances damage or, conversely, enhances post-stroke repair is not yet clear. It is also unknown whether the presence of lymphocytes can lead to the development of later CNS autoimmunity, as has been observed in adult injury models\textsuperscript{[89]}.

**Adhesion molecules**

The recruitment of leukocytes in the cerebral vasculature and the subsequent migration to the ischemic brain tissue are initially mediated by three main groups of cell adhesion molecules: selectins, the immunoglobulin superfamily and integrins\textsuperscript{[90]}. The recruitment process involves two stepwise stages, ie, an initial low affinity binding that is manifested as rolling and a later high affinity interaction that results in firm adhesion. Adhesion molecules may represent important therapeutic targets because inhibiting leukocyte adhesion with antibodies or inhibitors has improved histological and neurological outcomes in experimental stroke studies, whereas overexpression of adhesion molecules resulted in the exacerbation of infarcts\textsuperscript{[91]}. Very few neonatal studies have reported the role of and changes in adhesion molecules in HIE; we have summarized the available data from studies in both HIE and other inflammatory diseases in Table 1.

**Selectins**

Selectins play a key role in the early (rolling) stages of leukocyte/endothelial interactions in the ischemic cerebral microvasculature. Although all three selectins, L-, P-, and E-selectin, have been implicated in neutrophil rolling, P-selectin is the most important during the initial induction of neutrophil rolling after endothelial cell stimulation\textsuperscript{[92]}. Compared to adults, decreased P-selectin expression in neonates has been found in activated platelets\textsuperscript{[93]} and endothelial cells\textsuperscript{[94]}. Similarly, L-selectin expression in term infant neutrophils is significantly lower than that in adult neutrophils either stimulated or unstimulated\textsuperscript{[95]}. This may explain why the decreased adhesion of neutrophils to endothelial cells and delayed transendothelial cell migration of neutrophils have been consistently reported in neonatal animals and humans and may also contribute to susceptibility of neonates to infection\textsuperscript{[96, 97]}. In immature animal brains during acute inflammation, E-selectin appears to be less important than other selectins because the blockade of E-selectin has no effect on neutrophil recruitment to the brain parenchyma, whereas the administration of P-selectin blocking monoclonal antibody inhibited neutrophil recruitment by 85% compared with controls\textsuperscript{[98]}. 
Integrins and the immunoglobulin superfamily

The firm adhesion of leukocytes to the endothelium after rolling requires the activation and binding of leukocyte-expressed integrins to endothelial adhesion molecules[79]. Integrins are heterodimers consisting of a common β subunit and a variable α subunit[100]. The major integrins expressed on neutrophils are the β2 integrins LFA-1 (αLβ2, CD11a/CD18) and Mac-1 (αMβ2, CD11b/CD18). Monocytes adhere through the β1 integrins VLA-4 (α4β1, CD49d/CD29). To form a firm adhesion, integrins must bind to counter-receptors of the immunoglobulin superfamily expressed on inflamed endothelial cells, including ICAM-1, ICAM-2, VCAM-1, the mucosal vascular addressin cell adhesion molecule 1 (MadCAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1), and the receptor for advanced glycation end products (RAGE)[101–103]. Although no age-related differences in basal and stimulated LFA-1 surface expression were found in human neonatal and adult neutrophils[104–106], Mac-1 expression remains low during the prenatal and postnatal periods and reaches adult levels by 11 months[107, 108]. The lower surface expression of Mac-1 on neonatal neutrophils has been directly linked to impaired transendothelial migration under chemotactic stimulation[109,110] (Table 1).

Thus far, no data are available in neonates regarding the roles of integrins and the immunoglobulin superfamily in HIE. Experimental studies with adult stroke models have shown that blockade of LFA-1/Mac-1[111–113] and ICAM-1[114, 115] had beneficial effects on stroke outcomes. However, clinical trials of stroke patients given humanized antibodies against these adhesion molecules showed no effect[116, 117] or a worse outcome[118]. There are several reasons (see review[119]) for the failure of antibodies against these adhesion molecules to translate into a clinically relevant treatment strategy. For example, the study designs in the clinical trials did not mirror the laboratory models (such as late treatment or the absence of documented recanalization to the occluded vessel). Another possibility is that changes in neutrophil integrins are different between humans and rodents. Indeed, recent work has highlighted the differences in the immune system between species[120]. These differences emphasize the importance of clinical biomarkers and early phase studies to confirm the targets in both adult stroke and neonatal HIE, particularly using accessible sources such as peripheral blood. Although intervention strategies targeting adhesion molecules appeared to be effective in preclinical studies, moving this work into humans remains a tremendous challenge. It is encouraging that natalizumab, a humanized monoclonal antibody against α4-integrin, has been used to treat multiple sclerosis for more than 5 years[121] and has been reported to decrease the risk of disability progression by 42% to 54% and to reduce the annualized rate of relapse by 68%[122]. Natalizumab treatment is associated with a risk of progressive multifocal leukoencephalopathy (PML), an opportunistic brain infection caused by the JC virus[123]. However, because its clinical benefits outweigh the risks involved, natalizumab remains on the market in the US under a special prescription program using risk stratification algorithms and PML management strategies[124].

Cytokines

Cytokines are important inflammatory mediators, and cerebral ischemic injury can trigger a cascade of cytokine induction that acts to orchestrate an in situ inflammatory reaction[130] and maintains brain tissue homeostasis[131]. In general, the roles of cytokines are pleiotropic, and whether the overall effects are pro- or anti-inflammatory in the context of ischemic insults remains controversial even in adult models, for which there are more data than for HIE. The most studied cytokines related to the inflammatory responses to stroke are IL-1, IL-6, IL-10, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β)[132].

Table 1. Roles of adhesion molecules in pediatric inflammation.

| Mediators       | Investigated objects | Stimulation                     | Effects of stimulation | Compared to adults | References |
|-----------------|----------------------|---------------------------------|------------------------|--------------------|------------|
| L-selectin      | New born infants     | Acute bacterial infection       | Down-regulation        | N/A                | [126]      |
| New born infants| LPS                  |                                  | Up-regulation          | Lower              | [127]      |
| P-selectin      | 3–4 weeks rats       | IL-1β                           | Up-regulation          | No difference      | [98]       |
| P1 rats         | Thiglycollate        |                                  | Up-regulation          | Lower              | [94]       |
| E-selectin      | 3–4 weeks rats       | IL-1β                           | Up-regulation          | No difference      | [98]       |
| LFA-1           | New born infants     | IL-1                            | Up-regulation          | Lower              | [75]       |
| MAC-1           | New born infants     | LPS                             | Up-regulation          | Lower              | [127]      |
| VLA-4           | P7 rats              | Microglial activation           | Up-regulation          | N/A                | [128]      |
| ICAM-1          | P7 rats              | HIE                             | Up-regulation          | N/A                | [129]      |
| P2–3 mice       | Pneumocystis carinii |                                  | Trend in increase      | Lower              | [130]      |
| P2–3 mice       | TNF-α                |                                  | Up-regulation          | N/A                | [130]      |
| ICAM-2          | P4–10 mice           | Antigen-specific                | Up-regulation          | N/A                | [131]      |
| VCAM-1          | P2–3 mice            | Pneumocystis carinii            | Trend in increase      | Lower              | [130]      |
| P2–3 mice       | TNF-α                |                                  | Up-regulation          | N/A                | [130]      |
| PECAM-1         | P1 piglets           | HIE                             | Up-regulation          | N/A                | [132]      |
IL-1β and TNF-α are among the best-characterized early response cytokines and are often expressed concurrently\[136\]. Several types of CNS cells secrete IL-1β and TNF-α, including microglia, astrocytes, and neurons, and these cytokines share potent pro-inflammatory actions. Human newborns with HIE have higher levels of IL-1β and TNF-α in peripheral blood samples at P1, P3, and P7 compared to controls, and the IL-1β levels correlate positively with HIE severity\[137\]. The neurotoxic consequences of IL-1 activation have been shown in experimental studies with HIE\[138–140\] and other inflammatory disease models\[141–143\]. The most convincing evidence that IL-1 is functionally detrimental in the pathogenesis of HIE is provided by the neuroprotective potential of IL-1 receptor antagonist administration in HIE models in rodents\[144, 145\] (Table 2).

### Chemokines

Chemokines, or chemoattractant cytokines, also play a pivotal role in cerebral damage in ischemic stroke, HIE and excitotoxic brain injury models\[146\]. Chemokines are classified based on the positions of key cysteine residues (C): C, CC, CXC, and CX3C, and act through specific and shared receptors belonging to the superfamily of G-protein-coupled receptors\[147\]. As their name indicates, chemokins play a central role in leukocyte physiology by controlling inflammatory cell trafficking. HIE modeled in P7 rats induces the up-regulation of alpha-chemokines [growth related gene and macrophage inflammatory protein-2 (MIP-2)] and beta-chemokines (MIP-1α, MIP-1β, CCL-5) preceding the expression of markers for lymphocytes in the infarcted area\[150\]. In the neonatal brain, acute excitotoxic injury stimulates the expression of both monocyte chemotactic protein-1 [MCP-1, also called chemokine ligand 2 (CCL2)] and its receptor CCR2, suggesting that MCP-1 regulates the microglial/monocyte response to acute brain injury and contributes to the pathogenesis of acute neonatal brain injury\[147, 149\]. This has been confirmed by another study using the same model in which anti-MCP-1 antibody attenuated tissue injury in neonatal rats\[150\] (Table 2). Few data are available on the potential role of CXC chemokines in perinatal stroke. In experimental adult stroke models, stromal cell-derived factor 1 (SDF-1 or CXCL12) is expressed perivascularly in the injured region up to 30 d after the injury, suggesting that it could be a therapeutic target for tissue repair strategies\[151\]. However, in P7 mice, stroke induced up-regulation of CXCL12 was only observed up to 7 d after the injury but not at a later time point\[150\], indicating a significantly smaller temporal window for CXCL12-mediated repair after a perinatal stroke.

### Oxidative stress

Oxidative stress has recently been recognized as a common pathway in which different inflammatory cells mediate post-ischemic injury\[150, 160\]. After ischemic insults, the inflammatory cells in the brain are activated and then generate ROS via several enzyme systems to induce the expression of pro-inflammatory mediators including cytokines and adhesion molecules\[160\]. Superoxide is generated via cyclo-oxygenase (COX), xanthine dehydrogenase, xanthine oxidase, and NADPH oxidase, whereas myeloperoxidase (MPO) and monoamine oxidase (MAO) generate hypochlorous acid and \(H_2O_2\)\[121\]. Compared to adult mice, P7 pups show the increased accumulation of \(H_2O_2\) in the brain after a HI injury, suggesting that the neonatal brain may be more damaged even after a milder degree of acute hypoxic-ischemic injury\[161\] (Table 3). Glutathione peroxidase (GPX) is a key enzyme responsible for the degradation of \(H_2O_2\)\[162\]. The neonatal brain has limited

### Table 2. Roles of cytokines and chemokines in HIE.

| Mediators | Investigated objects | Stimulation | Expression after stimulation | Effects on HIE | References |
|-----------|----------------------|-------------|------------------------------|----------------|-----------|
| IL-1α     | P7 rats              | HIE         | Up-regulation                | Detrimental    | [144]     |
| IL-1β     | P7 rats              | HIE         | Up-regulation                | Detrimental    | [144]     |
| TNF-α     | P7 rats              | HIE         | Up-regulation                | Detrimental    | [152]     |
| IL-18     | P9 IL-18<sup>−/−</sup> mice | HIE         | N/A                          | Beneficial in KO | [154]     |
| IL-2      | Children 4.5 years (average age) | Perinatal stroke | Chronic up-regulation | N/A          | [155]     |
| IL-6      | P7 rats              | HIE         | Up-regulation                | Detrimental    | [152]     |
| IL-8      | Children 4.5 years (average age) | Perinatal stroke | Chronic up-regulation | N/A          | [155]     |
| IL-9      | P5 mice              | Ibotenate+IL-9 | N/A                          | Detrimental    | [156]     |
| IL-10     | P5 mice              | Ibotenate+IL-10 | N/A                         | Beneficial     | [157]     |
| IL-4      | P5 mice              | Ibotenate+IL-4 | N/A                          | Beneficial     | [156]     |
| IFN-γ     | P1-3 rats            | IFN-γ treated | N/A                          | Detrimental    | [158]     |
| CCL3/MIP-1α | P7 rats              | HIE         | Up-regulation                | Detrimental    | [35]      |
| CCL4/MIP-1β | P7 rats              | HIE         | Up-regulation                | Detrimental    | [35]      |
| CCL5/RANTES | P7 rats              | HIE         | Up-regulation                | Detrimental    | [35]      |
| CCL2/MCP-1 | P7 rats              | HIE         | Up-regulation                | Detrimental    | [150]     |
| CXCL12/SDF1 | P7 mice              | HIE         | Up-regulation                | Detrimental    | [63]      |

Data compared to the adults are not available.

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GPX activity and is more susceptible to oxidative damage, as described in a study showing that H$_2$O$_2$ rapidly accumulates in human-superoxide dismutase-1 (hSOD1) transgenic P7 mice, thus resulting in exacerbated HI brain injury, which is reversed in hGPX1-Tg mice$^{[163]}$. However, the role of ROS in neonatal inflammatory responses following HIE is controversial. Inhibition of NADPH oxidase, the most important source of ROS$^{[164]}$, increases HI injury and the level of IL-1β in P9 mice$^{[165]}$. In contrast, it has been well established that NADPH oxidase can exacerbate inflammatory responses and stroke outcomes in adult animal models (see review$^{[166]}$). Therefore, the results obtained in adult animals are not completely relevant to newborns and the role of oxidative stress in HIE remains to be fully investigated.

**Fetal inflammatory response syndrome (FIRS)**

Originally defined in fetuses who experienced preterm labor and preterm premature rupture of the membranes (PROM), FIRS is a unique condition characterized by the systemic activation of the fetal innate immune system and by an elevation in fetal plasma IL-6 concentrations$^{[171]}$. Currently, FIRS is characterized by a rapid increase in pro-inflammatory signaling (cytokines, chemokines, etc) and the mobilization of immune effector cells into the fetal circulation$^{[172]}$. These pro-inflammatory mediators readily cross the BBB and induce the activation of microglia, which initiates a detrimental cerebral inflammatory response. The unique circumstances of the “patient” (fetus) and the environment (uterus) in FIRS make it distinguishable from other diseases; however, by definition, FIRS and inflammatory responses after HIE partly overlap in pathophysiology, and they share similar inflammatory mechanisms in the brain. There are multiple putative mechanisms by which the neonatal brain can sense FIRS signals in the systemic circulation, which will then lead to neuroinflammation. These mechanisms include the interface of macrophages in the circumventricular brain area, without a BBB, with circulating inflammatory molecules$^{[173]}$, and the direct access of FIRS signals into the CNS through leakage of the BBB in the setting of peripheral inflammatory pain signaling through the vagal nerve$^{[174]}$. The manner in which FIRS influences the response to HIE and whether HIE can induce FIRS and subsequent peripheral immune activation is an area of active study.

**Summary**

HIE triggers a robust inflammatory response and accumulating data have linked post-ischemic inflammation to the exacerbation of brain damage. Many inflammatory mechanisms and pathways after cerebral ischemia have been assessed in various studies performed in adult subjects; however, caution should be exercised when attempting to extrapolate these findings to neonates. The mechanisms underlying cerebral ischemic injury and the following immune response are likely very different between the neonates and the adults.

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### Table 3. Roles of oxidative stress in HIE.

| Mediators                   | Investigated objects | Stimulation | Expression after stimulation | Effects on HIE | Compared to adults | References |
|-----------------------------|----------------------|-------------|------------------------------|----------------|-------------------|------------|
| H$_2$O$_2$                  | P7 rats              | HIE         | Up-regulation                | Detrimental    | Higher             | [161]      |
|                             | hSOD1-Tg P7 mice     | HIE         | Up-regulation                | Detrimental    | N/A               | [163]      |
| COX-2                       | P7 rats              | HIE+LPS     | Up-regulation                | Correlated     | N/A               | [167]      |
| NO                          | P7 rats              | HIE+LPS     | Up-regulation                | Correlated     | N/A               | [167]      |
|                             | P7 rats              | HIE         | Up-regulation                | Detrimental    | N/A               | [168]      |
|                             | Tyrosine nitration   | P1–3 piglets| HIE+INOS inhibitor           | Down-regulation| Detrimental       | [170]      |
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