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Age decreases macrophage IL-10 expression: implications for functional recovery and tissue repair in spinal cord injury

Bei Zhang¹, William M. Bailey¹, Kaitlyn J. Braun¹, and John C. Gensel^{1,*}

¹Spinal Cord and Brain Injury Research Center Department of Physiology University of Kentucky Lexington, KY 40536, United States.

Abstract

Macrophages with different activation states are present after spinal cord injury (SCI). M1 macrophages purportedly promote secondary injury processes while M2 cells support axon growth. The average age at the time of SCI has increased in recent decades, however, little is known about how different physiological factors contribute to macrophage activation states after SCI. Here we investigate the effect of age on IL-10, a key indicator of M2 macrophage activation. Following mild-moderate SCI in 4 and 14 month old (MO) mice we detected significantly reduced IL-10 expression with age in the injured spinal cord. Specifically, CD86/IL-10 positive macrophages, also known as M2b or regulatory macrophages, were reduced in 14 vs. 4 MO SCI animals. This age-dependent shift in macrophage phenotype was associated with impaired functional recovery and enhanced tissue damage in 14-month-old SCI mice. In vitro, M2b macrophages release anti-inflammatory cytokines without causing neurotoxicity, suggesting that imbalances in the M2b response in 14-month-old mice may be contributing to secondary injury processes. Our data indicate that age is an important factor that regulates SCI inflammation and recovery even to mild-moderate injury. Further, alterations in macrophage activation states may contribute to recovery and we have identified the M2b phenotype as a potential target for therapeutic intervention.

Keywords

microglia; contusion; brain; monocyte; IL-12p40; IL-12; neuroprotection; inflammation; macrophage polarization; macrophage polarity; traumatic; neurotrauma; aged; aging; adult; Digigait; grid walk; locomotor; BMS

Introduction

There is a growing appreciation that age alters the macrophage response to neurotrauma. For instance, the magnitude of intraspinal macrophage activation is significantly higher in aged

*correspondence to: John C. Gensel, B463 Biomedical & Biological Sciences Research Building (BBSRB), University of Kentucky, 741 S. Limestone Street, Lexington, KY 40536-0509, (859) 218-0516, gensel.1@uky.edu.

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vs. young rats following contusive spinal cord injury (SCI) (Hooshmand et al., 2014). Age also alters the phenotype of macrophages responding to traumatic brain injury (TBI) (Kumar et al., 2013). This is significant because the average age at the time of SCI has increased from 28.7 to 40.7 years old since the 1970s (National Spinal Cord Injury Statistical, 2013). Age is associated with increased CNS inflammation and elevated levels of pro-inflammatory cytokines (IL-1 β and IL-6); in contrast, anti-inflammatory cytokine levels (IL-10 and IL-4) decrease with age (Ye and Johnson, 1999, 2001; Maher et al., 2004; Maher et al., 2005; Nolan et al., 2005; Kuzumaki et al., 2010). Modest inflammatory changes in the brain during aging are correlated with deficits in cognition, motor coordination, and neuronal plasticity (Richwine et al., 2005; Hayashi et al., 2008).

Macrophages adopt different functional phenotypes that contribute to SCI repair processes (Kigerl et al., 2009; Kuo et al., 2011; Guerrero et al., 2012; Nakajima et al., 2012; Shin et al., 2013; Thawer et al., 2013; Huang et al., 2014). Neurotoxic, pro-inflammatory, classically-activated (M1) macrophages are identifiable by high levels of IL-12 while pro-reparative, alternative-activated (M2) macrophages secrete high levels of IL-10 (Edwards et al., 2006; Kigerl et al., 2009). IL-10 is common to most forms of alternative activation; it is released from IL-4-stimulated, arginase-positive M2a macrophages and is secreted from regulatory, M2b macrophages after activation through a combination of pro-inflammatory stimuli and immune complex-activated Fc-receptors (i.e. CD86, CD64). The phenotypic balance of activated macrophages is an important determinant of healing following trauma (Gensel and Zhang, 2015). Macrophage-mediated IL-10 release likely plays a significant role in this reparative balance. Indeed, SCI protective monocyte-derived macrophages express IL-10 and adoptive transfer of IL-10-deficient cells fails to promote SCI recovery (Shechter et al., 2009). Similarly, adoptive transfer of IL-10 secreting bone marrow-derived macrophages (BMDMs) significantly improves anatomical and functional SCI recovery (Ma et al., 2015). Additionally, chondroitinase ABC, a promising treatment for SCI, may be beneficial in part by increasing macrophage-specific IL-10 expression (Didangelos et al., 2014).

Recently, we determined that age impairs pro-reparative, M2a, macrophage activation. Specifically, we demonstrated that macrophage induction of IL-4 receptor α (IL-4R α) is impaired, and arginase expression is attenuated, in 18-20 month old mice after moderate to severe-SCI (Fenn et al., 2014). The purpose of the current study was to determine if the global effector molecule of alternative forms of macrophage activation, IL-10, is impaired with age. In order to model the current demographic trend of middle-aged individuals suffering an incomplete SCI, we performed a mild-moderate SCI in 14 and 4 MO mice (equivalent to human ages of 45 and 20 years old respectively (Quinn, 2005). Here, we report that after SCI, macrophage-mediated IL-10 expression is impaired with age, which is coincident with diminished functional recovery and potentiated tissue loss. Further, IL-10 positive macrophages in young animals co-expressed the Fc receptor CD86, indicative of an M2b phenotype. In vitro, M2b macrophages were not neurotoxic but secreted high levels of IL-10. Collectively, these data indicate that differences in age (4 vs. 14 MO) may impact the inflammatory response and repair processes after mild-moderate SCI. Further, we identify the regulatory, M2b macrophage phenotype as a promising target for improving SCI repair.

Materials and methods

Animals and surgery

4 and 14 month old (MO) C57BL/6, female mice (obtained from the National Institute on Aging) were used to model young (~18 years old) and middle-aged (~45 years old) humans respectively (Quinn, 2005). A total of 50 animals were used to generate data for the current study. One animal from each age group was excluded due to abnormalities in the force vs. time curve generated by the impact device at the time of SCI. These abnormalities are indicative of bone hits or instability in the spinal cord at the time of injury. Additionally, one animal from each group was excluded from anatomic quantification due technical difficulties in tissue blocking (the lesion epicenters were outside the cut tissue sections). The n for each outcome measure is indicated in the figure legends. Animals were anesthetized with intraperitoneal (i.p.) injections of ketamine (100 mg/kg) and xylazine (10 mg/kg). A laminectomy was performed to expose the T9 spinal cord and a mild to moderate-thoracic SCI was produced using the Infinite Horizons (IH) injury device (50 kdyn displacement) (Scheff et al., 2003). After injury, muscle and skin layers were closed using monofilament suture. Post-surgically, animals received one subcutaneous injection of buprenorphine-SR (1 mg/kg) in 1cc saline and recovered from the surgery in a warming cage overnight. Mice received subcutaneous injections of antibiotics (5mL/mg, Enrofloxacin (2.27%); Norbook Inc, Lenexa, KS) in 1ml of saline for 5 days. Wound healing and food and water intake were monitored throughout the course of the study. Bladder expression was performed on injured mice twice daily until autonomic bladder expression returned. Animals were housed in a standard environment with ad libitum access to food and water. All procedures were performed in accordance with the guidelines and protocols of the Office of Responsible Research Practices and with approval of the Institutional Animal Care and Use Committees at the University of Kentucky.

RNA isolation and gene expression

Mice designated for RNA analysis were anesthetized and then transcardially perfused with sterile DEPC-PBS. A 5-mm spinal cord segment, centered on the injury site, was then rapidly dissected, submerged in RNAlater stabilization solution (Life Technologies, Grand Island, NY), and stored at 4°C. For RNA isolation, spinal cord tissues were removed from RNAlater solution and homogenized in 1 ml Trizol reagent (Life Technologies). Total RNA was isolated based on the manufacturer's protocol, with an additional phase separation using BCP, precipitation with isopropanol (Sigma-Aldrich, St. Louis, MO), and wash of the isolated RNA in 70% ethanol. Then, 1 µg of RNA was reverse-transcribed using the high capacity cDNA reverse transcription kit (Life Technologies). Gene expression analysis was performed by using Taqman microarray cards loaded with a mixture of 100ng cDNA sample and Taqman Universal PCR Master Mix (Life Technologies, Grand Island, NY), and then run using the default thermal-cycling conditions by ViiA™ 7 Real-Time PCR System (Life Technologies). Expression of genes was normalized to 18S mRNA for each sample.

Behavioral analysis

The Basso Mouse Scale (BMS) was used to score hindlimb movements as previously described (Basso et al., 2006). Animals were assessed in an open field for 4 min pre-

surgically and at 1, 3, 7, 14, 21, and 28 days post injury (dpi) by two trained observers. The performance of left and right hindlimbs was rated separately and averaged to generate the BMS scores and subscores. The grid walk was adapted from (McEwen and Springer, 2006). Animals traversed a horizontal ladder (92 cm in length and 16.5 cm in width) over rungs 4mm in diameter spaced 1.2 cm apart from an open start platform to an enclosed black goal box. All experimental animals were habituated to the apparatus prior to surgery. The gridwalk was videotaped and the total number of foot slips over 30 continuous rungs was quantified. Gait analysis was conducted using the Digigait System (Mouse Specifics, Framingham, MA). Mice were pre-trained at a speed of 13-15 cm/s before SCI, and then tested at 27 dpi at a speed of 10-12 cm/s. For each test, at least 5 complete step cycles were recorded. A high-speed digital camera captured the movement of each paw and then footage was analyzed using the Digigait analysis software (Digigait 12.4).

Tissue processing and immunohistochemistry

Mice designated for histology were anesthetized and transcardially perfused with cold PBS (0.1 M, pH 7.4) then with cold 4% paraformaldehyde (PFA) in PBS. Spinal cords were dissected, post-fixed for 2 h in 4% PFA and then, rinsed, and stored in phosphate buffer (0.2 M, pH 7.4) overnight at 4°C. Tissues were then cryoprotected by immersion in 30% sucrose for 3-5 days at 4°C. Spinal cords were randomly, but evenly distributed by group into blocks in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA) and rapidly frozen on dry ice prior to sectioning. Transverse serial sections (10 µm) were cut through each block and mounted on coated slides (Fisher Scientific, Waltham, MA), then stored at -80°C before staining. Spinal cord sections were dried for an hour at 37°C and rinsed with 1×PBS. For IL-10/tomato lectin/CD86 triple staining and IL-10/GFAP double staining, sections were incubated with anti-IL-10 (1:300, Cat# ab9969, Abcam, Cambridge, MA), biotinylated tomato lectin (1:1000, Cat# L0651, Sigma-Aldrich), anti-CD86 (1:100, Cat# 553689, BD Pharmingen, Franklin Lakes, NJ) and anti-GFAP (1:200, Cat# GFAP, Aves, Tigard, Oregon) primary antibodies and Alexa Fluor (AF) 546 dye-conjugated goat anti-rabbit secondary antibody (1:1000, Cat# A11010, Life Technologies), streptavidin-AF488 (1:1000, Cat# S32354, Life Technologies), AF 488 dye-conjugated goat anti-rat secondary antibody (1:1000, Cat# A11006, Life Technologies), streptavidin-AF633 (1:1000, Cat# S21375, Life Technologies) or AF 488 goat anti-chicken (1:500, Cat# F-1005, Aves) secondary antibodies using standard techniques. Slides were coverslipped with Immu-Mount (Thermo Scientific, Waltham, MA). Fluorescent images were taken using a C2+ laser scanning confocal microscope (Nikon Instruments Inc, Melville, NY). To distinguish spared spinal cord axons and myelin, spinal cord sections were double-stained with eriochrome cyanine /neurofilament (EC/NF, anti-NF primary antibody 1:1000, Cat# NFH, Aves) as reported previously (Fenn et al., 2014). Brightfield images were captured with an Olympus Provis AX 80 photomicroscope (Olympus Optical).

Lesion volume was calculated by using Cavalieri method on images at 100 µm intervals centered on the lesion epicenter (Kigerl et al., 2006). The proportional area of macrophage activation and IL-10 expression was calculated as described previously (Donnelly et al., 2009). Background labeling was subtracted, and then a threshold value-based measurement within a predefined area was applied to quantify the density of labeling using the

MetaMorph analysis program (Molecular Devices, Sunnyvale, CA). The MetaMorph colocalization plugin was used to analyze colocalization of IL-10 and macrophages.

Cell culture

Bone marrow-derived macrophage (BMDM) cultures were obtained from C57BL/6 mice at 8-10 weeks of age as described previously (Longbrake et al., 2007). Femurs and tibias were isolated then bone marrow was flushed into sterile tubes with washing media (Dulbecco's Modified Eagle Medium (DMEM) /10% FBS, Life Technologies). Cells were triturated three to five times using syringes fit with 18 gauge needles and then centrifuged at 1200 rpm for 5 min. After removing supernatant, red blood cells were lysed in lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA, pH 7.4). The remaining cells were washed once in washing media, then plated at 1×10^6 cells/ml in DMEM supplemented with 1% penicillin/streptomycin, 1% HEPES, 0.001% β -mercaptoethanol, 10% FBS, and 20% supernatant from sL929 cells (a generous gift from Phillip Popovich, The Ohio State University). The sL929 supernatant (which contains macrophage colony-stimulating factor) is needed to promote differentiation of bone marrow cells into macrophages (7–10 d) (Burgess et al., 1985). Media was changed on days 2, 4 and 6 and then cells isolated on day 7. Cells were plated at a density of 1×10^6 /ml and differentiated into M1 macrophages with LPS (50 ng/ml; Invivogen) plus IFN γ (20 ng/ml; eBioscience, San Diego, CA), or M2b with LPS (50 ng/ml) plus immune complex (Mosser and Zhang, 2008; Kigerl et al., 2009). The immune complex was prepared by mixing a ten-fold molar excess of rabbit anti-chicken egg albumin (Anti-OVA IgG, Cat# 0220-1682G, AbD Serotec, Raleigh, NC) to chicken egg albumin (OVA; Cat# LS003059, Worthington Biochemical, Lakewood, NJ) for 30 min at room temperature (Mosser and Zhang, 2008). Stimuli were removed after 3 h incubation and then replaced with fresh Neuro2A media. Macrophage conditioned media (MCM) was collected 3 h later and filtered through a 0.2 μ m filter before being applied to cultured Neuro2A cells or being tested for levels of IL-10 and IL-12p40 using commercially available ELISA kits (Thermo Scientific, Rockford, IL).

Neuro-2a (N2a) cells (a generous gift from Chris Richard's laboratory at the University of Kentucky) were cultured in 45% DMEM and 45% Opti-MEM Reduced-Serum Medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Dlakic et al., 2007). To test cell viability, N2a cells were seeded in 48-well plates at a density of 2×10^4 cells/200 μ l. After 48 h, N2a growth media was replaced by MCM and incubated for another 24 h then the MTT assay performed according the manufacture instructions (Sigma-Aldrich and (Wang et al., 2007)). All measurements were done in triplicates with at least three independent experiments.

Statistical analysis

Investigators blinded to experimental conditions performed all data acquisition and analysis. Statistical analyses were completed using GraphPad Prism 6.0 (GraphPad Software). Data were analyzed using one- or two-way ANOVA followed by Tukey's, or Dunnett's test for multiple comparisons. Independent sample t-tests were used when appropriate. Grubb's test (extreme studentized deviate method; <http://www.graphpad.com/quickcalcs/grubbs1>) was used to remove a single subject from the study based upon a statistically significant outlier

value for IL-10 gene expression level that was greater than 11 standard deviations from the mean. Results were considered statistically significant at $p < 0.05$. All data are presented as mean $\pm$ SEM unless otherwise noted. Figures were prepared using Adobe Photoshop CS6 (Adobe Systems) and Prism 6.0.

Results

Age alters the phenotype of macrophages activated by SCI

In order to model middle-aged individuals suffering an incomplete SCI, the current demographic trend, we performed a mild-moderate (50kdyn) SCI in 14 and 4 MO mice (equivalent human ages of 45 and 20 years old respectively (Quinn, 2005)). The ratio of IL12:IL10 expression is a defining feature of M1 (high IL-12/low IL-10) vs. M2 (low IL-12/high IL-10) macrophages (Edwards et al., 2006). To first determine if macrophage phenotypes were altered with age after SCI, we examined gene expression levels in spinal cord tissue homogenates. There were no significant age differences in gene expression levels between 4 and 14 MO shams (Supplemental Fig. 1). Following SCI, there was an overall non-significant trend for higher IL-12 expression in 14 vs. 4 MO SCI mice ($p=0.06$ main effect of age; Fig. 1A). When examined over time, we found that IL-10 gene expression increased 5-fold in 4 MO mice from 3 to 7dpi ($p<0.05$) while there was no significant increase over time in 14 MO animals ($p>0.30$; Figure 1A). We further quantified IL-10 protein level in 14 and 4 MO mice. Similar to the gene expression data, IL-10 protein levels were comparable between 4 and 14 MO sham mice (Supplemental Fig. 1). After SCI, IL-10 expression was prominent in the lesion penumbra and co-localized with GFAP+ astrocytes (data not shown) and TomL+ macrophages, similar to what has been reported previously (Fig. 1) (Shechter et al., 2009). Overall levels of IL-10 protein were ~50% less in 14 vs. 4 MO animals at 7dpi ($p<0.01$; Fig 1B). Next, we examined colocalization between TomL and IL-10 to determine if macrophage-specific decreases in IL-10 contributed to the age differences. There was little evidence of macrophage IL-10 expression in the absence of SCI, regardless of age (Supplemental Fig. 1). After SCI, some presumably extracellular IL-10 (i.e. not associated with GFAP+astrocytes or TomL+macrophages) was detectable in both 4 MO (Fig 1D) and 14 MO mice (Fig 1J), however, IL-10/TomL colocalization was significantly decreased by ~25% in 14 MO animals ($p=0.03$; Fig 1C). High-powered confocal analysis confirmed a high degree of macrophage/IL-10 colocalization in the 4 (Fig 1F and 1G) vs. 14 MO animals (Fig. 1L and 1M). Interestingly, triple labeling revealed that IL-10 positive macrophages in 4 MO animals also co-expressed CD86 (Fig. 1H and 1I). CD86/TomL-positive macrophages in 14 MO lacked IL-10 expression (Fig 1L-O). The seemingly dichotomous co-expression of the pro-inflammatory marker, CD86 and the anti-inflammatory cytokine, IL-10 is a hallmark of the regulatory or M2b macrophage phenotype (Edwards et al., 2006).

Distinct effects of M1 and M2b macrophages on neuron survival

Our *in vivo* data suggest that age primes macrophage activation toward a pro-inflammatory (IL-12), M1-dominant phenotype with decreased M2b macrophage activation. In order to determine the potential consequences of this altered macrophage response with age, we examined the neurotoxic potential of M1 and M2b macrophages *in vitro* using bone marrow

derived macrophages (BMDMs). BMDMs have similar functional properties as SCI macrophages and the effects of stimulating BMDMs *in vitro* are predictive of spinal cord macrophage responses *in vivo* (Longbrake et al., 2007; Gensel et al., 2009). BMDMs were stimulated to adopt an M1 or M2b phenotype using LPS+IFN γ or LPS+immune complex (IC) respectively (Mosser and Zhang, 2008). The successful induction of macrophage phenotype was determined by examining IL-12p40 and IL-10 protein levels in macrophage-conditioned medium (MCM). As shown in Fig. 2, M1 macrophages secreted high levels of IL-12 and no detectable IL-10 whereas M2b macrophages secreted IL-10 with significantly less IL-12 production. We then exposed neurons to M1 or M2b MCM to determine the effect of macrophage phenotype on neuron survival. The addition of M1 MCM significantly reduced neuron survival by ~40% compared to MCM from unstimulated or M2b macrophages ($p<0.001$; Fig. 2B). Neuron survival did not decrease after exposure to M2b MCM compared to unstimulated MCM ($p=0.47$; Fig. 2B). Since macrophages are always present in some activated form in the injured CNS, these data indicate that age-related shifts in macrophage phenotype from M1 to M2b have the potential to impact pathology and neuroprotection.

Age worsens locomotor recovery and tissue pathology after SCI

We previously reported that age impairs locomotor recovery following a moderate-severe (75 kdyn) contusion SCI (Fenn et al., 2014). To determine if similar impairments occurred after milder injury with younger mice, we assessed functional recovery over a 28 dpi period after mild-moderate (50 kdyn) SCI in 14 and 4 MO mice. Age alone did not influence function since baseline scores for all outcome measures did not differ between 14 and 4 MO animals. Following SCI, both age groups had significant locomotor impairments that improved over time (time factor, $p<0.0001$; Fig. 3A) as measured by the Basso Mouse Scale (BMS), a comprehensive scale that scores the full range of locomotor functions (Basso et al., 2006). However, recovery was significantly reduced in 14 vs. 4 MO animals (age $\times$ time interaction $p<0.001$) (Fig. 3). These differences were significant by 3 dpi, and lasted throughout the 28 dpi survival period. At 28 dpi, 4 MO animals reached a BMS score of 6.7 ± 0.5 , indicating normal stepping with fore-hindlimb coordination and some trunk instability, whereas the average BMS of 14 MO mice was 5.1 ± 0.1 , indicating normal limb movement with some coordination but severe trunk instability (Fig. 3). These age-related deficits are reflected in the BMS subscore ($p<0.05$ overall affect of age and $p<0.001$ age $\times$ time interaction; Fig 3b) which quantifies fine aspects of locomotor recovery (paw/tail positions, trunk instability, etc. (Basso et al., 2006)).

An important aspect of SCI recovery is whether animals have coordinated movements between the fore and hindlimbs. As SCI animals recover, hindlimb stepping is gradually restored, but often with asymmetry between forelimb and hindlimb stepping frequencies. Coordination differences were observed at 28 dpi in 14 vs. 4 MO animals with the BMS (average BMS subscores of 1.3 ± 0 and 6.7 ± 1.3 for 14 and 4 MO animals respectively). In order to confirm these effects, we tested animals on the DigiGait system. The DigiGait is an objective locomotor analysis system that captures and digitizes footprints as animals walk on a transparent treadmill. Coordination, the ratio of forelimb-to-hindlimb stepping frequencies, is automatically calculated as the gait symmetry. Prior to SCI, both 14 and 4

MO animals had a gait symmetry score of roughly 1 indicating a normal 1:1 ratio between fore and hindlimb steps ($p>0.20$ for baseline 4 vs. 14 MO; Fig. 4B). At 27 dpi, gait symmetry scores for 4 MO animals were not significantly different from baseline ($p>0.20$; Fig. 4B). 14 MO animals however, had significantly impaired gait symmetry as compared to 4 MO SCI and baseline groups ($p<0.05$ vs. all other groups; Fig. 4B). Further, 4 weeks after SCI, 14 MO animals had increased hindlimb foot slips on the grid walk task compared to 4 MO animals ($p=0.05$; Fig. 5). The grid walk is a horizontal ladder used to assess sensory-motor coordination and performance disruption following TBI or SCI (Soblosky et al., 1997; Metz et al., 2000; McEwen and Springer, 2006).

Next, we measured anatomical tissue damage. Both the lesion volume and the lesion length were at least 1.5 times greater in 14 vs. 4 MO SCI mice at 28 dpi ($p=0.06$; Fig. 6). As shown in Figure 6A, the lesion is still apparent 0.6 and 1.2 mm rostral of the epicenter in 14 MO mice whereas the lesion is minimal 0.6 mm and undetectable 1.2 mm rostral of the epicenter in 4 MO mice. Even when correcting for potential differences in spinal cord volumes between 4 and 14 MO animals by normalizing sparing to total tissue area (Hooshmand et al., 2014), significantly less tissue was spared in the 14 vs. 4 MO animals ($p=0.02$; data not shown). Collectively, these results indicate that age impairs recovery and potentiates pathological processes after mild-moderate SCI.

Discussion

Aging-related immunological changes influence the recovery from acute injury (Gershkoff et al., 1993). In the current study we identify the M2b macrophage phenotype as a potential key contributor to age-related SCI deficits. This is based upon the observation that IL-10 production from CD86⁺ macrophages, key phenotypic features of M2b macrophages, was significantly reduced in 14 vs. 4 MO SCI mice (Fig. 1B). In vitro, M2b macrophages are not neurotoxic (Fig. 2). Since macrophages are present in some stimulated form after SCI, this suggests that an increased M1 vs. M2b response may increase secondary injury. Indeed, aged animals had worse tissue pathology (Fig. 6) and impaired functional recovery (Fig. 3-5) compared to younger animals. Collectively, these data strongly support the overall concept that age affects the macrophage response to neurotrauma and that the phenotypes of activated macrophages contribute to the pathophysiology of nervous system perturbations.

One important observation is impaired locomotor recovery and increased tissue pathology in 14 vs. 4 MO SCI mice (Figs. 3-6). This is consistent with our previous observations following a more severe (75 kdyn) SCI in older mice of a different strain (18-22 MO) (Fenn et al., 2014). The observed deficits in the current study in younger mice (14 MO) after a milder SCI (50 kdyn) suggest that subtle differences in age, even after mild-moderate SCI, may have profound effects on functional recovery and these effects occur regardless of strain. Indeed, 14 MO mice model 45 year old humans, (Quinn, 2005) and we have observed functional differences between 10 (~35 year old humans) and 4 MO SCI animals (unpublished results). While most clinical studies note differences in mortality and morbidity in geriatric vs. younger patient populations (Fassett et al., 2007; Furlan and Fehlings, 2009; Furlan et al., 2010) and age-related differences in recovery have been detected after moderate-severe rat SCI (Gwak et al., 2004b, a; Siegenthaler et al., 2008;

Fenn et al., 2014; Hooshmand et al., 2014), the current work is the first to report age-related deficits after a mild-moderate SCI. This mild-moderate SCI models the increased prevalence of fall-related, incomplete SCI among middle-aged individuals and highlights the importance of considering age in animal models and clinical trials.

One novel finding of the current study is the identification of the M2b macrophage as a potential target for improving SCI recovery. In vitro, M2b macrophages are not neurotoxic but release high levels of anti-inflammatory IL-10 (Fig 2). IL-10 SCI treatment decreases pro-inflammatory cytokine expression and improves recovery (Bethea et al., 1999). Loss of IL-10 potentiates SCI inflammation leading to worse functional recovery (Genovese et al., 2009). IL-10 also promotes activation of M2c macrophages that are associated with immunosuppression and uptake of apoptotic cells (Gordon, 2003; Mantovani et al., 2004). In addition, IL-10 facilitates neuroprotection through non-inflammatory mechanisms including direct neuronal activation of PI3K/AKT (Zhou et al., 2009; Thompson et al., 2013). Interestingly, a group from King's College in London recently reported that the beneficial effects of chondroitinase ABC treatment in SCI depend upon increases in IL-10 coincident with macrophage infiltration (Didangelos et al., 2014). It is possible that macrophage-associated changes in IL-10 expression, and therefore M2b macrophage activation, may be underlying the effectiveness of a range of SCI therapeutic interventions.

Age-related decreases in IL-10 have been reported previously (Ye and Johnson, 2001; Frank et al., 2006), however, the specific effects of IL-10 vary in response to different pathological insults. Specifically, in a choroidal neovascularization model, macrophage-mediated increases in IL-10 in aged mice (>18 MO) impair angiogenesis (Kelly et al., 2007). Peripheral LPS injection induces a significant increase in IL-10 expression in microglia isolated from the cortex of aged (26 MO) mice as compared to adult controls (Henry et al., 2009). These age-related increases in IL-10 are in contrast to our current observations and may be due to age accentuating the region specific differences in the inflammatory response to insults in the spinal cord vs. other brain areas (Zhang and Gensel, 2014).

The identification of M2b macrophages as a potential pro-reparative phenotype provides insight into seemingly conflicting results of mixed macrophage phenotypes promoting repair. For example, treatment with Pam2CSK4, a specific toll-like receptor 2 (TLR2) agonist, prevents axon dieback and induces a mixed macrophage activation profile with elevation of both M1 and M2 markers following dorsal column SCI (Stirling et al., 2014) (Gensel et al., 2015). Since M2b macrophages express markers typically associated with an M1 phenotype and the TLR2 agonists, zymosan, activates an M2b macrophage phenotype (Elcombe et al., 2013), it is possible that M2b macrophage activation may be driving the beneficial effects observed with Pam2CSK4. Further, this phenotype may be underlying macrophage-mediated axon regeneration and repair associated with zymosan-activated macrophages in the spinal cord (Yin et al., 2003; Gensel et al., 2009).

The balance of macrophage phenotypes may be a determinant for injury progression. In the current study, we identify age as an influential factor for regulating this balance in SCI. Similar macrophage-mediated imbalances have been reported for other age-related neurological conditions. For example, potentiating M2a activation in a mouse model of

prion disease slows neurodegeneration and disease progression (Gomez-Nicola et al., 2013). Lowering the M2a response while increasing M2b activation is associated with reductions in amyloid- β deposition in a mouse model of Alzheimer's disease (Sudduth et al., 2013). Additionally, increased M1 and decreased M2c activation is associated neurodegeneration in the hippocampus and thalamus of aged TBI mice (Kumar et al., 2013). The concept that subtle differences in macrophage phenotypes influence healing has been presented for atherosclerosis and SCI (Mantovani et al., 2009; Gensel and Zhang, 2015). Our results, along with the others presented above, provide evidence that age-related changes in macrophage phenotypes play a role in the pathophysiology of neurological conditions.

Recently we reported that IL-4 receptor α (IL-4R α) induction is impaired on microglia after SCI in aged animals (Fenn et al., 2014). IL-4 stimulates the M2a macrophage phenotype thus suggesting that age-related changes in macrophage plasticity may underlie the phenotypic differences we observed. However, age-dependent environmental stimuli also contribute to macrophage activation states (Stout and Suttles, 2005). For instance, age is associated with increased reactive oxygen species (ROS) production and impaired antioxidant buffering in the brain (Ansari et al., 2008b, a). The aged spinal cord creates a more oxidative microenvironment than the brain (Yonutas et al., 2015). ROS drive M1 macrophage activation and it is likely that a combination of intrinsic differences in macrophages and the microenvironment contribute to our current observations. The complex interactions among environmental stimuli and macrophages in the aged CNS remain to be elucidated. This is even more challenging considering that microglia- and monocyte-derived macrophages may adopt different healing properties after SCI (London et al., 2013).

In summary, our results demonstrate that there is an age-dependent switch in macrophage phenotype that strongly associates with functional recovery following SCI. These data provide insight into the mechanisms of tissue repair and functional recovery in different age groups and highlight that age may be a critical factor for consideration in the application of immunomodulatory therapies even after a mild-moderate SCI. This is important, as the average age at the time of SCI is increasing. Further, we provide evidence that the balance of macrophage phenotypes contributes to neuropathology and identify the M2b macrophage as a potential target for therapeutic exploitation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Mild-moderate spinal cord injury (SCI) in 4 and 14 month old (MO) mice
- Macrophage IL10 levels, tissue sparing, and recovery decrease with SCI age
- More CD86/IL-10-positive, M2b macrophages in 4 vs. 14 MO SCI mice
- Non-neurotoxic M2b macrophages release anti-inflammatory IL-10
- Novel report of the potentially beneficial M2b macrophage phenotype in SCI

Figure 1. Age impairs SCI-induced macrophage IL-10 expression

A) Gene expression of IL-10 and IL-12p40 at 3 and 7 dpi. Overall, IL-12 gene expression trended higher in 14 MO mice ($p=0.06$, ANOVA main effect of age, $n=4$). IL-10 is significantly increased at 7 dpi as compared to 3 dpi in 4 MO mice while the increase in IL-10 from 3 to 7 dpi in 14 MO mice is not significant ($p>0.30$). B) IL-10 protein expression is significantly reduced in 14 vs. 4 MO at the lesion epicenter at 7 dpi. C) The percentage of IL-10 that co-localizes with TomL+ macrophages is significantly decreased in 14 MO animals at 7 dpi. D-O) Representative images of the lesion epicenter from 4 (D-I) and 14 MO (J-O) mice at 7 dpi. Overall levels of IL-10 (red) are decreased in 14 MO mice although similar levels of macrophage activation (TomL, green) are evident in both groups. (F-I and L-O) High powered confocal reveals that CD86 (white) positive macrophages (green-white co-labelled indicated with arrows) co-express IL-10 in 4 MO (see arrows pointing at IL-10 positive cells in G) but not in 14 MO (M) animals. Z-plane projections are to the right and bottom of each confocal image. B-C, data are mean $\pm$ SEM, $n=5-8$; * $p<0.05$; ** $p<0.01$. Scale bar = 100 μ m in K; 10 μ m in O. Images are representative of group means.

Figure 2. M1 macrophages are neurotoxic while M2b macrophages release the anti-inflammatory cytokine IL-10 without neurotoxicity in vitro

A) Analysis of macrophage-conditioned media (MCM) reveals that M1 macrophages release high levels of IL-12 (solid bars, left axis) and no IL-10 (hatched bars, right axis) while M2b macrophage have significantly less IL-12 release and increased IL-10. Note that the level of IL-12p40 in M1 MCM is around 10-fold of that in M2b MCM whereas there is no IL-10 detected in M1 MCM. (B) M1 but not M2b MCM decreases neuron cell viability. N2a cells were treated with MCM for 24 h. The cell viability is determined by MTT assay. Data are mean $\pm$ SD and representative of 3 independent experiments. *** $p < 0.001$, **** $p < 0.0001$ vs. other conditions.

Figure 3. Age impairs SCI locomotor recovery

Time course of locomotor functional recovery assessed by BMS (A) and BMS subscore (B) in 4 and 14 MO SCI mice. 4 MO animals have significantly improved recovery over time with coordinated stepping (BMS ~7) and normal limb movement (BMS subscore ~7) at 28 dpi compared to 14 MO animals with some coordination (BMS ~5) and abnormal limb movement (BMS subscore ~2) (2-way ANOVA time $\times$ age interaction $p=0.002$; main effect of age = 0.004). Post-hoc comparisons reveal significant differences between groups starting at 3 and 14 dpi for BMS and BMS subscore respectively. Data are mean $\pm$ SEM, $n=7$, * $p<0.05$ for post-hoc comparisons on days indicated.

Figure 4. Coordination is impaired in 14 vs. 4 MO SCI mice

Gait symmetry analyzed using an automated treadmill (DigiGait). A) Representative dynamic gait signals of 4 MO and 14 MO mice at 27 days after SCI. The boxed region in (A) indicates a step with each paw area returning to 0 between steps. 4 MO animals have a comparable number of forelimb (left and right, LF/RF) and hind limb (LH/RH) steps with consistent area peaks over time. 14 MO mice have fewer hind limb steps with inconsistent paw area curves. B) Gait symmetry is expressed as the ratio of forelimb-to-hind limb stepping and is impaired in 14 MO mice compared to baseline and 4 MO mice (Data are mean $\pm$ SEM, $n=7$, $*p<0.05$). Gait signals in (A) are representative of group means.

Figure 5. Age impairs fine locomotor function after SCI

Grid walk score assessed by counting hind limb footfalls of 4 MO and 14 MO mice at 27 dpi. A-B) Representative sequential frames of foot placement in 4 and 14 MO animals. Notice that the hind limb falls below the rung of the horizontal ladder in the 14 MO mouse but not 4 MO mouse (Arrows). C) 14 MO animals had significantly more footfalls than 4 MO controls at 27 dpi (Data are mean $\pm$ SEM, $n=7$, $*p<0.05$).

Figure 6. Age is associated with increased tissue pathology after SCI

Quantification of lesion volume and lesion length using neurofilament (NF-brown) labeling combined with myelin (EC-blue) staining. (A) Representative images of NF/EC stained cross-sections from 1.2 mm rostral (–) to 1.2 mm caudal of the lesion epicenter in injured spinal cord of 4 MO and 14 MO mice. The lesion area is defined as lack of myelin or axon staining. Lesion volume (B) and lesion length (C) are significant greater in 14 MO as compared to 4 MO SCI mice at 28 dpi (mean $\pm$ SEM, * $p < 0.05$, $n = 6$). Scale bar = 200 μ m. Images are representative of group means.