REVIEW

Mechanisms and Potential Therapeutic Applications of Microglial Activation after Brain Injury

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SUMMARY

As the resident immune cells of the central nervous system, microglia rapidly respond to brain insults, including stroke and traumatic brain injury. Microglial activation plays a major role in neuronal cell damage and death by releasing a variety of inflammatory and neurotoxic mediators. Their activation is an early response that may exacerbate brain injury and many other stressors, especially in the acute stages, but are also essential to brain recovery and repair. The full range of microglial activities is still not completely understood, but there is accumulating knowledge about their role following brain injury. We review recent progress related to the deleterious and beneficial effects of microglia in the setting of acute neurological insults and the current literature surrounding pharmacological interventions for intervention.

Introduction

Inflammation within the central nervous system (CNS) can produce a variety of acute neurological insults including stroke, traumatic brain injury, and others that lead to brain cell damage and death [1]. Microglia are the resident macrophages of the brain, and they are a key player in its immune responses [2–4]. Microglia constitute 15% of the total glial population, with predominance in the gray matter [5]. They respond to extracellular signals and are involved in clearing debris and toxic substances by phagocytosis, thereby retaining normal cellular homeostasis in the CNS [6]. Consequently, under nonpathological states, there is continuous, low-level microglial activity in the CNS which is primarily involved in activity-dependent synaptic pruning and repair [2]. In the event of acute brain insults such as trauma, ischemia, and neurodegeneration, microglia quickly activate by undergoing morphologic transformation from a "ramified" resting state, characterized by many branching processes, to an active, motile "ameboid" state. In the ameboid state, microglia are difficult to distinguish from circulating macrophages and monocytes, not only morphologically, but also with regard to surface markers and function [7,8]. When activated, microglia have been shown to release a variety of inflammatory and cytotoxic mediators contributing to cell damage and cell death

leading to exacerbated brain injury [9,10]. Harmful effects appear to be predominant particularly in the acute stages of brain injury, while beneficial activities emerge in later stages. These studies indicate that different signals lead to two primary activation phenotypes: classically activated (M1) and alternatively activated (M2) [11–13] (Figure 1). The M1 phenotype, activated by lipopolysaccharide (LPS) and the pro-inflammatory cytokine interferonγ (IFNγ), promotes transcriptional activation of nuclear factor-κB (NF-κB) and generates high levels of pro-inflammatory cytokines and oxidative metabolites such as interleukin (IL)-12, tumor necrosis factor (TNF)-α, IL-6, IL-1β, and nitric oxide (NO), formerly indicated to cause additional damage. In contrast, the M2 phenotype is triggered by antiinflammatory cytokines such as IL-4 or IL-13 [14,15], which are thought to inhibit inflammation and enhance tissue repair and wound healing [16]. Microglial activation may initiate with an M1 phenotype that mediated an innate or an adaptive immune response and ultimately exacerbates neuronal damage [17,18]. Microglia may then adopt the M2 phenotype and facilitate repair-oriented functions by secreting growth factors and by clearing cellular debris via phagocytosis. M2 microglia also tend to limit pro-inflammatory signal production. This highlights the significance of identifying and understanding the different microglial phenotypes and their unique functions [19,20]. Furthermore, microglia present a
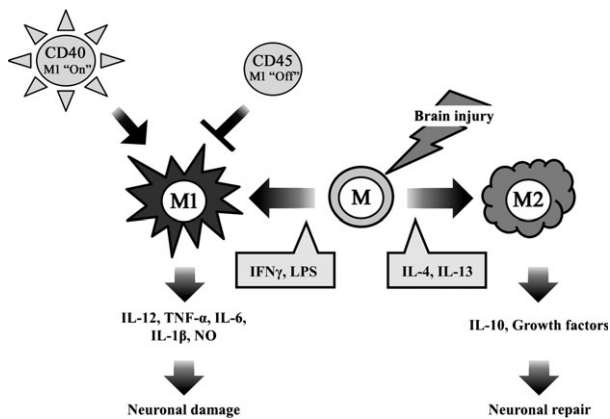
Figure 1 Microglial activation in brain injury. Microglia are activated by brain injury such as ischemic stroke and traumatic brain injury, which affect the polarization status of the cell. In brain injury, resting microglia (M) can be polarized to the M1 phenotype, or alternatively to the antiinflammatory, pro-phagocytic M2 phenotype by the some of factors shown in the gray boxes. M1 microglia take part in the neuronal damage by elaborating pro-inflammatory molecules, whereas M2 microglia promote neuronal protection, and through phagocytic functions, set the stage for recovery and repair. While not extensively studied in brain injury, CD40 leads to the M1 phenotype, whereas CD45 turns this "off."

potential target for therapeutic intervention with various time windows depending on the functions they contribute. Recent work has also shown that microglia can switch from the M1 phenotype to the M2 phenotype. In HIV-associated dementia [21] and spinal cord injury [22], microglia have been shown to initiate and maintain a M1 phenotype in the presence of CD40 ligation by CD40L and tumor necrosis factor (TNF), but may switch or "polarize" to the M2 phenotype through upregulation of CD45. In a study of aged rodents subjected to TBI, histological studies indicated that aged brains of injured mice had not only larger lesions and worsened outcome, but microglial polarization toward M1, compared to younger rodents [23]. The challenge will be to discover methods for selectively suppressing the detrimental effects of microglial activation without compromising the restorative properties such as repair and remodeling. In related areas such as Alzheimer's and HIV-related dementia, strategies to turn "off" CD40 or turn "on" CD45 and favoring a M2 phenotype appear to improve neurological outcome [21] and suggest potential therapeutic targets for stroke and brain trauma. However, very little work in this area has been carried out in stroke and brain trauma.

Microglial Receptors for Activation

Microglia respond to specific receptors in the extracellular space and adjacent cell surfaces that modulate their activation and effector function. Microglial phagocytosis may require different types of receptors to initiate function [24]. Microglial receptors are able to bind foreign microbial pathogens as well as cellular debris. Such receptors include the Toll-like receptors (TLRs), the purinergic receptors, which bind nucleotides, and a relatively newly recognized family, triggering receptor expressed on myeloid cells (TREM).

Toll-like receptors

The exact initiating stimulus of acute brain damage has not been fully characterized, although some recent research has now identified a number of candidate factors. Several studies have shown that brain damage does induce activation of the TLRs [25], a family of transmembrane proteins involved in the identification of and defense against microbes. TLRs are observed in several cell types in the CNS and are a critical activating mechanism of innate immunity. They have been found on microglia and perivascular macrophages. Microglia are activated by stimulation of TLR4, which in turn leads to the upregulation of several proinflammatory genes. Lehnardt et al. [26] have shown that TLR4 is necessary for microglial activation after hypoxia and TLR4-knockout mice have better neurological outcome following ischemic stroke [27–30].

Moreover, reports investigating the real trigger of microglial activation in brain injury have hypothesized that the ischemic brain produces substances which can bind TLRs. Substances released by dying cells which then bind immune receptors have been categorized as damage-associated molecular pattern molecules (DAMPs). There are also studies implicating the high mobility group box-1 (HMGB-1) [31,32], which is an endogenous TLR agonist and is related to cytokines. HMGB-1 is released by neurons as well as other immune cell types that contribute to cell death; HMGB-1 effectively functions as a pro-inflammatory cytokine [33]. Other examples of DAMPs are hyaluronan, surfactant protein, uric acid, and heat shock proteins. These substances can bind to and stimulate microglia and other immune cells, resulting in the upregulation of many immune mediators by activation of several pro-inflammatory transcription factors, including NF-κB [34], hypoxia inducible factor 1 (HIF-1), interferon regulator factor 1 and signal transducer and activator of transcription 3 (STAT3) [35].

Current research suggests that the presence of upstream TLR4 activators may indicate adverse outcomes after brain injury. In experimental stroke, HSP60 has been shown to activate TLR4 and thereby worsen brain injury [36]. TLRs are also involved in the phenomenon of tolerance, whereby stimulation of one or more of these receptors with ligands, such as lipopolysaccharide (TLR4) or CpG (TLR9), triggers protection from subsequent lethal insults [37–39]. Mice deficient in these receptors did not achieve tolerance [37].

Purinergic Receptors

Purinergic receptors have emerged as important sensors of brain injury. Of the numerous purinergic receptors identified, P2X$_7$ and P2Y$_{12}$ have been characterized as such, but P2X4 and adenosine receptors also contribute [40]. P2X$_7$ signaling generates the production and release of other cytokines including TNF-α and IL-1β via activation of several mitogen-activated protein (MAP) kinase family members [41–43], microglial proliferation [44,45], and superoxide production [46]. Furthermore, activated P2X$_7$ can escalate plasminogen release [47] and activation of the transcription factors nuclear factor of activated T cells and NF-κB, which are key regulators of pro-inflammatory genes [48,49]. In contrast, microglia also show decreased phagocytosis during P2X$_7$ receptor

stimulation [50]. A few recent studies have demonstrated that P2X$_7$ antagonists reduce injury and postinjury inflammation when administered acutely after experimental CNS injury model [51,52]. However, other reports have described experiments where stroke appeared to be exacerbated by P2X$_7$ antagonists [53]. These investigations are intriguing, but their results are difficult to interpret because these antagonists may also influence other purinergic receptors at concentrations found *in vivo*, and cell types other than microglia express P2X$_7$, as well as other purinergic receptors.

The earliest reports of P2Y$_{12}$ receptors described their involvement in microglial membrane ruffling and chemotaxis [54]. Activation of P2Y$_{12}$ contributes to process extension and subsequent microglial migration toward injury and involves the activation of the PI-3 kinase [55] and Akt [56] signaling pathway. Interestingly, just as P2X$_4$ is beginning to be associated with microglial movement, P2Y$_{12}$ has recently been implicated in neuropathic pain [57]. Clopidogrel, a widely used antiplatelet agent, is also a P2Y$_{12}$ receptor antagonist. Recent findings from our laboratory demonstrated that clopidogrel was protective in an experimental model of global cerebral ischemia [58]. In addition, P2Y$_{12}$ knockout mice showed neuroprotection and reduced microglial chemotaxis. Nevertheless, it is important to remember that some antagonists such as cangrelor, previously believed to be P2Y$_{12}$-specific, can also antagonize P2Y$_{13}$ [58,59]. As both receptors also have common agonists, and both are Gi-coupled, a role for P2Y$_{13}$ may not have been eliminated by these pharmacological investigations. Researchers continue to identify novel P2 receptor agonists and antagonists; with an increasing number of available pharmacological agents, there are many more opportunities to study the therapeutic potential of purinergic receptors in regulating microglial activities.

Both P2X7 and P2X12 receptors react to adenosine triphosphate (ATP) as an endogenous agonist. It has been hypothesized that ATP is released extracellularly as a result of tissue injury, but it is unlikely that this occurs simply as a passive result of membrane disruption. For instance, ischemic injury produces energy deficiency and ATP depletion, and ATP released into brain extracellular matrix is rapidly degraded by exonucleases [60]. Therefore, an active, ongoing release of ATP is more likely to be the stimulus source acting on microglial purinergic receptors. A feed-forward, ATP-induced release of ATP from astrocytes is one feasible mechanism [61].

In microglia, P2X$_4$ receptors are primarily related to pain; their expression is markedly increased in spinal cord microglia following peripheral nerve injury which directly contributes to tactile allodynia [46]. P2X$_4$ is thought to modulate pain by inducing increased production and release of brain-derived neurotrophic factor (BDNF) [62,63], a neurotrophin increased in pain models, which regulates neurotransmitter release and interneuron activity [41]. A recent study has also reported a role for P2X$_4$ in microglial chemotaxis [55], which formerly had been mainly associated with P2Y$_{12}$ [54].

PPARγ

Peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of nuclear hormone receptor, which mediates gene expression related to reproduction, metabolism, development, and immune responses. There are three different PPAR isotypes (α, β/δ, and γ), and each isotype exhibits distinct tissue distributions and ligand specificities [64,65]. In the CNS, PPAR β/δ is generally expressed, while PPARα only appears in astrocytes, and PPARγ is the dominant isoform in microglia [66]. In ischemic stroke, inflammation induces the release of cytokines, such as TNF-α and IL-1β, and adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule. These mediators drive the accumulation of macrophages and activated microglia in the ischemic tissue regions [67,68]. Infiltrating inflammatory cells express inducible nitric oxide synthases (iNOS) and produce generate large amounts of NO, with the subsequent formation of peroxynitrite [1]. The activation of PPARγ can antagonize these harmful effects, suggesting a promising neuroprotective role for PPARγ agonists in ischemic stroke [69,70].

Triggering receptor expressed on myeloid cells

Triggering receptor expressed on myeloid cells was initially identified on account of its ability to bind pathogens such as bacteria and viruses, as well as for its role in initiating phagocytosis [71]. Recent studies show the TREM family consists of two members, TREM1 and TREM2. Both receptors appear on myeloid cells, including microglia. After cross-linking, TREM recruits and engages its adapter protein, DNAX-activating protein of 12 (DAP12) and activates signaling, which differs depending on which TREM receptor is activated. In addition to binding anionic patterns on various microbes, TREM was also observed binding an as yet unknown ligand in the CNS [72–74].

TREM1 signaling is largely pro-inflammatory. The ligand is constitutively expressed on macrophages, neutrophils, and microglia and is upregulated by various mediators such as the TLR ligands LPS and lipoteichoic acid (LTA) and the pro-inflammatory cytokine TNF [75,76]. Activation of TREM1 on monocytes and neutrophils boosts secretion of pro-inflammatory cytokines and chemokines and mediates the upregulation of cell-surface molecules involved in extravasation, cell activation, and costimulation [77]. These phenomena have not been studied extensively. However, TREM1 is gaining recognition as a potentially important molecule in brain injury responses.

Contrary to TREM1, TREM2 signaling is primarily antiinflammatory, while also promoting phagocytosis. Activation of TREM-2 activates downstream signaling through pathways including phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ1, and p44-p42 extracellular signal-regulated kinase (ERK), but not by classical inflammatory pathways such as NF-κB and the p38 stress-activated protein kinase [78,79]. In addition, TREM2 inhibits secretion of pro-inflammatory factors such as cytokines and ROS [72,73].

Considerable efforts to identify the endogenous TREM ligand have been unsuccessful in definitively determining what causes TREM activation in the brain. One recent study using a model of autoimmune encephalomyelitis demonstrated that HSP60 is one possible TREM ligand [80]. A mitochondrial stress protein, HSP60, can translocate to the cell surface under appropriate conditions [81]. Activation of microglia by HSP60 was shown to trigger phagocytosis in TREM2 expressing microglia, but not among

microglia that were deficient in TREM2 [80]. In another study, microglia that appeared to bind TREM2 were activated to phagocytose-injured cells without stimulating a typical inflammatory response or the release of reactive oxygen species. Conversely, loss of TREM2 blocked phagocytosis and triggered inflammation [82]. TREM2 deficiency in cultured microglia was shown to interrupt phagocytosis and expression of microglial proinflammatory responses such as TNFα, while overexpression of TREM2 increased phagocytosis and TNF-α [82]. Restriction of TREM2 using a monoclonal antibody in experimental autoimmune encephalomyelitis resulted in exacerbation of immune responses with increased demyelination and worsened neurological function [83]. These findings indicate that TREM2 may play an important role in regulating microglial phagocytosis and inflammatory responses in brain injury.

Activated microglia become highly motile, migrating to the site of injury and phagocytosing remnants from dead or dying neurons. Relevant to brain injury, TREM2 seems to mediate phagocytosis of apoptotic neurons [84]. While there is yet no definitive conclusion as to whether this behavior is beneficial or harmful during various stages of brain injury, the common consensus appears to be that microglial and macrophage phagocytoses are important effectors in facilitating recovery and remodeling by clearing necrotic debris as well as infiltrating neutrophils that otherwise exacerbate damage [85,86]. Searches showed two recent articles reporting the effects of TREM2 after ischemic stroke. One study using a model of therapeutic hypothermia in experimental stroke showed that TREM2 positive microglia were increased under conditions of protection, indicating that TREM2 may be correlated with improved outcomes after ischemia [87]. However, in a study of TREM2-deficient mice, the knockouts had similar lesion sizes compared with wild-type animals, but also fewer activated microglia and overall reduction in inflammatory responses [88]—observations which seem to contradict those seen in a neuroinflammation model [83]. While the therapeutic potential of TREM2 and microglial phagocytosis yet requires further research, these early studies show that TREM2 could be an important target for treating inflammatory injuries at subacute time points.

Mechanisms of Microglial Cytotoxicity

Microglia, like macrophages, respond to invading pathogens by promoting rapid sequestration and inoculation of microorganisms and by restricting the effects of cell damage and necrosis [89]. These acute responses include migration, proliferation, and the release of a variety of effector substances including superoxide, NO, proteases, and cytokines. This is accompanied by phagocytosis of damaged cells. Some of these responses may exacerbate brain injury and thus provide potential therapeutic targets.

Superoxide

Superoxide is a reactive species which interacts with other molecules to form more highly reactive oxygen species, such as peroxynitrite, hypocholorus acid, carbonyl radical, and hydroxyl radical, all of which are directly cytotoxic to neurons and glia. In immune cells such as microglia, superoxide is generated by the partial reduction of molecular oxygen. The result is the production of O_2^- that are formed by the 1 electron reduction of oxygen using NADPH as the electron donor:$2O_2 + NADPH \rightarrow 2O_2^- + NADP + H^+$ [90]. Superoxide and other reactive species are also pro-inflammatory signaling molecules that stimulate microglial activation in a feed-forward manner [91,92]. The production of superoxide in microglia occurs mainly by NADPH oxidase (NOX), of which a number of isoforms have been identified [93,94]. The isoform predominantly detected in immune cells such as microglia is NOX2, or professional NOX. NOX expression and activation has been strongly linked to ischemic stroke and related brain disorders, and its inactivation or deficiency has been reported to be protective [95]. While NOX2 exists in both microglia and circulating immune cells, one study using a bone marrow chimera model indicated that the toxic effects of NOX-produced superoxide were specifically attributable to NOX expressed in brain cells [96]. Work from our own group using a similar experiment showed ischemic brain injury worsened after increases in superoxides that were produced by NOX from both microglia and circulating immune cells [97,98]. The involvement of NOX from circulating immune cells appeared to cause more severe damage than with microglial NOX alone.

The introduction of microglia to endothelial cell and astrocyte cocultures is also known to exacerbate ischemia-like injury. However, interrupting the production of superoxide protected these elements of the blood–brain barrier (BBB) in in vitro models of ischemia [98]. In addition, Walder et al. [96] reported that mice genetically modified to be deficient in the gp91 subunit of NOX2 were protected after experimental ischemia. A few others have investigated the therapeutic potential of treatment with the pharmacological NOX antagonists apocynin [99–102] and honokiol [103,104]. Their findings characterize NOX as a promising target for treating stroke and brain injury, but it is possible that the efficacy of this therapeutic intervention may be attributed largely or in part to inhibition of NOX in cell types other than microglia [105].

NO and NOS

The free radical gas NO is another substance generated by activated microglia which has been implicated in a wide range of functions following brain injury, including neuronal synaptic activity, host defense, modulating vascular tone, and as an inhibitor of platelet aggregation and leukocyte adhesion [106]. NO is produced from ʟ-arginine through nitric oxide synthases (NOS) [107]. At this time, three NOS have been closely examined 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 thought to be the most relevant to inflammation. iNOS is expressed mainly by microglia and other macrophages but has also been observed in astrocytes [108–111]. Active microglia increase the inducibility of iNOS and the consequent production of NO. NO generated in microglia may also react with superoxide to produce peroxynitrite, an even more reactive oxygen species that can damage cellular DNA [112,113]. Microglial NO may also be neuroprotective against brain injury [114,115], but more often, it has proven to be cytotoxic, especially at high levels. In an oligodendrocyte cell culture model of ischemia, one study showed considerable cell death occurred after exposure to

microglia-derived NO [116]. NO is also toxic to BBB constituents [117,118].

Studies investigating potential therapeutic applications have reported that pharmacological suppression of iNOS with aminoguanidine in mice decreases infarct volume after experimental stroke, and iNOS deficient mice also showed better outcomes after injury [110,119]. Moreover, therapeutic hypothermia and neuroprotection by estrogen and progesterone have been linked to reduced iNOS production, suggesting that NO/iNOS play a detrimental role in brain injury [120–122].

Matrix metalloproteinases

The matrix metalloproteinases (MMPs) are a family of at least 28 zinc-dependent endopeptidases that, when active, degrade the extracellular matrix. MMPs can cause disruption of the BBB, leading to further infiltration of circulating immune cells, serum proteins, and hemorrhage [123]. They are an important part of the inflammatory responses to brain injury. Resting MMPs are typically found in the cytosol in uninjured conditions, but in pathologic states, they transport extracellularly. Once situated outside the cell, they are cleaved to an active form and can degrade substrates of the extracellular matrix [1]. MMP-2, -3, and -9 have been extensively characterized in stroke and, to a lesser extent, in traumatic brain injury.

vMicroglia are the major main source of MMP release following various forms of brain injury, particularly MMP-3 and MMP-9 [124,125]. Fibronectin and vitronectin, substances commonly located in the plasma, can stimulate microglial cells to produce pro-MM-9 [126]. Neutrophils can also produce and secrete MMP-9 [127] and studies experiments of bone marrow chimeras have suggested that MMP-9 derived from circulating immune cells contribute to worsened ischemic injury. The damage contributed by MMPs secreted by leukocytes may even be more significant than those from microglia [128].

Cytokines and Chemokines

Cytokines and chemokines are two classes of proteins that function as signaling molecules for inter- and extracellular communication during inflammation. Inactive microglia release many different types of cytokines and chemokines at low levels. The pattern of this production changes drastically in the injured brain [129]. During early stages of injury, these factors act primarily as intercellular signaling molecules, and many have feed-forward effects in driving the inflammatory response. TNF-α, IFN-γ, IL-1, and IL-6 are some of the best-studied cytokines. They have been shown to simulate microglia [130] and to have direct cytotoxic effects [131]. They are also capable of affecting BBB integrity [132]. These molecules have been observed as early as 1 day after experiimental stroke ischemic stroke [133,134].

Although thought to be deleterious in the acute phases of injury, cytokines may have more beneficial effects at a later stage. IL-10 signaling ultimately inhibits proinflammatory cytokines such as IL-1 and TNF-α, as well as other cytokine receptor expression and downstream signals that are upregulated in pathologic states [135]. Another study showed that TGF-β1 overexpression by microglia improved neurobehavioral recovery after experimen-

tal ischemia, and these outcomes were correlated with a reduction in the inflammatory response, also attributed to TGF-β1 overexpression [136,137]. There have also been instances of *in vitro* work that have observed microglia protecting cultured neurons from ischemia-like injuries by releasing TGF-β1 [138]. In addition, microglia also generate a number of neurotrophic factors, such as TGFβ1, BDNF, and GDNF, and these cytokines are considered important in protecting neuronal integrity after brain injury [139–142].

Microglia are also activated by and are able to produce chemokines that perform a broad range of functions in the injured brain [143–146]. The expression of monocyte chemoattractant protein-1 (MCP-1, CCL2) is triggered by the proinflammatory stimuli, such as IFN-γ, TNF-α, and IL-1β [147]. Chemokines can also lead to the disruption of the BBB and are involved in the recruitment of mononuclear leukocytes into the CNS [147–149].

Mechanisms of Microglial Protection

Microglia play key roles in the tissue repair, remodeling, growth, and homeostasis by releasing antiinflammatory cytokines and growth factors and by clearing cell debris [150]. Less has been studied in this area, as it pertains to affecting outcome in stroke and brain trauma. However, recent studies demonstrate that microglia may be beneficial after stroke [151,152]. Transgenic mice in which proliferating microglia were ablated suffered increased lesion size and higher numbers of apoptotic cells after experimental ischemic stroke [153]. Moreover, Mac-2+ resident microglia release neurotrophic molecules such as insulin-like growth factor (IGF-1), which leads to neuroprotection [153]. In addition, organotypic hippocampal slice cultures exposed to ischemia-like injury led to decreased neuronal death when cocultured with microglial BV2 cells [150]. Other studies suggest that microglia may be beneficial because they engulf neutrophils [152] and release TNF-α [154] after brain injury. Microglia may also be beneficial through their phagocytic functions. Recent reports demonstrate that dead cells release nucleic acid remnants into the extracellular space where they associate with appropriate receptors on phagocytes. Some of these phagocytosis initiating signals have been referred to as danger-associated molecular patterns (DAMP) [155]. Those identified in stroke and related injury models include purines such as UTP, ADP, and ATP, and signal through purinergic receptor systems to lead to chemotaxis and phagocytosis [156,157]. However, phagocytosis through these signaling systems, while leading to the clearance of injured cells, may also worsen cell death either by causing microglia to phagocytose viable cells or generate more neurotoxic substances [158]. Preliminary work in our laboratory suggests that TREM2 may also bind purines, and TREM2 signaling in microglia, which leads to phagocytosis [84], tends to be antiinflammatory [83]. Deficiency of TREM2 led to worsened recovery following stroke [159]. Finally, no published works are yet available in stroke or brain trauma models regarding the microglial M1/M2 switch, and whether its modulation improves neurological outcome. These beneficial effects of microglia should be explored, as they may provide new therapeutic avenues in brain injury.

Microglia in Ischemia and Traumatic Brain Injury

Activated microglia are key players in neuroinflammation, which is present in many types of neurological disorders. Research in microglia points to many promising therapeutic targets for a broad range of neuroinflammatory diseases, including acute injuries such as ischemic stroke and traumatic brain injury [160]. A comparative review of microglial activation in stroke and brain trauma shows considerable overlap. In both injury models, neuroinflammation develops over a time period of hours to days after onset, presenting an ideal time window for therapeutic intervention during which microglia play a central and nuanced role in responding to injury, sustaining inflammatory signaling and gene transcription, and mediating cytotoxic mechanisms [161,162]. Identifying parallels and distinctions between stroke and TBI may also provide useful clues for future research in microglial response to acute injury, as well as help isolate the most robust microglial targets for therapeutic intervention.

Ischemic Stroke

Stroke is the second leading cause of death worldwide and the fourth leading cause of death in the United States. While improvements in acute stroke care have considerably reduced stroke death rates, postischemic morbidity and disability are yet a major and costly public health concern [163]. During ischemic stroke, major blood vessels in the brain are occluded, causing oxygen and glucose deprivation in brain tissues. This primary insult initiates subsequent secondary brain damage involving necrotic and apoptotic neuron death as well as the release of cytotoxic and proinflammatory substances such as glutamate, superoxide, NO, chemokines, and cytokines [161]. Secondary injury mechanisms are largely responsible for the high rates of poststroke morbidity and disability. Therefore, it is important to identify targets for treatments that can lessen the impact of secondary brain damage. Microglial activation occurs in the early stages of neuroinflammation [98,164]; activated microglia can be detected in lesions as early as 2 h postischemia and can be detected up to 1 week after injury [85]. Many studies have shown that the direct application of activated microglia has been shown to effect cell death in neurons [26,164,165]. The cytotoxic effects begin shortly after insult and can continue to exacerbate injury for a few days afterward. It is thought that the later effects of activated microglia may be important for tissue repair and wound healing [166,167]. Thus, timing is an important element to consider when manipulating microglial activation in stroke. Studies in therapeutic microglial targets will also need to find ways to suppress cytotoxic mechanisms without disrupting beneficial effects.

Traumatic Brain Injury

Brain trauma is another significant cause of morbidity and mortality worldwide, hospitalizing 1.7 million Americans each year [168]. As in stroke, microglial activation and neuroinflammation have been implicated as important mediators of secondary damage by releasing proinflammatory cytokines, cytotoxic superoxide, NO, and proteases such as MMP-9 [161,169]. Traumatic brain injury begins with a primary physical insult causing brain deformation [170]. Within seconds to minutes, acute injury can trigger a variety of secondary injury mechanisms, including ischemia, hemorrhage, CNS hypoxia, free radical formation, excitotoxicity, BBB disruption, necrotic and apoptotic cell death, and inflammation [162]. Microglial activation plays a central role in the inflammatory branch of secondary injury by promoting secretion of proinflammatory cytokines and recruiting circulating immune cells, thereby exacerbating injury [171,172]. One mechanism observed in brain trauma but not in stroke is that activated microglia rapidly migrate to the site of injury, where they fuse to form a barrier between damaged and healthy tissues by about half an hour after injury. This phenomenon appears to be mediated by ATP and P2Y-receptor-dependent mechanisms [173]. In humans, active microglia appear 72 h after injury, at which time they have been shown to localize at the site of injury [174,175]. These secondary mechanisms can continue long after the time of injury. Rat studies have shown that elevated levels of activated microglia persist long after initial trauma [176,177] and have been observed in human brains for up to 16 years after injury [174].

Prolonged microglial activation may be among the most neurotoxic consequences of neuroinflammation whether in stroke or TBI [178]. The presence of chronically activated microglia is associated with sustained increase in the expression of pro-inflammatory cytokines such as IL-1β and TNF-α [179]. Furthermore, a number of clinical studies have shown that brain trauma may increase the risk for dementia and Alzheimer's [180–182]. Based on these findings, experts have hypothesized that microglial activation may link TBI to these neurodegenerative conditions which tend to emerge later in life [169]. Identifying microglial targets for treating acute injury may additionally provide greater insight into preventing or mitigating chronic neurodegenerative diseases.

Microglial Targets for Treatment

To date, much work has been performed to identify microglial targets for therapy. The research has produced a considerable body of literature describing a broad repertory of potential pharmacological inducers and suppressors. Most of these approaches protect against the deleterious effects of neuroinflammation, whether by upregulating repressors of microglial activation or by blocking microglia-mediated proinflammatory signals or substances. Fewer studies focus on upregulating microglial repair and remodeling. While many of these drugs have produced promising preclinical results, there has been little headway in pharmacological treatments for acute injury at the clinical level [183,184]. The difficulty in identifying effective therapy is understandable given the complex, extensive web of immune responses in the injured brain. Successful intervention will most likely require a compound or combination of compounds that can modulate multiple targets. Tailoring such a drug or cocktail demands a detailed understanding of the secondary injury processes [185]. A central initiator and mediator of these processes, microglia—its activators, functions, and secretions—can serve as a useful focal point for organizing the current repertoire of known antiinflammatory drugs, highlighting key targets for future investigation.

Pharmacological Inhibition of Microglial Activation

One of the best-studied inhibitors of microglial activation, minocycline, is a second-generation tetracycline known for its ancillary function as an effective suppressor of brain inflammation. The earliest studies in minocycline neuroprotection reported the drug's ability to inhibit microglial activation in stroke and were studied as a promising antiinflammatory drug in many other neurodegenerative diseases, including brain trauma [184–192]. Minocycline inhibits microglia-mediated neuron death as well as several proinflammatory cytokines secreted by injury-activated microglia, thereby suppressing both downstream cytotoxic mechanisms in addition to disrupting the inflammatory feed-forward loop [193,194]. Minocycline is additionally known to block MMP-9, released by activated microglia, and reduces BBB disruption in stroke and TBI [123,124,195,196]. Studies have shown that minocycline treatment can reduce lesion sizes and improves neurobehavioral outcomes after injury [197].

Apocynin has antioxidant and antiinflammatory capabilities, specifically to block the activity of NOX. NOX exists on immune cells (microglia and leukocytes), and apocynin inhibits the release of superoxide through NOX by blocking migration of p47phox to the membrane, thus interfering with assembly of the functional NOX complex [198]. Immune cell-generated NOX 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 with apocynin preserved these BBB constituents *in vitro* [98]. Thus, NOX contributes to BBB disruption downstream events in ischemic stroke. In fact, apocynin attenuated brain edema formation MMP-9 expression [199], BBB disruption, and hemorrhagic transformation [100] as well as inhibiting immune cell responses [99].

TLR4

Upon exposure to signals released at the site of injury, the transmembrane receptor TLR4 activates microglia and triggers the upregulation of pro-inflammatory genes [161]. Thus, TLR4 may be a critical first step in microglial activation and injury response. Considerable work has been performed to more closely understand TLR4 mechanisms as well as its potential as a therapeutic target. In stroke, several groups have shown that TLR4 may be necessary for microglial activation, and animal studies describe better functional outcomes for TLR4-deficient mice following experimental stroke [26–29]. Work in trauma continues to compile a range of different neuroprotective TLR4 inhibitors that confer neuroprotection after TBI including resatorvid, curcumin, and ethyl pyruvate [200–202]. Studies have also associated TLR2 and TLR4 with increased inflammation and poorer outcomes among patients admitted for ischemic stroke, and support a clinical need for developing a therapy targeting TLRs [201,203].

TNF-α

Tumor necrosis factor (TNF) is an important immune signaling molecule released by activated microglia in acute injury and an especially promising therapeutic target for stroke and TBI [204]. Current findings in TNF studies using pharmacological inhibitors such as etanercept suggest that the therapeutic time window for treatments targeting components of microglia-mediated inflammation may extend long after injury [204]. Groups have demonstrated the efficacy of etanercept fused with a decoy BBB-receptor in mouse models of stroke, and still others have described the antiinflammatory and neuroprotective properties of TNF-α antagonist such as thalidomides and thalidomide derivatives [205–207].

PPARγ

Peroxisome proliferator-activated receptor-controlled transcription pathways have shown to be antiinflammatory in the brain in both stroke and TBI models. Upon dimerizing with an activating receptor, the PPARγ transcription factor translocates to the nucleus where it activates transcription [208]. Although PPARγ expression increases in microglia after stroke and trauma, a reduction in available ligand leads to fewer instances of DNA binding such that PPAR transcription is suppressed [209,210]. PPARγ suppression can be reversed by introducing endogenous ligand or by treatment with agonists such as rosiglitazone and pioglitazone [211,212]. Treatment with PPARγ ligand after stroke decreases microglia and macrophage activation and curbs the expression of inflammatory molecules such as ICAM-1, MMP-9, IL-1$β$, COX-2, TNFα, iNOS, and reactive oxygen species [213–215]. PPARγ agonists have also been shown to reduce inflammation and confer neuroprotection in animal models of traumatic injury [211,216,217].

Conclusion

The role of inflammation in brain injury has become an increasingly popular area of investigation, given its pleotropic roles in both acute damage and long-term recovery. Microglia are key players in neuroinflammation and are considered the major immunocompetent cells of the brain. For this reason, much effort has been directed toward an understanding of how microglia become activated and how they mediate neuroinflammation. These efforts have led to many studies which have shown the beneficial and detrimental effects of microglial functions in brain ischemia and trauma. Modulation of microglial behaviors is clearly an important topic of investigation and represents a huge potential for the development of new therapeutic strategies in acute brain injury.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest.

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