Inflammatory Disequilibrium in Stroke

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Abstract: Over the past several decades, there have been substantial advances in our knowledge of the pathophysiology of stroke. Understanding the benefits of timely reperfusion has led to the development of thrombolytic therapy as the cornerstone of current management of ischemic stroke, but there remains much to be learned about mechanisms of neuronal ischemic and reperfusion injury and associated inflammation. For ischemic stroke, novel therapeutic targets have continued to remain elusive. When considering modern molecular biological techniques, advanced translational stroke models, and clinical studies, a consistent pattern emerges, implicating perturbation of the immune equilibrium by stroke in both central nervous system injury and repair responses. Stroke triggers activation of the neuroimmune axis, comprised of multiple cellular constituents of the immune system resident within the parenchyma of the brain, leptomeninges, and vascular beds, as well as through secretion of biological response modifiers and recruitment of immune effector cells. This neuroimmune activation can directly impact the initiation, propagation, and resolution phases of ischemic brain injury. To leverage a potential opportunity to modulate local and systemic immune responses to favorably affect the stroke disease curve, it is necessary to expand our mechanistic understanding of the neuroimmune axis in ischemic stroke. This review explores the frontiers of current knowledge of innate and adaptive immune responses in the brain and how these responses together shape the course of ischemic stroke.  (Circ Res. 2016;119:142-158. DOI: 10.1161/CIRCRESAHA.116.308022.)

Key Words: adaptive immunity ▪ blood–brain barrier ▪ brain ischemia ▪ CD39; CD73 ▪ inflammation ▪ innate immunity

Stroke persists as a major cause of morbidity and mortality in the world today; beyond the human toll, the economic burden is tremendous, with annual costs to the United States estimated to be $34 billion. Over the past several decades, substantial progress has been made in our understanding and the management of patients having stroke. There are 2 main types of stroke: ischemic stroke and intracerebral hemorrhage. Because of the diversity of mechanisms and differing management approaches between the 2 types of stroke, this review will limit discussion to ischemic stroke. In ischemic stroke, tissue-type plasminogen activator (tPA) is the only Food and Drug Administration–approved medicine with proven clinical benefit. tPA works by inducing intravascular thrombolysis, which can restore blood flow to the ischemic brain and salvage dying neurons in the ischemic penumbra. Unfortunately, tPA must be administered with 4.5 hours of symptom onset to realize the clinical benefits.1,2 This narrow therapeutic window remains one of the major limitations of thrombolytic therapies in ischemic stroke. Newer management strategies focused on endovascular approaches to deliver thrombolytics (catheter-based approaches), and thrombectomy have shown promise, but still have limitations, including bleeding complications and limited accessibility to the specialized centers that offer these therapies. Because of these limitations, the search continues for alternative and synergistic therapeutic approaches.

There have been several strategies which have been studied in clinical trials to improve brain salvage and recovery from ischemic stroke, including antioxidant strategies, neuronal...
stroke reperfusion therapies, they also may underlie important biological differences that could mediate the nonvascular contributors to both AMI and ischemic stroke.

The early mechanistic work on ischemic stroke focused on neuron-specific drivers of stroke pathogenesis. Important insights on neuronal apoptosis, excitotoxicity, ionic imbalance, and oxidative stress were the mechanistic pillars that gave rise to thrombolytic therapeutics. As our understanding has advanced, we now appreciate that ischemic stroke not only involves neuronal dysfunction, but also is orchestrated by the complex interplay between many cellular players, including endothelial cells, the blood–brain barrier (BBB), the extracellular matrix, and the immune system. Early clinical observations suggested a link between inflammation and ischemic stroke. More recently, it has been recognized that inflammation not only predisposes to ischemic stroke, but inflammation can directly drive many pathogenic aspects of ischemic stroke. A stronger mechanistic understanding of the immune–brain relationships during ischemic stroke could pave the way for novel immune-modulating therapies that would be synergistic with antiischemic therapies like tPA.

Several excellent reviews have outlined the associations between inflammation and ischemic stroke. In light of recent mechanistic studies and clinical findings, in this review, we build on this mechanistic foundation and explore how the immune system drives aspects of ischemic stroke, including the cellular and molecular mechanisms that orchestrate immune compartmentalization and brain–immune networks. The first part of this review summarizes our current conceptual understanding of the BBB, leukocyte trafficking, and immune responses in the CNS. The second part of this review will build on these conceptual advances and relate the cellular components of each system to local and systemic inflammatory responses and how these responses drive different aspects of ischemic stroke.

### Mechanical and Functional Barriers Underlying CNS Immune Responses: Conceptual Framework

Because of their unique immune surveillance activities, several tissues are considered immune-privileged sites. The traditional view that the CNS is an immune-privileged site was rooted in what later proved to be the false assumption that the CNS lacks immune surveillance and that neuronal homeostasis was not compatible with typical immune cell patrolling. Specifically, mechanistic studies found that the CNS has distinct immune responses compared with other peripheral tissues, including (1) an absence of lymphatic vessels in the brain parenchyma that would allow for egress of immune cells from the CNS; (2) an inability of the CNS glial cells to propagate an effective immune response; and (3) low levels of dendritic cells (DC) which can dampen brain inflammatory responses.

Over the last several years, it has become clear that the distinct homeostatic mechanisms that characterize CNS immune responses and the CNS responses which underlie the inflammatory disequilibrium are common elements of ischemic stroke. The overarching concept of CNS immune privilege is not restricted to simple physical blockade of leukocyte
Table. Translational and Clinical Trials of Immunomodulatory Therapies in Acute Ischemic Stroke

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of Action</th>
<th>Immune Cells or Pathways</th>
<th>Animal Model</th>
<th>Effect on Animals</th>
<th>Clinical Trials</th>
<th>Clinical Results</th>
<th>Comments</th>
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<tr>
<td>Enlimomab</td>
<td>Murine anti-ICAM-1 Ab</td>
<td>EC/PMN</td>
<td>tMCAO rat</td>
<td>↓ infarct volumes and PMNs in cerebral cortex</td>
<td>Phase III, 625 patients</td>
<td>Detrimental day 5, 30, and 90 after stroke: ↑ neurodeficit, infarct volumes, and mortality</td>
<td>Unsafe, murine antibody caused PMN activation via complement-dependent mechanism; side effects fever, and infections</td>
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<td>E-selectin</td>
<td>Mucosal tolerance</td>
<td>EC/Sialyl Lewis A or X on Mono/PMN/T-cells</td>
<td>pMCAO in SHR-SP rats, intranasal rhE-selectin</td>
<td>(a) ↓ infarct volume and TNF-α; ↑ infiltrating T-regs and neuroblast survival</td>
<td>Phase II, 60 patients; nasal spray E-selectin</td>
<td>Phase II, not yet open for participants</td>
<td>(a) Study terminated; (b) Max. safe intranasal dose of rhE-selectin remains to be determined</td>
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<td>rhIL-1ra</td>
<td>IL-1 receptor antagonist</td>
<td>Selective antagonist of proinflammatory cytokines IL-1α/IL-1β</td>
<td>IMCAO Rat, ICV rhIL-1ra</td>
<td>(a) ↓ infarct volume 24 h and 48 h post stroke; ↓ brain cell death.</td>
<td>Phase I, 34 patients;</td>
<td>Acute and long-term protection; ↓ PMN count, C reactive protein and IL-6</td>
<td>Safe, effective, easily crosses BBB</td>
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<td>Phase Iib dose-ranging studies needed</td>
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<td>Natalizumab‡</td>
<td>Humanized mAb against α4 integrin (VLA-4)</td>
<td>VCAM-1, expressed on EC is ligand for VLA-4 on T lymphocytes</td>
<td>IMCAO mouse, prophylactic IP anti-VLA-4(CD49d) mAb</td>
<td>(a) ↓ infarct volume day 7 poststroke;↑ functional recovery; ↓ leukocyte infiltration</td>
<td>Phase II, ACTION Investigators Study, 77 patients</td>
<td>(a) NCT01955707: safe to use; infarct volume unchanged; global clinical gain</td>
<td>(a) Study tested safety of single dose in infarct volume using MRI</td>
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<td>(b) Safety and effect on infarct volume, neurological function and cognition</td>
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<td>(c) Infarct volume and functional outcome unaltered</td>
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<td>Abciximab</td>
<td>Chimeric mouse/human mAb with high affinity for glycoprotein (GPⅡb/Ⅲa)</td>
<td>Platelet GPIb/ GPⅡb-EC-collagen-activation of GPⅡb/Ⅲa-platelet aggregation</td>
<td>tMCAO mouse, prophylactic therapy</td>
<td>Fab fragment against GPⅡb and GPⅣ blockade; ↓ infarct volume; anti-GPⅡb/Ⅲa Ab had no effect</td>
<td>Phase III, AbESTT-II study; 1200 patients treated within 5 h of stroke onset, or 600 treated 6 h post stroke</td>
<td>NCT00073372: trial tested effectiveness of drug, 801 patients analyzed</td>
<td>Trial terminated-symptomatic ICH; Phase Iib proved safety of drug</td>
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<tbody>
<tr>
<td><strong>Minocycline</strong></td>
<td>Semi-synthetic second-generation tetracycline</td>
<td>Additional anti-inflammatory properties and protease inhibition</td>
<td>(a) IMCAO rat, prophylactic and therapeutic IP treatment¹⁹</td>
<td>(a) Both treatments ↓ infarct volume, microglia activation, COX-2 and II-1/J converting enzyme (ICE)</td>
<td>(a) Phase I and II, MINOS study, 60 patients, tested optimal IV dose of drug²⁷</td>
<td>(a) NCT00630396: safe, well-tolerated either alone or with tPA.</td>
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<td>(b) Study tested long-term efficacy of minocycline, but designated as futile and terminated</td>
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<td>(b) pMCAO mouse, prophylactic and therapeutic treatment up to 7 days post stroke¹⁸</td>
<td>(b) ↓ infarct size and neuronal apoptosis</td>
<td>(b) Open-label-phase IV, Acute Stroke Recovery Trial (NeuMAST), 152 patients</td>
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<td>(b) NCT0930020: study terminated</td>
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<td>(a) Fingolimod (FTY720)</td>
<td>Agonist for sphingosine-1 phosphate (S1P) receptors: (S1P1, S1P3, S1P4, S1P5)</td>
<td>Multifaceted phosphate form prevents egress of lymphocytes from the lymph nodes and into the brain</td>
<td>(a) IMCAO mouse, IP treatment at and after reperfusion, 24 h and 48 h poststroke²⁰</td>
<td>(a) ↓ infarct size and brain edema in acute and delayed stroke; ↓ neuro deficit, immune cell infiltration and ICAM-1 expression</td>
<td>(a) Open-labeled, 22 patients with acute and anterior cerebral circulation occlusion, 0.5 mg oral treatment for 3 consecutive days up to day 7²⁰</td>
<td>(a) NCT02002390: compared with tPA alone, combination associated with ↓ infarct size, blood leukocytes and hemorrhage and improved long term (90 day) recovery</td>
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<td>(a) Effective treatment for 72 h after stroke onset; crosses BBB²¹ and directly affects the CNS; pharmacodynamics assessed²²</td>
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<td>(b) Fingolimod+alteplase (tPA)—refer to clinical studies only</td>
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<td>(b) pMCAO mouse, therapeutic IP treatment¹⁹</td>
<td>(b) ↓ infarct size</td>
<td>(b) Phase II multicenter study, 47 patients (25 received tPA alone, 22 received fingolimod+IPA²³</td>
<td>(b) NCT02002390: compared with tPA alone, combination associated with ↓ infarct size, blood leukocytes and hemorrhage and improved long term (90 day) recovery</td>
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<td>(b) Promising drug for combination treatment of ischemic stroke</td>
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<td>(c) IMCAO rat, therapeutic IP treatment¹⁹</td>
<td>(c) ↓ infarct volume when given after reperfusion</td>
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<td>G-CSF (Filgrastim-AX200)</td>
<td>Granulocyte-colony stimulated factor secreted 20-kD hematopoietic molecule</td>
<td>Mobilization and maturation of bone marrow PMN precursors</td>
<td>(a) IMCAO Mouse, SC at the onset of reperfusion²⁴</td>
<td>(a) ↓ infarct volume 48 h postischemia; ↑ functional recovery and cognitive abilities</td>
<td>(a) Phase IIb, multicenter trial-AXIS; 44 patients (30 received AX20 IV in 4 escalating doses, 14 placebo)²⁵</td>
<td>(a) NCT00132470: results reported by SYGNIS’ AXIS study; safe to use and particularly efficient in patients with severe stroke</td>
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<td>(b) No long-term improvement</td>
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<td>(b) pMCAO Mouse, rh-CSF+ rm SCF injected for 20 consecutive days; 1–10 days: acute phase; 11–20 days: subacute phase²⁶</td>
<td>(b) ↓ infarct size with treatment in acute and subacute phase of ischemia; ↑ motor, higher brain function functions and tissue repair</td>
<td>(b) Phase II, AXIS-2; 328 patients, IV given over 72 h</td>
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<td>(b) NCT00927836: “G-CSF treatment failed to meet the primary and secondary end points of the trial”²⁷</td>
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trafficking, as was previously thought, but is rather a complex regulatory process comprised of both compartmentalization and active leukocyte trafficking mechanisms. Specifically, it is now recognized that CNS leukocytes perform continuous immunosurveillance under noninflammatory and homeostatic conditions and regularly traffic through the cerebrospinal...
fluid (CSF) and within subarachnoid space, whereas only few cells enter the neuropil. In addition, classical lymphatic vessels were discovered in the CNS meninges, providing a mechanism by which immune cells can drain from the brain interstitial fluid directly with the peripheral immune system.

Under inflammatory conditions, including those associated with stroke, the mechanisms that govern normal CNS immune surveillance are perturbed. CNS leukocyte trafficking can increase under these inflammatory conditions, leading to leukocyte penetration into the brain parenchyma through multiple barriers, including (1) blood to the subarachnoid space via leptomeningeal vessels; (2) blood to the parenchymal perivascular space through the neurovascular unit (NVU), with tight junctions forming the so-called BBB; (3) blood to the CSF, which bathes the brain and spinal cord, via the choroid plexus; and (4) blood to the CSF via meningeal spaces and ependymal cell layers, which line the cerebral ventricles. Only recently has it been suggested that these CNS barrier systems can actively control immune cell trafficking to immune-privileged organs, such as the CNS, through dynamic interactions between endothelial- and epithelial-selective gates. In the case of the CNS, the endothelial BBB is considered to be a true immunologic barrier, which in steady state blocks parenchymal leukocyte entry. In contrast, the blood to the CSF and choroid plexus epithelial gates represent permissive gates, enabling selective immune cell trafficking and skewing immune cells toward specific effector responses. This concept is based on the fact that these barriers are distinguishable by their (1) anatomic location because the BBB is positioned in deep brain parenchyma, whereas the blood to the CSF lines peripheral borders of brain tissue; (2) junctional formations–endothelial tight junctions versus epithelial intracellular gaps that lack tight junctions; and (3) immune-skewing capacities—an absolute immunologic endothelial barrier versus epithelial educational immunomodulatory gate. The meningeal microvessels, which separate the leptomeningeal space from the circulation, traditionally have been considered as part of the BBB, but are now defined as a discrete barrier with selective proinflammatory properties. These conceptual advances have important implications into understanding the immune mechanisms that predispose to the inflammatory disequilibrium which characterizes ischemic stroke, as detailed in subsequent sections of this review.

**Blood–Brain Barrier: Immunologic Barrier and Dysfunction With Stroke**

The BBB relies on continuous interactions with its surrounding extracellular matrix and other cellular elements; this network of molecular and cellular players now is commonly referred to as the NVU. The NVU comprises highly specialized brain endothelial cells (BECs), which are supported by an underlying basement membrane embedding a large number of pericytes, covered by the layer of astrocytic endfeet, neuronal processes, and extracellular matrix ensconcing the brain microvessels. The NVU itself has evolved in such a manner that its unique endothelial cells inhibit transcellular passage of large molecules, maintaining the BBB as a strong mechanical barrier of the CNS. BECs are tightly interconnected through specific proteins present in the form of tight junctions and adherent junctions. This complex network of tight junctional proteins functions to seal the interendothelial space, thereby restricting paracellular diffusion of hydrophilic molecules and immune cells. Perhaps one of the most unique features of the BBB is that it is exquisitely sensitive to signals from the local microenvironment and itself can activate intracellular signaling pathways by engaging signaling proteins or by capturing transcription factors at the plasma membrane of BECs. Several other features of the BBB support its strong barrier nature, which restricts leukocyte migration under normal conditions. These include endothelial expression of interleukin 25 (IL-25), which increases the expression of tight junction proteins, thereby preventing cytokine-induced BBB breakdown or expression of CXC-chemokine ligand 12 (CXCL12), which prevents CXC-chemokine receptor 4–dependent parenchymal leukocyte entry. In addition, the endothelium of the BBB under quiescent conditions expresses a paucity of selectins, resulting in an inability of T cells to migrate through typical mechanisms.

Under inflammatory conditions, such as stroke, the BBB loses parts of its leukocyte-mediated barrier properties, driving endothelial cell induction of selectins and integrins, and secretion of proinflammatory mediators, such as tumor necrosis factor-α (TNF-α), IL-1β, IL-6, monocyte chemotactant protein-1, cytokine-induced neutrophil chemoattractant protein-1, and prostaglandins. Under ischemic and hypoxic conditions, the elaboration of reactive oxygen metabolites causes swelling and detachment of BECs, leading to compromises in barrier function, increased protein extravasation, and interstitial edema. This cascade of events primarily affects postcapillary segments of the cerebral microvasculature, where leukocytes adhere to swollen endothelium causing a deleterious cycle of hypoxia and hypoperfusion. Perivascular cell activation initiates a vicious cycle of inflammatory activation, including the release of cytokines that additionally promote upregulation of adhesion molecules on both BECs and leukocytes. Because of the increased expression of leukocyte adhesion molecules and increased local production of reactive oxygen intermediates, peroxidation of cell membrane components further contributes to increased vascular permeability and vasogenic edema.

In ischemic stroke, not only are inflammatory pathways activated, but these contribute to friability of cerebral vasculature and a coagulation disequilibrium characterized by initial microvascular thrombosis and later by intracerebral hemorrhage. The final common pathway leading to hemorrhage includes activation of proteases, which are normally found in their latent forms in the CSF and in astrocytes. Yang and Rosenberg found that proteases, specifically matrix metalloproteinases (MMPs), participate in the biphasic opening of the BBB during ischemia–reperfusion injury. The initial phase is mediated by hypoxia-inducible factor 1α–gelatinase A (MMP-2) activation and interaction with tissue inhibitor of metalloproteinases-2 and membrane-type 1 MMP. The later phase of stroke (24–48 hours) is regulated by stromelysin-1 (MMP-3) and gelatinase B (MMP-9), which can also be activated during CNS injury. These proteases, through numerous extracellular matrix substrates, can lead to BBB dysfunction and hemorrhage during the
course of ischemic stroke. These MMPs are not only synthesized locally by brain parenchymal cells, but are also liberated from leukocytes. The biggest source of leukocyte MMPs are neutrophils, which releases activated MMP-9 on trafficking through the BBB in inflamed brain tissue. Interestingly, in early phases of ischemic stroke, molecules, such as tPA, migrate from the circulation to the brain, where it can also activate local cytokine release and further MMP activation. In addition to proteases, other proinflammatory mediators contribute to the inflammatory disequilibrium during ischemic stroke, including cyclo-oxygenase-1 and -2. Cyclo-oxygenase-1 and -2 have important but distinct roles in ischemic stroke, highlighting the importance of mechanistic preclinical models to inform the development of effective therapeutics. In addition, it has been shown that aquaporins, the ubiquitous pore-forming molecules which facilitate transport of water through the BBB, could be causally responsible for developing vasoactive edema after ischemia. For example, aquaporin-4 deletion reduces infarct sizes and reduces vasoactive edema in models where cytotoxic edema is major component of the pathobiology. However, under severe injuries, when vasoactive edema is significant, aquaporin-4 deletion exacerbates brain edema, as part of its passive water passage mechanism, which allows water to follow pressure gradients which under equilibrium conditions serves to remove extracellular fluid. 

Activated endothelial cells express adhesion molecules that provide a molecular platform for leukocyte ingress into inflamed brain tissue. The molecular mechanisms underlying leukocyte chemotaxis in the brain begins with leukocyte rolling through a tethering mechanism dependent on endothelial selectins and their counterpart sialylated glycoproteins on the leukocyte surface. During leukocyte rolling, leukocytes become activated, leading to morphological changes that result in a transition from a low to high avidity state. Activated high-avidity integrins interact with their corresponding endothelial ligands, including vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and activated leukocyte cell adhesion molecule. The importance of these pathways is highlighted by studies showing that P-selectin, E-selectin, and intercellular adhesion molecule-1–deficient mice are relatively resistant to cerebral damage after ischemia and reperfusion, confirming the role of leukocyte trafficking and endothelial activation in the pathobiology of stroke.

## Innate Immunity and Stroke

After ischemic CNS injury, local inflammatory responses occur through activation of innate immune responses. Specifically, CNS injury first triggers activation of microglia, the native resident brain macrophages, neutrophils, DCs, and an additional population of monocyte-derived macrophages, which traffic from the bone marrow to the CNS after ischemic insults. Until recently, infiltration of immune cells after CNS injury has been viewed as detrimental and a negative consequence of dysfunction of the BBB. However, recent investigations have revealed a much more nuanced appreciation of innate immune responses to CNS injury.

### Innate Immunity: Complement Activation

As a primordial and highly conserved defense mechanism, complement is activated to defend against certain pathogens which express on their surface repeating molecular patterns, the pathogen-associated molecular patterns. In ischemic stroke, damaged cells and recruited cells express other repeating patterns, damage-associated molecular patterns, which are recognized by this same primitive defense mechanism. There has been a large set of data accumulated over the years, suggesting that activation of complement in the setting of ischemic stroke contributes to the ongoing inflammatory disequilibrium and cerebral tissue damage. The classical complement pathway is triggered by initial deposition on damaged cell membranes of the complement component C1q, which clusters as a bouquet of roses which triggers a proteolytic cascade. This results in release of anaphylotoxins (C3a and C5a), which promote leukosequestration in the ischemic brain, as well as deposition of the membrane attack complex (complement components C5b-9) on damaged cells but also innocent bystander cells in the ischemic penumbra. At least in animal models, interfering selectively with specific complement components can reduce damage in the setting of frank cerebral ischemia triggered by arterial blockage or in neonatal models of hypoxic-ischemic cerebral injury.
Innate Immunity: Platelet-Mediated Immune Activation

Although it is well appreciated that platelets mediate thrombotic and coagulation complications associated with vascular disease, it is also clear that platelet activation can direct drive local and systemic inflammatory responses. The importance of platelet-mediated inflammatory effects in ischemic stroke is further supported by evidence that many clinically proven antiplatelet therapeutics have anti-inflammatory effects. Platelets are one of the first cell types to arrive at injured vasculature. Platelet accumulation in the vasculature of ischemic mouse brain has been associated with endothelial and immune cell activation through the liberation of inflammatory mediators, such as IL-1α; platelet IL-1α can trigger endothelial upregulation of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 and the release of chemokines, which amplify immune cell recruitment during ischemic brain injury.

Innate Immunity: Role of Microglia and Macrophages

Yolk sac–derived microglia are one of the main CNS innate immune cells. These unique cells arise early in development at the time of definitive hematopoiesis. Because of this unique developmental origin, microglia are maintained independently of circulating monocytes and are typically replenished by local proliferation from CNS precursors. As integral elements of the innate arm of the immune system within the CNS, microglia respond quickly to danger signals and act as a first line of defense against external pathogens, as well as being positioned to clear dying cells and cellular debris. They do so by recognizing damage-associated molecular patterns, either released by or expressed on the surface of damaged cells after ischemic brain insult. After initial exposure to these danger signals, microglia become activated and upregulate surface-specific molecules such as CD11b, Iba-1, CD40, CD80/86, and major histocompatibility complex class II, which are important to transact their functional responses to CNS injury. After CNS injury, microglia respond with a unique transcriptional program that is context-dependent and distinct from that of macrophages. The activation of microglia after CNS infarction can have both beneficial as well as harmful aspects. Their acute activation after a CNS ischemic insult can trigger local inflammatory responses that eventually become deleterious. After ischemic brain injury, or similar insults such as cerebral hypoxia and hypoperfusion, deleterious events transpire, such as oxidative stress, excitotoxicity, BBB dysfunction, microvascular injury, and posts ischemic inflammation. This inflammatory maelstrom exacerbates the ever-changing ischemic cerebral environment, which further alters the behavior of microglia and macrophages. Although acutely these changes can exacerbate injury, at later points, these set in motion processes which promote tissue repair.

In the inflamed CNS, microglia and macrophages share some common, but also some distinct features. Common characteristics include the expression of phenotypic markers, phagocytic behavior, and extraordinary plasticity necessary to respond to a diverse array of inflammatory inputs. However, recent studies have revealed distinct transcriptional mechanisms that control these unique functional responses. Development of microglia is dependent on the Csf-1-receptor, whereas macrophages require a distinct set of transcription factors, Myb and FLT3. The unique transcription factor signature of these innate immune cells leads to divergent transcriptional outputs. Genes highly and uniquely expressed by microglia include Cx3cr1-fractalkine receptor, MerTk, FCRLS, P2ry12, Gas6, and others, which are not expressed on CD11b-gated Ly6m myeloid monocyte/macrophages isolated from peripheral blood, spleen, or peritoneum. It is possible that the differential time course of recruitment to sites of CNS injury (microglia are first responders, macrophages later) may dictate phenotypic differences. Along this line of reasoning, the phenotype of monocytes–macrophages recruited in the later stages of an ongoing inflammatory response may depend partially on their route of trafficking to the injured CNS. Once in place, these cells work in concert with resident reactive microglia to remove debris and initiate tissue repair (Figure 1).

There are other differences between microglia and monocytes/macrophages. In microglia, hypoxia triggers hypoxia-inducible factor 1α–dependent autophagic cell death, with attendant release of proinflammatory cytokines IL-8 and TNF-α. In contrast, macrophages have evolved to function in hypoxic environments by switching to anaerobic metabolism. Thus, when hypoxia persists beyond the tolerance threshold of microglia, such as occurs in prolonged CNS ischemia, there can be irreversible microglial death in the ischemic core.

Blood-derived monocytes that are recruited to tissues on injury are typically divided into 2 subsets based on their expression of specific surface molecules: the first subset, CCR2–CX3CR1–Ly6C+ monocytes, are first recruited in response to ischemic injury and exhibit a proinflammatory phenotype, corresponding to classically activated M1 macrophages; the second subset, CCR2–CX3CR1+Ly6C– monocytes, correspond to the alternatively activated M2 macrophages. These M2 macrophages are instrumental in

![Figure 1. Cellular composition of a mouse brain 48 hours after middle cerebral artery occlusion. The nonischemic (contralateral) and ischemic (ipsilateral) hemispheres were digested, cells isolated, and characterized by flow cytometry using discrete surface markers to identify the cell type.](http://circres.ahajournals.org/doi/abs/10.1161/CIRCRESAHA.112.248618)
immune resolution and repair.\textsuperscript{101,102} According to our current framework, blood-derived monocytes are recruited to the CNS in response to ischemic injury, and the multiple waves are responsible for the initial, inflammatory response and the secondary reparative response that is required to clear debris, promote angiogenesis, and facilitate tissue healing. Because the CNS is an immune-privileged site, on its injury, the spontaneous ingress of beneficial macrophages may be insufficient to promote proper repair and heal the injured brain. We and others have found that myeloid-specific abnormalities in chemotaxis can hamper this reparative phase and in the context of ischemic stroke can lead to larger infarcts and worsen stroke outcomes.\textsuperscript{103} Thus, these studies demonstrate that inadequate tissue repair and resolution of CNS inflammation can be deleterious during ischemic stroke.

**Innate Immunity: The Role of Neutrophils**

One hallmark of CNS ischemic injury is the early infiltration of neutrophils.\textsuperscript{104} Clinical studies have demonstrated a massive early influx of neutrophils after ischemic stroke, which correlates with severity of the injury.\textsuperscript{105} Like other innate immune cells, neutrophils respond to damage-associated molecular patterns and pathogen-associated molecular patterns through toll-like receptors and, on activation by inflammatory mediators like TNF-α and interferon-γ, upregulate CD15 and CD11b adhesion receptors, which promote their adherence to endothelial cells and their migration into inflamed tissues.\textsuperscript{106} In response to CNS injury, neutrophils are first recruited from the bone marrow, through its receptor CXC-chemokine receptor 2, into the blood.\textsuperscript{107} From the blood they are recruited further into the brain tissue, attracted via specific interactions with chemokines such as CXCL1 and CXCL2/3.\textsuperscript{108} Studies that have deployed antibody (anti-Ly6G)-mediated neutrophil depletion or neutrophil-specific chemokine receptor (such as CXC-chemokine receptor 2) blockade has reduced ischemic brain injury.\textsuperscript{109} Once recruited, neutrophils can potentiate CNS injury by secreting inflammatory mediators, releasing lytic enzymes, and triggering cerebral capillary sludging.\textsuperscript{110} There may also be an important role for scavenger receptors, such as CD36, in neutrophil accumulation after ischemic CNS injury.\textsuperscript{111}

Neutrophil modulation of CNS ischemic responses can occur as early as 15 to 60 minutes after reperfusion.\textsuperscript{112} Early studies found that at these time points, neutrophils can become entrapped in cerebral microvessels, causing vascular sludging and microvascular hypoperfusion, including no-reflow in downstream tissues.\textsuperscript{112} Some controversy exists as to how much this phenomenon contributes to cerebral dysfunction. Some investigators think that because the cerebral capillaries (9 μM) are larger than heart capillaries (5 μM), this is less of a driver of CNS reperfusion injury.\textsuperscript{113} Further studies are needed to more clearly define the contribution on neutrophil sludging to ischemic stroke in both preclinical models and clinical populations, but the emerging evidence suggests that this phenomenon is multifactorial.\textsuperscript{114,115} Another novel neutrophil mechanism that could exacerbate ischemic stroke is intravascular neutrophil extracellular trap formation, caused by the expunging by neutrophils of intracellular contents (particularly sticky DNA that can entrap endogenous and exogenous structures, including bacteria). Some recent studies have shown that prolonged ischemia can elicit neutrophil extracellular trap formation, secondary microthrombosis, and additional brain tissue damage.\textsuperscript{116}

Numerous preclinical studies, including our own, have demonstrated improved neurological outcomes in stroke by reducing neutrophil infiltration through inhibition of adhesion receptors.\textsuperscript{117,118} However, targeted blockade of a specific leukocyte adhesion receptor was not promising in treatment of human stroke.\textsuperscript{119} It was assumed that the influx of inflammatory cells into the brain tissue after the stroke is facilitated by reperfusion and limited in the absence of reperfusion. However, new data, using permanent middle cerebral artery (MCA) occlusion in mice, have shown marked leukocyte infiltration as early as 3 hours post ischemia that is maintained for at least 24 hours.\textsuperscript{120} Among infiltrating cells, neutrophils were the most prevalent cell population (>50%) during the first 24 hours after the stroke. Comprehensive analysis of whole ischemic hemispheres, using flow cytometry, have revealed that neutrophils are 3-fold more numerous in permanent compared with 1 or 2 hours of transient MCA, suggesting that ongoing hypoxia in the infarct core is a major stimulus for infiltration of immune cells and does not absolutely depend of blood perfusion being restored.\textsuperscript{120} Although it remains still controversial as to whether neutrophil infiltration causes additional brain damage and can be effectively blocked in animals as well as in humans, studies have shown that, for instance, neutrophil depletion and intercellular adhesion molecule-1–deficient mice are resistant to brain ischemia–reperfusion injury.\textsuperscript{71}

**Innate Immunity: Role of DCs**

Under nonstress conditions, the brain lacks DCs or any functional counterparts that mediate antigen uptake and antigen presentation. This feature of the brain is one of the primary reasons that the CNS maintains its unique immune privileged characteristics.\textsuperscript{42} As a known link between innate and adaptive immunity, DCs are key cellular components of many immune responses; however, how DCs modulate CNS immune responses during ischemic stroke has not yet been fully elucidated.\textsuperscript{121} In clinical studies, numbers of circulating DCs are inversely correlated with clinical stage and ischemic infarct size.\textsuperscript{121} In agreement with the clinical studies, murine stroke models have also shown a strong correlation between brain parenchymal DCs and infarct volumes.\textsuperscript{122} Using a rat permanent MCA occlusion model, Kostulas et al were among the first to demonstrate the presence of DCs in the inflamed brain parenchyma, detectable as early as 1 hour after the initial ischemic insult.\textsuperscript{122} Using flow cytometry, Gelderblom et al have shown that DCs comprise a large portion of all infiltrating immune cells.\textsuperscript{123} Importantly, many studies suggest that DC amplification after cerebral ischemia exacerbates stroke outcomes.\textsuperscript{121,124} In mice, migration of DCs after transient MCA occlusion was found to be mediated by granulocyte-colony stimulating factor; in studies in which granulocyte-colony stimulating factor was suppressed, cerebral infarct volumes and inflammation were attenuated.\textsuperscript{124} Finally, murine studies using CD11c-GFP transgenic mice have provided further evidence of DC involvement in ischemic stroke.\textsuperscript{125} Though the exact mechanism through which DCs contribute to poorer...
stroke outcomes remains unknown, at least 2 possibilities are plausible. DCs present in the infarct zone could stimulate and activate T cells, induce a long-lasting immune response, and worsen stroke outcome. In addition, a transient decrease of DCs in the circulation might contribute to stroke-induced immunodepression.121

**Innate Immunity: The Roles of Mast Cells and Astrocytes**

The mast cell represents another cell of myeloid lineage, which is defined by the expression of c-kit and FcεRI, and is typically associated with allergic responses,126,127 Although mast cells primarily reside in organs exposed to the external environment (such as gut, lungs, and skin), they also are found in the brain, spinal cord meninges, and perivascular spaces.127,128 After brain ischemia, these cells release vasoactive and inflammatory mediators, such as histamine, proteases, TNF-α, and IL1-β, which can contribute to several aspects of the inflammatory disequilibrium after stroke, including vasoactive edema and tissue injury.129 Interestingly, mast cells are found in dura and pia mater, where they are involved in TNF-α secretion, BBB permeability regulation, and T cells and myeloid cells infiltration into the CNS.128 Moreover, studies of Mattila et al revealed that after transient cerebral ischemia, CNS mast cells secrete gelatinase-positive granules that can activate the cerebral microvasculature and cause BBB disruption.130 In addition, a recent study demonstrated that meningeal mast cells can also secrete IL-6 and participate in the CNS inflammatory and injury response after cerebral ischemia and reperfusion.131

The CNS has abundant resident cells of neuroepithelial origin that are categorized collectively as neuroglia. Under this umbrella designation, glial cell subtypes include astrocytes, oligodendrocytes, and polydendrocytes. Recent studies have demonstrated that glial cells are not simply passive support cells, but rather, they actively interact with and signal neurons and other CNS cells under both normal conditions and stress conditions like ischemic stroke.59 They are essential elements of the NVU, which contains endothelium, neurons, astrocytic endfeet, and even pericytes. The most numerous glial cell types in the CNS are astrocytes,132 which populate both white matter (such as the corpus callosum) and gray matter (such as cortex) and are best characterized as innate immune neuroglia. Astrocytes, as sentinels of the innate neuroimmune axis, are involved in modulating synaptic activity, regulating water homeostasis, and removing toxic metabolites.133 Additionally, the local endogenous fibrinolytic milieu can be regulated by the release of plasminogen activator inhibitor-1 from astrocytes, as well as the production of tPA by neurons.134 After CNS ischemia, astrocytes undergo numerous changes, including rapid swelling, enhanced Ca2+ signaling, and the increased expression of glial fibrillary acidic protein (a hallmark of all of reactive astrogliosis).135-137 After prolonged activation, reactive astrocytes will form a glial scar that denotes the demarcation zone between ischemic core and healthy surrounding brain tissue.135 In addition to these morphological alterations, astrocytes actively perpetuate immune response after CNS ischemia by producing inflammatory mediators (IL-6, IL-1β), complement components, and chemokines (CXCL12, CXCL1, CXCL10, and monocyte chemoattractant protein-1).138,139 Recent work sheds some light on the coordinated interplay between microglia and astrocytes, which may ultimately reveal potential therapeutic targets in to rescue the brain from stroke and neuroinflammation.140

**Adaptive Immunity and Stroke**

Ischemic CNS damage not only activates innate immune responses, but it also exposes latent CNS danger molecules that are typically secluded by the BBB and other mechanisms. These molecules can serve as antigenic substrates for the adaptive immune system. Unmasking of antigens in the CNS can lead to the development of cellular and humoral immune responses against the brain, responses which are a hallmark of autoimmunity. Additionally, there is growing evidence that acute ischemic injury to the brain can lead to immunosuppression. In murine ischemic stroke models, for instance, there is an apparent increase in the development of spontaneous bacterial infections within 24 hours.141 These infections are preceded by an acute suppression of peripheral adaptive immune responses, mainly characterized by lymphocyte dysfunction. These preclinical studies are supported by clinical associations between stroke-induced immunosuppression and postischemic infectious complications.142,143

With respect to autoimmune encephalitis, multiple sclerosis, and other inflammatory CNS diseases, antigen-specific adaptive immune responses are key drivers. In ischemic stroke, the role of adaptive immune responses is more ill-defined, and conclusive data are lacking; however, emerging evidence does implicate activation of the adaptive immune system as a mediator of particular phases of ischemic stroke.144,145 The major cellular modulators of adaptive immunity are T and B lymphocytes. These leukocyte subsets work in concert with other innate immune responses, forming the adaptive arm of the immune system responsible for antigen-specific responses as well as immunologic memory.146 Recent studies have found that patients with stroke have higher serum antibody titers147 and a higher number of circulating T cells compared with healthy controls, implicating adaptive immune responses functioning during ischemic stroke.148 Other reports document increases of myelin and neuronal antigens in secondary lymphoid organs of patients with stroke, consistent with the development of autoimmune responses.148 The ongoing T-cell immune response in these patients was associated with improved or impaired stroke outcome depending on the relative predominance of neuronal or myelin epitopes exposed.148 These and other studies implicate T-cell adaptive immune responses in ischemic stroke. Nevertheless, there remains controversy as to whether these antigen-specific immune responses are pathological in or simply markers for ischemic stroke.149

Ischemic CNS injury leads to lymphocytic infiltration, which may be directly contributory to smoldering brain injury after stroke onset.150,151 Interestingly, lymphocyte-deficient mice have been shown to be protected from stroke.152 This CNS protection has been attributed specifically to deficiencies in T-cell responses because B-cell–deficient mice and those reconstituted with B cells remain protected from ischemic CNS injury.152 A role for B cells may, therefore, be
more indirect because B-cell activation leads to secretion of antibodies against specific CNS self-epitopes, whereas T-cell activation leads to either a destructive autoimmune or tolerogenic response.153 Additionally, some studies have found that activated B cells accumulate in the weeks after ischemic stroke and can influence cognitive function and recovery.154 We and others have shown that both T cells and antigen-presenting cells are increased in murine models of ischemic stroke.97,123 The antigen-presenting cells present are associated with expression of major histocompatibility complex class II molecules and costimulatory molecules, such as CD80 antigen, suggesting that antigen presentation and T-cell responses modulate aspects of ischemic stroke (Figure 2).97,123 Recent work has further elucidated additional mechanisms through which T lymphocytes contribute to ischemic brain injury.151 Studies have found that in patients with stroke, the number of circulating γδT lymphocytes is decreased, but circulating levels of IL-17A are elevated.155 Shichita et al found that in the late phases of brain ischemia, γδT lymphocytes infiltrate into the CNS and, through IL-23-driven interactions with macrophages, may worsen stroke outcomes.156 It seems likely that γδT lymphocytes contribute to brain injury by secreting the proinflammatory cytokine IL-17.156

In addition to innate γδT lymphocytes, other T-cell populations have been implicated in the pathobiology of ischemic stroke.155 Studies in a murine MCA occlusion model have shown that CD8+ T lymphocytes are recruited as early as 3 hours after stroke onset. This CD8+ T-cell recruitment is also accompanied by the ingress of CD4+ T cells and NK T lymphocytes.120 Although the majority of studies suggest that T-cell populations are deleterious to ischemic stroke outcomes, several studies suggest a more nuanced interpretation of their role.157,158 Most studies have found that within the first 24 hours of ischemic injury, antigen-independent T cells exert a deleterious effect; this is in contrast to antigen-dependent T-cell responses, which peak 3 to 7 days after the ischemic insult.159 CD4+ T cells are also likely to have deleterious effects through the secretion of inflammatory cytokines, including interferon-γ and IL-21.159,160 In contrast, CD8+ T cells, through the perforin-granzyme pathway, can cause neuronal cell death and may worsen stroke outcomes.159

Interestingly, not all T-cell populations are deleterious after CNS ischemia. Forkhead box P3 (Foxp3)+ regulatory T cells (T-regs) seem to be neuroprotective after ischemic CNS injury.161 T-regs participate to maintain peripheral immune tolerance and keep autoimmune responses in check.161,162 Recently, Xie et al demonstrated that normal rat brains contain T-regs which suppress T effector cell responses, highlighting their key role in sustaining inflammatory equilibrium within the CNS.163 Extending these observations, it was found that during cerebral ischemia, there is a substantial accumulation of T-regs and that adoptive transfer of T-regs actually limited infarct size.161,164 In mice depleted of T-regs, intracerebroventricular administration of IL-10 concurrent with CNS ischemia reduced infarct volume at day 7.161 Interestingly, adoptive transfer of T-regs into lymphocyte-deficient mice also reduced infarct volume, but not if the T-regs were obtained from IL-10–deficient mice.164 These results suggest that in ischemic stroke, T-regs work in tandem with IL-10 to consummate neuroprotection. Interestingly, some recent studies have suggested that T-regs could be detrimental in the early phases of ischemic stroke through immune modulation and microvascular dysfunction.165,166 Further highlighting the complexity that underlies adaptive immunity and ischemic stroke, a recent study by Benakis et al demonstrated that alterations in the microbiome can alter γδT lymphocytes and T-reg functionality, affecting CNS ischemic injury responses.167 Altogether, the evidence suggests that adaptive immune responses provide for a delicate balance between CNS homeostasis and inflammatory disequilibrium after stroke. Further studies are needed to better define the antigen-dependent and antigen-independent adaptive immune responses and the disequilibrium that can occur between hypo- and hyperactive adaptive immune responses associated with ischemic CNS injury (Figure 3).

Conclusions and Future Perspectives
Ischemic stroke disrupts the delicate equilibrium that exits under quiescent conditions between coagulation and immune axes in the brain. Ongoing research continues to reveal a critical nexus between various immune mediators of ischemic stroke. Innate and adaptive immune responses triggered by ischemic stroke is fueled by local immune-activated cells, an influx of immune cells recruited from or transiting in through other brain compartments, such as the leptomeninges and choroid plexus, and those immune cells recruited from the bone marrow.154,168 Although there are many similarities between the cerebral and other vascular beds, the cerebral circulation has unique features, which warrant special consideration particularly after ischemic insult. The microvasculature of the brain is comprised of a tight apposition of cells in NVUs, which creates the BBB; the brain has an alternative circulation through leptomeninges and choroid plexus through which leukocytes can transit, and there is a rich and unique population of immune scavenger cells (microgliosis), which participate
in immune surveillance, debris removal, and tissue repair. The data provided in this review highlight the important role of inflammation also in amplifying and propagating neuronal damage after ischemic CNS injury. Although the inflammatory response is initially beneficial, serving to limit and resolve ischemic stress, unrestrained inflammatory CNS responses can impart significant damage to penumbral tissue after brain ischemic injury. Both the innate and adaptive arms of the immune system contribute to distinct aspects of the pathobiology of ischemic stroke. What is clear from recent studies is that the cellular mediators of immune responses often are both beneficial and detrimental depending on the phase of ischemic stroke and the microenvironment signals. This mechanistic understanding in parallel with exciting advances in our understanding of the BBB have the potential to transform our understanding of ischemic CNS injury and how this injury shapes local and systemic immune responses.

Although this mechanistic is foundational for developing novel therapeutic strategies for ischemic stroke, to truly realize the potential of novel therapeutics, it will be critical to validate preclinical findings in patient populations. Preclinical models of ischemic stroke have provided valuable insights but cannot...
recapitulate all features of human stroke. Preclinical models of ischemic stroke can be affected by variables as disparate as anesthesia and the surgical trauma associated with the model. Additionally, the murine immune system has important differences compared with the human immune system, including a preponderance of lymphocytes in rodents versus neutrophils in humans, and differential expression of key immune mediators, including toll-like receptors-2, costimulatory molecules, and chemokines. Going forward, it will be important to link preclinical stroke phenotypes to molecular signatures and cellular profiles, both local and systemic, and to validate these in clinical patient populations. In this way, the clinical arena will inform the mechanistic preclinical studies in parallel with the preclinical studies illuminating surrogate end points or biomarkers that could be linked to short- and long-term clinical outcomes.

As we learn more from clinical trials involving biologics and other small molecules in other immune/inflammatory diseases, we will be poised to combine our unique understanding of the architecture, cells, and molecular signatures in the brain to develop novel therapeutics to reverse the inflammatory disequilibrium and improve clinical outcomes in patients with ischemic stroke.

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None.

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