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Research Report

Macrophage activation and its role in repair and pathology after spinal cord injury

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ABSTRACT

The injured spinal cord does not heal properly. In contrast, tissue repair and functional recovery occur after skin or muscle injuries. The reason for this dichotomy in wound repair is unclear but inflammation, and specifically macrophage activation, likely plays a key role. Macrophages have the ability to promote the repair of injured tissue by regulating transitions through different phase of the healing response. In the current review we compare and contrast the healing and inflammatory responses between spinal cord injuries and tissues that undergo complete wound resolution. Through this comparison, we identify key macrophage phenotypes that are inaptly triggered or absent after spinal cord injury and discuss spinal cord stimuli that contribute to this maladaptive response. Sequential activation of classic, pro-inflammatory, M1 macrophages and alternatively activated, M2a, M2b, and M2c macrophages occurs during normal healing and facilitates transitions through the inflammatory, proliferative, and remodeling phases of repair. In contrast, in the injured spinal cord, pro-inflammatory macrophages potentiate a prolonged inflammatory phase and remodeling is not properly initiated. The desynchronized macrophage activation after spinal cord injury is reminiscent of the inflammation present in chronic, non-healing wounds. By refining the role macrophages play in spinal cord injury repair we bring to light important areas for future neuroinflammation and neurotrauma research.

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Receptor
CD206
Transcription factors
PPAR
TGF-beta
TNF-alpha
IL-1b
Remyelination
Myelination

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OPC
Oligodendrocyte
IL-10
LPS
IL-12
STAT6
STAT3
SLAM
MARCO
Proliferation
ECM
Ym1
Fizz-1
VEGF
IL-6
IL-4
Immune complex
Regulatory
M2b
M2c

1. Introduction

The injured spinal cord does not regenerate. Instead spinal cord injury (SCI) induces a chronic wound state that undergoes expansion and maintained demyelination resulting in impaired recovery and progressive tissue degeneration. Maladaptive inflammation, specifically macrophage activation, is likely a contributor. In mammals, macrophages, derived from blood monocytes and activated microglia, indefinitely persist at the site of SCI (Donnelly and Popovich, 2008). Macrophage depletion improves recovery (Popovich et al., 1999) and augmenting reparative macrophage phenotypes increases axon growth and motor function (Schwartz and Yoles, 2006). Collectively, these findings provide evidence that the natural macrophage response to SCI does not promote complete wound resolution.

In contrast, tissue repair and functional recovery occur after skin or muscle injuries through three sequential and overlapping wound healing phases. These include an inflammatory phase with phagocytic removal of cellular debris; a proliferative phase involving revascularization, angiogenesis and extracellular matrix deposition (ECM); and a remodeling phase involving wound retraction, inflammatory resolution, replacement of lost tissue and ultimately tissue homeostasis (Gurtner et al., 2008). Interruption of this delicate process leads to tissue destruction and a non-healing chronic wound state. Tissue repair has emerged as an active process in which macrophages play essential roles and orchestrate transitions within and among all three phases (Novak and Koh, 2013a).

Macrophages assume a wide spectrum of different functional states that can influence repair. Macrophage phenotypes are determined by the microenvironment and can change in response to new stimuli (Stout and Suttles, 2004). This "functional adaptivity" enables macrophage to contribute to all phases of repair by promoting inflammation, removing injurious triggers, depositing ECM, stimulating cell proliferation, and releasing anti-inflammatory cues. When activated out of sequence, however, macrophages have the potential to interrupt different phases of repair and persistent macrophage activation can lead to maladaptive, chronic inflammation and dysfunctional wound healing (Werdin et al., 2009; Nathan and Ding, 2010).

We now understand that SCI activates macrophages with different functional phenotypes (Kigerl et al., 2009; David and Kroner, 2011; Shechter and Schwartz, 2012; Ren and Young, 2013; Shin et al., 2013; Thawer et al., 2013). There are similarities between the types of macrophages activated during various phases of normal tissue repair (*i.e.* muscle and skin healing) and after SCI, however these similarities have not been examined in detail. Recent reviews have compared the overall wound healing responses between spinal cord and skin/muscle injuries and have examined the glial response to CNS injuries and disease (Shechter and Schwartz, 2013; Burda and Sofroniew, 2014). The role of resident *vs.* recruited macrophages (derived from microglia and monocytes respectively) is also a current focus and will not be discussed in detail (David and Kroner, 2011; Hawthorne and Popovich, 2011; London et al., 2013; Ren and Young, 2013). In this review we will compare and contrast the functions and phenotypes of macrophages needed during the progression of normal tissue repair with the macrophage response to SCI. Further, we will discuss factors that may contribute to different modes of macrophage activation after SCI. Throughout this review we will highlight areas that need to be explored in order to increase our understanding of inflammatory-mediated healing after SCI.

2. Overview of the macrophage response to spinal cord injury

A stereotypical, sequential inflammatory cascade is initiated after spinal cord trauma. Neutrophils are recruited from the circulation and CNS glia (astrocytes and microglia) are activated within the first 24 h after SCI. Shortly thereafter (2–3 days post-injury; dpi), blood monocytes migrate to the injury site where

they differentiate into macrophages that become phenotypically and morphologically indistinguishable from activated microglia. Subtle differences in the kinetics and magnitude of the macrophage response have been observed between different strains and species of animals (and humans); however, these cellular reactions are widely recognized as being pivotal in the pathological sequelae of all forms of mammalian SCI (Donnelly and Popovich, 2008). Regardless of the cause of SCI, in both basic science and clinical observations, macrophages persist at the injury site indefinitely (Donnelly and Popovich, 2008). The timing and distribution of monocyte- and microglia-derived macrophage activation after SCI has been reviewed previously and for more information readers are referred to Donnelly and Popovich (2008), David and Kroner (2011), Gensel et al. (2011) and Ren and Young (2013).

3. Macrophage activation and wound repair

The specific kinetics of the different healing phases depend upon the injury severity but are also affected by age of the individual at the time of injury, the specific organ/tissue injured, the health of the individual injured, and other factors. In the cases of normal tissue repair, after skin or muscle injury, the inflammatory phase lasts 1–2 days, the proliferation phase peaks ~1 week post-injury, and the remodeling is initiated during the proliferation phase but can last for months (Gurtner et al., 2008). Macrophages with specific phenotypes and functions are present in different phases of repair and contribute to processes and transitions within the wound repair program (see Fig. 1) (Novak and Koh, 2013a).

Specifically, macrophage activation varies along a continuum of pro-inflammatory, early stage, M1 macrophages, and pro-reparative, later stage, M2 macrophages (Fig. 1 and Table 1). M1 macrophages are observed during the acute response to trauma and release high levels of reactive oxygen species (ROS). Through increased phagocytosis and release of pro-inflammatory cytokines, M1-macrophages facilitate innate immunity to remove foreign microbes and wound debris from the injury site. M2-type macrophages exhibit tissue repair properties, show attenuated production of pro-inflammatory cytokines, and have less ROS production. They also secrete

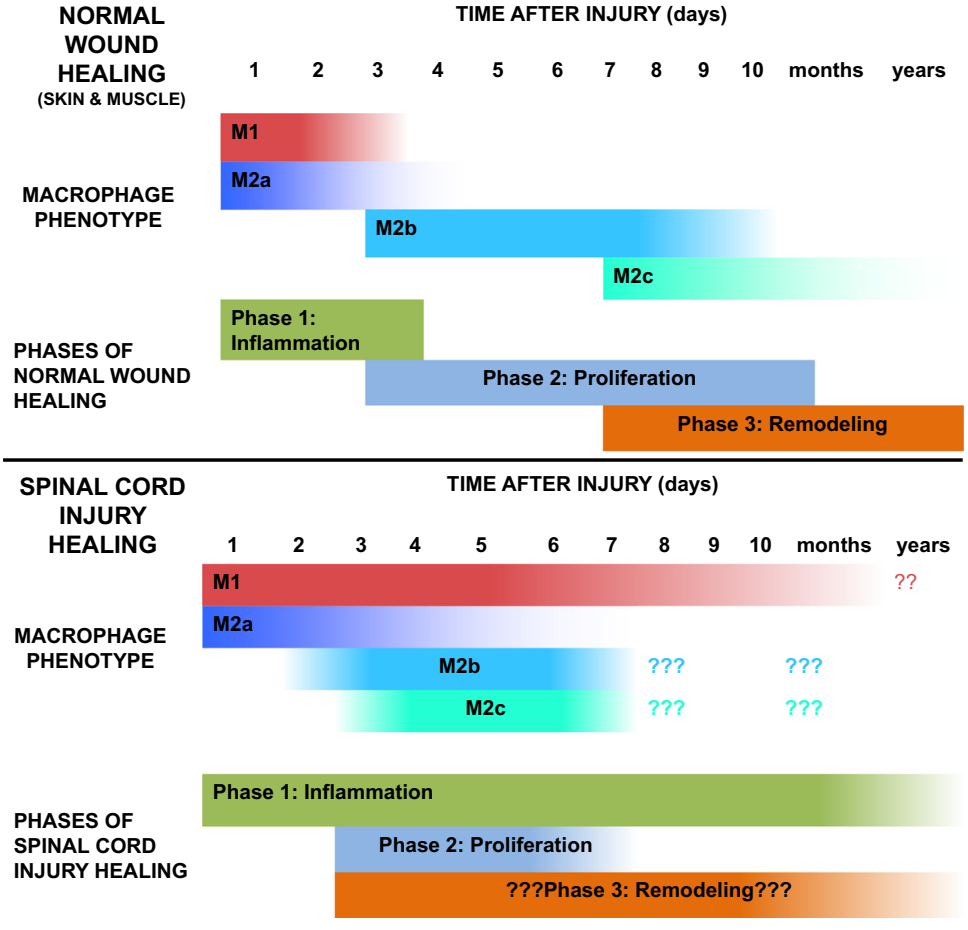

Fig. 1 – Discrete phases of phenotypically and functionally distinct macrophage subsets exist after normal wound healing and are dysregulated after SCI. (A) The phases of normal wound healing are orchestrated through sequential activation of M1 through M2c macrophages. A different pattern of macrophage activation and wound healing occurs after SCI compared to skin and muscle injury. Potentially reparative and immunosuppressive M2b and M2c macrophages (defined in Table 1) decrease over time while pathological M1 macrophages remain elevated. Changes in macrophage phenotypes over time were estimated from reviews and primary research papers referenced in the text and from quantitative-rtPCR of mouse spinal cord homogenates analyzed at 3 and 7 days after contusion SCI (data not shown).

Table 1 – Properties of wound healing macrophages. References and abbreviations can be found in the manuscript.

	M1 (classical)	M2 (alternatively-activated)		
Subtype	M1	M2a	M2b	M2c
Stimuli	INFγ+LPS/TNF-a	IL-4 or IL-13	TLRs+immune complexes	IL-10
Common features/ defining markers	**↑ROS, ↑IL-12, ↓IL10** IL-1B, TNF-α, IL-6, CD16, CD32, CCL2, CD86, MARCO, iNOS	**↓ROS** Arg-1, Ym1, CD206, Fizz-1, TREM2, IGF-1, IL1RN	**↓ROS, ↑IL-10, ↓IL-12** IL-6, VEGF, IGF-1, CD86, TNF-α, CD64	**↓ROS** TGF-B, CD206, CD163, SLAM, Sphk-1, THBS1, HMOX-1
Signaling factors	NFκ-B, STAT1, IRF5, AP-1	STAT6 KFL2, IRF4, PPARs		STAT3
Functions in normal healing	*Pro-inflammatory* Boost inflammation, debris removal, sterilization, apoptotic cell removal	*Wound healing* Anti-inflammatory, cell proliferation, cell migration, growth factors, apoptotic cell removal	*Immunoregulatory* Cell maturation, tissue stabilization, angiogenesis, ECM synthesis	*Immunosuppressive* inflammatory resolution, tissue repair, ECM synthesis, growth factors
Additional functions in CNS injury	Causes axon dieback	Remyelination, axon regeneration/reduces dieback	Axon regeneration/ reduces dieback???	Remyelination???

immunosuppressive cytokines (*e.g.* interleukin-10; IL-10) and chemokines (*e.g.* chemokine (C–C motif) ligand17 (CCL17), CCL18 and CCL22) to attract anti-inflammatory leukocytes, increase phagocytic receptors, and upregulate ECM components and growth factors (Mosser and Edwards, 2008; Van Assche, 2011; Galli etal., 2011). These mediators allow M2-type macrophages to tune inflammatory responses, scavenge debris, and promote tissue remodeling and repair. Collectively, this sequential M1–M2 macrophage response results in successful wound healing in the cases of skin and muscle injuries (Fig. 1).

Defining specific phenotypes *in vivo* is challenging due to various environmental and tissue specific stimuli that contribute to macrophage activation (Novak and Koh, 2013b; Martinez and Gordon, 2014). In addition, macrophages exhibit functional adaptivity and can change phenotypes in response to new stimuli (Stout and Suttles, 2004). Therefore, *in vitro*-defined phenotypes oversimplify the macrophage activation continuum but provide a useful platform of comparison among different wound repair systems. Using this classification system we can model macrophage-mediated wound repair as a progression through M1–M2c macrophage activation (see Fig. 1 and Table 1).

The *inflammatory phase* consists of macrophages with both M1 and M2a phenotypes (Fig. 1) (Lech and Anders, 2013). Evidence of M1 macrophages comes from the secretion of the pro-inflammatory cytokines IL-1, tumor necrosis factor α (TNF-α), and IL-6 (Novak and Koh, 2013a). M2a macrophages express high levels of arginase-1 and Ym1 during this early stage. Macrophages in the early *proliferative phase* continue to secrete pro-inflammatory cytokines but transition toward the release of IL-10 and some anti-inflammatory markers (Novak and Koh, 2013a). Interestingly, macrophages in this phase do not signal through signal transducers and activators of transcription 6 (STAT6), a traditional M2a activation pathway and instead adopt a different M2 phenotype (Daley et al., 2010). Given the mixed pro- and anti-inflammatory cytokines released and the increase in IL-10 expression, macrophages in the proliferative phase most easily map onto the M2b phenotype (Table 1 and Fig. 1) (Mantovani et al., 2004; Martinez et al., 2008; Lech and Anders, 2013). During later proliferative stages the M2b-mediated IL-10 release likely stimulates activation of M2c macrophages as evidenced by increased expression of prototypical M2c marker, transforming growth factor-β (TGF-β; Table 1) (Mantovani et al., 2004; Novak and Koh, 2013a). The *remodeling phase* is dominated primarily by M2c macrophages as indicated by high TGF-β and CD206 (*i.e.* mannose receptor) expression with concurrent decreases in arginase-1 (Lech and Anders, 2013; Novak and Koh, 2013a) (Fig. 1). Eventually, as remodeling is complete macrophages adopt a deactivated phenotype and inflammation resolves.

In successful wound repair macrophage numbers return to normal levels within weeks of injury in parallel with the timing of wound closure and healing (Sindrilaru et al., 2011). In contrast, wounds that do not heal within 3 months are considered chronic (Werdin et al., 2009) and persistent macrophage activation is a hallmark of this chronic condition (Sindrilaru et al., 2011). Sustained, improper macrophage activation disrupts transitions among different phases of repair. For example, chronic venous ulcers (CVU) are the most common type of chronic wound and in CVUs macrophages fail to switch from an M1 to an M2 phenotype (Werdin et al., 2009; Sindrilaru et al., 2011). The resolution interval, the point at which cells are reduced by 50% of peak activation, for macrophages after SCI is >7 weeks (Prüss et al., 2011). In addition, macrophage activation persists at 45% of peak activation levels into chronic time points (>2.5 months) (Prüss et al., 2011). Collectively, this highlights SCI as a chronic wound condition in which macrophage activation is a strong contributing factor (Fig. 1) (Shechter and Schwartz, 2013).

3.1. *Phase 1: inflammation*

The inflammatory phase of tissue repair is initiated by damage and disruption of tissue homeostasis. In SCI this is triggered by shearing and mechanical damage to cells and spinal cord tissue. Immediately after injury, inflammatory cells, including microglia and neutrophils, become activated and home to the site of injury. After skin, muscle, and spinal cord injuries, neutrophil accumulation begins within hours

and generally peaks within a day (Fleming et al., 2006; Donnelly and Popovich, 2008; Beck et al., 2010; Prüss et al., 2011; Rigamonti et al., 2014). The primary functions of inflammatory cells during this phase of repair are to remove damaged tissues, facilitate removal of neutrophils, and orchestrate the healing responses of fibroblasts (or astrocytes in the case of CNS injury), platelets, and endothelial cells through the release of pro-inflammatory cytokines (Novak and Koh, 2013a; Burda and Sofroniew, 2014). Unique features of the SCI during this phase include disruptions of the blood–spinal cord barrier (BSCB), activation and migration of NG2 positive, oligodendrocyte precursor cells (OPCs), and swelling of endogenous astrocytes (Burda and Sofroniew, 2014). In addition, myelin debris must be removed after SCI. The unique components of CNS *vs*. skin or muscle injury during this inflammatory phase has been reviewed previously (Shechter and Schwartz, 2013; Burda and Sofroniew, 2014).

Macrophages in the inflammatory phase have cytokine profiles similar to the M1 cells stimulated *in vitro* (Novak and Koh, 2013b). Specifically, early after skeletal muscle and skin injury, macrophages express high levels of TNF-α, IL-6, IL-12, and IL-1β (Daley et al., 2010; Brancato and Albina, 2011). Interestingly, macrophages in this phase also express high levels of arginase and Ym1, two hallmark indicators of an M2a macrophage activation (Table 1) (Mosser and Edwards, 2008; Daley et al., 2010). Collectively, this illustrates that macrophages during the inflammatory phase of normal wound repair adopt a mixture of M1 and M2a phenotypes (Brancato and Albina, 2011) (Fig. 1 and Table 1).

Conceptually, these phenotypes make sense for this phase of repair. Through the release of pro-inflammatory cytokines, M1 macrophages attract neutrophils and boost the inflammatory response to facilitate removal of damaged tissues. M1 macrophages have enhanced phagocytic abilities that further facilitate debris removal, bacterial removal and sterilization, and elimination of spent neutrophils. M2a cells, on the other hand, initiate the proliferative phase of repair through release of anti-inflammatory cytokines, increase cell proliferation and migration *via* release of arginase and Ym1, and promote the beginning of tissue formation through secretion of growth factors (Sindrilaru and Scharffetter-Kochanek, 2013; Novak and Koh, 2013a, 2013b).

A similar mixed M1 and M2a macrophage response occurs early after SCI (Kigerl et al., 2009). Expression of the pro-inflammatory markers IL-6, TNF-α, IL-1β, and IL-12 increases acutely in response to SCI (Nocentini et al., 2008; Sato et al., 2012). Macrophage arginase expression peaks within 1–3 dpi along with other markers of M2a activation, IL-4, CD206, and Fizz-1 (Kigerl et al., 2009; Kuo et al., 2011; Guerrero et al., 2012; Nakajima et al., 2012; Thawer et al., 2013) (Fig. 1). In addition, neutrophil accumulation begins within hours of SCI and, regardless of species, peaks within the normal timeframe of the inflammatory phase (1–3 days post-injury) (Kigerl et al., 2006; Beck et al., 2010; Prüss et al., 2011). Collectively, these data highlight that the macrophage and tissue responses are similar during the inflammatory phase between SCI and normally healing tissue.

3.2. *Phase 2: proliferation*

During normal healing, the proliferation phase consists of an initial cell proliferation period around 2 dpi, a peak in proliferation around 5 dpi, and decreases proliferation around 10–12 dpi (Gurtner et al., 2008). Cells important for tissue remodeling, *e.g.* endothelial cells and fibroblast-lineage cells, migrate and restore tissue integrity and barrier functions. During this phase in most tissue types, angiogenesis, collagen deposition, tissue granulation, and ECM formation occurs. The unique proliferative events after SCI include proliferation of glia and neural progenitor cells and induction of scar-forming astrocytes (Burda and Sofroniew, 2014). Analogous maturation initiated during the later proliferation phase after SCI includes differentiation of OPCs into oligodendrocytes, reduced axonal dieback and stabilization, astrocytic scar formation, Wallerian degeneration resolution, and BSCB repair. These events after SCI and neurotrauma have been compared to the wound healing responses after skin and muscle injury previously (Shechter and Schwartz, 2013; Burda and Sofroniew, 2014). The implications from those papers, and other comprehensive analyses of cellular events, indicate that the cellular responses occurring during the proliferative phase are delayed or incomplete after SCI (Donnelly and Popovich, 2008; Gensel et al., 2008; Mctigue and Tripathi, 2008; Zhang and Gensel, 2014).

After skin and muscle wounds, macrophages in the proliferative phase facilitate maturation of proliferating cells and stabilization of damaged tissue (Novak and Koh, 2013a). Specifically, macrophages facilitate initial cell proliferation during the later inflammatory phase and maintain proliferation during the proliferative phase then transition cells toward maturation at the end of the proliferative phase to start the remodeling phase (Novak and Koh, 2013a). Macrophage depletion during the proliferative phase in skin wounds disrupts vascular stability and the transition from granulation tissue into newly formed, healing scar tissue (Lucas et al., 2010). In circumstances of normal wound healing, macrophages with decreased pro-inflammatory cytokine profiles (decreased IL-12) and increased expression of anti-inflammatory cytokines and growth factors (IL-10, TGF-β, Insulin-like growth factor-1 (IGF-1)) drive the transition from inflammatory to remodeling phases of repair (Lech and Anders, 2013).

M2 macrophages in the proliferative phase have different phenotypic profiles than the M2 macrophages in the inflammatory phase (Daley et al., 2010). IL-10 is a key anti-inflammatory cytokine produced by macrophages during the proliferative stage of repair that facilitates tissue remodeling (Thompson et al., 2013; Novak and Koh, 2013a). IL-10 release is a hallmark of the M2b, or regulatory, macrophage phenotype (Edwards et al., 2006; Mosser and Edwards, 2008). M2b macrophage activation, therefore, is essential in the middle to later proliferative phase to trigger tissue remodeling (Edwards et al., 2006; Mosser and Edwards, 2008; Lech and Anders, 2013). In order to understand if the proliferative phase is properly initiated after SCI we examined the gene expression of a collection of markers associated with the M2b macrophage phenotype. We detected a significant decrease in genes associated with the M2b phenotype from the initiation (3 dpi) to the peak (7 dpi) of the proliferative phase (results summarized in Fig. 1). Similarly, gene expression of IL-10 after SCI is decreased by 6 dpi, a time of peak expression after normal wound healing (Kuo et al., 2011; Novak and Koh, 2013a). Collectively, these data highlight that the key macrophage phenotype regulating the proliferative phase of repair, the M2b macrophage phenotype, is improperly activated after SCI. It is likely, therefore, that SCI

macrophages do not facilitate proper transitions within the proliferative phase of repair (Shechter and Schwartz, 2013; Novak and Koh, 2013a; Burda and Sofroniew, 2014).

Similarly, a number of typical proliferative events are initiated within parallel time courses after skin/muscle injury and SCI, however, these events are incomplete and do not result in wound resolution. Proliferation of OPCs peaks within the first week of SCI and subsequent oligodendrocyte maturation occurs ~2 weeks (Tripathi and Mctigue, 2007; Mctigue and Tripathi, 2008). Nonetheless, remyelination of denuded axons is incomplete and signal transduction remains impaired chronically (Imai et al., 2008; Mctigue and Tripathi, 2008; Cao et al., 2010; James et al., 2011; Powers et al., 2012). ECM and collagen formation after SCI follows a similar time course to that of skin and muscle wounds (Gurtner et al., 2008; Burda and Sofroniew, 2014; Gaudet and Popovich, 2014), however, components of the ECM drive protracted inflammation and impair wound closure (Gaudet and Popovich, 2014). Similarly, the BSCB remains patent for over a month to small molecules after SCI which further illustrating that maturation events are not properly completed (Popovich et al., 1996; Schnell et al., 1999; Zhang and Gensel, 2014).

Altering macrophage phenotypes has the potential to facilitate a more constructive proliferate phase. OPC differentiation and maturation can be facilitated through activation of M2a and M2c macrophages (Miron et al., 2013). After peripheral nerve injury, macrophages are attracted to areas of Wallerian degeneration within 2–4 dpi (George and Griffin, 1994). As a result, myelin and axon clearance is complete within two weeks and subsequently, the injured nerve heals (George and Griffin, 1994). In contrast, the SCI macrophage response is delayed in areas of Wallerian degeneration and debris remains for months after SCI (George and Griffin, 1994). Debris can be removed in a timely fashion by boosting the macrophage response to SCI suggesting that manipulating macrophages may facilitate maturation events typical of normal wound healing (Perrin et al., 2005; Vallières et al., 2006). Following SCI, injured axons retract from the injury site over time. The initial trauma causes an early phase of axon retraction (1–2 dpi) but Silver and colleagues have demonstrated that later of phases of retraction are caused by activated macrophages (Horn et al., 2008; Busch et al., 2009, 2010, 2011; Evans et al., 2014). *In vitro* only M1, and not M2, macrophages physically engage dystrophic axons causing them to release supportive tethers and pull back from areas of high inhibitory growth substrates (Horn et al., 2008). Altering macrophage phenotypes by driving an alternative activation state indicative of an M2b phenotype (high IL-10, IL-1b and low Arg-1, CD206; see Fig. 9 in Stirling et al. (2014)) reduces axon dieback (Stirling et al., 2014). Collectively, these finding suggest that altering macrophages responses, specifically by boosting M2b and M2c macrophage phenotypes, may orchestrate the proliferative phase of wound healing to promote SCI repair (Fig. 1).

3.3. *Phase 3: remodeling*

The third and final phase of normal wound healing, the remodeling phase, begins 2–3 weeks after injury and can lasts for months to years (Gurtner et al., 2008). During this phase, cells that proliferated during the previous phase mature into new tissue. Processes initiated during previous phases generally wind down and eventually cease including inflammation, scarring, and angiogenesis. In muscle and skin wounds, collagen and ECM and breakdown reaches a steady state leading to wound contracture (Velnar et al., 2009). Growth factors (*e.g.* PDGF, TGF-β, FGF), matrix metalloproteinases, and others regulate this phase of the healing process and strengthen repaired tissue (Gurtner et al., 2008; Velnar et al., 2009). Ultimately the remodeling phase concludes with complete wound repair.

Although macrophages in the remodeling phase have an identifiable M2c phenotype, as indicated by increased expression of CD206, CD163, and TGF-β and decreased expression of the markers associated with an M2a or M2b phenotype: VEGF, arginase-1 and IGF-1 (Daley et al., 2010; Mirza and Koh, 2011; Novak and Koh, 2013a), their roles are not well understood. Macrophages likely play a greater role by inhibiting, rather than promoting, different aspects of the remodeling phase. Indeed, macrophage depletion during this phase does not affect scar formation and collagen production (Lucas et al., 2010) but instead a maintained pro-inflammatory macrophage presence is associated with a non-resolving, chronic wound state in various tissues (Wang et al., 2007; Hu et al., 2011; Sindrilaru et al., 2011; Rigamonti et al., 2014).

Little is known regarding the role macrophages play during the remodeling phase after SCI. This is due in part because remodeling events that occur endogenously after SCI do not lead to successful healing and thus the remodeling phase is not properly executed (Beattie et al., 1997). In addition, to the best of our knowledge, phenotypic characterization of M2c macrophages has not been performed after SCI with the exception of the preliminary short-term classification done to generate Fig. 1. Depletion studies similar to the ones examining the role of macrophages during different phases of repair after skin injury have also not been performed after SCI (Lucas et al., 2010). Additional studies in these areas could provide insight into the role of macrophages in later stages of SCI wound resolution.

Proper macrophage-mediated transitions through the phases of repair are influenced by injury severity, age, health of the individual, infections, and not of least importance, the biochemical milieu and tissue specifics of the injury. Changes in the mechanical, cellular, and biochemical makeup of the damaged tissue can influence macrophage phenotype and therefore the healing response. The relative contribution of specific factors present at the SCI site on macrophage phenotypes is not well understood. However, the initiation of secondary injury processes likely influences macrophage activation and contributes to the failure of macrophage-mediated wound healing. The effects of secondary injury processes on macrophage activation after SCI have been discussed previously (David and Kroner, 2011; Gensel et al., 2011, 2012; Hawthorne and Popovich, 2011; Ren and Young, 2013). Only a few of these processes are discussed below.

4. Secondary spinal cord injury processes and macrophage activation

4.1. *Reactive oxygen species*

Reactive oxygen and nitrogen species (ROS and RNS respectively) such as superoxide, hydroxyl radical, singlet oxygen, hydrogen peroxide, and peroxynitrite form after SCI (Bains and Hall, 2012). These free radicals participate in cell signaling under

normal physiological processes, however, excessive generation of ROS and RNS during pathophysiological conditions such as SCI may overwhelm cellular antioxidant defense and ultimately result in oxidative stress and secondary injury (Jia et al., 2012). ROS and RNS may also contribute to secondary injury process through maladaptive macrophage activation.

Major sources of ROS after SCI include mitochondria and phagocytic cells. Mitochondria consume approximately 90% of available oxygen during oxidative phosphorylation. Structural and functional mitochondrial changes following SCI can therefore augment ROS formation (Wingrave et al., 2003). Phagocytic neutrophils and macrophages also produce excessive ROS after SCI. In phagocytes, superoxide after SCI is mainly produced by the enzymatic activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Following SCI, phagocytic cells increase oxygen consumption and generate superoxide through activation of NADPH oxidase (Taoka et al., 1997). Other potential biological sources of ROS/RNS associated with the pathogenesis of SCI include cytosolic oxidases (*e.g.* xanthine oxidase), activation of the arachidonic acid cascade (*e.g.* Cyclooxygenase-2 (COX-2)), and activation of inducible nitric oxide synthase (iNOS) (Hall and Springer, 2004; Conti et al., 2007; Jia et al., 2012).

The impact of this ROS and RNS rich environment on macrophage phenotypes after SCI is largely unexplored. ROS are involved in the activation process of M1 macrophages, in part, through NF-κB (nuclear factor-kappa light-chain-enhancer of activated-B cells) (Brüne et al., 2013), however, specific ROS effects are difficult to discern, mainly due to the nonspecific effects of the antioxidants available (Gloire et al., 2006). A variety of redox-sensitive proteins participate in the signaling pathways that are triggered by inflammatory mediators (Forman and Torres, 2001). Thus, it is challenging to elucidate how oxidative stress, resulting from SCI, regulates the polarization of macrophages into different subtypes. It is possible that ROS or ROS-induced products generated upon SCI interfere with macrophage-mediated transitions between the different phases of wound repair.

4.2. *Lipid peroxidation*

Lipid peroxidation occurs when free oxygen radicals react with polyunsaturated fatty acids and cause oxidative degradation of lipids (Bains and Hall, 2012). Highly reactive hydroxyl radicals react with membrane polyunsaturated fatty acids, *i.e.* membrane lipids, and superoxide anions reacts with nitric oxide, to form peroxynitrite that then initiates lipid peroxidation. The oxygen radicals oxidize the double bonds of unsaturated fatty acids of phospholipids, resulting in the formation of toxic phospholipid byproducts such as oxidized phosphatidylcholine (OxPC), 4-hydroxynonenal (4-HNE), and acrolein. Peroxidation of lipids disrupts cellular membrane fluidity and permeability, interrupts metabolic process, and changes ion transport systems (Nigam and Schewe, 2000).

The time course of byproduct formation is well characterized after SCI; levels of 4-HNE increase as soon as 3 h, peaks at 24 h, and remain high for up to 2 weeks (Xiong et al., 2007). Elevation of acrolein, which is the strongest electrophile among all α,β-unsaturated aldehydes, has also been detected following SCI (Luo et al., 2005; Hamann et al., 2008). These overproduced aldehyde fragments in turn covalently bind to proteins and interrupt normal protein functions (Adibhatla et al., 2003).

The effects of oxidized lipid and lipoproteins on macrophage-mediated SCI wound healing processes are unclear. In atherosclerosis, macrophage phenotypic polarization can be modified by lipid mediators (Adamson and Leitinger, 2011). Oxidatively modified lipid and lipoproteins act as "danger signals" and activate macrophage toll-like receptors (TLRs). For instance, binding of oxidized low-density lipoprotein (oxLDL) to CD36 in combination with TLR heterodimers activates pro-inflammatory processes (Seimon et al., 2010; Stewart et al., 2010). Lipids-induced activation of TLRs in macrophages triggers NF-κB, MAP kinase, and ROS-dependent signaling pathways, resulting in the expression of pro-inflammatory genes and an M1-like phenotype (Adamson and Leitinger, 2011). In addition, accumulation of oxLDL attenuates the anti-inflammatory transcription factor, kruppel-like factor 2 (KLF2) and shifts M2 macrophages toward a pro-inflammatory phenotype (van Tits et al., 2011). Phospholipids may also alter macrophages phenotypes indirectly as OxPC on apoptotic cells enhances pro-inflammatory monocyte adhesion and activation (Chang et al., 2004; Bratton and Henson, 2005). Further studies are needed to clarify how these secondary injury processes affect macrophage-mediated transitions among different phases of wound healing.

4.3. *Transcriptional control of macrophage activation*

Macrophage phenotypes are regulated through transcriptional events. For example, signaling through the transcription factors: NF-κB, STAT1, and interferon regulatory factor 5 (IRF5) drive pro-inflammatory, M1 macrophage activation (Lawrence and Natoli, 2011). Specifically, a wide spectrum of pro-inflammatory cytokines and chemokines such as TNFα, IL-1β, IL-6 and CCL2, as well as ROS, are induced through these signaling cascades (Mosser and Edwards, 2008; Van Assche et al., 2011) (31, 32). In contrast, transcription factors regulating M2-type macrophage activation include STAT6, IRF4, and peroxisome proliferator-activated receptors (PPARs) (Lawrence and Natoli, 2011). We highlight a few transcription factors that may influence macrophage activation after SCI below. For further discussion regarding how transcription factors influence macrophage activation states after SCI readers are referred to the following reviews: (David and Kroner, 2011; Gensel et al., 2012; Mandrekar Colucci et al., 2013; Ren and Young, 2013).

PPARs are a family of ligand-activated transcription factors that play important roles in cellular processes including mitochondrial respiration, fatty acid metabolism, muscle lipid metabolism, and cell differentiation (Mandrekar Colucci et al., 2013). Three isoforms of this family have been described: PPARα, PPARβ (or PPARδ) and PPARγ. Their expression has been detected in microglia, astrocytes, neurons and oligodendrocytes in the CNS (Kliewer et al., 1994). Importantly, PPARβ and PPARγ have been implicated as critical transcriptional "gatekeepers" controlling the transcriptional components that influence macrophage phenotype activation. Activation of PPARs inhibits M1 gene expression in the presence of M1-type stimuli and enhances M2 gene expression in cells exposed to M2-type stimuli. Particularly, PPAR activation in macrophages causes the induction of M2 markers including Arg1, CD206, Ym1 and FIZZ1 and the effect is lost in PPAR deficient mice (Bouhlel

et al., 2007; Odegaard et al., 2007, 2008; Gallardo-Soler et al., 2008; Kang et al., 2008). Animals with a functional deletion of PPARγ in myeloid lineage cells also fail to generate wound-healing responses (Odegaard et al., 2007). Thus there appears to be a role for PPARγ in controlling alternative macrophage activation and ultimately facilitating macrophage-mediated wound repair.

In macrophages, PPARγ initiates a potent anti-inflammatory response. PPARγ transactivates M2 genes (*e.g.* CD206) by binding to the PPAR-responsive element (PPRE) sequence motif (Nicholson, 2004; Bouhlel et al., 2007). It also acts in a non-DNA-bound style, which involves protein-protein interactions between PPARγ and transcription factors participating in pro-inflammatory gene activation, such as AP-1, NF-AT, NF-κB, and STAT-1 (Ricote and Glass, 2007). By scavenging these transcription factors, PPARγ can block the production of pro-inflammatory genes. PPARγ is also able to bind to transcriptional coactivators, such as steroid receptor coactivator 1 or p300/CBP, inhibiting AP-1 activation and reducing NF-κB-dependent gene expression (Pascual et al., 2005; Brüne et al., 2013). In addition, PPARγ can interfere with protein kinase C-α (PKCα) cellular membrane translocation and thus block its activation. Inhibition of PKCα activity attenuates ROS production.

The anti-inflammatory effect of PPAR activation has been reported to be beneficial in several CNS injury and disease models, including traumatic brain injury (TBI), SCI, multiple sclerosis (MS), stroke, and amyotrophic lateral sclerosis (ALS) (Kiaei et al., 2005; Schütz et al., 2005; Drew et al., 2006; Yi et al., 2008; Villapol et al., 2012). For example, activation of PPARγ attenuates neuroinflammation and increases M2 macrophages in the model of Alzheimer's disease (AD), suggesting that administration of PPAR agonists may create an inflammatory milieu in the CNS favoring regeneration and recovery (Mandrekar Colucci et al., 2012). Activation of PPARβ in macrophages enhances the clearance of apoptotic cells through increased expression of macrophage opsonins (Mukundan et al., 2009). In models of MS and AD, activation of PPAR in microglia is neuroprotective by promoting phagocytosis of pathological protein aggregates (Mandrekar Colucci et al., 2012; Yamanaka et al., 2012). In addition, PPARγ activation increases the phagocytotic capability of microglia through upregulation of the scavenger receptor CD36 (Yamanaka et al., 2012). Because of the high production of myelin and cell debris following SCI, M2 activation induced by PPARs may be beneficial in promoting debris removal and thus facilitating macrophage-mediated transition from inflammatory to proliferative phases of repair.

5. Summary

It is unlikely that activation of a single macrophage phenotype can facilitate proper wound healing following SCI. Successful orchestration of macrophage-mediated tissue repair requires fine tuning different macrophage phenotypes to harmoniously facilitate transitions among different wound healing phases. Activating macrophage in concert may be achieved through transcriptional manipulations but secondary injury process, including the formation of reactive oxygen species and lipid peroxidation, promise dissonance. Determining which phenotypes we should be enhance and which we should abate to facilitate proper healing requires further investigation and will likely depend upon the injury-specific tissue microenvironment and the desired phase of repair.

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