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Hematogenous macrophage depletion reduces the fibrotic scar and increases axonal growth after spinal cord injury

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Abstract

Spinal cord injury (SCI) leads to formation of a fibrotic scar that is inhibitory to axon regeneration. Recent evidence indicates that the fibrotic scar is formed by perivascular fibroblasts, but the mechanism by which they are recruited to the injury site is unknown. Using bone marrow transplantation in mouse model of spinal cord injury, we show that fibroblasts in the fibrotic scar are associated with hematogenous macrophages rather than microglia, which are limited to the surrounding astroglial scar. Depletion of hematogenous macrophages results in reduced fibroblast density and basal lamina formation that is associated with increased axonal growth in the fibrotic scar. Cytokine gene expression analysis after macrophage depletion indicates that decreased *Tnfsf8*, *Tnfsf13* (Tumor necrosis factor superfamily members) and increased *BMP1-7* (Bone Morphogenetic Proteins) expression may serve as anti-fibrotic mechanisms. Our study demonstrates that hematogenous macrophages 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.

Keywords

spinal cord injury; fibrotic scar; fibroblasts; hematogenous macrophages; axonal growth; tumor necrosis factor; bone morphogenetic protein

Introduction

Spinal cord injury (SCI) typically leads to formation of scar tissue that can be categorized into glial and fibrotic components. This scar tissue is thought to play multiple roles including inhibition of axon regeneration and limiting infiltration of immune cells into the spinal cord parenchyma (Burda and Sofroniew, 2014; Cregg et al., 2014). Most of our

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current knowledge of the scar tissue has come from studies on the astroglial component, whereas our understanding of the fibrotic component is relatively lacking. In vitro assays indicate that fibroblasts are inhibitory/non-permissive for axon growth (Shearer and Fawcett, 2001) and genetic ablation of fibroblasts leads to cavitation at the injury site (Goritz et al., 2011). Therefore, a better understanding of fibrotic scar formation may provide novel therapeutic targets for SCI.

We recently reported that collagen1 α 1⁺ perivascular fibroblasts are a major cellular component of the fibrotic scar after SCI (Soderblom et al., 2013). While typically present around large diameter blood vessels in the normal spinal cord, after SCI, these perivascular fibroblasts detach and chronically reside in the injury site to form the fibrotic scar. However, the mechanism by which these fibroblasts are recruited from the perivascular niche into the scar is not known.

In this study, we demonstrate that macrophages are responsible for fibroblast recruitment to the injury site after SCI. Interestingly, these macrophages are of hematogenous origin rather than from microglia, which are present predominantly in the astroglial scar region surrounding the fibrotic scar. Accordingly, depletion of hematogenous macrophages from the injury site reduces fibroblast recruitment and is associated with greater axonal growth into this region. Cytokine expression analysis suggests that these effects may be due to decreased tumor necrosis factor superfamily (Tnfsf) member and increased bone morphogenetic protein (BMP) expression. Our study indicates that hematogenous macrophages recruit fibroblasts to the spinal cord injury site and macrophage depletion changes expression of multiple cytokines that makes the injury site less fibrotic and more conducive to axonal growth.

Materials and Methods

Animals

The following mice were obtained from Jackson labs: lysM-Cre (#004781), Cx3cr1^{GFP} (#005582). Col1 α 1^{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). 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^{tdTom} in which lysM-Cre is hemizygous and tdTomato is homozygous. Cx3cr1^{GFP} knock-in mice were used as heterozygotes.

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 these first four doses. PBS encapsulated liposomes were used as controls. Sunitinib malate (Selleck Chemicals, 0.3 mg/kg, 100 μ M in 2.5% DMSO) was delivered continuously for 2 weeks using an Alzet osmotic pump (model 1002) attached to an intrathecal catheter (Mouse Intrathecal Catheter, Alzet). After SCI, the catheter was inserted through the lumbar subarachnoid space (Lee et al., 2007) and the placement of the tip immediately caudal to the injury site was visualized through the laminectomy. The catheter and the pump were secured subcutaneously. In a separate group of animals, Evans blue dye was loaded into the osmotic pump to ensure that the drug delivery method was functional. All procedures were in accordance with University of Miami IACUC and NIH guidelines.

Histology and Light Sheet Fluorescent Microscopy (LSFM)

Mice were perfused transcardially with 4% paraformaldehyde (PFA). Brains and spinal cords were removed, post-fixed for two hours and placed in 30% sucrose overnight. Tissue was embedded in OCT compound (Tissue-Tek) and sectioned on a cryostat. Sagittal sections, 10 μ m, were cut serially from an 8 mm block surrounding the injury site and immunostained in PBS-0.3% TritonX-100 for CD11b (Invitrogen RM2800, 1:500), GFAP (Invitrogen 130300, 1:2000 or Abcam ab4674, 1:500), GFP (Abcam ab13970, 1:1,000), RFP (Rockland 600-401-379S, 1:4,000), laminin (Sigma L9393, 1:2000), and neurofilament (EnCor RPCA-NFM, 1:750). After primary antibody incubation, sections were incubated in the 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.

Tissue clearing and LSFM was performed as previously described (Erturk et al., 2012; Soderblom et al., 2013). Spinal cords were post-fixed in 4% PFA and washed in PBS (each overnight at 4°C). After removing the dura, spinal cord was incubated in tetrahydrofuran (Sigma 401757) gradients (50%, 70%, 80%, 90%, 100%, 2 hours per step and then 100% overnight) followed by 2–3 hours in BABB solution (1:2 ratio of benzyl alcohol, Sigma 305197, and benzyl benzoate, Sigma B6630). Peroxides were removed from tetrahydrofuran using activated aluminum oxide through column absorption chromatography as previously described (Becker et al., 2012). To label blood vessels, mice were transcardially perfused with DyLight-594-labeled *Lycopersicon esculentum* lectin (LEL) (Vector Laboratories) as previously described (Jahrling et al., 2009). After tissue clearing, spinal cords were imaged by LSFM (Ultramicroscope, LaVision Biotec) and analyzed using Imaris software (Bitplane) as described below.

Quantification

Quantification of immunohistochemical images were performed by unbiased observers using the Nikon IS software or ImageJ 1.47v. To quantify fibroblast and CD11b⁺ macrophage density at injury site after clodronate treatment, immunoreactivities of Col1 α 1_{GFP} 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 lysM_{tdTom} or Cx3cr1_{GFP} cells and neurofilament⁺ axons, 50 μ m 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 μ m 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 100 μ m apart were quantified, and the counts from each section were averaged.

Quantifications of LSM images were performed using specific tools in the Imaris software. The number of fibroblasts in a region of interest centered at the injury epicenter was determined using Imaris's spots tool at the automatically detected threshold level. Blood vessels at the same region of interest were quantified using Imaris's surface tool to calculate the volume of automatically thresholded vessels.

Bone marrow transplantation

Bone marrow transplantation was performed as previously described (Ashbaugh et al., 2013). Female lysM_{tdTom} 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 μ m 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 (5×10^5 cells/mouse) of female Cx3cr1_{GFP} recipient mice (6–8wks old) that received lethal whole-body irradiation (900 rad, Gammacell 40, ¹³⁷Cs source) 1d earlier. These lysM_{tdTom}>Cx3cr1_{GFP} 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 lysM_{tdTom}>Cx3cr1_{GFP} mice. Chimerism efficiency were tested using flow cytometry as described below.

Flow cytometry

For calculation of chimerism efficiency, 150 μ l of blood was collected from uninjured CD45.1>CD45.2 or lysM^{tdTom}>Cx3cr1^{GFP} mice through the tail vein and mixed with heparinized HBSS (1 IU/100 μ l). Immune cells from blood were enriched with Ficoll-Paque (GE Healthcare) according to manufacturer's instructions. Cell suspensions were 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 anti-CD45.1-PE/Cy7 (Biolegend 110729, 1:500), anti-CD11b-Brilliant Violet 650 (Biolegend 101239, 1:500) and anti-CD45.2-Pacific Blue (Biolegend 109819, 1:500). Cell suspensions were analyzed using BD LSR Fortessa-HTS flow cytometer and data were quantified using FACS-Diva software (BD Biosciences).

Gene 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 with ketamine/xylazine and perfused transcardially with cold DPBS (Gibco 14190-144). A 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 μ g 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 (http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-021Z.html). 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 gene and expression fold-changes of clodronate group over PBS group were calculated using the $2^{-\Delta\Delta C_t}$ method. Of the five housekeeping genes tested, Gusb (Glucuronidase beta) was selected because it showed the least change between PBS and clodronate groups.

Results

Fibroblasts and macrophages have similar spatiotemporal recruitment after SCI

Since macrophages (CD11b⁺ cells) are another major cellular component of the fibrotic scar, we sought to determine the spatiotemporal relationship between fibroblasts and CD11b⁺ cells after SCI. We utilized collagen1 α 1-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 were found only around blood vessels and in the meninges whereas CD11b⁺ cells were characteristic of microglia (Fig. 1A–D). At 3 days after injury, the injury site was mostly necrotic with very little fibroblasts and some circular CD11b⁺ cells in the periphery of the lesion (Fig. 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. 1I–L). At 14 days, when the fibrotic scar has started to mature, CD11b⁺ macrophages filled the fibrotic scar and were intimately associated with fibroblasts (Fig. 1M–P). This phenotype continued at 28 and 56 days (Fig. 1Q–X).

Therefore, our data demonstrates a close spatiotemporal relationship between fibroblast and CD11b⁺ cell recruitment to the injury site.

Hematogenous macrophages rather than microglia are present in the fibrotic scar

The close association between CD11b⁺ cells and fibroblasts suggested that CD11b⁺ cells may play a role in recruiting fibroblasts to the injury site. To determine whether these CD11b⁺ cells are monocyte-derived macrophages and/or activated microglia, we performed bone marrow transplantation using lysM^{tdTom} mice (lysM-Cre bred to Rosa26-tdTomato) as the donor and heterozygous Cx3cr1^{GFP} mice as the recipient to generate lysM^{tdTom}>Cx3cr1^{GFP} chimeric mice in which microglia are labeled with GFP and hematogenous myeloid cells are labeled with tdTomato. CD45.1>CD45.2 chimeric mice generated in parallel showed approximately 94% chimerism using flow cytometry of blood samples (Supplemental Fig. 1A). Although chimerism was not directly assessed in lysM^{tdTom}>Cx3cr1^{GFP} chimeras, the percent of CD11b⁺ cells in blood that were tdTomato⁺ or GFP⁺ was quantified prior to SCI. 0.4% ± 0.2% CD11b⁺ cells were GFP⁺ and 41.7% ± 2% were tdTomato⁺ in the chimeras (mean ± SEM, n=9), as compared to 59.1% ± 3% GFP⁺ cells in naïve heterozygous Cx3cr1^{GFP} (n=3) and 47.4% ± 4% tdTomato⁺ cells in naïve lysM^{tdTom} mice (n=3). Taken together, our data indicates a high level of bone marrow reconstitution in our chimeras and levels of myeloid cells in blood that are similar to naïve mice. Furthermore, the irradiation procedure did not have any visible effects on microglia and did not lead to infiltration of monocytes into spinal cord parenchyma (Supplemental Fig. 1B–I).

Using our lysM^{tdTom}>Cx3cr1^{GFP} chimeric mice, we performed SCI to determine the distribution of hematogenous macrophages (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⁺ and GFAP[−] regions (Fig. 2A–H). However, at 14 days when scar formation has stabilized, the fibrotic scar (GFAP[−] region) is comprised mostly of hMΦ while microglia is present mostly in the peripheral edges of the astroglial (GFAP⁺) scar region (Fig. 2I–L). Taken together, our data indicates that hMΦ, rather than activated microglia, are associated with the fibrotic scar after SCI, and suggest that hMΦ may play a role in recruiting fibroblasts to the injury site.

Depletion of macrophages reduces the fibrotic scar after SCI

To directly test the role of hMΦ in fibrotic scar formation, we depleted hMΦ by injecting Coll1α1^{GFP} mice with clodronate liposome after SCI. We confirmed the effect of clodronate liposome by demonstrating that F4/80⁺ cells were visibly decreased in the spleen, which was reported as the main source of macrophages acutely after SCI (Blomster et al., 2013) (data not shown). Accordingly, CD11b⁺ cells at the injury site were significantly reduced at both 7 and 14 days after SCI and this was associated with a significant reduction in the density of fibroblasts present at the injury site at both time points (Fig. 3). In addition, the dense population of fibroblasts at the peripheral rim was almost completely absent (Fig. 3E–G, M–O). Therefore, our data suggests that macrophages promote fibroblast recruitment after SCI.

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 (data not shown). However, at 14 days, fibrotic scar area was greater in clodronate treated mice (Fig. 3U). 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 below.

To test whether our results were due to off-target effects by clodronate liposome, we injected it into $\text{lysM}_{\text{tdTomato}} > \text{Cx3cr1}_{\text{GFP}}$ chimeric mice to confirm that only hMΦ, rather than microglia, were depleted (Fig. 4I–N). In addition, we injected DiI-labeled liposomes into $\text{Col1}\alpha 1_{\text{GFP}}$ mice after SCI to confirm that fibroblasts were not directly targeted by clodronate (absence of colabeling between DiI and GFP, Fig. 4A–H). These data gives us confidence that the reduced fibroblast accumulation was the result of hMΦ depletion.

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. 3H). However, after clodronate treatment, this distinct border was virtually absent and replaced by diffuse patches of laminin immunoreactivity observed throughout the fibrotic scar (Fig. 3P), suggesting that macrophages and/or fibroblasts are required for proper basal lamina formation around the fibrotic scar.

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), we sought to determine if 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, we 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 $\text{Col1}\alpha 1_{\text{GFP}}$ mice. We combined tissue clearing with light sheet fluorescent microscopy to obtain a three dimensional reconstruction of the blood vessels around the fibrotic scar. At 14 days after SCI, mice injected with clodronate liposome displayed significant reductions in the density of both fibroblasts (confirming our results above) and blood vessels at the injury epicenter (Fig. 5A–F, J–K, Video 1–4). While intrathecal infusion of sunitinib also decreased blood vessel density at the injury site, it did not have a significant effect on fibroblast density (Fig. 5G–I, L–M, Video 5, 6), suggesting that the effect of macrophage depletion on reducing the number of fibroblasts is independent of its effect on reducing angiogenesis.

Reduction of fibrotic scar is associated with increased axonal growth

To determine if the reduction in fibrotic scar and basal lamina formation after macrophage depletion leads to greater axonal growth, 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 (data not shown), although the level of reduction was not as large as mice euthanized at 7 and 14 days after SCI. However, the amount of Col1 α 1^{GFP} fibroblasts between clodronate and PBS treated animals was not visibly different (data not shown). 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. Nevertheless, neurofilament⁺ axons were much more prevalent in the fibrotic scar (GFAP⁺ region) of clodronate-treated mice than PBS-treated mice (Fig. 6).

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. 6), 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 better open-field locomotor recovery than PBS-treated controls as measured by the Basso Mouse Scale (Basso et al., 2006) (Supplemental Fig. 2). 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.

Macrophage depletion alters fibrotic cytokine gene expression

We hypothesized that macrophages recruit fibroblasts to the injury site through cytokine expression and tested this by comparing the cytokine gene expression profile between clodronate and PBS liposome treated mice at 7 days after SCI. We reasoned that macrophage depletion should lead to a decrease in the expression of cytokine(s) involved in fibroblast recruitment. Using a PCR array for 84 cytokines (listed in website link in Methods), we discovered three that showed significantly decreased expression, IL1RN, Tnfsf8 and Tnfsf13 (Fig. 7). 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 7). 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. 7A). 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 IL6 (Interleukin 6, increased) and

IL1RN (IL1 receptor antagonist, decreased), which would be predicted to increase interleukin signaling associated with fibrosis (Fielding et al., 2014).

Discussion

In this study, we sought to determine the mechanism of fibrotic scar formation after contusive SCI by focusing on the interaction between macrophages and fibroblasts. In order to differentiate between microglial and monocyte origin of macrophages associated with the fibrotic scar, we used chimeric mice to demonstrate that microglia was present mostly in the glial scar while 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 scar. 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 $lysM_{tdTomato} > Cx3cr1_{GFP}$ chimeric approach, these studies used heterozygous $Cx3cr1_{GFP}$ mice as bone marrow donors and irradiated wild type (WT) mice as recipients ($Cx3cr1_{GFP} > WT$ chimeras). Therefore, we repeated the spinal cord injury experiment with $Cx3cr1_{GFP} > WT$ chimeras and compared the results with those from naïve $Cx3cr1_{GFP}$ mice. Since both hMΦ and microglia are labeled with GFP in naïve $Cx3cr1_{GFP}$ mice, we expected to observe GFP⁺ cells throughout the spinal cord injury site. However, surprisingly, at 14 days after injury, despite noticeably more GFP⁺ cells present in $Cx3cr1_{GFP}$ mice than $Cx3cr1_{GFP} > WT$ 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 (Supplemental Fig. 3). Thus, our data indicates that data from previous studies using $Cx3cr1_{GFP} > WT$ 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 $Cx3cr1$ 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 recent developmental studies that have demonstrated that macrophages derived from $Cx3cr1_{GFP}$ precursor cells stop expressing GFP after entering certain tissue types to become tissue-resident macrophages (Yona et al., 2013). As a result, it is possible that hMΦ derived from blood-born $Cx3cr1_{GFP}$ 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 $Cx3cr1_{GFP}$ mice to generate $Cx3cr1_{GFP} > WT$ chimeras showed hMΦ present in the injury epicenter, consistent with our

results (Donnelly et al., 2011). This could be due to the possibility that a complete loss of function of the *Cx3cr1* gene in these homozygous *Cx3cr1_{GFP}* 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_{tdTomato}>Cx3cr1_{GFP}* 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*.

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 mechanistic insight by demonstrating that depletion of macrophages leads to 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. It has been shown that clodronate-mediated macrophage depletion leads to decreased axon 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 after SCI, 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 after SCI 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. While we propose 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 astroglial border, we cannot rule out the possibility that increased axon growth-promoting cytokine production may have also influenced this effect. In addition, 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 and ependymal cells also playing a role. Therefore, future studies will need to determine whether targeting the fibrotic scar directly can also lead to increased axonal growth after SCI.

What is the molecular signaling by which perivascular fibroblasts are recruited to the injury site by hMΦ? We hypothesized that macrophages (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 many interleukins; IL6 showed the largest increase and IL1RN showed the largest decrease of all genes tested. However, these changes would be expected to worsen SCI pathology (Kaplin et al., 2005; Nestic 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. In addition, the lack of behavioral improvement may also have been due to our chronic depletion of macrophages, which may offset the beneficial effects of macrophages that appear more sub-acutely after SCI. Therefore, while our studies demonstrate the beneficial effects of macrophage depletion, a more targeted approach may be needed to maximize the therapeutic benefits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Fibrotic scar is associated with hematogenous macrophages, whereas microglia occupy the astroglial scar
- Hematogenous macrophage depletion reduces the fibrotic scar and promotes axonal growth
- Macrophage depletion leads to decreased pro-fibrotic tumor necrosis factor superfamily members Tnfsf8 and Tnfsf13 as well as increased anti-fibrotic bone morphogenetic proteins BMP1-7.
- Macrophage depletion results in changes in multiple cytokines that make the injury site less fibrotic and more conducive to axonal growth.

Figure 1. Fibroblasts and macrophages display similar spatiotemporal distribution profile

In the uninjured spinal cord (A–D), Col1 α 1_{GFP} fibroblasts are around blood vessels while CD11b⁺ cells represent microglia. At 3 days after injury (EH), 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 μ m; D = 50 μ m.

Figure 2. Fibrotic scar is occupied by hematogenous macrophages

lysM_{tdTomato}>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 astroglial (GFAP⁺ area) scar. However, at 14 days (I–L), the fibrotic scar is occupied almost entirely by lysM_{tdTomato} cells whereas Cx3cr1_{GFP} cells are present mostly in the surrounding astroglial scar region. Majority of lysM_{tdTomato} 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 29 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. Hematogenous macrophage depletion reduces the fibrotic scar

Treatment with clodronate liposomes significantly reduced the amount of macrophages at both 7 (A, I, Q, n=6 for PBS, n=5 for CLO) and 14 (C, K, S, n=6 for PBS, n=7 for CLO) days after SCI. This reduction in macrophages was associated with a reduction in fibroblasts (B, J, D, L, R, T). Notably, the dense peripheral rim of fibroblasts bordering the astroglial scar was visibly diminished in clodronate treated animals (D, L, F, G, N, O). This reduction in peripheral rim fibroblasts was associated with diminished basal lamina formation between fibroblasts and astrocytes (H, P). The reduction in macrophages and fibroblasts led to a

significant increase in the GFAP⁺ area at 14 days (E, M, U). Boxed region in D, E is represented by F, G. Boxed region in L, M is represented by N, O. Q, R, S, T 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–E, I–M, H–P = 500 μm ; in F, G, N, O = 100 μm ; Error bars are s.e.m.

Figure 4. Liposomes specifically target hematogenous macrophages

At both 7 (A, B, E, F, n=3) and 14 (C, D, G, H, n=3) days after SCI in *Col1a1_{GFP}* mice, injection of DiI-labeled liposome demonstrates its localization in CD11b⁺ cells (A–D) and not in *Col1a1_{GFP}* fibroblasts (E–H). At 14 days after SCI, clodronate treatment in *LysM_{tdTom} > Cx3Cr1_{GFP}* chimeric mice (I–N, n=3 for clodronate, n=4 for PBS) shows a visible reduction in hMΦ (*LysM_{tdTom}*) but not microglia (*Cx3Cr1_{GFP}*). 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 5. Fibroblast reduction is not due to reduced angiogenesis after macrophage depletion

At 14 days after SCI, clodronate treatment (n=5, D–F) resulted in a significant decrease in both fibroblast density and blood vessel volume (J, K, Video 3, 4) as compared to PBS liposome treatment (n=4, A–C, J–K, Video 1, 2). While sunitinib treatment (G–I, n=6) also decreased blood vessel volume, it did not significantly affect fibroblast density (L, M, Video 5, 6). A–I are single optical sections from image stacks obtained using light sheet fluorescent microscopy (Video 1, 3, 5). Vehicle in the clodronate study was PBS liposome (A–C, J–K). Vehicle in the sunitinib study was 2.5% DMSO (n=4, images not shown). *p<0.05 compared to vehicle using two-tailed student's t-test. Scale bar = 300 μm in A–I. Error bars are s.e.m.

Figure 6. Fibrotic scar reduction is associated with increased growth of neurofilament⁺ axons
 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 smaller in clodronate liposome treated animals at 56 days after injury (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 μ m; in C = 50 μ m. PBS = PBS liposome. CLO = Clodronate liposome. Error bars are s.e.m.

Figure 7. 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)).