FTY720 Reduces Inflammation and Promotes Functional Recovery after Spinal Cord Injury

Kangmin D. Lee,¹ Woon N. Chow,² Carmen Sato-Bigbee,³ Martin R. Graf,¹
Robert S. Graham,¹ Raymond J. Colello,² Harold F. Young,¹ and Bruce E. Mathern¹

Abstract

A robust and complex inflammatory cascade is known to be a prominent component of secondary injury following spinal cord injury (SCI). Specifically, the concept of trauma-induced autoimmunity has linked the lymphocyte population with neural tissue injury and neurologic deficit. FTY720, a sphingosine receptor modulator that sequesters lymphocytes in secondary lymphoid organs, has been shown to be effective in the treatment of a variety of experimental autoimmune disorders. Accordingly, by reducing lymphocyte infiltration into the spinal cord following SCI, this novel immunomodulator may enhance tissue preservation and functional recovery. In the present study, a moderate to severe contusion SCI was simulated in adult Long-Evans hooded rats. Using flow cytometry we showed that daily FTY720 treatment dramatically reduced T-cell infiltration into the SCI lesion site at 4 and 7 days post-injury, while other inflammatory cell populations were relatively unaltered. To assess functional recovery, three groups of injured animals (treated, vehicle, and injury only) were evaluated weekly for hindlimb recovery. Animals in the treated group consistently exhibited higher functional scores than animals in the control groups after 2 weeks post-injury. This finding was associated with a greater degree of white matter sparing at the lesion epicenter when cords were later sectioned and stained. Furthermore, treated animals were found to exhibit improved bladder function and a reduced incidence of hemorrhagic cystitis compared to control counterparts. Collectively these results demonstrate the neuroprotective potential of FTY720 treatment after experimental SCI.

Key words: behavioral assessment; FTY720; inflammation; locomotor function; spinal cord injury

Introduction

THE PATHOPHYSIOLOGY OF SPINAL CORD INJURY (SCI) has classically been partitioned into primary and secondary injury processes. After the initial primary injury, secondary injury encompasses an insidious cascade of cellular and biochemical events, the magnitude of which is rivaled only by its complexity. Within this milieu of events, inflammation has been identified as an important component of secondary injury, and numerous cell populations have been implicated as mediators of inflammation. Among these cells, lymphocytes have generated interest due to their key involvement in the concept of trauma-induced autoimmunity (Popovich et al., 1996; Gonzalez et al., 2003; Ankeny et al., 2006). This concept is based on the hypothesis that traumatic injury results in the release of neural self-antigens and subsequent immunological activation. In support of this possibility, injection into naïve animals of T cells isolated from rats subjected to SCI resulted

in central nervous system (CNS) tissue injury and neurological deficit (Popovich et al., 1996).

Many of these studies of SCI and autoimmunity have drawn on parallels from our current knowledge of multiple sclerosis (MS), and the animal model of experimental autoimmune encephalomyelitis. In both of these processes, T cells are known to infiltrate the CNS, resulting in demyelination and neurologic dysfunction. Targeted therapies for MS have therefore focused on immunomodulation, specifically in the T-cell population.

FTY720 administration is one of the newest immunomodulatory therapies for MS, and is currently undergoing phase III clinical trials as monotherapy. FTY720 is a chemical derivative of the fungal metabolite myriocin, and in its phosphorylated form acts as a sphingosine-1-phosphate (S1P) mimetic and agonist of the S1P receptor (Brinkmann et al., 2002). Several groups have shown that administration of FTY720 results in a significant reduction in peripheral

¹Department of Neurosurgery, ²Department of Anatomy and Neurobiology, and ³Department of Biochemistry and Molecular Biology, Virginia Commonwealth University, Richmond, Virginia.

lymphocyte counts (Pinschewer et al., 2000; Brinkmann et al., 2000; Chiba et al., 1998). It has been postulated that FTY720 induces this lymphopenia by preventing lymphocyte egress from peripheral lymphoid organs. Once FTY720 binds to the S1P receptor, internalization and subsequent degradation of the receptor occurs (Matloubian et al., 2004). This degradation thereby eliminates the S1P signal necessary for lymphocyte egress and prevents recirculation into the CNS (Brinkmann et al., 2002; Matloubian et al., 2004; Mandala et al., 2002).

Independent of this ability to prevent lymphocyte migration, FTY720 has also been shown to have other cytoprotective capabilities. Studies have shown that FTY720 can prevent apoptosis in neural cells and act on endothelial cells to preserve vascular integrity (Brinkmann et al., 2004; Fujino et al., 2003; Coelho et al., 2007). The exact mechanisms of these effects are still under investigation.

In this study we examined the efficacy of FTY720 in a moderate to severe contusion model of SCI. We hypothesized that administration of FTY720 would reduce infiltration of T cells into the spinal cord lesion site and enhance functional recovery after SCI. Our results indeed demonstrate the efficacy of FTY720 in a model of CNS trauma, and show that administration enhances tissue preservation and functional outcome after SCI.

Methods

Animal model and surgical procedures

Adult Long-Evans hooded rats (70 $\pm$ 10 days of age) were used for the spinal cord injury protocols. All surgical and postoperative care procedures were performed in accordance with the Virginia Commonwealth University Institutional Animal Care and Use Committee. The animals were endotracheally intubated and maintained under inhalational anesthesia (2.5% isoflurane) during all surgical procedures. Depth of anesthesia was tested during surgical procedures by tail pinch and observation for reflexive movements. Body temperature was kept normothermic using a homeothermic blanket. Using sterile technique, a dorsal midline incision was made over the mid-thoracic spine and a standard T8 laminectomy was performed. The animals were then placed in a spinal frame and the exposed cord was contused with the MASCIS impactor using a 10-g weight dropped from a height of 25 mm. Animal injuries with unacceptable impact parameters were excluded from the study. Immediately after injury, the animals were randomized to receive an IP injection of FTY720 (1 mg/kg) (Cayman Chemical, Ann Arbor, MI) solubilized in 0.1% DMSO (Sigma-Aldrich, St Louis, MO), vehicle (0.1% DMSO), or no injection (injury only). Animals were then allowed to recover in a separate recovery area with a warming blanket.

All animals received prophylactic gentamicin (Butler Health Supply, Dublin, OH) 5 mg/kg before surgery and daily thereafter for 7 days. Manual bladder expression was performed three times daily for the first week after surgery and two times daily thereafter until spontaneous micturition was re-established. Urine volumes were recorded at each collection and spontaneous micturition was defined as established when daily volumes collected were <2 mL total for 3 consecutive days. Expressed urine was also monitored for evidence of urinary tract infection by the presence of elevated

urine pH or leukocytes, and treated with a 5-day course of gentamicin (5 mg/kg) if diagnosed.

Postoperatively, all animals were given easy access to food and water and housed two per cage. Analgesic medication (Tylenol oral suspension, 2 mg/mL) was mixed into the drinking water and administered for the first 3 days post-injury (dpi).

Flow cytometry

For flow cytometry, injured animals received daily IP injections of either FTY720 (n = 5) or vehicle (n = 5) until sacrificed. On the first, fourth, and seventh days post-injury, animals were sacrificed with a lethal dose of pentobarbital (Sigma-Aldrich). A 1-cm section of the cord peri-centric to the impact site as well as the spleen were then harvested. Both the cord and spleen tissue were individually dissociated by passage through a 70- μ m filter and washed twice with PBS. Erythrocytes were lysed with Tris-buffered ammonium chloride (PharmLyse; BD Biosciences, San Jose, CA). Leukocytes were identified as round, phase-bright entities with a diameter of $\sim$ 10 μ m that excluded trypan blue and were enumerated with a hemocytometer. Antibodies used for phenotypic analysis were anti-CD3, -CD4, -CD8 α , -CD11b, and -His48, and were biotinylated or conjugated to R-phycoerythrin or fluorescein isothiocyanate. Streptavidin conjugated to allophycocyanin was used for the detection of biotinylated antibodies (all from BD Biosciences). Approximately 1×10^6 cells were stained with 0.5 μ g of each monoclonal antibody as previously described (Graf et al., 2001). Cells were then fixed and permeabilized (Cytotfix/Cytoperm; BD Biosciences), washed with PBS, and stained with the DNA-specific dye 7-aminoactinomycin (7-AAD; 10 μ g/mL). Three-color fluorometric analysis was performed using a FACSCanto flow cytometer (BD Biosciences). Cells were identified as 7-AAD-positive events on a FL3 versus side-scatter histogram as described by Lipton and associates (2005), and 10,000 7-AAD-positive events were analyzed. T-cell subsets were identified as CD3⁺/CD4⁺ or CD3⁺/CD8⁺ double-positive cells. Monocytes and granulocytes were identified as CD11b⁺ and His48⁺ cells, respectively.

Behavioral outcome assessment

All animals were gentled in the open field twice a day for 7 consecutive days prior to injury. Animals randomized to the treated group (n = 11) received daily IP injections of FTY720 1 mg/kg for 4 weeks. Vehicle-group animals (n = 10) received daily IP injections of 0.1% DMSO for the same time period. The injury-only group (n = 9) received no injections. T8 laminectomies were performed on sham animals (n = 2), but no injuries were performed and no injections administered. At 2 and 7 dpi and once weekly thereafter for a total of 6 weeks, the rats were placed in the open field and observed for 4 min. Two researchers blinded to treatment group observed the animals in open-field testing. Gross locomotor recovery after SCI was assessed using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). Observations for each hindlimb were scored and averaged to provide a single score for each animal per session. All data were collected within the 4-min period. Once a plateau in locomotor recovery was evident ($\geq$ 28 dpi), a frequency analysis of plantar stepping and coordination was performed across all groups.

FIG. 1. FTY720 treatment reduces T-cell infiltration into the spinal cord after injury. After SCI, animals were injected with either vehicle or FTY720 for 1, 4, or 7 days. A 1-cm segment of the cord centered on the impact site was then harvested from each animal and prepared for flow cytometry. (A) Representative dot plots of the injury site of a vehicle-injected animal showing the 7-AAD+ population used for analysis (gate P5). (B) Representative dot plots of the injury site of a vehicle-injected animal showing the percentages of CD3⁺/CD4⁺ double-positive cells (gate Q2) and of (C) CD3⁺/CD8⁺ double-positive cells (gate Q2) within the 7-AAD+ gate. (D) A graphic summary of the mean percentages of 7-AAD+ events that were CD3⁺/CD4⁺ and CD3⁺/CD8⁺ for treated and vehicle animals at the various time points. As shown by this flow cytometric paradigm, FTY720 treatment reduces infiltration of both CD3⁺/CD4⁺ helper and CD3⁺/CD8⁺ cytotoxic T-cell populations into the cord after SCI (* $p < 0.05$ compared with vehicle-injected rats).

All animals were also tested on the inclined plane task. This task evaluates the animal's ability to maintain a horizontal body position on an inclined board. The angle of the board is incrementally raised until the animal is no longer able to maintain the horizontal position. Performance on the inclined plane correlates with the integrity of the rubrospinal tract (and other nonpyramidal pathways) after SCI. During testing, animals were placed in the right- and left-side-up positions. The maximum angle at which the animal was able to maintain position on the board for 10 sec was recorded for each side. Data were recorded as the average of the two sides to obtain a single score for each animal per session.

Analysis of myelin sparing

At 6 weeks post-injury, the animals were sacrificed with a lethal dose of pentobarbital and perfused transcardially with 125 mL of PBS (pH 7.4), followed by 250 mL of 4% paraformaldehyde. Spinal cords were harvested and post-fixed for 2 h in the same fixative before being immersed in PBS (0.2 M) overnight. The cords were then cryoprotected in 30% sucrose for 48 h. FTY720-treated ($n = 9$) and vehicle-treated ($n = 8$) cords were blocked and embedded together in optimal cut-

ting temperature compound (Thermo Shandon, Pittsburgh, PA), and sectioned at a thickness of 20 μ m. A complete series of axial sections spanning the injury site was collected and stained with eriochrome cyanine to assess for myelin sparing. Also, axial sections peripheral to the injury epicenter were collected at 0.5-mm intervals and stained with eriochrome cyanine. For each spinal cord, the lesion epicenter was qualitatively defined by a blinded researcher as the section containing the least amount of intact tissue. This section and sections located 100 μ m rostral and caudal were chosen to quantify the area of spared myelin at the epicenter. Light microscopy at 25 $\times$ magnification was used to visualize the sections, and images were obtained with an Olympus DP12 camera (Olympus America Inc., Center Valley, PA). These images were then imported into the NIH Image J program and white matter sparing analysis was performed by a blinded observer. The percentage of myelin spared was calculated by manually outlining the spared white matter regions, which then generated an area as denoted by the number of pixels. This area was then divided by the total cross-sectional area of the axial section. The average of the three sections was used to denote the percentage of myelin spared at the injury epicenter. In the same fashion as described above, sections rostral and

FIG. 2. FTY720 promotes hindlimb recovery after SCI. After SCI, the animals were randomly placed in one of three groups: injury only, vehicle, and FTY720-treated. Functional assessments were performed weekly for 6 weeks. **(A)** Open-field assessment of hindlimb locomotor function using the BBB scale. Starting at week 3, treated rats as a group performed significantly better than the control groups. No significant differences were noted between the vehicle and injury only groups at any time point. **(B)** The inclined-plane test. Treated animals outperformed members of the control groups starting at week 2, and continued to do so for the duration of the study. **(C)** For all time points, linear regression analysis revealed a very strong correlation between the BBB and inclined-plane scores ($r=0.87$; $*p < 0.05$ compared with injury-only and vehicle rats).

caudal to the epicenter at 0.5-mm intervals were analyzed for white matter sparing.

Statistical analysis

Data are reported as means $\pm$ standard error of the mean. All statistical analyses were performed using statistical software (SPSS 16.0; SPSS Inc., Chicago, IL). Data from open-field, inclined plane, and urine volumes were analyzed by repeated measures two-way ANOVA with the post-hoc pairwise comparison test. Days to spontaneous voiding was analyzed by one-way ANOVA with post-hoc Tukey analysis. Fisher's exact test was used for hematuria incidence and locomotor frequency analysis. For flow cytometry and myelin sparing analysis, data were compared using Student's *t*-test. Differences with a *p* value < 0.05 were considered statistically significant.

Results

FTY720 reduces T-cell infiltration into the spinal cord lesion site after injury

FTY720 is known to be a potent immunomodulator and numerous reports have shown that administration reduces peripheral lymphocyte circulation (Pinschewer et al., 2000; Brinkmann et al., 2000; Chiba et al., 1998). In order to establish the efficacy of FTY720 treatment in a model of SCI, we quantified inflammatory cell infiltration into the injured spinal cord using flow cytometry. Data from this analysis showed that FTY720 administration significantly reduced the infiltration of CD4⁺ T-helper cells to the spinal cord lesion site compared to vehicle administration at 4 dpi (7.56 ± 0.25 versus 3.80 ± 0.5 ; $p < 0.001$; Fig. 1D). This response was similar to the reduction seen in the CD8⁺ cytotoxic T-cell population (7.80 ± 1.01 versus 3.68 ± 1.31 ; $p < 0.01$; Fig. 1D). At 7 dpi, a similar reduction was seen in the CD4⁺ T-helper cells (10.01 ± 1.40 versus 6.40 ± 0.72 ; $p < 0.05$), and in the CD8⁺ cytotoxic T-cell population (8.51 ± 0.51 versus 6.01 ± 0.40 ; $p < 0.01$). However, there was no difference noted in either cell population at 1 dpi. For all time points, no statistical difference was noted between vehicle and FTY720 administration in the spleen tissue.

In order to evaluate for other inflammatory cell populations infiltrating the spinal cord after injury, antibody labeling for His48 (granulocytes) and CD11b (monocytes) was performed (data not shown). A slight trend toward reduction was observed with FTY720 administration in the His48+ (24 ± 2.4 versus 20.2 ± 1.9 ; $p = 0.29$) and the CD11b+ cell populations (40.3 ± 1.47 versus 32.8 ± 7.5 ; $p = 0.38$). However, neither of these differences were statistically significant, suggesting that FTY720 specifically inhibits T-cell infiltration.

FTY720 promotes hindlimb recovery after spinal cord injury

Once we established the efficacy of FTY720 in a model of SCI, our next step was to determine the extent to which administration would impact functional recovery. Hindlimb function was therefore evaluated for 6 weeks after SCI using the open-field and inclined-plane tests. During the acute and intermediate recovery stages, no differences in BBB locomotor scores were noted among groups (Fig. 2A). However, by

TABLE 1. ANIMALS TREATED WITH FTY720 ARE MORE LIKELY TO REACH LOCOMOTOR MILESTONES

	28 dpi		35 dpi		42 dpi	
	PS	C	PS	C	PS	C
Injury only	44.4% (4/9)	11.1% (1/9)	55.6% (5/9)	11.1% (1/9)	66.7% (6/9)	33.3% (3/9)
Vehicle	50% (5/10)	10% (1/10)	70% (7/10)	20% (2/10)	80% (8/10)	20% (2/10)
Treated	81.8% (9/11)*	63.6% (7/11)**	100% (11/11)*	63.6% (7/11)**	100% (11/11)*	81.8% (9/11)**

Frequency analysis of rats achieving consistent plantar stepping (PS) or occasional coordination (C) between 4 and 6 weeks after injury.

* $p < 0.05$ compared with injury-only and vehicle-treated rats.

** $p < 0.005$ compared with injury-only and vehicle-treated rats.

21 dpi the mean BBB score in the FTY720-treated animals surpassed those of the vehicle animals by 1.4 (11.5 versus 10.1; $p < 0.05$). Moreover, this difference was sustained throughout the remainder of the analysis, even after drug treatment was discontinued at 28 dpi. By the end of the analysis, the mean difference between treated and vehicle groups was 1.5 (12.5 versus 11.0; $p < 0.005$). No differences were noted between the vehicle and injury-only groups at any time point.

A frequency analysis of the open-field data is shown in Table 1, and reflects differences in locomotor milestones between the groups once recovery reached a plateau. For each time point post-injury analyzed, a significantly greater proportion of animals was consistently plantar stepping (BBB ≥ 11) or stepping with occasional coordination (BBB ≥ 12) in the FTY720-treated group compared to the vehicle and injury-only groups. By 42 dpi, all animals in the FTY720-treated group were consistently plantar stepping, and 81.8% exhibited at least occasional coordination (BBB ≥ 12). In contrast, only 80% of animals in the vehicle group exhibited consistent plantar stepping, and only 20% were at least occasionally coordinated.

Performance on the inclined plane mirrored open-field testing performance with the exception that differences were noted at earlier times along the study (Fig. 2B). By 14 dpi the mean difference between the treated and vehicle groups was 6.5° (35.5° versus 29° ; $p < 0.0001$). Average angles were consistently higher in the treated group for the duration of the study, and at 42 dpi the mean difference between the treated and vehicle groups was 8.6° (43.9° versus 35.3° ; $p < 0.0001$).

For both the open-field and inclined-plane tests, no significant difference in scores was detectable at any time point between the vehicle and injury-only control groups. Linear regression analysis revealed a very strong correlation between the BBB and inclined-plane scores for all time points (Fig. 2C; $r = 0.87$).

FTY720 improves bladder function after spinal cord injury

A well-known consequence of SCI in both animals and humans is bladder dysfunction. Using an animal model of SCI, Leung and associates (2007) showed that injury severity could be directly correlated with degree of bladder dysfunction, which was reflected in the volume of urine collected during manual expression. We therefore sought to utilize bladder function as another measure of functional recovery. Figure 3A shows that all animals had impaired bladder function immediately after SCI. However, animals in the treated group had significantly lower mean urine volumes

expressed compared to the vehicle and injury-only groups ($n = 10$ for each group; $p < 0.0001$). Post-hoc analysis also revealed no statistical difference between the vehicle and injury-only groups in terms of daily urine volumes collected.

For all animals, daily bladder expressions were performed until total daily volume collected was less than 2 mL for 3 consecutive days, indicating return of spontaneous bladder function. Intergroup comparison of the number of days required to reach this milestone revealed that animals in the treated group required fewer days compared to the vehicle and injury-only groups (Fig. 3B) (6.2 ± 0.4 versus 10.3 ± 0.6 and 9.8 ± 0.74 ; $p < 0.0001$). No statistically significant difference was noted between the vehicle and injury-only groups.

For each urine collection, urine color was also recorded. Retrospective analysis of the incidence of gross hematuria revealed that FTY720 treatment significantly reduced the incidence of gross hematuria compared to the vehicle and injury-only groups (Fig. 3C) (45.5% versus 90% versus 100%, respectively; $p < 0.005$ by Fisher's exact test). No difference was noted when the vehicle and injury-only groups were compared with each other.

FTY720 and body weight

One important aspect of assessing the utility of an immunosuppressant drug is determining whether the drug has an unacceptable level of toxicity. Many immunomodulators, although useful in their ability to reduce inflammation, are limited by their toxicity profile. Animal body weight was used as an indicator of general toxicity and monitored throughout the course of the study. Decreases in body weight can indicate failure to thrive, which would then imply drug toxicity. However, no significant differences in percentage change in body weight were noted among the three treatment groups studied (Fig. 4).

Locomotor recovery correlates with myelin sparing

In order to correlate locomotor recovery results with histological findings, we performed myelin sparing analysis of the lesion epicenter. Animals were sacrificed at the end of the final open-field testing and spinal cords were harvested for eriochrome cyanine staining. Due to the lack of differences detected in behavioral outcome between the vehicle and injury-only groups, we confined our analysis to the treated ($n = 9$) and vehicle groups ($n = 8$) for comparison. The data showed that FTY720 treatment resulted in a significant increase in the percentage of myelin spared (28.5 ± 1.6 versus 20.8 ± 1.7 , $p < 0.005$; Fig. 5C). By linear regression analysis, final BBB scores also had a very strong correlation with the

percentage of myelin spared at the epicenter (Fig. 5D; $r = 0.75$).

Discussion

The data presented in this study demonstrate that FTY720 administered after SCI dramatically reduces T-cell infiltration into the spinal cord. Furthermore, the results of behavioral outcome analyses revealed that treatment with FTY720 was effective in enhancing hindlimb and bladder recovery. Interestingly, the effect of FTY720 on hindlimb function was evident during the chronic phase of recovery, in contrast to the

FIG. 4. FTY720 treatment does not adversely affect body weight. Animal body weights were measured and recorded throughout the duration of the behavioral outcome study. Treatment with FTY720 did not affect body weight following SCI. The x-axis is presented on a logarithmic scale.

effect on bladder function, which was noted during the acute recovery phase. Results from morphometric analysis also provided support for the neuroprotective capacity of FTY720 and revealed that white matter preservation is enhanced with treatment.

Our initial step in establishing the efficacy of FTY720 in SCI was to determine whether treatment would affect lymphocyte infiltration into the spinal cord after injury. Analysis by flow cytometry revealed that treatment with FTY720 after SCI significantly reduced T-cell infiltration into the spinal cord (Fig. 1), whereas granulocyte and monocyte populations were relatively unaltered. These data are in agreement with previously reported studies that showed that FTY720 had differential effects on CD4⁺ and CD8⁺ T cells, and minimal effects on peripheral blood counts of granulocytes and monocytes (Kahan et al., 2003; Budde et al., 2003; Bohler et al., 2004; Quesniaux et al., 1999). The degree of lymphocyte reduction has been shown to be dose-dependent, but previously reported reduction ranges of 30–70% (Bohler et al., 2004; Quesniaux et al., 1999) are in good agreement with the data reported in this study of 50–59%.

FIG. 3. FTY720 improves bladder function after SCI. Manual bladder expression was performed three times daily for the first week after SCI, and two times daily thereafter until spontaneous micturition was re-established. The residual urine volumes of each animal were measured and recorded during each expression session to provide an average daily urine volume for the first 2 weeks post-SCI. Furthermore, during urine collection gross hematuria was noted when observed. (A) The mean residual urine volumes of treated rats were consistently and significantly lower than those of the control groups. (B) The number of days required until spontaneous micturition was re-established as defined by an average daily volume of <2 mL for 3 consecutive days. Treated animals did not require extended assistance in expelling urine and regained spontaneous bladder function by day 6, while control animals did not reach this milestone until day 10. (C) FTY720 treatment significantly reduced the incidence of gross hematuria compared to the control groups (* $p < 0.05$ compared with injury-only and vehicle-treated rats).

FIG. 5. Myelin sparing correlated with locomotor recovery. At the conclusion of the behavioral outcome study (6 weeks post-SCI), the animals were sacrificed and cords were harvested and cryosectioned. Sections at the level of the SCI epicenter were stained with eriochrome cyanine (EC) to determine the extent of white matter sparing. **(A)** A representative EC-stained cross section at the level of the SCI epicenter from a vehicle animal. **(B)** A comparable section from a FTY720-treated animal. **(C)** At the level of the SCI epicenter, FTY720 treatment resulted in a significant increase in the percentage of spared myelin compared to vehicle treatment. **(D)** A linear regression analysis of the final BBB locomotor scores and spared myelin percentages at the epicenter exhibited a very strong correlation ($r=0.75$). **(E)** In addition to the myelin-sparing analysis completed at the injury epicenter, adjacent cord sections at every 0.5 mm up to 5 mm away from the epicenter were similarly processed and analyzed. This analysis revealed that FTY720 treatment resulted in a significant increase in the percentage of spared myelin compared to vehicle treatment in adjacent areas of the injury epicenter, especially in regions caudal. Negative distances on the x-axis denote rostral direction, whereas positive distances denote caudal direction (scale bars 300 μ m; * $p < 0.05$ compared with vehicle rats).

Our flow cytometry results clearly demonstrated the efficacy of FTY720 as an immunomodulator in the post-SCI inflammatory response. However, the question remained as to whether this would have a beneficial or detrimental effect on functional recovery. Although inflammation is a well-established sequela of CNS trauma, its role in recovery has been somewhat controversial. Both beneficial and detrimental effects have been associated with the inflammatory process. Through the release of cytokines and other factors, inflammatory cell populations such as neutrophils and macrophages have long been associated with CNS toxicity (Bao and Liu, 2002; Chao et al., 1992; Donnelly and Popovich, 2008; Shamash et al., 2002). Various groups have further demonstrated that attenuation of these inflammatory mediators is beneficial and promotes neurological recovery (Beril et al., 2007; Blight, 1994; Giulian and Robertson, 1990; Gris et al., 2004; Pannu et al., 2005; Popovich et al., 1999). However, it has also been suggested that inflammation may play a dual role in neural recovery and may have significant beneficial aspects. As surveyors of the CNS, microglia have been hypothesized to have a clearing mechanism after SCI, eliminating debris and reducing the presence of any factors that might play an inhibitory role in CNS repair. These cells are known for their cytotoxic capabilities, but they have also been shown to secrete trophic factors for both neurons and glia (Banati and Graeber, 1994; Kreutzberg, 1996). There is also evidence that macrophages may have a neuroprotective role in the CNS (Yin et al., 2006; Rimaniol et al., 2000).

The concept of "protective autoimmunity" has been championed by some, with the implication that enhancement rather than attenuation of the immune response is necessary to promote CNS recovery (Schwartz and Kipnis, 2001, 2002; Yoles et al., 2001). Experimental data revealed that augmentation of the immune response via passive or active immunization with myelin basic protein limited secondary degeneration and improved functional recovery after SCI (Hauben et al., 2000, 2001). As a result, efforts have been made to design vaccines for various neurodegenerative diseases, as well as for CNS trauma (Lee et al., 1999; Schwartz, 2001). However, the concept of protective autoimmunity also runs counter to the classic concept of T cells as pathologic entities. Using T cells isolated from spinal-injured rats, Popovich and colleagues (1996) demonstrated that injection into naïve rats resulted in neurological deficits and pathology reminiscent of experimental autoimmune encephalomyelitis. Subsequent studies using transgenic mice and athymic nude rats also support the traditional view of the T cell as a pathogenic entity (Jones et al., 2002; Potas et al., 2006). Interestingly, independent efforts to replicate the original experimental vaccine protocols of Hauben and associates (2000, 2001) failed to demonstrate any benefit, and in fact revealed that functional recovery was impaired with vaccination (Jones et al., 2004). This has raised some questions regarding the safety and utility of intentionally activating CNS-reactive T cells (Popovich and Jones, 2003; Jones et al., 2004).

It is clear therefore that the only conclusion that can be made regarding the inflammatory response is that it is quite complex, and these studies only bolster the notion that the role of inflammation cannot be succinctly distilled as either beneficial or detrimental. In this study we showed that administration of FTY720 dramatically reduced T-cell infiltration into the spinal cord lesion site and enhanced locomotor recovery

as well as tissue preservation after SCI. Although the locomotor enhancement observed in the open-field and inclined-plane tests was modest, frequency analysis (Table 1) of the open-field data highlighted the differences between treatment groups. By the end of the study, the vast majority of animals in the treated group (82%) exhibited at least occasional coordination, whereas only a minority of animals in the vehicle group (20%) exhibited this milestone. A previously reported study by Gonzales and colleagues (2003), using a mouse spinal hemitranssection model, also showed similar results after T-cell infiltration was inhibited using a neutralizing antibody to a T-cell chemoattractant chemokine. Similarly, Ibarra and associates (2003) showed that pharmacological inhibition of T-cell responses to spinal cord antigens using cyclosporine improved functional recovery after SCI.

The results of this study would then imply that the T cells attenuated by FTY720 are deleterious to the process of recovery. Since FTY720 is not specific for all inflammatory cell populations, it may be that treatment is inhibitory to pathologic lymphocytic populations only, and not to that subset that may have a beneficial role. However, it is clear that the mechanism of action of FTY720 is not solely limited to immunomodulation. *In-vitro* studies have shown that FTY720 directly protects oligodendrocyte progenitor cells from apoptosis (Coelho et al., 2007). Studies have also shown that FTY720 acts on endothelial cells and helps to preserve vascular integrity and barrier function (Brinkmann et al., 2004). It is therefore possible that these actions of FTY720 on vascular integrity and oligodendrocyte cytoprotection could also play a role in the observed effects of the drug treatment.

Another observation that was made in our study was the effect of FTY720 on bladder function. Drug treatment dramatically reduced post-void residuals immediately after SCI, and reduced the number of days required to regain spontaneous bladder function. This observation suggests a direct drug effect on bladder function independent of spinal cord recovery. A link between FTY720 and bladder function is supported by *in-vitro* studies of rabbit bladder smooth muscle, which showed that FTY720 can regulate detrusor muscle tone (Waterson et al., 2007). We also noted a dramatic reduction in the incidence of hemorrhagic cystitis after SCI. This phenomenon is known to occur after experimental SCI, but the mechanism of this process has yet to be well characterized. Experimental studies have linked inflammatory changes in the spinal cord with disruption of bladder epithelium and hemorrhage (Apodaca et al., 2003; Doggweiler et al., 1998). Interestingly, studies have shown that FTY720 can act on endothelial cells and help to preserve vascular integrity and barrier function (Brinkmann et al., 2004). Thus it is possible that this preservation of vascular integrity is responsible for the reduced incidence of hemorrhagic cystitis observed in this study.

The effect of FTY720 on bladder function, although dramatic, represented a possible confounder and limitation of this study. Overall, fewer UTIs were diagnosed in the treated group than in the vehicle group, which was reflected in the number of antibiotic courses administered (6 versus 14, respectively). This difference is hypothesized to be due to the robust effect of treatment on bladder function. Animals that were able to void more efficiently would retain less urine and therefore be at lower risk for UTI. Theoretically, this could then confound our analysis, since improved overall systemic

status could result in improved clinical scores in the open-field and inclined-plane tests. However, there was a notable disparity in the time frame in which the effects of FTY720 on locomotor scores and bladder function were noted. If the above conjecture were true, then no disparity should be present, and locomotor improvement should be noted coincident with bladder function improvement. Furthermore, there were no differences noted among treatment groups in terms of body weight change, suggesting that overall systemic status was similar among the groups. Finally, morphometric analysis also supports the neuroprotective capabilities of FTY720, as improved bladder function would presumably not result in enhanced tissue preservation in the spinal cord. Taken together this leads us to conclude that administration of FTY720 enhances locomotor function independent of bladder function and systemic status.

Our results suggest that treatment with FTY720 not only reduces the adaptive immune response, but also affords neuroprotection and enhances bladder function after injury. Given the complexity of the posttraumatic inflammatory cascade, the results of this study do not clarify the role of T cells in CNS recovery. However, these results do reinforce the growing body of knowledge suggesting immunomodulation as a possible therapeutic strategy in SCI.

Acknowledgments

We acknowledge Salem P. Wilson and Grace Lee for technical assistance. We also acknowledge Anwar Ahmed and the Department of Biostatistics for assistance with statistical analysis, and we thank Dr. T. Parsons for his editorial review of the manuscript.

The funding for this study was provided by the Lind Lawrence Foundation, as well as NIH grants no. 5T32NS 007288-20 (W.N.C.), NIH-NCI R01-CA116695 (M.R.G.), and NMSS RG3432A/A2 (C.S.B.).

Author Disclosure Statement

No conflicting financial interests exist.

References

- Ankeny, D., Lucin, K., Sanders, V., McGaughy, V., and Popovich, P. (2006). Spinal cord injury triggers systemic autoimmunity: evidence for chronic B lymphocyte activation and lupus-like autoantibody synthesis. *J. Neurochem.* 99, 1073–1087.
- Apodaca, G., Kiss, S., Ruiz, W., Meyers, S., Zeider, M., and Birder, L. (2003). Disruption of bladder epithelium barrier function after spinal cord injury. *Am. J. Physiol. Renal Physiol.* 284, 966–976.
- Banati, R.B., and Graeber, M.B. (1994). Surveillance, intervention and cytotoxicity: is there a protective role of microglia? *Dev. Neurosci.* 16, 114–127.
- Bao, F., and Liu, D. (2002). Peroxynitrite generated in the rat spinal cord induces neuron death and neurological deficits. *Neuroscience* 115, 839–849.
- Basso, D.M., Beattie, M.S., and Bresnahan, J.C. (1995). A sensitive and reliable locomotor rating scale for open field testing in rats. *J. Neurotrauma* 12, 1–21.
- Beril, G., Solaroglu, I., Okutan, O., Cimen, B., Kaptanoglu, E., and Palaoglu, S. (2007). Metoprolol treatment decreases tissue myeloperoxidase activity after spinal cord injury in rats. *J. Clin. Neurosci.* 14, 138–142.
- Blight, A. (1994). Effects of silica on the outcome from experimental spinal cord injury: implication of macrophages in secondary tissue damage. *Neuroscience* 60, 263–273.
- Bohler, T., Waiser, J., Schuetz, M., Neumayer, H., and Budde, K. (2004). FTY720 exerts differential effects on CD4+ and CD8+ T-lymphocyte subpopulations expressing chemokine and adhesion receptors. *Nephrol. Dial. Transplant.* 19, 702–713.
- Brinkman, V., Cyster, J.G., Hla, T. (2004). FTY720: Sphingosine 1-phosphate receptor-1 in the control of lymphocyte egress and endothelial barrier function. *Am. J. Transplant* 4, 1019–1025.
- Brinkmann, V., Davis, M., Heise, C., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumrucker, T., Hiestand, P., Foster, C., Zollinger, M., and Lynch, K. (2002). The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J. Biol. Chem.* 277, 21453–21457.
- Brinkmann, V., Pinschewer, D., Chiba, K., and Feng, L. (2000). FTY720: a novel transplantation drug that modulates lymphocyte traffic rather than activation. *Trends Pharmacol. Sci.* 21, 49–52.
- Budde, K., Schmouder, R., Nashan, B., Brunhorst, R., Lucker, P., Mayer, T., Brookman, L., Nedelman, J., Skerjanec, A., Bohler, T., and Neumayer, H. (2003). Pharmacodynamics of single doses of the novel immunosuppressant FTY720 in stable renal transplant patients. *Am. J. Transplant.* 3, 836–854.
- Chao, C., Hu, S., Molitor, T., Shaskan, E., and Peterson, P. (1992). Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *J. Neuroimmunol.* 149, 2736–2741.
- Chiba, K., Tanagawa, Y., Masubuchi, Y., Kataoka, H., Kawaguchi, T., Ohtsuki, M., and Hoshino, Y. (1998). FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. *J. Immunol.* 160, 5037–5044.
- Coelho, R., Payne, S., Bittman, R., Spiegel, S., and Sato-Bigbee, C. (2007). The immunomodulator FTY720 has a direct cytoprotective effect on oligodendrocyte progenitors. *J. Pharmacol. Exp. Ther.* 323, 626–635.
- Doggweiler, D., Jasmin, L., and Schmidt, R. (1998). Neurogenically mediated cystitis in rats: An animal model. *J. Urol.* 160, 1551–1556.
- Donnelly, D.J., and Popovich, P.G. (2008). Inflammation and its role in neuroprotection, axonal regeneration, and functional recovery after spinal cord injury. *Exp. Neurol.* 209, 378–388.
- Fujino, M., Funesima, N., Kitazawa, Y., Kimura, H., Amemiya, H., Suzuki, S., and Li, X. (2003). Amelioration of experimental autoimmune encephalomyelitis in Lewis rats by FTY720 treatment. *J. Pharmacol. Exp. Ther.* 305, 70–77.
- Giulian, D., and Robertson, C. (1990). Inhibition of mononuclear phagocytes reduces ischemic injury in the spinal cord. *Ann. Neurol.* 27, 33–42.
- Gonzalez, R., Glaser, J., Liu, M., Lane, T., and Keirstead, H. (2003). Reducing inflammation decreases secondary degeneration and functional deficit after spinal cord injury. *Exp. Neurol.* 184, 456–463.
- Graf, M., Prins, R., and Merchant, R. (2001). IL-6 secretion by a rat T9 glioma clone induces a neutrophil-dependent antitumor response with resultant cellular, antiglioma immunity. *J. Immunol.* 166, 121–129.
- Gris, D., Marsh, D., Oatway, M., Chen, Y., Hamilton, E., Dekaban, G., and Weaver, L. (2004). Transient blockade of the CD11d/CD18 integrin reduces secondary damage after spinal cord injury, improving sensory, autonomic, and motor function. *J. Neurosci.* 24, 4043–4051.

- Hauben, E., Agranov, E., Gothilf, A., Nevo, U., Cohen, A., Smirnov, I., Steinman, L., and Schwartz, M. (2001). Post-traumatic therapeutic vaccination with modified myelin self-antigen prevents complete paralysis while avoiding autoimmune disease. *J. Clin. Invest.* 108, 591–599.
- Hauben, E., Butovsky, O., Nevo, U., Yoles, E., Moalem, G., Agranov, E., Mor, F., Leibowitz-Amit, R., Pevsner, E., Akselrod, S., Neeman, M., Cohen, I., and Schwartz, M. (2000). Passive or active immunization with myelin basic protein promotes recovery from spinal cord contusion. *J. Neurosci.* 20, 6421–6430.
- Ibarra A., Correa D., Willms K., Merchant M.T., Guizar-Sahagún, G., Grijalva, I., and Madrazo, I. (2003). Effects of cyclosporin-A on immune response, tissue protection, and motor function of rats subjected to spinal cord injury. *Brain Res.* 979, 165–178.
- Jones, T., Ankeny, D., Guan, Z., McGaughy, V., Fisher, L., Basso, M., and Popovich, P. (2004). Passive or active immunization with myelin basic protein impairs neurological function and exacerbates neuropathology after spinal cord injury in rats. *J. Neurosci.* 24, 3752–3761.
- Jones, T., Basso, M., Sodhi, A., Pan, J., Hart, R., MacCallum, R., Lee, S., Whitacre, C., and Popovich, P. (2002). Pathological CNS autoimmune disease triggered by traumatic spinal cord injury: Implications for autoimmune vaccine therapy. *J. Neurosci.* 22, 2690–2700.
- Kahan, B., Karlx, J., Feruson, R., Leichtman, A., Mulgaonkar, S., Gonwa, T., Skerjanec, A., Schmouder, R., and Chodoff, L. (2003). Pharmacodynamics, pharmacokinetics and safety of multiple doses of FTY720 in stable renal transplant patients: A multicenter, randomized, placebo-controlled, phase I study. *Transplantation* 76, 1079–1084.
- Kreutzberg, G.W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* 19, 312–318.
- Lee, M., Thangada, S., Claffey, K., Ancellin, N., Liu, C., Kluk, M., Volpi, M., Sha'afi, R., and Hla, T. (1999). Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell* 99, 301–312.
- Leung, P., Johnson, C., and Wrathall, J. (2007). Comparison of the effects of complete and incomplete spinal cord injury on lower urinary tract function as evaluated in unanesthetized rats. *Exp. Neurol.* 208, 80–91.
- Lipton, H., Kalio, P., and Jelachich, M. (2005). Simplified quantitative analysis of spinal cord cells from Theiler's virus-infected mice without the requirement for myelin debris removal. *J. Immunol. Meth.* 299, 107–115.
- Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G., Card, D., Keohane, C., Rosenbach, M., Hale, J., Lynch, C., Rupprecht, K., Parsons, W., and Rosen, H. (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* 296, 346–349.
- Matloubian, M., Lo, C., Cinamon, G., Lesneski, M., Xu, Y., Brinkmann, V., Allende, M., Proia, R., and Cyster, J. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* 427, 355–360.
- Pannu, R., Barbosa, E., Singh, A., and Singh, I. (2005). Attenuation of acute inflammatory response by atorvastatin after spinal cord injury in rats. *J. Neurosci. Res.* 79, 340–350.
- Pinschewer, D.O., Ochsenbein, A.F., Odermatt, B., Brinkmann, V., Hengartner, H., and Zinkernagel, R.M. (2000). FTY720 immunosuppression impairs effector T-cell peripheral homing without affecting induction, expansion, and memory. *J. Immunol.* 164, 5761–5770.
- Popovich, P., and Jones, T. (2003). Manipulating neuroinflammatory reactions in the injured spinal cord: back to basics. *Trends Pharmacol. Sci.* 24, 13–17.
- Popovich, P., Guan, Z., Wei, P., Huitinga, I., van Rooijen, N., and Stokes, B. (1999). Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. *Exp. Neurol.* 158, 351–365.
- Popovich, P., Stokes, B., and Whitacre, C. (1996). Concept of autoimmunity following spinal cord injury: possible roles for T lymphocytes in the traumatized central nervous system. *J. Neurosci. Res.* 45, 349–363.
- Potas, J., Zheng, Y., Moussa, C., Venn, M., Gorrie, C., Deng, C., and Waite, P. (2006). Augmented locomotor recovery after spinal cord injury in the athymic nude rat. *J. Neurotrauma* 23, 660–673.
- Quesniaux, V., Fullard, L., Arendse, H., Davison, G., Markgraaff, N., Auer, R., Ehrhart, F., Kraus, G., and Schuurman, H. (1999). A novel immunosuppressant, FTY720 induces peripheral lymphodepletion of both T- and B-cells and immunosuppression in baboons. *Transpl. Immunol.* 7, 149–157.
- Rimaniol, A., Haik, S., Martin, M., Le Grand, R., Boussin, F., Dereuddre-Bosquet, N., Gras, G., and Dormont, D. (2000). Na⁺-dependent high-affinity glutamate transport in macrophages. *J. Immunol.* 164, 5430–5438.
- Schwartz, M., and Kipnis, J. (2002). Multiple sclerosis as a by-product of the failure to sustain protective autoimmunity: a paradigm shift. *Neuroscientist* 8, 405–413.
- Schwartz, M., and Kipnis, J. (2001). Protective autoimmunity: regulation and prospects for vaccination after brain and spinal cord injuries. *Trends Mol. Med.* 7, 252–258.
- Schwartz, M. (2001). Harnessing the immune system for neuroprotection: therapeutic vaccines for acute and chronic neurodegenerative disorders. *Cell. Mol. Neurobiol.* 21, 617–627.
- Shamash, S., Reichert, F., and Rotshenker, S. (2002). The cytokine network of wallerian degeneration: tumor necrosis factor- α , interleukin-1 α , and interleukin-1 β . *J. Neurosci.* 22, 3052–3060.
- Waterson, K., Berg, K., Kapitonov, D., Payne, S., Miner, A., Bittman, R., Miltien, S., Ratz, P., and Spiegel, S. (2007). Sphingosine-1-phosphate and the immunosuppressant, FTY720-phosphate, regulate detrusor muscle tone. *FASEB J.* 21, 2818–2828.
- Yin, Y., Henzl, M., Lorber, B., Nakazawa, T., Thomas, T., Jiang, F., Langer, R., and Benowitz, L. (2006). Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells. *Nat. Neurosci.* 9, 843–852.
- Yoles, E., Hauben, E., Palgi, O., Agranov, E., Gothilf, A., Cohen, A., Kuchroo, V., Cohen, I. R., Weiner, H. and Schwartz, M. (2001). Protective autoimmunity is a physiological response to CNS trauma. *J. Neurosci.* 21, 3740–3748.

Address correspondence to:
Bruce E. Mathern, M.D.

Department of Neurosurgery
Virginia Commonwealth University
P.O. Box 980631
Richmond, VA 23298-0631

E-mail: bmathern@mcvh-vcu.edu