Research Article: New Research | Disorders of the Nervous System

Early Targeting of L-Selectin on Leukocytes Promotes Recovery after Spinal Cord Injury, Implicating Novel Mechanisms of Pathogenesis

D. A. McCreedy[1,3,4], S. Lee[1,2], C. J. Sontag[1], P. Weinstein[1], A. D. Olivas[1], A. F. Martinez[1], T. M. Fandel[1], A. Trivedi[1], C. A. Lowell[4], S. D. Rosen[5] and L. J. Noble-Haeusslein[1,6]

[1]*Department of Neurological Surgery, University of California, San Francisco, California 94143*
[2]*Brain and Spinal Injury Center, University of California, San Francisco, California 94143*
[3]*J. David Gladstone Institutes, University of California, San Francisco, California 94143*
[4]*Department of Laboratory Medicine, University of California, San Francisco, California 94143*
[5]*Department of Anatomy and Program in Immunology, University of California, San Francisco, California 94143*
[6]*Department of Physical Therapy and Rehabilitation Science, University of California, San Francisco, California 94143*

DOI: 10.1523/ENEURO.0101-18.2018

Received: 16 March 2018

Revised: 27 July 2018

Accepted: 3 August 2018

Published: 13 August 2018

Author contributions: D.A.M., S.M.L., C.J.S., P.W., A.D.O., T.M.F., A.T., C.A.L., S.R., and L.N.-H. designed research; D.A.M., S.M.L., C.J.S., A.D.O., A.F.M., T.M.F., and A.T. performed research; D.A.M., S.M.L., C.J.S., A.D.O., A.F.M., T.M.F., A.T., and L.N.-H. analyzed data; D.A.M., S.M.L., C.J.S., P.W., C.A.L., S.R., and L.N.-H. wrote the paper.

Funding: http://doi.org/10.13039/100000005U.S. Department of Defense (DOD): W81XWH-12-1-0563. http://doi.org/10.13039/100005191Craig H. Neilsen Foundation; http://doi.org/10.13039/100001068Dana Foundation; Oxnard Foundation of California; Roman Reed Foundation; http://doi.org/10.13039/100000065HHS | NIH | National Institute of Neurological Disorders and Stroke (NINDS): R01NSC39278; F32NS096883. UCSF Sandler Foundation;

The authors declare no competing financial interests.

This work was funded by the Department of Defense SCIRP W81XWH-12-1-0563, The Craig H. Neilsen Foundation, The Dana Foundation, The Oxnard Foundation of California, The Roman Reed Fund from the State of California, the National Institutes of Health, NINDS R01NSC39278, NINDS F32NS096883, and the UCSF Sandler Foundation in Basic Science Integrative Research Award.

D.A.M. and S.L.Co-first authors

Correspondence should be addressed to: Dr. Dylan McCreedy, University of California, San Francisco, 513 Parnassus Avenue HSW-1201A, San Francisco, California 94143. E-mail: Dylan.McCreedy@gladstone.ucsf.edu

Cite as: eNeuro 2018; 10.1523/ENEURO.0101-18.2018

Early targeting of L-selectin on leukocytes promotes recovery after spinal cord injury, implicating novel mechanisms of pathogenesis

Abbreviated title: L-selectin is a determinant of recovery after SCI

#*DA McCreedy[1,3,4], #S Lee[1,2], CJ Sontag[1], P Weinstein[1], AD Olivas[1], AF Martinez[1], TM Fandel[1], A Trivedi[1], CA Lowell[4], SD Rosen[5], LJ Noble-Haeusslein[1,6]
#Co-first authors
*Corresponding author

[1]Department of Neurological Surgery, [2]Brain and Spinal Injury Center, [3]J. David Gladstone Institutes, [4]Department of Laboratory Medicine, [5]Department of Anatomy and Program in Immunology, [6]Department of Physical Therapy and Rehabilitation Science, University of California, San Francisco, California 94143

Correspondence should be addressed to:
Dr. Dylan McCreedy
University of California, San Francisco
513 Parnassus Avenue HSW-1201A
San Francisco, California 94143
Dylan.McCreedy@gladstone.ucsf.edu

Number of Pages: 52

Number of Figures: 10

Number of Words: Abstract – 223, Introduction – 691, Discussion - 1429

The authors declare no competing financial interests.

Acknowledgements
This work was funded by the Department of Defense SCIRP W81XWH-12-1-0563, The Craig H. Neilsen Foundation, The Dana Foundation, The Oxnard Foundation of California, The Roman Reed Fund from the State of California, the National Institutes of Health, NINDS R01NSC39278, NINDS F32NS096883, and the UCSF Sandler Foundation in Basic Science Integrative Research Award. We would like to thank Tjoson Tjoa for his contributions to model generation, histology, and animal care and Mark Singer for his assistance during the early stages of the work. Behavioral data were obtained with support from the UCSF Neurobehavioral Core for Rehabilitation Research.

Abstract

L-selectin, a lectin-like receptor on all leukocyte classes, functions in adhesive and signaling roles in the recruitment of myeloid cells from the blood to sites of inflammation. Herein, we consider L-selectin as a determinant of neurological recovery in a murine model of spinal cord injury. Spinal cord-injured, L-selectin knockout mice (male) showed improved long-term recovery with greater white matter sparing relative to wildtype mice and reduced oxidative stress in the injured cord at 72 hours post-spinal cord injury. There was a partial and transient reduction in accumulation of neutrophils in the injured spinal cords of knockouts at 24 hours post-injury. To complement these findings with knockout mice, we sought a pharmacologic means for lowering L-selectin levels. We found that diclofenac, a nonsteroidal anti-inflammatory drug, induced the shedding of L-selectin from the cell surface of myeloid subsets, specifically neutrophils and non-classical monocytes, in the blood and the injured spinal cord. Diclofenac administration to injured wildtype mice enhanced neurological recovery to a level comparable to that of knockouts but did not improve recovery in knockouts. While diclofenac treatment had no effect on myeloid cell accumulation, there was a reduction in oxidative stress at 72 hours post-spinal cord injury. These findings implicate L-selectin in secondary pathogenesis beyond a role in leukocyte recruitment and raise the possibility of repurposing diclofenac for the treatment of spinal cord injury.

Keywords: spinal cord injury, L-selectin, leukocytes, myelin, oxidative stress, functional recovery, diclofenac, shedding

Significance Statement

In this study, we establish L-selectin, an adhesion and signaling receptor on immune cells, as a determinant of long-term recovery and tissue sparing after spinal cord injury. We demonstrate that L-selectin contributes to secondary pathogenesis during acute inflammation, and implicate L-selectin in novel roles other than recruitment. We also report a strategy to improve recovery by employing diclofenac, an FDA-approved NSAID that induces the shedding of L-selectin from the surface of innate immune cells. Our findings demonstrate a critical time-period for anti-inflammatory intervention in a murine model of spinal cord injury and suggest that diclofenac be tested as an acute therapy for attenuating neurological deficits following spinal cord injury in humans.

Introduction

Spinal cord injury (SCI) results in partial or complete loss of motor and sensory function below the site of injury. The neurological deficits resulting from SCI are not solely attributed to the initial mechanical damage, and there is a broad consensus that early infiltrating leukocytes, primarily myeloid lineage cells (i.e., neutrophils and monocytes), release neurotoxic substances including reactive oxygen species (ROS), proteases, and pro-inflammatory cytokines that cause secondary tissue damage (Chatzipanteli et al., 2002; Noble et al., 2002; Bareyre and Schwab, 2003; David and Kroner, 2011). Various strategies, directed at attenuating the early recruitment of myeloid cells from the blood into the injured spinal cord, have shown promising results for reducing cell injury and improving long-term neurological outcomes (Popovich et al., 1999; Gris et al., 2004; Popovich and Longbrake, 2008; Lee et al., 2011; Zhang et al., 2011). However, inconsistent benefits or deleterious consequences have been observed in other studies (Stirling et al., 2009; Hurtado et al., 2012). These discrepancies may be due to opposed reparative and damaging activities in the targeted myeloid subsets. In the present study, we have identified L-selectin, a leukocyte adhesion/signaling receptor, as a novel therapeutic target in SCI.

Selectins are C-type lectins that generally function sequentially with integrins during the multistep process of leukocyte recruitment from the blood into sites of inflammation (Ley et al., 2007; McEver, 2015). The vascular selectins, E- and P-selectin, are upregulated on inflamed vascular endothelium and bind to ligands such as PSGL-1 (P-selectin glycoprotein ligand-1) on leukocytes (McEver, 2015). L-selectin (CD62L) on lymphocytes mediates their rolling on high endothelial venules during homing to secondary lymphoid organs through the interaction of its lectin domain with carbohydrate-based ligands on this specialized endothelium (Rosen, 2004). L-selectin is also broadly expressed on circulating myeloid leukocytes and has adhesive and signaling activities that underlie various responses of these cells to inflammation (Ley et al., 2007; McEver, 2015). Studies employing KO mice or blocking antibodies have demonstrated that L-selectin, working in concert with vascular selectins, is involved in the recruitment of myeloid cells from the blood into various sites of inflammation (Lewinsohn et al., 1987; Pizcueta and Luscinskas, 1994; Tedder et al., 1995; Ley et al., 2007; Zuchtriegel et al., 2015).

It is now clear that L-selectin contributes to myeloid cell recruitment as a secondary adhesion molecule that mediates tethering between endothelial-adherent leukocytes and circulating leukocytes, via binding in trans to PSGL-1 on leukocytes (Walcheck et al., 1996; Sperandio et al., 2003), and as a signaling molecule that augments the activation of integrins on rolling leukocytes (Stadtmann et al., 2013; Morikis et al., 2017). Additional activities, apart from the recruitment of leukocytes from the blood, are indicated in that the ligation of L-selectin on neutrophils by soluble carbohydrate ligands, such as carcinoma-derived or salivary mucins, potentiates the degranulation of these cells (Shao et al., 2011; Mohanty et al., 2015). In the context of CNS inflammation, the possibility of a post-recruitment role for L-selectin has emerged based on the observation that L-selectin mediates the *in vitro* adhesion of leukocytes to myelinated fiber tracts in the CNS (Huang et al., 1991; Huang et al., 1994). In light of its potential recruitment and post-recruitment activities, we have investigated whether the reduction of L-selectin function through genetic or pharmacologic means has an impact on neurological recovery in a murine model of SCI.

We demonstrate that complete L-selectin deficiency results in a partial reduction of neutrophil accumulation and oxidative stress in the acutely injured cord as well as improved long-term neurological recovery that corresponds to greater sparing of white matter. We further show that diclofenac, an NSAID and an inducer of L-selectin shedding from the leukocyte cell surface (Diaz-Gonzalez et al., 1995; Gomez-Gaviro et al., 2002), has beneficial effects in SCI comparable to the genetic elimination of L-selectin. Since diclofenac is currently approved by the FDA (Altman et al., 2015), there could be an opportunity to repurpose this drug for the spinal cord injured patient. The beneficial consequences of reducing L-selectin levels cannot be attributed solely to reduced leukocyte recruitment, particularly in the case of diclofenac, highlighting the consideration of L-selectin in novel roles in secondary pathogenesis and subsequent long-term neurological deficits.

Materials and Methods

Animals. These studies were approved by the Institutional Animal Care and Use Committee at the University of California San Francisco and were in accordance with the United States

Department of Agriculture guidelines. Homozygous L-selectin KO mice and their WT littermates were generated by breeding heterozygous males and females on a C57Bl/6 background. We confirmed that mice from L-selectin KO and WT colonies did not contain the recently reported copy number variant in the *Dock2* allele (Mahajan et al., 2016). WT and KO littermates were then studied with the exception of flow cytometry experiments where WTs were purchased from Jackson Laboratories (Bay Harbor, ME). WT mice for diclofenac studies were purchased from Jackson Laboratories. Mice were housed in groups of two to five prior to injury and singly housed after SCI.

SCI. Adult male mice (~3-5 months of age) were anesthetized with 2.5% Avertin (0.02 ml/g body weight, i.p., tribromoethanol; Sigma, St. Louis, MO) or 2% isoflurane and subjected to a spinal cord contusion injury as described previously (Lee et al., 2011). Briefly, a laminectomy was performed at the ninth thoracic vertebra and a 3g weight was dropped 5-7.5 cm onto the exposed dura mater to produce the SCI. After injury, the skin was closed with wound clips. Body temperature was maintained at 37°C with a warming blanket throughout the surgery and during recovery from anesthesia. Postoperative care included subcutaneous administration of saline and antibiotics daily for ten days and manual expression of the bladder twice per day until euthanasia.

Treatment with Diclofenac. Diclofenac (Sigma) was dissolved in phosphate buffered saline (PBS) at 2.5 mg/mL and sterile filtered prior to use. To determine if diclofenac modulates neurological recovery after SCI, diclofenac (20, 30, or 40 mg/kg) was administered i.p.

immediately, 3 hrs, or 8 hrs after SCI. The dosing was based on previous studies in rodents (Grace et al., 2001). Behavioral tests were performed as described below.

Assessment of Neurological Recovery. Two behavioral tests, Basso Mouse Scale (BMS) and grid walk, were performed in the same mice to evaluate functional improvements after SCI. The nine-point BMS was used to examine locomotor recovery in an open field (53 cm x 108 cm x 5.5 cm) (Basso et al., 2006). This rating scale takes into account limb movement, stepping, coordination, and trunk stability. Mice were tested at one, three, and seven days and weekly thereafter until euthanasia at five to six weeks post-SCI. For studies examining diclofenac in WTs, mice achieving a BMS score ≥ 1 at one day post-SCI were considered insufficiently injured and were removed from the analysis. For grid walking, a mouse (with a BMS score of four or greater) was positioned on a grid, divided into 0.5 cm squares, and the number of foot faults was recorded over a period of three minutes. A foot fault was evident when a paw fully extended through a space in the grid. The grid walking test was performed over three days at approximately five weeks post-SCI with three trials per day.

Measurement of White Matter Sparing. Animals were euthanized at 35 or 42 days post-SCI and perfused with 50 ml of PBS followed by 50 ml of 4% paraformaldehyde (pH 7.4). The spinal cords were removed, postfixed overnight, and cyroprotected in 30% sucrose for four days. Cords were then embedded and frozen at -80 °C until sectioning. 20 μm transverse sections were made on a cryostat, and serial sections, 500 μm apart, were chosen for staining of white matter using either luxol fast blue (LFB) or eriochrome cyanine. Sections were evaluated by light microscopy and the one with the least spared white matter was selected as the lesion epicenter. For sections stained with LFB, the area of residual white matter was hand-traced using Neurolucida software (Microbrightfield Bioscience, Williston, VT) and the percent of spared white matter relative to the total cross sectional area of the cord at the epicenter was determined (Lee et al., 2011). This epicenter measurement has previously been demonstrated to correlate with injury severity and the degree of functional recovery (Kuhn and Wrathall, 1998). For sections stained with eriochrome cyanine, the area of residual white matter at the epicenter was traced and quantified using the StereoInvestigator (MBF Bioscience, Williston, VT) Cavalieri probe.

Immunoblotting. A 0.5 cm length of cord, centered over the site of impact and representing the epicenter, was homogenized in Glo lysis buffer (Promega, Madison, WI) or RIPA buffer (Thermo Fisher). The protein concentration in homogenates was determined by the BCA protein assay kit (Pierce, Rockford, IL). For detecting malondialdehyde (MDA), we used the OxiSelect Malondialdehyde immunoblot kit (Cell BioLabs, Inc., San Diego, CA) according to the manufacturer's protocol. Briefly, 20 μg of protein was loaded onto 12-15% SDS-PAGE gel and transferred onto a nitrocellulose or PVDF membrane. After blocking the membrane with 1% BSA solution, the membrane was incubated with rabbit anti-MDA antibody overnight. The membrane was washed with Tris-buffered saline including 0.1% triton-x and then incubated with anti-rabbit-HRP antibody for 1 hr. MDA positive bands were detected using Pierce SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific, IL, USA). Bands at ~65 kDa, including both bands for uninjured (UN) control samples, were measured. For all of the comparisons, β-actin (Sigma, RRID:AB_476744) or GAPDH (Millipore, RRID:AB_2107426) served as a loading control and was used for normalization.

Cell isolation and treatments. Neutrophils were isolated from peripheral blood of mice by Ficoll-Paque (Ficoll-PaqueTM PREMIUM 1.084, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to manufacturer's protocol. Briefly, 2 ml of peripheral blood were obtained by cardiac puncture from three mice and mixed with 10 ml of Hank's balanced salt solution. After addition of 3 ml of Ficoll-Paque, the sample as subjected to density-gradient centrifugation of 30 min at 400 g at room temperature (RT). Neutrophils were carefully collected from upper layers. To purify the neutrophil-enriched fraction, erythrocytes were lysed with RBC lysis buffer at RT (eBioscience, San Diego, CA). The purity of the neutrophil fraction was ≥ 90%.

To investigate whether NSAIDs induce L-selectin shedding, the isolated cells were treated with NSAIDs as previously described (Gomez-Gaviro et al., 2002). Cells were resuspended in PBS and incubated either alone or in the presence of diclofenac (20 to 500 µg/ml) or meclofenamic acid (MFA, 20 to 500 µg/ml, Sigma) for 30 min at 37 °C. PMA (250 ng/ml) served as a positive control. All NSAIDs were dissolved in PBS. Then cells were analyzed by flow cytometry and the supernatants were analyzed for soluble L-selectin by ELISA (see below). Data were collected from seven independent experiments for flow cytometry and two independent experiments for ELISA.

Flow Cytometry. For neutrophils and mononuclear cells exposed to NSAIDs, the cells were washed with PBS then incubated with anti-mouse CD16/32 Fc blocking antibody (1:10 dilution; eBioscience, RRID:AB_467133) for 10 min followed by anti-CD62L Ab conjugated with PE (1:10 dilution, Mel-14 clone, eBioscience, RRID:AB_465720) for 30 min at 4°C. After washing in PBS, the cells were analyzed in a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and FlowJo software (Tree Star Inc., Ashland, OR). In all *in vitro* experiments, at least 10^6 cells were analyzed for flow cytometry. Cell viability was determined by 7-Amino-Acinomycin D staining (7-AAD, BD Bioscience, San Jose, CA). Data were collected from seven independent experiments.

For *in vivo* experiments, blood samples were obtained by cardiac puncture using a heparin-primed syringe from uninjured and spinal cord injured mice. Recruitment of leukocytes from the blood into spinal cord can occur across blood vessels in the meninges or parenchymal tissue (Ransohoff et al., 2003). Injured mice were perfused with 25 mL of ice cold PBS to remove free leukocytes within blood vessels. From each animal, a 5 mm region of spinal cord (with associated meninges) centered over the injury site was removed and stored in ice cold RPMI media. The spinal cord segment was then mechanically dissociated using a plastic tissue pestle. The suspension was filtered through a 100 µm nylon mesh filter, and the filtrate was centrifuged at 300 g, 4°C for 5 min. Blood and spinal cord samples were then lysed with 1 mL of 1X RBC lysis buffer (eBioscience) for 5 min at 4°C, followed by the addition of 10 ml of FACS buffer (0.5 µM EDTA, 2% fetal bovine serum, HBSS, pH 7.4). The cell suspension was centrifuged and the pellet was re-suspended in 10 mL HBSS and centrifuged again. Live/dead cell staining was then performed by incubating samples with Ghost Dye Red 780 (1:1000, Tonbo Biosciences, San Diego, CA) for 30 min at 4°C. Samples were washed with 10 mL of FACS buffer, centrifuged, and the supernatant was discarded. Cells were then resuspended in FACS buffer and incubated with anti-mouse CD16/32 Fc blocking antibody (1:100 dilution; eBioscience, RRID:AB_467133) at 4°C for 20 min. Next, samples were washed with 10 mL of FACS buffer and 1 mL was partitioned for isotype control staining. Samples were then centrifuged and the pellets were incubated with a cocktail of the following antibodies: FITC conjugated rat anti-mouse CD62L (1:100 dilution, Mel-14 clone, BioLegend, San Diego, CA, RRID:AB_313093), Pacific Blue conjugated rat anti-mouse Ly-6G (1:200 dilution, 1A8 clone, BioLegend, RRID:AB_2251161), APC conjugated rat anti-mouse CD11b (1:200 dilution, M1/70 clone, BioLegend, RRID:AB_312795), PE conjugated rat anti-mouse CD45 (1:200 dilution, 30-F11 clone, Tonbo, RRID:AB_2621763) and PerCP/Cy5.5 conjugated rat anti-mouse Ly-6C (1:600 dilution, HK1.4 clone, BioLegend, RRID:AB_1659241). FITC conjugated rat IgG2a, κ (1:100 dilution, BioLegend, RRID:AB_2736919) served as the isotype control for CD62L. After incubating with antibodies for 30 min at 4°C, samples were washed with FACS buffer, centrifuged and the pellets were resuspended in 200 μL of FACs buffer. Flow cytometry was performed on the cell suspensions using a Fortessa flow cytometer (Becton Dickinson).

The data were analyzed using FlowJo software. At least 50,000 events were analyzed for blood samples and all of the collected events were analyzed for spinal cord samples. Cells (low SSC, high FSC) were gated from debris (high SSC, low FSC) as previously described (Stirling and Yong, 2008). The geometric mean fluorescent intensity (G-MFI) values for CD62L were calculated to determine the overall presence of L-selectin on the surface of leukocytes. Since staining histograms for L-selectin were often skewed, we used G-MFI instead of arithmetic MFI to represent the data. This parameter better reflects the central tendency of skewed distributions. Isotype G-MFI were determined for each sample and subtracted from the reported G-MFI values. Total leukocyte and myeloid lineage subset counts from spinal cord samples were extrapolated to the total volume of cells to account for the volume of cells removed for isotype staining.

Soluble L-selectin Enzyme-Linked Immunosorbent Assay (ELISA).

To determine if diclofenac treatment induces L-selectin shedding, blood was obtained by cardiac puncture at 0, 2, 8, 24, and 48 hrs after i.p. administration of this drug (1, 5, 10, 20, 40, or

60 mg/kg) immediately following SCI produced by dropping a 2g weight from a distance of 5cm. After centrifugation at 2,000 g for 20 min at RT, plasma was then collected for assessment using an ELISA kit (Quantikine®, mouse sL-Selectin, R&D system, Minneapolis, MN) according to the manufacturer's instructions. All incubations were conducted at RT and for 2 hrs unless otherwise indicated. 100 µl of supernatants were first incubated with assay diluent buffer in a microplate. After rinsing, the preparation was then incubated in 100 µl of soluble L-selectin conjugate, followed by addition of 100 µl of substrate for 30 min. The reaction was then stopped by addition of stop solution. Optimal density was read at 450 nm wavelength with correction at 540 nm using a microplate reader (Molecular Probes, NY).

Experimental Design and Statistical Analyses. The primary goal of this study was to determine if L-selectin contributes to inflammation and neurological deficits following SCI. We employed L-selectin KO mice and also evaluated the effect of diclofenac, an NSAID that induces L-selectin shedding, on inflammation and neurological recovery after SCI. For all studies, only adult male mice were used to eliminate gender as a variable. Time-points for collection of data and experimental endpoints were pre-determined based on previous experience. Sample sizes were determined by our previous experience with BMS scoring and immunoblotting (Lin et al., 2007; Lee et al., 2011; Whetstone et al., 2017), and by power analyses for flow cytometry studies and soluble L-selectin (sL-selectin) assays (effect sizes were 500 G-MFI units and 550 ng/mL, respectively, based on our own preliminary studies). For all studies we anticipated a ~10% mortality rate, based on previous experience in our lab (Whetstone et al., 2017) and substantiated in Fig. 10i, and we adjusted the samples sizes accordingly. To control for potential variation in the spinal cord injury surgery and behavioral analyses, all studies were conducted with the surgeon and observers blinded to the genotype and/or experimental condition. Simple or block randomization was used for all *in vivo* studies.

Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Flow cytometry was evaluated by unpaired two-tailed Student's t-tests or one-way ANOVA followed by Tukey's post-hoc test. ELISA data were evaluated by one-way ANOVA followed by a Dunnett's post-hoc test. A two-way, repeated measures ANOVA followed by Sidak's multiple comparisons test was used to evaluate BMS scores. Comparison of two groups (foot faults, spared white matter, and immunoblots) was by an unpaired two-tailed Student's t-test. Chi square analysis was used to compare the number of animals stepping in the open field followed by a one-sided Fisher's exact test. Comparison of two groups was performed by the Mann-Whitney test when at least one of the data sets failed the D'Agostino and Pearson omnibus normality test. Statistical significance was defined at $p \leq 0.05$. Data are expressed as means ± SEM.

Data availability. All data are available from the authors.

Results

Functional recovery is improved in L-selectin KO mice

To assess if L-selectin is a determinant of recovery after SCI, we first compared locomotion of spinal cord injured L-selectin KO mice with WT littermate controls using the BMS. L-selectin KO mice, subjected to SCI, showed a marked improvement in locomotor recovery compared to that of the WT group (Fig. 1A, p=0.006). A subsequent secondary analysis revealed that 79.2% of KO animals achieved weight supported plantar stepping (BMS ≥ 4) compared to 47.6% of WT mice by 42 days post-SCI (Fig. 1B, p=0.029). We also determined the number of foot-faults of mice crossing a wire grid, which reflects sensorimotor function and motor coordination (Schaar et al., 2010). Foot-faults were reduced by 57.9% in the KO relative to the WT group at 35 days post-SCI (Fig. 1C, p=0.023).

Functional recovery has been previously associated with greater white matter sparing at the lesion epicenter (Kuhn and Wrathall, 1998). To determine if L-selectin is associated with white matter damage, the percentage of spared white matter was compared between groups in transverse sections stained with LFB (Fig. 1D). Strikingly, the percentage of spared white matter at the lesion epicenter was 2.0 fold greater in the spinal cord injured KO mice as compared to injured WT mice at 42 days post-SCI (p=0.038, Fig. 1E).

L-selectin is a determinant of oxidative stress in the acutely injured cord

Newly recruited neutrophils and monocytes/macrophages from the blood promote oxidative stress in the acutely injured cord (Lee et al., 2011). Since L-selectin is expressed on blood neutrophils and monocytes, we examined the impact of the absence of L-selectin on oxidative stress. Immunoblotting verified that MDA, a product of lipid peroxidation (Liu et al., 2001) and marker for acute oxidative stress (Esterbauer et al., 1991; Azbill et al., 1997; Hichor et al., 2018), was similarly present at very low levels in the uninjured spinal cords of mice of both genotypes (Fig. 2, p=0.98). There was a 51% reduction in MDA in injured L-selectin KO mice compared to injured WT mice (p=0.026, three days post-SCI).

L-selectin is dynamically regulated on myeloid cells following injury

In light of our findings with L-selectin KO mice, we employed flow cytometry to quantify the expression of L-selectin on circulating and spinal cord-infiltrated leukocytes after SCI. Peripheral blood leukocytes from uninjured WT mice were also analyzed to establish baseline L-selectin levels. Because of their prominent recruitment during the acute phase of SCI (Beck et al., 2010), we focused on myeloid cells ($CD45^{+}$/$CD11b^{+}$). We identified leukocytes by gating for live (Ghost Dye^{-}) $CD45^{+}$ cells, then gated for myeloid cells ($CD11b^{+}$) and subdivided them into neutrophils ($Ly6C^{low}$/$Ly6G^{+}$), inflammatory/classical monocytes ($Ly6C^{hi}$/$L6G^{-}$), and patrolling/non-classical monocytes ($Ly6C^{low}$/$Ly6G^{-}$)(Fig. 3A). For the monocyte populations in peripheral blood, SCI resulted in over two-fold greater levels (G-MFI) of L-selectin (CD62L) on $Ly6C^{hi}$/$L6G^{-}$ cells at 24 hrs and 72 hrs post-SCI ($p<0.0001$) and an increase of 49.4% on $Ly6C^{low}$/$L6G^{-}$ cells at 72 hrs post-SCI ($p=0.048$) compared to uninjured mice (Figs. 3B-C). In contrast, L-selectin decreased by 25.3% on neutrophils ($Ly6C^{low}$/$Ly6G^{+}$ cells) at 24 hrs post-SCI ($p=0.007$) but returned to baseline (uninjured) values by 72 hrs post-SCI. Leukocytes that accumulated in the spinal cord parenchyma at 24 hrs post-SCI exhibited two to five-fold reduced levels of L-selectin on the myeloid subtypes relative to levels on the corresponding cells in the blood (Fig. 4A); however, considerable L-selectin remained on the infiltrated cells for all subtypes, as established by comparison with corresponding cells in KO mice (Fig. 4A) and isotype controls (Fig. 4B). These data demonstrate that L-selectin is present in varying degrees on all circulating myeloid lineages and is dynamically regulated after SCI. Furthermore, considerable L-selectin is retained on infiltrated myeloid cells, where it could potentially be involved in post-recruitment adhesive or signaling events.

L-selectin deficiency transiently reduces myeloid cell accumulation in the spinal cord

As reviewed in the Introduction, L-selectin has been implicated in the recruitment of myeloid cells in multiple inflammatory settings. We therefore asked whether the lack of L-selectin could reduce trafficking of myeloid cells into the injured cord. We compared the number of leukocytes in injured spinal cords of L-selectin KO and WT mice. The accumulation of leukocytes in a particular tissue reflects recruitment from the blood and their turnover in the tissue. Using flow cytometry, we first determined the total number of infiltrated myeloid lineage cells (CD45$^+$/CD11b$^+$). We then used Ly6C and Ly6G antibodies to discriminate neutrophils and monocyte/microglia subtypes (Fig. 5A-B), and further subdivided the monocyte and resident microglia populations based on CD45 levels. Few CD11b$^+$ cells were detected in the spinal cords of uninjured WT mice (650±103 cells) relative to injured WT mice at 24 hrs post-SCI (13970±1920 cells). At 24 hrs post-SCI, very few infiltrated CD11b$^+$ cells co-labeled for T-cells (213±60 TCRβ$^+$ cells) or B-cells (357±113 B220$^+$ cells), confirming that CD11b$^+$ cells were predominantly myeloid cells. Furthermore, no differences in CD45$^+$/CD11b$^-$ cells (lymphocytes) were observed in the spinal cords of uninjured vs. injured WT mice at 24 hrs post-SCI (485±186 cells vs. 532±178 cells, p=0.87). At 24 hrs post-SCI, we observed reductions of 29.9% and 26.4% of CD11b$^+$ cells (p=0.028) and Ly6C^{low}/L6G$^+$ cells (p=0.038), respectively, but no significant effects on Ly6C^{hi}/L6G$^-$ cells (p=0.10) or Ly6C^{low}/Ly6G$^-$ cells (p=0.72) in the spinal cords of L-selectin KO mice compared to WTs (Fig. 5A&C). Clearly, the decrease in CD11b$^+$ cells was mainly attributable to the Ly6C^{low}/L6G$^+$ cells (neutrophils), which comprised ~72% of this population. The contributions of L-selectin to myeloid cell and neutrophil accumulation were limited in duration as there were no differences in these populations between KO and WT mice at 72 hrs post-SCI (Fig. 5B&D). No differences in the total number of CD11b$^+$/CD45^{low}/L6G$^-$ cells (microglia) were observed between WT and KO mice at 24 hrs and

72 hrs post-SCI (Fig. 5E; p=0.10 and 0.38, respectively). No differences were observed in the proportion of CD11b$^+$ cells and myeloid lineage subsets among total CD45$^+$ leukocytes in the peripheral blood of WT and KO mice at 24 and 72 hrs post-SCI (Fig. 5F, p>0.25 for all subtypes). In summary, we found that SCI was associated with a massive influx of myeloid cells (21-fold increase) into the spinal cord and the absence of L-selectin resulted in a partial reduction in the accumulation of neutrophils at 24 hrs, which was not sustained through 72 hrs.

NSAIDS differentially induce L-selectin shedding on murine leukocytes

As an alternative to the genetic ablation of L-selectin, we sought a pharmacologic means to lower the cell surface levels of this receptor. A major mechanism for down-modulating L-selectin is through cleavage of the membrane proximal domain of L-selectin by cell surface metalloproteinases, such as ADAM17, causing the release (shedding) of the ligand-binding ectodomain into solution (Li et al., 2006). Certain NSAIDs can induce L-selectin shedding in human leukocytes *in vitro* and *in vivo* (Diaz-Gonzalez et al., 1995), which led us to investigate whether an NSAID could be used to down-modulate L-selectin levels on myeloid cells during SCI and produce benefit. We first performed *in vitro* experiments to test NSAIDs for their ability to induce L-selectin shedding from murine myeloid cells. We evaluated diclofenac and meclofenamic acid (MFA), which were shown to produce the most L-selectin shedding in human leukocytes (Gomez-Gaviro et al., 2002). Murine granulocytes (predominantly neutrophils), exposed *in vitro* to either diclofenac or MFA, exhibited a dose-dependent reduction in the level of L-selectin on the cell surface (Fig. 6A-B).

The *in vitro* effects of a drug on particular leukocytes do not necessarily predict its *in vivo* activities with critical factors being the pharmacokinetics of the drug and the turnover and anatomic compartmentalization of leukocytes. To determine if diclofenac induced the loss of cell surface L-selectin *in vivo*, uninjured WT mice were administered diclofenac (40 mg/kg, i.p.) and peripheral blood leukocytes were evaluated 2, 8, or 24 hours in separate cohorts of mice for each time point. At 2 hrs post-injection, increased L-selectin was detected on peripheral blood $CD11b^+$ cells from diclofenac-treated mice compared to saline-treated mice (Fig. 6C, 32.2% increase, p=0.03), possibly due to mobilization of myeloid cells from the bone marrow. At 8 hrs post-injection, there was a 38.3% reduction of L-selectin on $Ly6C^{low}/Ly6G^+$ cells (Fig. 6D, p=0.0006) and a reduction of 26.3% at 24 hrs (Fig. 6E, p=0.002).

Diclofenac improves long-term functional recovery

Having verified that diclofenac down-modulates L-selectin levels on myeloid cells *in vitro* and *in vivo*, we asked whether this drug could promote long-term recovery after SCI. Mice were treated with a single dose (20, 30, or 40 mg/kg) of diclofenac or vehicle (PBS) immediately after SCI, and neurological function was assessed using the BMS open field test. We found considerable improvement in locomotor recovery at the highest dose, but not at the lower doses. Enhanced recovery was apparent by seven days post-SCI and persisted throughout the remainder of the testing period (Fig. 7A). A subsequent secondary analysis revealed that by day 35, 61.5% of WT mice treated with 40 mg/kg diclofenac were occasionally stepping as opposed to 0.0% of PBS-treated mice (Fig. 7B, p=0.002).

Diclofenac as an NSAID with a multiplicity of anti-inflammatory activities (Scholer et al., 1986) could provide benefit independently of its effects on L-selectin. To address this issue, we compared neurological recovery and long-term white matter sparing in L-selectin KO mice subjected to SCI and treated with 40 mg/kg diclofenac or vehicle. As a positive control, we first examined diclofenac in WTs and again observed improved long-term neurological recovery (Fig. 7C-D). We then examined diclofenac- and vehicle-treated L-selectin KO mice and found no differences based upon the BMS scale (Fig. 7E). The percentage of spared white matter was also quantified at the lesion epicenter (Fig. 7F-G). Diclofenac-treated WT mice (40 mg/kg) showed five-fold greater white matter sparing compared to PBS-treated WT mice (p=0.030), whereas there was no difference in white matter sparing in KO mice treated with diclofenac vs. PBS (p=0.70). Thus, while diclofenac promoted recovery and neuroprotection in spinal cord injured WTs, it provided no added long-term benefit in the absence of L-selectin.

Diclofenac induces loss of L-selectin from specific leukocyte subsets but does not affect leukocyte accumulation in the injured spinal cord

We next determined if diclofenac treatment affected leukocyte accumulation after SCI. A single dose of diclofenac (40 mg/kg) or vehicle (PBS) was given immediately after SCI in WT mice. Peripheral blood and spinal cords were harvested 24 hrs later, and flow cytometry was performed to determine the levels of L-selectin as well as the accumulation of leukocyte subsets in the cord (Fig. 8A). Peripheral blood was also acquired from uninjured WT mice to compare basal expression levels of L-selectin in circulating myeloid lineages. Consistent with the results above (Fig. 3B), at 24 hrs post-SCI, injury itself (with saline injection) resulted in over two-fold greater levels of L-selectin on $Ly6C^{hi}/Ly6G^{-}$ cells (Fig. 8B&D, p=0.005) in blood. At 24 hrs post-SCI, diclofenac reduced L-selectin on $CD11b^{+}$ cells (38.2% reduction, p=0.004), $Ly6C^{low}/Ly6G^{-}$ cells (34.6% reduction, p=0.022), and $Ly6C^{low}/Ly6G^{+}$ cells (41.1% reduction, p=0.005), but not on $Ly6C^{hi}/Ly6G^{-}$ cells (p=0.12) in blood of injured mice compared to vehicle-treated controls (Fig. 8B&D). Importantly, diclofenac did not activate myeloid cells in the blood

(24 hrs post-SCI), as reflected by no change in CD11b levels (Fig. 8E). Thus, the actions of diclofenac do not appear to be attributable to a generalized activation of these cells.

No differences were apparent in the total numbers of $CD11b^{+}$ cells or myeloid lineage subsets in the spinal cords of diclofenac-treated mice at 24 hrs post-SCI compared to mice receiving the vehicle control (Fig. 8F). However, diclofenac reduced the level of L-selectin on infiltrated $CD11b^{+}$ cells (Fig. 8C&G, 26.7% loss, p=0.020), $Ly6C^{low}/Ly6G^{-}$ cells (28.8% loss, p=0.008) and $Ly6C^{low}/Ly6G^{+}$ cells (31.8% loss, p= 0.049). There was no effect on $Ly6C^{hi}/Ly6G^{-}$ cells (p=0.59). Thus, while diclofenac did not alter leukocyte accumulation in the injured cord, L-selectin was reduced on the same myeloid populations (i.e., neutrophils and non-classical monocytes) in both the blood and spinal cord of injured animals. To substantiate that diclofenac treatment resulted in shedding of L-selectin from leukocytes, we measured sL-selectin in blood plasma by ELISA. We employed a single dose of diclofenac at increasing doses (1, 5, 10, 20, 40 and 60 mg/kg) immediately following SCI. Elevated sL-selectin (above vehicle background) was detected at 8 and 24 hrs post-SCI with 40 and 60 mg/kg of diclofenac but not at the lower doses (Fig. 8H). The elevation did not persist at 72 hrs post-injection. Importantly, the high concentration of diclofenac required to induce L-selectin shedding paralleled the high concentration required to improve long-term neurological recovery (Fig. 7A).

Diclofenac reduces acute oxidative stress after SCI

Antibody-induced ligation of L-selectin has been shown to induce or potentiate the production of ROS by neutrophils (Crockett-Torabi et al., 1995). Therefore, the reduced level of L-selectin on these cells following diclofenac treatment could potentially mitigate oxidative stress in the acutely injured spinal cord. We therefore examined MDA levels in the injured spinal cord at 72 hrs post-SCI in mice receiving diclofenac (40 mg/kg) or vehicle (PBS) immediately following SCI. There was a 48.1% reduction in MDA between diclofenac and vehicle-treated mice (Fig. 9, p=0.045).

Diclofenac improves recovery when delivered within three hours after SCI

To determine the window for the beneficial effects of diclofenac, a single dose of diclofenac (40 mg/kg, i.p.) was administered at 0, 3, or 8 hrs following SCI. Again, we found that immediate delivery of diclofenac improved long-term neurologic recovery of hindlimb function, as seen by greater BMS scores within the first seven days post-SCI compared to vehicle-treated mice (Fig. 10A). Stepping was also improved with 66.7% of diclofenac-treated mice stepping at 42 days post-SCI compared to 7.7% of vehicle treated mice (Fig. 10D, p=0.003). When administration of diclofenac was delayed for 3 hrs, improved neurologic recovery was evident starting at seven days post-SCI compared to time-matched vehicle control mice (Fig. 10B). Although stepping was not improved (Fig. 10E and Table 2, 38.5% vs. 18.2%, p=0.26), white matter sparing was 2.3-fold greater in mice receiving diclofenac at 3 hrs post-SCI compared to vehicle treated mice (Fig. 10G, p=0.011). There was no long-term benefit in BMS scores or stepping when diclofenac treatment was delayed to 8 hrs post-SCI (Fig. 10C&F).

To determine if multiple doses of diclofenac conferred additional benefit, we randomly grouped mice into three cohorts and delivered diclofenac (40 mg/kg) at 0 hrs (single dose), 0 and 24 hrs (two doses), or 0, 24 and 48 hrs (three doses) post-SCI. Long-term neurological benefit was observed in the single and two-dose groups with improved BMS scores starting at seven days post-SCI compared to the vehicle-treated group (Fig. 10H). No additional benefit was observed for two doses compared to a single dose of diclofenac. BMS scores could not be determined for mice receiving three doses of diclofenac due to a high rate of morbidity and mortality. While a single dose of diclofenac exhibited a similar mortality rate to vehicle control injections, there was increased mortality for the double and triple dose regimens (Fig. 10I).

Discussion

The mechanisms underlying secondary pathogenesis, including the pathogenic activities of myeloid cells, after SCI are not fully understood, thereby limiting the development of clinical therapies. This is the first study to demonstrate the involvement of L-selectin in acute secondary pathogenesis in a murine model of SCI. We show that the genetic ablation of L-selectin markedly improves long-term neurologic recovery and white matter sparing. Pursuing a pharmacologic approach to reduce L-selectin, we demonstrate that the NSAID, diclofenac, induces partial L-selectin shedding from mouse myeloid cells *in vivo* and improves long-term neurologic outcomes when administered up to 3 hrs following injury. Our findings provide support for L-selectin shedding and the subsequent reduction of L-selectin-dependent activities other than myeloid cell recruitment, as an important anti-inflammatory activity of diclofenac during SCI.

Past studies have achieved success in limiting inflammation and secondary damage by targeting specific adhesion molecules (such as P-selectin, CD11d/CD18, and ICAM-1) and chemoattractants/chemokines involved in the migration of peripheral leukocytes into the spinal cord (Hamada et al., 1996; Taoka et al., 1997; Gonzalez et al., 2003; Gris et al., 2004; Popovich and Longbrake, 2008; Saiwai et al., 2010). In the present study, we have investigated L-selectin, which heretofore has not been considered in the context of SCI. A substantial body of evidence has established that L-selectin functions as adhesion/signaling molecule on myeloid cells and participates in their recruitment and activation at inflammatory sites (Lewinsohn et al., 1987; Pizcueta and Luscinskas, 1994; Tedder et al., 1995; Stadtmann et al., 2013; Zuchtriegel et al., 2015). We found considerable L-selectin levels on all circulating myeloid cell subtypes, including neutrophils and monocytes, in uninjured WT mice. After SCI, L-selectin was dynamically regulated on myeloid cells in blood. Notably, there was a 25.3% reduction of L-selectin on neutrophils 24 hrs after SCI, which may reflect a negative feedback mechanism to attenuate L-selectin-dependent inflammatory activities, as has been described in another setting of injury and inflammation (Strausbaugh et al., 1999). Reduced L-selectin has been previously observed in circulating neutrophils in spinal cord injured human patients (Bao et al., 2008); however, this study also found reduced L-selectin levels on circulating monocytes. The increase in L-selectin on circulating monocyte populations in our study in mouse may reflect species differences in L-selectin regulation during inflammatory responses to SCI.

L-selectin deficiency was associated with partially reduced neutrophil accumulation into the acutely injured spinal cord (24 hrs post-SCI). This is consistent with the participation of L-selectin in recruitment of neutrophils e.g., as an adhesive molecule in secondary tethering to other leukocytes or as a signaling molecule in activating integrins on neutrophils (Walcheck et al., 1996; Zarbock et al., 2011; Stadtmann et al., 2013; Morikis et al., 2017). At 72 hrs post-SCI, when neutrophil numbers in the cord were ~3-fold fewer than at 24 hrs (reflecting death or exit of the infiltrated neutrophils), there was no difference between WT and KO mice. However, at the same time point, we did find reduced oxidative stress in the acutely injured cord of L-selectin KO mice, suggesting the possibility of ROS as a component of L-selectin mediated pathogenesis. Many studies have established a role for L-selectin in modulating the internal signaling and secretome of neutrophils (Zarbock and Ley, 2008), including the potentiation of ROS production

(Crockett-Torabi et al., 1995). Elucidation of the signaling pathways by which L-selectin contributes to ROS production after SCI could identify novel targets to reduce acute secondary pathogenesis.

Myelin sheaths of CNS, but not peripheral nervous system, express ectopic ligands (presumed to be carbohydrate-based and structurally related to true biological ligands) for L-selectin that are sufficient to support leukocyte adhesion in an *in vitro* assay (Huang et al., 1991; Huang et al., 1994). We found that neutrophils that infiltrated the injured spinal cord at 24 hrs and 72 hrs post-SCI retained 19.2% and 14.8% of L-selectin levels, respectively, relative to those in peripheral blood. These levels are still appreciable, since L-selectin is normally present at high density on blood leukocytes (~10^5 molecules/cell) (Simon et al., 1992). Infiltrating neutrophils and monocytes could potentially interact with these "illegitimate" ligands via L-selectin to facilitate activation of effector functions that promote myelin degradation, a mechanism that has been invoked in a model of experimental allergic encephalitis (Grewal et al., 2001). Notably, engagement of L-selectin on neutrophils with incidental carbohydrate ligands (carcinoma or saliva mucins) potentiates the degranulation of these cells (Shao et al., 2011), even after considerable shedding of L-selectin (Mohanty et al., 2015). Thus, reduced L-selectin on spinal cord-infiltrated neutrophils may still be sufficient to drive post-recruitment activities.

NSAIDs are a heterogeneous group of compounds that continue to be an important intervention in patients with non-severe inflammatory disorders. Several NSAIDs provide neuroprotection in experimental models of SCI (Kwon et al., 2011). In the present study, we show that administration of diclofenac led to marked improvements in long-term recovery and sparing of white matter after SCI. The beneficial effects of diclofenac treatment following SCI were comparable to those seen in L-selectin KO mice. Our study thus adds diclofenac, a widely prescribed medication (Altman et al., 2015), to the group of NSAIDs that are beneficial in SCI.

We were initially drawn to diclofenac because it belongs to the subgroup of NSAIDs that are capable of inducing a high level of L-selectin shedding from the surface of leukocytes (Diaz-Gonzalez et al., 1995; Gomez-Gaviro et al., 2002). In the present study, diclofenac induced the loss of L-selectin in non-classical monocytes and neutrophils within the blood and spinal cord of injured mice. Our report is the first to show a differential effect of NSAIDs on loss of L-selectin from monocyte subtypes with non-classical monocytes being the susceptible population in the context of SCI. The diclofenac-induced reduction in L-selectin on non-classical monocytes could potentially impact signaling by these cells and diminish their deleterious activities in SCI (Donnelly et al., 2011). With respect to neutrophils, the diclofenac-induced reduction of L-selectin levels was not accompanied by reduced accumulation in the injured spinal cord (24 hrs), which contrasts with the observations in L-selectin KO mice at this time point. This difference may be ascribed to the fact that diclofenac treatment resulted in only a partial loss of L-selectin. Nonetheless, the diclofenac treatment reduced oxidative stress at 72 hours post-SCI, an effect that could plausibly be a consequence of reduced signaling and degranulation of neutrophils, as detailed above.

The anti-inflammatory activities of NSAIDs are usually attributed to inhibition of cyclooxygenase (COX), a key enzyme for prostaglandin production. However, Sanchez-Madrid, Diaz-Gonzalez and co-workers have also highlighted L-selectin shedding from neutrophils as a potential anti-inflammatory action of certain NSAIDs (Herrera-Garcia et al., 2013; Diaz-Gonzalez and Sanchez-Madrid, 2015). In our experiments, we cannot exclude that the benefit of diclofenac is due to COX inhibition or diclofenac-induced shedding of molecules other than L-selectin. However, our finding of equivalent neurological outcomes after SCI in L-selectin KO mice, treated with diclofenac compared to the vehicle control group, suggests that the beneficial effect of diclofenac is related to its ability to induce L-selectin shedding. Taken together, our diclofenac findings are consistent with the possibility that the partial loss of L-selectin induced by this drug reduces the deleterious activities of myeloid cells in secondary pathogenesis. Further mechanistic studies are needed to substantiate this scenario as opposed to other potential activities of diclofenac.

In our experiments, 40 mg/kg of diclofenac was required to improve long-term neurological recovery after SCI, but repeated dosing at this high level was associated with increased mortality. According to the FDA's guidelines for conversion to a human equivalent dose (Nair and Jacob, 2016), the dose for a 60 kg individual would be 3.25 mg/kg. In fact, oral administration of diclofenac to human subjects at a dose of ~2.5 mg/kg/day has been shown to promote robust loss of L-selectin on blood neutrophils (Baranda, 1998). Thus, it would be feasible to test diclofenac for efficacy in human SCI at an FDA-approved dose. Evaluation of diclofenac in the context of sex as a biological variable will be an important factor in such studies.

Our findings establish a therapeutic window for diclofenac administration with efficacy observed up to at least 3 hrs, but less than 8 hrs, after SCI. This timeframe is consistent with our observations that the pathological contributions of L-selectin are associated with early myeloid cell activities (< 72 hrs post-SCI). Recent clinical studies have also highlighted the importance of early intervention with anti-inflammatory strategies (Ahuja et al., 2016). Future exploration of more clinically relevant methods for delivery of diclofenac and development of faster acting structural analogues with improved safety profiles may extend the therapeutic window for strategies targeting L-selectin after SCI.

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Figure Legends

Figure 1. L-selectin KO mice show improved functional recovery and greater white matter sparing at 42 days after SCI.

(A) Functional recovery, as measured by the BMS open field test, was improved in KO mice. N=21 for WT and 24 for KO combined from 2 independent cohorts of mice. Two-way ANOVA (interaction p=0.11; time p<0.0001; genotype p=0.006; F(1, 43) = 8.42).

(B) Stepping was improved in KO mice compared to WT mice. N=21 for WT and 24 for KO combined from 2 independent cohorts of mice. Chi square analysis followed by a one-sided Fisher's exact test. *p=0.029.

(C) Performance on a grid, as measured by foot faults, was improved in the KO group. N=13 for WT and 8 for KO. Two-tailed Student’s t-test. *p=0.023, t(19) = 2.48.

(D) Representative transverse sections at the lesion epicenter, stained with luxol fast blue (LFB), of injured WT and KO mice at 42 days post-SCI. Scale bar indicates 500 µm.

(E) Greater spared white matter at the lesion epicenter, expressed as proportional area, was observed in the KO group. N=11 for WT and 8 for KO. Two-tailed Student's t-test. $*p=0.038$, $t(17) = 2.26$.

Figure 2. L-selectin is a determinant of early oxidative stress in the injured cord. Immunoblotting was performed on homogenates of uninjured (UN) and injured (SCI) cords from WT and L-selectin KO mice at 3 days post-SCI. All values were normalized to β-actin. By immunoblotting, malondialdehyde (MDA) was reduced in L-selectin KO mice by 51% compared to WT mice after SCI ($*p=0.026$, $t(8) = 2.73$). N=3/genotype for UN and 5/genotype for SCI. Unpaired two-tailed Student's t-test. For UN, $p=0.98$, $t(4) = 0.02$.

Figure 3. L-selectin is dynamically regulated after SCI.

(A) Flow cytometry gating of leukocytes and leukocyte subsets in the peripheral blood at 24 hrs and 72 hrs post-SCI.

(B) Representative flow cytometry histograms for CD62L staining of myeloid cells in the peripheral blood from uninjured mice (gray line) and injured mice at 24 hrs (red line) and 72 hrs (blue line) post-SCI. Representative isotype staining is shown in solid black.

(C) L-selectin (CD62L) levels on myeloid lineage cells in the peripheral blood from uninjured WT mice, and injured WT mice at 24 hrs and 72 hrs post-SCI. N=4 for UN, 8 for 24 hrs, and 6 for 72 hrs. One-way ANOVA with Tukey's post-hoc test ($p=0.004$, <0.0001, 0.046, 0.0001 and $F(2,15) = 7.97, 35.8, 3.80, 17.7$, respectively). $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$.

Figure 4. L-selectin is partially shed during infiltration into the spinal cord

(A) L-selectin levels (G-MFI) on circulating and infiltrated myeloid lineage cells from WT and KO mice at 24 hrs post-SCI. Background (isotype) values for CD62L were subtracted from each datapoint so that the y-axis represents the specific signal. Autofluorescence resulted in a faint signal in non-classical monocytes in the spinal cord but not in the blood of L-selectin KO mice. This was likely due to phagocytosed debris. N=8/group. One-way ANOVA with Tukey's post-hoc test ($p<0.0001$ for each cell type and $F(3, 27) = 5.49, 7.50, 4.71, 7.92$, respectively). **p<0.01, ****p<0.0001.

(B) Representative flow cytometry histograms for CD62L staining in leukocyte populations from the blood (white) and spinal cord (grey) of WT mice at 24 hrs post-SCI. Representative isotype staining is shown in solid black.

Figure 5. Leukocyte accumulation is transiently reduced in KO mice compared to WT mice after SCI.

(A-B) Flow cytometry gating of leukocytes and leukocyte subsets in the injured spinal cord at (A) 24 hrs and (B) 72 hrs post-SCI.

(C) Accumulation of myeloid cells ($CD11b^+$) and neutrophils ($Ly6C^{low}/Ly6G^+$), as determined by flow cytometry, was reduced in L-selectin KO compared to WT mice at 24 hrs post-SCI (*p=0.028 and 0.038, respectively). There were no differences in infiltrating inflammatory ($Ly6C^{hi}/Ly6G^-$, p=0.10), non-classical monocyte subsets ($Ly6C^{low}/Ly6G^-$, p=0.72) or microglia ($CD11b^+/CD45^{low}/Ly6G^-$, p=0.10) between WT and KO mice. N=7 for WT and 8 for KO. Mann Whitney test.

(D) There was no difference in the accumulation of any leukocyte subtype at 72 hrs post-SCI. N=6/group. Unpaired two-tailed Student's t-tests. p=0.82, 0.81, 0.81, 0.45 and t(10) = 0.23, 0.25, 0.24, 0.79, respectively.

(E) There were no differences in microglia ($CD11b^{+}/CD45^{low}/Ly6G^{-}$) between WT and KO mice at 24 or 72 hrs post-SCI (p=0.10 & 0.38, respectively).

(F) Prevalence of myeloid lineage subsets in the peripheral blood was similar between WT and KO mice at 24 and 72 hours post-SCI. N=7-8/genotype. Unpaired two-tailed Student's t-tests. No differences were detected (p=0.68, 0.25, 0.80, 0.70 and t(13)=0.42, 1.19, 0.25, 0.40, respectively).

Figure 6. NSAIDs induce L-selectin shedding.

(A) CD62L flow cytometry histograms for WT or L-selectin KO granulocytes treated with PMA or diclofenac (20 μg/ml or 500 μg/ml) and stained with an isotype control antibody (Iso Ctl) or a CD62L antibody.

(B) CD62L expression (G-MFI) on WT murine granulocytes was reduced relative to vehicle (N=7) by treatment with diclofenac (N=5-6) and MFA (meclofenamic acid, N=4-6). N=3/group for KOs. Data were obtained from 7 independent experiments. Cells were pooled from 3 mice for each experiment (21 total mice). One-way ANOVA with Dunnett's posthoc test. For diclofenac, p<0.0001 and F(4, 24) = 16.2. For MFA, p<0.0001 and F(4, 23) = 10.0. *p<0.05, **p<0.01, ***p<0.001.

(C-E) CD62L expression (G-MFI) was reduced on neutrophils ($Ly6C^{low}/Ly6G^{+}$) in uninjured mice receiving diclofenac relative to vehicle control at 8 and 24 hrs after injection. N =4/group at

8 hrs and 5/group at 2 hrs and 24 hrs. Unpaired two-tailed Student's t-tests. *p=0.031, 0.0006, 0.002, and t(8)=2.61, t(6)=6.64, t(8)=4.52, respectively.

Figure 7. Long-term functional recovery and white matter sparing are improved in spinal cord injured WT mice treated with diclofenac immediately after injury.

(A) Spinal cord injured mice treated with 40 mg/kg diclofenac showed improved recovery starting at 7 days post-SCI as determined by the BMS open field test. N=10 for 30 mg/kg diclofenac, and 13 for all other groups. Repeated measures two-way ANOVA with Tukey's post-hoc test (interaction $p<0.001$; time $p<0.001$; treatment p=0.003 and F(3,45)=5.50). ***$p<0.0001$ for vehicle vs 40 mg/kg diclofenac. No differences were observed between vehicle and 20 mg/kg or 30 mg/kg diclofenac.

(B) Stepping was improved in 40 mg/kg diclofenac-treated mice compared to mice receiving vehicle control. N=10 for 30 mg/kg diclofenac, and 13 for all other groups. Chi square analysis followed by a one-sided Fisher's exact test. **p=0.006 for vehicle vs 40 mg/kg. p=0.006 for 20 mg/kg vs 40 mg/kg and p=0.003 for 30 mg/kg vs 40 mg/kg.

(C) Spinal cord injured WT mice treated with 40 mg/kg diclofenac showed improved recovery compared to vehicle controls. N= 10 for vehicle and 11 for diclofenac. Repeated measures Two-way ANOVA with Sidak's post-hoc test (interaction $p<0.001$, time $p<0.0001$, treatment p=0.0004, and F(1,19)=18.8). ***$p<0.001$, ****$p<0.0001$.

(D) The number of foot faults was reduced in diclofenac-treated mice. N= 4 for PBS and 11 for diclofenac. Mann Whitney test. ***p=0.0007.

(E) Functional recovery was similar between L-selectin KO mice treated with diclofenac or vehicle (PBS). N= 6 for PBS and 5 for diclofenac. Repeated measures two-way ANOVA (interaction $p=0.54$, time $p<0.0001$, treatment $p=0.85$ and $F(1,9)=0.04$).

(F) Representative images of the lesion epicenter, stained with luxol fast blue (LFB), in mice treated with vehicle (PBS) or diclofenac. Scale bar indicates 500 µm.

(G) Spared white matter was increased in WT mice, but not L-selectin KO mice, treated with diclofenac compared to vehicle (PBS) at 42 days post-SCI. Data are expressed as proportional area. N=4 for PBS-WT, N=10 for diclofenac-WT, N=4 for PBS-KO and N=6 for diclofenac-KO. Unpaired two-tailed Student's t-test. *$p=0.030$ and $t(12)=2.46$. For KO, $p=0.70$ and $t(8)=0.40$.

Figure 8. Diclofenac treatment reduces L-selectin on peripheral blood and infiltrated leukocytes, but does not alter accumulation in the injured spinal cord.

(A) Flow cytometry gating for infiltrated myeloid cells and myeloid subsets.

(B-C) Representative flow cytometry histograms for CD62L staining in leukocyte populations from the blood **(B)** or spinal cord **(C)** of vehicle-treated (white) and diclofenac-treated (gray) mice at 24 hrs post-SCI. Representative isotype staining is shown in black.

(D) Flow cytometry analysis for CD62L expression on peripheral blood leukocytes from uninjured mice and injured mice treated with diclofenac or vehicle (PBS). Diclofenac induced a reduction of L-selectin on neutrophils and non-classical monocytes ($p=0.005$ and 0.02, respectively). N=4/uninjured and 6-7/SCI/treatment. One-way ANOVA with Tukey's post-hoc test ($p=0.004$, <0.0001, 0.019, 0.0004 and $F(2,14)=8.47$, 20.3, 5.30, 14.8, respectively). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

(E) Flow cytometry analysis for CD11b levels on peripheral blood leukocytes from spinal cord injured mice treated with diclofenac or vehicle (PBS). There were no differences in CD11b levels on myeloid cells or myeloid lineage subsets. N=6-7/treatment. Unpaired two-tailed Student's t-tests. p=0.15, 0.47, 0.99, 0.16 and t(11)=1.5, 0.75, 0.01, 1.5, respectively.

(F) There were no differences in the accumulation of total myeloid cells ($CD11b^+$) or any myeloid lineage subset in spinal cords of diclofenac-treated mice compared to vehicle-treated mice at 24 hrs post-SCI. N=7/treatment. Unpaired two-tailed Student's t-tests. p=0.37, 0.53, 0.92, 0.23 and t(12)=0.66, 0.64, 0.12, 1.27, respectively.

(G) Flow cytometry analysis demonstrated loss of L-selectin on total leukocytes ($CD45^+$, p=0.020 and t(12)=2.67), total myeloid cells ($CD11b^+$, *p=0.020 and t(12)=2.69), non-classical monocytes ($Ly6C^{low}/Ly6G^-$, **p=0.008 and t(12)=3.16), and neutrophils ($Ly6C^{low}/Ly6G^+$, *p=0.049 and t(12)=2.20) in the spinal cord of diclofenac-treated versus vehicle-treated mice at 24 hrs post-SCI. There was no effect on L-selectin on inflammatory monocytes ($Ly6C^{hi}/Ly6G^-$, p=0.59 and t(12)=0.56). N=7/treatment. Unpaired two-tailed Student's t-tests.

(H) ELISA for soluble L-selectin (sLselectin) in the peripheral blood at 8, 24, and 72 hrs post-SCI in mice receiving a vehicle (PBS) control injection or 1-60 mg/kg of diclofenac. Increased sL-selectin was observed at 8 and 24 hrs, but not 72 hrs, post-SCI in mice receiving 40 and 60 mg/kg diclofenac. N=5/group for 8 hrs and 72 hrs. N=5/group at 24 hrs except for vehicle (N=7) and 40 mg/kg diclofenac (N=10). One-way ANOVA followed by Dunnett's posthoc test (p=0.005, 0.006, 0.71, and F(6,28)=4.05, F(6,35) = 3.72, F(6,28) = 0.63, respectively). *p<0.05, **p<0.01.

Figure 9. Diclofenac treatment reduces acute oxidative stress at 72 hours post-SCI. By immunoblotting, malondialdehyde (MDA) was reduced by 29% in diclofenac-treated vs. vehicle-treated WT mice after SCI (*$p=0.045$, $t(8) = 2.37$). N=5/treatment. Unpaired two-tailed Student's t-test. All values were normalized to GAPDH.

Figure 10. Diclofenac treatment improves long-term recovery when delayed for 3 hrs, but not 8 hrs, post-SCI.

(A) BMS scores demonstrated improved neurologic recovery when diclofenac was delivered immediately following injury. N=13 for vehicle and 12 for diclofenac. Two-way ANOVA with Sidak's post hoc test (interaction $p<0.0001$, time $p<0.0001$, treatment $p<0.0001$ and $F(1,23)=26.0$). *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

(B) Recovery was also improved when administration of diclofenac was delayed to 3 hrs post-SCI. N=11 for vehicle and 13 for diclofenac. Two-way ANOVA with Sidak's post hoc test (interaction $p<0.0001$, time $p<0.0001$, treatment $p=0.0005$ and $F(1,22)=17.0$). *$p<0.05$, **$p<0.01$, ***$p<0.001$.

(C) No improvement was observed when diclofenac was delayed to 8 hrs post-SCI. N=13 for vehicle and 10 for diclofenac. Two-way ANOVA with Sidak's post hoc test (interaction $p=0.42$, time $p<0.001$, treatment $p=0.23$ and $F(1,21)=1.51$).

(D) Improved stepping ability was observed when diclofenac was delivered immediately after injury. Chi square analysis followed by a one-sided Fisher's exact test. N=13 for vehicle and 12 for diclofenac. **$p=0.003$.

(E) No benefit for stepping was observed when diclofenac was delivered 3 hrs after injury. Chi square analysis followed by a one-sided Fisher's exact test. N=11 for vehicle and 13 for diclofenac. *p=0.26.

(F) No benefit for stepping was observed with an 8 hr delay of diclofenac administration. Chi square analysis followed by a one-sided Fisher's exact test. N=13 for vehicle and 10 for diclofenac. p=0.69.

(G) Spared white matter at the lesion epicenter was increased at 42 days post-SCI in WT mice treated with diclofenac at 3 hrs post-SCI compared to the time-matched vehicle (PBS) control group. Data are expressed as total area. N=6 for PBS and N=12 for diclofenac. Two-tailed Student's t-test. *p=0.011, t(16)=2.87.

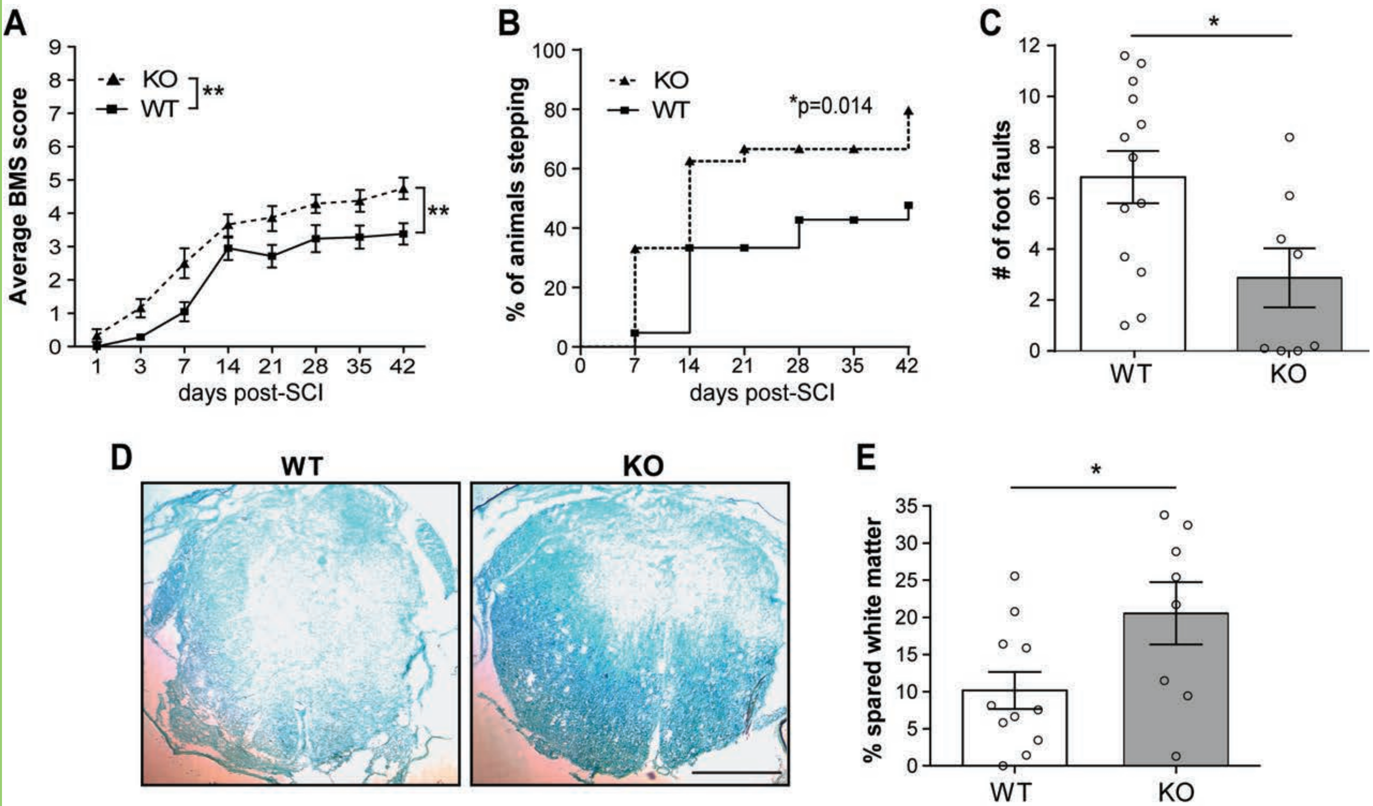
(H) No additional benefit in BMS score was achieved by administering a second dose of diclofenac. N=13 for vehicle, 15 for single dose, and 7 for 2 doses. Two-way ANOVA with Tukey's post hoc test (interaction p<0.001, time p<0.001, treatment p=0.002 and F(2,32)=8.04). p>0.55 for single vs 2 doses for all timepoints. *p<0.05, **p<0.01, ***p<0.001.

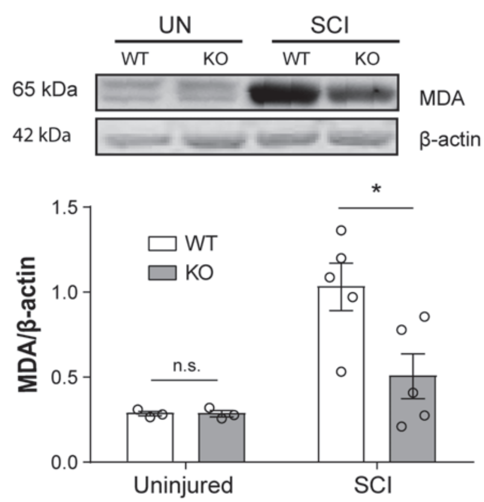
(I) Multiple doses of diclofenac resulted in higher mortality rates.

Table Legends

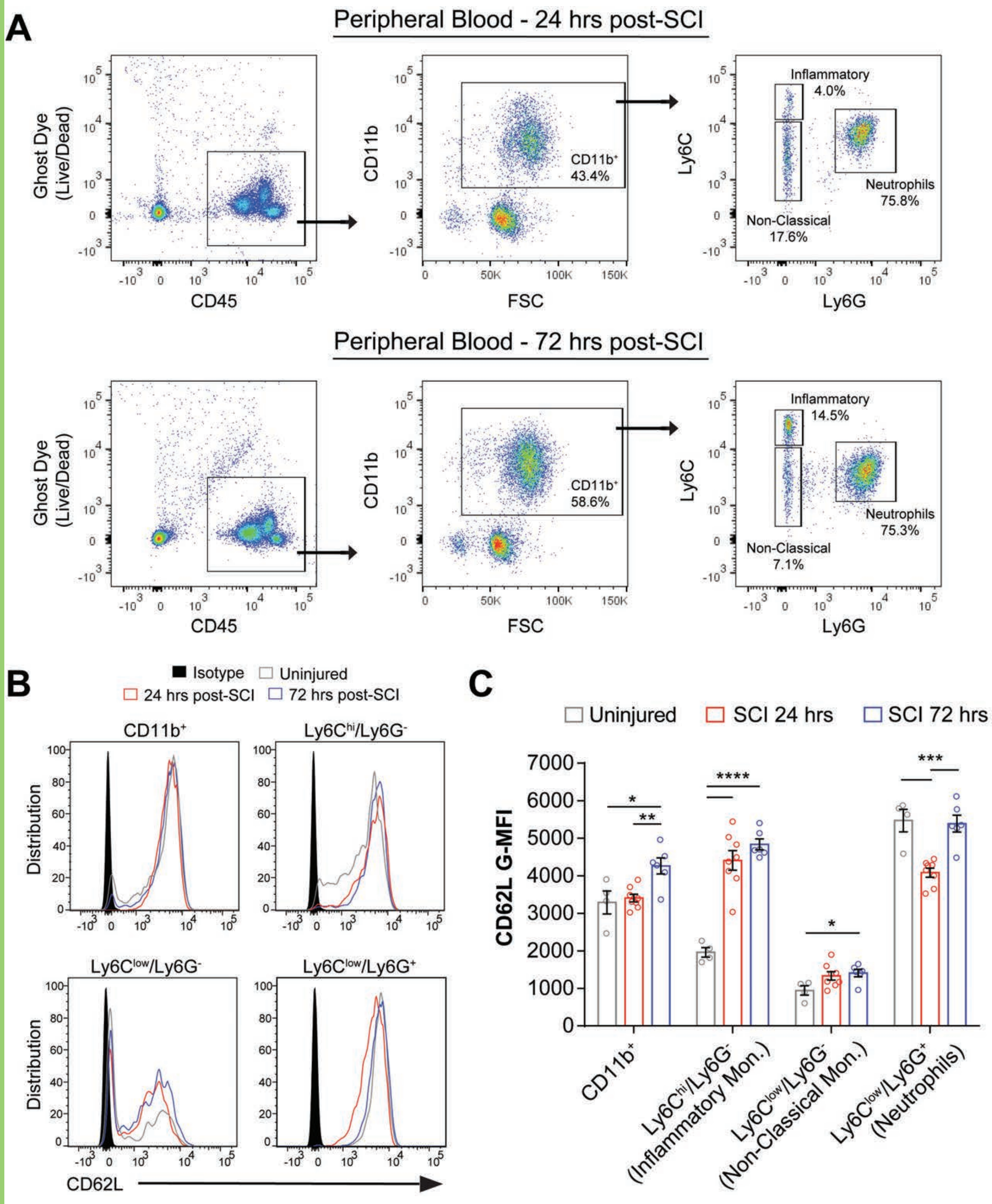
Table 1. Number of animals

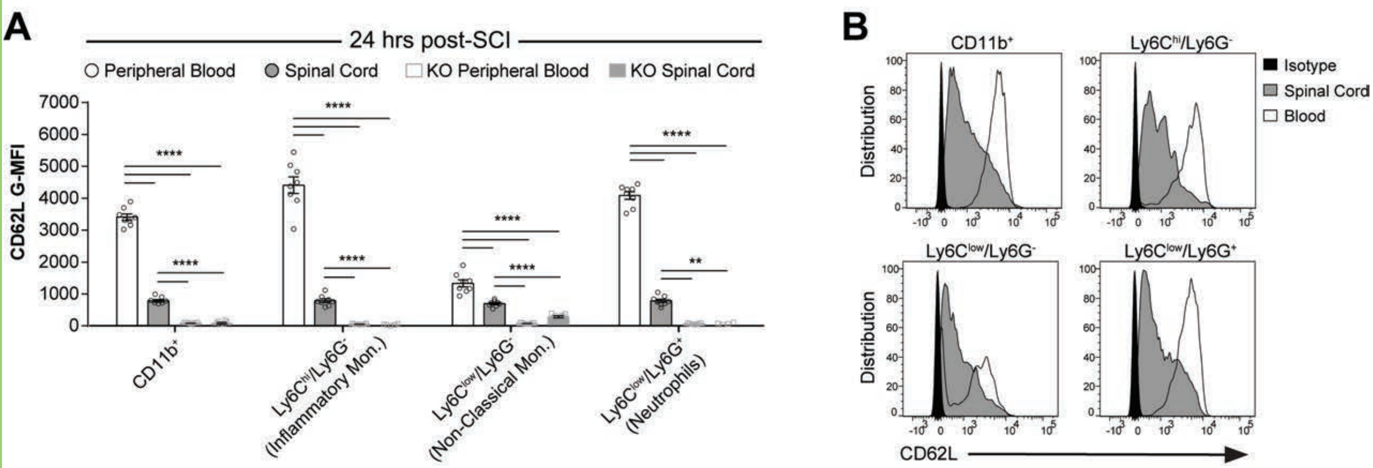
Table 2. Categorical analysis of BMS scoring

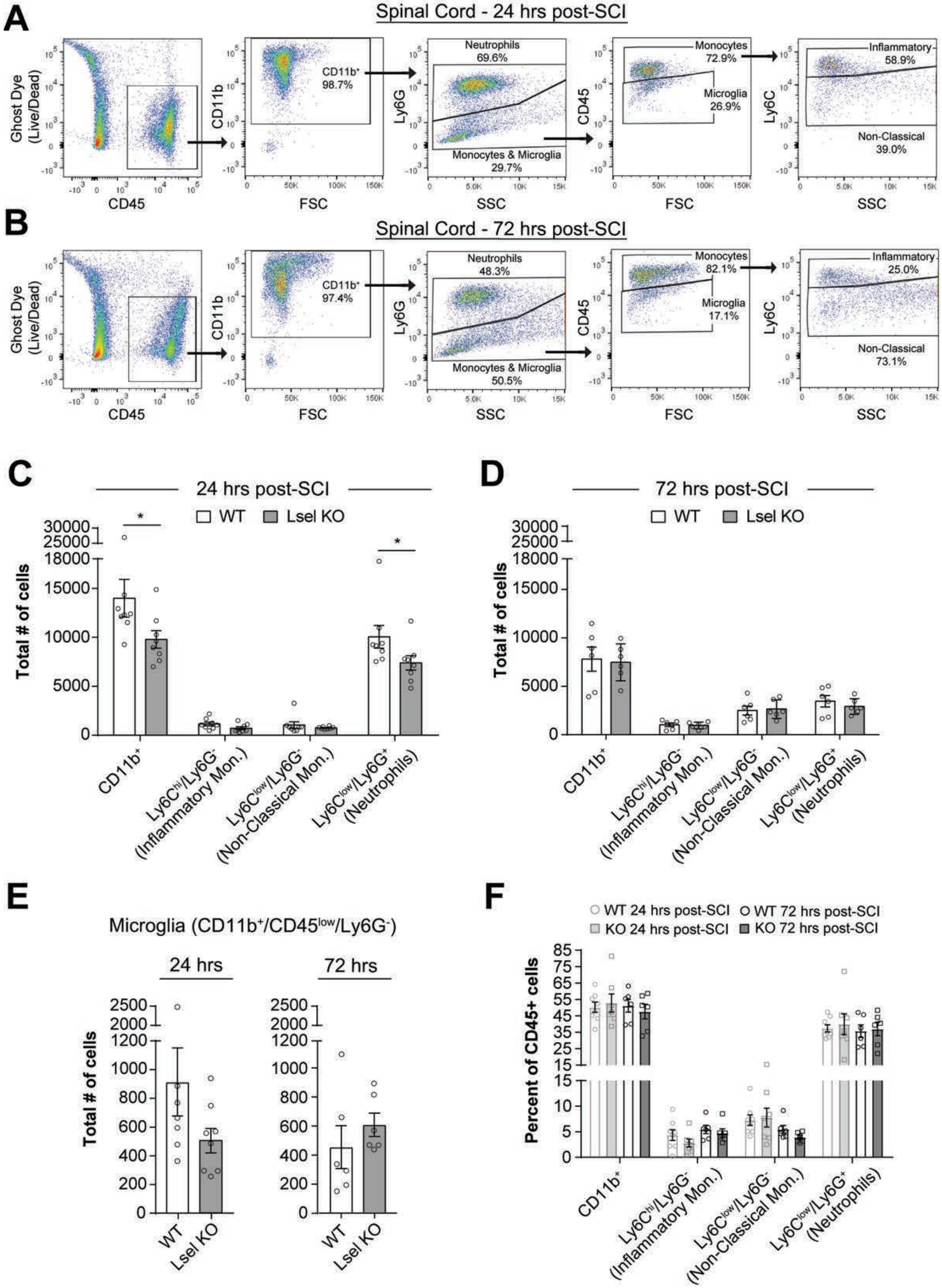

A
Average BMS score
KO
WT
**
**
days post-SCI
B
% of animals stepping
KO
WT
*p=0.014
days post-SCI
C
# of foot faults
*
WT
KO
D
WT
KO
E
% spared white matter
*
WT
KO

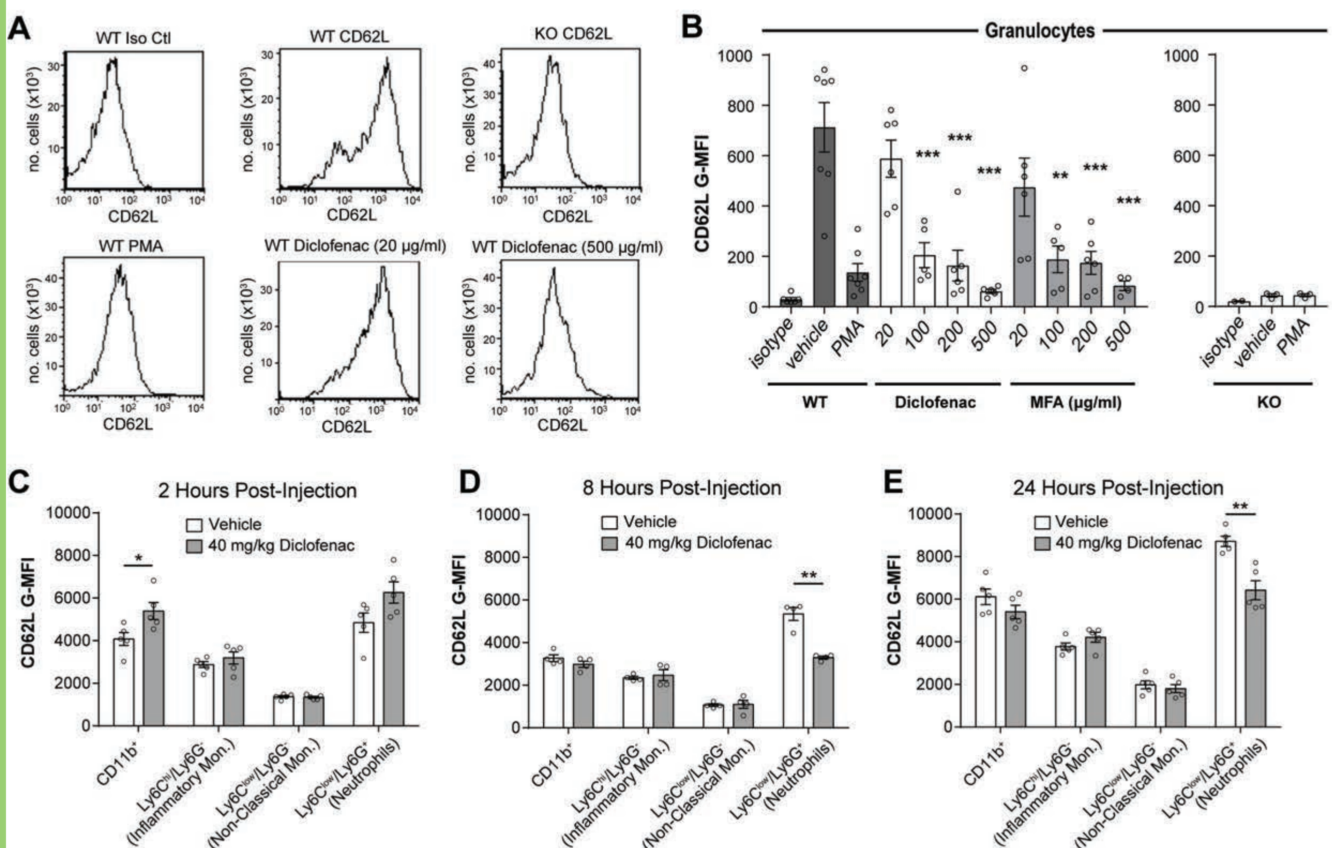


UN
SCI
WT
KO
WT
KO
65 kDa
42 kDa
MDA
β-actin
*
1.5
1.0
0.5
0.0
WT
KO
MDA/β-actin
n.s.
Uninjured
SCI

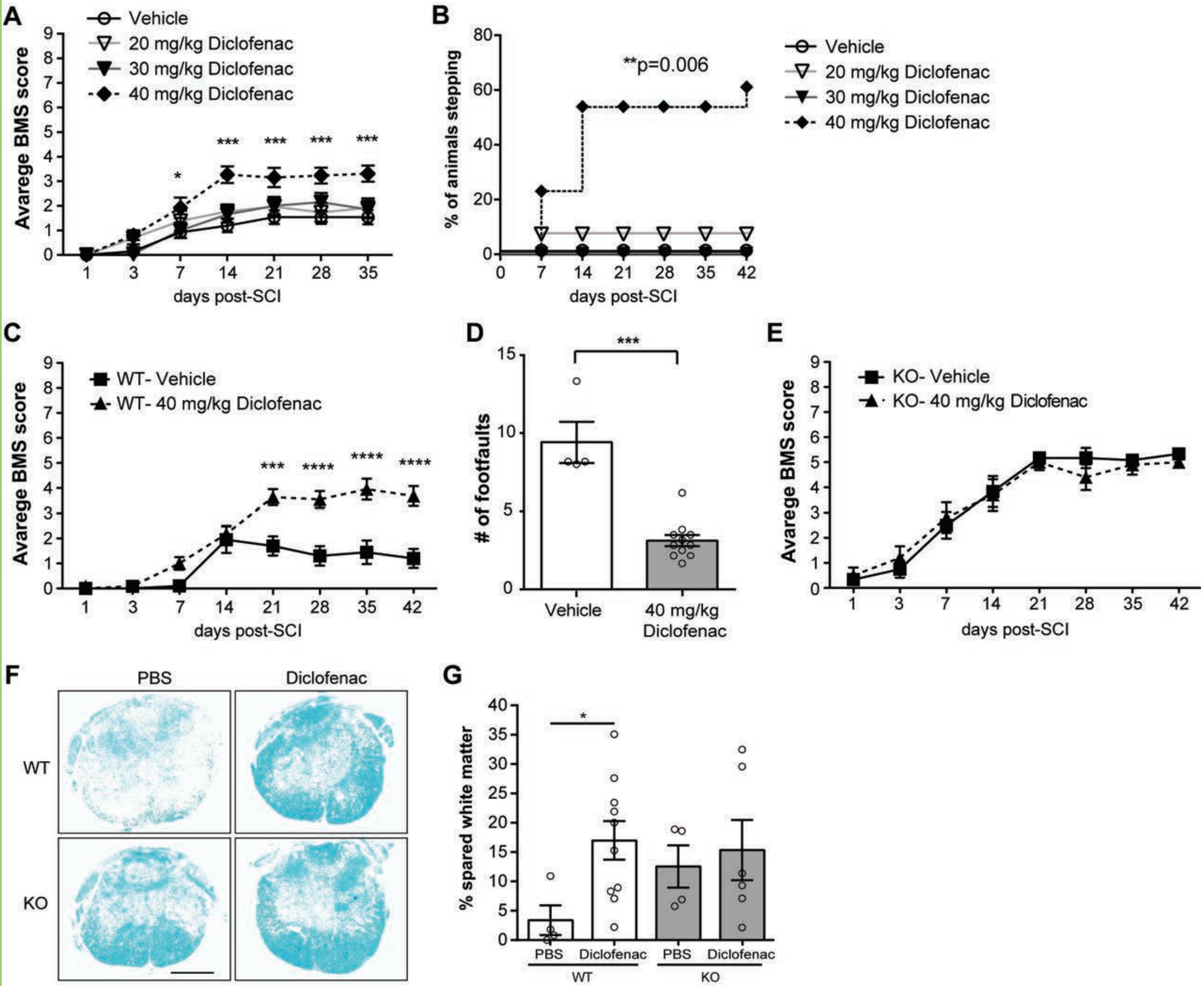

A
Peripheral Blood - 24 hrs post-SCI
Ghost Dye (Live/Dead)
CD45
CD11b
FSC
CD11b+
43.4%
Ly6C
Ly6G
Inflammatory
4.0%
Neutrophils
75.8%
Non-Classical
17.6%
Peripheral Blood - 72 hrs post-SCI
CD11b+
58.6%
Inflammatory
14.5%
Neutrophils
75.3%
Non-Classical
7.1%
B
Isotype
Uninjured
24 hrs post-SCI
72 hrs post-SCI
CD11b+
Ly6Chi/Ly6G-
Ly6Clow/Ly6G-
Ly6Clow/Ly6G+
Distribution
CD62L
C
Uninjured
SCI 24 hrs
SCI 72 hrs
CD62L G-MFI
CD11b+
Ly6Chi/Ly6G- (Inflammatory Mon.)
Ly6Clow/Ly6G- (Non-Classical Mon.)
Ly6Clow/Ly6G+ (Neutrophils)


A
24 hrs post-SCI
Peripheral Blood
Spinal Cord
KO Peripheral Blood
KO Spinal Cord
CD62L G-MFI
CD11b+
Ly6Chi/Ly6G- (Inflammatory Mon.)
Ly6Clow/Ly6G- (Non-Classical Mon.)
Ly6Clow/Ly6G+ (Neutrophils)
B
CD11b+
Ly6Chi/Ly6G-
Ly6Clow/Ly6G-
Ly6Clow/Ly6G+
Isotype
Spinal Cord
Blood
Distribution
CD62L

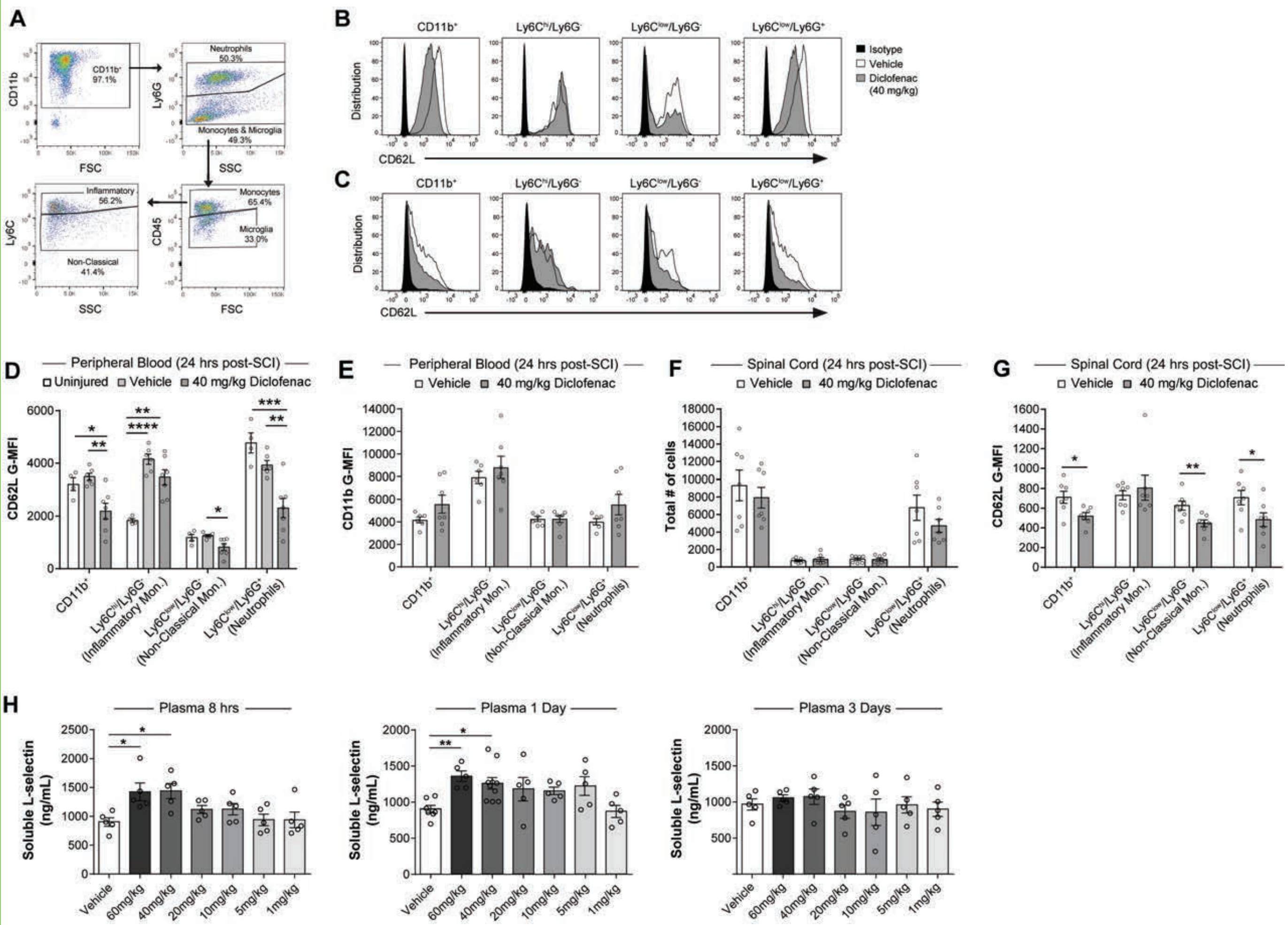

A
Spinal Cord - 24 hrs post-SCI
Ghost Dye (Live/Dead)
CD45
CD11b
CD11b⁺ 98.7%
FSC
Ly6G
Neutrophils 69.6%
Monocytes & Microglia 29.7%
SSC
CD45
Monocytes 72.9%
Microglia 26.9%
FSC
Ly6C
Inflammatory 58.9%
Non-Classical 39.0%
SSC
B
Spinal Cord - 72 hrs post-SCI
Ghost Dye (Live/Dead)
CD45
CD11b
CD11b⁺ 97.4%
FSC
Ly6G
Neutrophils 48.3%
Monocytes & Microglia 50.5%
SSC
CD45
Monocytes 82.1%
Microglia 17.1%
FSC
Ly6C
Inflammatory 25.0%
Non-Classical 73.1%
SSC
C
24 hrs post-SCI
WT
Lsel KO
Total # of cells
CD11b⁺
Ly6Chi/Ly6G⁻ (Inflammatory Mon.)
Ly6Clow/Ly6G⁻ (Non-Classical Mon.)
Ly6Clow/Ly6G⁺ (Neutrophils)
D
72 hrs post-SCI
WT
Lsel KO
Total # of cells
CD11b⁺
Ly6Chi/Ly6G⁻ (Inflammatory Mon.)
Ly6Clow/Ly6G⁻ (Non-Classical Mon.)
Ly6Clow/Ly6G⁺ (Neutrophils)
E
Microglia (CD11b⁺/CD45low/Ly6G⁻)
24 hrs
72 hrs
Total # of cells
WT
Lsel KO
WT
Lsel KO
F
WT 24 hrs post-SCI
WT 72 hrs post-SCI
KO 24 hrs post-SCI
KO 72 hrs post-SCI
Percent of CD45+ cells
CD11b⁺
Ly6Chi/Ly6G⁻ (Inflammatory Mon.)
Ly6Clow/Ly6G⁻ (Non-Classical Mon.)
Ly6Clow/Ly6G⁺ (Neutrophils)

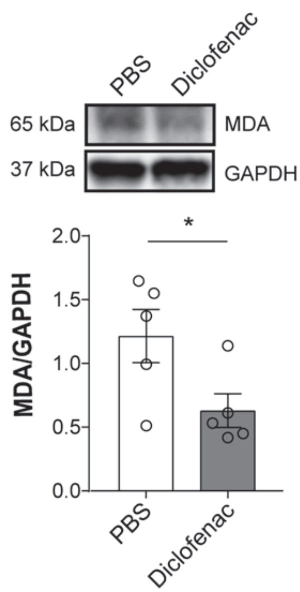


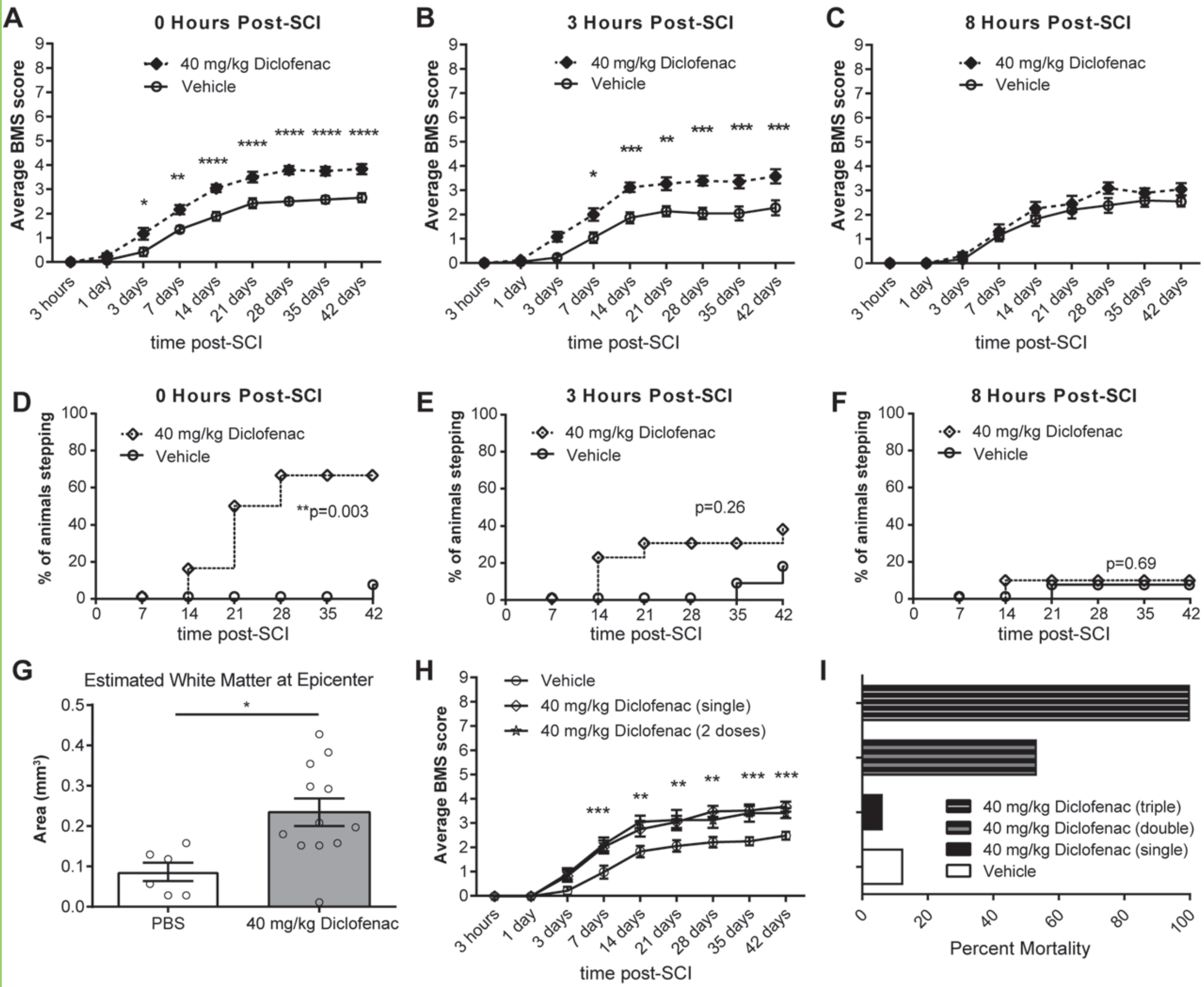
A
WT Iso Ctl
WT CD62L
KO CD62L
WT PMA
WT Diclofenac (20 µg/ml)
WT Diclofenac (500 µg/ml)
no. cells (x10³)
CD62L
B
Granulocytes
CD62L G-MFI
isotype
vehicle
PMA
WT
Diclofenac
MFA (µg/ml)
KO
C
2 Hours Post-Injection
D
8 Hours Post-Injection
E
24 Hours Post-Injection
Vehicle
40 mg/kg Diclofenac
CD62L G-MFI
CD11b⁺
Ly6Cʰⁱ/Ly6G⁻ (Inflammatory Mon.)
Ly6Cˡᵒʷ/Ly6G⁻ (Non-Classical Mon.)
Ly6Cˡᵒʷ/Ly6G⁺ (Neutrophils)



A
Vehicle
20 mg/kg Diclofenac
30 mg/kg Diclofenac
40 mg/kg Diclofenac
Avarege BMS score
days post-SCI
B
**p=0.006
% of animals stepping
days post-SCI
C
WT- Vehicle
WT- 40 mg/kg Diclofenac
Avarege BMS score
days post-SCI
D
# of footfaults
Vehicle
40 mg/kg Diclofenac
E
KO- Vehicle
KO- 40 mg/kg Diclofenac
Avarege BMS score
days post-SCI
F
PBS
Diclofenac
WT
KO
G
% spared white matter
PBS
Diclofenac
PBS
Diclofenac
WT
KO


A
CD11b
FSC
CD11b+ 97.1%
Neutrophils 50.3%
Ly6G
SSC
Monocytes & Microglia 49.3%
Inflammatory 56.2%
Ly6C
Non-Classical 41.4%
Monocytes 65.4%
CD45
Microglia 33.0%
B
CD11b+
Ly6Chi/Ly6G-
Ly6Clow/Ly6G-
Ly6Clow/Ly6G+
Distribution
CD62L
Isotype
Vehicle
Diclofenac (40 mg/kg)
C
D
Peripheral Blood (24 hrs post-SCI)
Uninjured
Vehicle
40 mg/kg Diclofenac
CD62L G-MFI
E
Peripheral Blood (24 hrs post-SCI)
CD11b G-MFI
F
Spinal Cord (24 hrs post-SCI)
Total # of cells
G
Spinal Cord (24 hrs post-SCI)
CD62L G-MFI
CD11b+
Ly6Chi/Ly6G- (Inflammatory Mon.)
Ly6Clow/Ly6G- (Non-Classical Mon.)
Ly6Clow/Ly6G+ (Neutrophils)
H
Plasma 8 hrs
Plasma 1 Day
Plasma 3 Days
Soluble L-selectin (ng/mL)
Vehicle
60mg/kg
40mg/kg
20mg/kg
10mg/kg
5mg/kg
1mg/kg


PBS
Diclofenac
65 kDa
MDA
37 kDa
GAPDH
*
2.0
1.5
1.0
0.5
0.0
MDA/GAPDH
PBS
Diclofenac


A
0 Hours Post-SCI
Average BMS score
40 mg/kg Diclofenac
Vehicle
*
**
****
****
****
****
****
3 hours
1 day
3 days
7 days
14 days
21 days
28 days
35 days
42 days
time post-SCI
B
3 Hours Post-SCI
Average BMS score
40 mg/kg Diclofenac
Vehicle
*
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**
***
***
***
time post-SCI
C
8 Hours Post-SCI
Average BMS score
40 mg/kg Diclofenac
Vehicle
time post-SCI
D
0 Hours Post-SCI
% of animals stepping
40 mg/kg Diclofenac
Vehicle
**p=0.003
time post-SCI
E
3 Hours Post-SCI
% of animals stepping
40 mg/kg Diclofenac
Vehicle
p=0.26
time post-SCI
F
8 Hours Post-SCI
% of animals stepping
40 mg/kg Diclofenac
Vehicle
p=0.69
time post-SCI
G
Estimated White Matter at Epicenter
Area (mm³)
*
PBS
40 mg/kg Diclofenac
H
Average BMS score
Vehicle
40 mg/kg Diclofenac (single)
40 mg/kg Diclofenac (2 doses)
***
**
**
**
***
***
time post-SCI
I
40 mg/kg Diclofenac (triple)
40 mg/kg Diclofenac (double)
40 mg/kg Diclofenac (single)
Vehicle
Percent Mortality

	WT (N)	KO (N)
Long-term studies		
SCI	21	24
BMS	21	24
Grid-walk	13	8
White matter sparing	11	8
Acute studies		
Uninjured	7	7
SCI	19	19
Immunoblotting	8	8
Flow cytometry	18	18

	Vehicle (N)	Diclofenac (N)
Long-term studies		
SCI (WT)	75	122
SCI (KO)	6	5
BMS	79	109
Grid walk	4	11
White matter sparing	14	28
Mortality (single dose)	2	1
Mortality (2 doses)	N/A	8
Mortality (3 doses)	N/A	9
Acute studies		
In vitro	21 (pooled)	
Uninjured	18	18
SCI	29	107
Flow cytometry	25	25
ELISA	17	95
Immunoblotting	5	5

BMS Category	Vehicle (3 hrs post-SCI, N=11)			Diclofenac (3 hrs post-SCI, N=13)		
	1 day	7 days	42 days	1 day	7 days	42 days
0	100% (11)	27.3% (3)		100% (13)	7.7% (1)	
1		45.5% (5)	27.3% (3)		38.5% (5)	
2		27.3% (3)	45.5% (5)		15.4% (2)	30.8% (4)
3			9.1% (1)		38.5% (5)	30.8% (4)
4			18.2% (2)			7.7% (1)
5						30.8% (4)