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Inflammatory Responses in Brain Ischemia

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Abstract

Brain infarction causes tissue death by ischemia due to occlusion of the cerebral vessels and recent work has shown that post stroke inflammation contributes significantly to the development of ischemic pathology. Because secondary damage by brain inflammation may have a longer therapeutic time window compared to the rescue of primary damage following arterial occlusion, controlling inflammation would be an obvious therapeutic target. A substantial amount of experimentall progress in this area has been made in recent years. However, it is difficult to elucidate the precise mechanisms of the inflammatory responses following ischemic stroke because inflammation is a complex series of interactions between inflammatory cells and molecules, all of which could be either detrimental or beneficial. We review recent advances in neuroinflammation and the modulation of inflammatory signaling pathways in brain ischemia. Potential targets for treatment of ischemic stroke will also be covered. The roles of the immune system and brain damage versus repair will help to clarify how immune modulation may treat stroke.

Keywords

Brain ischemia; inflammation; neuroprotection; stroke

1. INTRODUCTION

Stroke encompasses conditions caused by occlusion and/or rupture of the brain's blood vessels. It is a leading cause of death and disability worldwide with significant clinical and socioeconomic impact. Ischemic stroke represent the majority seen in industrialized nations [1]. Besides its high incidence, approved effective therapies are limited. There are many mechanisms involved in the pathogenesis of stroke, but recent work has focused on the duality of post stroke inflammation. That is, inflammation contributes both negatively and positively to neurological outcome [2–4]. Brain inflammation leads to secondary injury following stroke (Fig. 1). Brain ischemia triggers inflammation as a response necrotic cells

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CONFLICT OF INTEREST

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followed by the generation of reactive oxygen species (ROS), although many other factors have yet to be identified. Once activated, these initiators of inflammation lead to activation of microglia, the brain's resident immune cell. Microglia then generate more pro-inflammatory cytokines which in turn leads to adhesion molecules induction in the cerebral vasculature [5–7]. These events have been documented to occur within the first 24 h of stroke onset. Chemokine upregulation leads to chemotaxis of circulating immune cells into ischemic brain especially around the penumbra. Adhesion molecules mediate leukocyte adhesion to vascular endothelia leading to microvascular occlusion and leukocyte entry into the ischemic brain parenchyma [8, 9]. Inflammatory cells release several cytotoxic agents such as matrix metalloproteinases (MMPs), nitric oxide (NO) and also are also an independent source of ROS. These molecules exacerbate brain cell damage, and lead to disruption of the extracellular matrix and blood-brain barrier (BBB) [10]. This disruption permits blood and other potentially neurotoxic serum substances to enter the Brain Res.ulting in brain edema and hemorrhagic transformation. Brain ischemia may also activate the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal axis (HPA) and further influence circulating immune cells to enter the brain. This in turn may lead to fewer circulating immune cells, and increase the risk of infectious complications [11]. Leukocyte plugging of the brain's microvasculature has also been implicated in microvascular stasis leading to hypoperfusion or the so-called 'no-reflow' phenomenon. Blocking inflammation has shown to ameliorate injury in stroke models [12].

What makes these inflammatory processes difficult to understand is that some of these responses may be detrimental by facilitating cell death, but also beneficial to the ischemic brain as immune cells are also capable of secreting neurotrophic factors and by scavenging necrotic debris allowing for the reorganization of a new environment for neural repair [13–16].

There has been significant progress over past years regarding inflammatory signaling pathways, cells and proteins as they pertain to post-ischemic inflammation. This review will focus on recent findings of the innate immune system and related mechanisms, and discusses ischemia-associated inflammation and possible therapeutic targets.

2. CELLS INVOLVED IN POST STROKE INFLAMMATION

The post stroke inflammatory response consists of cells intrinsic to the brain, as well as cells of the peripheral circulation which gain access through activated endothelium and/or disrupted blood brain barrier (BBB). Accumulation of circulating immune cells begins when ischemia damages and activates endothelial cells with rapid upregulation of adhesion molecules [17]. Circulating immune cells can then gain access into the injured brain and elaborate immune molecules that exacerbate ischemic cell death. Ischemic brain cells also activate endogenous immune cells such as microglia. A few studies implicate astrocytes as another brain resident immune cell which can also activate in response to ischemia.

2.1. Cerebral Endothelial Cells and the Blood Brain Barrier (BBB)

The cerebral endothelium is unique in that it contains a network of tight junctions, pericytes and astrocyte end-foot processes which make up the BBB [18, 19]. The interconnection of

the cells is facilitated by integrins [20]. Endothelial cells are among the first cells which face the impact of ischemia related inflammation. When damaged by ischemic stimuli, endothelial cells swell or detach from the underlying basement membrane, leading to compromises in barrier function. This leads to increased BBB permeability causing protein extravasation and interstitial edema as well as entry of immune molecules and cells [17, 21]. Hypoxia also activates the arachidonic acid cascade leading to the generation of pro-inflammatory molecules such as prostaglandins, leukotrienes, and platelet-activating factor (PAF) [22]. These substances lead to platelet and neutrophil activation and adhesion, followed by changes in local cerebral blood flow and increases in BBB permeability [6, 22–24]. Ischemic endothelial cells are also known to express and release pro-inflammatory cytokines and chemokines. These mediators have been shown to up-regulate additional mediators in the ischemic endothelium, such as selectins and immunoglobin superfamily members leading to leukocyte adhesion and transmigration into the brain [25, 26].

2.2. Leukocytes

Leukocytes promote cerebral ischemic injury through several different mechanisms. They can adhere to the endothelium and obstruct the microvasculature. Such ‘leukocyte pluggins’ can impair local blood flow causing the ‘ no-reflow’ phenomenon and leads to additional ischemic injury [21]. Activated leukocytes near the surface of the endothelium also produce toxic ROS, proteases, gelatinases, and collagenases, and damage potentially salvageable blood vessels and brain tissues. Further, phospholipase activation in leukocytes leads to the production of leukotrienes, eicosanoids, prostaglandins, and PAF, causing vasoconstriction and increased platelet aggregation. Finally, infiltrated leukocytes elaborate proinflammatory cytokines and other immune molecules in around the penumbra surrounding the infarct core causing further neuronal injury [27–29].

2.2.1. Neutrophils—Following ischemia, neutrophils are thought to be the earliest type of leukocyte to enter the ischemic brain. Neutrophils have been observed as early as 4–6 h in models of transient focal cerebral ischemia [30, 31], and at around 12 hours after permanent focal cerebral ischemia [32]. They have been shown to exacerbate injury by producing inflammatory mediators, many of which are thought to be toxic to the injured brain [4, 33]. In transient focal cerebral ischemia, several reports have shown reduced infarct size when neutrophils are depleted or their infiltration is prevented [29, 34–38]. Many of these immune mediators appear to contribute to immune mediated damaged to adjacent viable tissue. By blocking neutrophil entry into the brain, studies have documented improved neurological outcome, thus indicating that neutrophils contribute negatively in this setting [39].

2.2.2. Lymphocytes—While the data are conflicting, lymphocytes appear to play an active role in ischemic brain pathogenesis. Like neutrophils, lymphocytes are also capable of producing pro-inflammatory cytokines and cytotoxic substances. A few studies in stroke models have shown that lymphocytes are elevated in the ischemic brain later than neutrophils (3 to 6 days post stroke) [40, 41]. Strategies to block lymphocyte entry into ischemic brain reduced injury, and like neutrophils, indicate that lymphocytes play a damaging role [42]. T, but not B lymphocytes are thought to be central to the development of inflammation in stroke models [43, 44]. Several studies have evaluated the role of T

lymphocyte deficient mice in the transient focal ischemia model, and have shown reduced infarct volumes and reduced neurological deficits compared to controls [44–47]. In lymphocyte-deficient mice, protection from experimental stroke was found to be explained by the lack of T lymphocytes, rather than B lymphocytes. When investigators administered B lymphocytes to these lymphocyte deficient mice, protection from lymphocyte deficiency was not affected, whereas administration of T lymphocytes led to loss of protection [44, 46, 47]. Although, T lymphocytes appear worsen outcome in transient focal cerebral ischemia models, they do not appear to play a role in permanent focal cerebral ischemia models. In experimental permanent focal cerebral ischemia, Saino *et al.* [48] did not observed any change of infarct size in T and B lymphocyte deficient mice versus wildtype. The reasons for these differences are unclear.

However, not all T lymphocyte subtypes are detrimental in brain ischemia models. A recent study showed that neither γδ T lymphocytes nor natural killer (NK) cells contribute to ischemic injury [44]. There are conflicting reports concerning regulatory T (T_{reg}) lymphocyte participation in stroke. Liesz *et al.* [49] showed that ischemic lesion size and neurological deficit were increased in mice treated with an antibody to neutralize T_{reg} lymphocytes compared to controls, suggesting that they contribute positively. They also suggested that IL-10 signaling may be essential for this immunomodulatory effect. However, Ren *et al.* [50] could not find any effect of T_{reg} cells in similar stroke models. There is also evidence that hepatic NK cell function is impaired due to augmented sympathetic neurotransmission after stroke. This loss of invariant NK (iNK) cell activity contributed to immunosuppression and susceptibility to infections following stroke [51].

Clinical studies suggest that increased serum lymphocytes are observed in patients with increased stroke risk and mortality [52]. However, *in vitro* studies have shown that while neutrophils potentiate neuronal injury due to necrotic insults, lymphocytes did not actually increase astrocyte proliferation [53].

Typically, T lymphocytes kill pathogen-infected cells through the release of cytokines, or cytotoxins [54], and similar actions probably occur at the site of ischemic injury. Alternatively, T lymphocytes may cause cell death via interaction with the Fas receptor [55], and a few groups showed that blocking T lymphocyte-derived cytokines decreased infarct volume and improved neurological outcome in experimental stroke [46, 49, 56]. Furthermore, perforin-deficient mice were protected against stroke, suggesting that perforin, which is released by T lymphocytes, potentiates ischemic damage [57]. In addition to these studies, other work indicates that T lymphocytes increase oxidative stress through superoxide produced by NADPH oxidase type 2 (Nox-2). T lymphocytes produce 7 to 15 times more super-oxide through Nox-2 following experimental stroke in mice compared to uninjured mice [58]. Thus, the role of lymphocytes in stroke may depend on the subtype involved.

2.3. Microglia/Macrophages

Microglia constitute approximately 5–10% of all of the cells of the brain, and are often considered the brain's resident immune cell. Microglia modulate immune responses and phagocytic activity in the brain [59]. They participate in the brain's defense against

infection, and are activated under conditions of trauma, ischemia, and neurodegeneration [60]. Activated microglia undergo morphologic transformation from a resting state referred to as “ramified” to an “amoeboid” state, where they become virtually indistinguishable from circulating macrophages [59, 61]. Activated microglia are often called resident brain macrophages. Through their phagocytic properties, microglia will clear foreign organisms as well as injured neurons [30, 62–64]. Cerebral ischemia also induces microglial activation, but like the circulating immune systems, the precise mechanisms of activation following ischemia are not completely understood. Accumulating data show that CD14 receptors, followed by toll-like receptor 4 (TRL4) have been documented in microglia of the infarcted brain and could be one of the mechanisms involved in activation [65–67].

Activated macrophages have been observed as early as 2 hours following ischemia in rodents, while circulating macrophages appear in the brain after 10 hours. By 24–48 hours post insult, activated microglia and macrophages are present throughout the entire lesion and persist out to 1 week and longer [31, 68–71]. Once activated, microglia are thought to release a variety of toxic substances which lead to cell injury and death [62, 72]. Edaravone, a glutathione peroxidase mimic and free radical scavenger, appears to protect against experimental stroke by reducing microglial activation [73]. Hyperbaric oxygen treatment similarly decreased infarct volume through reduction of microglial activation [74]. Phagocytic microglia have been documented in ischemic brains following transient focal cerebral ischemia [63, 75]. Others have shown that minocycline, a tetracycline family antibiotic, protects against brain ischemia through the inhibition of microglial activation and proliferation [76, 77]. Co-culture models of microglia and neurons have mostly shown that the addition of microglia increases neuron death, and provides evidence for a damaging role of these immune cells [63, 67, 78, 79]. However, some microglial products are also neuroprotective [62, 80]. When microglial proliferation was inhibited in transgenic mice, infarct size was increased following ischemia, and suggests that proliferating microglia are also important in limiting ischemic damage [81]. There are a few explanations underlying these latter observations. Microglia are known to generate neurotrophic factors which have the potential to neurogenesis and plasticity. Microglia are known to produce TGF-β1, a growth factor. Microglia may also prevent release of toxic immune mediators by invading leukocytes by phagocytosing neutrophils [82, 83]. Phagocytic microglia/macrophages may remove necrotic debris and other potentially damaing substances [83].

2.4. Astrocytes

In addition to the above described inflammatory cells, astrocytes have been found to produce various inflammatory molecules [84, 85]. Astrocytes activate after brain ischemia leading to increased glial fibrillary acidic protein (GFAP) expression. Astrocytes also contribute to reactive gliosis [86], which can be destructive after injury [30, 87]. An astroglial response begins in the center of the ischemic lesion beginning 4–24 hours post insult, and reaches a peak around 4 days and is persists even 28 days later [88, 89]. Activated astrocytes lead to glial scar formation which have both neurotoxic and neurotrophic properties. The scar can function as a barrier which prevents axonal ingrowth and reinnervation, thus impeding recovery. This scar isolates damaged tissue from viable tissue, and prevents additional damage to the surrounding brain [88]. Astrocytes can also directly participate in the brain’s

immune response as evidenced by expression of major histocompatibility complex (MHC) and respective costimulatory molecules. Astrocytes are also capable of developing Th2 (anti-inflammatory) responses; however, this has not been shown in ischemia [90]. Astrocytes are also known to elaborate cytokines, chemokines and inducible nitric oxide synthase (iNOS) [90, 91]. iNOS was detected in reactive hippocampal astrocytes following forebrain ischemia [91]. Furthermore, astrocyte-generated iNOS may increase injury to neurons om response to ischemia-like insults [92]. Astrocytes have also been shown to signal through TWEAK (tumor necrosis factor like weak inducer of apoptosis), which is a member of the tumor necrosis factor superfamily. TWEAK is secreted by neurons, astrocytes and endothelial cells, and when it binds its Fn14 receptor which is expressed on astrocytes, leads to proinflammatory molecule production [93–95]. Expression of TWEAK and Fn14 has been documented in a murine model of stroke, and a soluble decoy to Fn14 reduced infarct volume [94]. These observations suggest that activated astrocytes may also exacerbate injury to ischemic brain in a manner that is reminiscent of traditional immune cells.

3. ADHESION MOLECULES

Adhesion molecules involved in acute inflammatory responses permit interactions between endothelial cells, platelets, leukocytes, and lymphocytes, and are important in mediating events that lead to the infiltration of immune cells into the brain parenchyma [96]. Rolling, adhesion, and transendothelial migration of immune cells are three major steps, involved in leukocyte entry into the brain [97]. Immune cells interact with the vascular endothelium via three classes of adhesion molecules: selectins (P-selectin, E-selectin, and L-selectin), the immunoglobulin superfamily (intercellular adhesion molecules, e.g. ICAM-1, 2 and vascular cell adhesion molecule-1, or VCAM-1) and integrins (CD11a-c) [98, 99]. Inhibiting leukocyte adhesion by targeting various adhesion molecules and preventing leukocytes entry into ischemic brain led to reduced neurologic injury [35, 100]. Mice lacking various adhesion molecules have reduced infarct volume after transient focal cerebral ischemia [101–103]. Although many of these adhesion molecules are upregulated in both permanent and transient middle cerebral artery occlusion (MCAO)models [104–106], blocking these targets are effective largely when reperfusion occurs (i.e., transient, rather than permanent MCAO) [100, 107, 108].

3.1. Selectins

Selectins are calcium-dependent, transmembrane glyco-proteins that bind to carbohydrate residues (sialyl-Lewis^{x}). They mediate adhesion between cells and leukocyte rolling. The three major selectins include E-selectin, P-selectin, and L-selectin [9, 32, 109]. There is very little E-selectin [104, 106], or P-selectin [8, 110] expression in uninjured brain vessels, but are present on the outer cell membrane of activated endothelium. E- and P-selectin contribute to leukocyte rolling and recruitment to areas of injury, reaching a peak 4–24 hours post stroke, and return to baseline by 72–96 hours [8, 104, 106, 111]. In contrast, L-selectin acts to guide leukocytes to activated endothelium, and sheds from the cell surface when the leukocyte binds the endothelial cell’s surface [112].

P- and E-selectin upregulation have been observed in several stroke models. Their presence appears to promote inflammatory responses and increase ischemic injury [8, 92, 104, 113–115]. Mice overexpressing P-selectin had larger infarcts compared to wildtype mice, whereas P- and E-selectin inhibition led to better neurological outcomes [113, 116, 117]. P-selectin's role appears to differ in focal versus global cerebral ischemia. In focal ischemia, neutrophils accumulated in the ischemic cortex of wild-type mice compared to P-selectin deficient mice [101]. P-selectin knockout mice also had reduced neurological deficits compared to control mice. P-selectin inhibition also improved early restoration of cerebral blood flow and this led to better stroke outcome [101]. In contrast, antibody-mediated blocking of P-selectin in models of global cerebral ischemia, paradoxically reduced survival while still reducing leukocyte rolling [118]. Reasons for these conflicting outcomes are unclear, but indicate that inflammation after focal and global ischemia may be different. It is less clear whether L-selectin plays a significant role in brain ischemia. L-selectin mediates leukocyte transmigration, but existing work in stroke models does not indicate that it influences stroke outcome. In a rabbit stroke model, L-selectin inhibition with an antibody failed to influence stroke outcome [119]. Selectins have also been studied as a target for potential vaccines against stroke. In a few studies, animals given intranasal E-selectin induced immune tolerance to subsequent exposure to brain antigens, and led to improved outcome from experimental stroke and even prevent their occurrence [120, 121]. Thus, E-selectin exposure could lead to immune tolerance, and prevent subsequent leukocyte trafficking into the brain.

3.2. Immunoglobulin Superfamily

Immunoglobulin superfamily members include intercellular adhesion molecules (ICAM-1, ICAM-2), vascular cell adhesion molecule (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PECAM-1), the mucosal vascular addressing cell adhesion molecule 1 (MAdCAM-1), and activated leukocyte cell adhesion molecule (ALCAM). ICAM-1 is constitutively expressed at low levels on endothelial cells, leukocytes, epithelial cells, and fibroblasts [6]. Upon activation by immune stimuli, ICAM-1 attaches immune cells to the endothelial wall in order to facilitate diapedesis thorough the vessel wall through integrin interactions (see sectin 4.3) into the site of injury [33, 122]. Its expression is increased soon after ischemia or cytokine exposure [6, 10, 123]. TNF-alpha and IL-1 are capable of inducing VCAM-1, while PE-CAM-1 (CD31) participates in the adhesion of endothelial cells to one another, as well as leukocyte transmigration across the endothelium. MAdCAM-1, typically expressed on mucosal endothelium, is a ligand for L-selectin and the $\alpha_4\beta_7$ integrin.

Of the immunoglobulin superfamily members, ICAM-1 and VCAM-1 are the most studied in stroke models [6]. ICAM-1 increases within the brain's vasculature hours after stroke onset, peaking at about 12–48 h. Its initial expression is followed by leukocyte infiltration and returns to near base line levels by 1 week [6, 10, 23, 68, 123]. Several groups have demonstrated that antibody inhibition of ICAM-1 [29, 57, 124–126], inhibiting ICAM-1 at the mRNA level or studying ICAM-1 deficient mice all lead to reduced leukocyte infiltration into the brain and improved neurological outcome from experimental stroke [6, 36, 102]. Interestingly, other studies have shown that nitric oxide donors blocked ICAM-1 expression

and also led to neuroprotection [115]. In diabetic rat models, ischemia-induced ICAM-1 elevations were higher than that seen in non-diabetic rats, and could partially explain why hyperglycemia worsens stroke outcome [127].

Reports of VCAM-1 expression in stroke are conflicting. VCAM-1 mRNA and protein have been shown to increase after cerebral ischemia [128, 129], while other reports have failed to observe significant changes [126]. Nevertheless, a study of the leukotriene receptor antagonist, ONO-1078 in a model of rat global cerebral ischemia led to improved neurological deficits and reduced neuron death by inhibiting VCAM-1 upregulation [130]. Treatment of experimental stroke with fractionated heparin decreased VCAM-1 expression and reduced infarct size [131]. Leisz *et al.* [57] also found that gene knockdown of VCAM-1 via small interfering RNA demonstrated reduced T lymphocyte infiltration into the brain and decrased infarct volume after the ischemic insult. However, there are other studies that did not show any change in outcome using anti-VCAM-1 antibodies treatment [57, 129].

In humans, soluable ICAM-1 and VCAM-1 (sICAM-1 and sVCAM-1, respectively) have been documented to increase in the blood and cerebral spinal fluid of patients with recent stroke, and these increases correlated to neurological severity [10, 128]. Increased VCAM-1 expression in brain blood vessels and astrocytes has been observed in autopsy specimens of stroke victims [128].

3.3. Integrins

Integrins are surface proteins involved in cell-cell binding. They are composed of heterodimeric combinations of a and b subunits, and are activated in response to signaling molecules similar to other adhesion molecules. Integrins which associate with a common β2 chain (CD18) and are thus known as β2 integrins. Along with CD18, the majority of leukocytes also express a integrin chains CD11a (also known as LFA-1) and CD11b (also known as Mac-1). α and β integrins form het-erodimers which participate in the binding of leukocytes to endothelial cells via ICAM-1 [132]. Lymphocytes and monocytes integrins include a4b1 (CD49d/CD29 also known as VLA-1, very late antigen-1) and a4b7 (CD49d/CD103 or LPAM-1, lymphocyte Peyer's patch adhesion molecule-1). Lymphocytes bind to the endothelium through these a integrins and either VCAM-1 or MAdCAM-1 [133].

In an *in vitro* model, hypoxia increased neutrophil CD11b expression, and aprotinin, which reduces CD11b expression, led to cytoprotection [134]. Reducing CD11b expression using 3-aminobenzamide (3-AB) to block poly-ADP ribose polymerase (PARP) was also protective [135]. At the *in vivo* level, antibodies to block CD11b and/or CD18 or studying mice lacking CD18 similarly reduced damage from MCAO, and was correlated to decreased numbers of neutrophils in the brain [34, 38, 57, 107, 136]. Blocking lymphocyte and monocyte integrins also limited damage due to ischemic injury. Treating rodents with antibodies against the α_4 integrin led to smaller infarct volumes compared to control treatment [42, 137], and this was associated with reduced neurological deficit [42].

4. INFLAMMATORY MEDIATORS

Several immune mediators have been studied in stroke models, and many have been specifically studied for potential therapeutic value.

4.1. Cytokines

Cytokine expression increases in the brain after stroke, and are expressed by cells of the immune system, as well as brain cells such as glia and neurons [45, 138]. Cytokine upregulation has also been documented in blood, cerebrospinal fluid and brain of humans with stroke [139]. Interleukin-1 (IL-1α and β), TNF-α, interleukin-6 (IL-6), interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) have been the most extensively studied cytokines in stroke models [12]. IL-1β appears to potentiate ischemic brain injury, while TNFα appears to contribute to and ameliorate neuronal injury. The collective literature surrounding IL-6, IL-10 and TGF-β suggest that these cytokines may be neuroprotective [140]. Many cytokines studied in stroke do not appear to be directly neurotoxic, since their administration to the uninjured brain does not lead to any obvious brain cell death. However, in the setting of injury, certain cytokines have been shown to both worsen ischemic brain damage.

4.1.1. IL-1—IL-1 is generally thought to potentiate ischemic brain damage. IL-1 exists in two isoforms, IL-1α and IL-1β. IL-1 also has an endogenous inhibitor, IL-1 receptor antagonist (IL-1ra). These three molecules have been extensively studied in stroke models [141]. IL-1β, rather than IL-1α is considered to be more engaged in ischemia pathogenesis [142]. IL-1β mRNA elevations occur within minutes following brain ischemia [123, 143], and the corresponding protein increases a few hours later starting from the infarct core and spreading to the peri-infarct area [45, 144]. In global cerebral ischemia models, IL-1β mRNA and protein expression appear biphasic, with an initial peak occurring as early as 1 h into reperfusion (following 20 minutes of forebrain ischemia), with a second peak occurring 6–24 h later [145]. Consistent with a damaging effect, IL-1β administered to rats exposed to focal cerebral ischemia led to increased infarct size [146], while mice lacking IL-1β had smaller infarcts compared to controls [142]. Reperfusion in diabetic rats also led to higher protein levels of IL-1β compared to non-diabetic rats, indicating that IL-1β may contribute to the worsening of ischemic damage by diabetes [127]. IL-1β results from conversion from its pro-form by IL-1β converting enzyme (ICE-1, a member of the caspase family) to its active form, and inhibiting ICE-1 is also protective [147]. IL-1R1 and IL-1R2 are IL-1's two main receptors; however, only IL-1R1 appears to be involved in signal transduction [148]. IL-1R1 deficient mice suffered less brain damage due to hypoxic-ischemia (H/I) with reduced neurological deficits [149]. Overexpression of or treatment with IL-1ra, the endogenous inhibitor of the IL-1 receptor, also reduced infarct size [150, 151]. Similarly, IL-1ra deficient mice suffered worsened ischemic damage [141]. When mixed neuronal cultures from these mice were exposed to glutamate toxicity, cells from IL-1ra deficient mice experienced more death compared to wildtype cells [141].

4.1.2. TNF-α—Brain ischemia also leads to TNF-α upregulation. Like IL-1β, TNF-α expression is biphasic, with initial increases 1–3 hours post ischemia, and an initial peak at

12 h [123], followed by a second peak 24–36 h later [152, 153]. TNF-α expression has been observed in neurons [138], astrocytes [154] and in immune cells [153] in stroke models.

Although TNF-α and IL-1β often work synergistically, TNF-α seems to have both neurotoxic and neuroprotective effects in the brain, while IL-1β seems generally neurotoxic [155, 156]. Consistent with a damaging role, TNF-α inhibition reduces ischemic brain injury [157], and administration of recombinant TNF-α protein after stroke onset exacerbates damage [158]. However, TNF-α has also been shown to protect the brain in other situations. TNF-α has been implicated in ischemic tolerance [159], because mice lacking TNF receptors have larger infarcts [160]. TNF-α may also be region specific, as TNF-α in the striatum leads to neurodegeneration, while TNF-α in the hippocampus is neuroprotective [161]. TNF-α also leads to vasogenic edema as a consequence of endothelial cell death, and is involved in trafficking of circulating immune cells. However, TNF-α also activates repair processes and mediates neuronal plasticity [161]. The reasons for this disparity are still unknown, and several hypotheses have been proposed. First, TNF-α ‘s actions may depend on the timing. Acutely, TNF-α may have a detrimental effect, but may be beneficial at later time points [162], but this does not explain why TNF-α seems to underlie the phenomenon of tolerance. Other explanations for these disparate observations may have to do with the receptors to which TNF-α binds, and the fact that TNF-α is present in soluble and membrane-bound forms with potentially different functions [163]. Soluble TNF-α binds to TNF receptor 1, and potentiates damage, whereas membrane bound TNF-α binds to TNF receptor 2 (TNFR2) is associated with neuroprotection [62]. However, TNFR2 is primarily found on microglia and endothelial cells. To further complicate the picture, other studies indicate that TNF receptor 1 (TNFR1) signaling pathways are neuroprotective [164].

4.1.3. Other Inflammation-Related Cytokines—The role of IL-6 in the ischemic stroke is even less clear than that of the previously discussed cytokines. IL-6 has been shown to increase its expression continuously up to 24 hours after ischemia onset [123]. However, IL-6 deficient mice or mice treated with an IL-6 receptor antagonist were no different than wildtype or untreated controls, and suggests that IL-6 does not actively participate in ischemia pathogenesis [165, 166]. Conflicting data on IL-6 also exists. Herrmann *et al.* [167] reported beneficial effects of IL-6, while Smith *et al.* [168] showed detrimental effects. There are also reports showing strong correlation between serum IL-6 levels and inhospital mortality rates in stroke patients[168, 169]. Further, brain derived IL-6 appears to contribute to angiogenesis and neuronal survival through STAT3 activation and manganese-superoxide dismutase [170, 171].

IL-10 is an anti-inflammatory cytokine which inhibits proinflammatory cytokines including IL-1 and TNF-α. It also suppresses cytokine receptor expression and activation. In the brain, it is mostly produced by microglia and astrocytes, and is upregulated by ischemia and related insults [172]. Studies of exogenous IL-10 administration [173] and its gene transfer [174] in cerebral ischemia models lead to improved neurological and histological outcomes. Mononuclear cells of patients with acute stroke secrete increased IL-10 compared to patients without stroke [175] and elevated IL-10 levels have been found in the cerebrospinal fluid of stroke victims [176]. Consistent with a beneficial role of IL-10, patients with decreased IL-10 levels are thought to have higher stroke risk [177].

In brain, TGF-β1 has been observed in microglia and astrocytes. Low levels have been documented in neurons [178]. Viral vector mediated TGF-β1 overexpression led to decreased ischemic lesion sizes and reduced inflammation in a mouse stroke model [179]. A study in neurons cultured with microglia were protected by microglia, and this protection was thought to be due to TGF-β1secreted by the microglia [180].

4.2. Chemokines

Chemokines, or chemotactic cytokines, and their corresponding receptors are involved in leukoctye migration and extravasation. Chemokines are expressed by injured neurons, astrocytes, microglia, and endothelial cells, and circulating immune cells [45, 85, 181, 182]. The classes of chemokines are based on their structures: CXC, CC, C, CX3C, with “Cs” refer to two cysteine residues, and the X’s referring to intervening amino acids. Members of the chemokine family tend to bind to several receptors, and individual chemokine receptors bind multiple ligands [183–185]. The CXC subfamily also includes ELR+ or ELR− subgroups, based on whether there is a glutamate–leucine–arginine sequence between the N-terminus and the first cysteine [185, 186]. The ELR+ CXC chemokine subfamily are generally attract neutrophils, whereas the CC chemokines attract monocytes and T lymphocytes [45, 187]. Several chemokines in CXC group have been shown to participate in stroke pathogenesis [43, 188] by increasing leukocyte infiltration [99]. Ischemic brain insults lead to large increases in ELR+ chemokines, along with CXCR2 and its ligands CXCL1 and CXCL2 [43]. As predicted, CXCR2 inhibition following transient focal ischemia prevented increases in these genes, and decreased leukocyte infiltration into the brain; however, this was not associated with any improvement in infarct size, brain edema or neurological outcome.

Recent data also showed that CC chemokines monocyte chemoattractant protein-1 (MCP-1, CCL2) and macrophage inflammatory protein-1α (MIP-1α, CCL-3) led to expression and secretion of chemokines RANTES and CCL5 on T cells, and macrophage inflammatory protein-3 alpha (MIP-3α) was induced in animal stroke models [85, 123, 189–191]. At baseline, MCP-1 mRNA expression was almost absent, but ischemia led to a increases in MCP-1 mRNA within the ischemic cortex after either permanent or temporary MCAO around 12 h to 2 days and remained elevated 5 days later [181, 182]. CC chemokine inhibition or deficiency led to reduced brain injury, consistent with a deleterious role [37]. Similarly, MCP-1 overexpression led to exacerbated injury and increased recruitment of inflammatory cells [189].

Fractalkine (CX3CL1), unique in that it is expressed on neurons, acts through its CX3CR receptor found on microglia. Its expression has been localized to neurons and endothelial cells following experimental stroke. The finding of fractalkine on neurons and its receptor on microglia suggests that fractalkine may be involved in signaling between these cells [192]. Fractalkine deficient mice are protected from experimental stroke, indicating that fractalkine somehow exacerbates neuron death [193].

Chemokines also directly influence BBB permeability. MCP-1 enhanced the permeability of brain derived endothelial cells cocultured with astrocytes (a model of the BBB) and led to redictopms in tight junction (TJ) proteins, implicating MCP-1 in ‘opening’ the BBB [194].

Chemokines may also be important for stem cell migration into regions of injury, a property which could be potentially used to this advantage [195]. In fact, MCP-1 and SDF-1 and their corresponding receptors have been documented in ischemic brain and transplanted stem cells [196]. In stroke models, MCP-1 and other chemokines have been implicated in bone marrow derived stromal cell (BMSC) migration into sites of injury [197–199].

4.3. Arachidonic Acid Metabolites

Once immune cells are activated, phospholipase A2 (PLA2) is released and initiates the arachidonic acid (AA) cascade [22]. Stroke leads to cessation of blood flow and energy failure, which results calcium entry and accumulation in brain cells. Increased intracellular calcium activates PLA2 which, in turn, hydrolyses glycerophospholipids, and AA is released. PLA2 activity increases following experimental stroke [200]. AA metabolites contribute to worsening of ischemic brain injury by potentiating immune responses [201]. Consistent with this, PLA2 deficient mice exposed to experimental stroke developed less brain edema and had better outcomes compared to their wild type littermates [202]. AA undergoes metabolism through the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, both of which are independently active in stroke pathogenesis.

4.3.1. Cyclooxygenase Pathways—Following arachidonic acid release, COX converts AA to prostaglandin H2 (PGH2). COX exists in two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in microglia, leukocytes and other cell types [203]. COX-1's role in brain injury is unclear. Consistent with a beneficial role, COX-1 deficient mice have worse outcomes in brain ischemia models [204]. However, in a forebrain ischemia model, COX-1 inhibition led to increased numbers of healthy neurons in the CA1 region of the hippocampus, and would point to a damaging role of COX-1 in this model [205]. These reasons for these discrepancies are unknown, but suggest inherent differences in inflammatory responses between focal and global cerebral ischemia models.

COX-2 is induced following brain ischemia [206]. In autopsy specimens from stroke patients, COX-2 was observed within areas of ischemic brain [207], as well as regions remote from the infarct [208]. There are many COX metabolites that are biologically active, but accumulated data suggest that they are most likely deleterious in stroke. Prostaglandin E2 EP1 receptors have been identified as an effector involved in neurotoxicity [209]. Collectively, several studies now show that COX-2 inhibitor treatment or the study of COX-2 deficient mice led to improved neurological outcome after stroke [206, 210, 211], whereas COX-2 overexpression worsens outcome [212]. COX-2's toxic effect appears to be mediated though PGE2 rather than ROS, even though COX-2 is capable of generating both [213].

4.3.2. 5-Lipoxygenase Pathway—In brain, less is known about the role of the lipoxygenase pathway, compared to that of the COX pathway. AA may alternatively be converted to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) via 5-lipoxygenase (5-LOX). 5-LOX is metabolized to leukotriene A4 (LTA4), a precursor to cysteinyl leukotrienes (cysLTs). LTA4 is converted to leukotriene C4 (LTC4), which is a potent chemoattractant thought to contribute to BBB disruption, as well as tissue edema and cell death. In the

setting of stroke, AA and LTC4 increases are biphasic, and correlate to BBB disruption [214]. 5-LOX has also been documented in autopsy specimens from stroke victims, and its expression localized to perivascular monocytes [215]. In a stroke model, the 5-LOX inhibitor AA861 led to decreased LTC4 levels and reduction in brain edema and cell death [216]. Furthermore, exposure of a neuron cell line to oxygen glucose deprivation led to attenuated cell death with treatment by 5-LOX inhibitor caffeic acid [217]. In contrast, at the *in vivo* level, 5-LOX deficiency failed to have any effect on stroke outcome in both permanent and temporary MCAO [218]. The reasons for this disparity might be due to an unforeseen phenotype in lifelong 5-LOX deficient mice, but further studies are needed to more precisely clarify.

AA may also be converted to other biologically active leukotrienes via 12-LOX. 12-LOX is found in platelets, but nevertheless leads to oxidative stress which can be toxic to brain tissue. In a rabbit model of multiple small embolic infarcts, baicalein, an inhibitor of 12/12-LOX, was found to improve neurological outcomes when administered as late as 2 h post insult [219]. However, there are known interspecies differences within 12/15-LOX making drug development challenging. Yet, a recent study showed that novel compound ML351 which blocked 12/15-LOX in both humans and rodents, was protective in a murine stroke model [220]. Thus, there is a clear need for more work in the area of the lipoxygenase pathways.

4.4. Nitric Oxide/Nitric Oxide Synthase

Nitric oxide (NO) is involved in a variety of processes including as host defense, regulation of vascular tone, and modulation of the immune response. NO enters cells readily, and reacts with various molecular targets. Through the enzyme nitric oxide synthase (NOS), NO is generated from L-arginine. To date, three nitric oxide synthases (NOS) isoforms have been studied in stroke: endothelial NOS (eNOS or NOS-3), neuronal NOS (nNOS or NOS-1), and inducible NOS (iNOS or NOS-2). iNOS is found almost exclusively in inflammatory cells, including circulating leukocytes, microglia, but also in astrocytes. iNOS mRNA and protein both increase in experimental stroke [221–223]. In addition to its participation in various signaling pathways, NO is thought to cause direct DNA damage through the formation of peroxynitrite, when NO reacts with superoxide [224–226]. Pharmacological inhibition or genetic deletion of iNOS leads to reduced infarct volume and improved neurological outcomes [222, 227]. Further, in therapeutic hypothermia, where cooling conditions leads to neuroprotection, microglia have been shown to generate less NO and iNOS [228], and estrogen and progesterone appears protect the brain from experimental stroke by modulating iNOS expression [229, 230].

4.5. Reactive Oxygen Species

Immune cells generate reactive oxygen species (ROS) through several enzyme systems. These include COX, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine dehydrogenase and xanthine oxidase. The myeloperoxidase (MPO) and monoamine oxidase (MAO) systems lead to the production of hypocholorous acid and hydrogen peroxide, respectively. The most studied ROS in the ischemic brain is superoxide

anion. Superoxide anion has been shown to cause direct brain injury, or indirect injury when it reacts with NO to generate peroxynitrite [231].

NOX is a multi-component enzyme originally identified in immune cells. It contains both cytoplasmic subunits ($p45^{phox}$, $p67^{phox}$, and $p40^{phox}$ and Rac2) and plasma membrane subunits ($p91^{phox}$, $p22^{phox}$). After NOX activation and phosphorylation, the cytosolic subunits translocate to the membrane and form a complete complex on the cell surface, enabling activated NOX to generate superoxide from oxygen by transferring electrons from NADPH [232]. Experimental stroke leads to increases in brain NOX [233], and NOX generated superoxide may be the major effector molecule which cause microglia to exacerbate ischemic injury [234]. In fact, mice lacking the gp91 subunit of NOX have reduced ischemic lesion size compared to wildtype controls [235]. By transplanting NOX deficient bone marrow in to wildtype host mice, or vice versa, NOX in circulating immune cells was found to contribute more to stroke pathology compared to NOX found in microglia [236]. NOX has also been implicated as an important factor by which hyperglycemia exacerbates stroke outcome [237].

Found largely in neutrophils and monocytes, myeloperoxidase (MPO) is a bactericical enzyme whose actions are through H_2O_2 and hypochlorous acid. Increases in serum MPO may predict stroke risk, and MPO has been observed in experimental stroke models of both temporary and permanent MCAO [37]. Interestingly, infarct size in MPO deficient mice actually increased after stroke, suggesting a beneficial role [238]. Products of nitrosylation were also increased in MPO deficient mice, suggesting that any protective effect of MPO may be through scavenging nitrotyrosine, which is produced via peroxynitrite reactions [238]. Thus, MPO may paradoxically attenuate ROS-mediated damage.

4.6. Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) comprise a group of proteases involved in the breakdown of proteins of the extracellular matrix. These proteases are normally involved in a variety of physiological processes including development, wound healing, cone growth, ovulation and angiogenesis. However, under injury conditions such as brain ischemia, MMPs are upregulated and activated especially within peri-infarct areas, and are part of the neuroinflammatory response. MMPs exist in a pro-form, that is, they are present in an inactivated state. However, MMPs are activated when cleaved by proteases such as plasmin or other MMPs [239]. Tissue plasminogen activator (tPA) disrupts the extracellular matrix as well as components of the BBB through activation of plasmin. This may result in extrusion of serum protein and even hemorrhage [240, 241]. Microglia are a major source of MMPs, and ischemia can also cause MMP activation in astrocytes [242].

In models of focal ischemia, MMP-9 activity increases at 15–48 h) and returns to baseline around 15 days. MMP-2 peaks at 5 days and returns to baseline around 15 days [243, 244]. MMPs contribute to apoptotic neuronal cell death by processing death receptor ligands, TNF-α and FasL. Not surprisingly, pharmacologic MMP inhibition reduces infarct size, brain edema and hemorrhage [245]. However, not all MMPs appear to contribute to stroke pathology. In fact, mice lacking MMP-9, but not MMP-2 had smaller infarcts compared to wildtype controls [246, 247]. MMP-2 deficient mice fared no differently than control mice

following focal cerebral ischemia. Observations at the clinical level seem to parallel findings in rodent stroke models. In stroke patients, serum MMP-9 levels appeared to correlate to stroke severity, whereas MMP-2 did not [248]. Considering that MMP-9 is produced by immune cells, but MMP-2 is not, emphasizes the significance of the immune system in stroke pathogenesis. Like NOX, MMP-9 derived from circulating leukocytes, rather than MMP-9 from brain, appears to contribute more to ischemic brain injury. Chimeric mice transplanted with bone marrow from MMP-9 deficient mice suffered less injury than chimeric MMP-9 deficient mice transplanted with marrow containing intact MMP-9 [249].

Not all MMP functions are detrimental. MMPs may also be involved in plasticity and recovery. Mice subjected to focal cerebral ischemia and treated with the broad spectrum MMP inhibitor (GM6001) had decreased migration of neuronal precursor cells from the subventricular zone (SVZ) into the striatum [250], indicating that MMPs are important in neurogenic migration. MMPs also associate with angiogenic factors including vascular endothelial growth factor (VEGF). MMP inhibition suppressed neurovascular remodeling post stroke, and reduced VEGF signaling [251].

5. TRANSCRIPTIONAL REGULATION OF INFLAMMATION

Several transcription factors are active following cerebral ischemia leading to changes in gene expression. Those transcription factors known to be involved in the ischemic inflammatory response will be discussed here.

5.1. Nuclear Factor κB (NF-κB)

Nuclear factor-kappaB (NF-κB) is largely associated with the initiation of inflammatory responses [252]. It has been widely studied in stroke models. It contains dimers of the Rel family subunits: Rel (cRel), RelA (p65), RelB, NF-κB1 (p50 and the p105 precursor) and NF-κB. NF-κB most commonly exists as a heterodimer composed of Rel A (p65) and p50. It is located in the cytoplasm in an inactivated state where it is bound to IκB, its endogenous inhibitor protein. Several pro-inflammatory stimuli activate IκB's upstream kinase (IKK). Once activated, IKK phosphorylates IκB at serines 32 and 36 leading to its phosphorylation, ubiquitination and degradation by the cell's proteasome. NF-κB, now free from IκB, is able to translocate into the nucleus, where it binds to concensus κB sites, sites contained within the promoters of many inflammatory genes previously discussed. While therapeutic hypothermia correlated to reduced IKK and NF-κB activation, suggesting a damaging role for NF-κB [253], its role in stroke is still conflicting [254]. Mice lacking NF-κB's p50 subunit subjected to stroke are protected [255], observations consistent with a damaging role of NF-κB. Pharmacologically inhibiting NF-κB with S-nitrosoglutathione (GSNO) similarly led to improved outcome [115]. IKK inhibition also reduced infarct size in stroke experiments [256], and constitutive activation of IKK led to larger infarct size [256]. Similar observations were made in a global ischemia where treatment with NF-κB decoy oligodeoxynucleotides reduced neuronal damage [257]. However, diethyldithiocarbamate (DDTC), a NF-κB inhibitor, given to rats subjected to experimental stroke led to increased infarct sizes compared to controls, consistent with a beneficial role of NF-κB [258]. It is unclear why these conflicting observations exist, but some have speculated over whether this

may depend on the cell type in which NFkB is activated, the experimental model used, or an off target effect of the pharmacological inhibitors used.

5.2. Mitogen-Activated Protein Kinase (MAPK)

Mitogen-activated protein kinases (MAPK) are a cascade of kinase phosphorylation and transcription factor activation enzymes which ultimately regulate inflammatory gene production [2, 259]. Three interlinked MAPK signaling pathways have been studied in cerebral ischemia. They include the stress-activated protein kinases/c-Jun N-terminal kinases (SAPK/JNK), p38 MAPKs and extracellular signal-regulated kinases (ERKs) [259–261]. p38 MAPK is involved in the upregulation of many proinflammatory genes [262]. In global cerebral ischemia, MLK3-MKK4-JNK activation has been documented as early as 30 min post injury onset, with a second period of activation 3 days later [263]. Knockdown of POSH (plenty of SH3s) using antisense oligodeoxynucleotides protected neurons of the hippocampal CA1 region, and reduced POSH interactions with MLK3, MKK4 and phospho-JNKs. JNK signaling was also attenuated. In a model of *in vitro* ischemia in astrocytes, the addition of bone marrow stromal cells led to MAPK activation and subsequently exerted protection to astrocytes [264]. Using a neuronal membrane lipid precursor, CDP-choline, others have demonstrated improved recovery after ischemic stroke, and this was associated with decreased phosphorylation of MAP-kinase family members, ERK1/2, MEK1/2, and Elk-1 transcription factor. In global cerebral ischemia, activated p38 MAPK has been detected in hippocampal neurons [261] and microglia [265], and suggests a role in inflammation. Consistent with a damaging role of inflammation in brain ischemia, treatment with p38 MAPK inhibitor lead to less brain injury and decreased neurological deficits [2].

6. INITIATION OF INNATE IMMUNE RESPONSES

The initiation of the immune response following stroke is still not fully clear, but recent studies have focused on various pro-inflammatory factors elaborated by the ischemic brain that might act on receptors involved in innate immune responses. The two main groups of innate immune receptors studied in brain ischemia are found on microglia and circulating immune cells, and include the Toll-like receptors (TLRs) and purinergic receptors. The ischemic brain is thought to generate extracellular nucleic acid following cell lysis. These and other ligands are often referred to as danger associated molecular pattern molecules (DAMPs). When bound to their respective ligands, an inflammasome is formed consisting of nucleic acids such ATP, UTP, adenosine and other pro-inflammatory molecules such as caspase 1, leading to the maturation and elaboration of pro-inflammatory cytokines and a full blown inflammatory response [13, 266] Toll-like receptors (TRLs) and purinergic receptors are widely expressed on microglia [13, 267].

6.1. Toll-Like Receptors

Toll-like receptors (TLRs) exist on the cell surface in order to recognize extracellular pathogens through pathogen-associated molecular patterns (PAMPs). They are also known to recognize similar patterns on injured or altered cell surfaces, and these are patterns are collectively known as death associated molecular patterns (DAMPs). They have been the focus of recent investigation, and are considered to play pivotal roles in the initiation of the

inflammatory response in stroke and related injuries [268, 269]. In addition to traditional immune cells, TLRs have also been described on other cells of the nervous system, including microglia, astrocytes, neurons, and cells of the cerebral vasculature [270, 271]. Reports in the brain ischemia literature point to DAMPs, including high mobility group box 1 protein (HMGB1) [272], heat shock proteins, extracellular peroxire-doxin [273] and nucleic acids [13] as likely ligands for TLRs.

Microglial activation following cerebral ischemia is associated with signaling through TLRs, particularly TLR2 and TLR4. TLR activation leads to increased cytokine expression, and other immune responses ultimately leading to brain cell damage. Increased brain TLR2 expression has been documented in both *in vitro* and *in vivo* stroke models [274–276]. Genes upregulated as a result of TLR2 activation include NF-κB, Cyclooxygenase-2 (COX2), IL-1β, IL-17, IL-23, and TNF-α, all of which have been shown to increase after ischemia [274, 276–278]. Yao *et al.* [278] showed that upregulation of TLR2 and IL-1β expression in an *in vitro* ischemia model led to neuronal cell death. The ischemic insult induced IL-1β, but cell death was prevented in TLR2 knockout mice. However, Hua *et al.* [279] reported that mice lacking TLR 2 had larger infarcts and increased mortality compared to wildtype mice. Brain ischemia also leads to increased TLR4 expression. In some reports, TLR4 deficient mice had smaller infarcts and better neurological function compared to wild-type mice. This was not the case for TLR3 or TLR9, however. TLR4 co-localized to microglia and TLR4 deficient mice had few numbers of activated microglia compared to mice with intact receptor [280, 281].

6.2. Purinergic Receptors

Purinergic receptors have been detected in both brain and periphery. They are a family of receptors that execute various cellular functions. P2 purinoreceptors are both ionotropic and metabotropic. The P2X family are ionotropic and regulate ion flow, whereas the P2Y subfamily are metabotropic and are G-protein coupled. In microglia and other immune cells, purinergic receptors are involved in migration and phagocytosis [282]. Purinergic receptors on microglia are of relevance to understanding how inflammation may be initiated in brain injury because they are capable of binding nucleotides released by injured cells. Microglial purinergic receptors has mostly focused on P2X7, where it has been shown to activate microglia in experimental brain ischemia. Pharmacologic blockade of P2X7 led to decreased ischemic damage [283–286]. Less work has been carried out on the P2Y receptor; however, of clinical relevance is the P2Y12 receptor [282, 287]. P2Y12 found on platelets as well as immune cells, and is inhibited by clopidogrel, a widely prescribed antiplatelet agent. P2Y12 is present on the surface of microglia and is activated by ATP or ADP. Once activated, P2Y12 promotes microglial chemotaxis and is also involved in the phosphorylation of Akt [288]. We have recently shown that P2Y12 mediates microglial migration in brain ischemia, and its deficiency or inhibition is neuroprotective [289].

7. INFLAMMATORY RESPONSES TO THE OTHER ORGANS FOLLOWING ISCHEMIC STROKE

Recent studies indicate that inflammatory responses following stroke affect the entire body, and not simply the brain. For example, splenectomy has been shown to confer neuroprotection against experimental stroke. The spleen is an important lymphatic organ, and sequesters red and white blood cells. It is involved in antibody synthesis, and also removes antibody coated cells from the circulation. Ajmo *et al.* [290] showed that splenectomy reduced lesion size in a stroke model and resulted in decreased numbers of activated microglia, macrophages, and neutrophils in the brain. These data suggest that spleen-mediated peripheral immune responses contribute to the inflammation in stroke, and that splenectomy could improve stroke outcome.

Evidence suggests that stroke renders the body in a state of immunodepression which could be detrimental. Soon after stroke, circulating immune cells are quickly reduced, thus increasing the risk of developing infections. This systemic immunodepression has been documented as early as 12 hours post stroke, and continues out to several weeks [11, 291, 292]. Immune cells of the spleen, thymus, liver and lymph node are reduced following stroke, and this may explain the observed immunosuppression [11, 153, 291–293]. The immunosuppression has been attributed to increased activity of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal axis (HPA) [11, 294]. As a result, immune cells undergo increased apoptosis in these organs after stroke, and cause these secondary organs to atrophy [11, 153, 291]. However, whether lower numbers of immune cells in the circulation are due to their entry into ischemic brain has yet to be definitively shown.

A consequence of this immunodepression is thought to lead to infectious complications which often arise after stroke [11, 153, 291] and lead to worsened outcome [295–297]. While hyperactivity of the SNS and the HPA is thought to underlie this phenomenon, the precise signals and mechanisms that trigger this immunodepression remain unclear. Blocking the SNS and HPA in experimental stroke models prevented splenocyte apoptosis and decreased circulating lymphocytes, along with decreased infection and reduced mortality [11, 294]. In line with experimental studies, evidence of SNS-mediated immunosuppression in stroke patients has been reported [3]. T and B lymphocytes undergo apoptosis in stroke, and this can increase susceptibility to infection [298]. The vagal cholinergic anti-inflammatory pathway appears to be another means of cross talk between the CNS and immune system. The vagus nerve can be activated by pro-inflammatory cytokines to release acetylcholine. This in turn inhibits the release of pro-inflammatory mediators by macrophages [299, 300]. Vagal nerve stimulation has been shown to inhibit the release of pro-inflammatory cytokines and improves outcomes in different stroke models [300]. Thus, this vagal cholinergic anti-inflammatory pathway may also be involved in post stroke immunodepression.

8. BENCH TO BEDSIDES: TRIALS, TRIBULATIONS AND PROMISING THERAPIES

8.1. Clinical Trials

Following several promising preclinical studies, a few clinical trials were carried out to determine whether anti-inflammatory strategies were beneficial. However, some of these trials did not meet with success either due to unanticipated effects of the agents tested or inappropriate study design [301–303]. Yet, with increased knowledge of the complexities of inflammation in stroke, a few treatments may be on the horizon.

8.1.1. Anti-Integrin—In order to limit leukocyte adhesion and migration into the infarcted brain, a few clinical studies treated stroke patients with anti-integrin therapies. LeukArrest studied a humanized CD11/CD18 antibody in stroke patients who presented within a 12 hour window [302]. The Acute Stroke Therapy by Inhibition of Neutrophils (ASTIN) trial studied treatment with a nonantibody peptide, recombinant neutrophil inhibiting factor (rNIF). rNIF was given within 6 hours of symptom onset [304]. Unfortunately, in both cases, studies were stopped early due to a lack of effect in primary endpoints. Both drugs seemed to have a beneficial effect in animal stroke models [38, 136], but the lack of benefit in humans may be explained by later treatment windows in humans, or inherent differences in neutrophil integrins between humans and other species. Consistent with this line of thinking, CD11b decreases after stroke in humans [305], but has been shown to increase in rodent stroke models [306].

8.1.2. Anti-ICAM1—Antibodies against the adhesion molecule ICAM-1 was studied at the phase III clinical trial level in stroke patients. The results of the study showed that ICAM-1 antibody failed to improve outcome in ischemic stroke, but it also showed that such antibody treatment actually made outcome worse [301, 303]. Unfortunately, the antibody given to study participants was a murine antibody, and had not been adapted for use in humans. This may have lead to complement activation by the presence of a non-humanized antibody, and the development of clinically significant fever and other adverse immune events, thus negating any potential neuroprotective benefit of ICAM-1 blocking [98]. Further, the rationale for treatment with anti-adhesion strategies was based on the observation of the ‘no-reflow’ phenomenon in animals models, but this phenomenon had not been clearly documented in humans. Thus, the “no-reflow” phenomenon, thought to be due to leukocyte plugging of the brain’s microvasculature, might be relevant in rodent stroke models, but of limited or unknown relevance in humans [307].

8.1.3. Minocycline—Minocycline is tetracycline family antibiotic. It has also been shown to inhibit both inflammatory and apoptotic pathways in stroke models. Of its anti-inflammatory properties, minocycline has been shown to suppress microglial activation, and inhibit MMP and NO production. Its anti-inflammatory effect appears to be due to a mechanism inhibiting MAPK activation in microglia [308]. As such, minocycline has been shown to protect the brain against cerebral ischemia and lessen functional impairment [77, 309–311]. Lampl *et al.* [312] conducted a clinical trial giving oral minocycline to ischemic patients and found that functional endpoints were improved amongst minocycline-treated

patients. However, this study was an open-label, evaluator-blinded study and the total number of the patients was relatively small. A second study established safety, dose ranging and feasibility in combination with rt-PA [313], and that intravenous minocycline administration could decrease plasma matrix metalloproteinase-9 in stroke patients [314]. Clinical studies to determine minocycline's efficacy in stroke and long term recovery (Neuroprotection with minocycline therapy for acute stroke recovery trial, NeuMAST) are also ongoing.

8.2 Future Therapies

8.2.1. FTY 720—FTY720 (Fingolimod) is a sphingosine-1-phosphate (S1P) receptor agonist, and was developed as an anti-inflammatory agent [315, 316]. It is already FDA-approved for the treatment of multiple sclerosis. FTY720 crosses the blood-brain barrier where it can be phosphorylated by sphin-gosine kinase (SphK) [317, 318], into the active compound phospho-FTY720, which then acts on S1P receptor subtypes $S1P_1$, $S1P_3$, $S1P_4$, $S1P_5$. It has been shown to be neuroprotective effect against many central nervous system diseases including cerebral ischemia [318–322]. FTY720 regulates myelination of axons and activation of microglia following injury, as well as proliferation and migration of neural precursor cells and neuronal differentiation. It has also been shown to have antiapoptotic and anti-inflammatory properties [321–327]. FTY720 exerts its immunomodulatory actions by affecting lymphocyte production, trafficking, and apoptosis through S1P receptors leading to the depletion of circulating lymphocytes, and prevents release of lymphocytes from the lymph nodes. Most of the past reports have shown beneficial effects of S1P in brain ischemia, but Liesz *et al.* [57] showed opposite results. These authors found that while FTY720 treatment did show a reduction of lymphocyte brain infiltration, they did not observe significant reduction in infarct volumes and improvement in neurological deficits [57]. Liu *et al.* [328] recently published a meta-analysis of the FTY720's efficacy in animal models of cerebral ischemia. In this analysis, they concluded that FTY720 reduced infarct volume and improved functional outcome. However, the authors also indicated that more experimental studies should be performed to evaluate the safety of FTY720 in the future. Thus, the S1P pathways are a promising area of investigation for stroke treatment.

9. BRIEF SUMMARY

Inflammation following ischemic stroke is increasingly recognized as a key element in its progression. In this review we have focused on many key players which are involved in the inflammatory responses following stroke, such as cellular components including microglia and leukocytes, adhesion molecules, inflammatory mediators and chemokines. While acute inflammatory responses appear to exacerbate ischemic injury, later responses may be essential in recovery and repair. This may explain some of the conflicting reports in the scientific literature. Regardless, future work towards a better understanding of this field are needed, with the hopes of translating discoveries into treatments for stroke patients.

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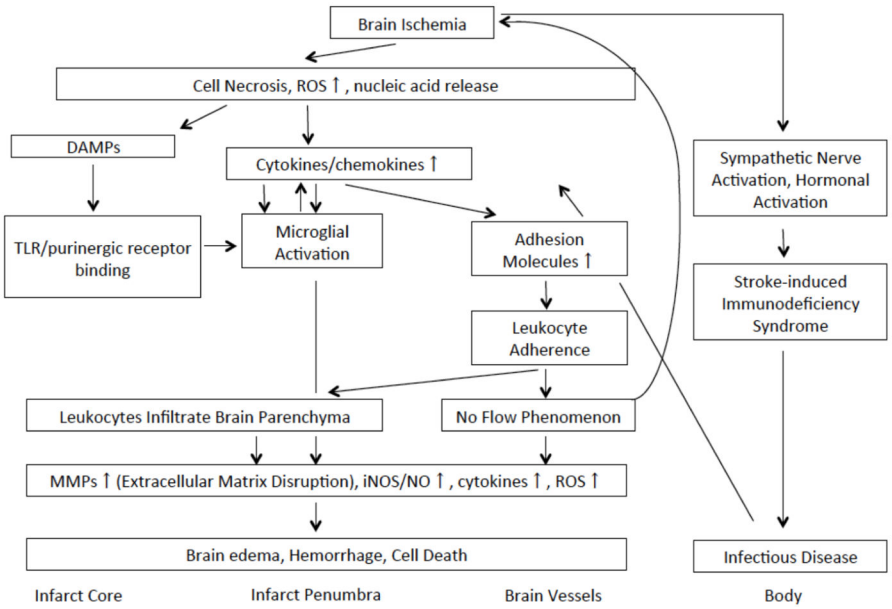

Fig. 1.

Post stroke inflammation pathway. Brain ischemia initiates inflammation in response to necrotic cells within the infarct. Necrotic cells then cause inflammatory cells to generate reactive oxygen species (ROS) and inflammatory cytokines. Necrotic cells lyse and release nucleic acids which can act as danger associated molecular pattern molecules (DAMPs). DAMPs and other immune molecules activate immune receptors on microglia, which in turn, leads to more cytokine production. Cytokines upregulate adhesion molecule expression on the surfaces of cells of the cerebral vasculature. The production of chemokines by cells of the immune system and brain recruit circulating immune cells to the ischemic brain. Adhesion molecules traffic circulating leukocytes to activated the endothelium where they infiltrate the brain parenchyma. After entering the brain, activated leukocytes along with activated microglia, generate inflammatory mediators, including the matrix metalloproteinases (MMPs), inducible nitric oxide synthase (iNOS) leading to nitric oxide (NO) generation, cytokines and more ROS. These pro-inflammatory molecules ultimately result in brain edema, hemorrhage and cell death. Brain infarction also affects the rest of the body, leading to a state of immunodeficiency, and is thought to be mediated through the sympathetic nervous system, resulting in lowering circulating immune cells. This can subsequently lead to infections which complicates stroke by further increasing immune activation in the brain.