American Journal of BioMedicine
AJBM 2015;3(3): 453–463
Research Article doi: 10.18081/2333-5106/015-03/453-462

Critical role of microglia in the inflammatory response after spinal injury

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Abstract

Spinal cord injury induces a robust neuroinflammatory response that includes marked changes in the variety of endogenous CNS cell types specially microglia. In response to spinal injury, microglia undergo dramatic changes in cell morphology and promote inflammatory responses, which result in production of inflammatory factors and oxidative stress including reactive oxygen species. Further pro-inflammatory cytokines and chemokines are also rapidly up-regulated and likely contribute to microglial activation. This topic review will explore the current research on microglial responses to spinal injury and the recent progress in the pharmacologic and molecular targeting of microglia in spinal injury. Finally, we explore the argument for a positive versus negative role of microglia after spinal cord injury.

Keywords: Spinal cord injury; Microglia; pro-inflammatory cytokines; CNS; Immune cells

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Received 11 March 2015; accepted June; 09, 2015, Published July 11, 2015
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Introduction

Spinal cord injury (SCI) is associated with devastating neurological outcomes and limited therapeutic opportunities. It has three phases: acute, secondary and chronic [1]. The outcomes of SCI are mainly influenced by the secondary phase. SCI causes inflammatory responses through the activation of innate immune responses that contribute to secondary injury [2]. Macrophages accumulated within the epicenter and the hematoma of the injured spinal cord play a significant role in this inflammation [3]. Microglia/macrophages associated inflammation appears to be a significant mechanism related to neuronal degeneration and regeneration. Macrophages in the central nervous system (CNS) derived from blood monocytes and resident microglia, are pervasive in the injured spinal cord and change their phenotypes and functions in response to signals in the lesion environment [4]. In this review, we discuss the role of microglia in the inflammatory response after spinal injury.

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Types of microglia

Several macrophage subsets have been classified based on the expression of cell surface markers, intracellular enzymes, and secreted molecules, including M1 (classical activation), M2 (alternative activation), regulatory macrophages, tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), and so forth [5]. M1 and M2 are often seen as the two primary subsets of macrophages at the injured site. Depending on the phenotypes and activation status of macrophages, they may not only initiate secondary damage, but also initiate repair. The phenotypes and functions of macrophages in the injured spinal cord are dynamic and can change according to the microenvironment in the spinal lesion [6]. As reported, M1 (CD86-positive) and M2 (arginase-1-positive) macrophages coexist at the lesion epicenter during the first week after SCI, but only M1 macrophages persist until day 28 post-injury in mice [7]. Our unpublished data showed that macrophages phagocytosis of myelin debris are detected in injury site from 1–2 weeks after SCI. These myelin-laden macrophages exhibit M1 like phenotype and persist for long period of time. It was also reported that M1 microglia appear immediately after injury and secrete pro-inflammatory cytokines and chemokines that both lead to further damage following primary mechanical injury [8]. The appearance of M2 macrophages and secreted anti-inflammatory cytokines and chemokines lead to the suppression of excessive inflammatory responses around the injured spinal cord and regeneration of injured spinal tissues [9]. In addition, a switch from M1 to M2 in the injured spinal cord, induced by transplantation of stem cells (neural and other), prevents axonal damage and improves locomotor function [10].

Classically activated microglia/macrophages (M1)

Classical activation involves the induction of M1 macrophages by Th1 cell-derived cytokines. Generally, the properties of M1 macrophages in inflammation during secondary damage are neurotoxic and growth inhibitory. Because of these properties, M1 macrophages contribute to the formation of axonal growth-inhibitory glial scar and production of pro-inflammatory radicals/mediators, leading to a hostile environment at the lesion site, which results in the limited regenerative nature of the injured spinal cord [11]. Markers for M1 macrophages in the inflammation phase of secondary damage include NOX, NOS2, CD16/32 and CD86 [12]. Activated M1 macrophages produce a high level of pro-inflammatory molecules such as IL-1β, IL-6, IL-12, IL-23, TNF-α, IFN-γ, chemokine (C-C motif) ligand 5 (CCL5), nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, inducible nitric oxide synthase (iNOS), toxic intermediates, and opsonic receptors such as immunoglobulin Fcγ receptors [13]. Classical activation also causes the release of proteolytic enzymes that can lead to deterioration of the extracellular matrix, such as metalloproteinases, collagenases, and furin, thus degrading cellular integrity and leading to easier destruction of the cell [14]. Meanwhile, these cells secrete low levels of anti-inflammatory mediators [15]. M1-polarized macrophages show the ability to

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induce neuron death directly through iNOS activity and the capacity to obliquely contribute to secondary degradation [16]).

Phagocytosis of Microglia

Debris is considered one of the obstacles for axonal outgrowth after nerve injury in the CNS. Cortical neurons that were transected by cutting axons of the cortical explants, regrew after the clearance of axon debris by LPS- or interferon-β (IFN-β)-activated microglia co-cultured with rat cortical neurons in an *in vitro* Wallerian degeneration model [17]. This suggests that the phagocytic ability of microglia and infiltrating peripheral macrophages for removing damaged tissue after nerve injury has an important role in axonal outgrowth. Activated microglia efficiently process phagocytic material, and phagocyte-damaged and degenerating tissue in the early stage after nerve injury. Compared with resident microglia, macrophages play a crucial role in phagocytosis in the peripheral system but are less efficient at processing phagocytic responses in the early phase after CNS injury. However, they become the predominant cells in contact with degenerating axons and damaged tissue in the late stage after CNS injury [18], which was shown by using lysozyme M EGFP-knockin mouse to label myelomonocytic cells, but not microglia. Accumulating evidence has revealed that several factors such as toll-like receptor 4 (TLR4), glia cell line-derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF) are involved in the phagocytosis of axonal debris by microglia and macrophages. Microglial phagocytosis of axonal debris was inhibited by pharmacologic blockade of TLR4 *in vitro*, and genetic disruption in TLR4 knockout mice [20].

The TLR4-dependent microglial clearance of unmyelinated axonal debris facilitated axon outgrowth, which indicates that TLR4 plays a key role in axonal debris clearance by microglia, thus creating a more permissive environment for axonal outgrowth [19]. GDNF can increase the phagocytic ability of microglia through a distinct mechanism via upregulation of α5 integrin expression. CNTF also improved the phagocytic function of microglia via a calcium-mediated pathway. Galectin (Gal)-1 is a small carbohydrate-binding protein and immune modulatory cytokine that drives axon regrowth by regulating macrophage inflammatory signalling, accumulation and phagocytosis.

A recent study examined the spatiotemporal distribution of Gal1 in rats with SCI. Compared with non-expression of Gal1 in intact rat, the expression of Gal1 was significantly upregulated in spinal cords after SCI. Gal1 was expressed in ~40% of lesion-localized macrophages at 3–28 days post-injury (dpi), and in ~45% of astrocytes in the lesion border at 7–28 dpi [21]. However, most lesion-localized Gal1-positive macrophages did not express the phagocytosis marker ED1, and Gal1-positive cells contained less phagocytosed lipids. These data suggest that time- and location-dependent regulation of Gal1 by infiltrating peripheral macrophages could be important for modulating phagocytosis after SCI. Bone marrow-derived M1 and M2 macrophages have a distinct phagocytic capacity. Phagocytosis of myelin and neuronal fragments was increased in M1 macrophages compared with M2 macrophages [22].

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Microglial Repair of Leaky Blood Vessels Is Blocked by a Fibrinogen-Derived Inhibitory Peptide.

In light of previous studies that the interaction between extravascular fibrinogen and the microglial cell surface receptor Mac-1 integrin promotes microglial clustering around leaky blood vessels in an EAE model of MS [23], researchers exposed mice to hypoxia for periods of up to 7 d, while at the same time treating them with daily intraperitoneal (i.p.) injections of the fibrinogen-derived inhibitory peptide $\gamma^{377-395}$ that blocks the interaction of fibrinogen with its Mac-1 integrin receptor, and then after 4 or 7 d CMH, quantified the number of leaky blood vessels in spinal cord sections. researchers showed that spinal cords of mice receiving the inhibitory $\gamma^{377-395}$ peptide contained significantly higher numbers of leaky blood vessels compared to control mice that received a scrambled peptide at all time-points (Fig. 1 A and B). In addition, the $\gamma^{377-395}$ inhibitory peptide prevented the migration and accumulation of activated microglia around leaky blood vessels (Fig. 1*C*). These findings demonstrate that microglial recruitment and repair of leaking blood vessels is dependent on a fibrinogen-Mac-1–mediated mechanism.

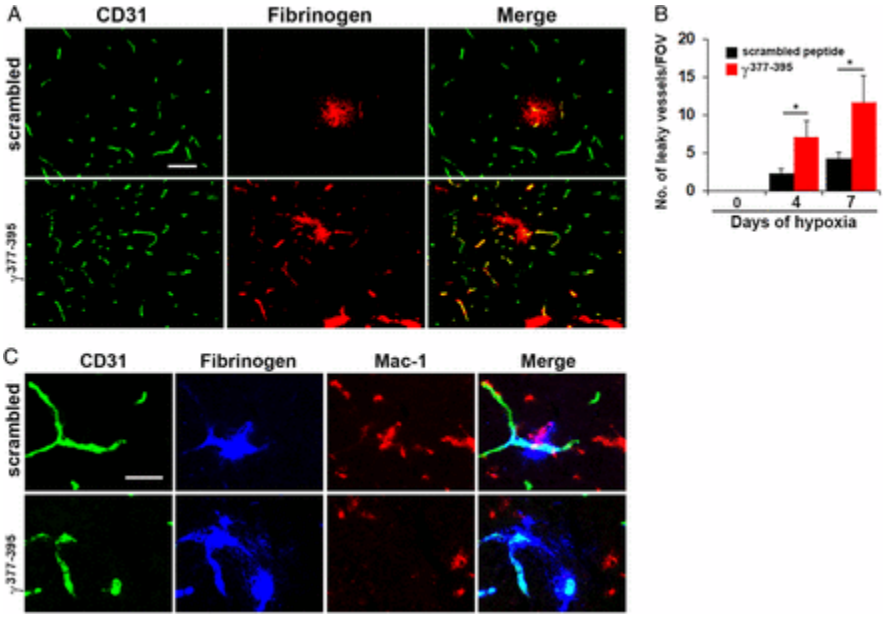

Figure 1.

Microglial vascular repair is blocked by a fibrinogen-derived inhibitory peptide. (*A* and *C*) Frozen sections of lumbar spinal cord taken from mice maintained under hypoxic conditions for 7 d that received daily injections of either the fibrinogen-derived inhibitory peptide $\gamma^{377-395}$ or a scrambled peptide, were stained for CD31 (Alexa Fluor 488) and fibrinogen (Cy-3) in *A* or CD31 (Alexa Fluor 488), fibrinogen (Cy-5, blue), and Mac-1 (Cy-3) in *C*. (*B*) Quantification of the number of leaky vessels/FOV. Results are expressed as the mean ± SEM (n = 6 mice per group). *P < 0.05. One-way ANOVA followed by Tukey's multiple comparison test. Note that mice treated with the inhibitory peptide $\gamma^{377-395}$ showed a greater number of leaky blood vessels and the peptide prevented the migration and accumulation of activated microglia around leaky blood vessels (*C*). (Scale bars, *A*, 50 μm; *C*, 25 μm.)

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Phenotypes of Microglia

The contradictory functions of microglia/macrophages might attribute to the existence of distinct phenotypes during dynamic phases of the pathological microenvironment in the injured CNS. Microglia that maintain homeostasis are sensitive to small changes in CNS environment, whereas microglia 'housekeepers' immediately respond to inflammation caused by foreign materials and CNS injury [14].

The activated microglia are classically polarized into M1 and M2 phenotype cells. M1 microglia and macrophages produce destructive pro-inflammatory factors. In contrast, M2 microglia and macrophages produce numerous protective and trophic factors, and have higher phagocytic ability for clearance of cellular debris. Recent data showed that adult human M1 microglia express higher levels of CD80 and CCR7 than M2 microglia and unpolarized microglia, whereas M2 microglia express higher levels of CD209 than M1 microglia and unpolarized microglia [15]. Gene expression was also examined via PCR analysis in this study. Gene expression of CCL8, CXCL10, CXCL12, HLA-DQB1 and TLR2 was upregulated in M2 microglia.

In *in vitro* primary microglia culture, LPS- or IFN- β-stimulated M1 microglia expressed high levels of inflammatory cytokines, such as IL-6 and TNF-a, as well as chemokine CCL2. Levels of IL-1β, NO synthase-II, cyclooxygenase-2 and CCL2 were also slightly increased in IL-10 stimulated M2 microglia. These data suggest that M1 and M2 microglia have different properties related to different functions in response to different pathological environments in the injured CNS [16].

Investigation of temporal changes in microglial subsets has revealed that the number of M2 microglia increased at 1 week after traumatic brain injury (TBI) in adult mice, and the number of M1 microglia increased at 4 weeks post-TBI. These results suggest that different phenotypes of microglia increased in the different phases (early and late phases) after TBI [17]. This indicates that compared with M1 microglia, M2 microglia might have more potential for phagocytosis in the early stage after CNS injury, whereas M1 phenotype cells attribute to the chronic inflammation in the late injured stage.

Increasing evidence has revealed that M2 microglia perform beneficial functions in neuronal repair after SCI, such as clearing of cellular debris through phagocytosis and release of numerous protective and trophic factors; in contrast, M1 microglia release destructive pro-inflammatory factors [18]. For example, LPS-stimulated M1 microglia inhibited neurite and axonal growth through repulsive guidance molecule a (RGMa) [19].

M2 microglia exert a neuroprotective effect on tissue repair via phagocytosis of cellular debris, and reduce inflammation by regulating the inflammatory response [20]. In addition, recent data showed that bone marrow-derived M1and M2 macrophages have a distinct migratory capacity towards medium conditioned by various cell types of the CNS. M2 macrophages are preferentially attracted by the low weight (<10 kDa) fraction of the neuronal conditioned

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medium, while M1 macrophages are attracted in higher numbers by astrocyte- and oligodendrocyte-conditioned medium. Adhesion to ECM molecules was significantly enhanced in M1 macrophages compared with control and M2 macrophages. The actin cytoskeleton was differentially organized between M1 and M2 macrophages, possibly due to greater activity of the GTPases RhoA and Rac in CA macrophages. These data indicate that intrinsic motility of M2 macrophages is higher than that of M1 macrophages [21].

Microglia/macrophages and demyelination

Astrogliosis is a pervasive response to different insults to the adult CNS, including trauma, toxicity, and genetic and degenerative diseases [22]. Astrogliosis is responsible for the failure of remyelination in many experimental models of demyelination and demyelinating pathologies [23].

The pathological process of demyelination due to the loss of oligodendrocytes is particularly active during the sub-acute (secondary) and chronic phase of SCI [24]. Recent studies suggested that immunological demyelination is accompanied by a robust activation of macrophage/microglial cells without an astrogliosis response [25]. The activities of macrophages and microglia following SCI are maximal between 3 and 7 days post-injury. Notably, activated macrophages and microglia were reported to exclusively locate to regions of immunological demyelination, with only a few of them outside of the region. In spinal lesions during secondary injury after SCI, the activities of microglia and macrophages were significantly higher within regions of immunological demyelination [26].

Immunological demyelination creates a unique environment in which astrocytes do not form a glial scar and provides a unique model to understand the putative interaction between astrocytes and activated macrophage/microglial cells. However, during the process of demyelination, axons are directly exposed to damaging effects such as inflammatory cytokines and free radicals, leading to neuronal loss. As a result, demyelination leads to conduction delays and conduction block [27].

Cytokines of Microglia

Both pro- and anti-inflammatory factors produced by activated microglia under pathological CNS conditions have been well studied. Major anti-inflammatory cytokines, including IL-4 and IL-10 released from microglia in the injured CNS have been well studied [28]. A recent study showed that in adult IL-4 receptor α (IL-4Rα)-deficient mice, IL-4/IL-4Rα dysfunction in microglia is a crucial contributing factor to impaired functional recovery after SCI. Other study, used aged mice (18–19 months) showed poorer functional recovery than adult (3–4 months) mice after SCI; this was proposed to be due to the decrease in the expression of IL-4Rα in microglia. IL-10, another well-known anti-inflammatory cytokine produced by microglia, plays an important role in mediating the inflammatory response and immune reactions. In the CNS, IL-10 is mainly produced by astrocytes and microglia, and it is upregulated after various insults including traumatic CNS injury. Recent data showed that expression of IL-10 mRNA is constant

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in both intact wild-type (WT) and GFAP-IL-10 transgenic (GFAP-IL-10Tg) mice, whereas the expression of IL-10 mRNA is substantially higher in GFAP-IL-10Tg mice than in WT after facial nerve axotomy [28].

A change in microglia morphology from resting-ramified to activated-amoeboid, and an increase in the infiltration of CD3-positive T-cells were observed in these transgenic mice. In addition, motor neuronal survival was improved by the activated microglia and infiltrating CD3-positive T-cells after facial nerve axotomy. This indicates that IL-10 interaction with microglia and CD3-positive T-cells has beneficial effects on post-injury CNS repair [28]. Moreover, recent data indicated that a significant reduction in IL-10 expression occurs in macrophages in 14-month-old mice compared with that in 4-month-old mice; this possibly explains the reduced functional recovery and enhanced tissue damage after SCI in old mice compared with young mice [6].

Other studies highlight an important role for microglia in maintaining vascular integrity in the hypoxic spinal cord and suggest that a fibrinogen–Mac-1 interaction underpins this response. We demonstrate that in the mouse spinal cord, CMH results in a transient vascular leak in a small minority of blood vessels, primarily in white matter, an effect that peaks within the first 7 d of CMH exposure, and then recedes. The timing of this vascular leak correlates closely with the vascular remodeling events induced by CMH whereby endothelial cells uncouple from their neighbors and migrate to extend new sprouts [9].

How can an initial vascular leak translate into a longer-term beneficial effect that actually enhances vascular integrity? Our data support the notion that the initial leak is only transient but then stimulates adaptive responses within blood vessels that lead to enhancement of vascular integrity, including up-regulated endothelial expression of tight junction proteins and expression of ECM proteins such as laminin in the vascular basement membrane [3].

It was notable that in mice with normal microglial levels, vascular leak in spinal cord vessels typically occurred only in white matter, so what accounts for this specificity? Most likely this is related to the fact that CMH induces a stronger vascular remodeling response in spinal cord white matter compared with gray matter, consistent with the idea that more vascular remodeling is associated with greater uncoupling of endothelial cells, leading to higher levels of vascular leak [33].

Exactly why white matter is the site of greater vascular remodeling and vascular leak is still an open question, but this may be related to the low vascular density in white matter (approximately only 25% of that in gray matter) [4], thus with a low functional reserve any drop in the rate of oxygen delivery has to be compensated by a stronger remodeling response in white matter.

Several studies have described microglial accumulation around disrupted CNS blood vessels, although in each study, different conclusions have been drawn. For instance, in the EAE mouse model of MS, the Akassoglou laboratory found that extravascular leak of fibrinogen attracted microglia to leaking blood vessels, concluding that fibrinogen stimulates microglia into an

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activated destructive phenotype, leading to demyelination [8]. Other findings are similar in showing that microglial surround extravascular leaked fibrinogen and are dependent on the fibrinogen–Mac-1 integrin interaction, but whereas the former study proposed this as a pathological mechanism.

Other studies demonstrate that microglia play an important protective role in reducing vascular leak from spinal cord vessels under hypoxic conditions. Based on this, we propose that the distinction between microglia playing a protective or pathogenic role could depend on the timing and/or severity of insult.

In this model, during the early phase of vascular leak, microglia mediate a protective response to maintain vascular integrity, but in the face of continued vascular leak, microglia may become inappropriately stimulated into a phagocytic phenotype. This model would also explain the other findings [6], who showed that in the early stages of a mouse model of ischemic stroke, microglia congregate around damaged blood vessels, consistent with our findings, but in this severe model of ischemic/hypoxic insult, microglia eventually switch from a repair into a phagocytotic phenotype that remove the dying endothelial cells.

The trigger that tips microglia from protective into phagocytic mode may be a function of time or degree of stimulation, perhaps mediated by the overall integral of incoming activation signals that microglia transduce, which would include signals from cytokines and chemokines as well as serum ECM proteins such as fibrinogen.

Alternatively, another possibility is that the generation of oxygen free radicals that accompanies reperfusion following temporary ischemic stroke also plays a key role in triggering microglial switch into phagocytic mode. Our findings are also consistent with other reports in diabetic mouse and laser ablation models, demonstrating an important function for microglia in repairing damaged cerebral blood vessels [5].

In these studies, we showed that CMH results in a transient vascular leak in a small minority of spinal cord blood vessels, an effect that peaks within the first 7 d of CMH exposure, and then recedes. As fibrinogen is a relatively large molecule (molecular mass ~340 kDa), this is most likely a relatively late indicator of vascular breakdown and only labels quite severe disruptions in vascular integrity.

Therefore, in future studies we plan to use molecular probes of varying size (sodium fluorescein, FITC-dextrans) to more closely characterize the time-course of this hypoxic-induced vascular leak. At the same time, we will also chart the dose–response of this vascular leak by exposing mice to different levels of hypoxia (from 8 to 16% O_2). Using this approach, we will also seek to understand whether the size of the leak equates to the efficacy or magnitude of the microglial repair response.

Unlike previous studies that examined microglial–vascular interactions in animal models of severe pathological disease involving extensive vascular damage (MS, stroke, diabetes mellitus), others experiments were performed in a subclinical model of mild hypoxia. This is important because people experience relative hypoxia in a wide variety of situations, including

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visiting high altitude locations, lung disease (e.g., chronic obstructive pulmonary disease [COPD], obstructive sleep apnea, and experiencing age-related ischemia/hypoxia [27], thus it seems likely that microglia are constantly monitoring and sealing these vascular leaks. From a clinical viewpoint, this has several implications. First, drugs that interfere with microglial function, e.g., minocycline, could suppress this protective effect and thereby predispose to worse vascular leak and CNS damage [30].

Second, people with COPD or sleep apnea or those predisposed to ischemic stroke most likely suffer varying levels of intermittent hypoxia for months or years before any clinically detectable event. Based on our studies, it seems likely that microglia may be playing a vital role during this time by repairing hypoxic-induced vascular leaks as they occur. Third, during the process of aging, CNS vascular integrity is gradually diminished [31], which raises the question: Is this a result of age-related vascular degeneration or is it due to reduced microglial repair function, and irrespective of the cause, could vascular integrity in the elderly be enhanced by promoting this protective microglial response? A greater understanding of these age-related events would facilitate improved design of therapeutic strategies aimed at influencing microglial function to optimize their vasculo-protective activity.

Conclusion

Important implications regarding the critical role of microglia in maintaining CNS vascular integrity.

Competing interests

The authors declare that they have no competing interests.

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American Journal of BioMedicine
Journal Abbreviation: AJBM
ISSN: 2333-5106 (Online)
DOI: 10.18081/issn.2333-5106
Publisher: BM-Publisher
Email: editor@ajbm.net