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Innate Inflammatory Responses in Stroke: Mechanisms and Potential Therapeutic Targets

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Abstract: Stroke is a frequent cause of long-term disability and death worldwide. Ischemic stroke is more commonly encountered compared to hemorrhagic stroke, and leads to tissue death by ischemia due to occlusion of a cerebral artery. Inflammation is known to result as a result of ischemic injury, long thought to be involved in initiating the recovery and repair process. However, work over the past few decades indicates that aspects of this inflammatory response may in fact be detrimental to stroke outcome. Acutely, inflammation appears to have a detrimental effect, and anti-inflammatory treatments have been been studied as a potential therapeutic target. Chronically, reports suggest that post-ischemic inflammation is also essential for the tissue repairing and remodeling. The majority of the work in this area has centered around innate immune mechanisms, which will be the focus of this review. This review describes the different key players in neuroinflammation and their possible detrimental and protective effects in stroke. A better understanding of the roles of the different immune cells and their temporal profile of damage versus repair will help to clarify more effective modulation of inflammation post stroke.

Keywords: Brain ischemia, inflammation, neuroprotection, stroke.

INTRODUCTION

Stroke refers to conditions caused by occlusion and/or rupture of blood vessels in the brain, and is a leading cause of death and disability in the industrialized world.

Ischemic strokes represent more than 80% of all cases of stroke and are characterized by the occlusion of a brain arterial blood vessel due to a thrombus or embolus [1]. In spite of its high prevalence, effective therapies are few [2, 1]. Brain inflammation has been implicated as a secondary injury mechanism following ischemia and stroke [3-5]. Stroke triggers this inflammatory response as a result of several factors, such as necrotic cells and debris and reactive oxygen species (ROS) and many other factors which have yet to be precisely identified. These triggering factors lead to microglial activation, leading to more cytokine generation and induction of adhesion molecules within the cerebral vasculature, all within 24 hours of the ischemic insult [6-8]. Chemokine upregulation stimulates inflammatory cell chemotaxis into ischemic brain, especially around the penumbra, or the infarct's border.

Adhesion molecules on activated endothelia in turn leads to the adhesion of circulating leukocytes causing microvascular occlusion and infiltration of immune cells into the brain parenchyma [9, 10].

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Activated inflammatory cells then elaborate a variety of cytotoxic molecules such as more pro-inflammatory cytokines, matrix metalloproteinases (MMPs), nitric oxide (NO) and more ROS. These molecules in turn potentiate brain cell damage, including disruption of the blood-brain barrier (BBB) and extracellular matrix [11]. A disrupted BBB can further exacerbate brain injury, contributing to secondary ischemic damage by permitting serum elements and blood to enter the brain. This often leads to brain edema and hemorrhagic transformation. Brain ischemia is also thought to influence immune cells in the circulation possibly through increased activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal axis (HPA). This may lead to fewer circulating immune cells, and increase the risk of infectious complications [12]. Leukocyte plugging of the brain's microvasculature has also been implicated in microvascular stasis leading to hypoperfusion. Inhibiting variousinflammatory molecules has shown to reduce injury in experimental stroke models [13], although this has yet to be shown in human stroke patients.

In contrast, other immune responses may be beneficial to the ischemic brain by secreting neurotrophic factors and by scavenging necrotic debris allowing for the reorganization of a new environment for neural repair [14-17]. These reparative responses are thought to be especially relevant during the chronic phases of stroke. Thus, complete inhibition of immune responses could be predicted to impede recovery. While both acutely damaging and chronically reparative properties of post stroke inflammation have been embraced for quite some time, the precise nature of these dual properties has not been well defined. A better understanding of this

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tight balance may help better tailor both anti- and pro- immune therapies for maximum effectiveness.

Over the past few years, there have been advances in inflammatory signaling molecules in brain ischemia. This review focuses on recent findings as they largely pertain to innate immune responses and mechanisms in brain ischemia, plus possible therapeutic targets.

ENTRY OF IMMUNE CELLS INTO THE ISCHEMIC BRAIN

Inflammation is traditionally defined in histological terms by the presence of immune cells in the tissue of interest. Inflammatory cells have long been documented in autopsy specimens of patient who suffered stroke. Circulating immune cells gain access to the ischemic brain beginning with rapid upregulation of adhesion molecules [18]. 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 also activate in response to ischemia.

The initial vascular response to ischemic includes activation of the endothelium within and around the ischemic brain, as well as activation of circulating leukocytes. Ischemia leads to the upregulation and activation of a family of adhesion molecules involved in acute inflammatory responses, and permit interactions between endothelial cells, platelets, leukocytes, and lymphocytes, and leads to the infiltration of immune cells into the brain parenchyma after stroke [19]. Migration of these cells into the brain is mediated by cell adhesion molecules: selectins, integrins, and immunoglobulins, the expression of which is regulated both intracellularly and by cytokine signaling [20]. Activated leukocytes, namely neutrophils in the acute stages, home to the site of ischemic injury through interactions between the various adhesion molecules including the selectins (P-selectin, E-selectin, and L-selectin), immunoglobulin superfamily (e.g., ICAM-1, or intercellular cell adhesion molecule-1) and integrins (CD11a, b and c) [21, 22].

Selectins are calcium-dependent, transmembrane glycoproteins that bind to carbohydrate residues (sialyl-Lewisx), and mediate rolling and adhesion to vascular endothelium. E-, P-, and L-selectin have all been shown to be involved in leukocyte trafficking in brain ischemia [23, 10, 24]. E-selectin [25, 26], or P-selectin [9, 27] have been shown to participate in initial leukocyte rolling and recruitment [9, 25, 26, 28], whereas L-selectin guides unstimulated leukocytes to areas of activated endothelium [29]. P- and E-selectin expression have been documented in various experimental stroke models and their upregulation appears to be involved in promoting both ischemic inflammatory responses and injury [9, 26, 30-33]. In mice overexpressing P-selectin, larger infarcts have been documented. Similarly, P- and E-selectin inhibition or blocking was associated with improved neurological outcome [34, 30, 35]. However, how L-select participates in brain ischemia is less clear. L-selectin is involved in the immediate steps leading to leukocyte transmigration. When it encounters activated endothelium, it sheds from the leukocyte surface prior to entry into ischemic brain. Yet, inhibiting L-selectin shedding does not consistently influence stroke outcome. In a rabbit model of transient focal brain ischemia, treatment with an L-selectin antibody did not affect stroke outcome [36].

Once leukocytes have entered the ischemic brain, members of the immunoglobin superfamily permit binding of activated leukocytes to activated endothelium. These molecules contribute to the inflammatory response by attaching immune cells tightly to the endothelial wall, and facilitate and even stimulate diapedesis through the vessel wall to the site of injury [20, 37]. Among the immunoglobulin superfamily members, ICAM -1 and vascular cellular adhesion molecule-1 (VCAM-1) have been the most investigated in brain ischemia [7]. Expression of ICAM-1 increases soon after ischemia following activation by pro-inflammatory cytokines [7, 38, 11]. ICAM-1 is increased in ischemic brain within hours after stroke onset, and peaks at about 12- 48 h [7, 38, 11, 39, 40]. Animals deficient in adhesion molecules, or treated with strategies that block ICAM-1 have decreased ischemic damage and less brain leukocyte infiltration in experimental stroke [41-48, 7]. Further, mice lacking ICAM-1 had smaller infarcts compared to wild-type mice [49, 42].

VCAM-1's contribution to stroke is less clear. Some groups have shown that VCAM-1 mRNA and protein both increase after cerebral ischemia [50, 51], whereas others found no change in expression [47]. ONO- 1078, a leukotriene receptor antagonist, improved neurological deficits and reduced neuronal death in a model of forebrain ischemia, and this was correlated to decreased VCAM-1 upregulation in the hippocampus [52]. Another study showed that unfractionated heparin reduced infarct size in a stroke model, and this was associated with decreased VCAM-1 expression and suppression of the inflammatory response [53]. Liesz el al. [48] also found that knocking down VCAM-1 mRNA with siRNA led to reduced T lymphocyte infiltration in to the brain and decreased infarct volume after experimental stroke. However, there are conflicting results, as other studies did not show any beneficial outcome using anti-VCAM-1 antibodies [51, 48].

Integrins are adhesion molecules composed of heterodimeric combination of various α and β subunits. Prior to leukocytes binding, integrins are expressed on cell surfaces and will subunits will associate in order to recognize endothelial cell adhesion molecules [54]. Almost all leukocytes express CD11a/CD18 (leukocyte function–associated antigen-1 [LFA-1]) and CD11b/CD18, also known as Mac-1, which are integrins that contain a common β2 chain (CD18) and are thus known as β2 integrins. These integrins allow the cells to bind to endothelial ICAM-1 and migrate through the vessel. In addition to the β2 integrins, lymphocytes and monocytes express α4β1 (CD49d/CD29) and α4β7 (CD49d/CD103).

Several groups have shown that preventing neutrophil improves outcome from experimental stroke [55-59], but blocking integrins involved in lymphocyte and monocyte trafficking has also similar effects [60, 48]. Treatment of experimental stroke with anti-α 4 integrin antibody led to smaller infarct size and reduced neurological deficits [60, 61].

BLOOD BRAIN BARRIER DISRUPTION: ENTRY OF IMMUNE CELLS AND BRAIN EDEMA AND HEMORRHAGE

Disruption of the blood-brain barrier (BBB) after ischemic stroke permits infiltration of circulating immune cells into the brain, and also exacerbates edema and hemorrhage. This leads to functional impairment of the so-called 'neurovascular unit' (the functional unit including neurons, supporting glia and the cerebral vasculature as an integrated whole), which includes basement membrane tight junction proteins, transport proteins, endothelial cells, astrocytes and neurons. Molecules involved in these pathologies include the matrix metalloproteinases MMPs), cyclooxygenase (COX), nitric oxide (NO) and reactive oxygen species (ROS).

Endothelial cells are actively engaged in processes of microvascular stasis and are the first cells which face the impact of ischemia. When damaged by ischemic stimuli, endothelial cells swell or detachment from the underlying basement membrane, leading to compromises in barrier function. This leads to increased BBB permeability causing serum protein extravasation and interstitial edema as well as entry of immune molecules and cells [18, 62].

Endothelial cells from the BBB through tight junction (TJ) proteins. Among a number of TJ proteins (claudins, occludin, and zonula occludens proteins ZO-1, ZO- 2, and ZO-3), major TJ proteins are occludin and claudins, closing to the blood and junctional adhesion molecules deeper in the endothelial cell clefts [63]. Stabilization of TJ involves a complex of claudins and occludins and ZO proteins linking the transmembrane to the actin cytoskeleton. A basal lamina is composed mainly of type IV collagen, fibronectin, heparan sulfate, and laminin enveloped the abluminal surface of the endothelial cell. These function as a charge and molecular weight barrier and interact in complex ways with integrins to regulate permeability and cellular transport across the BBB [64]. Pericytes, which embedded in the basal lamina, functions a hybrid cells with both macrophage and smooth muscle properties [65]. The disruption of the BBB facilitates the entry of free water and serum from the intravascular compartment, and increases brain edema. At its most extreme, BBB disruption can lead to hemorrhage. Experimental strategies to prevent BBB disruption have largely involved the inhibition of matrix metalloproteinases (MMPs) and endogenous tissue plasminogen activator (rt-PA), proteases known to cleave and disrupt the extracellular matrix. Treatment with MMP inhibitors [66, 67] or neuroserpin, an endogenous inhibitor of rt-PA [68-70], led to improved neurological outcome and decreased cerebral hemorrhage.

LEUKOCYTE DAMAGE TO THE ISCHEMIC BRAIN

Following ischemia, infiltrated leukocytes release proinflammaroty mediators into the area of ischemia.Neutrophils initially enter the ischemic brain having been observed at about 6-12 h in models of transient focal ischemia [71, 72, 24]. Leukocytes promote cerebral ischemic injury in several different ways. First, adhesion of leukocytes to the endothelium can impair the flow of erythrocytes through the microvasculature causing the cerebral no-reflow phenomenon and additional ischemic injury [62]. Second, activated leukocytes at the surface of the endothelium produce ROS, proteases, gelatinases, and collagenases, and damage potentially salvageable blood vessels and brain tissues. Third, phospholipase activation in leukocytes results in the production of biologically active substances like leukotrienes, eicosanoids, prostaglandins, and platelet-activating factor, which can cause vasoconstriction and increase platelet aggregation. Finally, infiltrated leukocytes elaborate proinflammatory cytokines and other immune molecules in around the penumbra surrounding the infarct core causing further neuronal injury [73, 74, 45].

While the data are conflicting, lymphocytes have largely been shown to negatively contribute to ischemic brain pathogenesis. Like neutrophils, lymphocytes are also sources of pro-inflammatory cytokines and cytotoxic substances, such as ROS. 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) [75, 76].

Blocking lymphocyte entry into ischemic brain decreased injury, and suggests that like neutrophils, lymphocytes also play a deleterious role [60]. T lymphocytes, but not B lymphocytes are now considered to be the central to the development of inflammation in stroke models [77, 78]. Several recent studies have evaluated the role of T lymphocyte deficient mice in the transient focal ischemia model, and have consistently reported a smaller infarct volume and improved functional outcome than in control groups [79, 78, 80, 81]. Protection observed in lymphocyte- deficient mice subjected to stroke appears to be due to the lack of T lymphocytes, and not B lymphocytes, as the reconstitution of B lymphocytes does not affect the protection observed. By contrast, when T lymphocytes are transplanted back in to Rag1-/- mice, this protection was lost [78, 80, 81]. However, Saino el al. [82] failed to see significant differences infarct size between immunodeficient mice (deficient in both T and B lymphocytes) and wildtype. The reason for these differences is unclear, although the latter study used a model of permanent rather than temporary focal cerebral ischemia.

However, not all T lymphocytes subtypes are detrimental to acute stroke outcome. A recent study showed that neither T lymphocytes nor natural killer (NK) cells contribute to stroke injury [78]. Furthermore, the role of regulatory T (Treg) lymphocytes is still in question. Liesz *et al*. [83] showed that infarct volume and neurological deficit were significantly increased in mice given an antibody to neutralize Treg lymphocytes compared to controls. They also suggested that IL-10 signaling may be essential for this immunomodulatory effect. However, Ren *et al.* [84] could not find any modulatory effect of Treg cells. In addition, there is now evidence that resident NK cell function in the liver is profoundly impaired due to augmented sympathetic neurotransmission following stroke, and that this loss of NK cell activity substantially contributes to the immunosuppression and susceptibility to infections that occur following stroke [85].

Clinical studies also support a damaging role for lymphocytes. One study showed that increases in circulating lymphocytes correlated to an increased risk of stroke recurrence and death [86]. However, in a model of ischemia-like necrotic injury to cultured primary hippocampal neurons, isolated neutrophils worsened neuronal injury due to excito-

toxin exposure, whereas lymphocytes were not neurotoxic and actually increased astrocyte proliferation [87].

The precise mechanisms of lymphocyte-mediated brain injury are currently unclear. Classically, T lymphocytes kill bacteria- and virus-infected cells either by the release of cytokines, or cytotoxins [88], and similar actions probably occur at the site of ischemic injury. Alternatively, T lymphocytes may cause cell death via interaction with the Fas receptor [89], and a few groups showed that neutralization of T lymphocyte-derived cytokines (IL-17, IL-12, IL-23, interferon gamma) decreased infarct volume and improved neurological outcome in stroke models [90, 80, 83]. Moreover, stroke in perforin-deficient mice show significant neuroprotection, suggesting that perforin, released by T lymphocytes also contributes to ischemic damage [48]. In addition to these studies, the recent evidence showed that T lymphocytes may contribute to oxidative tissue injury following stroke, potentially via NADPH oxidase type 2 (Nox-2)-derived superoxide. T lymphocytes are known to contain a functional Nox-2 oxidase and, soon after the ischemic stroke, circulating T cells produce 7 to 15 fold greater amounts of Nox2-derived superoxide than from the control mice [91].

MICROGLIA/MACROPHAGES & ASTROCYTES

Microglia represent anywhere from 5-20 % of the total glial population and are key modulators of the immune response in the brain [92]. Microglia are often considered the brain's resident immune cell [93]. Of myeloid origin, microglia can undergo morphologic transformation from a resting state referred to as "ramified" to an "amoeboid" state, where they become virtually indistinguishable from circulating macrophages [92, 94]. Therefore, activated microglia are often called brain macrophages. Through its phagocytic properties, microglia clear foreign organisms as well as injured brain cells [71, 95-97]. Cerebral ischemia also induces microglial activation, however, the precise mechanisms of its activation following ischemia are not completely understood. Activated microglia have been documented in the cerebral cortex of the ischemic hemisphere of animals exposed to transient focal cerebral ischemia [107, 96]. Accumulating data shows that CD14 receptors, followed by toll-like receptor 4 (TRL4) have been documented in activated microglia in the infarct brain and could be one of the mechanisms involved in its activation [98-100]. Activated macrophages can be detected as early as 2 hours after ischemia, whereas blood- derived macrophages do not enter the brain before 10 hours. By 22-46 hours after the insult, activated microglia and macrophages are distributed throughout the entire lesion and are detectable up to 1 week after the insult [39, 72, 101-103]. Once, activated, microglia are thought to release a variety of inflammatory and cytotoxic mediators contributing to cell damage and cell death [104, 95]. Treatment with edaravone, a free radical scavenger which acts as a mimic to glutathione peroxidase (GPx), was found to be neuroprotective against brain ischemia mice by decreasing microglial activation [105]. Multiple hyperbaric oxygen (HBO) treatments similarly reduced infarct volume by suppressing microglia activation [106]. Minocycline, a tetracycline family antibiotic, has now been shown by a few grops to provide significant protection against brain ischemia through its ability to inhibit microglial activation [108, 109]. More direct evidence of a damaging role of microglia/macrophages was demonstrated when their direct application potentiated neuron cell death [110, 100, 96, 111]. However, microglia are also a major producer of the growth factor TGF-β1, which is generally believed to be neuroprotective [112, 95]. When microglial proliferation was inhibited in transgenic mice, infarct size was increased following ischemia, and suggests that proliferating microglia cells exert a beneficial role [113]. There are some possible mechanisms underlying these observations. First, microglia produce neurotrophic factors which stimulate neurogenesis and plasticity. Secondly, phagocytosis of neutrophils by activated microglia may prevent the release of toxic mediators [114, 115]. Finally, resident macrophages scavenge and remove necrotic debris and other potentiall harmful substances [115].

Astrocytes exert many active roles in brain homeostasis, including the regulation of immune reactions. In addition to traditional immune cells, astrocytes have also been documented to express various inflammatory mediators [116, 117]. Following brain ischemia, astrocytes are capable of activation which leads to upregulation of glial fibrillary acidic protein (GFAP). Astrocytes also contribute reactive gliosis and glial scar formation [118]. Astrocytic gliosis can be destructive after injury [119, 71]. A massive astroglial response starts in the core of the lesion from 4 hours to 1 day after the insult, and reaches a peak around 4 days and is observed up to 28 days after stroke onset [120, 121]. This glial scar has both neurotoxic and neurotrophic properties. The scar can function as a barrier which prevents axonal ingrowth and reinnervation, thus impending recovery. However, while this scar also isolates damaged tissue from viable tissue, and prevents additional damage to the surrounding brain [120]. Astrocytes are also capable of expressing various immune molecules, such as cytokines, chemokines and indicible nitric oxide synthase (iNOS) and can develop a Th2 (anti-inflammatory) immune response, although this has yet to be demonstrated in brain ischemia [122]. In a model of global cerebral ischemia, iNOS was detected in reactive hippocampal astrocytes [123]. Furthermore, astrocyte generated iNOS has been shown to exacerbate ischemia-like injury to neurons [32]. The inflammatory role of astrocytes was demonstrated in a study of tumor necrosis factor like weak inducer of apoptosis (TWEAK), a member of the tumor necrosis factor superfamily. TWEAK has been detected on neurons, astrocytes and endothelial cells, and, through interaction with the astrocytic Fn14 receptor, stimulates proinflammatory molecule production [124-126]. TWEAK and Fn14 expression have been documented in rodent stroke models, and Fn14 blockade led to reduced ischemic injury [125]. These data indicate that while astrocytes have long been viewed to play scaffolding and supportive roles for neurons, activated astrocytes can play a similarly detrimental role as traditional immune cells.

MEDIATORS OF INFLAMMATION

Activated immune cells elaborate numerous substances which mediate inflammation. They include a variety of bioactive molecules including ROS, NO, cytokines, chemokines, prostaglandins, leukotrienes, and platelet-activating factor (PAF) [127] [40, 7, 128]. These molecules all appear to act directly or indirectly to lead to worsened brain cell death.

However, other molecules, such as families of trophic factors, appear important in recovery and repair, and several anti- inflammatory cytokines have been documented to limit the pro-inflammatory response and improve outcome from stroke.

Cytokines

Increased cytokine production has been documented in the brain following various acute insults including stroke. Microglia and infiltrating leukocytes are known to elaborate cytokines, but some reports have shown that cytokines can also be produced by resident brain cells following brain ischemia, including glia and neurons, [129, 79] and in humans [130]. Interleukin-1 (IL-1), TNF- α, interleukin-6 (IL-6), interleukin-10 (IL-10) and transforming growth factor-β (TGF- β) have been the most extentively studied cytokines in brain ischemia [13]. Of these cytokines, IL-1 has been shown to mediate ischemic, excitotoxic and traumatic brain injury, probably through multiple actions on glia, neurons and the vasculature, while TNF-α might contribute to both neuronal injury and protection. IL-10 and TGF-β may be neuroprotective [131]. Reports of the role of IL-6 appear to be conflicting [132, 133, 54, 134].

IL-1 has been strongly implicated in the pathogenesis of ischemic brain as a neurotoxic mediator.

There are two IL-1 isoforms, IL- 1α and IL- 1β, plus an endogenous inhibitor, IL-1 receptor antagonist (IL-1ra) which have been studied in brain ischemia models [135, 136]. IL-1 has been shown to increase after ischemia [137, 138], and IL-1β was found to have a biphasic expression pattern, increasing initially during early reperfusion (1 h), then peaked again at a later timepoint (6–24 h) [139, 140]. IL-1β, rather than IL-1α is considered to be more engaged in the ischemic pathogenesis [141]. Administering IL- 1β to rats exposed to focal cerebral ischemia led to worsened outcome, indicating a damaging role for the protein [142]. Similarly, mice deficient in IL-1 had smaller infarcts compared to wildtype [141]. Reperfusion in diabetic rats also demonstrated higher protein level of IL-1 compared to wild type and may explain the worsening of ischemic damage by diabetes [143]. IL-1β generation results from conversion from its pro-form by IL-1β converting enzyme (ICE-1, a member of the caspase family) to its active form [144]. IL-1 has two receptors, IL-1R1, which is involved in signal transduction and IL-1R2, which has no known function but may serve as a decoy receptor [138]. In a model of brain hypoxia-ischemia, IL- 1R1 deficiency or inhibition decreased brain damage and improved neurological function [145]. Overexpression or treatment with the IL-1ra, IL-1's endogenous inhibitor, also led to neuroprotection [146, 147]. Similarly, mice deficient in ILL-1ra had worsened ischemic damage after experimental stroke [135]. Further, excitotoxic injury due to NMDA or AMPA was higher in neuron-astrocyte co-cultures derived from IL-1ra deficient mice [135]. TNF-α has also been shown to have similar expression patters and IL-1 β following brain ischemia [140], with initial upregulation 1-3 h post ischemia, and a second and peak at 12-24h [38, 148, 149]. TNF-α expression has been observed in neurons [129], astrocytes [150] in addition to the peripheral immune system [149] in stroke models.

Although TNF-α and IL-1β often work synergistically, TNF-α seems to have both neurotoxic and neuroprotective effects, while IL-1β seems generally neurotoxic [151, 152].

TNF-α inhibition has been shown to be neuroprotective against ischemic brain injury [153], while treatment with recombinant TNF-α protein post ischemia onset appears to exacerbate brain damage [154]. However, other work indicates that TNF-α may also be neuroprotective under certain circumstances. TNF- α has been linked to the phenomenon of ischemic tolerance, whereby preischemic treatment leads to improved outcome [155]. Consistent with a beneficial role of TNF, mice lacking TNF receptors have larger infarcts [156]. TNF-α released in the striatum leads to neurodegeneration, while release in the hippocampus may promote neuroprotection [157]. TNF-α stimulates apoptosis of endothelial cells and contributes to vasogenic edema and infiltration of circulatory immune cells are stimulated by this BBB breakdown. On the other hand, TNF-α activates repair processes of the cerebral microvasculature and mediates neuronal plasticity [157]. The reasons for this disparity are still unknown, but a few hypotheses have been proposed. First, TNF-α 's actions may depend on the timing. It appears to contribute to detrimental effects in the early phase of the inflammatory response, but may have more beneficial effects at a later stage [158], although this does not explain why TNF-α seems to underlie the phenomenon of tolerance. Another hypothesis relates to the receptors to which TNF-α binds. Soluble TNF-α which binds to TNF receptor 1 primarily leads to detrimental effects, whereas membrane bound TNF-α which binds to TNF receptor 2 leads to neuroprotection [95]. Other studies suggest that TNF receptor 1 signal pathways are also neuroprotective [159].

The precise role of IL-6 in the ischemic stroke has not been clearly identified. IL-6 has been shown to increase its expression continuously up to 24 hours after ischemia onset [38]. However, ischemic brain damage was not attenuated in IL-6 deficient mice or in IL-6 receptor antagonist treated mice compared to wildtype, and suggests that it probably does not contribute significantly to ischemic pathogenesis [132, 134]. Yet, Herrmann *et al.* [133] reported a beneficial effect of IL-6, while Smith *et al.* [160] showed detrimental effects. There are also reports showing strong correlation between serum IL-6 levels and in-hospital mortality rates in stroke patients [160, 161]. There has recently been a new focus on brain derived IL-6 where it appears to contribute to neoangiogenesis and neuronal survival through STAT3 activation and manganese-superoxide dismutase [162, 163].

IL-10 and IL-4 are anti-inflammatory cytokines. IL-10 acts by inhibiting proinflammatory cytokines such as IL-1 and TNF- α. IL-10 also by suppresses cytokine receptor expression and downstream signalling, and is upregulated in microglia and astrocytes following experimental stroke [164]. Both exogenous administration [165] and overexpression by gene transfer [166] of IL-10 in stroke models appears to be neuroprotective. IL-4 acts to differentiate T lymphocytes towards a Th2, or anti-inflammatory phenotype. IL-4 deficient mice were observed to have worsened outcome after experimental stroke with exacerbated pro-inflammatory responses [167].

TGF-β1 has been observed in microglia and astrocytes, with low levels in neurons [168]. TGF- β1 overexpression using an adenoviral vector improved outcome from experimental stroke, and this correlated to a reduced inflammatory response [169]. In some model systems, microglia were observed to protect cultured neurons from ischemia-like insults by secreting TGF-β1 [170].

Chemokines

Chemokines are chemotactic cytokines and, together with their receptors expressed on leukocytes, they play a crucial role in the extravasation and migration of leukocytes under inflammatory conditions. Chemokines are expressed by injured neurons, astrocytes, microglia, and endothelial cells, as well as circulating immune cells [117, 171, 172, 79]. Different classes of chemokines are differentiated by their structures, the main classes being CXC or CC, C, CX3C. The "Cs" refer to the two N-terminal cysteine residues, and the classes are divided depending on whether there is an amino acid between them (CXC), or whether they are adjacent (CC). Like cytokines, chemokines act through both unique and overlapping receptors, and these receptors are a part of a superfamily of G-protein-coupled receptors [173, 174]. The CXC subfamily can be further split into ELR+ or ELR− groups based on whether the glutamate–leucine– arginine motif is present between the N-terminus and the first cysteine [174, 175]. Members of the chemokine superfamily tend to bind to several receptors, and a chemokine receptor possibly binding multiple ligands [176]. However, the ELR+ CXC chemokine subfamily are thought to be mainly neutrophil chemoattractants, whereas the CC chemokines more typically attract monocytes and T lymphocytes [177, 79]. Several chemokines in CXC group have been shown to participate in stroke pathogenesis [178, 77] by mediating leukocyte infiltration. Because of this, chemokines have also been implicated in the worsening of stroke outcome [22]. Thus, chemokine ligands and receptors are potential therapeutic targets. Brait el al. [77] showed large increases in expression (by 10- to 300-fold) of key members of the ELR+ CXC chemokine subfamily, the neutrophil receptor CXCR2, and its ligands CXCL1 and CXCL2 which are mostly reached maximum at 24 to 72 hours. As predicted, pharmacological inhibition of CXCR2 following transient focal ischemia prevented the increases in the expression of these genes as well as neutrophil infiltration into the brain; however, this treatment had no effect on functional outcome, infarct or edema volume at 72 h after stroke.

In stroke models, CC chemokines such as monocyte chemoattractant protein-1 (MCP-1,CCL2), macrophage inflammatory protein- 1α (MIP-1α, CCL-3), regulated on activation, normal T-cell expressed and secreted (RANTES, CCL5), and macrophage inflammatory protein-3 alpha (MIP-3α) have been documented to increase in expression [38, 117, 179, 180]. At baseline, MCP-1 mRNA expression was almost absent, but ischemia led to a significant increase in MCP-1 mRNA expression in the ischemic cortex after either permanent or temporary MCAO around 12 h to 2 days and remained elevated up to 5 days [171] [172]. Inhibition or deficiency of these chemokines leads to reduced injury [181]. Similarly, overexpression of MCP-1 worsened ischemic brain injury, and this was associated with increased infiltration of inflammatory cells [182].

Fractalkine (CX3CL1) is a neuronally expressed chemokine, acts through its receptor CX3C. Its expression has been localized to viable neurons, and in brain ischemia model, it is largely localized to neurons of the infarct periphery. Its receptor, CX3CR1 was observed exclusively on microglia/macrophages, and suggests that fractalkine may be involved in neuron-microglial signaling [183]. Fractalkine deficient mice subjected to focal cerebral ischemia also have smaller infarct sizes and better outcomes compared to wild-type mice, and indicate that fractalkine contributes negatively to ischemic pathology [184]. In addition to their chemotactic properties, chemokines were discovered to directly affect the BBB. For example, when MCP-1 was added to cocultures of endothelial cells and astrocytes, this was associated loss of tight junction (TJ) proteins, suggesting that MCP-1 may be involved in BBB permeability [185]. Chemokines may also play a role in cell based therapies for stroke and related conditions. For example, they appear to be involved in honing stem cells to regions of injury [186, 188-190], and MCP-1 and SDF-1 and their corresponding receptors have been documented in the vicinity of ischemic brain tissue and transplanted cells [187]. Optimization of these signals may improve the successful application of such therapies.

Arachidonic Acid Metabolites

Following immune cell activation, the arachidonic acid (AA) cascade is initiated as a result of the release of phospholipase A2 (PLA2) [127]. Upstream of PLA2 release is the increased intracellular calcium accumulation due to energy failure and loss of ion concentration gradients. High intracellular calcium activates PLA2, which in turn hydrolyses glycerophospholipids to release AA. Increased PLA2 activity has been documented in experimental stroke models [191]. AA metabolites are signialing molecules which contribute to many post ischemic immune responses [192]. In line with a detrimental role in brain ischemia, PLA2 deficient mice had smaller infarcts and more favorable neurological outcome than wild type controls [193].

AA is further metabolized through the cyclooxygenase (COX) and lipoxygenase (LOX) pathways. Once released from brain phospholipids, AA is converted to prostaglandin H2 (PGH2) by COX, of which there are two isoforms. COX-1 is constitutively expressed, whereas COX-2 is inducible. In brain ischemia, COX-1 has been described in many cells types, including microglia and leukocytes [194]. COX-1 deficient mice have worsened outcome against brain ischemia, consistent a protective role possibly through a favorable effect on cerebral blood flow [195]. However, in a model of global cerebral ischemia, pharmacologic inhibition of COX-1 enhanced hippocampal neuron survival suggesting a damaing role [196]. The reasons for these differences are unclear, but could point to slight differences between focal and global cerebral ischemia.

COX-2, the inducible COX isoform, is essential for prostanoid synthesis. It is upregulated within ischemic borderzone areas in focal cerebral ischemia models [197]. Autopsy specimens from stroke patients have also docu-

mented the presence of COX-2 in ischemic brain regions [198, 199]. There are many functions of COX and its metabolites, but the collective literature suggests that most of these molecules are deleterious in stroke. Several studies have now shown that COX-2 inhibition improves neurological outcome in brain ischemia [197, 201]. In addition, COX-2 deficient mice are protected from injury due to N-methyl-D- aspartate (NMDA) exposure [202], whereas COX-2 over expression worsens brain injury [203]. Further, COX-2 appears to act through PGE2 rather than ROS, even though COX-2 generates both [204].

Compared to the COX pathway, less is known about the LOX pathway in brain ischemia. AA is converted to 5- hydroperoxyeicosatetraenoic acid (5-HPETE) by 5-lipoxygenase (5-LOX). 5-LOX is then metabolized to leukotriene A4 (LTA4), a precursor of cysteinyl leukotriene (cysLTs). LTA4 acts as a chemoattractant implicated in BBB dysfunction and neuronal death in ischemia. Like cytokines and other immune moleculars, biphasic AA and LTC4 expression patterns have been documented and also seem to correlate to the biphasic patterns of BBB opening [205]. 5-LOX has also been observed in post mortem ischemic human brains, typically localizing to perivascular monocytes [206]. In a brain ischemia model, treatment with AA861, a 5-LOX inhibitor, led to decreases in LTC4 levels and amelioration of ischemic brain injury [207]. In a model of *in vitro* ischemia (OGD), the 5-LOX inhibitor caffeic acid attenuated PC12 cell death [208]. However, the role of LOX in brain ischemia is not entirely clear since no protection in 5-LOX deficient mice could be observed in various experimental stroke models [209]. There are no obvious explanations for these conflicting observations, but more work in this area is clearly needed.

Nitric Oxide/Nitric Oxide Synthase

Oxidative stress can damage the organism if the physiological balance between oxidants and anti-oxidants is disrupted in favor of the former. Nitric oxide (NO) has been implicated in a variety of functions following brain ischemia. It has been documented to be involved in neuronal synapses, host defense, regulation of vascular tone, and as an inhibitor of platelet aggregation and leukocyte adhesion. Nitric oxide is generated from L-arginine through nitric oxide synthases (NOS). To date, three NOS have been studied in brain injury models. Endothelial NOS (eNOS, NOS-3), neuronal NOS (nNOS, NOS-1), and inducible NOS (iNOS, NOS-2). Of these isoforms, iNOS is perhaps the most relevant to inflammation. iNOS expression is limited almost exclusively to immune cells such as leukocytes and microglia, but has been observed in astrocytes as well [210-212]. In addition to its signaling properties, NO may also react with superoxide to form peroxynitrite, and even more reactive specia that may cause DNA damage [213, 30]. Several studies have now shown that iNOS inhibitors are neuroprotective [211], and iNOS deficient mice have better outcomes from stroke [214].

Furthermore, therapeutic hypothermia and neuroprotection by estrogen and progesterone is associated with reduced iNOS generation, indicating that NO/iNOS play a damaging role [215-217].

Reactive Oxygen Species

Reactive oxygen species (ROS) production by inflammatory cells occurs via several enzyme systems. Superoxide is generated via COX, xanthine dehydrogenase, xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Hypochlorous acid and H2O2 are generated through myeloperoxidase (MPO) and monoamine oxidase (MAO) [218]. ROS are an important underlying factor in delayed neuronal death induced by cerebral ischemia–reperfusion. During reperfusion, robust oxidants are generated and are directly involved in the damage to cellular macromolecules, such as lipids, proteins, and nucleic acids, eventually leading to cell death [219].

Superoxide can be produced in phagosomes, which contain ingested bacteria and fungi, or it can be produced outside of the cell. In a phagosome, superoxide can spontaneously form hydrogen peroxide that will undergo further reactions to generate ROS. Vascular ROS are produced in endothelial, adventitial, and vascular smooth muscle cells and derived primarily from NADPH oxidase (NOX), a multisubunit enzyme catalyzing a $O2^{\bullet-}$ production by the 1 electron reduction of oxygen using NADPH as the electron donor: 2O2 + NADPH → 2O2- + NADP + H+ [220]. NOX was originally identified in immune cells as playing an important microbicidal role. NOX consists of cytoplasmic subunits (p45phox, p67phox, and p40phox and Rac2) and upon phosphorylation, these subunits can form a complex and translocate to the plasma membrane to dock with the plasma membrane subunits (p91phox, p22phox) [221]. Catalysis of NOX occurs in the p91phox subunit (Nox2) and is initiated by transferring of electrons from molecular oxygen through redox coupling with NADPH, FAD and heme to produce superoxide anion [222] (Fig. **1**).

Immune cell generated NOX (NOX2) also appears important in the maintenance of vascular integrity. The addition of microglia to endothelial cell and astrocyte cocultures worsens ischemia-like injury, and inhibiting superoxide production preserved these BBB constituents in an *in vitro* model [223], and reduced brain edema formation, matrix metalloproteinase-9 (MMP-9) expression [224], BBB disruption, hemorrhagic transformation [225] and immune cell responses in *in vivo* stroke models [226]. Thus, NOX contributes to BBB disruption.

NOX is also expressed in the central nervous system. *In vitro* studies have shown NOX expression in neurons, astrocytes, and in microglia [222]. Immunohistochemical studies have shown that NOX subunits are widely distributed in the cortex, the hippocampus, and in the cerebellum *in vivo* [227-230]. NOX has been documented to increase in the brain after experimental stroke [231] and we have shown that NOX derived from circulating cells contributes significantly to stroke pathogenesis compare to the brain resident cells [232]. Walder el al. [233] showed that NOX2 deficient mice are protected from experimental stroke, and work from our lab has shown that microglia derived NOX2 leads to BBB damage [223]. Further, NOX appears to significantly contribute to reperfusion injury, as reperfusion permits the restoration of glucose to the ischemic brain. The restoration of glucose (rather than oxygen, which is traditionally thought to be a source of ROS in this setting) appears to 'fuel' NOX by

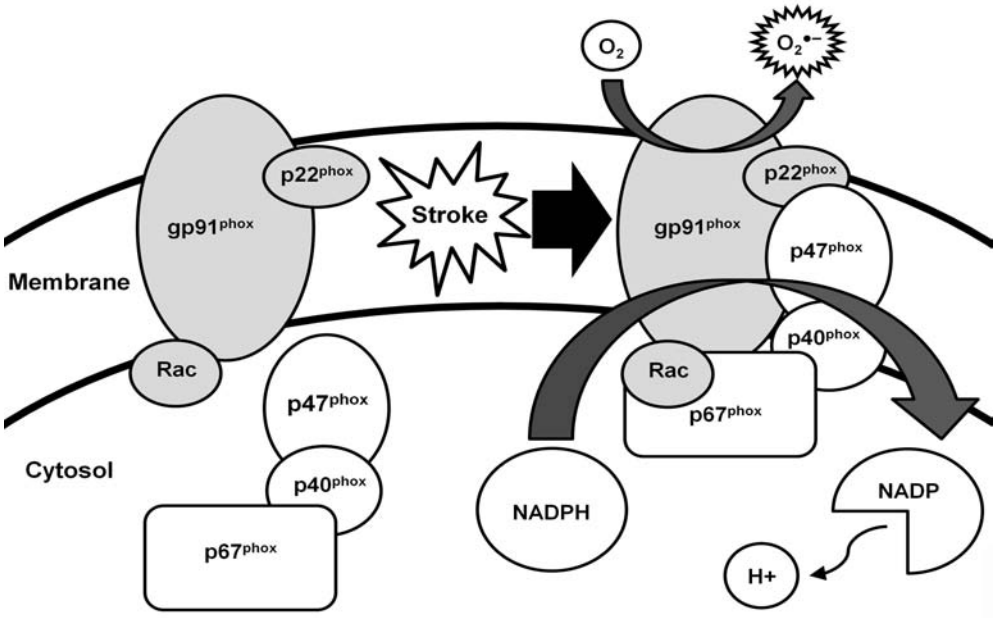

Fig. (1). The structure of the active NADPH oxidase complex in stroke. The NADPH oxidase comprises a cytosolic subunits (p47phox, p67phox, p40phox and Rac) and membrane subunits (gp91phox and p22phox) which associate with this complex in the activated enzyme. The NADPH-binding domain is predicted to be on one side of the membrane, whereas O2●– generation is predicted to occur on the other in stroke.

serving as an electron donor to produce damaging levels of superoxide [234]. Interestingly, reperfusion in the presence of glucose appears to increase neuronal NOX activity and NOX deficiency or inhibition prevents this. NOX also appears to be a primary source of ROS generated by NMDA receptor activation [235].

A few studies have examined the therapeutic potential of treatment with the NOX inhibitor apocynin. Apocynin, the active compound found in Picrorhiza kurroa, a botanical plant used as an herbal medicine for treatment of a number of inflammatory diseases. From our own lab, we found that a dose of 2.5 mg/kg given parenterally just prior to reperfusion, or 1.5 h after ischemia onset, resulted in reduced infarct volume and improved neurological outcome [225]. We also found that $O2^{\bullet-}$ is largely generated in neurons and some microglia/monocytes, with no generation in brain vascular endothelial cells. Apocynin markedly reduced $O2^{\bullet-}$ in the brain. However, apocynin at higher doses (3.75 and 5 mg/kg) failed to show any benefit, and actually increased the severity of brain hemorrhage. Thus, this rather narrow therapeutic dose range may limit its translation to the clinical level. However, other groups have shown salutary effects of apocynin at doses as high as 50 mg/kg [236, 237]. In global cerebral ischemia, 5 mg/kg apocynin attenuated hippocampal injury when given prior to ischemia onset [238].

Myeloperoxidase (MPO), present in leukocytes including neutrophils and monocytes, mediates bactericidal killing through H2O2 and hypochlorous acid. MPO has been documented within infiltrating neutrophils following both permanent and transient MCAO [181]. However, MPO deficient mice had worsened outcome following experimental stroke, suggesting a protective role rather than a damaging one [239]. There were also more products of nitrosylation within the ischemic brain of MPO deficient mice, suggesting that MPO's protective effect may be due to its ability to scavenge nitrotyrosine (a by product of peroxynitrite reactions) [239]. Thus, MPO may decrease ROS induced ischemic injury, rather than potentiate it.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of essential proteases that break down components of extracellular matrix. Physiologically, these proteases participate in tissue development, wound healing, cone growth, ovulation and angiogenesis, however, after brain ischemia, MMPs are upregulated, activated, and involved in both neuroinflammatory response and extracellular matrix remodeling. Under physiologic conditions, many MMPs exist as a pro- or inactivated protein, but undergo activation after cleavage by other proteases such as plasmin or other MMPs [240]. Tissue plasminogen activator (tPA) has been shown to disrupt the BBB due to MMP-9 resulting in hemorrhagic transformation [241, 242]. Microglia are a major source of the MMPs in brain ischemia. The y also stimulate astrocytes to generate active MMPs [243]. In models of focal ischemia, MMP-9 activity increases at early time points (15-48 h) and returns to baseline around 15 days, followed by MMP-2 (peaks at 5 days and return to baseline around 15 days) [244, 245]. MMPs are also involved in receptor mediated (extrinsic pathway) apoptotic neuronal cell death by processing TNF-α and FasL. In stroke models, MMP inhibition is beneficial and not only decreases infarct size, but ameliorates brain edema and hemorrhage a well [246]. Similarly, mice lacking MMP-9 had smaller infarcts compared to wildtype mice [247]. However, no such effect was observed in MMP-2 deficient mice [248], suggesting that MMP-9 may be primarily involved with edema, while MMP-2 may correlate more with neovascularization [249]. Further, bone marrow chi-

mera models showed that circulating immune cells, rather than brain derived MMP-9 may contribute significantly to ischemic brain injury. Mice transplanted with MMP-8 deficient bone marrow suffered less injury and BBB disruption than mice transplanted with marrow containing intact MMP-9 [250]. MMPs may be involved in neuron migration. Animals given the broad spectrum MMP inhibitor GM6001 were found to have less migration of newly formed neurons in the migratory stream after transient focal cerebral ischemia in mice [251].

MMPs also seem to play a differential roles depending on the phase of ischemic injury. During the later phases, they appear to participate in plasticity and recovery. MMPs were found to associate with factors involved in angiogenesis, including vascular endothelial growth factor (VEGF). Treatment with the MMP inhibitor FN-439 suppressed neurovascular remodeling in a stroke model, and impaired functional recovery while reducing VEGF signaling [252].

Transcriptional Regulation of Inflammation

Cerebral ischemia is well known to upregulate gene expression. Transcription factor activation has been studied in several brain ischemia models, and some of these factors are involved in the inflammatory response (Fig. **2**).

Nuclear Factor κB (NF- κ B)

NF-κB is a classic transcription factor involved in the activation of inflammatory responses [253]. It is a heteromeric transcription factor consisting of various combinations of subunits from the Rel family: Rel (cRel), RelA (p65), RelB, NF- κB1(p50 and its precursor p105) and NF- κB. The most common form is composed of Rel A (p65) and p50. NF- κB is normally sequestered in the cytoplasm bound to its inhibitor protein, IκB. Phosphorylation of IκB by IκB kinase (IKK) leads to IκB phosphorylation, ubiquitination and degradation. This liberates NF- κB to translocate to the nucleus, and bind to concensus κB sites, promoter domains present in many pro-inflammatory genes, such as tumor necrosis factor- α (TNF-α), intercellular adhesion molecule-1 (ICAM-1), cyclooxygenase-2 (COX-2), iNOS and interleukin-6 (IL-6). Therapeutic hypothermia in a stroke model was correlated to IKK and NF- κB inactivation [13]. However, NF- κB also regulates genes involved in neuroprotection, and NF-κB's role in stroke is controversial [254]. Mice deficient in the p50 subunit of NF-κB or treated with a NF-κB inhibitor are protected from brain ischemia [255, 33, 257]. Similarly, inhibition of IKK reduced infarct size [256], and activation of IKK enlarged the infarct size [256]. However, NF-κB inhibition with diethyldithiocarbamate (DDTC) increased infarct size, suggesting a beneficial role [258]. The reasons for these differences are not entirely clear, but have been postulated to be dependent on the cell type in which NF-κB is activated. It is also possible that these differences are due to the experimental model studied, or off target effects of the pharmacological inhibitors.

Mitogen-activated Protein Kinase (MAPK)

MAPKs are known to transduce stress-related signals through a cascade of interlinked signaling pathways which lead to inflammatory induction [3, 259]. In brain ischemia models, the stress-activated protein kinases/c-Jun N-terminal kinases (SAPK/JNK), the p38 MAPKs and extracellular signal-regulated kinases (ERKs) have been described [259-

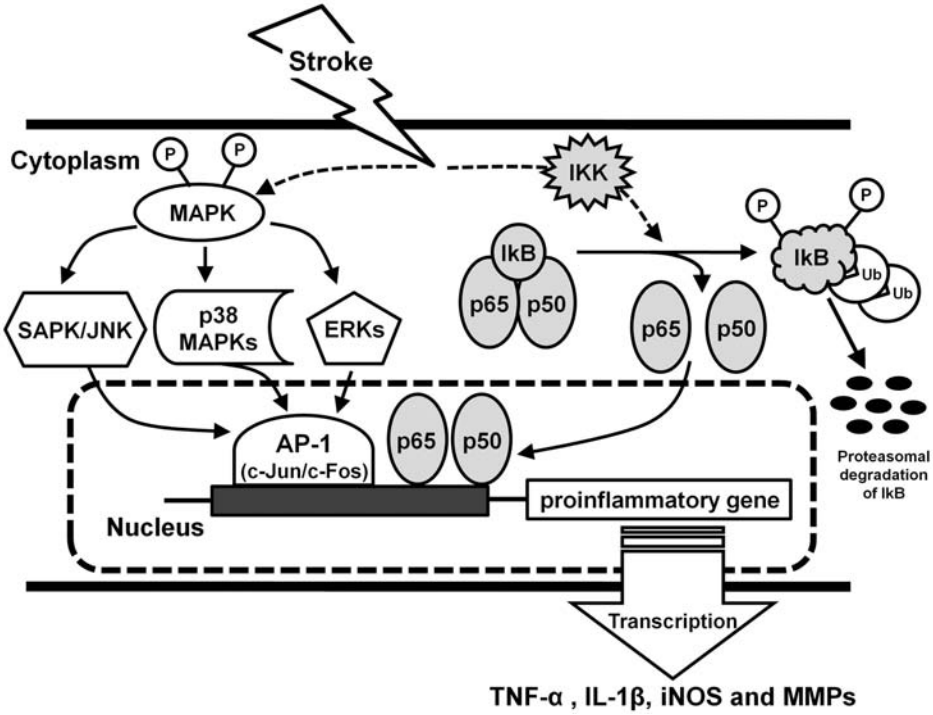

Fig. (2). Several transcription factors have been documented in the inflammatory response, Ischemic injury activates IKK and MAPK cascade to stimulate IkB, SAPK/JNK, p38 MAPKs and ERK phosphorylation, leading to transcription factors; NF-kB (subunit p50 and p65) and AP-1 (phosphorylation of c- Jun and upregulation of c-Fos) activation and then enhances pro-inflammatory genes expression.

261]. p38 MAPK is involved in the stabilization and translation of several proinflammatory mRNAs [262], and activation of this pathway was observed to occur 30 min and 3 days following brain ischemia [263]. Phosphorylated p38 MAPK was detected in neurons [261] and microglia [265] of ischemic brain tissue, and suggests its role in the inflammatory response. Interruption of this pathway at its apex by knockdown of POSH (Plenty of SH3s) attenuated downstream signaling and significantly increased neuron survival. In cultured astrocytes exposed to *in vitro* ischemia, the addition of bone marrow stromal cells activated MAPK, and subsequently led to cytoprotection [264]. Treatment with CDP-choline, a neuronal membrane lipid precursor, led to improved recovery after ischemic stroke with reduction in phosphorylation of MAPK family members, ERK1/2 and MEK1/2, and Elk-1 transcription factor. Similar studies of p38 MAPK inhibitors have shown reduction in brain injury and improvement in neurological deficits against focal cerebral ischemia along with reduction in ischemic-induced cytokine production [3].

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 [14, 266]. Toll-like receptors (TRLs) and purinergic receptors are widely expressed on microglia [14, 267].

Toll-like receptors (TLRs) are a large of pattern recognition receptors that recognize exogenous pathogen-associated molecular patterns (PAMPs) and endogenous DAMPs. They have been the focus of recent investigation, and are considered to play critical roles in the initiation of the immune response in stroke and related injuries [268, 269]. TLRs have traditionally been found on immune cells, but they have also been described in various cell types of the central nervous system (CNS), including microglia, astrocytes, neurons, and cerebral vascular cells [270, 271]. Reports in the brain ischemia literature indicate that TLRs are most likely activated by DAMPs, such as heat shock proteins, high mobility group box 1 protein (HMGB1) [272], extracellular peroxiredoxin [273] and nucleic acids [14].

Activation of microglial cells in response to cerebral ischemia is associated with signaling through several TLRs, especially TLR2 (TNF-α, IL-6, IL-10), TLR3 (TNF-α, IL-6, IL-10, IL-12, CXCL-10, IFN-β), and TLR4 (TNF-α, IL-6, IL-10,CXCL- 10, IFN-β), yet astrocytes initiate only minor IL-6 responses to all but TLR3 stimulation [274]. The TLRs signal through intracellular pathways leading to transcription factor and activation and the generation of cytokines and chemokines [275].

Among TLR family, TLR2 and 4 has been shown to a key player in cerebral ischemic damage. Brain TLR2 expression is increased in transient and permanent focal ischemia models as well as *in vitro* ischemia models [276-278]. TLR2- deficient mice have less CNS injury compared with controls in a model of focal cerebral ischemia [237]. TLR2 was expressed mainly in microglia in post-ischemic brain tissue, but also in selected endothelial cells, neurons, and astrocytes; TLR2- related genes with pro-inflammatory and pro-apoptotic, such as NF-κB, Cyclooxygenase-2 (COX2), IL-1β, IL-17, IL-23, were also induced after ischemia [276, 278-280]. In a model of transient focal cerebral ischemia, infarct volume in TLR2 -deficient mice was significantly smaller compared to wild-type mice. Therefore, TLR2 upregulation and signaling are important events in focal cerebral ischemia and contribute to neuronal damage [276]. A recent study demonstrated that inflammatory signaling of the TLR2 heterodimer TLR2/1 in the post-ischemic brain requires the scavenger receptor CD36 [281]. The link between CD36 and TLR2/1 was specific for brain inflammation because CD36 is required for TLR2/6 (another TLR2 heterodimer) signaling. Another study demonstrated that TLR2 mediates leukocyte and microglial infiltration and neuronal death, which can be attenuated by TLR2 inhibition [282]. TLR 2 knockout mice also experienced higher mortality and increased infarct size compared to wildtype mice [283].

Brain expression of TLR4 in ischemic brain is also up regulated [284, 285] and plays a crucial role in the innate immunity of the CNS [286]. Numerous studies demonstrate that TLR4 participates in ischemic injury. Several studies confirm that cerebral ischemia results in the upregulation of TLR4 mRNA in neurons as early as one hour after initiation of cerebral ischemia model [237, 287]. TLR4-deficient mice exhibit reduced infarct size compared with wild-type mice after cerebral ischemic injury [288, 289, 285, 290]. Following MCAO and forebrain ischemia, these mice exhibited improved neurological behavior and reduced edema, as well as reduced secretion of proinflammatory cytokines such as TNF-α and IL-6. In addition, TLR4 knock-out mice have reduced expression of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2), and IFN-γ [285, 289]. Moreover, after MCAO, loss of TLR4 function is associated with reduced expression of p38 and Erk1/2 in damaged neurons, implicating TLR4 in MCAO injury. Taken together, these studies indicate that TLR4 signaling contributes to the severity of ischemia-induced neuronal damage. Thus, targeting TLR signaling may be a novel therapeutic strategy of inflammatory for cerebral ischemic injury.

The purinergic receptors are found in numerous cell types, both in brain and peripherally, and mediate a variety of cellular functions. The P2 purinoreceptors consist of two families: the ionotropic receptors (P2X) contain channels that permit ion flow, whereas the metabotropic receptors (P2Y) are G-protein coupled second messenger systems. In immune cells, they are involved in pro-inflammatory responses, migration, and phagocytosis [291]. The family of

purinergic receptors on microglia have recently become of interest because they bind to nucleotides that may be released by injured cells, and may initiate proinflammatory signaling. The role of the purinergic receptors in the microglial inflammatory response has largely focused on P2X7, where it has been shown to modulate microglial activation following experimental brain ischemia and stroke, and its pharmacologic blockade led to decreased ischemic damage [292-295]. However, little has been studied on the Gi coupled ATP receptor, P2Y12 [296, 291]. P2Y12 is present on microglia and is expressed on the membrane surface in the resting state and activated by ATP, ADP, or neighboring neurotoxicity. It promotes microglial migration toward the source of these nucleotides and involves in the phosphorylation of Akt [297]. Because P2Y12 is also the target of a widely used antiplatelet agent, clopidogrel, it is an attractive target for modulating the microglial inflammatory cascade. We have recently shown that P2Y12 participates in ischemia related inflammation by mediating microglial migration and potentiation of neurotoxicity using P2Y12 knockout mice [298].

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 also synthesizes antibodies in its white pulp and removes antibody coated blood cells from blood and lymph node circulation. Ajmo *et al.* [299] have shown that removal of the spleen significantly reduced neurodegeneration after brain ischemia. Rats splenectomized 2 weeks before permanent middle cerebral artery occlusion had a >80% decrease in infarction volume and splenectomy also resulted in decreased numbers of activated microglia, macrophages, and neutrophils present in the brain tissue. They concluded that these results demonstrate that the peripheral immune response as mediated by the spleen is a major contributor to the inflammation that enhances ischemic damage after stroke.

Yet, 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 occurs as early as 12 hours after ischemic stroke, and may continue out to several weeks [12, 83, 300]. This phenomenon involves reduced numbers of T cells and other immune cells present in the spleen, thymus, liver and lymph node [300, 83, 301, 12, 149], and is considered to be mediated by hyperactivity of the sympathetic nervous system (SNS) and the hypothalamic- pituitary-adrenal axis (HPA) [12, 302]. This leads to increased apoptosis of immune cells in these organs and as a result, these secondary lymphatic organs undergo atrophy [83, 149, 12]. However, it has yet to be shown whether the infiltration of these cells into the brain is what contributes to the lower circulating cell numbers.

As a consequence of this phenomenon, infectious complications often arise after stroke, predominantly lung and urinary tract infections in animal models [83, 149, 12] which lead to worsened outcome [303-305]. Hyperactivity of the sympathetic nervous system (SNS) and the hypothalamic pituitary axis (HPA) is thought to underlie this phenomenon, although the precise signals and mechanisms that trigger this immunodepression remain unclear. Blocking the SNS and HPA significantly reversed the percentage of apoptotic splenocytes to control levels and prevented the decrease in circulating lymphocytes, bacterial infections and mortality following stroke in the animal model [302, 12]. Consistent with experimental reports, several recent clinical studies have found evidence that SNS- mediated stroke-induced immunodepression and subsequent susceptibility to post stroke infections also occurs in patients. Chamorro *et al.* [4] found that acute ischemic stroke is associated with an early activation of the sympathetic adrenomedullar pathway that lowers the threshold of infection and increases the mortality. Urra *et al.* [306] reported increased apoptosis and a reduction in the circulating levels of T and B lymphocytes following stroke. They also found a correlation between SNS and HPA activation, lower level of T lymphocytes and infection. In addition to this, another pathway of communication between the nervous and immune systems, known as the vagal cholinergic anti-inflammatory pathway has been indentified. When the vagus nerve is activated by pro- inflammatory cytokines, it releases acetylcholine, which results in inhibition of the release of more pro-inflammatory mediators by macrophages [307, 308]. Experimental studies have shown that vagal nerve signaling inhibits the release of pro-inflammatory cytokines and improves outcomes following different models of ischemic stroke [308]. This vagal cholinergic anti-inflammatory pathway is another potential mediator of immunodepression.

Clinical Approaches

Following several promising preclinical studies, a few clinical trials were carried out to determine whether anti-inflammatory strategies were beneficial (Table **1**). However, some of these trials did not meet with success either due to unanticipated effects of the agents tested or inappropriate study design [309-311]. Yet, with increased knowledge of the complexities of inflammation in stroke, a few treatments may be on the horizon. In order to limit the leukocyte adhesion and migration into the infarct brain, clinical studies have studied anti-integrin therapies in stroke patients. In the HALT study, a humanized antibody against the integrin CD11/CD18 was given to patients who presented within 12 h of symptom onset [310]. There was also a phase IIb study of recombinant neutrophil inhibiting factor (rNIF), a nonantibody peptide, in stroke patients (Acute Stroke Therapy by Inhibition of Neutrophils or ASTIN) who presented within 6 h of symptom onset [312]. Neither study showed any beneficial effect, as determine by study endpoints, and were terminated prematurely, although both compounds showed benefit in animal models [313, 58], The reasons for an absence of a beneficial effect in humans may multifold, stemming from incomplete preclinical testing, to the heterogeneity of clinical stroke. Another possibility is that neutrophil integrins are

Table 1.

Agent	Representative Name	Design	Phase	n	Status	Results	Ref.
Anti-ICAM1	Enlimomab Acute Stroke Trial	Efficacy and safety of enlimomab versus placebo	III	625	Completed	Not effective, may worsen outcome.	[309, 311]
Anti-integrin	The HALT Stroke Study	Trial of Hu23F2G anti-adhesion to limit cytotoxic injury in acute ischemic stroke	III	-	Aborted	Stopped early for futility	[310]
	ASTIN	Trial of recombinant neutrophil inhibitory factor	II	966	Aborted	Stopped early for futility	[312]
Minocycline		Trial of 200 mg oral minocycline to evaluate its efficacy	Open-Label	152	Completed	Treated patients showed better NIHSS, mRS and B.I.	[335]
	MINOS	Dose-Finding study of minocycline	nonrandomized, dose-escalation trial	60	Completed	Showed safe up to dose of 10 mg/kg i.v.	[336]
	MINOS (Sub-analysis)	Determine the impact of i.v. minocycline of MMP-9 expresion	nonrandomized, dose-escalation trial	60	Completed	Showed lower MMP-9 expression with the minocyline administration	[336]
	NeuMAST	Determine the efficacy of minocyline in long term recovery	IV	-	Aborted	Stopped early for futility	NCT00930020

Abbreviations: NIHSS, NIH Stroke Scale; mRS, modified Rankin Scale; B.I., Barthel Index.

different in acute ischemic stroke patients compared to rodents. While CD11b is thought it increase in animal stroke models [315], it is actually decreased in human stroke [314]. Therefore, this approach would not be expected to work in humans. Nevertheless, more work in this area including improved trial design and guidelines for preclinical development are needed.

An antibody against ICAM-1 (enlimomab) was studied at the phase III level in stroke patients. However, it, too, was not an effective treatment for ischemic stroke [309, 311]. Not only did this approach not benefit patients, but those who received treatment had worsened stroke outcome. Reasons for this worsened outcome have been attributed to the fact that the Enlimomab antibody is a murine antibody, and possibly not suited for use in humans. The thought being that the murine antibody itself could lead to unwanted neutrophil and complement activation. It also could be said of these trials that the therapies interfered with endogenous immunoregulatory defenses to promote the development of clinically significant infections and fever, thereby negating any potential cerebroprotective effects. This deleterious immunomodulation is not entirely unexpected, because ICAM-1 and integrin are critical to numerous host defenses such as leukocyte adhesion, diapedesis, oxidative burst, and selectin expression [21]. Second, proinflammatory microvascular failure leading to "no-reflow" might be important in rodent stroke, yet of limited relevance to primate stroke, due to differences in cerebrovascular collateralization [316].

Therapeutic Hypothermia

The role of the hypothermia in brain ischemia is well described in our recent review [317]. It has largely been embraced at the clinical level, as it has been shown to improve neurological outcome following cardiac arrest. Because therapeutic hypothermia affects pathways leading to excitotoxicity [318], apoptosis [319], inflammation and free radical production, as well as blood flow, intracranial pressure [320], metabolism [320] and blood-brain barrier integrity in acute, subacute and chronic stages of ischemia, it is likely that no single factor can explain the neuroprotection provided by hypothermia, however, from the concept of this review, here we will discuss the effect of hypothermia from the inflammation aspect.

Hypothermia affects many aspects of immune response. It lowers numbers of neutrophils and activated microglia in the ischemic area [321, 322] and reduces levels of many inflammatory mediators including ROS [323] and reactive nitrogen species [215], adhesion molecules [321, 322], proimflammatory cytokines (such as IL-1), TNF-α, and IL-6, IL-10) [324] and the Chemokine such as macrophage inflammatory protein-3α (MIP3) and its only receptor C-C chemokine receptor 6 (CCR6) [180]. Hypothermia also suppresses the activation of NF-κB [13, 325], one of the most important transcription factors playing a pivotal role in activating many inflammation-related genes. However, NF-κB also regulates genes involved in cell survival and growth; thus, the net effect of hypothermia-induced suppression of NF- κB activity is difficult to predict. Hypothermia also af-

fects the mitogen-activated protein kinase (MAPK) pathway, another important enzyme system that regulates inflammation [326, 327].

However, anti-inflammatory cytokines such as IL-10 and TGF-β are also reduced by hypothermia as well, indicating that hypothermia does not have a purely anti-inflammatory effect [328, 329]. Regardless, hypothermia has a largely suppressive effect on inflammation, and this anti-inflammatory property might serve as a major protective mechanism under ischemic conditions.

Minocycline

Minocycline is a member of the tetracycline family of antibiotics with recently recognized anti-apoptotic, anti-inflammatory properties, reduction of microglial activation, MMP reduction, and NO production. The anti-apoptotic properties of Minocycline appear to be due to tis ability to inhibit caspase-3 [330], whereas its anti-inflammatory effect appears to be due to a mechanism inhibiting MAPK activation in microglia [331]. As such, Minocycline has been shown to protect the brain against ischemic insults and improve functional impairment [332, 109, 333, 334]. According to these results, Lampl *et al.* [335] have conducted a clinical trial using oral Minocycline administration to the ischemic patients and found that both NIHSS and mRS were significantly lower and BI scores were significantly higher in minocycline-treated patients. This pattern was already apparent on day 7 and day 30 of follow-up. Deaths, myocardial infarctions, recurrent strokes, and hemorrhagic transformations during follow-up did not differ by treatment group. However, this study was an open-labeled, evaluator-blinded study and the total number of the patients were relatively small. A second study established safety, dose ranging and feasibility in combination with rt-PA [336]. Clinical studies to determine minocycline's efficacy in stroke and long term recovery (Neuroprotection with minocycline therapy for acute stroke recovery trial, NeuMAST), but was recently terminated due to futility. However, large prospective randomized trials are still needed.

Fingolimod (FTY 720)

FTY720 is a novel immunomodulatory agent, which in its phosphorylated form acts as a high affinity agonist of Sphingosine-1-phosphate (S1P) receptors [337, 338]. It became the first oral drug to be FDA-approved for clinical use in the treatment of multiple sclerosis. FTY720 readily crosses the blood-brain barrier and exerts a number of direct effects in the central nervous system. FTY720 is phosphorylated by Sphingosine kinase (SphK), mainly by SphK2 [339, 340], into the active compound phospho-FTY720, which then acts on 4 of the 5 known S1P receptor subtypes (S1P1, S1P3, S1P4, S1P5), and shows neuroprotective effect against many central nervous system disease including cerebral ischemia [341-344]. Mechanisms include regulation of myelination and microglial activation following injury, proliferation and migration of neural precursor cells toward injury sites, and potentiation of growth-factor regulated neuronal differentiation, survival, and process extension, and also antiapoptotic and anti-inflammatory pathways [344-349, 343]. FTY720 also exerts immunomodulatory actions by affecting lymphocyte production, trafficking, and apoptosis through S1P receptors which induces a depletion of circulating lymphocytes by preventing the egress of lymphocytes from the lymph nodes. Mechanistically, this is due to a down regulation of the S1P type 1 receptor (S1P1). Expression levels of endothelial adhesion molecules such as E-selectin, P-selectin, ICAM-1 or VCAM-1 were shown to be induced by FTY720 treatment, and therefore might contribute to the prevention of early infiltration of neurotrophils and activation of microglia/macrophages. These findings suggest that anti-inflammatory mechanisms, and possibly vasculoprotection, rather than direct effects on neurons, underlie the beneficial effects of fingolimod after stroke. Most of the past reports have shown beneficial effect of S1P in the field of ischemia, but by contrast, Liesz *et al.* [48] showed opposite results. These authors found that S1P treatment did show a reduction of lymphocyte brain invasion but could not achieve a significant reduction of infarct volumes and behavior dysfunction [48]. Liu *et al.* [350] recently published a systematic meta-analysis of the efficacy of FTY720 in animal model of stroke. In this study, they concluded that FTY720 reduced infarct volume and improve functional outcome. However, the authors also indicated that more experimental studies should be performed to evaluate the safety of FTY720 in the future. Thus, taken this recent scientific highlights together, it is obvious that S1P receptor pathways and sphingolipids regulating enzymes are a highly promising target in stroke treatment.

CONCLUSION

Inflammation following ischemic stroke is increasingly recognized as a key element in its progression. The role of inflammation in stroke has become an increasingly popular area of investigation, given its pleotropic roles in both acute damage and long term recovery. In the early phases, accumulated research indicates that it appears to play a mostly detrimental role. However, inflammatory processes are also important to recovery and repair, processes that occur weeks to months later. Thus, immune modulating interventions should be tailored to the specific phase of stroke, as most certainly, inhibiting the same processes at the wrong time could prove damaging. However, what these time windows are, and how to best intervene are largely unknown, but areas ripe for future investigation.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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