Role of Hematogenous Macrophages in Formation of the Fibrotic Scar after Spinal Cord Injury

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ROLE OF HEMATOGENOUS MACROPHAGES IN FORMATION OF THE FIBROTIC SCAR AFTER SPINAL CORD INJURY

By

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After spinal cord injury (SCI), a fibrotic scar forms at the injury site that is best characterized by accumulation of perivascular fibroblasts and microglia/macrophages as well as deposition of extracellular matrix proteins such as fibronectin. While the fibronectin matrix plays a critical role in non-central nervous system (CNS) organ wound healing, whether or how fibronectin forms a matrix after SCI is not known. The mechanism by which perivascular fibroblasts are recruited to the fibrotic scar after SCI is also not clear. Using mutant mice and bone marrow transplantation in a mouse model of contusive SCI, we demonstrate that the fibrotic scar is associated with hematogenous macrophages (hMΦ) rather than microglia, which are limited to the surrounding glial scar. We also demonstrate that fibronectin is initially present in a soluble form and is assembled into a matrix after SCI. Assembly of the fibronectin matrix may be mediated by the canonical fibronectin receptor, integrin α5β1, which is primarily expressed by hMΦ in the fibrotic scar. Depletion of hMΦ leads to a reduction in fibroblast density, basal lamina formation and ECM deposition that is associated with increased axonal growth in the fibrotic scar. Cytokine expression analysis suggests that these effects may be due to decreased tumor necrosis factor...
superfamily (Tnfsf) members and increased bone morphogenetic protein (BMP) expression. In conclusion, our study demonstrates that hMΦ are necessary for fibrotic scar formation and macrophage depletion results in changes in multiple cytokines that make the injury site less fibrotic and more conducive to axonal growth.
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Chapter 1

Introduction

1.1 Spinal Cord Injury Overview

Epidemiology and social impact

Spinal cord injury (SCI) is a devastating condition that is prevalent across the world. SCI can lead to permanent disability, such as loss of voluntary movement, sensory and autonomic functions as well as chronic pain and spasticity. According to the National Spinal Cord Injury Statistical Center (NSCISC) report, in the United States there are approximately 276,000 people living with SCI, and approximately 12,500 new cases reported annually (NSCISC, 2014). However, the actual incidence of SCI may be far greater than what is recorded, attributable to two main reasons: 1) a substantial number of patients suffering from SCI die at the scene of accident or before they reach the hospital; 2) patients who do reach the hospital are at risk of high mortality rate caused by complications related to SCI, such as respiratory and renal infections (Cadotte and Fehlings, 2011). The most common causes of traumatic SCI include land transport, falls, violence/self-harm and sports/recreation accidents, affecting primarily males aged 18-32 years in both developed and developing countries, and elderly people over 65 years in developed countries, due to population aging (Lee et al., 2014). In addition to the physiological suffering that SCI patients endure, SCI also poses an enormous burden on life expectancy, financial status and quality of life. According to the NSCISC report, life expectancy for people with SCI is significantly lower than those without SCI and has not improved since
the 1980’s. The estimated lifetime costs associated with SCI in the United States is $1.1-2.3 million for a person with incomplete paraplegia and $2.1-4.6 million for one with complete tetraplegia, and this does not include any indirect costs such as loss in wages, fringe benefits and productivity which average $70,575 per year (NSCISC, 2014). SCI also results in extensive negative psychological effects. Approximately 30% of people with SCI developed a depressive disorder during rehabilitation, and approximately 27% had depressive symptoms when living in the community (Craig et al., 2009). To determine what functions are most important to the SCI population, a study showed that most people ranked recovery of bladder/bowel function, eliminating autonomic dysreflexia, and regaining sexual function as the top priorities to improve quality of life (Anderson, 2004). Despite the tremendous socioeconomic impacts of SCI, currently available treatment options offer modest benefits, thus prevention (e.g. seat belts, weapon control and sports safety regulations) is still the best measure to reduce SCI occurrence (Thuret et al., 2006). Therefore, research focusing on understanding the pathological changes and mechanisms of regeneration failure after SCI holds promise for more effective therapies to enhance spinal cord function.

General pathological mechanisms of spinal cord injury

The pathology of traumatic SCI is a consequence of both primary and secondary injuries. Primary injury is the actual mechanical damage, resulting from tear, compression and/or distortion of the neural tissue, often leading to
immediate hemorrhage and cell death at the injury site. Secondary injury is responsible for expansion of the primary injury through vascular and biochemical events, starting from minutes and persisting to years after injury. About 25 well-established mechanisms for the secondary injury after SCI have been categorized and reviewed (Oyinbo, 2011). It is worth noting that those mechanisms are not independent of each other, but rather they are highly related. The major secondary injury mechanisms are briefly summarized below. 1) Inflammation: The neuroinflammatory response after SCI is primarily orchestrated by neutrophils, microglia/macrophages and lymphocytes, and is discussed in detail in section 1.5 of this chapter. While exerting a neuroprotective role by clearing out tissue debris, the inflammatory response can be over-activated and exacerbates the primary injury. 2) Oxidative stress: SCI-induced inflammation and metabolic dysfunction contribute to elevated levels of free radicals in the lesion, which include reactive oxygen and nitrogen species. Free radicals not only directly damage proteins and nucleic acids, but also compromise the integrity of cell membranes by lipid peroxidation. This could also aggravate mitochondria stress and result in calcium overload, eventually leading to cell death. 3) Glutamate excitotoxicity: SCI results in release of glutamate from damaged astrocytes and synaptic terminals, increasing glutamate levels in and around the injury site. As an excitatory neurotransmitter, glutamate binds to glutamate receptors such as NMDA receptors and mediates calcium influx. High intracellular calcium levels can trigger a wide range of biological processes, including mitochondria swelling and caspase-mediated apoptosis. Neurons and
oligodendrocytes are especially vulnerable to glutamate excitotoxicity because they express high levels of glutamate receptors. 4) Ionic imbalance: Ionic imbalance is a result of compromised cell membrane and glutamate excitotoxicity, characterized by increased intracellular levels of potassium and calcium. Unregulated ionic flux disrupts cell homeostasis and is detrimental to cell survival. 5) Vascular disorders: Vascular disturbances after SCI include hemorrhage, microcirculation derangements, impaired autoregulation, and ischemia/reperfusion damage. Injury to the spinal cord immediately destroys small blood vessels, leading to reduced microcirculation and hemorrhage. Autoregulation ensures constant blood flow when systemic blood pressure or CO$_2$ level fluctuates. However, SCI causes failure of autoregulation, together with the loss of microcirculation, all of which contribute to ischemia around the injury site. While lack of energy supply caused by ischemia itself induces cell death, reperfusion of the ischemic tissue at later stages results in further tissue damage by release of free radicals and other toxic metabolic byproducts (Cao and Dong, 2013; Martirosyan et al., 2011; Mortazavi et al., 2015; Oyinbo, 2011).

Rodent models of spinal cord injury

Human SCI can be grossly classified into four groups: solid cord injury, contusion/cavity, laceration and massive compression (Norenberg et al., 2004). Among them, contusion injury has the highest occurrence (49% of cases) and is often caused by blunt, non-penetrating trauma. Human contusion is usually associated with hemorrhage, necrosis and cavitation in the injured spinal cord.
parenchyma with no disruption of the meninges. Laceration has the second highest occurrence (21% of cases), characterized by a clear-cut penetration through the meninges, with damage to the underlying parenchyma. The lesion of laceration injury is usually filled with a fibrous connective tissue that is adherent to the meninges. To mimic the pathology of human SCI, a range of models such as contusion, compression and transection-based injuries have been developed and applied to different mammalian species (Kundi et al., 2013). Historically, SCI has been studied in larger animals such as cats, pigs and non-human primates, but now rodent models of SCI are more widely used due to reasons such as size, cost, availability, housing facilities, medical care and ethics (Lee and Lee, 2013).

Because contusion is the most common type of SCI in humans, rodent models of contusive SCI are considered clinically relevant and are preferred in pre-clinical studies. Reproducible experimental contusion injury depends on precise devices. Three types of devices are most commonly used: the Ohio State University impactor, the New York University impactor and the Infinite Horizon impactor. All of these devices are capable of delivering a computer-adjustable force to the exposed spinal cord to control injury severity (Kim et al., 2009). Contusion injury typically results in a lesion irregular in shape that expands both rostrally and caudally beyond the primary injury site (Kigerl et al., 2006), while leaving the meninges largely intact. Interestingly, in mice the contusion injury site is completely filled by a fibrotic scar tissue characterized by excessive deposition of extracellular matrix proteins and accumulation of perivascular fibroblasts as well as microglia/macrophages. However, in rats and other larger mammals
including humans, the injury site gradually degenerates and develops fluid-filled cavities. Why mice and rats display such distinct scarring responses after contusive SCI is unknown. We postulate that the initial scarring mechanisms between mice and rats (and possibly humans) are similar, evident by the presence of a fibrotic scar rich in connective tissue in rats at 7 days after contusion (Zhang et al., 2005). However, at later stages after contusion, the fibrotic scar in rats is only present lining the cavity wall (Zhu et al., 2014b), indicating that species-specific responses may have altered scarring progression chronically after SCI. Given the observation that the fibrotic scar is always present along the cavity wall in rats, we could infer that a diminished fibrotic scarring may be the cause of the significant cavitation after rat SCI. This is in sharp contrast to the dense fibrotic scar in mice where cavitation does not happen. A possible explanation for this could be due to that rats and mice have different inflammatory reactions after contusive SCI (Sroga et al., 2003). Thus, mouse model of contusive SCI is advantageous for the study of central nervous system (CNS) fibrosis, which can provide clues to why fibrotic scarring is not as prominent and why cavitation occurs in human patients of SCI. Lastly, the disadvantage of contusion injury is that it often leaves a peripheral rim of uninjured white matter, in which spared axons are difficult to distinguish from regenerating axons, causing confusion especially when investigating axon regeneration after SCI (Lee and Lee, 2013).

Similar to laceration injury in humans, rodent penetrating SCI tears the meninges and leads to deposition of a connective tissue mass in the lesion
This connective tissue scar is similar to the fibrotic scar formed after contusion in regards to the extracellular matrix composition. However, these two types of scar tissues may be derived from different origins. The scar tissue after penetrating injury is likely from a meningeal origin, based on its continuous association with the dura. While the dura remains intact after contusion, the fibrotic scar is formed by fibroblasts that come from the perivascular niche (Soderblom et al., 2013). The most frequently used penetrating SCI models include complete transection, focal myelotomy (incision), and dorsal or lateral hemisection (Onifer et al., 2007). Unlike contusion injury that often spares axon tracts, penetrating injury models can effectively sever descending or ascending axon tracts of interest, and thus are suitable for studying mechanisms that inhibit or promote axon regeneration after SCI.

In summary, by using different rodent models of SCI to study cellular events such as scar formation after SCI, we can better understand the pathology in humans in order to promote repair.

1.2 Scar formation after spinal cord injury

Axon regeneration failure after CNS injury

It has been known that injured axons have limited regenerative ability in the adult mammalian CNS. A prevailing theory is that both the presence of an extrinsic inhibitory environment and the lack of intrinsic growth ability contribute to this failure of axonal regrowth after CNS injury. The scar tissue at the injury site as well as oligodendrocyte-derived myelin are the two main sources of
extrinsic factors that contribute to the inhibitory environment in the SCI lesion. While triple knockout of myelin-associated inhibitors Nogo, MAG, and OMgp did not lead to increased axonal regeneration after SCI (Lee et al., 2010; Lee and Zheng, 2012), altering scar-associated extracellular matrix (ECM) molecules such as chondroitin sulfate proteoglycans (CSPGs) has resulted in enhanced regenerative outcomes after SCI (Ramer et al., 2014). Although modulation of intrinsic pathways such as PTEN/mTOR increased growth ability of neurons and successfully improved axonal regeneration after SCI (Liu et al., 2010; Park et al., 2008; Zukor et al., 2013), limited functional recovery was achieved by stimulating neuron-intrinsic growth ability alone, suggesting that combinatory strategies targeting both extrinsic and intrinsic factors are required to maximize restoration of function after SCI. Therefore, a better understanding of the cellular and molecular mechanisms underlying scar formation after SCI is an essential step toward development of better treatment strategies for SCI.

**CNS versus non-CNS wound healing**

Tissue damage typically triggers a natural reparative response called wound healing that attempts to restore tissue homeostasis and recover physiological function. The wound healing process has been extensively investigated in the context of cutaneous injury. However, whether wound healing contributes to the pathology of SCI is poorly studied. Skin wound healing shares many similarities with that of CNS injury. For example, the major stages of skin wound healing include homeostasis, inflammation, proliferation, and scar
remodeling, which are all present after SCI (Bielefeld et al., 2013; Eming et al., 2014; Shaw and Martin, 2009). Immediately after injury in both scenarios, blood vessels are destroyed and coagulation is initiated to maintain tissue homeostasis. This is followed by inflammation that is orchestrated by infiltrating and tissue-resident immune cells which mediate clearance of tissue debris. Next, cells including endothelial cells, fibroblasts and many others at the injury site undergo extensive proliferation, migration and deposition of an ECM, which result in formation of a granulation tissue that serves as a provisional replacement for the damaged tissue. The final stage, scar remodeling, is where CNS and non-CNS wound healing diverge tremendously. While in skin injury the scar tissue can normally resolve and become replaced by newly generated epidermal cells, the scar tissue in the CNS persists in the injury site and does not go away. This situation resembles pathological wound healing in the skin where the injury site develops a chronic wound or fibrosis, characterized by excessive deposition of ECM proteins, prolonged accumulation of fibroblasts and immune cells (Leask and Abraham, 2004). Indeed, these features are commonly seen after SCI (Soderblom et al., 2013; Zhu et al., 2014a; Zhu et al., 2014b). Why CNS scarring is unable to resolve is not known, but one speculation is that this could be due to CNS-unique features following injury, such as extended blood-CNS barrier leakage, prolonged gliosis, inefficient clearance of myelin debris, and limited regenerative ability of CNS-resident cells (Shechter and Schwartz, 2013). My dissertation work focused on a particular aspect of CNS scarring, namely, the fibrotic scar formation after SCI, which will be covered in more detail below.
Glial and fibrotic scars

The scar tissue that forms at the SCI site has been categorized into glial and fibrotic components. The glial scar is present at the penumbra of the injury site, and is comprised of GFAP\(^+\) reactive astrocytes, NG2\(^+\) cells (also known as oligodendrocyte progenitor cells), and activated microglia/macrophages which are distributed throughout the injury site. Astrocytes have been extensively studied and are known as the primary cells that undergo gliosis to form the glial scar (Cregg et al., 2014). NG2\(^+\) cells are less understood than other cells after CNS injury. NG2\(^+\) cells have been shown to respond to CNS injury by short-distance migration towards the lesion (Hughes et al., 2013) and local proliferation (McTigue et al., 2001), and are capable of differentiating into oligodendrocytes as well as astrocytes after CNS injury (Komitova et al., 2011; Sellers et al., 2009; Tripathi et al., 2010; Zawadzka et al., 2010). However, whether NG2\(^+\) cells play a role in scar formation is not clear. While microglia/macrophages are present in both the glial and fibrotic scars, their contribution to scar formation after SCI is not completely understood. A more detailed review of the roles of microglia/macrophages in CNS injury will be discussed in Section 1.4 of this chapter.

The glial scar has been traditionally regarded as the primary inhibitory barrier to regenerating axons. Upon injury, astrocytes become reactive in response to many factors, such as pro-inflammation cytokines (e.g IL-1\(\beta\) and TNF-\(\alpha\)) produced by activated microglia/macrophages and other molecules including TGF-\(\beta\), fibrinogen, and CNTF (Sofroniew, 2014). As a result, astrocytes...
become hypertrophic and rapidly increase the expression of intermediate filament proteins such as GFAP, vimentin and nestin, and form a dense meshwork surrounding the lesion core (Burda and Sofroniew, 2014). While it has been long postulated that the large number of astrocytes at the injury site is a result of both migration and proliferation, recent studies have demonstrated that astrocyte aggregation around the lesion mostly relies on local proliferation instead of migration, after both brain (Bardehle et al., 2013) and spinal cord (Wanner et al., 2013) injury. This astrocytic glial scar exerts beneficial functions by restricting inflammatory cells and sealing off the injury site to prevent spreading of secondary damage to the nearby intact tissue (Faulkner et al., 2004; Wanner et al., 2013). However, reactive astrocytes also produce ECM proteins such as laminin and collagen to form a basal lamina surrounding the injury site, which is regarded as a physical barrier that blocks axon regeneration (Liesi and Kauppila, 2002). Furthermore, the glial scar is also considered a potent chemical barrier because many inhibitory ECM molecules are secreted and deposited in the glial scar, including tenascin C, semaphorin 3A, Ephrin-B2 and a series of CSPGs (Cregg et al., 2014; Sandvig et al., 2004). CSPGs are the most well-characterized inhibitors of axon growth after SCI, and delivery of Chondroitinase ABC, a bacterial enzyme that degrades CSPGs, can promote axonal growth as well as partial recovery of functions that are lost after SCI (Bradbury and Carter, 2011).

In addition to the glial scar, the fibrotic scar is another inhibitory barrier that prevents axons from regenerating. The fibrotic scar occupies the injury
epicenter which lacks GFAP expression, and is best characterized by accumulation of fibroblasts and macrophages, and excess deposition of ECM molecules such as fibronectin (Soderblom et al., 2013; Zhu et al., 2014a; Zhu et al., 2014b). Most studies of the scar tissue have focused on the glial scar while the role of the fibrotic scar is poorly understood. One reason for this gap in our knowledge is that there are no specific antigenic markers for fibroblasts. Using genetically modified mice, it was recently demonstrated by our laboratory that collagen1α1+ perivascular fibroblasts detached from large diameter blood vessels and chronically resided in the injury site to form the fibrotic scar (Soderblom et al., 2013). The non-permissive effect of fibroblasts on axonal growth was first revealed by in vitro astrocyte-fibroblast co-culture experiments, in which neurons plated on top of this co-culture preferred to extend axons over astrocytes and avoided fibroblasts (Shearer and Fawcett, 2001). Moreover, similar results were also observed under in vivo conditions. Recent studies showed that axons with stimulated intrinsic growth ability can grow on GFAP+ astrocyte processes to cross the SCI lesion, but they always avoided the GFAP− fibrotic scar (Liu et al., 2010; Zukor et al., 2013). This suggests that the glial scar is not always non-permissive for axonal growth, while the fibrotic scar may be a more potent inhibitory barrier in the scar tissue that impedes axonal growth. Therefore, understanding the mechanisms of fibrotic scar formation after SCI may provide insights into finding therapeutic strategies that reduce fibrotic scarring and encourage axon regeneration.
1.3 Fibronectin in injury and repair

Roles of fibronectin in non-CNS tissues

Fibronectin is a ubiquitous ECM protein that plays essential roles in cell growth, adhesion and migration, and has been extensively studied in embryonic development and tissue repair responses. Fibronectin is produced from a single gene with multiple isoforms generated by alternative splicing. There are two major types of fibronectin. One is plasma fibronectin, which circulates in the blood at a high concentration in an inactive form, and is almost exclusively synthesized by liver hepatocytes. The other type is cellular fibronectin, which is a mixture of different isoforms that contain alternative splicing domains including EIIIA, EIIIB and a variable region (Schwarzbauer and DeSimone, 2011; To and Midwood, 2011). Cellular fibronectin could come from multiple sources such as fibroblasts, macrophages, and reactive astrocytes (Gratchev et al., 2001; Stoffels et al., 2013). Both plasma fibronectin and cellular fibronectin are initially expressed in a deoxycholate-soluble form but are irreversibly converted into a deoxycholate-insoluble form as they are incorporated into an ECM (Singh et al., 2010).

In fibrosis and wound healing of non-CNS tissues, the general role of fibronectin is to form a scaffold that binds other ECM proteins and also facilitates cell migration to promote tissue remodeling and repair. In the case of skin injury, plasma fibronectin is deposited at the injury site after blood vessel damage and interacts with platelet and fibrin to form a provisional clot matrix which stops bleeding. As repair of the injury site progresses, endothelial cells, fibroblasts,
macrophages and many other types of cells repopulate the wound, degrade the provisional clot matrix and deposit cellular fibronectin to form a more stable matrix. The fibronectin matrix has been shown to direct endothelial cell migration and proliferation, and promote angiogenesis (Zhou et al., 2008; Zou et al., 2012). It also regulates fibroblast-mediated matrix contraction (Liu et al., 2006; Meckmongkol et al., 2007; Midwood et al., 2004). To be a functional matrix, soluble fibronectin needs to polymerize into a fibrillar network, which is initiated by binding to cellular integrin receptors. Upon binding to fibronectin, integrin receptors cluster and bring adjacent fibronectin fibrils close together thereby promoting their association and formation into an insoluble matrix (Rhett et al., 2008; Singh et al., 2010; To and Midwood, 2011). In addition to skin injury, fibronectin is also found to attract fibroblasts in glomerular fibrosis (Gharaee-Kermani et al., 1996) and support migration and survival of both circulating monocytes and microglia (Abshire et al., 2011; Nasu-Tada et al., 2005; Trial et al., 1999). Inhibition of fibronectin matrix assembly by applying a fibronectin polymerization inhibitor pUR4 in vivo significantly reduced ECM deposition, cell proliferation and leukocytes infiltration in atherosclerosis (Chiang et al., 2009). Thus, the critical functions of fibronectin depend on assembly of secreted soluble fibronectin into a matrix. Studies in non-CNS organ systems provide clues to the roles of fibronectin after SCI.
Roles of fibronectin in CNS injury

Our current knowledge of fibronectin in the CNS is mostly based on plasma rather than cellular fibronectin probably because plasma fibronectin is structurally more homogenous and has a high concentration in the blood plasma, thus making it much easier to isolate than cellular fibronectin. Plasma fibronectin is frequently incorporated with other ECM molecules such as collagen and laminin into biomaterials to facilitate cell adhesion, migration and survival after CNS injury (Alovskaya A, 2007; Geller and Fawcett, 2002). For example, plasma fibronectin was absorbed into a collagen I-based scaffold to support neural stem cell implants in the rat brain and this promoted cell migration and survival and improved behavioral performance after traumatic brain injury (Tate et al., 2009). In a rat SCI model, plasma fibronectin was mixed with soluble fibrin in a liquid gel and injected into the lesion cavity in the spinal cord to eliminate the formation of cavities. This gel also promoted Schwann cell migration and axonal growth into the implants (King et al., 2010a). Moreover, plasma fibronectin is implicated in a neuroprotective role in the CNS. Application of plasma fibronectin mats to the rat SCI site reduced lesion size, apoptosis, the number of damaged axons and promoted functional recovery (King et al., 2010b). This result is consistent with previous reports that mice lacking plasma fibronectin had greater cell death and larger lesion size after cerebral ischemia and traumatic brain injury (Sakai et al., 2001; Tate et al., 2007). In addition, fibronectin is also involved in neuropathic pain after SCI. Intrathecal injection of plasma fibronectin in rat resulted in up-regulation of P2X4 receptors on microglia which enhanced tactile allodynia that
was rescued by administration of antagonists of integrin receptors (Tsuda et al., 2008). This result suggested that fibronectin-integrin signaling in the spinal cord caused neuropathic pain, which contradicted with a recent study in which a single injection of plasma fibronectin immediately after SCI alleviated chronic allodynia and increased axon sprouting in rats, probably through integrin α5β1 and/or α4β1 pathways (Lin et al., 2012). While most studies have focused on blood-derived plasma fibronectin, cellular fibronectin produced locally at the injury site may be more important in forming the ECM in the fibrotic scar. One line of evidence in support of this is that the blood-spinal cord barrier breached following SCI becomes re-established and non-permeable to large proteins like plasma fibronectin around 2 weeks after injury (Noble and Wrathall, 1989; Whetstone et al., 2003), while the fibronectin matrix is still present in the fibrotic scar after 14 days and for up to 6 months (Camand et al., 2004; Zhu et al., 2014b), indicating that fibronectin in the fibrotic scar may come from a cellular source. Thus, the mechanism of fibronectin matrix assembly after SCI and its functional role in the fibrotic scar is largely unexplored.

**Integrin receptors involved in fibronectin assembly**

ECM matrix assembly is an essential process during fibrotic scar formation. Assembly of the fibronectin matrix is initiated by integrin receptors, which are transmembrane receptors that consist of heterodimeric α and β subunits. The main function of integrins is to attach cells to the ECM by coupling their cytoskeleton to the ECM and transduce both mechanical and chemical
signals from outside environment to the cell. This provides a cell with contextual information including cell location and adhesive state, which affects the cellular response to other inputs (Harburger and Calderwood, 2009; Hynes, 2002). For example, integrins and PDGF are known to signal through collaborative pathways to regulate cell proliferation and migration. Loss of cellular attachment to the ECM induced a reduction in both protein expression and auto-phosphorylation of PDGFR-β, and this can be reversed by plating the cells on a fibronectin substrate again (Baron and Schwartz, 2000). Thus, cells can sense surrounding environmental information by integrins binding to the ECM, which leads to activation of shared downstream elements and crosstalk with other pathways that regulate cellular functions such as adhesion, movement and growth.

As a ubiquitous ECM protein, fibronectin binds to many receptors including syndecans, integrins α5β1, αvβ3, αvβ6, α4β1, and αIIbβ3. Among them, integrin α5β1 mediates fibronectin assembly most efficiently in vitro while other receptors require addition of stimulation reagents to facilitate formation of fibronectin fibrils (Schwarzbauer & Desimone, 2011). Genetic knockout studies in mouse embryonic development have provided evidence that α5 and αv integrins are key receptors that mediate fibronectin assembly. Mice lacking either α5 or αv integrin exhibited normal fibronectin matrix assembly. However, double knockout of both αv and α5 dramatically reduced the amount of the fibronectin matrix, resembling the phenotype of the fibronectin-null mice (Bader et al., 1998; George et al., 1993; Yang et al., 1999). Furthermore, recent study revealed that integrin
αvβ3 assembled a normal fibronectin matrix in vivo when the RGD binding domain for α5β1 was mutated (Takahashi et al., 2007). Since other fibronectin binding αv integrins including αvβ6 and αvβ1 do not support fibronectin fibrillogenesis in vivo (Huang et al., 1996; To and Midwood, 2011), these evidences indicate that α5β1 and αvβ3 may be the most important integrin receptors responsible for fibronectin matrix assembly in vivo. Thus, targeting integrin receptors responsible for fibronectin assembly may be another potential way to manipulate fibrotic scar formation after SCI.

1.4 Macrophage and microglia responses after spinal cord injury

Overview of the immune responses after spinal cord injury

The mammalian CNS is relatively immune-privileged under normal physiological conditions, due to the lack of professional antigen-presenting cells (such as dendritic cells) in the CNS parenchyma and the presence of a tight blood-CNS barrier that limits entry of peripheral immune mediators (Galea et al., 2007). However, trauma to the spinal cord leads to blood vessel rupture that greatly compromises the blood-spinal cord barrier. As a consequence, extravasation of plasma proteins and immune cells from the hemorrhage, along with primary cell death induced by mechanical injury, initiates a series of complex neuroinflammatory responses, which further contribute to secondary tissue damage after injury.

A principal component of the neuroinflammatory response after SCI is the innate immune response mediated primarily by myeloid cells. The major myeloid
effector cells after SCI include neutrophils, microglia and macrophages. As CNS resident surveillance cells, microglia are among the first myeloid cells to respond to CNS injury. After a focal laser injury to the spinal cord or brain, microglia re-orient and extend their fine processes rapidly towards the injury site, potentially sealing off the lesion to prevent the spread of damage. This process happens within minutes to hours (Davalos et al., 2005; Dibaj et al., 2010; Hines et al., 2009). During the next few hours, injury-triggered inflammation starts to attract circulating leukocytes to infiltrate the lesion. Neutrophils are the first to arrive. They arrive at the injury site within hours and peak at 1~2 days. Macrophages reach the injury site later, at around 3 days and peak at 7~14 days with a second wave of infiltration at 6~8 weeks after injury (Beck et al., 2010; Fleming et al., 2006; Kigerl et al., 2006; Stirling and Yong, 2008). Neutrophils have generally received less attention than macrophages in SCI studies, probably because they are present in significant numbers mostly at an acute time point and are replaced by macrophages at sub-acute phases (~14 days), when most of the pathological processes at the injury site are occurring (Benowitz and Popovich, 2011; Hawthorne and Popovich, 2011). Macrophages present at the CNS injury site are derived from microglia and/or blood monocytes, and are often collectively referred to as microglia/macrophages, because they are morphologically and antigenically similar in histological sections. Macrophages at the injury site are highly phagocytic and are capable of clearing out necrotic debris to promote tissue repair. However, they also mediate axonal dieback and produce inflammatory cytokines and free radicals that exacerbate SCI pathology.
The dual roles, both reparative and destructive, of macrophages and microglia will be discussed in detail below. Mast cells are another type of myeloid cells which are mainly present in the meninges, the choroid plexus, and perivascular regions of the CNS parenchyma (Nelissen et al., 2014). Mast cells have been shown to aggravate the pathology of experimental autoimmune encephalomyelitis (Secor et al., 2000; Tanzola et al., 2003). They are also believed to be the first responders after stroke that mediate ischemic damage in the brain (Jin et al., 2009; Lindsberg et al., 2010; Strbian et al., 2006). Role of mast cells in SCI has not been thoroughly studied. One study showed that mast cells were protective by production of proteases that degrade pro-inflammatory cytokines after SCI (Nelissen et al., 2014).

SCI also affects the adaptive immune system, which has received less attention probably because its response is not as prominent as the innate immune response. The number of infiltrating lymphocytes is 10~20 times fewer than microglia/macrophages after both spinal cord and brain injury (Beck et al., 2010; Jin et al., 2012). After SCI, T and B lymphocytes are activated in the spleen and bone marrow within 24 hours, and they infiltrate the SCI site starting from a few days and lasting until months after injury (Ankeny et al., 2006; Beck et al., 2010; Sroga et al., 2003). Although SCI can activate cells of the adaptive immune system, SCI generally leads to a systemic depression of the immune system. This is supported by reports that bacterial infections are the leading cause of mortality in patients in the post-acute phase following SCI (Riegger et al., 2009). Although the mechanism underlying post-SCI immune depression is
not known, compelling evidence has indicated that SCI disrupts autonomic control of lymphoid organs such as the spleen, which results in dysfunction of lymphocytes that comprise the adaptive immune response. It has been shown that SCI suppresses effector functions of CD4\(^+\) T cells to control viral infection (Held et al., 2010), and chronic SCI impairs both CD4\(^+\) and CD8\(^+\) T cell functions by inducing T cell exhaustion and altering splenic sympathetic activity (Zha et al., 2014). SCI also changes germinal center B cell function and impairs primary antibody responses (Oropallo et al., 2012). In addition, SCI leads to release of many self-antigens that activate autoreactive lymphocytes, causing autoimmune reactions which may exacerbate the injury. It has been reported that autoantibodies against a range of CNS proteins such as GM1 gangliosides and myelin-associated glycoprotein were detected in serum of SCI patients (Davies et al., 2007). In mice, antibody-secreting B cells, immunoglobulins and complement component 1q were present in the cerebrospinal fluid and accumulated at sites of axon loss and demyelination in the injured spinal cord (Ankeny et al., 2009). Finally, although these evidences suggest that lymphocytes are effector cells after SCI, it is not known whether they directly contribute to the pathophysiology of SCI. Thus, future studies will be needed to elucidate the beneficial and pathologic effects of the adaptive immune response in order to find better treatments for SCI.
Detrimental and beneficial effects of microglia/macrophages after SCI

As one of the most prevalent class of effector cells involved in neuroinflammation, activated microglia/macrophages are generally attributed with a detrimental role after SCI. Numerous studies have indicated that activation of microglia/macrophages exacerbates the primary traumatic injury, leading to disruption of the blood-spinal cord barrier, expansion of the lesion, destruction of spared axons and neurons and persistent chronic inflammation. For example, activated microglia/macrophages secrete several classes of factors including pro-inflammatory cytokines, proteases and oxidative metabolites, all of which act on other immune cells, glia and endothelial cells as part of the inflammatory cascade that ultimately leads to cell damage and death (Mortazavi et al., 2015; Oyinbo, 2011). For example, IL-1β and TNF-α are two major pro-inflammatory cytokines expressed by microglia/macrophages that are cytotoxic to neurons and oligodendrocytes (Esposito and Cuzzocrea, 2011). Matrix metalloproteinases are a class of proteases secreted by microglia/macrophages, which contribute to blood-spinal cord barrier breakdown, oxidative stress and apoptosis after SCI (Zhang et al., 2011). Activated microglia/macrophages also release free radicals such as nitric oxide, which can cause oxidative damage to organelles and nucleic acids within neurons and glia (Oyinbo, 2011). The destructive effects of macrophages after SCI have led to many studies using various reagents to inhibit or deplete microglia/macrophages. Counteracting macrophage responses during the first 1-2 weeks after SCI has consistently resulted in decreased tissue damage and improved functional recovery (Silver et al., 2014). These include use
of anti-inflammatory drugs such as minocycline (Stirling et al., 2004) and FK506 (Lopez-Vales et al., 2005) that inhibit macrophage activation, clodronate liposomes that systematically deplete monocytes and macrophages (Popovich et al., 1999), and mouse genetic manipulations such as myeloid-targeted deletion of the CD95-ligand which blocks macrophage infiltration (Letellier et al., 2010).

Macrophages can also have beneficial roles after SCI. Besides scavenging cell debris such as damaged myelin sheaths, microglia/macrophages can also promote neuroprotection as well as reduce inflammation. Activated microglia/macrophages are known to express a variety of neurotrophic factors such as CNTF, BDNF, NGF, NT-3, PDGF and oncomodulin after CNS injury (Benowitz and Popovich, 2011; Donnelly and Popovich, 2008). These factors have neuroprotective functions and can potentially support neural survival and sprouting of spared axons after SCI. Macrophages also produce cytokines to resolve inflammation. For example, a subset of macrophages trafficking through the choroid plexus of the brain was found at the SCI site. They exhibited M2-like characteristics, expressed anti-inflammatory cytokines IL-10 and TGF-β, suppressed the pro-inflammatory cytokine milieu, and promoted functional recovery (Shechter et al., 2013). Interestingly, it was also reported that macrophages supported myelination. Systematic macrophage depletion reduced oligodendrocyte progenitor cells (OPCs) recruitment and compromised remyelination in a focal spinal cord demyelination model (Kotter et al., 2005). Further studies showed that zymosan-activated microglia/macrophages secreted endothelin 2 to promote OPC-mediated remyelination in the retina (Setzu et al.,
Although it has not been documented, macrophages may also promote repair of myelin sheaths through similar mechanisms after SCI. This can be critical for promoting regeneration after SCI, since chronic loss of oligodendrocytes is a common feature in SCI pathology.

**Macrophage heterogeneity and spinal cord injury**

Studies in peripheral organ systems have uncovered a continuum of macrophage activation or polarization states, with classically activated (M1) and alternatively activated (M2) macrophages being the extremes. These distinct activation states were first documented *in vitro*, where type 1 T helper cell (Th1) related cytokines, such as interferon-γ, and microbial toxin lipopolysaccharide (LPS) drive macrophages to the M1 phenotype and induce production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, and TNFα as well as other inflammatory mediators such as reactive oxygen and nitrogen intermediates. Contrary to M1 macrophages, type 2 T helper cell (Th2) related cytokines, such as IL-4 and IL-13, activate macrophages to an M2 phenotype that has a relatively low expression level of pro-inflammatory cytokines. *In vivo* characterization of the phenotypes and functions of the M1/M2 cells has been controversial, but it is largely believed that M1 macrophages are pro-inflammatory, possess higher phagocytic and antigen-presenting abilities and exert microbicidal functions, whereas M2 macrophages are anti-inflammatory and play important regulatory roles in wound healing and fibrosis (David and Kroner, 2011; Murray and Wynn, 2011). The controversy is that under pathological conditions, macrophages can
show mixed or overlapped M1/M2 phenotypes rather than clear-cut polarization states (Martinez and Gordon, 2014), probably due to the fact that the immunological microenvironment in tissue is complex and there are no clear stimuli for M1 and M2 phenotypes as compared to *in vitro* conditions. Thus, the current M1 and M2 classification is oversimplified and a wide range of macrophage activation states may exist *in vivo*. As a consequence, understanding macrophage function at the population level is limited by its heterogeneous nature and use of single-cell approaches may help to better define distinct macrophage phenotypes.

What has added more complexity to the M1/M2 paradigm is the presence of multiple subsets of monocytes. Monocytes are blood-circulating precursor cells of dendritic cells and macrophages. At least two discrete subsets of monocytes exist in mice and human under steady state, which are referred as inflammatory versus resident monocytes (or classical versus non-classical monocytes). The inflammatory monocytes in mice are Ly6c\textsuperscript{hi}/CD43\textsuperscript{lo}/Ccr2\textsuperscript{hi}/Cx3cr1\textsuperscript{lo}, while resident monocytes are Ly6c\textsuperscript{lo}/CD43\textsuperscript{hi}/Ccr2\textsuperscript{lo}/Cx3cr1\textsuperscript{hi}, corresponding to the CD14\textsuperscript{hi}/CD16\textsuperscript{lo} inflammatory and CD14\textsuperscript{lo}/CD16\textsuperscript{hi} resident monocytes found in humans (Ziegler-Heitbrock et al., 2010). Recruitment of different monocyte subsets during non-CNS wound healing happens in a sequential manner: Ly6c\textsuperscript{hi} inflammatory monocytes invade the inflamed tissue first and mediate acute inflammation; Ly6c\textsuperscript{lo} resident monocytes become dominant at later resolution phases where they express lower levels of pro-inflammatory cytokines and promote healing (Arnold et al., 2007; Crane et al., 2014; Nahrendorf et al., 2007).
After CNS trauma such as SCI, Ly6c\textsuperscript{hi} and Ly6c\textsuperscript{lo} monocyte/macrophages exhibiting pro- and anti-inflammatory characteristics were also identified at the injury site (Shechter et al., 2013). Although the inflammatory properties of different monocyte subsets resemble the M1 and M2 macrophage polarization states, there is no direct evidence that M1/M2 macrophages are direct progenies of Ly6c\textsuperscript{hi}/Ly6c\textsuperscript{lo} monocytes. In fact, studies have shown that either Ly6c\textsuperscript{hi} or Ly6c\textsuperscript{lo} monocytes could give rise to both M1 and M2 macrophages (Arnold et al., 2007; Misharin et al., 2014).

A growing number of studies have started to focus on macrophage polarization after SCI. A time course study of macrophage phenotypes using immunohistochemistry showed that microglia/macrophages at the SCI site are predominately M1 subtype, which are CD86- and CD16/32- positive, whereas arginase 1- and CD206-positive M2 subtype comprises only a small fraction (~40% at 1-3 days and reducing to less than 10% at 28 days). However, microarray analysis of the entire injury site for various M1 and M2 markers did not show coordinated expression changes (Kigerl et al., 2009), which does not support a clear phenotypic switch of macrophages after SCI. This is probably due to the fact that heterogeneous subsets of microglia/macrophages have different timing of activation after SCI, and there is a lack of specific markers to define macrophage subsets – intermediate subsets can express both M1 and M2 markers (Martinez and Gordon, 2014). Overall, although previous studies indicate that cytokine-elicited M1 macrophages are toxic to neurons and induce axonal dieback, while M2 macrophages promote axon outgrowth on inhibitory
substrates (Busch et al., 2011; Horn et al., 2008; Kigerl et al., 2009), the functional significance of microglia/macrophages polarization after SCI is not fully understood.

**Macrophage versus microglia after spinal cord injury**

Microglia are tissue-resident macrophages of the CNS. Under homeostatic conditions, microglia have highly ramified morphology and are constantly extending and retracting numerous cytoplasmic processes to survey the surrounding microenvironment. When exposed to insult to the CNS such as SCI, microglia lose their processes and convert to an amoeboid morphology to become activated, functional phagocytes (David and Kroner, 2011). Activated microglia are morphologically similar to monocyte-derived macrophages, which are referred to as hematogenous macrophages (hMΦ), and the two cell types express many of the same myeloid markers including CD11b, Iba1, CSF1R and F4/80. For these reasons, morphological or phenotypic distinction between activated microglia and hMΦ in histological sections has been difficult, leading to ambiguities of where macrophages at the SCI site are derived from. Although a few studies using bone marrow chimeras, flow cytometry and *in vivo* live imaging have reported distinct spatiotemporal infiltration profiles between microglia and hMΦ after SCI (Evans et al., 2014; Fenrich et al., 2013; Mawhinney et al., 2012; Popovich and Hickey, 2001), none of them have investigated the contribution of microglia and hMΦ to formation of the glial and fibrotic scar. Therefore, our study
aimed to fill in this gap in knowledge in order to better understand the mechanism of scar formation after SCI.

These morphological and phenotypic similarities between microglia and hMΦ are partially due to the fact that both types of cells are from the myeloid lineage. However, microglia and hMΦ have distinct origins developmentally. Genetic fate-mapping studies in mice have demonstrated that microglia arise from the primitive ectoderm of the yolk sac at around embryonic day 8.5, while blood monocytes are derived from hematopoietic stem cells in the fetal liver at around embryonic day 12, and later the bone marrow takes over to generate monocytes postnatally. Contrary to the short-lived circulating monocytes (half-life of a mouse monocyte is about 1-2 days) (Yona et al., 2013), microglia have very long life-span and stay in the CNS since their genesis. Microglia are insensitive to irradiation and undergo limited self-renewal by local proliferation (Ginhoux and Jung, 2014; Prinz and Priller, 2014; Wynn et al., 2013).

After injury to the CNS, microglia are the first responder cells while monocytes are newly recruited from the circulation at a later time point. Once monocytes enter the injury site, they differentiate into macrophages and dendritic cells in response to various inflammatory signals. Although it is known that bone marrow is the source of blood monocytes in adult animals, recent studies indicated that the spleen serves as a major reservoir for monocytes and rapidly releases them into the circulation after tissue damage (Swirski et al., 2009). An acute myocardial infarction mouse model showed that monocytes migrated from the spleen, but not bone marrow, to infiltrate the infarct site, and splenectomy
reduced 75% of monocytes influx into the ischemic myocardium. The process of monocyte mobilization from the spleen may be dependent on angiotensin II signaling and independent of CCR2 (Swirski et al., 2009). Similarly, in a rat model of stroke, splenectomy significantly reduced the number of activated microglia/macrophages but not neutrophils at the infarct zone, and this is associated with greater tissue preservation (Ajmo et al., 2008). In addition to ischemic injury, splenic monocytes also seem to contribute to infiltrating macrophages after contusive SCI. A study using mice bone marrow transplantation method reliably identified hMΦ in the spinal cord, and when splenectomy was performed after SCI, hMΦ at the injury site were almost completely eliminated at 7 days, which was also accompanied by improved locomotion recovery at 42 days (Blomster et al., 2013).

Due to the distinct origins from which microglia and hMΦ are generated and recruited, it is logical to postulate that these cells are intrinsically different, and thus contribute differently to the neuroinflammatory effects after SCI. However, whether this idea is true remains unclear, although a few studies have provided clues. In line with the fact that microglia react to injury almost immediately whereas hMΦ do not infiltrate the injured spinal cord until around 3 days (Beck et al., 2010; Dibaj et al., 2010; Fleming et al., 2006; Kigerl et al., 2006; Stirling and Yong, 2008), a mouse contusive SCI study confirmed that microglia were associated with phagocytosis of tissue debris acutely after injury while hMΦ took over at later stages. Interestingly, microglia seemed to process phagocytic materials more efficiently, remained viable and proliferated 7-fold greater than
hMΦ, whereas higher number of hMΦ were associated with engulfed materials and hMΦ were more susceptible to cell death (Greenhalgh and David, 2014). These results suggest that microglia may contribute more positively than hMΦ to SCI recovery, which is consistent with the observation that hMΦ but not microglia, are responsible for secondary axonal dieback after dorsal column crush (Evans et al., 2014). However, conflicting data indicated that hMΦ rather than microglia, promoted spontaneous hindlimb recovery after SCI through production of the anti-inflammatory cytokine IL-10 (Shechter et al., 2009). Therefore, the differential roles microglia and hMΦ may play after SCI require further investigation. A better understanding of this notion may enable us to boost the beneficial effects of neuroinflammation in order to maximize functional recovery after SCI.

1.5 Study objective and specific aims

SCI leads to formation of a fibrotic scar that is highly inhibitory to regenerating axons. The fibrotic scar is best characterized by accumulation of microglia/macrophages and perivascular fibroblasts, and deposition of a fibronectin matrix. While fibronectin plays essential roles in non-CNS wound healing, it is not known how the fibronectin matrix is formed after SCI. It is also not clear whether macrophages derived from different origins contribute differently to the fibrotic scar and what is the mechanism by which perivascular fibroblasts are recruited to the injury site.
The overall objective of our study was to investigate the mechanism of the fibrotic scar formation after contusive SCI and explore how we can reduce this inhibitory barrier to promote axon regeneration. Our central hypothesis was that perivascular fibroblasts are recruited to the injury site by hMΦ, which are the major cells responsible for formation of the fibronectin matrix in the fibrotic scar. To test our central hypothesis, the following specific aims and hypotheses were investigated.

**Aim 1**: Determine the spatiotemporal expression of fibronectin and its integrin receptors in the fibrotic scar after contusive SCI. Hypothesis: fibronectin is associated with integrin receptor α5β1 which is primarily expressed by microglia/macrophages in the fibrotic scar.

**Aim 2**: Distinguish between microglia and hMΦ in formation of the fibrotic scar after contusive SCI. Hypothesis: Macrophages occupying the fibrotic scar are mostly of hematogenous origin.

**Aim 3**: Deplete macrophages in the fibrotic scar and determine the effects on scar formation and axon regeneration. Hypothesis: Since hMΦ attract infiltration of fibroblasts and assemble fibronectin into a matrix to form the fibrotic scar, depleting macrophages will result in decreased fibrotic scarring and increased axon regeneration.

Using genetically modified mice and a bone marrow transplantation approach, we demonstrated that hMΦ rather than microglia, are associated with the fibrotic scar and may be responsible for recruiting perivascular fibroblasts to the injury site. In addition, the fibronectin receptor integrin α5β1 is mostly
expressed by hMΦ, suggesting that hMΦ may play a role in fibronectin assembly in the fibrotic scar. By depleting hMΦ, we discovered that the overall cytokine profile of the lesion site becomes less fibrotic and this is correlated with decreased fibroblast accumulation, basal lamina formation and ECM deposition as well as increased axonal growth. Overall, our results filled a gap in knowledge about the role of macrophages in fibrotic scarring after SCI and highlighted hMΦ as a potential target to promote SCI repair.
Chapter 2
Fibronectin assembly after spinal cord injury

As a ubiquitous ECM protein, fibronectin plays an important role in tissue repair and remodeling during wound healing and fibrosis in non-CNS organs. However, whether or how fibronectin assembly occurs after SCI is poorly understood. In this chapter, we demonstrate that fibronectin is an ECM molecule intimately associated with fibroblasts in the fibrotic scar. By deleting fibronectin in myeloid cells, we demonstrate that myeloid cells are not the major contributors of fibronectin at the injury site and fibroblasts are most likely the major source. Finally, we demonstrate that fibronectin is initially present in a soluble form and is assembled into a matrix after SCI. Assembly of the fibronectin matrix may be mediated by the canonical fibronectin receptor, integrin α5β1, which is primarily expressed by macrophages/microglia in the fibrotic scar. Taken together, our results provide novel insight to the mechanism of fibrotic scar formation.

2.1 Fibronectin matrix and fibroblasts have similar spatiotemporal distribution after SCI

To study the mechanism of fibrotic scar formation after SCI, we used a mouse model of contusive SCI throughout our study because contusion injury is considered more clinically relevant than other SCI models (see Section 1.1). It was previously reported that fibronectin, collagen and laminin are the major ECM components of the fibrotic scar in the rat SCI lesion (Klapka and Muller, 2006; Weidner et al., 1999). To compare the expression pattern of these ECM markers
in our mouse model, we stained the injury site tissue with antibodies against collagen IV, laminin and fibronectin. At 14 days after SCI, all three ECM proteins were present in the GFAP\textsuperscript{−} region (Fig. 2.1), suggesting their presence in the fibrotic scar. While fibronectin was distributed throughout the fibrotic scar (GFAP\textsuperscript{−} regions), the expression of collagen IV and laminin was less prominent in the lesion epicenter. Instead, they were preferentially expressed in regions of the basal lamina present at blood vessels and the border between the glial (GFAP\textsuperscript{+}) and the fibrotic (GFAP\textsuperscript{−}) scars (Fig. 2.1). These observations are consistent with a previous study using a similar mouse contusion model (Jakeman et al., 2000). Thus, fibronectin is a reliable ECM marker of the fibrotic scar after contusive SCI in mice.

Since the fibrotic scar is best characterized by regions of high fibronectin expression and fibroblast infiltration, we sought to determine the spatiotemporal relationship between fibronectin and fibroblasts after SCI. We utilized Col1α1\textsuperscript{GFP} transgenic mice, which we have previously described as labeling perivascular fibroblasts in the spinal cord (Soderblom et al., 2013). In the uninjured spinal cord, fibroblasts and fibronectin were found only around blood vessels and in the meninges (Fig. 2.2A-D). At 3 days after injury, the injury site was mostly necrotic with very few fibroblasts, suggesting that the fibrotic scar has not formed yet. While fibronectin expression was increased compared to the uninjured spinal cord, its staining pattern was very diffuse throughout the injury site, suggesting that fibronectin has not assembled into a matrix in the ECM (Fig. 2.2E-H). The fact that only few fibroblasts are present in the lesion site suggests that
Figure 2.1. Fibronectin is a better ECM marker for the fibrotic scar. At 14 days after contusion, ECM proteins collagen IV (A), laminin (C) and fibronectin (E) are present in the fibrotic scar (GFAP− regions). While fibronectin fills the entire fibrotic scar, collagen IV and laminin preferentially label the basal lamina present at blood vessels and the fibrotic-glial border (edges of the GFAP+ regions). Col IV = collagen IV, LN = laminin, FN = fibronectin. Scale bar =500 μm. n=3 per group.

Fibronectin at this stage most likely come from the blood plasma as a result of the hemorrhage after injury. However, at 7 days, there were a large number of fibroblasts present at the injury site and their distribution pattern closely matched that of fibronectin expression (Fig. 2.2I-L). In addition, while fibronectin expression appeared very diffuse at 3 days after injury, it became much more condensed and fibrillar by 7 days, indicating that fibronectin was starting to form into a matrix. At 14 days, when the fibrotic scar has matured, fibronectin
Figure 2.2. Fibrotic scar formation after contusive SCI. In the uninjured spinal cord (A-D), fibronectin (red) expression and fibroblasts (green) are found only around blood vessels (D) and in the dura. At 3 days after injury (E-H), there is diffuse fibronectin expression throughout the injury site (E) while fibroblasts are not present in significant numbers (F). At 7 days (I-L), when fibroblasts densely populate the injury site (J), the area of fibronectin expression closely matches that of fibroblasts (K) and appears more condensed (L). At 14 days (M-P, U-X), when the fibrotic scar has matured, it is characterized by a dense population of mostly fibroblasts and CD11b+ leukocytes (W) and a fibronectin expression network that closely follows the distribution of fibroblasts throughout the injury site. This pattern continues at 28 days (Q-T). n=5 per group. Region of interest (ROI) represents boxed region in the left. Scale bar in A, E, I, M, Q = 500 μm; in D, H, L, P, T = 50 μm; in U = 200 μm.
expression became much more organized into a fibrillar network that closely resembled the fibroblast distribution pattern (Fig. 2.2M-P, U-X). At this time, CD11b+ leukocytes also filled the fibrotic scar and were intimately associated with fibroblasts. This phenotype continued at 28 days (Fig. 2.2Q-T). Hence, these results demonstrate a matching distribution pattern between the fibronectin matrix and infiltration of perivascular fibroblasts at the injury site.

### 2.2 Fibroblasts are likely the primary source of fibronectin

Although the expression pattern of fibronectin suggests that fibroblasts are the source of fibronectin, it is possible that activated microglia/macrophages could also contribute. Thus, we sought to determine whether microglia/macrophages are a source of fibronectin by deleting fibronectin in these cells. The lysM-Cre knock-in mouse line expresses Cre recombinase in myeloid cells including most macrophages and granulocytes (Clausen et al., 1999). While this mouse line has been well-characterized in terms of recombination in peripheral organs, no one has reported its recombination efficiency in mouse spinal cord. Thus, our laboratory used lysM<sup>tdTom</sup> mice (lysM-Cre mice crossed with Rosa26-tdTomato reporter mice) to determine the recombination efficiency of lysM-Cre in the uninjured spinal cord. We found that in the uninjured spinal cord, tdTomato labeled approximately 34% of all CD11b<sup>+</sup> microglia (Zhu et al., 2014b). This is in accordance with a recent report showing about 40% lysM-Cre efficiency in microglia isolated from the uninjured spinal cord using flow cytometry (Goldmann et al., 2013). This recombination efficiency for microglia is
relatively low compared to the 80-90% recombination efficiency reported for mature macrophages (Abram et al., 2014; Clausen et al., 1999). Surprisingly, we also observed that around 6% of neurons also underwent recombination. Of all the tdTomato+ cells, approximately 66% were microglia and the rest were neurons (Zhu et al., 2014b).

Next we injured lysM<sup>tdTom</sup> mice to determine the expression pattern of lysM-Cre recombinase after SCI. We found that the fibrotic scar (GFAP<sup>-</sup> area) was filled with tdTomato<sup>+</sup> cells (Fig. 2.3A-C), suggesting that the recombination efficiency is very high in myeloid cells that occupy the fibrotic scar. We then performed SCI on lysM<sup>Cre</sup>+/FN<sup>flox</sup> mice (lysM-Cre mice crossed with floxed fibronectin mice). We found that while CD11b<sup>+</sup> cells were densely concentrated in the fibrotic scar (Fig. 2.3H, I), there were no visible differences in fibronectin expression level as compared to Cre<sup>-</sup> controls (Fig. 2.3D-G). Our results rule out microglia/macrophages as a major source of fibronectin and suggest that fibroblasts are most likely the primary source of fibronectin in the fibrotic scar.

### 2.3 Fibronectin is assembled into a matrix

While the fibrillar organization of fibronectin at 7 days and beyond suggested its assembly into a matrix, we confirmed our observation using a biochemical approach by comparing the deoxycholate (DOC)-soluble and insoluble fractions of the injury site using western blot analysis. Fibronectin that has not been assembled into a matrix is DOC-soluble, while assembled fibronectin is
*Figure 2.3. Activated microglia/macrophages are not a major source of fibronectin after SCI.* Breeding lysM-Cre mice to Rosa26-tdTomato reporter mice demonstrates the distribution of myeloid cells at the injury site that have undergone recombination (A-C, n=3, courtesy of Michelle Trojanowsky). Genetic deletion of fibronectin (FN) in myeloid cells (lysM-Cre bred to floxed FN mouse, D,F,H, n=5) does not show visible differences in CD11b⁺ leukocytes infiltration and FN expression at the injury site as compared to Cre⁻ controls (E,G,I, n=5). Scale bar in A, D = 500 μm.
DOC-insoluble. In the uninjured spinal cord, both soluble and insoluble fibronectin were below detectable levels (Fig. 2.4A, B), consistent with our immunohistochemistry results above (Fig. 2.2A). There was a large increase in soluble fibronectin at 3 days after injury, peaking at 7 days and then dramatically decreasing at 14 and 28 days (Fig. 2.4A). Insoluble fibronectin is indicated by multiple bands above the expected 250 kD monomer band. These multiple bands are only present in the insoluble fraction and contain fibronectin polymers that may be still in an interconnected matrix form. Insoluble fibronectin showed dramatic increases at 7 and 14 days after injury and decreased by 28 days (Fig. 2.4B). This suggests that soluble fibronectin expressed acutely after injury (3 days) is replaced by insoluble matrix fibronectin at later time points and that there is a decrease in overall fibronectin expression by 28 days after injury. In addition to the multiple bands above 250 kD, there are 125 kD lower bands that are also only specific to the insoluble fraction from 7 through 28 days (Fig. 2.4B). This temporal distribution coincides with fibronectin matrix formation revealed by immunohistochemistry (Fig. 2.2A, E, I, M, Q), suggesting that the 125 kD bands may be proteolytic products of the fibronectin matrix as a result of ECM dynamics after SCI. Taken together, our data indicates that while fibronectin is highly expressed as early as 3 days after injury, its assembly into a matrix occurs at around 7 days when there is a large accumulation of fibroblasts and microglia/macrophages at the injury site.
Figure 2.4. Fibronectin matrix assembly after SCI. Soluble fibronectin is significantly increased at 3 and 7 days after SCI and then decreases over time, albeit still at higher levels than the uninjured spinal cord (A, n=5 per group). Insoluble fibronectin (indicated by multiple bands above the 250 kD monomer band) is highest at 7 and 14 days after injury (B, n=5 per group). Purified plasma fibronectin (pFN) was used as a positive control and loaded in equal amounts in both blots. Note the absence of >250 kD bands in the soluble fraction (A) even after a longer film exposure time (as indicated by darker pFN band in A as compared to B). *p<0.05 compared to 14d, 28d in A; 3d, 28d in B using one way ANOVA with Tukey’s post-test. Error bars are s.e.m. AU = Arbitrary Units.

2.4 Fibronectin is associated with integrin receptor α5β1 which is mainly expressed by microglia/macrophages

To investigate the mechanism of fibronectin matrix assembly, we studied the expression of α5β1 and αVβ3 integrin receptors, which are the two major fibronectin receptors that have been described to participate in fibronectin assembly in vivo (Takahashi et al., 2007; van der Flier et al., 2010; Yang et al., 1999). Using qRT-PCR, we discovered that in the uninjured spinal cord, α5 subunit had the lowest expression compared to the other subunits (Fig. 2.5A), confirming barely detectable levels of α5β1 immunoreactivity in the uninjured spinal cord tissue sections (data not shown). However, after SCI, there was a 30 to 40 fold increase in α5 gene expression at 7 and 28 days after injury (Fig. 2.5B).
There was also a significant increase in β1 expression while αV and β3 subunits showed an increasing trend that did not reach statistical significance (Fig. 2.5B). Therefore, α5 and β1 expression showed the largest changes after injury and virtually all fibronectin co-localized with α5β1 (Fig. 2.5E-G), suggesting that the α5β1 integrin receptor may be the primary fibronectin receptor after SCI. It should be noted, however, that not all α5β1 receptor co-localized with fibronectin, suggesting that this receptor may have functions other than fibronectin assembly in the fibrotic scar.

To determine the cell types that express α5β1, we performed immunohistochemistry to co-label α5β1 with different cellular markers. Since the fibrotic scar is comprised mainly of fibroblasts and immune cells, we focused on Col1α1\(^{GFP}\)+ fibroblasts and CD11b\(^+\) cells. Inside the fibrotic scar, approximately 80% and 20% of α5β1\(^+\) cells were CD11b\(^+\) and Col1α1\(^{GFP}\)+ respectively (Fig. 2.5H-M, D). Since most CD11b\(^+\) cells are microglia/macrophages at this time after SCI (Kigerl et al., 2006), our data suggests that activated microglia/macrophages may play a role in fibronectin matrix assembly in the fibrotic scar.

2.5 Discussion

In this chapter, we sought to determine the mechanism of fibrotic scar formation after SCI by focusing on fibronectin matrix assembly. Our data indicates that while fibronectin is present at the injury site as early as 3 days after SCI, it starts to form a matrix around 7 days. This coincides with the time of peak
Figure 2.5. Integrin receptor α5β1 is expressed by microglia/macrophages and fibroblasts after SCI. In the uninjured spinal cord, αV and β1 integrin subunit mRNA levels are higher than α5 and β3 (A, n=3 per group). After SCI, both α5 and β1 mRNA are significantly increased, while αV and β3 mRNA levels are not significantly altered (B, n=3 per group). Within the fibrotic scar at 14 days after injury (C), fibronectin is present mostly in regions of α5β1 expression (E-G), which is present on CD11b+ microglia/macrophages (H-J, D, n=3 per group) and fibroblasts (K-M, D, n=3 per group). E-M represents an enlarged region in the fibrotic scar (e.g. boxed region in C). C, E, H, K are from different animals. Scale bar: C=500 μm, E, H, K=50 μm. A: *p<0.05 compared to α5, β3 using one way ANOVA with Tukey’s post-test. B: *p<0.05 compared to uninjured (uninj), #p<0.05 compared to 7d, two way ANOVA with Tukey’s post-test. Error bars are s.e.m.
fibroblast (Soderblom et al., 2013) and macrophage/microglia (Donnelly and Popovich, 2008) infiltration, both of which express the prototypical fibronectin receptor integrin α5β1. We determined that activated microglia/macrophages are not a source of fibronectin by deleting fibronectin in myeloid cells, suggesting that fibroblasts are likely the primary source of fibronectin after SCI. Although we cannot completely rule out possible contributions from plasma fibronectin, we think this is less likely because that plasma fibronectin can no longer enter the spinal cord lesion site at 2-3 weeks after SCI due to re-establishment of the blood-spinal cord barrier (Noble and Wrathall, 1989; Whetstone et al., 2003), whereas the fibronectin matrix is still prominent during this time.

Much of our knowledge of fibronectin comes from studies of cutaneous wound healing and peripheral organ fibrosis, in which fibronectin is suggested as a scaffolding protein that regulates ECM deposition and support cell adhesion, migration and survival. For example, in later phases of skin wound healing, the fibronectin matrix regulates the deposition of other ECM molecules such as fibrinogen, collagen, laminin, CSPGs and tenascin-C (Rhett et al., 2008; Rolls et al., 2008). Fibronectin is also found to attract fibroblasts in glomerular fibrosis (Gharaee-Kermani et al., 1996) and support migration and survival of both circulating monocytes and microglia (Abshire et al., 2011; Nasu-Tada et al., 2005; Trial et al., 1999). In CNS injury, plasma fibronectin is frequently used as a component in biomaterial-based and/or cell-based grafts to facilitate cell migration, adhesion and survival and promote axon regeneration (Geissmann et al., 2003; King et al., 2010a; King et al., 2010b; Tate et al., 2009). Moreover,
plasma fibronectin has been shown to have a neuroprotective role in brain injury (Tate et al., 2007) and ischemia (Sakai et al., 2001). Although fibronectin is the most commonly used marker of the fibrotic scar, virtually nothing is known about its role in the pathophysiology of SCI. Since fibronectin itself seems to be neuroprotective and growth permissive for axons, why is the fibronectin-abundant fibrotic scar so inhibitory to axon regeneration? A possible explanation would be that inhibitory molecules bind to the fibronectin matrix after SCI and result in an inhibitory environment in the fibrotic scar. This is supported by reports that the fibronectin matrix is closely associated with inhibitory proteins such as NG2 proteoglycan and tenascin-C in astrocytes/fibroblasts co-culture (Kimura-Kuroda et al., 2010) and in SCI lesion epicenter (Camand et al., 2004; Tang et al., 2003). Furthermore, fibronectin molecule has binding sites for the glycosaminoglycan (GAG) chains of CSPGs and it has been demonstrated that CSPGs interact with fibronectin in vitro using various binding assays (Evans et al., 2014; Horn et al., 2008; Perkins et al., 1979). It is important to note that in order for these interactions to occur, fibronectin typically needs to form into a matrix, which we have demonstrated in this study. Therefore, understanding how the fibronectin matrix is assembled after SCI may allow us to target multiple inhibitory molecules in the fibrotic scar in order to promote axon regeneration.

Based on the functions of fibronectin described above, we hypothesize that fibronectin present at 3 days after SCI is most likely plasma fibronectin from the blood, which is involved in formation of the provisional clot to stop bleeding. This provisional matrix provides a substrate for fibroblast and
macrophage/microglia migration, which reaches a peak by 7 days. We propose that fibroblasts express cellular fibronectin that is assembled into a matrix mostly by activated macrophages/microglia. This peak in cellular migration also corresponds to peak soluble and insoluble fibronectin expression and regulation as indicated by matrix assembly and degradation. At 14 days after injury, matrix fibronectin remains abundant while the level of soluble fibronectin significantly decreases, perhaps due to a combination of re-establishment of the blood-spinal cord barrier (Noble and Wrathall, 1989; Whetstone et al., 2003) and degradation of plasma fibronectin by infiltrating cells. By 28 days after injury, matrix fibronectin levels also decline significantly and this could be due to a decrease in the number of fibroblasts at this time (Soderblom et al., 2013). However, more functional studies are needed to support this working model. For example, since it remains possible that fibroblasts simply assemble the fibronectin that is expressed by another cell type, future studies will need to delete fibronectin specifically in fibroblasts to address this issue. In addition, this loss-of-function study will also address what role, if any, fibronectin play in fibrotic scar formation. In addition, while the expression of integrin α5β1 mostly on microglia/macrophages suggests that these cells might play a role in assembly of fibronectin into a matrix, a genetic deletion of the receptor specifically in these cells is necessary to reach a more firm conclusion.

In conclusion, our data indicates that fibronectin is assembled into a matrix after SCI and that it is most likely expressed by fibroblasts at the injury site. Assembly of the fibronectin matrix may be mediated by the canonical fibronectin
receptor, integrin α5β1, which is primarily expressed by activated microglia/macrophages in the fibrotic scar.
Chapter 3

Distinguishing between macrophages and microglia in the scar tissue after spinal cord injury

Microglia and hMΦ are the predominant types of innate immune cells after SCI. Since activated microglia and hMΦ are antigenically and morphologically indistinguishable using conventional histological methods, they are often collectively referred to as CNS microglia/macrophages. This has led to the question of whether or how they contribute differently to scar formation after SCI. In this chapter, we used mutant mice and bone marrow transplantation to determine the cellular distribution of hMΦ and microglia in the glial and fibrotic scars after SCI. We found that whereas hMΦ are associated with the fibrotic scar, microglia are mostly limited to the glial scar. Thus, hMΦ rather than microglia, may be responsible for recruiting fibroblasts to form the fibrotic scar after SCI.

3.1 Fibroblasts and microglia/macrophages have similar spatiotemporal recruitment after SCI

Since activated microglia/macrophages are CD11b⁺ and they are a major cellular component of the fibrotic scar (Fig. 3.1), we sought to determine the spatiotemporal relationship between fibroblasts and CD11b⁺ cells after SCI. We utilized Col1α1GFP transgenic mice to track accumulation of perivascular fibroblasts (Soderblom et al., 2013). In the uninjured spinal cord, fibroblasts were found only around blood vessels and in the meninges whereas CD11b⁺ cells were characteristic of microglia (Fig. 3.1A-D). At 3 days after injury, the injury site
**Figure 3.1. Fibroblasts and macrophages/microglia display similar spatiotemporal distribution profile.** In the uninjured spinal cord (A-D), Col1α1GFP fibroblasts are around blood vessels while CD11b+ cells represent microglia. At 3 days after injury (E-H), fibroblasts are not present in significant numbers (E) and round CD11b+ cells occupy mostly the peripheral edges of the injury site (F). At 7 days (I-L), fibroblasts and CD11b+ macrophages densely populate the injury site. At 14 days (M-P), when the fibrotic scar has matured, it is characterized by a dense population of mostly fibroblasts and CD11b+ macrophages. Similar expression pattern is found at more chronic time points (Q-X). Region of interest (ROI) represents boxed region in the left. n=5 per group. Scale bar in A = 500 μm; D = 50 μm.

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was mostly necrotic with very little fibroblasts and some circular CD11b⁺ cells in the periphery of the lesion (Fig. 3.1E-H). However, at 7 days, there were a large number of fibroblasts present at the injury site that coincided with a significant infiltration of CD11b⁺ cells (Fig. 3.1I-L). At 14 days, when the fibrotic scar has started to mature, CD11b⁺ cells filled the fibrotic scar and were intimately associated with fibroblasts (Fig. 1M-P). This phenotype continued at 28 and 56 days (Fig. 3.1Q-X). Therefore, our data demonstrates a close spatiotemporal relationship between fibroblast and CD11b⁺ microglia/macrophages recruitment to the injury site.

3.2 Distinguishing hematogenous macrophages from microglia using bone marrow chimeras

Generation of bone marrow chimeric mice

The close association between CD11b⁺ cells and fibroblasts suggested that CD11b⁺ microglia/macrophages may play a role in recruiting fibroblasts to the injury site. To determine whether these CD11b⁺ microglia/macrophages are monocyte-derived macrophages and/or activated microglia, we performed bone marrow transplantation using lysM<sup>tdTom</sup> mice (lysM-Cre bred to Rosa26-tdTomato) as the donor and heterozygous Cx3cr1<sup>GFP</sup> mice as the recipient to generate lysM<sup>tdTom</sup> > Cx3cr1<sup>GFP</sup> chimeric mice, in which microglia are labeled with GFP and hematogenous myeloid cells are labeled with tdTomato (Fig. 3.2A). CD45.1>CD45.2 chimeric mice generated in parallel showed approximately 94% chimerism using flow cytometry of blood samples (Fig. 3.2B). Although
Figure 3.2. Generation of bone marrow chimeric mice. Schematic diagram (A) showing generation of the lysM<sup>tdTom</sup>\textgreater{}Cx3cr1<sup>GFP</sup> chimeric mice, in which hematogenous macrophages are labeled with tdTomato and microglia labeled with GFP. Blood samples taken from CD45.1>CD45.2 chimeric mice showed approximately 94% chimerism (B, n=9 for naïve, n=6 for chimera). Comparison of lysM<sup>tdTom</sup>\textgreater{}Cx3cr1<sup>GFP</sup> chimeric mice (G-L, n=3) to naïve Cx3cr1<sup>GFP</sup> animals (C-F, n=3) did not show visible effects of irradiation on microglia morphology and number at 8 weeks after bone marrow transplantation. lysM<sup>tdTom</sup> monocytes were limited to PECAM<sup>+</sup> blood vessels (H-L) and did not enter the spinal cord parenchyma in uninjured (uninj) lysM<sup>tdTom</sup>\textgreater{}Cx3cr1<sup>GFP</sup> chimeric mice. D-F represent boxed region in C. H-L represent boxed region in G. Scale bar in C, G = 500 μm; in D-F, H-L = 50 μm.
chimerism was not directly assessed in lysM<sup>tdTom</sup>&gt;Cx3cr1<sup>GFP</sup> chimeras, the percent of CD11b<sup>+</sup> cells in the blood that were tdTomato<sup>+</sup> or GFP<sup>+</sup> was quantified prior to SCI. 0.4% ± 0.2% CD11b<sup>+</sup> cells were GFP<sup>+</sup> and 41.7% ± 2% were tdTomato<sup>+</sup> in the chimeras (mean ± SEM, n=9), as compared to 59.1% ± 3% GFP<sup>+</sup> cells in naïve heterozygous Cx3cr1<sup>GFP</sup> (n=3) and 47.4% ± 4% tdTomato<sup>+</sup> cells in naïve lysM<sup>tdTom</sup> mice (n=3). Taken together, our data indicates a high level of bone marrow reconstitution in our chimeras and the percentage of myeloid cells in the blood is similar to that of naïve mice. Furthermore, very few lysM<sup>tdTom</sup> cells were present in the spinal cord of uninjured chimeras and they were all associated with blood vessels, suggesting that the irradiation procedure did not lead to chronic infiltration of monocytes into spinal cord parenchyma. Irradiation did not cause any noticeable change to microglia morphology or number either (Fig. 3.2C-L).

**Hematogenous macrophages rather than microglia are present in the fibrotic scar**

Using our lysM<sup>tdTom</sup>&gt;Cx3cr1<sup>GFP</sup> chimeric mice, we performed SCI to determine the distribution of hMΦ and microglia in the fibrotic scar region. At 5 and 7 days after SCI when macrophage infiltration reaches peak levels (Kigerl et al., 2006), microglia and hMΦ are interspersed throughout the injury site in both GFAP<sup>+</sup> and GFAP<sup>−</sup> regions (Fig. 3.3A-H). However, at 14 days when scar formation has stabilized, the fibrotic scar (GFAP<sup>−</sup> region) is comprised mostly of hMΦ while microglia is present mostly in the peripheral edges of the glial (GFAP<sup>+</sup>) scar region (Fig. 3.3I-L). Meanwhile, integrin α5β1 co-localizes with CD11b
Figure 3.3. Fibrotic scar is occupied by hematogenous macrophages. lysM_{tdTom}^{+}Cx3cr1_{GFP}^{+} chimeric mice in which hematogenous macrophages are labeled with tdTomato and microglia are labeled with GFP show that at 5 (A-D) and 7 (E-H) days after SCI, both cell types are interspersed throughout the fibrotic (GFAP⁻ area) and glial (GFAP⁺ area) scar. However, at 14 days (I-L), the fibrotic scar is occupied almost entirely by lysM_{tdTom}^{+} cells whereas Cx3cr1_{GFP}^{+} cells are present mostly in the surrounding glial scar region. Majority of lysM_{tdTom}^{+} cells are present in the fibrotic scar (GFAP⁻ region) at 5, 7 and 14 days after SCI (M, n=3), while the presence of Cx3cr1_{GFP}^{+} cells preferentially in the GFAP⁺ region appears at 14 days but not earlier after injury (N, n=3). *p<0.05 compared to GFAP⁻ region in M, compared to 14d GFAP⁺ region in N using one way ANOVA with Tukey’s post-test. Error bars are s.e.m.
Figure 3.4. Both hematogenous macrophages and microglia express α5β1 after SCI. At both 7 (A-E, n=3) and 14 (F-J, n=3) days after injury in the lysMtdTom>Cx3cr1GFP chimeras, lysMtdTom+ and Cx3cr1GFP+ cells comprise majority of CD11b+ cells (E, J) at the injury site. Both cell types express the fibronectin receptor integrin α5β1 (D, I). D and J are from adjacent serial sections from the same animals, respectively. Scale bar = 500 μm. n represents biological replicates.
expression throughout the injury site (Fig. 3.4D-J), and is mostly concentrated in the regions of high amount of hMΦ (Fig. 3.4B, G, D, I), suggesting that hMΦ may participate in fibronectin matrix assembly in the fibrotic scar. Notably, some integrin α5β1 is also expressed in the regions adjacent to the fibrotic scar (Fig. 3.4D, I), suggesting that other cell types such as microglia may also contribute to fibronectin assembly at the periphery of the fibrotic scar. Taken together, our data indicates that hMΦ, rather than microglia, are associated with the fibrotic scar after SCI, and thus suggests that hMΦ may play a role in recruiting fibroblasts to the injury site.

3.3 lysM<sup>tdTom<sup>+</sup></sup> macrophages are representative of the overall population of infiltrating hematogenous macrophages

Since only lysM<sup>tdTom<sup>+</sup></sup> cells are present in the fibrotic scar (Fig. 3.3) and lysM-Cre is not expressed in all macrophages (Abram et al., 2014; Clausen et al., 1999), it is possible that the lysM<sup>tdTom<sup>+</sup></sup> cells represent a novel subset of scar-forming macrophages. To identify the type of hMΦ that comprise the fibrotic scar, we performed flow cytometry on cells dissociated from the injury sites of lysM<sup>tdTom<sup>-</sup></sup>Cx3cr1<sup>GFP</sup> chimeric mice at 7 and 14 days after SCI. We first identified CD11b<sup>+</sup> cells and then further separated these cells based on CD45<sup>hi</sup>, CD45<sup>low</sup>, tdTomato and GFP fluorescent intensity. CD11b<sup>+</sup> cells were separated into CD45<sup>hi</sup> and CD45<sup>low</sup> populations that represent infiltrating myeloid cells and activated microglia, respectively (Sedgwick et al., 1991). As expected, tdTomato<sup>+</sup> cells were distributed among the CD45<sup>hi</sup> population while GFP<sup>+</sup> cells were among
the CD45<sub>low</sub> population. Overall, lysM<sup>tdTom</sup> labeled 49.3 ± 2% of all infiltrating myeloid cells (CD11b<sup>+</sup>/CD45<sup>hi</sup>) (mean ± SEM, n=9). To determine if tdTomato<sup>+</sup> cells represent a distinct subclass of hMΦ, we compared the antigenic profile of CD11b<sup>+</sup>/tdTomato<sup>+</sup> cells against all CD11b<sup>+</sup>/CD45<sup>hi</sup> cells (Fig. 3.5A, B). Ly6G<sup>+</sup> cells comprised less than 3% of these cell populations, indicating that CD45<sup>hi</sup>/CD11b<sup>+</sup> cells were mostly macrophages rather than neutrophils at 7 and 14 days, consistent with the kinetics of neutrophil infiltration after SCI, which peaks at 1-2 days and decreases by 3 days (Beck et al., 2010; Stirling and Yong, 2008). At 7 days after SCI, the antigenic profile using Ly6C, CD11c, CD86, CD206 and CD43 between these two groups was similar (Fig 4C-H). At 14 days, the two populations continued to be mostly similar with the exception of Ly6C that was expressed by a larger percentage of tdTomato<sup>+</sup> cells (24 ± 0.4% vs 15.4 ± 0.6%, n=5, Fig 4I-T). Taken together, our data indicates that the lysM<sup>tdTom</sup> macrophages at the injury site are representative of infiltrating hMΦ in general.

3.4 Discussion

The data we presented in this chapter revealed a close spatiotemporal association between the fibrotic scar and CD11b<sup>+</sup> cells, which indicated a possible interaction between fibroblasts and microglia/macrophages after SCI. Since microglia/macrophages present at the injury site consists of heterogeneous populations of cells, we sought to investigate the origins and antigenic profile of these cells using bone marrow transplantation and flow cytometry. We determined that macrophages associated with the fibrotic scar are predominantly
Figure 3.5. Comparison of antigenic profile of lysM<sup>tdTom+</sup> and CD45<sup>hi</sup> macrophages after SCI. Immune cells dissociated from injury sites of lysM<sup>tdTom+</sup>&gt;Cx3cr1<sup>GFP</sup> chimeric mice were separated based on CD11b and CD45 expression (A). CD11b<sup>+</sup> cells (boxed region in A) were further separated based on tdTomato expression (B). These CD11b<sup>+</sup>/tdTom<sup>+</sup> cells (B) were compared to CD11b<sup>+</sup>/CD45<sup>hi</sup> cells (gray region in A). At 7 days after injury (C-H, n=4), the percent of cells expressing Ly6G, Ly6C, CD11c, CD86, CD206 and CD43 were similar between CD11b<sup>+</sup>/tdTom<sup>+</sup> (red bars) and CD11b<sup>+</sup>/CD45<sup>hi</sup> cells (gray bar). At 14 days (I-T, n=5), a higher percentage of CD11b<sup>+</sup>/tdTom<sup>+</sup> cells expressed Ly6C as compared to CD11b<sup>+</sup>/CD45<sup>hi</sup> macrophages (J). O-T: Representative relative fluorescence histograms of CD11b<sup>+</sup>/tdTom<sup>+</sup> (red) and CD11b<sup>+</sup>/CD45<sup>hi</sup> (gray) cells at 14 days. Positive gates (regions between two solid lines) in the histograms were determined based on corresponding isotype controls. *p<0.05 compared to CD11b<sup>+</sup>/tdTom<sup>+</sup> using two-tailed Student’s T-test.
of hematogenous origin, while microglia are restricted to the glial scar, suggesting that that hMΦ may play a role in recruiting fibroblasts to form the fibrotic scar.

In order to differentiate between microglial and monocyte origins of macrophages that are associated with the fibrotic scar, we generated chimeric mice in which microglia were labeled with GFP and hematogenous myeloid cells were labeled with tdTomato. While microglia was present mostly in the glial scar, the fibrotic scar was comprised almost solely of hMΦ. Our results are consistent with previous studies that demonstrated different spatial distribution between microglia and hMΦ (Evans et al., 2014; Fenrich et al., 2013; Mawhinney et al., 2012; Popovich and Hickey, 2001). However, our study is the first to demonstrate their distribution in distinct portions of the glial and fibrotic scars. The functional significance of the specificity of microglia to the glial scar is not clear, but it could underscore the interdependence of these two cell types in providing neuroprotection after injury (Min et al., 2006; Shinozaki et al., 2014). Furthermore, even though microglia is developmentally more similar to hMΦ than neural cells, our data demonstrates that microglia occupies the CNS region (glial scar) as opposed to the non-CNS region (fibrotic scar) at the injury site.

Interestingly, two studies using similar bone marrow chimeric approach and injury paradigm reported opposite findings to ours; hMΦ are only present in the injury penumbra while microglia occupy the injury epicenter (Rolls et al., 2008; Shechter et al., 2009). In contrast to our lysM<sup>tdTom>GFP</sup>Cx3cr1<sup>GFP</sup> chimeric approach, these studies used heterozygous Cx3cr1<sup>GFP</sup> mice as bone marrow donors and
irradiated wild type (WT) mice as recipients (Cx3cr1^GF>P WT chimeras). Therefore, we repeated the SCI experiment with Cx3cr1^GF>P WT chimeras and compared the results with those from naïve Cx3cr1^GF>P mice. Since both hMΦ and microglia are labeled with GFP in naïve Cx3cr1^GF>P mice, we expected to observe GFP^+ cells throughout the SCI site. However, surprisingly, at 14 days after injury, despite noticeably more GFP^+ cells present in Cx3cr1^GF>P mice than Cx3cr1^GF>P WT chimeras, the distribution of GFP^+ cells at the injury site looked similar between the two groups; GFP^+ cells were concentrated mostly around the edges of the injury epicenter even though immunostaining clearly showed the injury epicenter filled with CD11b^+ cells (Fig. 3.6). Thus, our data indicates that data from previous studies using Cx3cr1^GF>P WT chimeras may be open to alternative interpretations.

We believe that a likely explanation is that since GFP expression is under the regulation of the endogenous Cx3xr1 promoter in these knock-in mice (Jung et al., 2000), GFP expression is being down-regulated as hMΦ enter the fibrotic scar. This is supported by a recent fate mapping study of macrophage development demonstrating that macrophages derived from Cx3cr1^GF>P precursor cells stop expressing GFP after entering certain tissue types to become tissue-resident macrophages (van Rooijen and Hendrikx, 2010). As a result, it is possible that hMΦ derived from blood-borne Cx3cr1^GF>P monocytes may have down-regulated GFP expression upon infiltrating the SCI lesion site, giving the appearance that these cells were absent from the injury epicenter. Interestingly, another study using homozygous Cx3cr1^GF>P mice to generate Cx3cr1^GF>P WT
Figure 3.6. Cx3cr1-GFP is not a reliable marker for hematogenous macrophages at the injury site. Despite a dense distribution of CD11b+ cells at 14 days after SCI (C, D and Fig. 3.1), both Cx3cr1<sup>GFP</sup> >WT chimeras (n=5) and naïve Cx3cr1<sup>GFP</sup> mice (n=3) showed GFP+ cells preferentially occupying the periphery of the lesion epicenter (A, B). Scale bar in A = 500 μm.

Chimeras showed the presence of GFP+ hMΦ in the injury epicenter, consistent with our results (Weisser et al., 2012). This could be due to the possibility that a complete loss-of-function of the Cx3cr1 gene in these homozygous Cx3cr1<sup>GFP</sup> mice could have interfered with the endogenous regulation of the Cx3cr1 gene so that GFP expression in hMΦ remained active after SCI. Alternatively, having two copies of GFP in the homozygous mice could have led to better detection of GFP signal at the injury site as compared to the heterozygous mice. Importantly, an advantage of our lysM<sup>TdTom</sup> >Cx3cr1<sup>GFP</sup> chimeras is that since tdTomato is
constitutively expressed in myeloid cells (lysM-Cre mice crossed with Rosa26-tdTomato reporter mice), macrophages are less likely to be susceptible to these types of alterations in gene expression, including lysM.

Since the lysM-Cre mouse line labels approximate 40% peripheral blood monocytes (Abram et al., 2014) and 80-90% peritoneal macrophages (Abram et al., 2014; Clausen et al., 1999), we determined that lysM\textsuperscript{tdTom}\textsuperscript+ cells comprised about 50% of all infiltrating hMΦ (CD11b\textsuperscript+/CD45\textsuperscript{hi}) at the SCI site. We further investigated whether the lysM\textsuperscript{tdTom}\textsuperscript+ cells at the injury site were a unique subclass of macrophages by comparing their antigenic profile to the CD11b\textsuperscript+/CD45\textsuperscript{hi} cell population. Overall, lysM\textsuperscript{tdTom}\textsuperscript+ cells displayed a similar antigenic profile to the general infiltrating hMΦ population, except for a higher percentage of lysM\textsuperscript{tdTom}\textsuperscript+ cells expressing Ly6C at 14 days (24 ± 0.4% vs 15.4 ± 0.6%) after injury. Interestingly, Ly6C\textsuperscript{hi} cells were reported to give rise to a subset of pro-fibrotic macrophages that contribute to kidney fibrosis (Lin et al., 2009). Although Ly6C\textsuperscript{hi}CD43\textsuperscript{lo} and Ly6C\textsuperscript{lo}CD43\textsuperscript{hi} have been used to distinguish between inflammatory and resident monocytes (Ziegler-Heitbrock et al., 2010), we did not find a clear separation in Ly6C or CD43 expression in the injured spinal cord. The fact that the percent of Ly6C\textsuperscript{+} macrophages declining from 7 to 14 days (70% at 7d vs 15% at 14d) coincides with the decreasing amount of fibroblasts from 7 to 14 days (Soderblom et al., 2013), suggests that Ly6C\textsuperscript{+} macrophages may also play a pro-fibrotic role that regulates fibroblast accumulation after SCI. In addition, our data also demonstrated that hMΦ at the injury site predominately expressed the M1 macrophage marker CD86 (56% at 7d and 88% at 14d) in contrast to the
M2 macrophage marker CD206 (0.4% at 7d and 7% at 14d) (Fig. 3.5F-M), suggesting that the injured spinal cord favors inflammation over wound healing at these acute to sub-acute stages. Our data is consistent with the M1-dominant macrophage response at the SCI site as reported (Kigerl et al., 2009; Kroner et al., 2014). Overall, the difference in antigenic expression of Ly6C between 7 and 14 days after injury correlates with maturation of the fibrotic scar. Despite a modest difference in the proportion of cells that express Ly6C, the lysM<sup>ldTom+</sup> macrophages are overall representative of infiltrating hMΦ at the injury site. A more detailed analysis of the role of Ly6C in future studies may provide additional insight into the mechanism of fibrotic scar formation after SCI.

In conclusion, our study determined a distinct differential distribution of hMΦ and microglia after SCI. While the fibrotic scar is comprised almost exclusively of hMΦ, microglia are limited to the glial scar. Moreover, the recruitment of hMΦ and fibroblasts exhibits a strong spatiotemporal association after SCI, raising the possibility that hMΦ may attract infiltration of fibroblasts to the injury site.
Chapter 4

The effect of hematogenous macrophage depletion on fibrotic scarring after spinal cord injury

As we have discussed in chapter 3, infiltration of hMΦ exhibits a close spatiotemporal association with fibroblasts accumulation in the fibrotic scar. These results led us to hypothesize that hMΦ might be responsible for attracting perivascular fibroblasts to the injury site by producing specific cytokines. In this chapter, we sought to test this hypothesis by performing hMΦ depletion as well as analyzing changes in cytokine expression after macrophage depletion. We demonstrated that depletion of hMΦ results in increased axonal growth that is associated with a reduction in overall fibrotic scarring, and changes multiple cytokine expression that makes the injury site less fibrotic. Thus, our data highlights hMΦ as a potential cellular target to promote SCI repair.

4.1 Clodronate liposome specifically targets hematogenous macrophages

To directly test the role of hMΦ in fibrotic scar formation, we sought to deplete hMΦ by injecting Col1α1\textsuperscript{GFP} mice with clodronate liposome after SCI. Clodronate liposome is clodronate encapsulated liposome that can be engulfed by phagocytic cells, especially monocytes/macrophages. Once internalized in the cytoplasm, the phospholipid membranes of the liposome are disrupted by lysosomal enzymes to release clodronate, which is processed into a non-hydrolyzable ATP analog that causes mitochondria dysfunction and subsequent...
Figure 4.1. Clodronate liposomes deplete macrophages in spleen. Mice subjected to SCI received three intraperitoneal injections of clodronate liposome (CLO) or PBS liposome (PBS) at 1, 3, and 6 days. At 7 days after injury, F4/80$^+$ macrophages in the spleen were visibly decreased in CLO treated animals (C-D). PBS = PBS liposome. CLO = clodronate liposome. Scale bar in A = 200 μm.

cell apoptosis (Frith et al., 1997; Lehenkari et al., 2002). Free clodronate released by leakage from liposomes or dead macrophages cannot cross cell membranes and has an extremely short half-life in body fluids (Van Rooijen and Sanders, 1994). We first confirmed the effect of clodronate liposome by demonstrating that F4/80$^+$ cells were visibly decreased in the spleen (Fig. 4.1),
which was reported as the main source of macrophages acutely after SCI (Blomster et al., 2013).

To trace the distribution of clodronate liposome \textit{in vivo}, we injected DiI-labeled PBS liposome, in which the fluorophore DiI is incorporated into the liposome bilayer membranes, into Col1α1\textsuperscript{GFP} mice after SCI. We showed that Dil signal was largely restricted to the GFAP\textsuperscript{−} fibrotic scar region characterized by accumulation of fibroblasts (Fig. 4.2E, G). However, there was no co-localization between GFP and Dil fluorescence and almost all Dil signal was enclosed in CD11b\textsuperscript{+} cells (Fig. 4.2B, D, F, H), suggesting that liposome directly targeted CD11b\textsuperscript{+} macrophages rather than fibroblasts. Next, we injected clodronate liposome into lysM\textsuperscript{IddTom}\textgreater Cx3cr1\textsuperscript{GFP} chimeric mice to determine whether clodronate liposome selectively targets hMΦ over microglia. Our results showed that only hMΦ were depleted while microglia were not visibly affected after SCI (Fig. 4.2I-N). Taken together, our results indicate that clodronate liposome specifically depletes hMΦ in the fibrotic scar.

### 4.2 Acute macrophage depletion reduces the fibrotic scar

To determine the acute effect of hMΦ depletion on fibrotic scar formation, we gave Col1α1\textsuperscript{GFP} mice multiple injections of clodronate liposome at 1, 3 and 6 days or 1, 3, 6 and 11 days after SCI, corresponding to the time course of macrophage infiltration after contusive SCI (Kigerl et al., 2006; Popovich et al., 1999). We found that CD11b\textsuperscript{+} cells at the injury site were significantly reduced at both 7 and 14 days, and this was associated with a significant reduction in the
Figure 4.2 Clodronate liposome specifically targets hematogenous macrophages. At both 7 (A, B, E, F, n=3) and 14 (C, D, G, H, n=3) days after SCI in Col1α1<sup>GFP</sup> mice, injection of Dil-labeled liposome demonstrates its localization in CD11b<sup>+</sup> cells (A-D) but not in Col1α1<sup>GFP</sup> fibroblasts (E-H). At 14 days after SCI, clodronate treatment in LysM<sup>tdTom</sup> >Cx3Cr1<sup>GFP</sup> chimeric mice (I-N, n=3 for clodronate, n=4 for PBS) shows a visible reduction in hMΦ (LysM<sup>tdTom</sup>) but not microglia (Cx3Cr1<sup>GFP</sup>). B, D, F, H are single planes from a confocal z-stack series and represent boxed region in A, C, E, G respectively. Scale bar in A, I = 500 μm; B = 50 μm.
Figure 4.3. Acute depletion of hematogenous macrophages reduces the fibrotic scar. Treatment with clodronate liposomes significantly reduces the amount of macrophages at both 7 (a, G, L, n=6 for PBS, n=5 for CLO) and 14 (C, I, O, n=6 for PBS, n=7 for CLO) days after SCI. This reduction in macrophages is associated with a reduction in fibroblasts (b, H, d, J, M, P). Notably, the dense peripheral rim of fibroblasts bordering the glial scar is visibly diminished in clodronate treated animas (D, J, E, F, K, L). This reduction in peripheral rim fibroblasts is associated with diminished basal lamina formation between fibroblasts and astrocytes (see Fig 4.4). Boxed region in d is represented by E, F. Boxed region in J is represented by K, L. L, M, O, P were calculated based on percent of GFAP area. *p<0.05 compared to PBS using two-tailed Student’s T-test. PBS = PBS liposome. CLO = clodronate liposome. Scale bar in a-D, G-J = 500 μm; in E, F, K, L = 100 μm; Error bars are s.e.m.
density of fibroblasts present at the injury site at both time points (Fig. 4.3). In addition, the dense population of fibroblasts at the peripheral rim was almost completely absent (Fig. 4.3D-F, J-L). Therefore, our data suggests that hMΦ promote fibroblast recruitment after SCI.

Since basal lamina that forms between astrocytes and fibroblasts is thought to inhibit axon regeneration (Bundesen et al., 2003), we sought to determine if reduced fibroblast accumulation would affect basal lamina formation. At 14 days after injury, basal lamina (detected using antibody against laminin) in PBS liposome treated mice was observed as a distinct border surrounding the fibrotic scar (Fig. 4.4A-C). However, after clodronate treatment, this distinct border was virtually absent and replaced by diffuse patches of laminin immunoreactivity observed throughout the fibrotic scar (Fig. 4.4D-F), suggesting that macrophages and/or fibroblasts are required for proper basal lamina formation around the fibrotic scar.

While the density of fibroblasts decreased after macrophage depletion, the total area of the fibrotic scar (as determined by GFAP⁺ area) varied with time. At 7 days after injury, fibrotic scar area was similar between clodronate and PBS treated groups. However, at 14 days, fibrotic scar area was greater in clodronate treated mice (Fig. 4.9N). Interestingly, the opposite trend was observed at 56 days with clodronate treated mice displaying smaller fibrotic scar area (Fig. 6N). Possible reasons for the opposite trend between 14 and 56 days are discussed in the Discussion section of this chapter.
Figure 4.4. Acute depletion of hematogenous macrophages reduces basal lamina formation. At 14 days after injury, a basal lamina (laminin+) typically present at the astrocyte-fibroblast border (A-C) is disrupted after macrophage depletion (D-F, n=6 for PBS, n=7 for CLO). The reduction of basal lamina formation is also associated decreased density of fibroblasts at the peripheral rim (also see Fig 4.3 E, F, K, L). Note that depletion of macrophages chronically (56 days after injury) does not visibly change basal lamina formation at the peripheral rim (Fig 4.8). PBS = PBS liposome. CLO = clodronate liposome. Region of interest (ROI) represents boxed region in the left. Scale bar in a = 500 μm; in C = 100 μm.

Since the fibronectin receptor integrin α5β1 is expressed mainly by hMΦ at the injury site (Fig. 2.5), we hypothesized that macrophage depletion will lead to a reduction in fibronectin matrix assembly. At 14 days after injury, immunohistochemistry showed a typical network of fibrillar fibronectin expression in the fibrotic scar, suggesting the presence of a fibronectin matrix (Fig. 4.5A, C-E). However, after macrophage depletion, the staining pattern of fibronectin was much more diffuse, suggesting the lack of matrix formation (Fig. 4.5B, F-H). Western blot analysis of soluble and insoluble fibronectin indicated greater soluble fibronectin level after macrophage depletion (Fig. 4.5I, K). The level of
insoluble fibronectin after macrophage depletion showed a decreasing trend that did not reach statistical significance as compared to controls (Fig. 4.5I, K). Since collagens are also present in the fibrotic scar (Fig. 2.1) and are postulated to serve as a backbone of the ECM (Klapka and Muller, 2006), we performed the Masson's trichrome staining, which should stain all collagens in blue, cytoplasm in pink and nuclei in black. At 14 days after SCI, there was a visible decrease in collagen deposition in clodronate liposome treated injury site tissue compared with controls (Fig. 4.6). Taken together, we conclude that hMΦ depletion disrupts deposition of the ECM in the fibrotic scar overall. In addition, our data also supports the hypothesis that hMΦ contribute to fibronectin matrix assembly after SCI, but other cells such as microglia and fibroblasts that also express α5β1 may also make significant contributions.

**Figure 4.5. Acute depletion of hematogenous macrophages disrupts the fibronectin matrix (see next page).** Clodronate liposome treated mice displayed more diffuse fibronectin immunoreactivity at the injury site as compared to the more fibrillar organization that is typically present at 14 days after injury (A, B, n=6 for PBS, n=7 for CLO). Boxed regions in A and B are enlarged in C-E and F-H. Demonstration of the entire western blot film along with Coomassie stained membrane for deoxycholate (DOC)-soluble (I) and insoluble (J) fibronectin (indicated by multiple bands above the 250 kD monomer band) in the injured spinal cord after clodronate liposome (CLO) or PBS liposome (PBS) injections. Purified plasma fibronectin (pFN) was used as a positive control and loaded in equal amount in both blots. Note that β-actin is uniformly present in the soluble fraction and is not present in the insoluble fraction, as expected. Soluble fibronectin (I, K, n=5 for PBS, n=4 for CLO) was significantly increased after clodronate treatment at 14 days. Although insoluble fibronectin levels showed a decreasing trend, it did not reach statistical significance (J, L). Scale bar in A = 500 μm; in C = 50 μm. *p<0.05 compared to PBS using two-tailed Student’s T-test. AU = Arbitrary Units.
Figure 4.6. Acute depletion of hematogenous macrophages reduces collagen deposition. Masson’s trichrome staining stains cytoplasm in pink, nuclei in black and collagen in blue. At 14 days after injury, there is visibly less collagen deposition in clodronate liposome treated animals compared to controls. PBS = PBS liposome. CLO = clodronate liposome. n=6 for PBS, n=7 for CLO. Scale bar in A = 500 μm.
4.3 Chronic macrophage depletion promotes axonal growth

To determine if the reduction in fibroblast infiltration, ECM deposition and basal lamina formation after hMΦ depletion leads to greater axonal growth, we waited 8 weeks after injury to allow sufficient time for axons to grow into the fibrotic scar. We injected clodronate liposome during the first 14 days as described above and weekly thereafter until the mice were sacrificed at 56 days after SCI. Histological sections of the injury site showed a reduction in CD11b+ cells in the fibrotic scar (Fig. 4.7A, C), indicating a continued depletion of macrophages, although the level of reduction was not as large as mice euthanized at 7 and 14 days after SCI. However, the amount of Col1α1GFP fibroblasts between clodronate and PBS treated animals was not visibly different (Fig. 4.7B, D). This suggests that either infiltration of fibroblasts is acutely, but not chronically, dependent on macrophages after SCI, or that the level of macrophage depletion achieved chronically may have been insufficient to affect fibroblast recruitment. Accordingly, differences in the distinct basal lamina border surrounding the fibrotic scar were no longer as robust as compared to 14 days post-SCI (Fig. 4.7E-J). However, the fact that the size of the fibrotic scar (GFAP+ region) became smaller after clodronate treatment (Fig. 4.9N) suggests that although fibroblasts reinvade the fibrotic scar after macrophage depletion, the overall effect of fibrotic scar reduction is maintained chronically after SCI.

We assessed the growth of serotonergic axons, which originate from the brainstem, and neurofilament+ axons, which is a marker of many different types of axons. In both PBS and clodronate liposome treated mice, serotonergic axons
Figure 4.7. Chronic depletion of hematogenous macrophages does not change fibroblast accumulation or basal lamina formation. Treatment with clodronate liposome reduces the amount of macrophages in the fibrotic scar (GFAP+ regions) at 56 days (A, C), but the reduction is not as robust as in 7 and 14 days (Fig 4.3). Correspondingly, the amount of fibroblasts is not visibly changed at 56 days (B, D), suggesting that the fibrotic scar has reformed as macrophages come back. The basal lamina between fibroblasts and astrocytes are also similar between PBS and clodronate liposome treated animals at 56 days (E-J). Region of interest (ROI) represents boxed region in the left. PBS = PBS liposome. CLO = clodronate liposome. n=7 in each group. Scale bar in A, E = 500 μm; in G = 100 μm.
Figure 4.8. Chronic depletion of hematogenous macrophages is not sufficient to promote serotonergic axon growth beyond the injury site. At 56 days after injury, serotonergic axons cannot grow across the astrocyte-fibroblast border in both PBS liposome (A-C) and clodronate liposome (D-F) treated animals. Boxed regions in A-B and D-E are enlarged in C and F. n=7 in each group. Scale bar in A = 500 μm; in C = 100 μm.

were distributed throughout the entire spinal cord and stopped at the astrocyte-fibroblast border after SCI (Fig. 4.8), indicating that reduction in the fibrotic scar by macrophage depletion is insufficient to promote serotonergic axon growth into the fibrotic scar. However, neurofilament+ axons were much more prevalent in the fibrotic scar of clodronate-treated mice than PBS-treated mice (Fig. 4.9).

To determine if this increase was due to neuroprotective effects of macrophage depletion, we compared the number of neurofilament+ axons between 14 and 56 days after injury. Our data indicates that at 14 days after SCI, the density of axons between PBS and clodronate treated animals did not differ from each other (Fig. 4.9M), suggesting that the greater axon density observed after clodronate treatment at 56 days after SCI was most likely due to axonal
growth rather than neuroprotection. Even though there were more axons in the clodronate-treated animals, they did not display a better open-field locomotor recovery than PBS-treated controls as measured by the Basso Mouse Scale (Basso et al., 2006) (Fig. 4.10). Taken together, our data indicates that the reduction in fibrotic scar and basal lamina after macrophage depletion is associated with increased axonal growth after SCI.

4.4 Macrophage depletion alters gene expression of fibrotic cytokines

We hypothesized that macrophages recruit fibroblasts to the injury site through cytokine expression. Since 7 days post injury is when we observed the largest reduction in macrophage number and fibroblast density after clodronate treatment (Fig. 4.3), we chose this time point to test our hypothesis by comparing the cytokine gene expression profile between clodronate and PBS liposome treated mice. We reasoned that macrophage depletion should lead to a decrease in the expression of cytokine(s) involved in fibroblast recruitment. Using a PCR

Figure 4.9. Fibrotic scar reduction is associated with increased growth of neurofilament+ axons (see next page). At 14 days after SCI, the number of neurofilament+ axons in the fibrotic scar (GFAP- region) was similar between PBS (A-C, n=3) and clodronate liposome (D-F, n=4) treated animals (M). However, at 56 days after SCI, the number of neurofilament+ axons in the clodronate liposome treated animals (J-L, M, n=7) was significantly higher than PBS liposome treated controls (G-I, M, n=7). The GFAP- area was unchanged at 7 days, larger at 14 days and smaller at 56 days after injury in clodronate treated animals (N). Region of interest (ROI) represents boxed region to the left. *p<0.05 compared to the other groups using two way ANOVA with Tukey’s post-test in M or two-tailed Student’s T-test in N. Scale bar in A = 500 μm; in C = 50 μm. PBS = PBS liposome. CLO = clodronate liposome. Error bars are s.e.m.
array for 84 cytokines (Table 4.1), we discovered three that showed significantly decreased expression, IL-1RN, Tnfsf8 and Tnfsf13 (Fig. 4.11). Both Tnfsf8 and Tnfsf13 have been associated with fibrosis in patients (Matsushita et al., 2007; Shao et al., 2005), raising the possibility that recruitment of fibroblasts by macrophages after SCI is dependent on these TNF superfamily members.

Surprisingly, most of the differentially expressed cytokine genes (26 out of 29) showed increased expression after clodronate treatment (Fig 4.11). We focused on cytokines with anti-fibrotic properties and discovered that all bone morphogenetic proteins (BMP1-7) (Dendooven et al., 2011; Gao et al., 2014)
tested in this array were significantly increased after macrophage depletion and comprised a large portion (7 out of 21) of the genes that showed the highest fold increase (upper right sector in Fig. 4.11A). This suggests that in addition to decreasing pro-fibrotic Tnfsf8, Tnfsf13 cytokine expression, macrophage depletion can also lead to increased expression of anti-fibrotic cytokines that may have additive/synergistic effects on reducing fibrosis after SCI. However, it is important to note that the genes that showed the largest fold-changes were IL-6 (Interleukin 6, increased) and IL-1RN (IL1 receptor antagonist, decreased), which would be predicted to increase interleukin signaling associated with fibrosis (Fielding et al., 2014). Nevertheless, our demonstration of decreased fibroblast infiltration and reduced ECM deposition indicates that the overall effect of altered cytokine expression after macrophage depletion is anti-fibrotic.
Table 4.1. List of all 84 cytokines and 5 housekeeping genes tested in the PCR array. See more information at: http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-021Z.html.

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Figure 4.11. Macrophage depletion alters both pro- and anti-fibrotic cytokine expression. A) Volcano plot of all 84 cytokines tested in the PCR array comparing the injury sites of clodronate and PBS treated mice at 7 days after injury (n=5 per group). Dotted vertical line represents 1.5 fold-change and solid horizontal line represents p=0.05 (unpaired t-test comparing clodronate and PBS treated mice for each gene). Green squares are BMPs and magenta triangles are Tnfsf. B) Bar graph showing fold-change in differentially expressed genes between clodronate and PBS treated mice (each bar represents average of clodronate (n=5) / average of PBS (n=5)).
4.5 Discussion

In this chapter, we directly tested the hypothesis that hMΦ are responsible for recruiting fibroblasts to form the fibrotic scar by performing macrophage depletion after SCI. In addition to confirming previous studies using clodronate liposome after SCI (Iannotti et al., 2011; Lee et al., 2011; Popovich et al., 1999), our study provides novel mechanistic insight by demonstrating that depletion of hMΦ results in changes in both pro- and anti-fibrotic cytokine expression and reduced fibrotic scar formation. Accordingly, reduced fibrotic scar was associated with increased growth of neurofilament+ axons, supporting the notion that the fibrotic scar is inhibitory to axon regeneration.

To interpret our data rigorously, we carefully considered several alternative possibilities that may confound our conclusion. First, since we previously reported that blood vessels are the major source of fibroblasts at the injury site (Soderblom et al., 2013), and since macrophages are known to promote angiogenesis (Jetten et al., 2014), it was possible that the reduction in fibroblast number after macrophage depletion could be an indirect effect of reduced angiogenesis after macrophage depletion. We reasoned that if this is true, then other anti-angiogenic treatments should also lead to reduced fibroblast numbers. To test this, other members in my laboratory compared the effects of clodronate liposome and sunitinib, an anti-angiogenic broad spectrum receptor tyrosine kinase inhibitor (de Bouard et al., 2007), on blood vessel formation and fibroblast accumulation in Col1α1GFP mice. We found that at 14 days after SCI, mice injected with clodronate liposome displayed significant reductions in the
density of both fibroblasts and blood vessels at the injury epicenter (Zhu et al., 2014a). While intrathecal infusion of sunitinib also decreased blood vessel density at the injury site, it did not have a significant effect on fibroblast density (Zhu et al., 2014a), suggesting that the effect of macrophage depletion on reducing the number of fibroblasts is independent of its effect on reducing angiogenesis.

Second, it has been shown that clodronate-mediated macrophage depletion leads to decreased axonal retraction after SCI (Horn et al., 2008). To discern this neuroprotective effect from actual axonal growth, we compared the number of neurofilament+ axons between 14 and 56 days after injury. We reasoned that if the increased density of neurofilament+ axons after clodronate treatment is solely due to lack of axon retraction compared with PBS controls at 56 days, there should be no difference in axon density between 56 and 14 days in clodronate-treated animals. Our data showed that after clodronate treatment, the density of neurofilament+ axons at 56 days was higher compared to 14 days. This indicated that the greater density of neurofilament+ axons at 56 days was most likely a consequence of enhanced axonal growth rather than less axonal retraction.

Third, while we attempted to address the possibility of indirect effects of macrophage depletion on reduction of the fibrotic scar by investigating blood vessel formation, we cannot rule out other cell types such as lymphocytes also playing a role. Since lymphocytes regulate fibroblast function by expressing trophic factors and cytokines in fibrosis condition of multiple non-CNS organs (Lo
Re et al., 2013; Wei, 2011; Yhee et al., 2008), it is possible that macrophage depletion may have an effect on lymphocytes present in the fibrotic scar, which may in turn indirectly affect fibroblasts response after SCI. Further analysis of lymphocyte infiltration and composition by flow cytometry at the injury site following macrophage depletion could address this issue. In addition, while we proposed that the increased axonal growth was the direct result of reduced fibrotic scar formation, especially the reduction in the dense peripheral rim of fibroblasts and basal lamina at the border between the glial and fibrotic scars, we cannot rule out the possibility that increased axon growth-promoting cytokine production may have also influenced this effect. For example, vascular endothelial growth factors (VGEFs) and BMPs are known to stimulate axon regeneration through direct or indirect mechanisms (Facchiano et al., 2002; Pereira Lopes et al., 2011; Sondell et al., 1999; Sondell et al., 2000; Zhong and Zou, 2014), and both cytokines were up-regulated after macrophage depletion. Therefore, future studies will need to determine whether targeting the fibrotic scar directly can also lead to increased axonal growth after SCI. One way is to use a Col1α1-Cre mouse that specifically expresses Cre recombinase in fibroblasts, and crosses it to a Cre-inducible diphtheria toxin receptor transgenic mouse (Buch et al., 2005). This will lead to expression of diphtheria toxin receptor on fibroblasts, and by injecting diphtheria toxin at desired time points, we should be able to specifically ablate fibroblasts after SCI and study the effect on axon regeneration.
Since we previously inferred that hMΦ may play critical roles in assembling the fibronectin matrix through expression of the prototypic fibronectin receptor, integrin α5β1, we hypothesized that depletion of hMΦ should decrease the level of fibronectin matrix assembly after SCI. Indeed, at 14 days after SCI when the fibrotic scar has stabilized, the distinct fibrillar matrix of fibronectin was largely disrupted after clodronate treatment. Concomitantly, deposition of collagens, another class of ECM proteins that are known to interact with fibronectin (Rhett et al., 2008; Rolls et al., 2008) and are also postulated to form a matrix at the SCI site (Klapka and Muller, 2006), was greatly reduced in mice injected with clodronate. These results support the idea that the fibronectin matrix may serve as a scaffold that binds many different ECM proteins, possibly including those that are inhibitory to axon regeneration. Additional studies are needed to further investigate the composition and function of the ECM occupying the fibrotic scar. We sought to quantify the level of fibronectin assembly using western blot and the DOC solubility assay as we described in Chapter 2. The fact that the intracellular cytoskeletal protein β-actin was only present in the soluble but not insoluble fraction provided confidence in our method. Although we found a significant increase in soluble fibronectin level as expected, insoluble fibronectin only showed a decreasing trend that was not statistically significant. One possible explanation is that integrin α5β1 is expressed by cells other than hMΦ, such as microglia at the periphery of the fibrotic scar. These other cell types may have also contributed significantly to fibronectin assembly around the fibrotic scar, and contributed to the insoluble fraction in our tissue homogenates.
of the injury sites that included these surrounding regions. In summary, our data suggests that hMΦ is required for proper formation of the ECM in the fibrotic scar. However, we have not convincingly demonstrated that hMΦ are directly responsible for assembly of the fibronectin matrix. Further studies that generate loss-of-function mutation of α5β1 in macrophages would provide us with a better answer.

Although we observed an increased number of neurofilament+ axons in the fibrotic scar after chronic hMΦ depletion, it was insufficient to promote meaningful locomotor recovery. Since neurofilament preferentially labels large diameter axons in tissue sections, we believe that these axons are most likely large diameter sensory afferents or bulbospinal axons. We examined the serotonergic axons, which are descending axons originating from the raphe nuclei in the brain stem and are known to have a high growth capacity following injury (Saruhashi et al., 1996). However, these axons failed to grow into the fibrotic scar following macrophage depletion (Fig. 4.8). Our finding largely agrees with previous studies using acute clodronate liposome treatment after contusive SCI (Iannotti et al., 2011; Lee et al., 2011; Popovich et al., 1999). All three studies demonstrated overall improved neurological outcomes such as reduced oxidative stress, decreased lesion volume, and increased axon sparing at the injury site. While Iannotti et al. showed a significant behavioral improvement following clodronate treatment, Lee et al. and Popovich et al. reported no or only modest recovery as reflected by the BBB locomotor (Basso et al., 1995) subscores. A possible explanation for this lack of functional improvement in our
study and others would be that since macrophages are comprised of heterogeneous populations of cells that play both detrimental and beneficial roles after SCI (Alexander and Popovich, 2009), indiscriminately depleting all hMΦ chronically may eliminate the anti-inflammatory macrophages that putatively appear chronically after injury, and offset the beneficial effects of depleting pro-inflammatory macrophages that appear more sub-acutely after SCI. Evidence for this hypothesis comes from a study using osmotic pumps to continuously deliver ganciclovir to deplete thymidine kinase expressing, CD11b+ macrophages after sciatic nerve injury, which led to severely worsened axonal growth, injury histopathology and behavioral recovery (Barrette et al., 2008). These results highlight the dual role of macrophages in axon regeneration. Furthermore, another study demonstrated that infiltration of monocytes or hMΦ to the SCI site came in two waves—a pro-inflammatory, M1-like population arrived first and dominated at 1-3 days, whereas an anti-inflammatory, M2-like population took over at 5-14 days (Shechter et al., 2013). This kinetics of monocytes influx resembles that in non-CNS wound healing (Arnold et al., 2007; Crane et al., 2014; Nahrendorf et al., 2007). Given these evidence, we performed a pilot study that targets hMΦ more acutely by administrating clodronate liposome for 3 weeks and scarificing the animals at 8 weeks after SCI. Interestingly, acute hMΦ depletion (3600 ± 315 axons/mm², n=3) resulted in approximately twice the amount of neurofilament+ axons in the fibrotic scar compared to chronic depletion (1719 ± 201 axons/mm², n=7), which supports the hypothesis that destructive macrophages may infiltrate the injury site early. Thus, the timing of macrophage
depletion may be critical to maximize the beneficial regenerative effects after SCI, and warrants further future investigation.

What is the molecular signaling by which perivascular fibroblasts are recruited to the injury site by hMΦ? We hypothesized that hMΦ (directly or indirectly) increase expression of cytokines responsible for recruiting fibroblasts and that we would be able to identify these cytokines by looking for those that show decreased expression upon macrophage depletion. Paradoxically, of the cytokines that were differentially expressed (29 out of 84), only 3 were decreased while the rest were increased. The fold-change ranged from approximately 3 fold decrease to a 4 fold increase in cytokine expression. Although these are relatively small changes, it is most likely due to the fact that the depleted macrophages represent only a minor population of cells in the analyzed tissue (4mm centered at the injury site). From the decreased cytokines, we identified Tnfsf8 and Tnfsf13 as potential pro-fibrotic molecules that could be involved in fibroblast recruitment by macrophages. In addition, Tnfsf13 is closely related to another pro-fibrotic cytokine Tnfsf13b (also known as B-cell Activating Factor) (Hasegawa and Takehara, 2012). This raises the possibility that TNF superfamily members may be important mediators of fibrosis after SCI.

Interestingly, from the list of increased cytokines, we discovered that BMP1-7, many of which have been well-characterized as having anti-fibrotic properties, were among the most highly expressed cytokines in our array. In addition to being anti-fibrotic, BMPs are also known to promote astrogliogenesis from progenitor cells (Haas et al., 2012; Hampton et al., 2007), raising the
possibility that macrophage depletion may also promote generation of astrocytes after SCI. This is consistent with our observation of decreased GFAP\(^-\) (i.e. increased GFAP\(^+\) area) area chronically (56 days) after injury. However, it is important to note that the GFAP\(^-\) area at 14 days after injury was actually increased after macrophage depletion. We believe that this is most likely the result of the reduced fibrotic scar leading to less contraction of the injury site, which is a unique (as compared to humans and rats) feature of the mouse SCI histopathology. Therefore, while the GFAP\(^-\) region at 14 days was in fact larger after macrophage depletion, we believe that the accurate interpretation of this observation is the failure of the injury site to contract as much as PBS-treated controls rather than the region becoming actively larger after macrophage depletion. Accordingly, the decreased GFAP\(^-\) area observed at 56 days could be due to a combination of injury site contraction as the fibrotic scar is reestablished and increased astrogliogenesis due to increased BMP expression.

In addition to BMPs, we also observed expression changes in various interleukins; IL-6 showed the largest increase and IL-1RN showed the largest decrease of all genes tested. However, these changes would be expected to worsen SCI pathology (Akuzawa et al., 2008; Kaplin et al., 2005; Mukaino et al., 2010; Nesic et al., 2001), suggesting that the effect of macrophage depletion on cytokine expression has both beneficial and detrimental effects, and may explain why we did not observe improved behavioral outcome even after reduced fibrotic scar formation and increased axonal growth. Therefore, while our studies
demonstrate the beneficial effects of macrophage depletion, a more targeted approach may be needed to maximize the therapeutic benefits.

In conclusion, depletion of \( h\text{M}\Phi \) results in reduced fibrotic scarring and increased axonal growth, potentially due to changes in multiple cytokines in the global environment of the injury site tissue. Thus, \( h\text{M}\Phi \) are necessary for fibrotic scar formation and can serve as a potential therapeutic target to promote wound healing and axonal growth after SCI.
Chapter 5

Concluding remarks

SCI is a devastating condition that has high socioeconomic impacts. However, current understanding of the pathological mechanisms of SCI is incomplete and insufficient to yield effective treatment strategies that restore spinal cord function. One of the major barriers that impedes axon regeneration after SCI is presence of the fibrotic scar at the injury site. This dissertation work answers critical questions regarding the mechanism of fibrotic scar formation and identifies hMΦ as a potential therapeutic target to promote axonal growth and wound healing after SCI.

Fibronectin is a ubiquitous ECM protein that has critical functions in wound healing and fibrosis of non-CNS organs. However, while fibronectin has been found to be abundantly expressed after contusive SCI, it is not known what role it plays in the fibrotic scar. We sought to explore this broad question by first investigating fibronectin matrix assembly after SCI. We demonstrated that fibronectin is assembled into a matrix that is closely associated with fibroblasts spatiotemporally in the fibrotic scar. Assembly of the fibronectin matrix is associated with the fibronectin receptor integrin α5β1, which is primarily expressed by hMΦ in the fibrotic scar. Although we provide evidence that fibroblasts are most likely the primary source of fibronectin by ruling out myeloid cells as contributors, we cannot exclude plasma fibronectin as an alternative source. Future study that deletes fibronectin specifically in fibroblasts or in the blood plasma should provide us with a clearer answer. This loss-of-function study
will also address what role, if any, fibronectin plays in fibrotic scar formation. Moreover, while we suggest that hMΦ are primarily responsible for fibronectin assembly based on their expression of integrin α5β1, our fibronectin solubility assay after hMΦ depletion did not fully support this hypothesis. Since integrin α5β1 is expressed by cells other than hMΦ including fibroblasts and microglia, it is possible that these other cells also contributed to the insoluble fraction in the injury site homogenates that included the regions surrounding the fibrotic scar. Future studies aiming to genetically disrupt integrin α5β1 in hMΦ and other surrounding cell types are required to reach a more solid conclusion. Nevertheless, our results provide novel insight into the mechanism of ECM regulation in the fibrotic scar after SCI.

The intimate spatiotemporal association between CD11b+ cells and fibroblasts accumulation led us to hypothesize that microglia/macrophages may play a role in recruiting fibroblasts to the fibrotic scar through production of specific cytokines. Using a bone marrow transplantation approach, we demonstrated that hMΦ rather than microglia, are present in the fibrotic scar, suggesting that hMΦ may be responsible for fibroblasts recruitment. The finding that microglia are limited to the glial scar is intriguing, suggesting that microglia-astrocyte interaction may have a critical role in regulating the glial scar. By depleting hMΦ, we detected a reduction in fibroblast accumulation, basal lamina formation, and ECM deposition that was associated with increased axonal growth. Since we previously found that fibroblasts originate from perivascular regions, we specifically confirmed that the reduction in fibroblast accumulation was
independent of the decreased angiogenesis that resulted from macrophage depletion. Lymphocytes present in the fibrotic scar are another likely source of indirect effects since they are known to regulate fibrosis in non-CNS organs. Future studies will be required to determine the effects of macrophage depletion on recruitment and composition of lymphocytes subpopulations. While we proposed that the increased axonal growth was the direct result of reduced fibrotic scar formation, other possibilities such as increased axon growth-promoting cytokine production may have also influenced this effect. Therefore, future studies that ablate fibroblasts after SCI will determine whether targeting the fibrotic scar directly can also lead to increased axonal growth. Overall, our data demonstrate that hMΦ are necessary for fibrotic scar formation after SCI.

Since we hypothesized that hMΦ increase expression of cytokines that promote fibroblasts recruitment, we tried to address this by determining which cytokines decreased in expression after macrophage depletion. Surprisingly, cytokine levels at the injury site were generally up-regulated after macrophage depletion. An interesting finding we discovered was that all the BMPs tested in our cytokine array, BMP1-7, were among the most highly up-regulated genes. In addition to being anti-fibrotic, BMPs are also known to promote astrogliogenesis, suggesting that macrophage depletion may also promote generation of astrocytes after SCI, which could explain why we observed decreased GFAP\(^-\) (i.e. increased GFAP\(^+\) area) area chronically after injury. It is also worth noting that although we did observe decreased expression of three cytokines, which could be candidate cytokines expressed by macrophages to attract fibroblasts, it still
remains possible that these were indirect effects of macrophage depletion. For this reason, we have initiated a RNA sequencing study of hMΦ specifically from the injury site to compare the translational profile of hMΦ across various time points after SCI. In addition to determine the types of cytokines directly produced by hMΦ, this study also provides a broader scope to determine the functional transition of hMΦ over time during fibrotic scar formation, and can serve as a basis for future studies that aim to explore the diverse role that macrophages play after SCI.

In conclusion, this dissertation contributes significantly to the gap in knowledge about what role macrophages play in formation of the fibrotic scar after SCI. By depleting hMΦ, we demonstrate that the overall cytokine response at the injury site environment becomes less fibrotic, which is associated with a decrease in fibrotic scarring and increase in axonal growth. Our novel findings demonstrate that targeting hMΦ after SCI has therapeutic potentials in promoting wound healing and functional recovery.
Chapter 6
Material and Methods

5.1 Animals

The following mice were obtained from Jackson labs: lysM-Cre (#004781), Cx3cr1\textsuperscript{GFP} (#005582), wild type C57BL/6-CD45.1 (Jackson #002014) and wild type C57BL/6 (Jackson #000664) mice. Col1\alpha1\textsuperscript{GFP} mice were kindly donated by Dr. David Brenner and have been previously described (Yata et al., 2003). Rosa26-tdTomato reporter mice were kindly donated by Dr. Fan Wang and have been previously described (Arenkiel et al., 2011). Floxed fibronectin mice were kindly donated by Dr. Sakai Takao and have been previously described (Moriya et al., 2011). All mice were backcrossed to C57BL/6 for at least 6 generations. lysM-Cre mice were bred to Rosa26-tdTomato reporter mice to generate lysM\textsuperscript{tdTom} in which lysM-Cre is hemizygous and tdTomato is homozygous. Same strategy was used to generate lysM-FN\textsuperscript{fl/fl} mice. Cx3cr1\textsuperscript{GFP} knock-in mice were used as heterozygotes. Mice were housed in a virus/antigen-free facility with a 12 h light/dark cycle, controlled temperature and humidity, and provided with water and food ad libitum.

5.2 Surgery and behavioral assessment

Mouse contusive SCI was performed as previously described (Lee and Lee, 2013). Eight- to ten-week old female mice were anesthetized (ketamine/xylazine, 100 mg/15 mg/kg i.p) before receiving mid-thoracic (T8)
contusive spinal cord injuries. Mice received a laminectomy at T8 and then the spinal column was stabilized using spinal clamps and positioned on an Infinite Horizon impactor device (Precision Systems and Instrumentation, LLC). The exposed spinal cord was visually aligned with the impactor tip, and then given a moderate (75 kDynes) contusion via computer-controlled delivery. All SCI mice received fluid supplements (Lactated Ringer’s solution, 1ml), antibiotics (Baytril, 10 mg/kg), and analgesics (buprenorphine, 0.05 mg/kg) subcutaneously for the first week (twice per day) following surgery. Twice daily bladder expressions continued for the duration of the study. Locomotor recovery was assessed using the Basso Mouse Scale (Basso et al., 2006) open field test at 1 day and weekly after injury. To deplete macrophages, clodronate encapsulated liposomes (Clodrosome, Encapsula Nanosciences, 50 mg/kg) were injected i.p at different time points after SCI. For 7d end point, liposomes were injected at 1, 3 and 6 days after SCI; for 14d end point, liposomes were injected at 1, 3, 6 and 11 days after SCI; and for 56d end point, liposomes were given weekly after the first four doses. PBS encapsulated liposomes (Encapsome, Encapsula Nanosciences, 50 mg/kg) were used as controls. Dil-labeled liposomes (Fluoroliposome-Dil, Encapsula Nanosciences, 50 mg/kg) were used to trace the distribution of liposomes. All procedures were in accordance with University of Miami IACUC and NIH guidelines.
5.3 Histology

Mice were perfused transcardially with cold PBS followed by 4% paraformaldehyde (PFA). Brains and spinal cords were harvested, post-fixed for two hours and placed in 30% sucrose overnight. 8 mm mouse spinal segment centered at the injury site was embedded in OCT compound (Tissue-Tek) and sectioned on a cryostat. Sagittal or horizontal sections were cut serially at 10 µm. Sections were blocked with 5% normal goat serum and immunostained in PBS-0.3% TritonX-100 for mouse fibronectin (Millipore AB2033, 1:500), integrin αβ1 (Millipore MAB1984, 1:250), PDGFR-β (Abcam AB32570, 1:200), CD11b (Invitrogen RM2800, 1:500), GFAP (Invitrogen 130300, 1:2000) or GFAP (Abcam ab4674, 1:500), NeuN (Millipore MAB377, 1:300), Olig2 (Millipore AB9610, 1:250), RFP (Rockland 600-401-379S, 1:2000), GFP (Abcam ab13970, 1:1,000), laminin (Sigma L9393, 1:2000), collagen IV (AbD Serotec, 1:200) and neurofilament (EnCor RPCA-NFM, 1:750). Primary antibody incubation was followed by appropriate Alexa Fluor secondary antibodies (Invitrogen, 1:500). Sections were mounted in Vectashield containing DAPI (Vector Laboratories), and images were collected with a Nikon Eclipse Ti fluorescent microscope or an Olympus FluoView 1000 confocal microscope. Trichome connective tissue stain (Abcam, ab150686) of frozen sections was performed according to the manufacturer's instructions. Bright filed images were collected with a Zeiss Axiovert 200M microscope.
5.4 Western blot

Mice were anesthetized (see above) and perfused transcardially with cold PBS. 4 mm spinal segment centered at the injury site was dissected and meninges removed. Spinal cord tissues were then chopped into 100 µm slices using a McIlwain Tissue Chopper and lysed using a deoxycholate (DOC) extraction protocol as previously described (Wierzbicka-Patynowski et al., 2004). Chopped tissue in freshly prepared 2% DOC lysis buffer (2% DOC, 20mM Tris.Cl, 2mM EDTA, 2mM iodoacetic acid, 2mM N-ethylmaleimide, 2mM PMSF) was passed through a 25G needle and centrifuged (4°C, 15,000g) to obtain DOC-soluble (supernatant) and DOC-insoluble (pellet) components. The DOC-insoluble pellet was solubilized in freshly prepared 1% SDS lysis buffer (1% SDS, 20mM Tris.Cl, 2mM EDTA, 2mM iodoacetic acid, 2mM N-ethylmaleimide, 2mM PMSF) and centrifuged (room temperature, 15,000g) to obtain supernatant that contained DOC-insoluble ECM. Protein concentration was measured using the BCA assay (Thermo Scientific). Protein sample from individual animals (mixed in β-mercaptoethanol) were separated on a 4-15% Criterion TGX precast gel (Bio-rad) and transferred onto a PVDF membrane that was blocked in 5% milk (in TBS-T) and incubated in rabbit anti-fibronectin antibody (Millipore AB2033, 1:2000) overnight at 4°C. Next day, membranes were washed and incubated in appropriate HRP-conjugated secondary antibodies (Jackson ImmunoResearch, 1:50,000) and detected using chemiluminescent substrate (SuperSignal West Pico) and exposed to film. To control for loading variations, transferred PVDF membranes were incubated in 0.1% Coomassie blue R250 in 50%
methanol/water for 30 min and de-stained in acetic acid/ethanol/water (1:5:4) for 20 min as previously described (Welinder and Ekblad, 2011). Band densities were quantified via ImageJ. Destained membrane was dried completely overnight and scanned. Each complete lane was then selected and band densities quantified using ImageJ 1.47v. Densities of fibronectin bands were normalized to densities of Coomassie blue.

5.5 Quantitative real-time PCR

Mice were anesthetized (see above) and perfused transcardially with cold DEPC PBS. 4 mm spinal segment centered at the injury site was dissected and homogenized and total RNA extracted using Qiagen RNeasy Plus Micro Kit. Total RNA was treated with DNAase (AMPD1, Sigma-Aldrich) and cDNA synthesized using the Advantage RT-PCR Kit (ClonTech). qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and the Corbett Research RG 3000 thermal cycler. A standard curve was generated for each primer pair using serial dilutions of liver cDNA to optimize PCR conditions for each primer set. Expression level of each gene was calculated using the ΔΔCt method and normalized to GAPDH. Each sample was tested in triplicates. The primer sequences were: itga5 (forward: GGACCAAGACGGCTACAATGATGT, reverse: ACCTGGGAAGGTTTAGTGCTCAGT), itgb1 (forward: TTCAGACTTCCGCATTGGCTTTGG, reverse: TGGGCTGGTGCAGTTTTGTTCAC), itgav (forward: GGGCCTATTGTTCAGCACACAT, reverse: GATTCCACAGCCCAAGTGT). The
primer set for itgb3 was purchased from Qiagen (QT00128849, QuantiTect Primer Assay).

5.6 Bone marrow transplantation

Bone marrow transplantation was performed as previously described (Ashbaugh et al., 2013). Female lysMtdTom donor mice (8-12 wks old) were anesthetized as described above and euthanized by cervical dislocation. Bone marrow cells from femur and tibia were flushed out with sterile HBSS using a 27.5 G needle attached to a 10ml syringe. Red blood cells from bone marrow were lysed with Tris-buffered ammonium chloride (140 mM NH₄Cl and 17 mM Tris, pH 7.65) for 1 min at 37°C. After three washes with sterile HBSS, bone marrow cells were passed through 40 µm cell strainer to prepare single cell suspension. The number of live cells was estimated by trypan blue staining. Donor cells were injected through the tail vein (5 x 10⁵ cells/mouse) of female Cx3cr1GFP recipient mice (6-8wks old) that received lethal whole-body irradiation (900 rad, Gammacell 40, ¹³⁷Cs source) 1d earlier. These lysMtdTom>Cx3cr1GFP chimeras were kept on antibiotics (gentamicin in drinking water, 0.5 mg/ml) for 2 weeks after irradiation/transplantation and allowed at least 8 weeks for reconstitution before receiving SCI as described above. To test for chimerism efficiency, bone marrow from CD45.1 C57BL/6 donor mice (Jackson #002014) were injected into CD45.2 C57BL/6 recipient mice to generate CD45.1>CD45.2 chimeras in parallel with generating lysMtdTom>Cx3cr1GFP mice. Chimerism efficiency was tested using flow cytometry as described below.
5.7 Flow cytometry

For calculation of chimerism efficiency, 150 ul blood was collected from uninjured CD45.1>CD45.2 or lysM<sup>tdTom</sup>>Cx3cr1<sup>GFP</sup> mice through the tail vein and mixed with heparinized HBSS (1 IU/100 µl). Immune cells from the blood were enriched with Ficoll-Paque (GE Healthcare) according to manufacturer’s instructions. To assess immune cell infiltration after SCI, mice were anesthetized and perfused transcardially with cold PBS at specified time points post injury. 4 mm spinal segment centered at the injury site was dissected and meninges removed. Spinal cord tissue was dissociated and passed through a 70 µm cell strainer. Cell suspension was mixed with myelin removal beads (Miltenyi Biotec, 130-096-733) and ran through a MACS LS column (Miltenyi Biotec, 130-042-401) according to manufacturer’s instructions. The number of live cells was quantified by trypan blue staining. Cell suspension was Fc blocked with anti-mouse CD16/32 (Biolegend, 1:200) for 10 min on ice and subsequently incubated for 30 min at 4°C with the following surface antigens: anti-CD45.1-PE/Cy7 (Biolegend 110729, 1:500), anti-CD45.2-Pacific Blue (Biolegend 109819, 1:500), anti-CD11b-Brilliant Violet 650 (Biolegend 101239, 1:500), anti-Ly6G-PerCP/Cy5.5 (Biolegend 127615, 1:200), anti-Ly6C-Brilliant Violet 510 (Biolegend 128033, 1:200), anti-CD11c-APC/Cy7 (Biolegend 117323, 1:100), anti-CD86-PE/Cy5 (Biolegend 105015, 1:200), and anti-CD43-APC (Biolegend 143207, 1:200). After surface staining, cells were fixed and permeabilized using Foxp3 staining Kit (eBioscience) before incubation with anti-CD206-PE/Cy7 (Biolegend 141719, 1:200). The following isotype control antibodies were used to determine flow
cytometry gating: Rat IgG2a-PerCP/Cy5.5 (Biolegend 400531, 1:200), Armenian Hamster IgG- APC/Cy7 (Biolegend 400927, 1:100), Rat IgG2a-PE/Cy5 (Biolegend 400509, 1:200), Rat IgG2b-APC (Biolegend 400611, 1:200), Rat IgG2a-PE/Cy7 (Biolegend 400521, 1:200). Cell suspensions were analyzed using BD LSR Fortessa-HTS flow cytometer and data were quantified using FACS-Diva software (BD Biosciences).

5.8 Cytokine expression analysis

Mice received moderate contusive SCI and were treated with PBS or clodronate liposomes as described above (n=5 per group). At 7 days after injury, mice were anesthetized and perfused transcardially with cold DPBS (Gibco 14190-144). 4 mm spinal cord segment centered at the injury site was dissected, homogenized and total RNA extracted using Qiagen RNeasy Plus Micro kit as per manufacturer’s directions. cDNA was synthesized using Qiagen RT² First Strand Kit. cDNA (0.4 ug per sample) was added to RT² SYBR Green Mastermix and aliquoted across a 384-well (4 X 96) Qiagen Mouse Common Cytokine RT² Profiler PCR Array to assess 84 cytokine and 5 housekeeping genes (Table 4.1). The PCR plate was loaded onto a QuantStudio 6 Real-Time PCR machine to perform qPCR. Data was analyzed using manufacturer-provided Excel templates. Briefly, PCR reproducibility, genomic DNA contamination and reverse transcription efficiency were examined for each sample based on Ct values of control wells in the PCR array. Ct value of each gene was normalized to housekeeping genes and expression fold-changes of clodronate group over PBS
group were calculated using the ΔΔCt method. Of the five housekeeping genes tested, Gusb (Glucuronidase beta) was selected because it showed the least change between PBS and clodronate groups.

5.9 Quantification

Quantification of immunohistochemical images were performed by unbiased observers using the Nikon IS software or ImageJ 1.47v. To quantify the number of α5β1+ cells that are also CD11b+ or GFP+, co-localized cells were counted in three randomly selected 100μm X 100μm squares in the GFAP- region (fibrotic scar). Only DAPI+ cells were counted. To quantify fibroblast and CD11b+ macrophage density at injury site after clodronate treatment, immunoreactivities of Col1α1GFP and CD11b were determined by thresholding above background level and calculating the area covered by the thresholded regions using imageJ. Immunoreactivities of GFP and CD11b were then normalized to the area of GFAP- region. To quantify the number of lysMldTom or Cx3cr1GFP cells and neurofilament+ axons, 50 μm square grids were generated over the entire image. Regions were determined based on GFAP staining; GFAP- regions were considered the fibrotic scar and GFAP+ regions considered the astroglial scar. Every 6th square was quantified. For quantification of cell density, only DAPI+ cells were counted and cells touching the left and bottom limits of a square were disregarded. For quantification of axon density, only neurofilament signal that is linear and at least 1 μm in length was counted as one axon. Axon density was only quantified in the GFAP- regions (fibrotic scar). Co-localization
was determined using Olympus FV10-ASW 3.0 viewer software to examine each of the ten one-micron Z-stack slices. For each animal, sections including the injury epicenter and two adjacent sagittal sections spaced 100μm apart were quantified, and the counts from each section were averaged.

5.10 Statistics

For single comparisons, two-tailed Student’s T-test was performed. For multiple comparisons, one-way or two-way ANOVA with Tukey’s post-test was performed. P values equal to or less than 0.05 were considered statistically significant.
References


