Author

Michelle Tu's interest in stem cells began when she read about the promise of stem cell research; she was amazed by their unique features, especially their ability to differentiate into any cell type in the body. She was drawn to Professor Keirstead's study on human-embryonic stem cell-derived oligodendrocyte progenitor cells in spinal cord injury. Michelle describes her research experience as eye-opening and inspiring. Through her experience, she has acquired a genuine passion for stem cell research. Michelle spent the summer of 2007 conducting stem cell research at UC San Francisco and graduated from UCI in spring 2008. She hopes to continue into graduate school and ultimately become a stem cell scientist.

Key Terms

- Demyelination
- Human-Embryonic Stem Cell
- Inflammatory Response
- Macrophages
- Oligodendrocyte
- Remyelination
- Spinal Cord Injury (SCI)

Quantification of Macrophage Response in Contusion and Laceration Spinal Cord Injuries

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Abstract

Spinal cord injury (SCI) is damage to the spinal cord that leads to physiological impairment. This loss of function can be partly attributed to the chronic progressive demyelination of axons following SCI. We have previously shown that transplantation of human embryonic stem cell-derived oligodendrocyte progenitor cells after contusion injury results in enhanced remyelination and locomotor recovery. We recently demonstrated that contusion and laceration SCI pathologically differ. Examining the inflammatory response following SCI may reveal different responses to contusions and lacerations, which may explain the difference in myelin pathology. Our study examined macrophages, which have been shown to play a role in both demyelination and remyelination. We also looked at macrophages in grey and white matter following SCI. Eight female rats received contusion injuries and eight more received laceration injuries. Animals were sacrificed three and fourteen days post injury. Spinal cords were removed and embedded in optical coherence tomography compound. Through immunohistochemistry, the temporal and spatial response of macrophages was quantified. We found more macrophages in contusion than laceration injuries and more macrophages in grey than white matter, suggesting that the inflammatory and macrophage response correlates with the extent of different pathologies. This knowledge will aid in developing better targeted SCI therapies.

Faculty Mentor

Michelle Tu's "Quantification of Macrophage Response in Contusion and Laceration Spinal Cord Injuries" demonstrates differences in the macrophage response between two spinal cord injury types. This work is important in delineating the pathological differences between contusion and laceration spinal cord injuries. Her discovery in the differences of the macrophage response following the two injuries is a plausible explanation for the differences in myelin pathology seen between the two injury types. Her contribution to the field of spinal cord injury research augments findings that suggest that therapies must be developed separately for laceration and contusion spinal cord injuries, considering the pathological differences. This is an important and clinically relevant series of studies.

Hans S. Keirstead
School of Medicine

Introduction

The spinal cord is composed of a bundle of axons, which allow nerve cells, called neurons, to transmit electrical signals from the brain toward the periphery of the body. When a spinal cord injury (SCI) occurs, physical damage to the cord disrupts the cellular signals that travel through the axons. Because the signals from the brain cannot be properly transmitted to the rest of the body, physiological impairment occurs at or below the injury site. There are approximately 11,000 new incidents of SCI per year (NSCISC, 2006). Since June 2006, it is estimated that 253,000 persons are living with SCI. SCI occurs predominantly in males, and the average age of incident is 38 years old. In 2000, 46.6% of SCI were caused by motor vehicle crashes and 13.3% were a result of violent acts, predominantly gunshot wounds. Vehicle crashes and gunshot wounds can be categorized as contusion and laceration injuries, respectively (Bunge et al., 1993). A contusion injury is caused by blunt impact that displaces the spinal cord and causes bruising. A laceration injury is characterized as a localized open lesion, such as a stab or gunshot wound. Both types of SCI result in physical spinal cord damage that prevents axons from conducting electrical impulses from the brain to the rest of the body, leading to limb paralysis. These axons are unable to function properly in part due to their demyelination, or the loss of the myelin sheath. The myelin sheath is responsible for insulating axons and is produced by glial cells called oligodendrocytes (Bambakidis and Miller, 2004).

Since demyelination impedes the recovery of locomotor function, one therapeutic approach to treating SCI is to target demyelination (McDonald and Howard, 2002). One type of therapy under development is to transplant human embryonic stem cell (hESC)-derived oligodendrocyte progenitor cells (OPCs) into the site of injury. We have previously shown that transplantation of hESC-derived OPCs in contusion injuries resulted in improved remyelination and locomotor recovery (Keirstead et al., 2005; Faulkner, 2006). However, it is uncertain if transplantation of hESC-derived OPCs into adult rats with laceration sites would show enhanced remyelination or motor function. Evidently, targeting demyelination is a viable approach to treating contusion SCIs, but it may be ineffective in treating laceration SCIs due to differences in the pathology between the two injury types. Contusion injuries are more widespread; damage is distributed along the rostral (towards the nose) and caudal (towards the tail) direction. In addition, contusion injuries sustain greater tissue loss at the epicenter but maintain a rim of spared tissue (Kalb and Strittmatter, 2000). In contrast, laceration injuries incur less tissue damage at the epicenter and the lesion is confined to the area close to the epicenter (Figure 1).

Mechanical injury to the spinal cord leads to the immediate death of cells, but also triggers a delayed response in which surrounding tissue is destroyed. This response is called secondary degeneration and results in widespread demyelination and increased physiological impairment (Haag and Oudega, 2006). Secondary degeneration may be more devastating than the initial trauma because it causes extensive damage to the areas surrounding the injury site. This widespread destruction can be linked to the inflammatory response, which is fundamental to secondary degeneration after injury (Ahn et al., 2006). The inflammatory response is characterized by the influx of immune cells and fluid accumulation. This response has been known to exacerbate secondary injury processes, but has also been implicated in injury repair following SCI (Bethea and Dietrich, 2002). During the inflammatory response there is a release of macrophages, which are immune cells that are responsible for engulfing and digesting cellular debris. Macrophages have been shown to be present following SCI and presumably play a role in disease pathology as well as tissue regeneration after SCI (Frisen et al., 1994).

One study reveals that reducing invading macrophages using

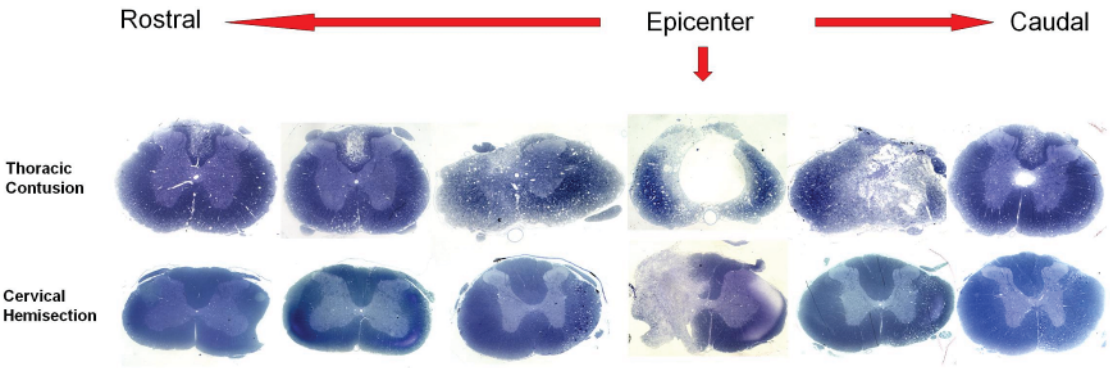

Figure 1

Siegenthaler et al., 2007 demonstrate differences in the extent of pathology, apoptosis, and demyelination in contusion and laceration (hemisection) injuries.

clodronate liposomes enhances remyelination and improves motor function in adult rats following SCI (Popovich et al., 1999). Additionally, macrophages have been shown to contribute to the demyelination process. A cuprizone-induced model for demyelination revealed that macrophage accumulation occurs prior to gross demyelination. Macrophages were also shown to increase in numbers until the demyelination process was finished. These results suggest that macrophages react to demyelinating lesions and may contribute to the exacerbation of demyelination (Hiremath et al., 1998). In contrast, several studies have revealed that macrophages may play a beneficial role following spinal cord injury. One investigation demonstrates how activated macrophages in acute inflammation may produce a favorable environment for OPCs to expand and differentiate. The expansion and differentiation of OPCs stimulates remyelination in regions of chronic demyelination (Foote and Blakemore, 2005). It has also been shown that transplanting activated macrophages into the SCI site of an adult rat promotes axonal regeneration. This may be due to macrophages releasing cytokines and communicating with nonneuronal cells within the surrounding area (Prewitt, et al. 1997). As can be seen, the role of macrophages following SCI is quite controversial, and we hope to further elucidate macrophage response in SCI, specifically in contusion and laceration injuries. We hypothesize that there will be a difference in macrophage response in the two distinct injury types that will correlate with their different pathologies.

We also speculate that there might be a difference in macrophage response in grey and white matter following the distinct SCI types. Grey matter is located in the central region of the spinal cord and is surrounded by white matter. Grey matter mainly consists of glial cells, nerve cell bodies, and capillaries, whereas white matter consists of only axons. A recent study reveals that distinct types of microglial activation occur in the grey and white matter following mid-thoracic spinal transection in rats (McKay, et al. 2007). This difference in inflammatory response in the two regions of the spinal cord demonstrates a potential relationship between macrophage response and grey and white matter. Because little is known about the role of the inflammatory response in grey and white matter after SCI, we are interested in examining macrophage activation in the grey and white matter following contusion and laceration SCI. To characterize the macrophage response, we will use immunohistochemistry to identify macrophages in the rat spinal cord tissue and use light microscopy to analyze and quantify the macrophage response. We will quantify the macrophage response in distinct regions within the contused and lacerated spinal cord tissue and subsequently compare the macrophage response in the grey and white matter. This data will elucidate the spatial and temporal response of macrophages in the two distinct injury types.

Methods and Materials

Spinal Cord Injury

Sixteen female Sprague-Dawley (S-D) rats were randomly divided into two time groups, 3-day and 14-day. Each group had eight animals; four were inflicted with contusion injury, while the other 4 received a lateral hemisection injury. All the rats were anesthetized by injection of 80 mg/kg ketamine (Phoenix Pharmaceuticals, St. Joseph, MO) and 10 mg/kg xylazine (Phoenix Pharmaceuticals, St. Joseph, MO). Laminectomies were carried out at the thoracic level (T10 level) in all the rats. The rats receiving contusion injuries were clamped and stabilized with a stereotactic device at spinal sections T9 and T11. The contusion injury was produced using the Infinite Horizon Impactor (Precision Systems and Instrumentation LLC, Fairfax, VA) with a force of 200 kDynes for a single hit injury. Two additional uninjured S-D rats were used as controls.

Rats receiving laceration injury underwent a complete lateral hemisection with the use of a #11 feather stainless steel surgical blade (Feather Safety Razor Co., Ltd., Japan) on a #3 scalpel holder (Fine Scientific Tools, North Vancouver, Canada) on the left side of the spinal cord. The incision began at the midline, with the tip of the blade next to the dorsal blood vessel. The blade was lowered to the ventral surface of the spinal cord and pulled laterally, until a full lateral hemisection was produced. The incision was then examined underneath a microscope to confirm that the lesion was complete. After each contusion or laceration injury, the muscles of the injured rats were sutured in layers and stainless steel clips were used to seal the skin. Animals were placed on an isothermal pad until they regained awareness and then were injected with a mixture for hydration, pain relief and bacterial protection. All animals received manual bladder expression two times per day until their bladder response function was restored. All procedures were approved by the Institute of Animal Care and Use Committee at University of California, Irvine (IACUC# 2000-2168).

Histology

Animals were sacrificed 3 and 14 days post injury by intracardiac perfusion with 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA). The spinal cord was removed and post fixed in 4% paraformaldehyde for 24 hours and then transferred into 30% sucrose for 3 days. Transverse

sections of cords were cut 1 mm rostral and caudal to the injury site for embedding in OCT compound. The cryostat Leica CM1850 was used to slice embedded tissue at 20 μm. Sections were placed on bond rite slides.

Immunohistochemical Staining

OX-42 (or CD 11b) immunohistochemical staining was used to detect macrophages on tissues from the 3- and 14-day groups. Slides were washed with PBS 3x5 minutes and then tissue was blocked with 2% goat serum, 2% BSA in PBS for 1 hour. Afterward, tissue was incubated overnight at 4 °C in primary antibody mouse anti-rat CD11b diluted 1:100 in blocking solution (Serotec, Raleigh, NC). The following day, tissue was washed 4x5 minutes in PBS and incubated for 1 hour at room temperature in secondary antibody, Alexafluor 594 goat anti-mouse diluted in 1:200 PBS (Chemicon, Temecula, CA). Tissue was then washed 4x5 minutes in PBS and incubated in 1:5000 dilution of Hoescht for 5 minutes. Following incubation, tissue was washed 2x5 minutes in de-ionized water. Lastly, slides were air dried for 1 hour and coverslipped with permafluor.

Quantification of Macrophages

Macrophages were quantified in tissue sections at distances 80 μm, 680 μm and 880 μm from the epicenter of injury in both the rostral and caudal direction. Distances were chosen based on proximity to the injury site; 80 μm is close to the injury site, while 680μm and 880 μm are farther away from the injury site. The 880 μm distance was also chosen because we had previously transplanted OPCs at approximately 880 μm rostral and caudal in the contusion SCI (Keirstead et al., 2005). Digital images of the dorsal column, dorsal horn, lateral column, ventral column and ventral horn were taken in tissue from the 3- and 14-day groups (Figure 2). Images were captured at 40x magnification using an Olympus AX80 microscope and Olympus Microsuite Software. Images were sized to 200 μm and a 250x250 μm grid was produced to assist in touch counting. Macrophages were only counted in the six center boxes of the grid: B2, B3, B4, C2, C3, and C4 (Figure 3). The number of macrophages in each box and the average of the six boxes were recorded in an Excel spreadsheet.

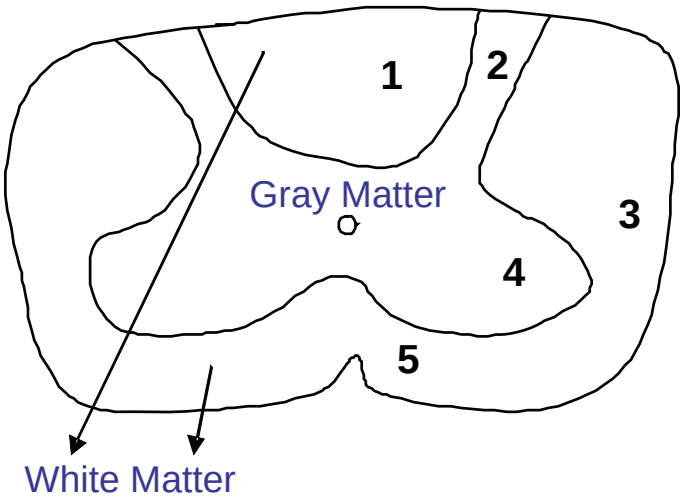

Figure 2

Illustration depicting the regions where images were captured: dorsal column (1), dorsal horn (2), lateral column (3), ventral (horn), and ventral column (5).

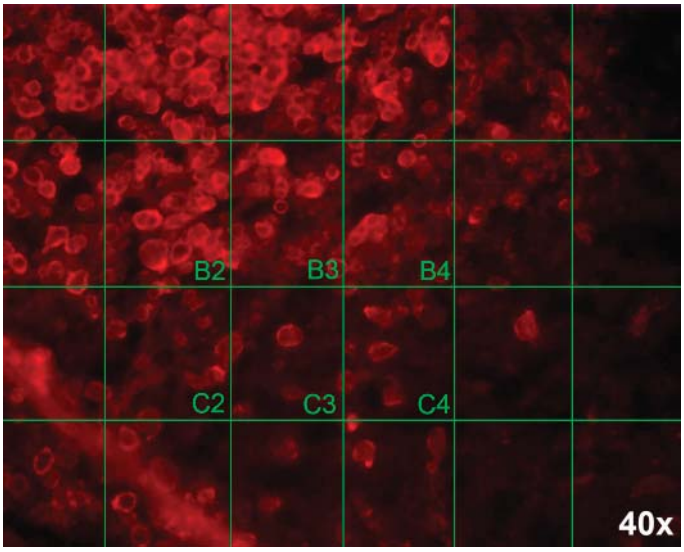

Figure 3

Macrophages found in contusion injury at day 3, in the ventral horn region towards the rostral direction. Grid illustrates quantified region. Image taken at 40x magnification.

Results

Quantification of macrophages reveals that the average number of macrophages per 0.25 mm² in all regions of the spinal cord is greater at three days post injury as compared to fourteen days post injury for both injury types. Additionally, contusion injury results in a significantly greater number of macrophages as compared to hemisection both 3 and 14 days post injury (Figure 4).

Macrophages were quantified in tissue sections at distances of 80 μm, 680 μm and 880 μm from the epicenter of the injury site in both the rostral and caudal direction. At 3 days post injury, there were more macrophages in the contusion injury than the hemisection injury in both the rostral and caudal direction. There were significantly more macrophages found in the contusion injury than the hemisection

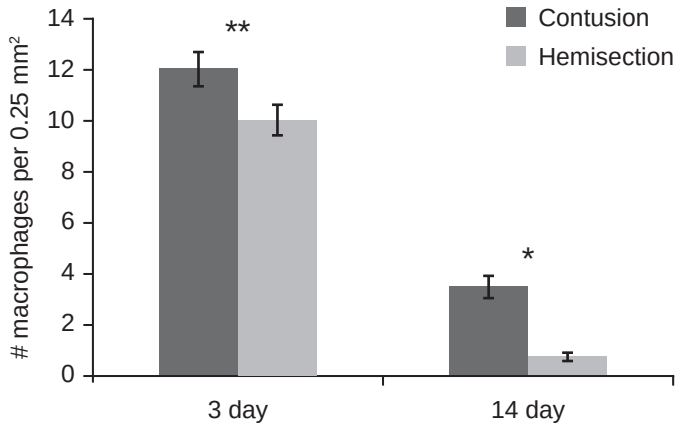

Figure 4

More macrophages are found in contusion SCI than hemisection SCI in both day 3 and day 14. ** p<0.05, * p<0.01

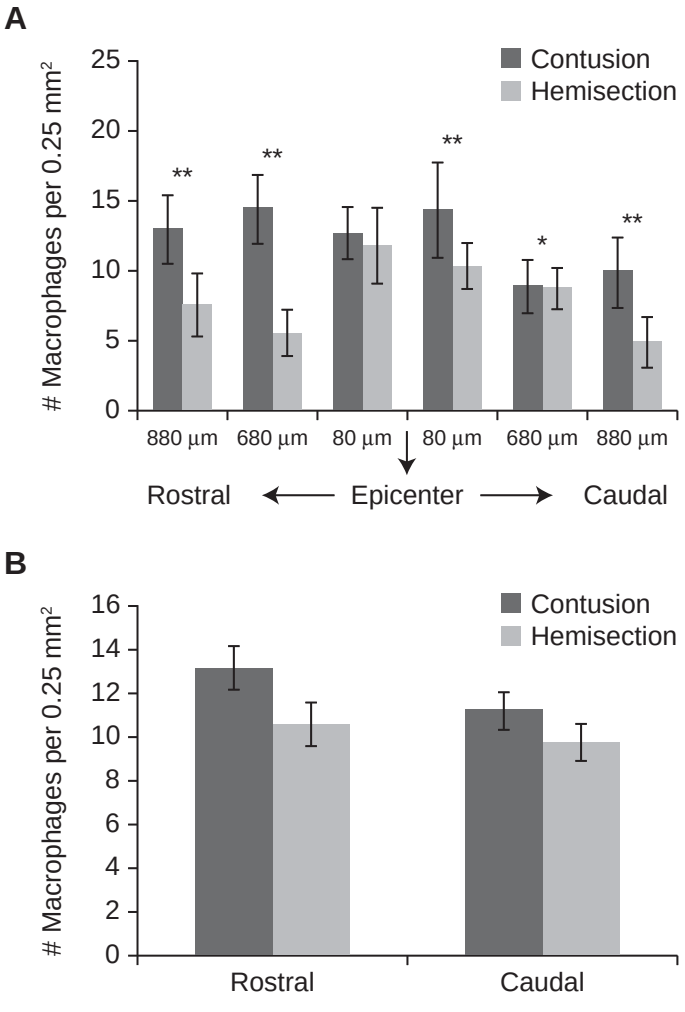

Figure 5

A) Macrophage response is significantly higher in contusion injury than hemisection for day 3 at various distances rostral and caudal from epicenter. B) There is no significant difference in the overall macrophage response between contusion and hemisection SCI in the rostral and caudal direction for day 3. p<0.05, * p<0.01

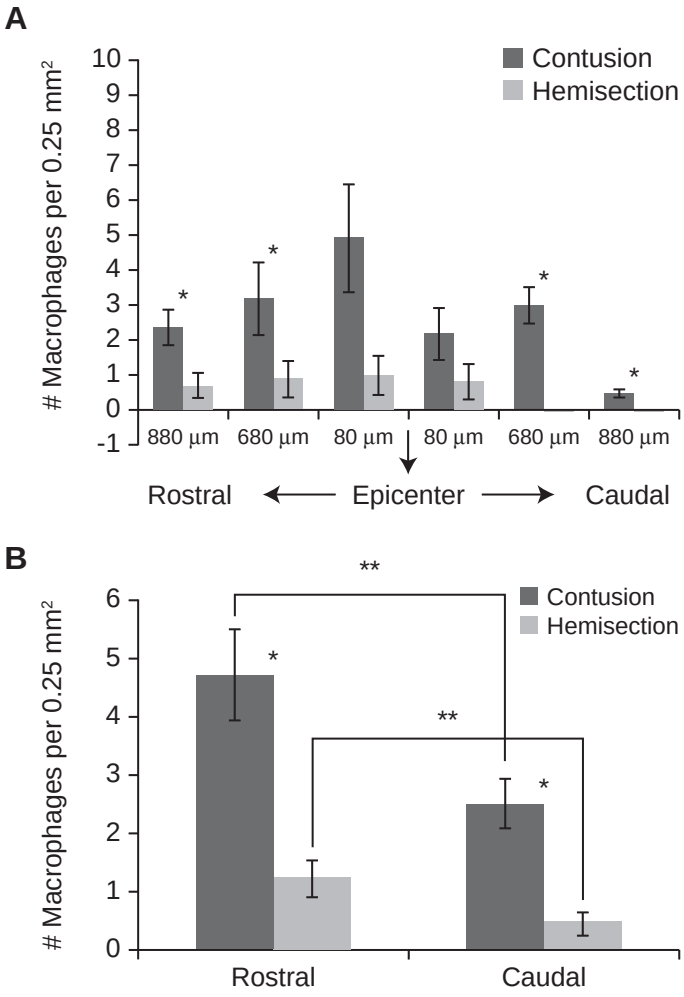

Figure 6

A) More macrophages are found in contusion than hemisection SCI at day 14 at different distances rostral and caudal from the epicenter. B) At day 14, there are significantly more macrophages found in contusion than hemisection SCI in both the rostral and caudal direction. There are also significantly more macrophages found in the rostral direction than caudal direction in contusion SCI. ** p<0.05, * p<0.01

injury 680 µm and 880 µm from the epicenter towards the rostral direction and 80 µm and 880 µm from the epicenter towards the caudal direction (Figure 5a). Subsequently, we averaged the number of macrophages in the rostral direction and the number of macrophages in the caudal direction for both injury types (Figure 5b). Comparing the two injury types with t-test analysis, we observed no significant difference in the overall number of macrophages between the contusion and hemisection SCI in both the rostral and caudal direction. There was also no significant difference in the number of macrophages in either the hemisection or contusion injury between the rostral and caudal directions.

At 14 days post injury, there were significantly ($p<0.01$) more macrophages in the contusion injury than the hemisection injury 680 μm and 880 μm from the epicenter towards both the rostral and caudal directions (Figure 6a). When we averaged the data for day 14 there were significantly ($p<0.01$) more macrophages found in the contusion SCI than the hemisection SCI in both the rostral and caudal directions as determined by t-test analysis (Figure 6b). Furthermore, there were significantly ($p<0.05$) more macrophages observed in the contusion SCI at the rostral end than the caudal end. There were also significantly ($p<0.05$) more macrophages found in the hemisection SCI at the rostral end than the caudal end. Thus, there are significantly more macrophages found in the contusion injury than hemisection 14 days after injury. In contrast, there are no significant differences observed three days after injury; however, there is an observable trend.

Data on white matter versus grey matter 3 days post injury (Figure 7a) showed that there were significantly ($p<0.05$) more macrophages in the grey matter region in both contusion and hemisection SCI than in the white matter. Furthermore, the contusion injury had statistically ($p<0.05$) more macrophages than the hemisection injury in the grey and white matter. Similarly, at 14 days post injury there were significantly ($p<0.01$) more macrophages in contused SCI than hemisection in both grey matter and white matter (Figure 7b).

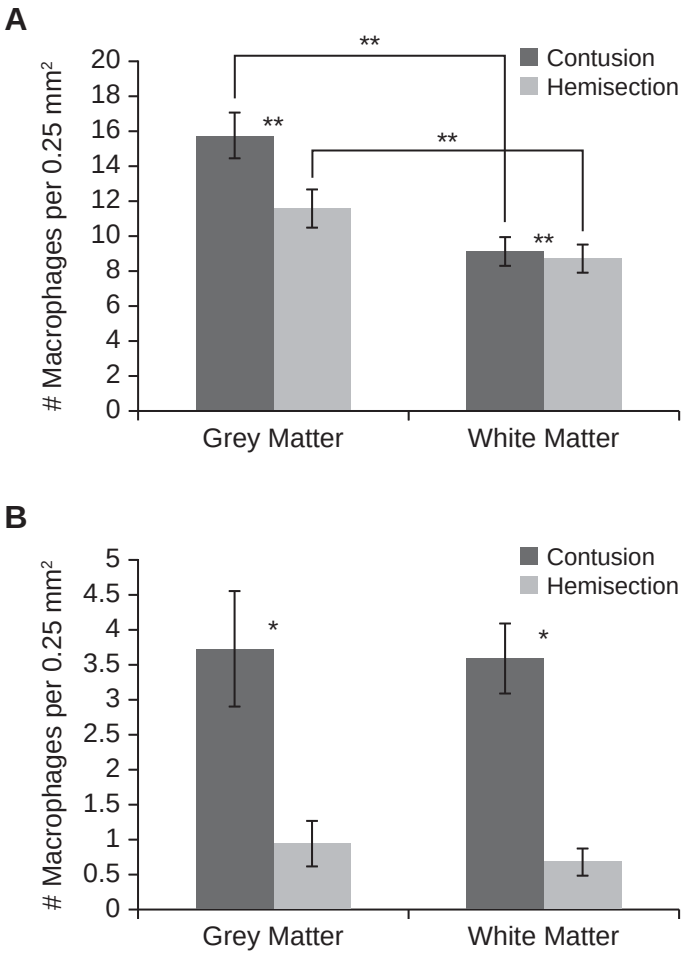

Figure 7

A) Macrophage response is significantly higher in grey matter than white matter for both SCI types at day 3. Additionally, there are statistically more macrophages in contusion than hemisection SCI in grey matter and white matter at day 3. B) There are significantly more macrophages found in contusion than hemisection SCI in grey matter and white matter at day 14. ** $p<0.05$, * $p<0.01$

Discussion

It has previously been shown that contusion and laceration injuries result in a different extent of pathologies (Siegenthaler et al., 2007). Contusion injury results in widespread pathology and demyelination, whereas laceration injury results in localized pathology and demyelination. One plausible explanation for this is differences in the extent of mechanical damage following these two injury types, which would lead to differences in the extent of the inflammatory response. The inflammatory response triggers immune cells, such as macrophages, to invade the injury site. Our findings indicate that there are significantly more macrophages in contusion injury than in hemisection injury. The robust macrophage response in the contusion injury correlates with the increased inflammatory response. The greater inflammatory response may contribute in turn to the enlargement of the area of pathology, increase in cell death, and greater amount of demyelination. Additionally, the fact that the inflammatory response is more widespread in contusion suggests that the initial mechanical damage is more widespread than in laceration. Differences in macrophage and inflammatory response in contusion and laceration SCI may elucidate their distinct pathologies and degree of demyelination. This information allows us to better classify the SCI types and may lead to the development of therapies tailored to each injury type.

In addition to examining the overall macrophage response in the two injury types, we analyzed the macrophage response in the grey matter and white matter. Our study reveals that there are more macrophages found in grey matter than white matter. In addition, there are more macrophages in the grey matter of the contusion injury than the laceration injury. These findings are consistent with greater blood vessel innervation in the grey matter than the white matter. When an SCI occurs, the blood-brain barrier

is broken and the immune system cells that usually circulate in the blood invade the surrounding tissue. As a result, an inflammatory response is triggered, which activates a macrophage response (Yamauchi et al., 2004). A blood-spinal cord barrier (BSB) disruption study found that BSB permeability was greatest at three days and greater in grey matter than in white matter, which correlates with our data (Popovich et al., 1996). The relationship between the BSB and grey matter might explain the increase in the number of macrophages in the grey matter and suggests that the macrophages are more likely to be blood-borne macrophages, rather than activated resident microglia.

Although data was only collected up to 0.88 mm rostral and caudal from the injury site, we found a high number of macrophages within this range. There were significantly more macrophages in the contusion than laceration injury, at distances farther from the injury site. This finding suggests that macrophage accumulation correlates with the extent of mechanical damage. We previously transplanted OPCs at 0.88 mm rostral and caudal from the epicenter of a contusion injury and found that the OPCs facilitated remyelination and locomotor recovery. Interestingly, there is a greater accumulation of macrophages in contusion SCI at 0.88 mm rostral and caudal from the injury site. Macrophages are known to release growth factors that are crucial for both neuronal survival and remyelination, and digest cellular debris after injury (Bouhy et al., 2006). This may indicate that macrophages found in the contusion injury produce a favorable environment for OPC differentiation and expansion and, in turn, enhance remyelination and locomotor function. Therefore, it appears that transplantation of OPCs may be a feasible approach to treating contusion SCI. However, this may not be the case for laceration SCI, which has less extensive mechanical damage and demyelination and, consequently, a weaker inflammatory response. As a result, a weaker macrophage response is produced that may not be sufficient to facilitate OPC differentiation, expansion, and migration. We therefore need to consider alternative approaches to treating laceration SCI.

Because we examined only a small range of distances surrounding the injury site, it would be wise to conduct additional studies on macrophage response beyond this region. Observing the macrophage response at greater distances may support our current findings or contradict the correlation between macrophage response and the extent of pathology in contusion and laceration SCI. It would also be beneficial to conduct studies that focus on the complete inflammatory response following the two SCI types. One study found that the immune system contributes to lesion expansion and simultaneously activates inflammatory cells that assist in repair processes, such as remyelination (Jones et al., 2005). Clearly, investigating the role of the immune response following SCI is critical in understanding the distinct pathology of different SCI models and the development of novel treatments.

In summary, there is a greater macrophage response in contusion injuries than laceration injuries, which may be attributed to a greater inflammatory response following the initial injury. Because laceration injury results in a localized lesion, we suspect that the injury produces a weaker inflammatory response than contusion injury and, therefore, fewer macrophages are observed in the laceration SCI. This may also indicate that the inflammatory response and macrophage response influence remyelination in SCI. After examining the macrophage response in grey and white matter, we found that there were more macrophages in the grey matter. This finding suggests that the macrophages are blood-borne, rather than microglial. It would be of interest to further investigate the relationship between grey matter and macrophages following SCI and its possible involvement in remyelination. More importantly, our data suggests that there is a correlation between the macrophage response and the different extent of pathologies in the two distinct injury types. This information is useful in further categorizing the two injury types and facilitating the design of better targeted SCI therapies.

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