Predictability of Lumbar Spinal Cord Microenvironment after Thoracic Injury based on the Gene Expression Profile of Blood Neutrophils and Monocytes

Undergraduate Research Thesis

Shijia Li 1,4

Diana Norden1,2, Timothy D. Faw1,2,3, Rochelle J Deibert1,2 Kristina Witcher 1,2 Lesley C. Fisher1,2, Jonathan P. Godbout1,2 and D. Michele Basso1,2

1School of Health and Rehabilitation Sciences, 2Center for Brain and Spinal Cord Repair, 3Neuroscience Graduate Program, 4Department of Neuroscience

Abstract:

Profound locomotor impairments exist with human spinal cord injury (SCI) even after intensive neuro-rehabilitation. Animal studies show that active recruitment of myeloid cells from the bloodstream into the spinal cord can induce a toxic microenvironment which impedes neuroplasticity and functional recovery. Evidence also shows elevated pro-inflammatory gene expression within infiltrating monocytes in the lumbar cord likely drives inflammation in locomotor networks and impairs recovery. Clinical effective training can only be attained early after SCI and in combination with reduced inflammation. Unfortunately, knowing the real-time microenvironment of the human spinal cord and when it is best to deliver rehabilitation is currently impossible. Therefore, this study aims to predict the lumbar cord microenvironment from gene expression profiles of myeloid cells in blood samples after murine SCI. I hypothesize that changes in gene expression in blood neutrophils and monocytes among four categories–inflammatory, trafficking, extracellular matrix (ECM) remodeling, and growth and repair–will correlate with and predict the lumbar microenvironment. NanoString nCounter technology was used to analyze 60 genes in samples from mice 24 hours after SCI. The expression level of lymphocyte antigen 6 complex (Ly6C) by neutrophils, protein tyrosine phosphatase receptor type C (ptprc/CD45) by monocytes were correlated with expression levels within lumbar cord. Therefore, by measuring their concentration of in blood, we may be closer to being able to predict and assess the microenvironment of lumbar cord in human subjects in a clinically feasible way and to improve the outcome of rehabilitation.

Key Words: Spinal Cord Injury, NanoString, Biomarkers, Neutrophils, Lumbar spinal cord

Introduction

Approximately 282,000 people are living with spinal cord injury (SCI) in the United States (National Spinal Cord Injury Statistical Center 2016). The substantial loss of locomotor function persists even after years of intensive neuro-rehabilitation. To date, no treatment has been identified to reverse the physical deficits fully, and the mechanism underlying the limited improvements remain unclear.

Motor neuron damage and blood-spinal cord barrier (BSCB) disruption caused by the mechanical trauma usually initiates a complex inflammation response following SCI. Ruptured blood vessels and the upregulation of proinflammatory cytokines Interleukin-1 beta (IL-1b) and tumor necrosis factor (TNF) increase the permeability and infiltration of bone marrow-derived immune cells (myeloid cells) to the lesion epicenter (Schnell et al 1999). Together with resident inflammatory microglia, the recruited myeloid cells contribute to the production of proinflammatory cytokines, reactive oxygen species, nitric oxide, and other vasoactive substances which increase the neurotoxicity at the injury site (Donnelly & Popovich 2008).

Bone marrow derived neutrophils and monocytes are both involved in active parenchymal infiltration. Neutrophils are the first inflammatory cells to arrive at the site of injury, reaching peak numbers at 24 hours, and are associated with the development of neuropathic pain (Zhang et al 2013). Bone marrow derived monocytes infiltrate the spinal cord starting at 24 hours after injury (Letellier et al 2010) and dominate the injury site at 7 days (Beck et al 2010). Additionally, most depletion studies reported increased locomotor recovery when trafficking of myeloid cells is regulated (Lee et al 2011, Wohleb et al 2014). Together, these studies support the pathological alterations after SCI and the detrimental effect of neuroinflammation induced by infiltrating myeloid cells on the functional recovery of injured animals.

Although the inflammation response at the lesion center after SCI has been well studied (Greenhalgh & David 2014, Popovich et al 1996, Whetstone et al 2003), few studies have been done to characterize the microenvironment distant from injury site and its potential influence on recovery. Literature is limited on chemokine production in remote regions and its contribution to mechanical hypersensitivity and impaired locomotion recovery. However, since the lumbar cord houses the central pattern generator which regulates rhythmic patterned motor outputs and sensory function (Detloff et al 2008, Hansen et al 2013), this area is of high importance to protect from inflammation in order to optimize rehabilitation results.

Furthermore, the outcome of behavioral interventions depends on an interaction between the timing of rehabilitation training and the local microenvironment at the lumbar segment of the spinal cord. Recent studies have suggested the level of inflammatory cytokines in the lumbar cord was attenuated after SCI in mice models with depleted matrix metalloproteinase 9 (MMP9) gene. The delivery of acute treadmill training in the absence of this potent inflammatory regulator was proven to facilitate neuroplasticity and robust locomotor recovery (Hansen et al 2013). However, neither training nor MMP9 depletion alone promoted recovery. The late delivery of the same training was also ineffective to induce robust recovery. Therefore, it is important to assess the expression profile of myeloid cells within the spinal cord after injury at different time points to determine the ideal onset time of behavioral therapies accordingly. Unfortunately, temporal assessment of the spinal cord microenvironment after human SCI is clinically infeasible.

The term “biomarker” refers to the objective indications of a medical state observed from outside the patient which can be measured accurately and reproducibly (Strimbu & Tavel 2010). In the past decades, biomarkers have been identified and applied widely in cancer research. Studies identify germline mutations, such as BRCA1, as increasing the risk of developing breast or ovarian cancer and then specifically design treatment with amplified efficacy (Ford et al 1995). Biomarkers have also been used in the field of neuroscience to assist in the prognosis of neurological disease or injuries and in predictions of outcome from rehabilitation interventions. Studies found levels of inflammatory markers measured from the cerebral spinal fluid of acute SCI patients were significantly different between individuals with improved American Spinal Injury Association Impairment Scale (AIS) grade and those who did not improve (Kwon et al 2017). Proinflammatory cytokines from the blood have also been used as biomarkers to predict the neurological improvement in patients with SCI after 12 weeks. The study successfully correlates higher cytokine expression levels with patients without neurological improvements (Ferbert et al 2017).

Previous studies rarely adopted mRNA as a measure of neuroinflammatory biomarkers due to its natural tendency of rapid degradation despite the fact that it is highly sensitive and specific (Karimi et al 2015). However, the development of NanoString techniques can enable the accurate detection of mRNA debris. NanoString has high sensitivity, reproducibility, and efficiency in measuring genetic content within samples (Kulkarni 2011). It used “target-specific, color-coded probe pairs” to search for desired mRNA molecules and process up to 800 genes in a single run. Studies have shown the feasibility in using NanoString to detect the changes of microglia transcription profile in response to myelin debris by measuring the expression level of 24 genes (Siddiqui et al 2014). Therefore, we believe the use of NanoString techniques can leverage the detection and prediction of the lumbar cord microenvironment after SCI.

In this study, we hypothesize that gene expression levels of myeloid cells in blood will correlate with and predict the lumbar microenvironment after SCI. We analyzed the mRNA expression profiles of both bone marrow derived neutrophils and monocytes in blood to identify potential genetic biomarkers to predict the microenvironment of spinal cord at 24 hours after injury, evaluating the changes of individual genes in reaction to the injury and the similarity among activation patterns of genes in different families following SCI.

Methods

Mice

Experiments were conducted in accordance with The Ohio State University Institutional Laboratory Animal Care and Use Committee. Thirty-six mice (2-3 months of age) female C57BL/6J wild-type mice were obtained from Jackson Laboratories. Mice were housed up to 5 per cage and provided food and water ad libitum.

Thirteen mice were put in the naïve group and they would not receive any surgical procedure. Twenty three mice underwent SCI and were randomly assigned into three groups based on time of tissue collection after injury – 24 hours (n=11), 3 days (n=7) and 7days (n=5).

Spinal Cord Injury

Contusion of the spinal cord was performed as previously described (Hansen et al., 2013). In short, mice were anesthetized with a ketamine (138 mg/kg)-xylazine (20 mg/kg) cocktail and given prophylactic antibiotics (gentocin, 1mg/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 kilodyne of force to induce a severe contusion injury. Mean force (76.71±0.11 kilodyne) and displacement (615.24± 5.18 microns). The incision was closed in layers and 2cc of sterile saline was given subcutaneously to prevent dehydration. During recovery, mice received antibiotics and saline for five days, and bladders were manually expressed twice per day (Hoschouer et al 2010).

The severity of locomotor deficits was screened and recorded on day 1, day 3, and day 7 post injury (dpi) using the Basso Mouse Scale for Locomotion (BMS). During testing, mice were placed individually in a plastic-bottomed open field and rated by two observers. The scale ranged from 0–9 based on the behavioral patterns of ankle movement, plantar placing, stepping, coordination, paw position, trunk instability, and tail.

Tissue Collection

At previously determined endpoints, each animal was anesthetized with a terminal dose of ketamine (207 mg/kg)-xylazine (30 mg/kg) cocktail. The hub space of 1mL syringes were filled with 0.5M ethylenediaminetetraacetic acid (EDTA, pH 8.0) to prevent coagulation. These syringes were then used to collect at least 100 µl of blood, from the left ventricle.

Phosphate buffered saline (PBS) perfusion was administrated to exsanguinate blood from the animal system. The T12-L2 laminas were removed and fresh lumbar tissue (L1-L6) was collected and placed in 1mL of PBS in a centrifuge tube. Both blood and spinal cord tissue were stored on ice for future processing.

Sorting of Monocytes and Neutrophils from Spinal Cord and Blood Samples

The lumbar cord was then homogenized and centrifuged to collect a cell pellet. Lumbar cord cell pellets were then suspended in a Percoll gradient, which is a low viscosity media used to separate cells based on their density. A discontinuous Percoll density gradient was layered as 70%, 35%, and 0% isotonic Percoll. The gradient was centrifuged and cells expressed Integrin alpha M (Itgam/CD11b), which is a marker for bone marrow derived leukocytes, were collected from the interface between the 70%/35% Percoll gradient as previous described (Fenn et al 2014)

Flow cytometry of myeloid cells surface markers were performed as described previously, but with a few modifications (Donnelly et al 2011, Henry et al 2008). Both blood and spinal cord samples were stained with rat anti-mouse Ly6C-PE, Ly6G-PE, CD45-PerCP-Cy5.5 and CD11b-APC antibodies (eBioscience, CA) and cells were sorted using a Becton-Dickinson FACSAria III cell sorter at the OSU Comprehensive Cancer core facility. Spinal cord neutrophils were identified by CD11b+/ CD 45 +./Ly6G+ expression and bone marrow derived monocytes were identified by CD11b+/ CD 45 +/Ly6G-/ $Ly6C^{hi}$ expression. Blood neutrophils expressed CD11b+/Ly6G+ markers and blood monocytes showed CD11b+/Ly6G-/$Ly6C^{hi}$ markers.

RNA Extraction from Spinal Cord and Blood Samples

Cells were then lysed immediately following the sorting procedures using lysis buffer provided in Arcturus Pico Pure RNA isolation kit and frozen until extraction. During extraction, the Arcturus PicoPure RNA isolation kit was used to isolate and recover mRNA from samples. Then, RNA quality and integrity was determined using Bioanalyzer 2100 (Agilent Technologies).

NanoString and nSolver analysis of mRNA copy Number

Qualified RNA samples were then analyzed by NanoString nSolver technology (www.nanostring.com) which enabled sensitive expression analysis (<1 copy per cell) of a large amount of genes from a single sample. It used molecular "barcodes" and single molecule imaging to detect and count hundreds of unique transcripts. NanoString analysis was performed by the Nucleic Acid Core Facility at OSU.

In this experiment, nCounter profiled the expression levels of 61 custom genes. Customized plates were designed with selected neutrophil and monocyte related genes that are relevant to inflammation, growth/repair, trafficking, and extracellular matrix remodeling.

Monocyte and neutrophil RNA samples were run on separate cassettes and, therefore, cannot be directly compared. Normalization was based on positive controls, which are the expression of housekeeping genes of GAPDH, ACTB and OAZ. Data are expressed as mRNA copy numbers. Heat maps were generated by unsupervised clustering analyses using Pearson correlation in the NanoString nSolver software program.

Group Average and Paired Sample Analysis

Based on the results produced by Bioanalyzer and NanoString nSolver, data collected from 6 animals in the 24 hours group were analyzed using excel spreadsheet. Four sets of data were produced for spinal cord neutrophils, blood neutrophils, spinal cord monocytes, and spinal cord neutrophils separately. In the naïve group, four animals produced data indicating mRNA expression levels in blood neutrophil and monocytes. (Detailed sample information can be obtained from Appendix 1)

Group The group average was calculated for a particular gene in each tissue type. among the same tissue across the 4 data sets and the result indicated the typical mRNA expression level for the gene. This value was used to compare with the expression levels of other genes, the same gene in another cell type, and the changes across conditions.

Paired sample analysis was used when the blood and spinal cord samples were acquired from the same animal after injury. Three sets of data were acquired for neutrophils and monocytes separately. These analyses examined the relationship between blood and spinal cord gene expression levels which enables the prediction of spinal cord microenvironment.

Gene Analysis

Fifty-eight genes were selected based on their functions in maintaining typical cellular homeostasis. Under both injured and naïve conditions, if the mRNA copy number of a particular gene was lower than 35, it was excluded from the study (Figure 1). The cut-off value 35 was determined by discovering the natural break in histograms of the copy numbers of neutrophil or

monocytes across conditions. Low expression levels below 35 can demonstrate that these genes are less likely to impact the environment.

Coefficient of variation (COV) was used to look for extreme variations between the values for each gene. By mapping the values of COV across all genes using a histogram to identify the natural gap in the range of COV, most COV levels fell under 0.72 and it was determined as the threshold number to classify whether a set of data was too variable or not. The copy number that was farthest away from the mean of a variable gene was excluded and a new COV was then calculated. If the new COV is lower than 0.72, this gene would remain in the data set for future analysis after excluding the single outlier. If the new value is still greater or equals to 0.72, the gene would be excluded from further calculation due to low reliability (Figure 1). Finally, the qualified genes were divided into four different groups- trafficking, inflammation, growth and repair, and extracellular matrix- based on their primary known activity. (Detailed gene information can be obtained from Appendix 2)

Statistics

All data was analyzed using Pearson Product Moment Correlation Test or a t-test ($p<0.05$) when appropriate. The correlation tests for paired animal samples were unable to be performed in the paired sample analysis due to the small paired animal sample size (n=3) at 24 hour-time point. The coefficient of determination, R-square, were calculated to measure how well the regression line can be used to represent the data in such linear correlations.

Sample Exclusion

Due to a known error of the genome sequence on the NanoString customized gene expression panel, TNFa was excluded from analysis.

Based on the data yielded by RNA bioanalyzer, five mice from the 24 hours group and three mice from the naïve group were excluded based on their poor RNA quality and integrity. One animal from the 3 days group died during surgeries and another animal died following anesthesia reaction. During tissue collection, the injury levels was assessed for every animal to be T9 levels. One animal was excluded from 3 day group due to an injury level at T11.

Samples acquired from the 3 and 7 days groups had low cell counts identified by FACSAria III cell sorter and were not able to be processed by

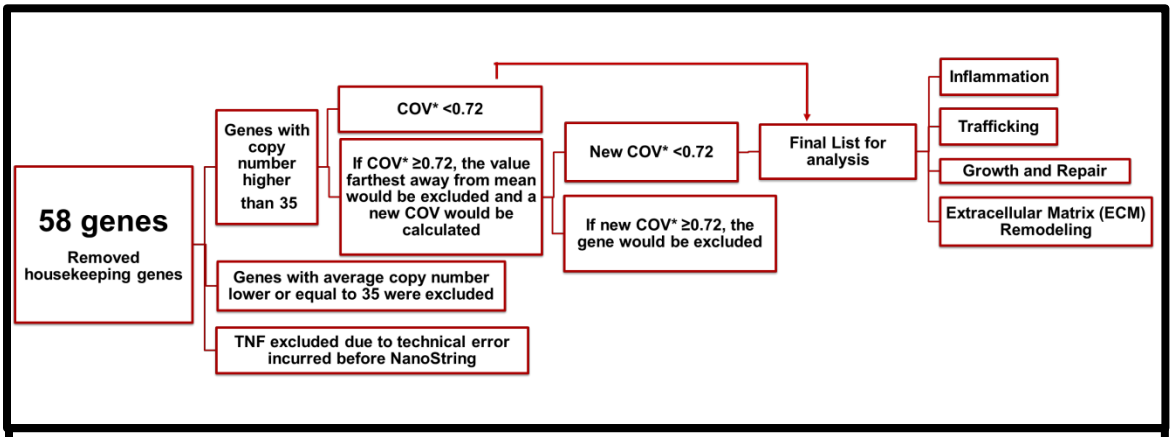

Figure 1: Exclusion Process Flow Chart; The housekeeping genes are GAPDH, ACTB and OAZ1. (*) COV stands for Coefficient of Variation

the NanoString technology.

Results

Lower than Expected Cell Counts Found in 3d and 7d Tissue

Due to the lower than expected cell counts in 3d and 7d tissues, qPCR measuring GAPDH, 18S or beta actin need to be done for all samples to better understand the RNA content before going through the NanoString process. Therefore, data from these timepoints are not included for this thesis defense. The results included in the following section will rely exclusively on the 24 hour time point.

Downregulation Prominent in Gene Expression in Blood after SCI

Comparing the changes in copy numbers in blood before and after SCI, 72.3% of neutrophil genes and 64.3% of monocytes genes had decreased copy numbers. The rest of the genes were upregulated after injury and the increase was most prominent in ECM remodeling genes with 45% of neutrophil and 80% of monocyte genes showing increased copy numbers in blood.

To confirm our findings about the prevailing downregulation across genes, we produced heat maps using nSolver software to examine the expression patterns after SCI in blood. Again, they verified the decrease in expression levels was more pervasive among genes in neutrophils compared to monocytes (Figure2). Heat maps were also used to compare the expression profile of monocytes and neutrophils under naïve condition. Even before any injuries, we can identify blood neutrophils have more mRNA copies compared to monocytes in the levels of growth and repair genes but not in trafficking and inflammatory genes (Figure 2). This phenomenon might help to explain the predisposed role of the two different cell types.

Genes Changed significantly were Identified after SCI in blood

Comparing the naïve and blood samples after SCI, specific genes changed significantly after SCI at 24 hour time point and were identified by t-test ($p<0.05$) (Figure 3). In neutrophils, receptors CX3C chemokine receptor 1(Cx3cr1), transforming growth factor beta receptor I (TGFbR1), and C-C chemokine receptor type 2 (CCR2) related to cell adhesion and migration were all downregulated significantly after the injury in the blood. Inflammation response associated gene protein tyrosine phosphatase receptor type C (CD45/ptprc), Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) and Interleukin 1 beta (IL-1B) were downregulated significantly. Meanwhile, proinflammatory gene Chemokine ligand 2 (Cxcl2) and lymphocyte antigen 6 complex, locus C1 (Ly6C) are elevated significantly in blood. Only the expression of chitinase-like protein 3 (Chi31a/YM1), an anti-inflammatory cytokine marker, was increased significantly.

For monocytes, Cx3cr1 and Syntaxin 6 (Stx6), which relate to proliferation, decreased significantly. Colony stimulating factor 1 receptor (CSF1R/CD115), CD45/ptprc, and a gene belonging to major histocompatibility complex, histocompatibility 2, class II antigen E beta (H2-EB1), which transduce inflammatory signals and initiate immune response all decreased significantly in blood. Genes related to proliferation and differentiation, Transforming growth factor beta 1 (Tgfb1) and Mannose Receptor C-Type 1 (Mrc-1) both decreased after injury while

proinflammatory inhibitor interleukin-1 receptor antagonist (IL-Ra) increased significantly. It is interesting to notice that A Disintegrin and metalloproteinase domain-containing protein 8 (ADAM8) expression was increased in both cell types. Since ADAMs are characterized by the

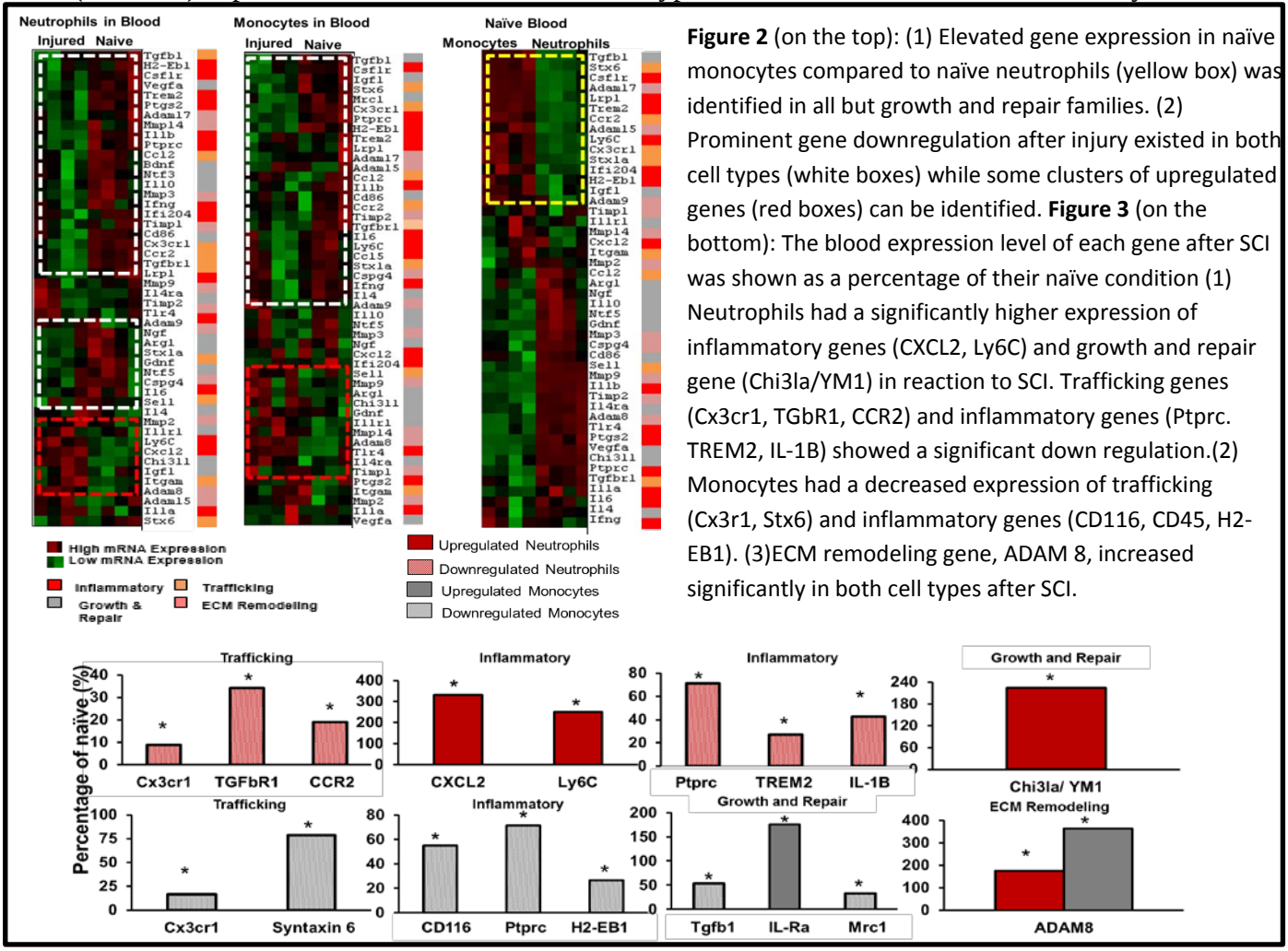

Figure 2 (on the top): (1) Elevated gene expression in naïve monocytes compared to naïve neutrophils (yellow box) was identified in all but growth and repair families. (2) Prominent gene downregulation after injury existed in both cell types (white boxes) while some clusters of upregulated genes (red boxes) can be identified. **Figure 3** (on the bottom): The blood expression level of each gene after SCI was shown as a percentage of their naïve condition (1) Neutrophils had a significantly higher expression of inflammatory genes (CXCL2, Ly6C) and growth and repair gene (Chi3la/YM1) in reaction to SCI. Trafficking genes (Cx3cr1, TGbR1, CCR2) and inflammatory genes (Ptprc. TREM2, IL-1B) showed a significant down regulation.(2) Monocytes had a decreased expression of trafficking (Cx3r1, Stx6) and inflammatory genes (CD116, CD45, H2-EB1). (3)ECM remodeling gene, ADAM 8, increased significantly in both cell types after SCI.

presence of metalloprotease and integrin receptor, they are actively involved in cell adhesion during neurodegeneration or inflammation. Thus, the increased levels suggests similarity in the underlying cell properties in the blood stream after injury.

Injury Caused Significant Changes between Blood and Spinal Cord Gene Expression

Comparing the average mRNA copy numbers across animal samples, 12 genes in neutrophils and 9 genes in monocytes were identified to have high copy numbers in the naïve condition (over 8000). It is possible that genes with consistent high expression level across conditions might play important roles in the maintenance of normal cellular environment as well as shape microenvironment after injury significantly.

Indicated by Figure 4A, even the high expression level of these genes was maintained in blood and lumbar cord after SCI, their expression levels altered dramatically compared to naïve condition. Inflammatory genes IL-1B, CD45/Ptprc, CSF1R, and IFI204 all decreased in blood expression level after injury. Most of their spinal cord expression level was lower than naïve

condition, while CSF1R has increased spinal cord expression level as well as different significantly from its blood expression level after injury (p=0.039). It is noticeable that

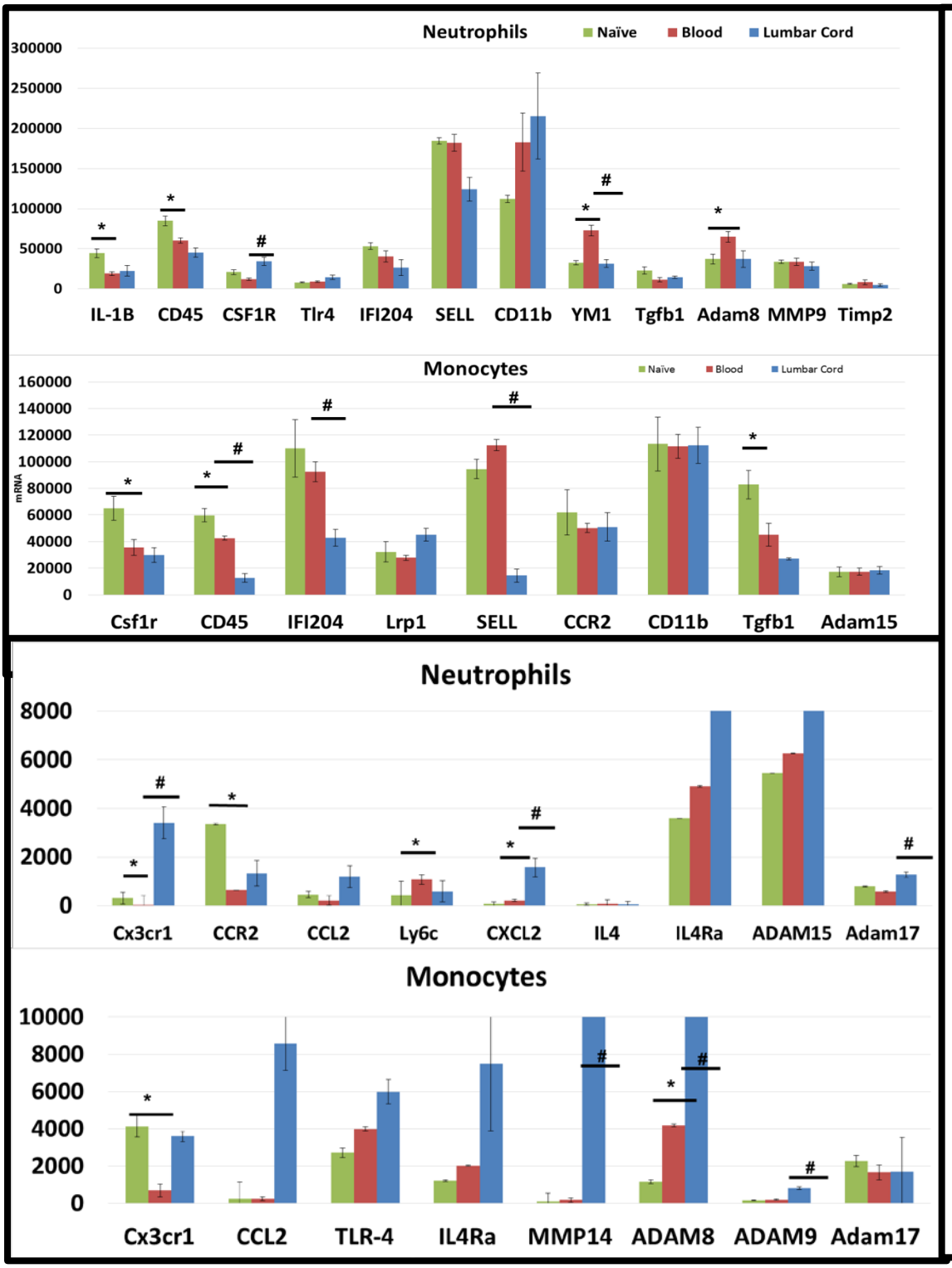

Figure 4A: (on the top) Gene expression levels (mRNA) of high copy number genes (over 8000 mRNA) under naïve condition, in blood samples, and in injured spinal cord samples after injury**. Figure 4B**: (On the bottom) gene with less than 8000 mRNA copies in at least two different conditions. They might exemplify significant difference between conditions or trend to show significant difference. (*) indicates significant changes in blood after injury. (#) indicates significant different between blood and spinal cord

trafficking genes, Itgam/CD11b and L-selectin (SELL) expressed a very high level in both cell types. (Figure 4A) However, SELL maintained similar levels in blood before and after injury, however it expressed at a lower level in spinal cord in both cell types. In addition, monocytes

expressed it at a much lower level within the lumbar cord compared to neutrophils. For CD11b, it was expressed by neutrophils in blood and lumbar cord at a much higher level than its naïve condition. However, in monocytes, the expression of CD11b maintained similar in naïve blood samples, blood, and lumbar cord samples after injury. Its expression level in spinal cord was only half of the amount expressed by neutrophils. Growth and repair gene had various expression pattern in the two cell types. Neutrophil YM1/Chi31a showed a significant increase in blood stream and differed from its spinal cord content significantly; monocyte Tgfb1 gene had a significant decrease after injury in the blood. Most high expression ECM remodeling genes remained similar levels across samples (MMP9, Timp2 in neutrophils, and ADAM15 in monocytes), however a significantly increase was seen in ADAM metallopeptidase domain 8 (ADAM8) in the blood stream.

Genes did not have high expression (mRNA>8000) across three samples, but differ or trend to differ significantly from naïve conditions were also identified (Figure 4B). They reacted to the injury actively and were likely to play a role in shaping the microenvironment after SCI. It is interesting to notice the significant decrease of the trafficking gene Cx3cr1 in blood after injury across cell types. It also had significantly higher expression in lumbar spinal cord after injury compared to blood stream in neutrophils. In addition, CCL2 expression, though not significant, upsurge largely in lumbar spinal cord after SCI in both neutrophils and monocytes. In addition, CXCL2, a pro-inflammatory gene, increased significantly after injury in blood and its spinal cord expression was significantly higher than its expression level in blood. Moreover, ECM remodeling genes (Neutrophils: ADAM15, ADAM17; Monocytes: MMP14, ADAM8, ADAM9) all had prominent higher expression levels compared to their blood expression levels after injury.

Predictive Abilities of Genes Expressed by Blood Myeloid Cells at 24 hours

In paired animal sample analysis (Figure 5A), we found that blood mRNA level of two genes expressed by neutrophils and four genes expressed by monocytes correlate with their spinal cord mRNA level consistently using three matched animal samples. Genes related to inflammatory and trafficking in both cell types seem to have the strongest and most stable relationship between blood and spinal cord copy numbers. Interestingly, more positive correlations existed in neutrophil genes compared to monocyte genes overall.

In neutrophils, mRNA copies of inflammatory gene lymphocyte antigen 6 complex (Ly6C) and trafficking gene Syntaxin 6 (Stx6) in the blood are positively correlated with the copies in lumbar cord. R-square of 0.7016 was calculated for Ly6C and 0.9335 was calculated for Stx6 (Figure 5A). In monocytes, positive relationships also exist in inflammatory protein tyrosine phosphatase receptor type C (Ptprc/CD45) and trafficking gene chemokine Cx3C motif receptor 1 (cx3cr1). Their R-square was calculated to be 0.9958 and 0.8715, respectively. Additionally, some blood mRNA copies correlate with spinal cord negatively, including inflammatory gene low density lipoprotein receptor related protein (Lrp1) (R2=0.9419) and ECM remodeling gene ADAM17 (R2=0.5955) (Figure 5A).

Myeloid Cells Have Differential Expression Pattern in Spinal Cord at 24 hours

Based on the heat map generated by NanoString nSolver (Figure 5B), gene expression profiles in the blood are clearly different from the profile in the lumbar cord after SCI. More transcriptional activities were observed within the lumbar cord in both cell types. Genes with high mRNA count

in relationship to all other genes were determined by the nSolver software and were assigned red colors. Indicated by the red boxes, higher lumbar cord expressional activities were more distinct and prevalent in neutrophils than monocytes. Additionally, the majority of trafficking genes expressed by neutrophils (indicated by orange in figure 5B) had higher copy numbers in the lumbar cord compared to blood after SCI. On the other side, more ECM remodeling genes (labeled by light pink in figure 5B) were upregulated in monocytes compared to neutrophils within lumbar cord. This comparison might suggest the differential functions associated with neutrophils and monocytes and their relation towards the injury at 24 hours' time point.

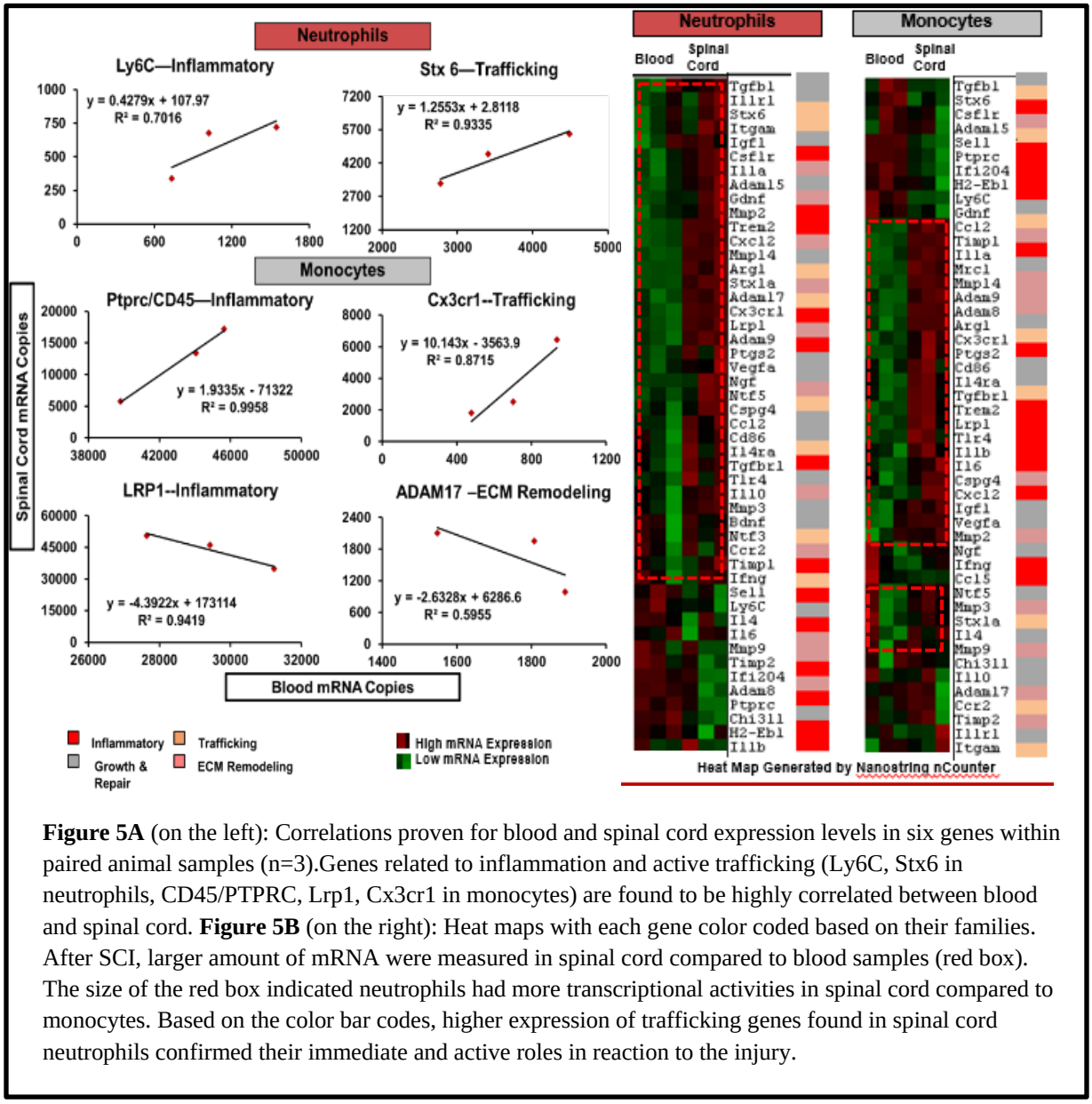

Figure 5A (on the left): Correlations proven for blood and spinal cord expression levels in six genes within paired animal samples (n=3).Genes related to inflammation and active trafficking (Ly6C, Stx6 in neutrophils, CD45/PTPRC, Lrp1, Cx3cr1 in monocytes) are found to be highly correlated between blood and spinal cord. **Figure 5B** (on the right): Heat maps with each gene color coded based on their families. After SCI, larger amount of mRNA were measured in spinal cord compared to blood samples (red box). The size of the red box indicated neutrophils had more transcriptional activities in spinal cord compared to monocytes. Based on the color bar codes, higher expression of trafficking genes found in spinal cord neutrophils confirmed their immediate and active roles in reaction to the injury.

Correlations between Blood and Spinal Cord Gene Expression Varied between Families

As previous described, genes were organized into four families--inflammatory, trafficking, growth and repair, and ECM remodeling--that best characterized their functions in reaction to the injury or maintain normal cellular homeostasis according to previous studies. All four families in neutrophils presented a statistically significant correlation between their blood and spinal cord expression level (Table 1). Inflammatory family genes were identified to possess the weakest association (r=0.848), compared to ECM remodeling family which has the strongest association (r = 0.949). However, in monocytes, only the inflammatory and growth and repair families presented to be statistically significant. Growth and repair genes have the strongest association in expression levels within the family (r= 0.970) while ECM remodeling family rendered the weakest association (r= 0.589) (Table 1).

Cell Type / Families	Neutrophils (R^2)	Neutrophils (r)	Monocytes (R^2)	Monocytes (r)
Trafficking	0.845	0.919**	0.497	0.705
Inflammatory	0.719	0.848**	0.699	0.836**
Growth and repair	0.859	0.927**	0.941	0.970**
ECM remodeling	0.901	0.949**	0.347	0.589

Table 1: Correlation coefficient (r) was calculated to demonstrate the overall similarity of genes in a family and their abilities to predict the spinal cord gene expression level based on blood expression levels after SCI. Statistically significant correlations (**) existed in all neutrophil gene families while only inflammatory and growth and repair genes presented significant correlation in monocytes.

A stronger association indicates that more similarities are shared within the same family. Therefore, after SCI, genes in the same families in myeloid adopted similar activities and had a comparable relationship between the blood mRNA and spinal cord mRNA expression. In both neutrophils and monocytes, inflammatory and growth and repair related genes work in parallel with others; therefore, it suggested neutrophils and monocytes have overlapped roles in reaction to the injury. However, it is also clear that significant correlations only existed within the trafficking genes and ECM remodeling genes in neutrophils. Hence, it demonstrated neutrophils' unique involvements in the alteration of membrane properties, cellular migration, adhesion, and mediation of early inflammatory responses.

Discussion

Proinflammatory Genes Increased in Spinal Cord but Decreased in Blood at 24 hours

Recognized genes associated with inflammatory responses expressed large copies in spinal cord at 24 hours after injury, we can attribute their fast accumulation in the lumbar cord to their sensitivity towards injury and critical role in initiating the negative microenvironment.

Gene markers associated with type 1 cell activation (IL-1B, CD45/PTPRC), which are generally associated with a pro-inflammatory response, all decreased in blood but largely increased in lumbar cord after SCI. Interleukin-1 beta (IL-1B) is a well-studied pro-inflammatory cytokine

that contributes to the induction and maintenance of pain and inflammatory conditions. (Sato et al 2012). Previous study in our laboratory has indicated that IL-1B had high expression levels in lumbar cord and was even higher in infiltrating macrophages compared to naïve microglia. However, the expression level was higher in epicenter compared to lumbar cord (Norden et al 2017). Similarly, in this study, the highest IL-1B expression was seen in neutrophils in naïve samples. It is possible that IL-1B actively participated in the inflammatory response localized at the epicenter instead of mediating lumbar microenvironment at 24 hour-time point. In addition, IL-1B has been recognized to enhance early proliferation and differentiation of neural progenitor cells during development by previous study (Sato et al 2012). Therefore, a lower than naïve expression might reflect its decreased capacity in promoting cellular regeneration and tissue repairing.

Common leukocyte antigen (CD45/PTPRC) also presented large copy numbers across conditions in both cell types. Its decreased expression after injury in blood stream matched with previous studies which indicated that highly activated monocyte-derived macrophages down-regulate CD45 expression responding to central nervous system injury (Trahanas et al 2015). As a result, we can critically hypothesize that along with a lower CD45 levels, more monocytes and neutrophils are being activated in both the blood stream and trafficked into the spinal cord.

As shown in Figure 4A, high gene expression of inflammatory genes (IL-1B, CD45/PTPRC) exists in naïve blood. Even though their expression level in blood decreased greatly, the inflammatory family has the most genes expressing high copy numbers within spinal cord. It is possible that the myeloid cells expressing high amounts of inflammation factors will be recruited or infiltrated to the lumbar region first to create the toxic microenvironment with a huge increase in spinal cord expression level (mRNA of more than 20000 copies) at 24 hours. Prior to the injury, no myeloid cells were found within the spinal cord. Considering their large decrease in expression number in blood and increased expression within the spinal cord after SCI, we can rationalize that myeloid cell proliferation and infiltration happened almost simultaneously after injury. Previous studies also found a transient increase in leukocyte populations in blood early after SCI followed by the orchestrated invasion of neutrophils into the injured cord starting from the 24 hour time point (Stirling & Yong 2008). This study suggested that immune cells are directly infiltrated from the blood and the process takes place earlier than 24 hours-hour time point and their gene expression profile maintained the same within lumbar cord. Therefore, gene expression profiles of myeloid cells need to be evaluated at earlier time points to trace the starting point and to identify the activation patter of the first infiltrated cells occurring in the lumbar spinal cord. Interestingly, even though neutrophil infiltration occurs due to and more rapidly than monocyte infiltration, the average expressions of inflammatory genes in monocytes are higher than neutrophil genes in spinal cord. This indicated the more toxic and proinflammatory role associated with monocytes/macrophages after injury.

However, different from the genes mentioned above, some inflammatory genes have higher expression in lumbar cord compared to blood sample before or after injury. In one way, this discrepancy indicates their active role in initiating the negative microenvironment while disable them to be used as reliable predictors based on their blood expression levels. Colony-stimulating factor 1 receptor (CSF1R) plays important roles in hematopoiesis, regulation of proliferation, cell survival and maturation of microglia and monocytes. According to the result, despite CSF1R expression decreased in blood after injury in both cell types, neutrophils' CSF1R expression in

spinal cord was significantly greater than its expression level in blood after SCI. The result validated neutrophils responded to the injury more actively, infiltrated more pro-inflammatory factors into the microenvironment at an early time point, and potentially initiated future microglia activation.

Toll-like receptor 4 (TLR4) remained at high expression levels, unchanged from naïve levels in blood but SCI increased expression to higher levels in the spinal cord. TLR4 functions as a signal-transducing receptor for the activation of innate immunity pathways and has been associated with the induction of inflammatory cytokines (Lehnardt et al 2002). Its unchanged blood expression and elevated spinal cord expression might suggest a distinct aggregation or production pattern specific to the spinal cord. As we known from previous studies, the production of CXC chemokine ligands 2 (CXCL2) is TLR4 dependent. Therefore, the upsurge of TLR4 genes in both neutrophils and monocytes (Figure 4A&B) suggested the synthesis of CXCL2. Indeed, CXCL2 expression in neutrophils was significantly more in lumbar cord compared to its blood concentration. In addition, we confirmed the chemoattractants of CCL2 and CXCL2 can infiltrate neutrophil actively and the important role of neutrophils to induce inflammatory response. Therefore, TLR4 seems to be able to initiate a proinflammatory and trafficking cascade that enables neutrophils to enter into the spinal cord early on (De Filippo et al 2013).

Trafficking Genes Increased Significantly in Spinal Cord

Trafficking genes are most responsible for the migration of myeloid cells from the bloodstream to sites of inflammation through a series of interaction between immune cells, endothelial cells, and extracellular matrix ligands (Bevilacqua 1993). It is noticeable that the expression of the trafficking gene l-selectin (SELL) was one of the highest across both cell types. Additionally, its expression level in neutrophils within the lumbar cord is over five times more than their concentration levels in monocytes. Previous studies shows SELL are the first molecules involved in cellular migration. The higher expression of SELL genes within neutrophils was responsible for the infiltration of neutrophils into the lumbar cord prior to monocytes. Also, integrin alpha M (Itgam/CD11b) are proteins expressed by leukocytes, and they mediated their migration together with selectins. A simultaneous rapid up-regulation of Itgam/CD11b and shedding of L-selectin on leukocytes upon activation has been demonstrated by previous studies which enables leukocytes to rapidly transition between the rolling and firmly adherent states (Ley et al 2007). Their rapid increase within the spinal cord indicated the active infiltration of neutrophils and monocytes. At 24 hours, neutrophil infiltration is more prominent which corresponds to the elevation of Itgam/CD11b in this study.

The chemokine ligand 2 (CCL2) genes encodes a chemoattractant protein that regulates the migration and infiltration of monocytes after injury. It allows monocytes to travel across vascular endothelium in reaction to inflammation (Deshmane et al 2009). Previous study has demonstrated CCL2 increases at 24 hours after SCI within the lumbar cord and returns to baseline at 7 days (Hansen et al 2016a). In this study, CCL2 were elevated in the spinal cord compared to both blood samples in neutrophils and monocytes. Specifically, its expression in monocytes was 40 times higher than its naïve or blood concentration after injury. Previous study also demonstrated that CCL2 were elevated in both microglia and macrophages after injury

(Norden et al 2017). The 24 hour data in this study replicated previous results and reinforced the distinct inflammatory response occur at a remote region of the spinal cord, away from the injury center. The CCL2 upsurge contributes towards the initiation of the lumbar cord inflammatory microenvironment driven by infiltrating monocytes.

ECM Genes Increased Significantly in Spinal Cord

ECM remodeling genes have a prevailing increase in the injured blood, and it is best characterized by monocytes instead of neutrophils at the 24 hour time point. They contributed to the alteration of membrane properties and the interaction within the neurovascular junction. Changes in extracellular matrix composition in the lumbar enlargement segment after SCI have been identified to form a negative microenvironment for plasticity in locomotor interneuron networks (Andrews et al 2012).

ADAM (A Disintegrin and Metalloproteinase) participates in the activation of membrane-anchored growth factors, cytokines and receptors through protein-protein interaction. The expression of ADAM8 is required for the adhesion of activated neutrophils to the endothelial cells, and it played a significant role in proliferation and/or migration of endothelial cells during angiogenesis following SCI (Mahoney et al 2009). In addition, the enzymatic activity of ADAM8 is not regulated by tissue inhibitors of metalloproteinase (TIMPs) (Amour et al 2002), which allows it to sustain high expression levels and to continue driving leukocytes to the lumbar cord. Additionally, it had been found to correlate with the inflammation levels within synovial fluid of patients with rheumatoid arthritis (Gomez-Gaviro et al 2007) which suggested its potential to function as a biomarker. In this study, ADAM8 had very high expression in all tissues, and it doubled its blood concentration after injury in neutrophils; however, for monocytes, a four times increase was seen in spinal cord after injury compared to its expression in naïve blood. It indicated the active role of ADAM 8 in infiltrating monocytes after injury. This result corresponded to previous studies that infiltrating macrophages have increased ADAM 8 in lumbar cord compared to resident microglia (Norden et al 2017). However, interestingly, the results also indicated decreased expression of ADAM15, 17 in infiltrating macrophages. In the current study, ADAM 15 was unchanged by injury and remained high in all monocyte samples while ADAM17 was slightly decrease in monocytes in spinal cord. Alternately, neutrophils had an elevated ADAM 15 and 17 gene expression in the spinal cord compared to the blood samples. Knowing that ADAM17 and ADAM8 can both cleave L-selectin, the differential activation patterns in neutrophils and monocytes might all serve the same goal of interacting with the endothelial cells and increasing myeloid cells infiltration from the blood stream to the spinal cord. In conclusion, the quick accumulation of ADAMs within the spinal cord in both neutrophils and monocytes after SCI suggests their active involvement in cellular adhesion, infiltration, and the formation of a negative microenvironment.

Another ECM remodeling family gene, Matrix Metalloproteinases (MMPs), regulates diverse functions including tissue remodeling, inflammation, and learning after injury (Ethell & Ethell 2007, Zhang et al 2011). MMP9 expresss very high copy numbers by neutrophils in naïve blood, injured blood, and spinal cord (Figure 4A). A recent study has demonstrated their capacities in increasing blood spinal cord barrier (BSCB) permeability, introducing the infiltration of CD45/PTPRC cells into the lumbar cord, and amplifying proinflammatory cytokine production (Hansen et al 2016b). MMP-9 upregulation in the lumbar enlargement was also proved to cause

remote inflammation during the first week. The administration of training during this period caused greater locomotion deficit. These findings have demonstrated the relationships among MMP9, inflammation, and rehabilitation outcome. Moreover, previous publications indicate that neutrophil infiltration highly contributes to enhanced MMP9 in the brain by releasing MMP9 proform, which can directly participate in the inflammatory reaction (Justicia et al 2003). Therefore, we are able to theorize that the concentration of MMP9 within spinal cord at the 24 hour time point was driven by neutrophils. The deletion of MMP9 on neutrophils can attenuate remote microglial activation, decrease the production of TNF, and therefore restore the homeostatic within spinal lumbar cord. Also, when MMP9 deletion was combined with early treadmill training for SCI mice, robust behavioral improvements were seen by 7 days (Hansen et al 2013). Again, results from this study suggested neutrophils are most responsible for the accumulation of MMP9 in the lumbar spinal cord, which makes neutrophils of high potential value as a biomarker predicting the lumbar microenvironment. Besides MMP9, we observed a significant increase of MMP14 expression in monocytes. Previous studies have demonstrated the significant contribution of MMP14 in the attraction and infiltration of monocytes to sites of inflammation in skin wounds (Atkinson et al 2007). It is possible that MMPs might have differential roles for infiltrating specific myeloid cells. As a result, a variety of targets need to be selected to decrease the overall infiltration of immune cells and to decrease the level of inflammation.

Therefore, these alterations indicate thoracic spinal cord injury markedly changed the blood brain barrier in the lumbar enlargement region. They might lead to barrier breakdown and increased barrier permeability, which will lead to increased active recruitment of myeloid cells at the lumbar site and impede functional recovery. ***Diversified Growth and Repair Genes Expression at 24 hour***

Few growth and repair genes had large copy numbers or changed significantly after SCI within blood and spinal cord. Their expression pattern in responding to injury at the 24 hour time point remained diverse.

In neutrophils, Chitinase-like protein 3 (Chi3la/YM1), a gene associated with anti-inflammatory activation, was expressed highly in naive blood, blood, and spinal cord after injury. Its concentration in blood increased significantly after injury compared to naïve blood and is significantly more than its lumbar cord concentration. Previous studies indicated its involvement in tissue remodeling and regeneration (Roszer 2015). It is likely that the increase Chi31a/YM1 in blood predicts its future increase in spinal cord and facilitates the infiltration of neutrophils in the lumbar cord. Due to the high COV within monocyte samples, we do not have reliable data regarding Chi3la/YM1 expression in monocytes in this study. As found in previous work from our lab (Norden et al 2017), infiltrating macrophages expressed elevated Chi31a/YM1 expression in the lumbar cord at 3-7 days after injury compared to microglia at the same time. Therefore, we can reasonably expect that the expression of Chi3la/YM1 in monocytes and possibly neutrophils will continue to increase and remain high for at least 7 days after SCI. However, we also cannot completely rule out the possibility that neutrophils expressed Chi3la/YM1 will tend not to infiltrate into the cord but stay in the circulatory system. Therefore, the role of Chi3la/YM1 cannot be articulated without analyzing its expression levels in different cells at later time points. More importantly, Chi3la/YM1 gene is specific to murine, therefore, studies need to be done using a similar chitinase-like lectin gene in human (Raes et al 2005).

Interleukin-four (IL-4) maintained stable levels in blood after SCI; however, its downstream receptor IL4Ra is greatly increased in the blood and cord after the injury. This increase seemed to be inconsistent compared to the interleukin level. Previous studies showed IL4 and its receptor reduces proinflammatory macrophage density and enhances myelination (Psachoulia et al 2016). Additionally, IL-4 and IL4Ra production could also be seen as an upstream stimulation signal for inflammatory gene expression, including YM1/Chi31a, Arg1, and Fizz1 (Raes et al 2002). According to the rapid increase of Chi3la/YM1, the salient increase of IL4Ra within the spinal cord might contribute to the Chi3la/YM1 pathway instead of reacting to the IL-4 signals directly.

Transforming growth factor beta1 (Tgfb1) gene encoded protein regulates cell proliferation, differentiation and growth. It has been associated with type 2 activation and is frequently upregulated in tumor cells. Tgfb1 has very high expression in blood under the naïve condition which decreases by half after SCI in both monocytes and neutrophils. The spinal cord expression level is similar or less than the blood expression level after injury. Similar findings have been reported in a study that showed decreased expression of Tgfb1 was seen after injury in microglia or infiltrated macrophages compared to naïve microglia in lumbar spinal cord (Norden et al 2017). These results suggested that the expression of growth and repair genes Tgfb1 will likely stay suppressed within the spinal cord which permits an increased expression of pro-inflammatory cytokines by neutrophils (de Oliveira et al 2016).

Genes from the Same Family are Expressed More Similarly in Neutrophils

Neutrophil genes seem to have more similar functions within the same family compared to monocyte genes 24 hours after injury due to their larger amount of transcriptional activities in the spinal cord and their consistent functional similarity between genes from the same category. It supported the findings of previous studies for the active role of neutrophils in initiating the toxic microenvironment (Neirinckx et al 2014).

The infiltration of monocytes also took place at or before 24 hours after injury, but will not peak until 7 days after injury. At this point, monocytes presented an interesting mix of properties: they are likely to drive inflammation as well as enhance neuroprotection to the spinal cord. Previous studies have indicated that immune cells can polarized into two different functional types corresponding to local microenvironment signals. Monocytes and neutrophils undergo type 1 activation (M1 and N1) are usually associated with cytotoxic properties while type 2 (M2 and N2) is associated with angiogenesis and tumor growth (Martinez & Gordon 2014, Selders et al 2017). Even M1 cells are often found within the lesion center while M2 cells are rarely seen, however, this study produce a mix activation signals regarding the polarization in both neutrophils and monocytes at a region remote to the injury site. Therefore, at 24-hour after SCI, it appeared that the infiltrated myeloid do not segregate into a certain classifications (M1/M2, N1/N2), they tended to activate along a spectrum to regulate inflammation or regeneration capacity. (Mosser & Edwards 2008) Therefore, the spinal lumbar cord seems to have greater neuroplasticity and the outcome of neuro-rehabilitation can be enhanced if we can increase the protective features of monocytes and neutrophils at the appropriate time window (Gliem et al 2016).

Expression Level of Blood Biomarkers Predict the Lumbar Cord Microenvironment

Based on the data, we can theorize that genes changing significantly after spinal cord injury in blood have more reactivity compared to the genes which stayed stable in blood concentration. Additionally, strong correlations between blood and lumbar cord expression after injury indicate their capability to be biomarkers in predicting the microenvironment of the lumbar spinal cord. As a result, we identified potential genetic biomarkers in Ly6C for neutrophils and CD45/PTPRC for monocytes at 24 hours after injury.

In the current study, there was a significant increase of Ly6C in blood, which implied its expression was influenced by the injury and is likely to play a role in creating a toxic microenvironment in the lumbar cord. Also, in paired animal comparisons, the correlation between cord expression and blood was consistent in three different animals. It indicated that greater Ly6C within the blood stream will predict more Ly6C expression in the spinal cord at least in these small samples. According to previous literatures, despite its function as a cell-sorting marker for monocytes, Ly6C also expressed consistently on neutrophils (Fleming et al 1993) and other blood derived myeloid cells in previous studies (Rose et al 2012). Therefore, we can infer that Ly6C in blood stream can be used as a biomarker to predict the lumbar microenvironment. Whether their blood levels will continue to be a sensitive predictor of lumbar levels will require further testing in larger sample sizes and across longer time points.

High expression of CD45/PTPRC in both neutrophils and monocytes indicated their importance in maintaining normal cellular function and their significant change in reaction to the injury indicated their active involvement in injury. The positive correlation was seen between the blood and cord expression in monocytes. It indicated more CD45/PTPRC production in blood was associated with higher spinal cord expression. This finding indicated the early activation of macrophages after injury within 24 hours and the active infiltration in the spinal cord lumbar region. Similarly, previous studies indicated the robust recruitment of myeloid cells within spinal locomotor network starts 24 hours post injury and has a detrimental role in activity-dependent recovery (Hansen et al 2016a). Together with previous findings using CD45/PTPRC as a common cellular marker for bone marrow derived monocytes, its expression implied the number of infiltrated monocytes from the blood stream at lumbar cord (Wohleb et al 2011).

Some other genes also had consistent relationship between spinal cord and blood expression levels, however, their blood concentration change after the injury was not significant. It might indicate their lack of reaction towards the injury and minimal roles in altering the spinal cord microenvironment. However, they still have the potential to be considered as good bio-markers at other time points with more experimental data. For example, Chemokine receptor Cx3cr1 was ruled out due to its variability across paired animal comparison. One animal can be seen as an outlier to influence the relationship established between blood expression and spinal cord expression. However, as suggested by previous studies, it has been used as a genetic marker to highlight the monocytes heterogeneity in mouse blood. In studies using trans-genetic GFP reporter mice, a subset of monocytes can be identified and actively recruited to inflamed tissues by using Cx3cr1 marker (Geissmann et al 2003). Therefore, it is possible that Cx3cr1 can be used to indicate the process and amount of monocyte infiltration with more available animal data.

Limitations in Experimental Design and Animal Samples

Throughout the experiment, we used a classification system to sort genes into four major families for the sake of overall expression patterns. However, these categories are somewhat arbitrary as many genes have overlapping capabilities and could participate in multiple cellular processes after injury. Therefore, it is important to be flexible in order to understand the bigger pictures. Additionally, as we only measured a small portion of the whole myeloid cells genome profile, whole transcriptome shotgun sequencing techniques (RNAseq) might be used in the future to aid in our knowledge regarding to the expression and correlation of genes participating in the infiltration of myeloid cells after SCI.

We also suspected that the mRNA expression levels do not correlate with the translated protein levels in the spinal cord. Therefore, there might be a mismatch between the transcriptional activities and the actual products which affect the lumbar microenvironment. As a result, we might conduct experiments measuring and correlating the protein and mRNA levels in the lumbar cord and gain more insight into the feasibility of using mRNA in blood as a biomarker.

In addition, for the 24 hour time point, the generalizability of the result is significantly limited by sample size. Only 3 SCI animals contributed both blood and spinal cord samples. The data has a large level of variability, and it is heavily affected by outliers. There is no precise way to identify the outlier effect and to adjust the data towards an ideal direction. Also, the heatmap was produced without taking out particular genes that are classified as a rule-out due to technical difficulties. In the future study, a larger sample size and more time points need to be included to confirm the accuracy and to expand the clinical feasibility of the finding.

Unfortunately, the low cell counts in spinal cord and blood tissues at 3 days and 7 days has prevented or at least complicated future NanoString analysis. It is possible that human errors might have occurred during tissue collection or processing. However, cell markers used for 24 hours group analysis might not be the most reliable options for later time points--3 days and 7 days. Therefore, GFP chimera mice might be used in the future study to better sort and count bone marrow derived cells. In addition, natural changes among the numbers of infiltrated cells might influence their detection. It is known that the numbers of neutrophils would have decreased by 24 hours post injury. (Nguyen et al 2011) As a result, fewer neutrophils should be expected in the 3 days and 7 days lumbar cord sample.

Conclusion

Overall, this study has identified genetic biomarkers Ly6C expressed by blood neutrophils and CD45 expressed by monocytes to be reliable predictor of the lumbar spinal cord microenvironment 24 hours after SCI. We also confirm that neutrophils have differential activation patterns compared to monocytes, contribute, and determine the early toxic microenvironment in lumbar cord at 24 hours. We also gain better understanding towards the active players of myeloid infiltration at 24 hours, provide new targets for future medical intervention (KO study), and insight into future biomarkers at different time point.

Appendix

1. Summary of samples used in the 24 hours group

Animal #	16142	16143	16165	16180	16181	16183
sample type	Surgery	Surgery	Surgery	Surgery	Surgery	Surgery
Spinal Cord Neutropils	x	x	x		x	
Blood Neutrophils	x	x			x	x
Spinal Cord Monocytes		x	x	x	x	
Blood Monocytes	x	x		x	x	
Animal #	16163	16160	16185	16162	16189	16187
sample type	naive	naive	naive	naive	naive	naive
Blood Neutrophils	x		x	x	x	
Blood Monocytes		x	x	x		x

2. Summary of Included Genes at 24 hour time point

	inflammatory												
Neutrophils	CSF1R	CD45	LRP	TLR-4	TREM2	IL-1B	IL6	COX2	IFN-y	CXCL2	Ly6C	MHC-II	IFI204
Monocytes	CSF1R	CD45	LRP	TLR-4	TREM2	IL-1B	IL6	Ly6C	MHC-II	IFI204			
	Trafficking												
Neutrophils	Itgam	Cx3cr1	TGFbR1	L-selectin	Syntaxin 1	Syntaxin	CCR2	CCL2					
Monocytes	Itgam	Cx3cr1	TGFbR1	L-selectin	Syntaxin 6	CCR2	CCL2						
	Growth and Repair												
Neutrophils	Chi3la/ YM1	IL10	Tgfb1	IL4	IL-1Ra	IL4Ra	CD86	GDNF	BDNF	NGF	NTF5	VEGF	
Monocytes	IL10	Tgfb1	IL-1Ra(Il1	IL4Ra	Mrc1	CD86	GDNF	Igf1	NGF	NTF5			
	ECM Remodling												
Neutrophils	MMP2	MMP3	MMP9	MMP14	TIMP2	ADAM8	ADAM9	ADAM15	ADAM17	Cspg4			
Monocytes	MMP3	MMP9	MMP14	TIMP1	TIMP2	ADAM8	ADAM9	ADAM15	ADAM17				

References

Amour A, Knight CG, English WR, Webster A, Slocombe PM, et al. 2002. The enzymatic activity of ADAM8 and ADAM9 is not regulated by TIMPs. *FEBS Lett* 524: 154-8

Andrews EM, Richards RJ, Yin FQ, Viapiano MS, Jakeman LB. 2012. Alterations in chondroitin sulfate proteoglycan expression occur both at and far from the site of spinal contusion injury. *Exp Neurol* 235: 174-87

Atkinson JJ, Toennies HM, Holmbeck K, Senior RM. 2007. Membrane type 1 matrix metalloproteinase is necessary for distal airway epithelial repair and keratinocyte growth factor receptor expression after acute injury. *American journal of physiology. Lung cellular and molecular physiology* 293: L600-10

Beck KD, Nguyen HX, Galvan MD, Salazar DL, Woodruff TM, Anderson AJ. 2010. Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment. *Brain* 133: 433-47

Bevilacqua MP. 1993. Endothelial-leukocyte adhesion molecules. *Annual review of immunology* 11: 767-804

De Filippo K, Dudeck A, Hasenberg M, Nye E, van Rooijen N, et al. 2013. Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. *Blood* 121: 4930-7

de Oliveira S, Rosowski EE, Huttenlocher A. 2016. Neutrophil migration in infection and wound repair: going forward in reverse. *Nature reviews. Immunology* 16: 378-91

Deshmane SL, Kremlev S, Amini S, Sawaya BE. 2009. Monocyte chemoattractant protein-1 (MCP-1): an overview. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* 29: 313-26

Detloff MR, Fisher LC, McGaughy V, Longbrake EE, Popovich PG, Basso DM. 2008. Remote activation of microglia and pro-inflammatory cytokines predict the onset and severity of below-level neuropathic pain after spinal cord injury in rats. *Exp Neurol* 212: 337-47

Donnelly DJ, Longbrake EE, Shawler TM, Kigerl KA, Lai W, et al. 2011. Deficient CX3CR1 signaling promotes recovery after mouse spinal cord injury by limiting the recruitment and activation of Ly6Clo/iNOS+ macrophages. *J Neurosci* 31: 9910-22

Donnelly DJ, Popovich PG. 2008. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Exp Neurol* 209: 378-88

Ethell IM, Ethell DW. 2007. Matrix metalloproteinases in brain development and remodeling: synaptic functions and targets. *J Neurosci Res* 85: 2813-23

Fenn AM, Hall JCE, Gensel JC, Popovich PG, Godbout JP. 2014. IL-4 signaling drives a unique arginase+/IL-1β+ microglia phenotype and recruits macrophages to the inflammatory CNS: consequences of age-related deficits in IL-4Rα after traumatic spinal cord injury. *J Neurosci* 34: 8904-17

Ferbert T, Child C, Graeser V, Swing T, Akbar M, et al. 2017. Tracking Spinal Cord Injury: Differences in Cytokine Expression of IGF-1, TGF- B1, and sCD95l Can Be Measured in Blood Samples and Correspond to Neurological Remission in a 12-Week Follow-Up. *J Neurotrauma* 34: 607-14

Fleming TJ, Fleming ML, Malek TR. 1993. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *Journal of immunology (Baltimore, Md. : 1950)* 151: 2399-408

Ford D, Easton DF, Peto J. 1995. Estimates of the gene frequency of BRCA1 and its contribution to breast and ovarian cancer incidence. *Am J Hum Genet* 57: 1457-62

Geissmann F, Jung S, Littman DR. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19: 71-82

Gliem M, Schwaninger M, Jander S. 2016. Protective features of peripheral monocytes/macrophages in stroke. *Biochim Biophys Acta* 1862: 329-38

Gomez-Gaviro M, Dominguez-Luis M, Canchado J, Calafat J, Janssen H, et al. 2007. Expression and regulation of the metalloproteinase ADAM-8 during human neutrophil pathophysiological activation and its catalytic activity on L-selectin shedding. *Journal of immunology (Baltimore, Md. : 1950)* 178: 8053-63

Greenhalgh AD, David S. 2014. Differences in the phagocytic response of microglia and peripheral macrophages after spinal cord injury and its effects on cell death. *J Neurosci* 34: 6316-22

Hansen CN, Faw TD, Buford JA, White S, Grau JW, Basso DM. 2016a. Sparing of descending axons rescues interneuron plasticity in the lumbar cord to allow adaptive learning after thoracic spinal cord injury. *Front Neural Circuits* 10: 10.3389/fncir.2016.00011

Hansen CN, Fisher LC, Deibert RJ, Jakeman LB, Zhang H, et al. 2013. Elevated MMP-9 in the lumbar cord early after thoracic spinal cord injury impedes motor relearning in mice. *J Neurosci* 33: 13101-11

Hansen CN, Norden DM, Faw TD, Deibert R, Wohleb ES, et al. 2016b. Lumbar Myeloid Cell Trafficking into Locomotor Networks after Thoracic Spinal Cord Injury. *Exp Neurol* 282: 86-98

Henry CJ, Huang Y, Wynne A, Hanke M, Himler J, et al. 2008. Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. *J Neuroinflammation* 5: 15

Hoschouer EL, Basso DM, Jakeman LB. 2010. Aberrant sensory responses are dependent on lesion severity after spinal cord contusion injury in mice. *Pain* 148: 328-42

Justicia C, Panes J, Sole S, Cervera A, Deulofeu R, et al. 2003. Neutrophil infiltration increases matrix metalloproteinase-9 in the ischemic brain after occlusion/reperfusion of the middle cerebral artery in rats. *J Cereb Blood Flow Metab* 23: 1430-40

Karimi S, Mohamadnia A, Nadji SA, Yadegarazari R, Khosravi A, et al. 2015. Expression of two basic mRNA biomarkers in peripheral blood of patients with non-small cell lung cancer detected by real-time rt-PCR, individually and simultaneously. *Iran Biomed J* 19: 17-22

Kulkarni MM. 2011. Digital multiplexed gene expression analysis using the NanoString nCounter system. *Current protocols in molecular biology* Chapter 25: Unit25B.10

Kwon BK, Streijger F, Fallah N, Noonan VK, Belanger LM, et al. 2017. Cerebrospinal Fluid Biomarkers To Stratify Injury Severity and Predict Outcome in Human Traumatic Spinal Cord Injury. *J Neurotrauma* 34: 567-80

Lee SM, Rosen S, Weinstein P, van Rooijen N, Noble-Haeusslein LJ. 2011. Prevention of both neutrophil and monocyte recruitment promotes recovery after spinal cord injury. *J Neurotrauma* 28: 1893-907

Lehnardt S, Lachance C, Patrizi S, Lefebvre S, Follett PL, et al. 2002. The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 22: 2478-86

Letellier E, Kumar S, Sancho-Martinez I, Krauth S, Funke-Kaiser A, et al. 2010. CD95-ligand on peripheral myeloid cells activates Syk kinase to trigger their recruitment to the inflammatory site. *Immunity* 32: 240-52

Ley K, Laudanna C, Cybulsky MI, Nourshargh S. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nature reviews. Immunology* 7: 678-89

Mahoney ET, Benton RL, Maddie MA, Whittemore SR, Hagg T. 2009. ADAM8 is selectively up-regulated in endothelial cells and is associated with angiogenesis after spinal cord injury in adult mice. *J Comp Neurol* 512: 243-55

Martinez FO, Gordon S. 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime reports* 6: 13

Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology* 8: 958-69

National Spinal Cord Injury Statistical Center. 2016. 2016 Spinal Cord Injury Facts and Figures at a Glance. University of Alabama at Birmingham.

Neirinckx V, Coste C, Franzen R, Gothot A, Rogister B, Wislet S. 2014. Neutrophil contribution to spinal cord injury and repair. *J Neuroinflammation* 11: 150

Nguyen HX, Beck KD, Anderson AJ. 2011. Quantitative assessment of immune cells in the injured spinal cord tissue by flow cytometry: a novel use for a cell purification method. *Journal of visualized experiments : JoVE*

Norden DM, Faw TD, McKim D, Deibert R, Kerr S, et al. 2017. Infiltrating Macrophages Promote Inflammation Rapidly at Epicenter and Remote Regions after Thoracic SCI.

Popovich PG, Horner PJ, Mullin BB, Stokes BT. 1996. A quantitative spatial analysis of the blood-spinal cord barrier. I. Permeability changes after experimental spinal contusion injury. *Exp Neurol* 142: 258-75

Psachoulia K, Chamberlain KA, Heo D, Davis SE, Paskus JD, et al. 2016. IL4I1 augments CNS remyelination and axonal protection by modulating T cell driven inflammation. *Brain* 139: 3121-36

Raes G, De Baetselier P, Noel W, Beschin A, Brombacher F, Hassanzadeh Gh G. 2002. Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. *J Leukoc Biol* 71: 597-602

Raes G, Van den Bergh R, De Baetselier P, Ghassabeh GH. 2005. Arginase-1 and Ym1 Are Markers for Murine, but Not Human, Alternatively Activated Myeloid Cells. *The Journal of Immunology* 174: 6561-62

Rose S, Misharin A, Perlman H. 2012. A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* 81: 343-50

Roszer T. 2015. Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediators of inflammation* 2015: 816460

Sato A, Ohtaki H, Tsumuraya T, Song D, Ohara K, et al. 2012. Interleukin-1 participates in the classical and alternative activation of microglia/macrophages after spinal cord injury. *J Neuroinflammation* 9: 65

Schnell L, Fearn S, Schwab ME, Perry VH, Anthony DC. 1999. Cytokine-induced acute inflammation in the brain and spinal cord. *J Neuropathol Exp Neurol* 58: 245-54

Selders GS, Fetz AE, Radic MZ, Bowlin GL. 2017. An overview of the role of neutrophils in innate immunity, inflammation and host-biomaterial integration. *Regenerative biomaterials* 4: 55-68

Siddiqui T, Lively S, Ferreira R, Wong R, Schlichter LC. 2014. Expression and contributions of TRPM7 and KCa2.3/SK3 channels to the increased migration and invasion of microglia in anti-inflammatory activation states. *PLoS One* 9: e106087

Stirling DP, Yong VW. 2008. Dynamics of the inflammatory response after murine spinal cord injury revealed by flow cytometry. *J Neurosci Res* 86: 1944-58

Strimbu K, Tavel JA. 2010. What are biomarkers? *Curr Opin HIV AIDS* 5: 463-6

Sutherland TE, Logan N, Ruckerl D, Humbles AA, Allan SM, et al. 2014. Chitinase-like proteins promote IL-17-mediated neutrophilia in a tradeoff between nematode killing and host damage. *Nature immunology* 15: 1116-25

Trahanas DM, Cuda CM, Perlman H, Schwulst SJ. 2015. Differential Activation of Infiltrating Monocyte-Derived Cells After Mild and Severe Traumatic Brain Injury. *Shock (Augusta, Ga.)* 43: 255-60

Whetstone WD, Hsu JY, Eisenberg M, Werb Z, Noble-Haeusslein LJ. 2003. Blood-spinal cord barrier after spinal cord injury: relation to revascularization and wound healing. *J Neurosci Res* 74: 227-39

Wohleb ES, Hanke ML, Corona AW, Powell ND, Stiner LTM, et al. 2011. β-Adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. *J Neurosci* 31: 6277-88

Wohleb ES, Patterson JM, Sharma V, Quan N, Godbout JP, Sheridan JF. 2014. Knockdown of interleukin-1 receptor type-1 on endothelial cells attenuated stress-induced neuroinflammation and prevented anxiety-like behavior. *J Neurosci* 34: 2583-91

Zhang H, Chang M, Hansen CN, Basso DM, Noble-Haeusslein LJ. 2011. Role of Matrix Metalloproteinases and Therapeutic Benefits of Their Inhibition in Spinal Cord Injury. *Neurotherapeutics* 8: 206-20

Zhang ZJ, Cao DL, Zhang X, Ji RR, Gao YJ. 2013. Chemokine contribution to neuropathic pain: respective induction of CXCL1 and CXCR2 in spinal cord astrocytes and neurons. *Pain* 154: 2185-97