Bone Marrow-Derived Monocytes Drive the Inflammatory Microenvironment in Local and Remote Regions after Thoracic Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) produces a toxic inflammatory microenvironment that negatively affects plasticity and recovery. Recently, we showed glial activation and peripheral myeloid cell infiltration extending beyond the epicenter through the remote lumbar cord after thoracic SCI. The presence and role of infiltrating monocytes is important, especially in the lumbar cord where locomotor central pattern generators are housed. Therefore, we compared the inflammatory profile of resident microglia and peripheral myeloid cells after SCI. Bone marrow chimeras received midthoracic contusive SCI, and trafficking was determined 1–7 days later. Fluorescence-activated cell (FAC) sorting showed similar infiltration timing of both neutrophils and macrophages in epicenter and lumbar regions. While neutrophil numbers were attenuated by day 3, macrophages remained unchanged at day 7, suggesting that macrophages have important long-term influence on the microenvironment. Nanostring gene array identified a strong proinflammatory profile of infiltrating macrophages relative to microglia at both epicenter and lumbar sites. Macrophages had elevated expression of inflammatory cytokines (IL-1 β , IFN γ), chemokines (CCL2, CXCL2), mediators (COX-1, MMP-9), and receptors (CCR2, Ly6C), and decreased expression of growth promoting genes (GDNF, BDNF). Importantly, lumbar macrophages had elevated expression of active trafficking genes (CCR2, L-selectin, MMP-9) compared with epicenter macrophages. Further, acute rehabilitation exacerbated the inflammatory profile of infiltrated macrophages in the lumbar cord. Such high inflammatory potential and negative response to rehabilitation of infiltrating macrophages within lumbar locomotor central pattern generators likely impedes activity-dependent recovery. Therefore, limiting active trafficking of macrophages into the lumbar cord identifies a novel target for SCI therapies to improve locomotion.

Keywords: acute rehabilitation; inflammation; macrophage; spinal cord injury

Introduction

INFLAMMATION has both positive and negative effects after spinal cord injury (SCI).^{1,2} Increasing evidence, however, identifies inflammation as a major contributor to impaired recovery after SCI. High levels of inflammation in the spinal cord after injury can impair functional locomotor recovery and negatively impact quality of life—for instance, causing neuropathic pain.^{3–5} Recent evidence showed that inflammation spreads past the epicenter through the lumbar cord after a thoracic SCI.^{6,7} This is important because the lumbar segments house a dynamic network of pattern generating interneurons that mediate walking.^{8–10} Rehabilitation studies from our laboratory indicate that cytokine production and elevated matrix metalloproteinase-9 (MMP9) activity in the lumbar cord impede recovery.⁷ Moreover, cytokine production in these segments induces mechanical hypersensitivity after injury.^{4,11,12} Thus, modulating inflammation within the

lumbar microenvironment may provide an intervention to potentiate the recovery of locomotion and limit the development of neuropathic pain.

After injury, resident microglia and peripherally derived immune cells both contribute to inflammatory processes and upregulation of cytokines.^{13,14} At the lesion epicenter, injured blood vessels increase permeability allowing parenchymal infiltration of peripheral immune cells.^{15–19} Recently, we showed that peripherally derived myeloid cells also enter the spinal cord in the remote lumbar cord acutely after thoracic injury.²⁰ This trafficking was not associated with increased permeability of blood vessels. Instead, the injury induced a reactive endothelium and chemokine gradient that recruited myeloid cells into the lumbar tissue. The specific cell type and phenotypes of these cells remain unknown, and it is unclear what role these cells play in recovery.

Other studies have indicated a detrimental role of peripheral immune cells entering the spinal cord after injury. Depletion studies

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report increased locomotor recovery and axonal sparing when trafficking of myeloid cells was inhibited.^{18,21–23} Importantly, both neutrophils and monocytes are recruited to the injury site. While some studies show improved recovery with monocyte depletion alone,²¹ another study showed that inhibition of both neutrophil and monocyte trafficking was necessary to improve recovery.²³ Overall, depletion studies indicate that peripheral myeloid cells recruited to the injured cord may impair recovery. It is less well understood, however, how myeloid cells recruited to epicenter or lumbar regions contribute to inflammation and recovery.

Our previous work identified attenuated infiltration of myeloid cells in the lumbar cord of injured MMP9 deficient mice.²⁰ Importantly, eliminating MMP9 also attenuated remote cytokine expression and enhanced activity-dependent recovery.⁷ Treadmill training, however, surprisingly worsened recovery in wild-type mice where MMP9 was highly expressed in the lumbar cord. Overall, these studies suggest a relationship between elevated MMP9 activity, infiltrating myeloid cells, and responsiveness to rehabilitation.

The purpose of this study was to characterize the gene profile of infiltrating myeloid cells to better understand their contribution to the inflammatory microenvironment of the lumbar cord. We compared gene expression in resident microglia and infiltrating macrophages in the lumbar cord after thoracic SCI using NanoString gene arrays. In addition, macrophages recruited to the lumbar cord were compared with macrophages present at the injury site, because the inflammatory profile of monocytes recruited to intact tissue may be altered compared with those at the lesion. Last, the effect of acute rehabilitation on lumbar macrophage gene expression was explored.

Methods

Mice

Experiments were conducted in accordance with The Ohio State University Institutional Laboratory Animal Care and Use Committee. Adult (3–4 months of age) female C57BL/6J wild-type (WT) mice were obtained from Jackson Laboratories. Mice were housed 3–4 per cage and provided food and water *ad libitum*. The chimeric mice were engineered by adoptively transferring bone marrow (BM) from a C57BL/6-Tg (CAG-enhanced green fluorescent protein [EGFP]) donor (3–4 months of age, Jackson) into a busulfan-treated C57BL/6J recipient mice (1–2 months of age, Jackson).

Engineering of GFP⁺ BM-chimera

BM recipient C57BL/6 female mice (6–8 weeks old) were injected intraperitoneally once daily for two consecutive days with busulfan in a 1:1 solution of dimethyl sulfoxide and deionized H₂O (30 mg/kg/100 μ L). The selected dosage of busulfan results in partial BM ablation and limited morbidity.^{20,24} Donor mice were euthanized with carbon dioxide, and the femur was extracted. Donor BM-derived cells were isolated from the femur and passed through a 70 μ m cell strainer. Total number of cells was determined with a BD Coulter Particle Count and Size Analyzer (Beckman Coulter). BM-derived cells (1×10^6) were transferred to recipient mice by tail vein injection (100 μ L) 48 h after the second dose of busulfan. Mice were left undisturbed for four weeks to allow engraftment. Engraftment was verified by determining the percentage of GFP⁺ cells in the blood.

SCI

Contusion of the spinal cord was performed as described previously.⁷ In brief, mice were anesthetized with a ketamine (138 mg/kg)-xylazine (20 mg/kg) cocktail and given prophylactic antibiotic agents (gentocin, 1 mg/kg). Using aseptic techniques, removal of the

spinous process and lamina of T9 exposed the dura. After stabilizing the vertebral column, the Infinite Horizon (IH) device delivered 75 kilodynes of force to induce a severe contusion injury. The incision was closed in layers and 2 mL of sterile saline was administered subcutaneously (SC) to prevent dehydration. Randomized group assignment occurred. During recovery, mice received antibiotic agents (1 mg/kg gentocin, SC) and saline for five days, and bladders were expressed manually twice per day until tissue harvest.²⁵

Training paradigm and locomotor recovery

Treadmill training was delivered in subgroups of mice at early time points as described previously.⁷ Starting two days after injury, training consisted of six consecutive days of manually delivered, weight-supported stepping during quadrupedal locomotion on a custom-built treadmill (Columbus Instruments). Hindlimb stepping was assisted manually as needed using small rounded pestles to achieve toe clearance and plantar placement of the paw on the treadmill belt. Adjustable harnesses provided partial body weight support while maintaining the trunk in a typical horizontal murine posture. Each session included two 10 min bouts separated by a 20 min rest interval to prevent delayed onset muscle soreness. Locomotor recovery was assessed in the open field using the Basso Mouse Scale²⁶ before injury and at one, three, and seven days post-SCI. Two raters, blind to group, assessed joint movement, weight support, plantar stepping, coordination, paw position, trunk and tail control over a 4-min period. Scores range from no hindlimb movement (0) to normal locomotion (9).

Flow cytometry on blood

To confirm chimerism, blood was collected by cardiac stick, and red blood cells were lysed. Blood leukocytes were washed, and the Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience). Cells were incubated with CD11b (eBioscience) and Ly6C (BD Biosciences) antibodies for 1 h at 4°C. Cells were washed and resuspended in FACS buffer for analysis. Antigen expression was determined using a Becton-Dickinson FACSCaliber four-color cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star), and gating for each antibody was determined based on isotype stained controls and percent GFP⁺ cells was determined. Chimerism resulted in 60–65% of all circulating cells and 95% of Ly6C^{high} monocytes being positive for GFP.

Purification of CD11b⁺ cells from spinal cord

Enriched CD11b⁺ cells were isolated from the spinal cord as described previously.²⁷ In brief, the spinal cord was removed, and the epicenter and lumbar regions were dissected. Each segment was homogenized and centrifuged to collect a cell pellet. Cells were then suspended in a discontinuous Percoll density gradient (70%/35%/0%) and centrifuged. Microglia and peripherally derived CD11b⁺ cells were collected at the 70%/35% Percoll interphase.

Sorting of microglia and macrophages from spinal cord tissue

Percoll enriched CD11b⁺ cells were incubated with an Fc receptors block (anti-CD16/CD32 antibody) followed with rat anti-mouse Ly6G-PE, CD45-PerCP-Cy5.5, and CD11b-APC antibodies (eBioscience, CA). Cells were sorted using a Becton-Dickinson FACSARIA III cell sorter at the OSU Comprehensive Cancer Center core facility. Neutrophils were identified by CD11b⁺/GFP⁺/Ly6G⁺ expression and were excluded (Fig. 1A). Microglia were identified by CD11b⁺/CD45^{low}/GFP⁺ expression, and peripherally derived macrophages were identified by CD11b⁺/CD45^{high}/GFP⁺ expression (Fig. 1A). Microglia and macrophages (10–5000 cells) were sorted into separate collection tubes. Flow data were analyzed using FlowJo software (Tree Star, CA).

FIG. 1. Recruitment of peripheral macrophages and neutrophils into the spinal cord. **(A)** FACS-sorting of neutrophils, macrophages, and microglia. C57BL/6 mice underwent busulfan treatment to ablate the bone marrow (BM). Next, busulfan treated mice received a BM transfer of cells from a green fluorescent protein (GFP)+ C57BL/6 donor mouse. After BM reconstitution (3 weeks), GFP+ BM chimeric mice were naïve or underwent a thoracic spinal cord injury (SCI). The epicenter region and lumbar region was collected 24 h, here days, and seven days after the injury. Myeloid cells were isolated by Percoll gradient and neutrophils (CD11b⁺Ly6G⁺), macrophages (CD11b⁺Ly6G⁺GFP⁺), and microglia (CD11b⁺Ly6G⁺GFP⁺) were identified by FACS. **(B)** The percentage of neutrophils in the epicenter and lumbar region is shown. **(C)** Neutrophil infiltration into the lumbar gray matter was confirmed by immunohistochemistry in a subset of chimeric mice. **(D)** The percentage of macrophages in the epicenter and lumbar region is shown. Bars represent the mean + standard error of the mean. Means with (*) are significantly different than controls. ($n=5$ per group)

NanoString and nCounter analysis of mRNA copy number

FACS-sorted microglia and macrophages were pelleted and lysed in RNA lysis buffer. Norgen Biotek Single Cell RNA Purification

Columns were used to purify and concentrate total RNA. RNA quality and integrity was determined using Bioanalyzer 2100 (Agilent Technologies). RNA was analyzed using NanoString nCounter technology (www.nanostring.com), which allows expression analysis of multiple genes from a single sample. This

approach adds confidence in reaching conclusions on expression patterns that would be limited if using quantitative polymerase chain reaction (qPCR) to validate single gene messenger RNA (mRNA) expression. Here, the low number of cells sorted and low RNA concentration would necessitate pooled samples for qPCR, potentially increasing variability. Hence, Nanostring is a more sensitive assay. NanoString analysis was performed by the Nucleic Acid Core Facility at OSU ($n=84$).

nCounter multiplexed target profiling of 60 custom genes was performed. Customized plates were designed with selected microglia or macrophage related genes that are relevant to inflammation/trafficking, extracellular matrix (ECM) remodeling, and growth/repair. Importantly, several genes within each category were chosen so that a trend in expression could be determined. Data were analyzed using nSolver 2.6. After technical normalization of positive and negative controls, RNA was normalized based on expression of housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta-actin (ACTB), and ornithine decarboxylase antizyme 1 (OAZ1). Samples with correlation coefficients <0.8 within the biological group were removed from the study ($n=6$). Heat maps were generated by unsupervised clustering analyses using Pearson correlation in the nSolver program.

Immunohistochemistry and digital image analysis

GFP+ chimeric mice were deeply anesthetized and transcardially perfused with 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (pH 7.2). Spinal cord segments from spinal levels L1–L6 were post-fixed for 1 h in 4% paraformaldehyde, rinsed overnight in 0.2M phosphate buffer (PB, pH 7.4), then cryoprotected in 30% sucrose before being embedded in optimal cutting temperature compound (ThermoFisher Scientific) and frozen on dry ice.^{7,28,29} Lumbar blocks were sectioned in their entirety at 20 μ m on a Microm HM505E cryostat.

Sections were stained for neutrophils using Ly6G antibody (ThermoFisher Scientific). In brief, sections were blocked (1% bovine serum albumin [BSA], 5% normal goat serum [NGS]) and then incubated with rabbit polyclonal Ly6G antibody (1:500) overnight at 4°C. Sections were washed with PBS and incubated with a fluorochrome-conjugated secondary antibody (Alexa Fluor 594) (1:500). Control sections were processed by eliminating the primary antibody and replacing with blocking solution to ensure positive labeling. Fluorescent images were visualized using a Zeiss 510 Laser Confocal Microscope (The Ohio State University Confocal Microscopy Imaging Facility).

Statistical analysis

All data were analyzed using one way analysis of variance with Tukey *post hoc* testing or Student *t* test when appropriate (IBM SPSS). Means and standard error of the mean (SEM) are reported throughout. Significance set at $p < 0.05$.

Results

Recruitment of peripheral macrophages and neutrophils into the spinal cord

We showed previously that BM-derived peripheral immune cells infiltrate the epicenter and lumbar region after thoracic SCI.²⁰ To further characterize the cell types that infiltrate the spinal cord after injury, we generated GFP+ BM chimeric mice and analyzed peripheral immune cells infiltrating epicenter or lumbar regions by flow cytometry. Of the chimeric cells, only infiltrating neutrophils and macrophages will be CD11b⁺, GFP⁺, and CD45^{high}. To distinguish between these cell types, cells were also labeled with Ly6G to identify neutrophils. The FACS schematic is shown in Figure 1A.

Quantification of neutrophils showed infiltration into the cord acutely after SCI but not later. Figure 2B shows significant neutrophil infiltration into the epicenter at 24 h and surprisingly into the lumbar cord as well ($p < 0.001$). At later time points, however, there was no increase in neutrophils at the epicenter or lumbar cord. To confirm the presence of neutrophils within the lumbar cord parenchyma, GFP+ chimeric mice were perfused one day after SCI, and the lumbar cord was stained for Ly6G. Figure 2C shows infiltration of BM derived GFP+ cells that co-label with Ly6G.

Unlike neutrophils, macrophages infiltrated both the epicenter region and lumbar cord at 24 h and persisted for seven days after SCI. Figure 2D shows a robust infiltration of macrophages into the epicenter and the lumbar cord 1–7 days after injury ($p < 0.05$). Importantly, the proportion of macrophages recruited to the lumbar cord was similar to the proportion at the epicenter, because both of these populations were between 30–35% of all CD11b+ cells. While the percentages of macrophages were similar between the two regions, the absolute number of macrophages present at the lesion site was significantly higher compared with that in the lumbar region. For example, at 24 h after SCI, the absolute number of macrophages collected at the epicenter region was 5880 ± 390 compared with 985 ± 269 at the lumbar region ($p < 0.05$). Overall, because the number of neutrophils was attenuated, the number of macrophages remained stable in both the epicenter and lumbar cord.

Macrophages promote inflammation in the lumbar cord after thoracic SCI

The sustained presence of macrophages in the lumbar cord after thoracic SCI suggests that macrophages play an important long-term role in influencing the remote microenvironment. Therefore, we determined the gene profile of infiltrating macrophages compared with resident microglia. After FAC-sorting of microglia and macrophages, the RNA was collected and analyzed using a customized NanoString gene array. Genes were selected based on relevance to inflammation, trafficking, ECM remodeling, and growth/repair. First, the gene profiles of microglia and macrophages recruited to the lumbar cord were determined. Figure 2 shows a heat map of all the genes included in the array for microglia and macrophages. Because there are few macrophages in the non-injured spinal cord, the naïve condition is only represented by microglia isolated from naïve mice. The heat map shows relative expression of genes in microglia from naïve mice (Naïve), microglia at 24 h after SCI (MGL), and macrophages at 24 h after SCI (MAC). This heat map shows distinct clusters of genes that were downregulated (green) or upregulated (red) in infiltrating macrophages (Fig. 2). This profile of the infiltrating macrophages did not cluster with microglia. In fact, resident microglia after SCI were more similar to microglia in naïve mice.

The heat map in Figure 2 shows several interesting comparisons between infiltrating lumbar macrophages and microglia. Infiltrating macrophages had decreased expression of several growth factors and anti-inflammatory mediators. These included transforming growth factor beta 1 (TGF β 1), nerve growth factor (NGF), neurotrophic factor 5 (NTF5), NTF3, glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) ($p=0.08$), insulin-like growth factor 1 (IGF1) ($p=0.06$), C-X3-C motif chemokine receptor 1 (CX3CR1), and colony stimulating factor 1 receptor (CSF1R) ($p < 0.05$). A few growth/repair genes, however, had high expression in macrophages. This was arginase (Arg1), YM-1, and vascular endothelial growth factor A (VEGFA) ($p < 0.05$). Compared with naïve, only infiltrating macrophages had elevated expression of these mediators.

Infiltrating macrophages also had altered expression of ECM remodeling mediators and enzymes compared with resident microglia. In the lumbar cord, macrophages had decreased expression of a disintegrin and metalloproteinase domain (ADAM)15, ADAM17, ADAM28, MMP2, MMP3, and tissue inhibitor of metalloproteinase (TIMP)2 ($p < 0.02$). While MMPs are involved in ECM remodeling, many ADAM genes are involved in cell-cell interactions—for example, ADAM17 can cleave L-selectin on the surface of cells.³⁰ Both MMP2 and TIMP2 have inhibitory effects of ECM remodeling; thus, their decreased expression could lead to increased ECM remodeling. Other genes were increased, including ADAM8, TIMP1, and MMP9 ($p < 0.01$). Of these genes, only ADAM8 was elevated in microglia after SCI compared with naïve ($p < 0.05$). These expression results indicate that macrophages have a higher capacity to contribute to ECM remodeling after SCI compared with resident microglia in the lumbar cord.

In the lumbar cord, the pro-inflammatory response with elevated expression of inflammatory cytokines, chemokines, and mediators was primarily attributed to infiltrating macrophages 24 h after SCI (Fig. 2). For example, interleukin (IL)-1 β , cyclooxygenase-2 (COX-2), interferon gamma (IFN γ), C-X-C motif chemokine ligand 2 (CXCL2), and CC motif chemokine ligand (CCL)5 were only elevated in macrophages compared with naïve ($p < 0.01$). CCL2 was elevated in both microglia and macrophages after injury ($p < 0.05$). Macrophages recruited to the lumbar cord also had elevated expression of inflammatory receptors and trafficking related genes. These included C-C motif chemokine receptor (CCR)2, L-selectin, major histocompatibility complex (MHC) class II, and Ly6C ($p < 0.01$). These data show that at 24 h after SCI, macrophages recruited to the lumbar cord have high expression of inflammatory related genes and genes involved with active trafficking of monocytes and neutrophils.

Infiltrating macrophages influence lumbar microenvironment in the first week after SCI

Next, we evaluated how expression of these genes changed through seven days after injury. Time-course graphs for genes involved in inflammation and trafficking are shown in Figure 3. Microglia and macrophages at one, three, and seven days after SCI are compared with naïve microglia. Interestingly, IL-1 α expression was low in both microglia and macrophages through the seven days. Other proinflammatory mediators had increased expression initially that decreased with time. IL-1 β , COX-2, and IFN γ expression was elevated in macrophages at one day ($p < 0.01$), but by seven days, expression of these mediators was similar to that of microglia and naïve. The chemokine CCL2 was increased in both microglia and macrophages only at day 1 and had decreased expression at three and seven days. Other chemokines, including CCL5 and the neutrophil chemotactic factor CXCL2, were increased in macrophages but not microglia at day 1 ($p < 0.01$), and expression decreased through seven days.

Other markers of inflammation and mediators involved in trafficking remained distinctly different in macrophages compared with

FIG. 2. Macrophages infiltrating the lumbar cord have a distinct gene expression profile compared with resident microglia. Chimeric C57BL/6 mice were naïve or underwent a thoracic spinal cord injury (SCI), and after 24 h, FAC-sorted macrophages and microglia were collected from the lumbar cord. The RNA was isolated and analyzed by NanoString gene array. Heat map shows genes upregulated (red) or downregulated (green) in microglia from naïve mice (Naïve), microglia from injured mice (MGL), and macrophages from injured mice (MAC). ($n = 3$ per group)

Lumbar Microglia/Macrophage Gene Expression (mRNA counts)

FIG. 3. Macrophages promote inflammation in the lumbar cord after thoracic spinal cord injury (SCI). Chimeric C57BL/6 mice were naive or underwent a thoracic SCI. The lumbar region was collected 24 h, three days, and seven days after the injury, and macrophages and microglia were FAC-sorted. RNA was isolated and analyzed by NanoString gene array. Bars represent the mRNA count, mean + standard error of the mean. Means with (*) are significantly different than naive microglia. Means with (+) have a tendency ($p=0.1$) to be different than naive microglia. Macrophage means with (#) are significantly different than microglia at the corresponding time point. ($n=4$ per group). IL, interleukin; COX, cyclooxygenase; IFN, interferon; CCL, C-C motif chemokine ligand; CCR2, C-C motif chemokine receptor; CXCL, C-X-C motif chemokine ligand; CX₃CR1, C-X₃-C motif chemokine receptor; MHC, major histocompatibility complex.

microglia (Fig. 3). Infiltrating macrophages had elevated expression of the inflammatory markers MHC II, Ly6C, and L-selectin, and expression of these markers did not decrease through time ($p<0.01$). Expression of CCR2 was largely elevated in macrophages, and through seven days decreased in expression but still remained significantly greater than resident microglia after SCI ($p<0.01$). Similarly, expression of the chemokine and anti-inflammatory receptor CX₃CR1 was decreased in macrophages ($p<0.01$). Expression of CX₃CR1 increased with time but did not reach levels similar to microglia over seven days. These data indicate that infiltrating macrophages have some capacity to adopt a more microglia-like phenotype with decreased CCR2 and increased CX₃CR1 expression.

Several ECM remodeling genes also showed time-dependent expression patterns (Fig. 4). Macrophage expression of MMP2 and MMP3 was decreased compared with resident microglia at one day, and this decrease remained at seven days ($p<0.05$). MMP9 was highly elevated in macrophages at one day and remained elevated through seven days ($p<0.01$) while there was no increase in MMP9 expression in microglia at any of the time points. MMP14 expression was increased in both microglia and macrophages at one day, but only macrophages had increased expression at seven days ($p<0.01$).

Expression of the inhibitor TIMP1 was significantly elevated at one day in macrophages. By seven days, expression was decreased

and similar to that of microglia. Another inhibitor, TIMP2 was lowly expressed in macrophages at all time points ($p<0.01$). ADAM8 was increased acutely after injury in both microglia and macrophages but remained elevated in macrophages only at seven days ($p<0.01$). Unlike ADAM8, ADAM15 was decreased in macrophages at all time points ($p<0.01$). Overall, these findings show that macrophages have differential expression pattern of ECM remodeling genes compared with resident microglia and several of these differences were observed through the seven days.

Several genes associated with growth/repair were also differentially regulated and changed through time (Fig. 4). Many of the classical neuronal trophic and growth factors were decreased in macrophages through the seven days, including BDNF, GDNF, NGF, and NTF5 ($p<0.05$). Arg1 was increased significantly at one day in macrophages, but expression was attenuated at later time points. VEGFA was increased in macrophages, but not microglia, at one and seven days ($p<0.05$). Unlike Arg1 that decreased with time, the anti-inflammatory or damage associated gene YM-1 was increased in macrophages 1–7 days after injury ($p<0.01$). YM-1 was not changed in microglia at any time point.

Overall, these data show that macrophages have a distinct gene expression profile compared with resident microglia, with high inflammatory gene expression, high ECM remodeling capacity, and

Lumbar Microglia/Macrophage Gene Expression (mRNA counts)

FIG. 4. Macrophages have differential expression of remodeling and repair genes compared with microglia. Chimeric C57BL6 mice were naïve or underwent a thoracic spinal cord injury (SCI). The lumbar region was collected 24 h, three days, and seven days after the injury, and macrophages and microglia were FAC-sorted. RNA was isolated and analyzed by NanoString gene array. Bars represent the mRNA count mean + standard error of the mean. Means with (*) are significantly different than naïve microglia. Means with (+) have a tendency ($p=0.1$) to be different than naïve microglia. Macrophage means with (#) are significantly different than microglia at the corresponding time point. ($n=4$ per group). MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ADAM, a disintegrin and metalloproteinase domain; BDNF, brain-derived neurotrophic factor; GDNF, glial cell-derived neurotrophic factor; IGF, insulin-like growth factor; NGF, nerve growth factor; Arg, arginase; VEGFA, vascular endothelial growth factor A; NTF, neurotrophic factor.

low expression of growth factors. Through the one-week period, the CNS microenvironment altered some aspects of the macrophage profile. Macrophages started to adopt a more microglia-like profile with decreased inflammatory gene expression. These adaptations, however, were only partial because ECM remodeling capability was high and several growth factors remained lowly expressed in macrophages through the seven days.

Microglia and macrophages contribute to inflammation at the epicenter

Next, the gene profiles of microglia and macrophages at the site of the lesion (epicenter) were determined (Fig. 5). Similar to the lumbar cord, there were distinct clusters of genes that were downregulated (green) or upregulated (red) in infiltrating macrophages. More genes were altered in microglia after injury at the

epicenter than the lumbar cord where microglia were similar to naïve. In the epicenter after injury, microglia had decreased expression of anti-inflammatory genes $TGF\beta1$, $CSF1R$, and $CX3CR1$ ($p<0.02$). Expression of $IL-1\alpha$, $MMP2$, and $TIMP2$ was also decreased ($p<0.02$). More genes were upregulated in microglia at the epicenter compared with the lumbar cord. At the epicenter, microglia had increased expression of $CCL2$, $COX-2$, $VEGFA$, $Arg1$, $ADAM8$, $MMP9$ ($p<0.05$).

These data show that microglia respond more to the injury at the epicenter compared with the lumbar cord. Although microglia at the epicenter had altered gene expression after injury, infiltrating macrophage gene expression was also altered. Similar to the lumbar cord, the majority of inflammatory gene expression was driven by infiltrating macrophages. Compared with microglia, macrophages had elevated expression of inflammatory cytokines, chemokines, and mediators including $IL-1\beta$, $COX-2$, $CCL2$, $CCL5$,

1d SCI (Epicenter)

CXCL2, and IL-4R α ($p < 0.05$). Macrophages also had elevated expression of inflammatory receptors and trafficking related genes including CCR2, L-selectin, MHCII, and Ly6C ($p < 0.01$). Macrophages had exaggerated expression of TIMP1 and attenuated expression of TIMP2, MMP2, MMP3, ADAM15, and ADAM28 ($p < 0.01$). Similar to macrophages in the lumbar cord, epicenter macrophages had decreased expression of growth factors including NGF, GDNF, BDNF, NTF5, NTF3, CX₃CR1, and CSF1R ($p < 0.01$). Although expression of VEGFA and Arg1 was increased in microglia after injury, this expression was exaggerated in macrophages ($p < 0.05$).

Macrophages in the lumbar cord have a gene profile associated with active recruitment

Gene expression comparisons showed that macrophages have a higher inflammatory pattern compared with resident microglia after SCI. This result was observed in both the epicenter and lumbar region. These two regions, however, have a distinctly different microenvironment. While the epicenter region has a lesion and damaged vasculature, the lumbar cord remains relatively intact. Therefore, we hypothesized that there are differences in the population of macrophages recruited to the lumbar cord, compared with those that enter the lesion site.

Using the same NanoString gene array results mentioned previously, we compared the expression profiles of macrophages at the epicenter to those recruited to the lumbar cord (Fig. 6). Macrophages at the lumbar cord had decreased expression of inflammatory cytokines and chemokines, including IL-1 β , COX-2, CCL2, and CXCL2 ($p < 0.01$). IFN γ expression, however, was low in both groups but was increased in lumbar macrophages ($p < 0.01$). mRNA expression of inflammatory receptors and genes associated with active trafficking was increased in lumbar macrophages. These included MHC II, Ly6C ($p = 0.1$), CCR2, CX₃CR1, and L-selectin ($p < 0.03$). Increased expression of these genes suggests that macrophages in the lumbar cord rely on mechanisms of active trafficking more so when compared with those at the epicenter.

Expression of ECM remodeling genes followed a similar trend. Lumbar macrophages had decreased expression of ADAM8 and TIMP1 while expression of ADAM15, TIMP2, and MMP9 were elevated ($p < 0.04$). We have shown previously that MMP9 is required for macrophages to recruit into the lumbar cord.²⁰ Therefore, these data indicate that MMP9 is more important for macrophage recruitment into the lumbar region but not the epicenter region. There was also differential expression of growth and repair genes. Arg1 and VEGFA were decreased in lumbar macrophages, while YM1 was increased ($p < 0.02$). There was no difference in several of the classic neuron growth factors, including GDNF and NTF5. These differences in expression of inflammatory and growth promoting genes such as Arg1 and VEGFA are likely because of the macrophages at the epicenter becoming highly activated in response to the lesion.

FIG. 5. Microglia and macrophages contribute to inflammation at the epicenter. Chimeric C57BL6 mice were naïve or underwent a thoracic spinal cord injury (SCI), and after 24 h FAC- sorted macrophages and microglia were collected from the lesion. The RNA was isolated and analyzed by NanoString gene array. Heat map shows genes upregulated (red) or downregulated (green) in microglia from naïve mice (Naïve), microglia from injured mice (MGL), and macrophages from injured mice (MAC). ($n = 3$ per group)

1d SCI Macrophage Gene Expression (mRNA counts)

FIG. 6. Macrophages recruited to the lumbar cord have increased expression of trafficking related genes compared with macrophages at the epicenter. Chimeric C57BL6 mice were naïve or underwent a thoracic SCI. At 24 h after the injury, macrophages (MAC) were collected from the epicenter and lumbar regions. RNA was isolated and analyzed by NanoString gene array. Bars represent the mRNA count mean + standard error of the mean. Means with (*) are significantly different from epicenter macrophages. ($n=4$ per group). IL, interleukin; COX, cyclooxygenase; IFN, interferon; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; MHC, major histocompatibility complex; CCR, C-C motif chemokine receptor; CX₃CR, C-X₃-C motif chemokine receptor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ADAM, a disintegrin and metalloproteinase domain; GDNF, glial cell-derived neurotrophic factor; Arg, arginase; VEGF, vascular endothelial growth factor; NTF, neurotrophic factor.

Acute rehabilitation exaggerates the inflammatory profile of infiltrating macrophages

We showed previously that early treadmill training led to significant locomotor improvements in MMP9 null mutant mice, while poor responses occurred in WT mice.⁷ In addition, we showed decreased macrophage recruitment in MMP9 knockout mice.²⁰ Thus, we hypothesized that an interaction exists between macrophage infiltration and behavioral deficits after acute rehabilitation. We determined the effects of early treadmill training on macrophage recruitment into the lumbar cord and on macrophage gene expression. Mice underwent treadmill-based rehabilitation 2–7 days after SCI, and macrophages were collected at seven days post-injury (Fig. 7A).

Consistent with our previous work,⁷ acute rehabilitation had no immediate effect on open field locomotor performance (Fig. 7B). In addition, there was no effect of rehabilitation on the number of cir-

culating monocytes (Fig. 7C) nor infiltrating macrophages in the epicenter or lumbar cord (Fig. 7D). For gene expression, however, several changes occurred (Fig. 8). Macrophages from treadmill trained mice had increased expression of several trafficking and inflammatory related genes including CCL5 ($p=0.1$), Ly6C ($p<0.05$), and CCR2 ($p=0.1$). Expression of ECM related genes ADAM8 ($p<0.05$) and MMP9 ($p<0.03$) increased while MMP2 decreased ($p=0.06$). The only growth or repair related gene that decreased after rehabilitation was Arg1 ($p=0.1$). These data indicate that acute rehabilitation alters the gene profile of infiltrating macrophages to a higher level trafficking and inflammatory gene expression.

Discussion

Infiltration of inflammatory myeloid cells into the injured spinal cord may contribute to high levels of inflammation that ultimately

FIG. 7. Early treadmill training does not affect the number of macrophages trafficking into the injured spinal cord. Chimeric C57BL/6 mice were naïve or underwent a thoracic spinal cord injury (SCI). (A) Starting at two days after the injury, a subset of mice received assisted treadmill training daily. (B) At seven days after the injury, circulating myeloid cells were determined. (C) Infiltration of macrophages in the epicenter and lumbar regions are shown. Bars represent the mean + standard error of the mean. Means with (*) are significantly different from naïve. ($n = 6$ per group). BM, bone marrow; GFP, green fluorescent protein; MAC, macrophage; MGL, microglia.

impede functional locomotor recovery. This is especially important in the lumbar cord where recruitment of inflammatory myeloid cells can induce damage to otherwise intact tissue distant from the lesion. Here, we characterized myeloid cell trafficking by determining the specific cell types that infiltrate the spinal cord after injury. We found that macrophages were recruited to the lumbar cord acutely after thoracic SCI and remained in the cord at least seven days. These macrophages had a gene expression profile associated with elevated inflammation, while expression of growth/repair genes was attenuated compared with resident microglia. Such a high inflammatory microenvironment within the central pattern generators for locomotion in the lumbar cord is likely to impede activity-dependent plasticity and recovery. We found that treadmill training delivered acutely after SCI further magnified the inflammatory profile of infiltrating macrophages. These data show the necessity of inhibiting macrophage recruitment to the lumbar cord to maximize the beneficial effects of rehabilitation.

One important finding of this study was that both macrophages and neutrophils were recruited to the epicenter and lumbar cord. The infiltration of neutrophils to the epicenter has been shown previously and is not surprising because this is an active lesion.²³ We did not expect, however, that neutrophils would also be recruited into the lumbar cord after T9 SCI. Neutrophils can exert toxic effects on neural tissue through reactive oxygen species formation.³¹ Therefore, their presence in the lumbar cord may cause direct damage and alterations to the microenvironment that are not favorable for recovery.

Similar to neutrophils, macrophages were recruited to both the epicenter and lumbar regions acutely after SCI. In the lumbar cord, where there is no vascular leakage,²⁰ the initial recruiter of these peripheral cells may be resident glia (microglia or astrocytes) because these cells have been shown to increase CCL2 after SCI.³² Here we show that microglia in the lumbar cord have increased CCL2 acutely after injury. Once macrophages are recruited initially, they may further amplify inflammation and recruitment of myeloid cells. Indeed, macrophages in the lumbar cord had elevated expression of both CCL2 to further recruit macrophages and elevated expression of CXCL2, which is a chemotactic for neutrophils. Therefore, it is possible that macrophages increase both macrophage and neutrophil recruitment.

Consistent with previous studies,³³ however, neutrophils were not present in the lumbar spinal cord beyond one day post-injury. Moreover, in our previous study, we confirmed that there is no infiltration of peripheral immune cells into rostral regions acutely after T9 SCI.²⁰ Future studies will investigate differential infiltration patterns in these remote cervical and lumbar regions over time to better understand recruitment mechanisms.

There is significant interest in understanding macrophage heterogeneity after SCI. Macrophages play diverse roles in CNS trauma and elicit mechanisms associated with both damage and repair.^{1,2,34,35} Here, we used GFP chimeras to unequivocally separate resident versus BM-derived macrophages within the cord. Given the high GFP rate in monocytes, we were able to compare the gene profile of infiltrating macrophages with that of resident microglia after SCI with high confidence.

Effects of Exercise on Lumbar Macrophage Gene Expression (mRNA counts)

FIG. 8. Early treadmill training increases the inflammatory gene profile of infiltrating macrophages in the lumbar cord. Chimeric C57BL6 mice were naïve or underwent a thoracic spinal cord injury (SCI). Starting at two days after the injury, a subset of mice received assisted treadmill training daily. At seven days after the injury, infiltrating macrophages (MAC) were collected and RNA was analyzed by NanoString gene array. Bars represent the mean + standard error of the mean. Means with (*) are significantly different from MAC (no exercise). Means with (+) tend to be different ($p=0.1$) from MAC (no exercise). ($n=4$ per group). MGL, microglia; IL, interleukin; COX, cyclooxygenase; IFN, interferon; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; MHC, major histocompatibility complex; CCR, C-C motif chemokine receptor; CX₃CR, C-X₃-C motif chemokine receptor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ADAM, a disintegrin and metalloproteinase domain; GDNF, glial cell-derived neurotrophic factor; Arg, arginase; VEGFA, vascular endothelial growth factor A; NTF, neurotrophic factor.

Macrophages had high expression of inflammatory genes and lower expression of several growth/repair genes. Of the M2 markers, YM1, Arg1, and VEGFA were elevated in macrophages. Although these mediators are classified as “M2” markers, their upregulation may not be beneficial fully. YM1 can promote neutrophil recruitment and tissue damage in lungs,³⁶ and its function in CNS tissue has yet to be determined fully. VEGFA has been shown to increase vascular permeability in a mouse model of multiple sclerosis,³⁷ and thus increased VEGFA expression by macrophages may contribute to delayed vascular permeability in the lumbar cord.²⁰

This study aimed to understand the role of macrophage infiltration into the spinal cord after injury. Here, we describe their gene expression profile as proinflammatory with high expression of several inflammatory cytokines, chemokines, and mediators. The functional consequence of macrophages recruited to the injured spinal cord remains controversial, however. Previous studies have shown improved functional recovery, decreased lesion size, and increased axonal growth when macrophages were depleted or recruitment was inhibited.^{18,21,322,38} Alternatively, studies have shown a beneficial

role of infiltrating macrophages in recovery after SCI.^{39,40} Moreover, these studies focused on macrophage recruitment to the epicenter, where the macrophage profile is known to be important.

Inflammatory, or M1, macrophages can promote axonal damage, whereas anti-inflammatory, or M2, macrophages can promote axonal growth.^{34,39} Here, we focused on macrophages recruited to the lumbar cord. This region is particularly interesting because there is no direct lesion in the lumbar cord after T9 contusion; thereby avoiding blood vessel shearing, neuronal necrosis, and apoptosis in lumbar gray matter. Lesion affects are transferred caudally via severed axons of descending pathways that enter into Wallerian degeneration, thereby establishing a different type of lesion remotely. The caudal projections of thoracic-injured axons likely contribute to some of the lumbar immune activation we report. Wallerian degeneration, however, likely develops too slowly in the lumbar cord to explain the rapid macrophage infiltration (24 h) we report.^{41,42} Therefore, we propose that macrophages do not infiltrate the lumbar cord in response to degenerating axons, but are actively recruited to the lumbar cord well ahead of degenerative processes.

Given this timing discrepancy between macrophage infiltration and Wallerian degeneration in the lumbar cord, the presence of highly inflammatory macrophages in the absence of overt damage likely contributes to the detrimental effects described previously⁷ and the benefits conferred by depletion.^{18,21,22,38} Further studies will be required to delineate the role of recruited lumbar macrophages in Wallerian degeneration at later time points.

In addition, after injury, resident microglia in the lumbar cord had minimal changes in gene expression. This was an unexpected finding because previously we found significant morphological activation in microglia after SCI.⁷ It appears that morphological assessment alone is insufficient to characterize immune activation after SCI, especially in remote regions. Therefore, these novel findings indicate the inflammatory environment of the lumbar cord is significantly influenced by the infiltrating macrophages. The impact of these cells in altering the lumbar microenvironment may have large implications for below-level deficits.

Our NanoString data support the hypothesis that the populations of macrophages present at the lesion versus the lumbar cord have distinct profiles and possible functions. Macrophages in the lumbar cord had elevated expression of trafficking mediators (CCR2, L-selectin, MMP9), suggesting that the mechanisms of macrophage recruitment to the lumbar cord may be substantially different than the passive infiltration at the lesion epicenter.^{34,35,43} Although this is an interesting finding, it is not too surprising. The epicenter has damaged blood vessels, which facilitates neutrophils and macrophages entry to the cord through passive infiltration. In the lumbar cord, however, the vasculature is intact,²⁰ and cells likely rely on active trafficking to enter the cord. Therefore, these findings identify a novel target for SCI therapies to specifically inhibit macrophage infiltration to the lumbar cord, which may improve locomotion and other below-level functional deficits.

Another important finding in this study is that we identified gene expression patterns of infiltrating macrophages over time. Previously, we showed that infiltrating macrophages increase Iba1 expression and adopt a more ramified morphology after entrance into the CNS.²⁰ Here, we show that infiltrating macrophages also change their gene expression profile to more closely resemble resident microglia. Through seven days, macrophages decreased expression of inflammatory genes (IL-1 β , COX-2, CCR2) while increasing CX₃CR1 expression. This is an important finding, because it showcases how difficult it can be to distinguish resident microglia to infiltrating BM derived macrophages.

Overall, these data, in addition to previous studies, suggest that macrophages respond to the CNS environment and alter their gene expression profile accordingly.³⁴ Although the initial recruitment of macrophages at one day after injury increases the inflammatory environment of the lumbar cord, the altered profile of macrophage through time may not influence the environment as substantially. The long-term consequence of peripherally derived macrophages in the lumbar cord still needs to be explored.

Implications for neurerehabilitation focus on rehabilitation-induced changes in inflammatory gene-expression of infiltrating macrophages within lumbar central pattern generators. Acute treadmill training produced functional declines early after SCI.⁷ When macrophage recruitment and inflammation were decreased through MMP9 deletion, however, treadmill training markedly improved locomotor recovery.^{7,20} Here, we show in wild-type mice that early treadmill training increased MMP9 expression in lumbar macrophages, suggesting behavioral worsening may be because of acute rehabilitation-induced gene profile changes.

A long-term evaluation of macrophage phenotype within lumbar gray matter in response to activity-based training seems warranted. In this study, we only determined macrophage profiles at seven days after SCI and acutely after intervention. Because macrophages adopt a more microglia-like profile after seven days, it is possible that they would also respond differently to later treadmill rehabilitation. In support of this hypothesis, previous studies showed improved locomotor function in injured rats after delayed treadmill training.^{44,45} Delayed treadmill training also increased gene expression for neuroplasticity in the lumbar cord,⁴⁴ greater IGF1 in the cerebrospinal fluid, and decreased reactive oxygen species formation at the lesion in rats.⁴⁵ Together with our work, these studies identify the great potential of treadmill training and highlight the importance of the timing of the delivery of rehabilitation. To be most effective, rehabilitation should begin once the acute inflammatory response has subsided and macrophages have decreased their inflammatory profile.

Conclusion

The cellular and molecular changes caused within the first week of SCI extend well caudal into the lumbar cord. BM-derived macrophages infiltrate into the lumbar cord and have a greater inflammatory genetic profile than resident microglia. A potential barrier to improving recovery is that acute treadmill training heightens inflammatory profiles and recruitment of macrophages. Therefore, limiting active trafficking of macrophages into the lumbar cord identifies a novel target for SCI therapies to improve locomotion.

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