Prevention of Both Neutrophil and Monocyte Recruitment Promotes Recovery after Spinal Cord Injury

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Abstract

Strategies that block infiltration of leukocytes into the injured spinal cord improve sparing of white matter and neurological recovery. In this article, we examine the dependency of recovery on hematogenous depletion of neutrophils and monocytes. Mice were depleted of neutrophils or monocytes by systemic administration of anti-Ly6G or clodronate-liposomes. A third group was depleted of both subsets. Neurological improvement, based on a battery of tests of performance, and white matter sparing, occurred only in animals depleted of both neutrophils and monocytes. We also attempted to define the nature of the environment that was favorable to recovery. Hemeoxygenase-1 and malondialdehyde, markers of oxidative stress and lipid peroxidation, respectively, were reduced to similar levels in animals depleted of both neutrophils and monocytes, or only monocytes, but remained elevated in the group only depleted of neutrophils. Matrix metalloproteinase-9, a protease involved in early damage, was most strongly reduced in animals depleted of both leukocyte subsets. Finally, disruption of the blood–spinal cord barrier and abnormal nonheme iron accumulation were reduced only in animals depleted of both neutrophils and monocytes. Together, these findings indicate cooperation between neutrophils and monocytes in mediating early pathogenesis in the contused spinal cord and defining long-term neurological recovery.

Key words: functional recovery; monocytes; mouse; neutrophils; spinal cord injury

Introduction

THE EARLY INFILTRATION of leukocytes contributes to secondary pathogenesis in the injured spinal cord (Bao et al., 2008; Fleming et al., 2008; Gris et al., 2004; Letellier et al., 2010; Noble et al., 2002). This is exemplified in studies showing that blockade of α D β 2 or α 4 β 1 integrins on leukocytes, which function in endothelial adhesion (Davenpeck et al., 1998; Shanley et al., 1998; Yednock et al., 1995), reduces migration of these immune cells into the injured spinal cord and results in neuroprotection with enhanced neurological recovery (Bao et al., 2008; Fleming et al., 2008; Gris et al., 2004).

Less is known about which subsets of leukocytes are key determinants of long-term recovery after spinal cord injury (SCI). Neutrophils and monocytes are the first to infiltrate the spinal cord. Neutrophils are increased by 12 to 24 h post-injury (Beck et al., 2010; Stirling and Yong, 2008) and are neurotoxic *in vitro* (Nguyen et al., 2007). They are likely mediators of damage because of their abilities to generate

oxidative stress (DiStasi and Ley, 2009; Juurlink and Paterson, 1998), release proteases (Hsu et al., 2008; Noble et al., 2002), recruit further classes of inflammatory cells through chemokine networks (Soehnlein et al., 2009), and induce vascular leakage (DiStasi and Ley, 2009). Nonetheless, controversy remains regarding an overall adverse role for neutrophils in the injured cord. Notably, a strategy to deplete neutrophils resulted in poorer neurological recovery and a greater loss of white matter after SCI (Stirling et al., 2009).

Monocytes, sources of cytokines, chemokines (Christophi et al., 2009; Kigerl et al., 2009) and oxidative stress (Laskin, 2009), infiltrate the cord, beginning as early as 24 h post-injury (Letellier et al., 2010), reaching peak levels between 4 and 7 days (Beck et al., 2010). Animals, treated systemically with clodronate liposomes to deplete these monocytes, show an improvement in neurological recovery after SCI (Popovich et al., 1999). Notably, this benefit is not as great as that reported in studies that generally block leukocyte infiltration into the injured cord (Bao et al., 2008; Fleming et al., 2008; Gris et al., 2004; Letellier et al., 2010). Whereas this difference may

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reflect variations in the injury model or differences in species, it is also possible that there is cooperativity between leukocyte classes in producing pathogenesis. In support of that hypothesis, Kim and associates (2009), studying a murine model of viral meningitis, showed complementary activities for neutrophils and monocytes in mediating pathological vascular permeability.

In the present study, early pathogenesis and long-term measures of neurological recovery were studied after SCI in mice that were depleted of neutrophils, monocytes, or a combination of both. We found a reduction in abnormal iron accumulation and blood-spinal cord barrier disruption and an improvement in long-term neurological recovery only in those animals that had been depleted of both subsets of leukocytes. Collectively, these data support additive pathogenic roles for neutrophils and monocytes in the acutely injured cord, which probably influence long-term neurological recovery.

Methods

SCI

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. Adult female mice (3 months; C57Bl/6; Charles River) were anesthetized with 2.5% Avertin (0.02 mL/g body weight, i.p., tribromoethanol; Sigma, St. Louis, MO) and subjected to a moderate spinal cord contusion injury (Noble et al., 2002). Briefly, a laminectomy was performed at the ninth thoracic vertebra, and a 2 g weight was dropped 5 cm onto the exposed dura mater. After injury, the skin was closed with wound clips. The body temperature of the animals was maintained at 37 °C with a warming blanket throughout the surgery and during the recovery from anesthesia. Postoperative care included subcutaneous administration of antibiotics twice per day for 10 days and manual expression of the bladder twice per day until euthanasia.

Monocytes and neutrophil depletion

Clodronate was gift of Roche diagnostics GmbH (Germany) and encapsulated in liposomes as described (Van Rooijen and Sanders, 1994). To generate monocyte-depleted mice, anesthetized animals were intravenously administered 100 μ L of liposome-encapsulated clodronate or phosphate-buffered saline (PBS) at 1, 3, and 6 days post injury. We selected this dosing strategy not only because it has been shown to support recovery in injured rodents (Popovich et al., 1999) but also because it precedes peak levels of macrophages in the injured cord. To deplete neutrophils, anti-Ly6G mAb (hybridoma and monoclonal antibody core facility, University of California, San Francisco) was given to mice at 100 μ g by intraperitoneal injection at 1 day before injury, so as to maximally reduce the number of infiltrating neutrophils that typically occur within hours post-injury (Stirling and Young 2008). An irrelevant IgG was used as a control. To deplete both monocytes and neutrophils, mice were treated with anti-Ly6G 1 day before injury followed by clodronate-liposomes at 1, 3, and 6 days post-injury. Controls were as described in the preceding paragraph.

Verification of leukocyte depletion

To confirm depletion of neutrophils, three blood smears, prepared 1 day before and 1, 3, 7, and 14 days post-injury ($n=4-5$ per each time point) were stained with Hema 3[®] stain set (Fisher Scientific). At least 300 white blood cells were counted and the percentage of neutrophils relative to total white blood cells was determined. To confirm depletion of monocytes, blood samples were taken at 4, 7, and 14 days post-injury ($n=5$ per each time point). A hematology automated white blood cell analyzer (HemaVet[®] 850, DREW Scientific Inc., Oxford, CT) was used to quantify monocytes. Data are presented as relative percentages relative to total white blood cells. For the baseline levels of white blood cells, blood samples were prepared from uninjured mice ($n=3-4$ per each time point).

Assessment of recovery

Three different behavioral tests were performed to evaluate functional improvements after SCI. The 9-point BMS was used to examine locomotor recovery in an open field (53 $\times$ 108 $\times$ 5.5 cm) (Basso et al., 2006). This rating scale takes into account limb movement, stepping, coordination, and trunk stability. Mice were tested at 1 and 3 days and weekly thereafter until euthanasia at 6 weeks post-injury. Performance on a rotarod and the ability to traverse a wire grid were evaluated, in sequence, at 35, 36, and 37 days after injury. There were three trials daily, with a total of nine trials for each test. In each of these tests, the average score was used to calculate the mean.

Tissue preparation

Animals were anesthetized with 2.5 % Avertin and perfused with 4% paraformaldehyde (PFA) in PBS, pH 7.4. The spinal cord was removed, immersed in 4% PFA over night, and then transferred into 30% sucrose in PBS. A 0.5-cm segment of spinal cord, centered over the area of maximal damage, was embedded in OCT compound (Thermo Scientific, Kalamazoo, MI), cut transversely at 20 μ m in thickness using a cryostat (Leica CM1900, Leica Microsystem, Bannockburn, IL) and mounted onto Superfrost Plus[®] microscope slides (Fisher Scientific, Pittsburgh, PA).

Measurement of white matter sparing

To assess white matter sparing, transverse sections were stained for luxol fast blue at 42 days after SCI ($n=10$ per each group) as previously described (Noble et al., 2002). Briefly, sections were dehydrated with 75% ethyl alcohol and then incubated with 0.1% luxol fast blue (Kodak, Rochester, NY) in 0.05% acetic acid (Fisher Scientific) and 95% ethyl alcohol (Fisher Scientific) at 50 °C for overnight. After rinsing with 95% ethyl alcohol, sections were differentiated in 0.05% lithium carbonate (Sigma). Two sections, representing the area of maximal damage, were selected for analysis. The total cross-sectional area and the area of residual white matter area was determined using the NeuroLucida imaging system (MicroBrightField, Williston, VT). Residual white matter was calculated by the following formula: % white matter sparing = (white matter area/the total area cross-sectional area) $\times$ 100.

Flow cytometry: preparation and analysis

Blood samples were obtained by cardiac puncture using a heparin-primed syringe. Each sample was lysed in 1X RBC lysis buffer (eBioscience, San Diego, CA) according to the manufacturer's protocol. Briefly, 200 μ L of each sample were treated with 2 mL of 1X RBC lysis buffer for 5 min at room temperature with occasional shaking, followed by the addition of 20 mL of PBS. The cell suspension was centrifuged at 300 $\times$ g, 4 $^{\circ}$ C for 5 min. The supernatant was discarded and the pellet was re-suspended in FACS buffer (0.02% NaN_3 , 1% fetal bovine serum, 0.1 M PBS, pH7.4). Spinal cord samples were prepared as previously described with some modifications (Stirling and Yong, 2008; Tjoa et al., 2003). Briefly, a 3-mm segment of injured spinal cord was harvested and then mechanically dissociated with the edge of a syringe. Cells were suspended in FACS buffer and then passed through a 70- μ m nylon cell strainer (Becton Dickinson, San Jose, CA) to isolate tissue debris. The cell suspension was centrifuged at 300 $\times$ g, 4 $^{\circ}$ C for 5 min. The cell pellet was re-suspended in FACS buffer and then lysed in 1X RBC lysis buffer. Both blood and spinal cord samples were incubated with anti-mouse CD16/32 Fc blocking antibody (1:10 dilution; eBioscience) at 4 $^{\circ}$ C for 10 min and then split into 4 tubes. Samples were incubated with 1:10 dilution of antibodies: fluorescein isothiocyanate (FITC) conjugated rat anti-mouse CD11b (eBioscience), FITC conjugated rat anti-mouse Gr-1 (eBioscience), FITC conjugated rat anti-mouse F4/80 (eBioscience) and phycoerythrin (PE) conjugated rat anti-mouse CD45 (eBioscience). FITC conjugated rat IgG2b, κ (eBioscience) and PE conjugated rat IgG2b, κ (eBioscience), served as isotype controls. After incubating with antibodies for 30 min at 4 $^{\circ}$ C, samples were washed with PBS and re-suspended in fixative (1% PFA). To avoid debris, prepared spinal cord cells were stained with 7-Amino-Acinomycin D (7-AAD, BD Bioscience, San Jose, CA), which is a dye for dead cells. Flow cytometric analysis of cell suspensions was performed on a FACS Sort Cell Sorter flow cytometer (Becton Dickinson). The data were analyzed using FlowJo software. At

least 100,000 events were analyzed for blood samples and 500,000 events were analyzed for spinal cord samples. Blood cells were initially gated by their characteristic forward and side scatter profiles, which represent size and granularity, respectively, in blood leukocytes. Viable cells were determined by gating on 7-AAD non-labeled cells. Spinal cord cells were initially gated by live cells. Gated cells were then analyzed for fluorescent intensity. To determine the absolute numbers of cells, the cell concentration was calculated with CountBrightTM absolute counting beads (Molecular Probes, Carlsbad, CA) according to manufacturer's protocol.

Immunoblotting

A 0.5-cm length of cord, centered over the site of impact and representing the epicenter, was homogenized in Glo lysis buffer from Promega (Madison, WI). The protein concentration of homogenates was determined by the BCA protein assay kit (Pierce, Rockford, IL) and 20 μ g of protein was loaded onto 8 or 12% SDS-PAGE gel, electrophoresed, and transferred onto a nitrocellulose membrane. After blocking the membrane with blocking solution (Li-COR Bioscience, Lincoln, NE), the membrane was incubated with antibodies directed against hemeoxygenase-1 (HO-1) (Stressgen, Ann Arbor, MI), matrix metalloproteinase-9 (MMP-9) (Abcam, Cambridge, MA), or malondialdehyde (MDA) (OxiSelect Malondialdehyde immunoblot kit; Cell BioLabs, Inc., San Diego, CA). After incubating with LR-680 conjugated secondary antibodies (Li-COR Bioscience), the membrane was washed with PBS and then visualized with a Li-COR scanner (Li-COR Bioscience). β -actin (Sigma) served as a loading control. Values were expressed relative to the uninjured samples ($n=3-5$ per group).

Gelatin zymography

Gelatin zymography was performed as described previously (Noble et al., 2002). Briefly, the epicenter of injured spinal cord (0.5 cm in length) was homogenized in lysis buffer

FIG. 1. Treatment with anti-Ly6G Ab or clodronate liposomes reduces neutrophils and hematogenous macrophages (monocytes), respectively, in blood. **(A)** To deplete neutrophils, anti-Ly6G Ab was administered i.p. 1 day before injury. Neutrophils (NE) were manually counted in blood smears stained with Hema 3[®] Stain kit. Two-way ANOVA with Bonferroni post-hoc test; $n=4-5$ per group; $**p<0.001$. **(B)** To deplete hematogenous macrophages (MO), clodronate liposomes were administered i.p. at 1, 3, and 6 days post-injury. Hematogenous macrophages (monocytes) were quantified using an automated white blood cell analyzer (HemaVet). Two-way ANOVA followed by Bonferroni post-hoc test; $n=4-5$ per group; $^{\#}p<0.05$ and $***p<0.0001$.

FIG. 2. Flow cytometric analysis of leukocytes in blood in uninjured (UN) and injured animals at 2 (A–C) and 7 (D–F) days post-injury. (A–C) Treatment with the anti-Ly6G Ab alone or in combination with clodronate liposomes significantly reduced the number of Gr-1+ /CD45+ (A), CD11b+ /CD45+ (B), and F4-80+ /CD45+ cells at 2 days post-injury. (D–F) By 7 days post-injury, treatment with anti-Ly6G Ab alone resulted in an increase in Gr-1+ /CD45+ cells (D), whereas treatment with clodronate liposomes alone or in combination with anti-Ly6G Ab resulted in a reduction in both CD11b+ /CD45+ and F4-80+ /CD45+ cells (E, F). Unpaired *t*-tests; *n* = 4–5 per group; #*p* < 0.05, ##*p* < 0.005, and ***p* < 0.001.

FIG. 3. Flow cytometry analysis of neutrophils and macrophages in the injured cord. Representative flow cytometric plots for spinal cord samples (A[a to h]). 7-Amino-Actinomycin D (7-AAD) was used to determine the cell viability of the cell suspensions from uninjured spinal cord. Live cells (not labeled with 7-AAD) were gated (R1, A[a]) and identified by FITC+ and PE+ labeling (A[c]–A[h]). To determine absolute numbers of cells, CountBright™ absolute counting beads (A[a]) were gated (R2) and then the cell concentration was calculated according to manufacturer's instructions. Cells were stained with isotype control (A[b]). Cell suspensions from injured spinal cord at 2 days after injury were stained with Gr-1 with CD-45 (A[c] enclosed in rectangle). Cell suspensions from injured spinal cords treated with Ly6G Ab (A[f]) and clodronate liposomes (A[g and h]) were reduced relative to controls. Flow cytometric analysis of leukocytes in homogenates of uninjured (UN) and injured spinal cords at 2 (B) and 7 (C) days post-injury. (B) At 2 days post-injury, treatment with anti-Ly6G Ab alone or in combination with clodronate liposomes results in a significant reduction in Gr-1+ /CD45+ and CD11b+ /CD45+ cells (a, b). Treatment with anti-Ly6G Ab in combination with clodronate-liposomes also reduces the number of F4-80+ /CD45+ cells (c). (C) At 7 days post-injury, Gr-1+ /CD45+ cells are similar to controls in all treated groups (a) whereas treatment with anti-Ly6G Ab, clodronate liposomes, or a combination of both results in a reduction in CD11b+ /CD45+ cells (b). Whereas F4-80+ /CD45+ cells were unchanged in the vehicle versus the anti-Ly6G Ab treated group, they were significantly reduced in the clodronate liposome group or in combination with anti-Ly6G Ab (c). *n* = 4–5 per group; unpaired *t*-tests; #*p* < 0.05, ##*p* < 0.005, **p* < 0.01, and ***p* < 0.001.

containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS. 50 μ g of protein were loaded onto 10% SDS-polyacrylamide gels, copolymerized with gelatin (1 mg/mL, Sigma). After electrophoresis, renaturation was achieved by incubation of the gel in 2.5% Triton X-100 for 30 min and in substrate buffer (50 mM Tris-HCl, pH 8.5, 5 mM

CaCl₂) for 48 h at 37°C. The gel was stained with Coomassie blue solution for 4 h and then de-stained with 40% methanol/10% acetic acid. For quantitative analysis, gels were scanned and the positive band was measured using NIH Image J software (NIH). Data were expressed as the values related to uninjured samples ($n = 4-5$ per group).

Nonheme iron staining

Histochemistry for nonheme iron was performed as described previously (Yoneyama-Sarnecky et al., 2010). Transverse sections from the epicenter of injured spinal cord were incubated in modified Perl's solution (2% potassium ferrocyanide/6% hydrochloride) for 30 min, washed with PBS, and then reacted with liquid DAB⁺ substrate chromogen system (DAKO, Carpinteria, CA) for 10 min. The sections were mounted on slides and photographed using a Nikon Bright-field microscope (Nikon, Tokyo, Japan) equipped with a digital camera (SPOTTM Imaging solutions, Sterling Heights, MI). For the quantitative analysis, integrated density was determined using NIH Image J software ($n=4-5$ per group).

Immunohistochemical analysis

The following primary antibodies were used: rabbit anti-HO-1 (Stressgen), rabbit anti-MMP-9 (Abchem), rat anti-F4/80 (Caltag, Burlingame, CA), rat anti-CD11b (Serotec, Kidlington, Oxford), rat anti-Gr1 (Millipore, Billerica, MA), hamster anti-CD31 (Chemicon, Millipore, Billerica, CA), rabbit anti-GFAP (DAKO). Secondary antibodies were as follows: anti-rabbit-Cys3 (Jackson ImmunoResearch, Tucker, GA), anti-rabbit FITC (Jackson ImmunoResearch), anti-rat-Cys3 (Jackson ImmunoResearch), and anti-hamster-Cys3 (Jackson ImmunoResearch). Sections were rinsed with PBS and incubated with blocking solution containing 1% normal serum and 0.1 % bovine serum albumin in PBS at room temperature for 1 h and incubated with primary antibodies overnight at 4°C. After rinsing with PBS, secondary antibodies were applied and incubated at room temperature for 1 h. The sections were then rinsed with PBS and mounted with ProLong[®] Gold antifade reagent (Invitrogen, Eugene, Oregon). 4',6-diamidino-2-phenylindole, dilactate (DAPI) (Invitrogen) was used for nuclear staining. All images were captured on a fluorescent microscope (Optiphot EF-D3; Nikon, Tokyo, Japan) equipped with a SPOT camera (SPOTTM Imaging solutions).

Blood–spinal cord barrier measurements

To quantify vascular leakage, Evans blue permeability was measured as previously described (Kim et al., 2009). Evans blue dye (20 mg/kg body weight) was injected intravenously at 2 days post-injury. After 4 h, mice were perfused with PBS. One-half centimeter of injured spinal cord was homogenized in 50 μ l of N,N-dimethyl formamide (Sigma) and then the supernatant was obtained by centrifugation at 16,000 $\times$ g for 20 min at 4 °C. For quantification, supernatant was measured with a ND-3300 fluorospectrometer (NanoDrop Technologies Inc. Wilmington, DE). The supernatant from injured cords that were not injected with Evans blue dye served as negative controls. Representative images were taken using a digital camera (Cyber-shot, Sony) and a confocal microscope (LSM510). For immunohistochemical analysis, tissues were prepared as described previously. Tissues were incubated with PECAM antibody and DAPI for nuclear staining. All images were captured on a Confocal microscope (LSM510, Zeiss).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA). Repeated two-way AN-

OVA was used to evaluate performance in BMS open field tests. Immunoblots (MMP-9 and HO-1) and gelatin zymography were analyzed by two-way ANOVA. Two-group comparisons were by unpaired *t*-tests. Statistical significance was defined at $p \leq 0.05$. Data are expressed as means $\pm$ SEM.

Results

Verification of leukocyte depletion

Depletion of circulating neutrophils and monocytes was first confirmed by differential blood cell counts (Fig. 1). The number of neutrophils in blood was reduced for at least the first 3 days after a single pretreatment with the Ly6G Ab and returned to baseline values by 7 days (Fig. 1A). Treatment with clodronate liposomes on 1, 3, and 6 days post-injury reduced circulating monocytes for at least the first 14 days post injury (Fig. 1B).

Flow cytometry, conducted at 0, 2, and 7 days, was next used to profile CD45⁺ subsets in the blood of each of the groups. As expected, Gr-1⁺/CD45⁺ cells were reduced in animals treated with either anti-Ly6G Ab or anti-Ly6G Ab/clodronate liposomes (by 81.3% and 86.3%, respectively) (Fig. 2A). CD11b⁺/CD45⁺ and F4-80⁺/CD45⁺ cells were reduced in animals treated with either clodronate liposomes (by 60.5 % and 66.7%, respectively) or anti-Ly6G Ab/clodronate-liposomes (by 84.7% and 92%, respectively) (Fig. 2B and C). At 7 days post-injury, there was an increase in Gr-1/CD45 cells in animals that had been treated with anti-Ly6G Ab, whereas numbers of CD11b⁺/CD4⁺ and F4-80⁺/CD45⁺ cells remained depressed in clodronate liposome treated groups (by 65.5% and 48%, respectively) and the anti-Ly6G Ab/clodronate liposomes treated group (by 91.4% and 85.7%, respectively) (Fig. 2D-F).

A similar flow cytometric analysis was conducted for spinal cord tissue at 0, 2, and 7 days post-injury (Fig. 1A–C). At 2 days post-injury, reductions in CD45⁺ subsets paralleled that seen in blood with fewer Gr-1⁺/CD45⁺ cells in the anti-Ly6G Ab (by 48.8%) and anti-Ly6G Ab/clodronate liposome treated groups (by 48.8%). Similarly, there were reduced numbers of CD11b⁺/CD45⁺ and F4-80⁺/CD45⁺ cells in the anti-Ly6G Ab/clodronate liposome treated groups (by 83% and 59.5%, respectively) (Fig. 3B). At 7 days post-injury, differences in leukocyte subsets were limited to monocytes where reductions were noted in both CD11b⁺/CD45⁺ and F4-80⁺/CD45⁺ in groups treated with clodronate liposomes (by 38% and 29.2%, respectively) and anti-Ly6G Ab/clodronate liposomes (by 51.9% and 39.3%, respectively) (Fig. 3C).

Depletion of both neutrophils and monocytes improves long-term functional recovery and white matter sparing

To determine if the number of infiltrating leukocytes was related to long-term recovery, performance was evaluated in the BMS open field test (Basso et al., 2006), on a rotarod, and on a grid walking task. Although all groups showed a spontaneous improvement in locomotor recovery over time based upon the BMS open field test, this recovery was markedly higher (by 121–123 %) between 28 and 42 days post-injury in the group depleted of both neutrophils and monocytes (Fig. 4A). Subsequent assessment of the subscore for this test, as well as the ability to maintain position on a rotarod and walk across a grid, likewise revealed an improvement (by 333%, 182%, and 55%, respectively) in animals depleted of both

FIG. 4. Depletion of both neutrophils and macrophages improves long-term functional recovery after spinal cord injury. (A) Functional recovery as determined by the BMS in mice treated with anti-Ly6G Ab, clodronate-liposomes, or both anti-Ly6G Ab and clodronate-liposomes. Repeated two-way ANOVA followed by Bonferroni post-hoc test. (B) BMS subscores in anti-Ly6G Ab and/or clodronate-liposomes treated, spinal cord injured mice post-injury. Circles indicate individual animals. Repeated two-way ANOVA followed by Bonferroni post-hoc test. (C) Performance on a rotarod in anti-Ly6G Ab and/or clodronate liposome treated, spinal cord injured mice. Unpaired *t*-tests. (D) Comparison of grid walking in anti-Ly6G Ab and/or clodronate liposome treated, spinal cord injured mice. Unpaired *t*-tests; $n=12-14$ per group; $\#p<0.05$, $*p<0.01$, and $***p<0.0001$.

neutrophils and monocytes, relative to their respective controls (Fig. 4B–D).

After the completion of the behavioral assays, animals were euthanized and white matter sparing was evaluated in all groups. Total cross-sectional areas of the injured cord were comparable among all groups at 42 days post-injury (data not

shown). A comparison of white matter sparing within control groups demonstrated no differences (IgG vs. IgG + PBS-liposomes [$p=0.18$] and Ly6G versus IgG + PBS-liposomes [$p=0.10$]). Consistent with the behavioral outcomes, white matter sparing was greater in the group depleted of both neutrophils and monocytes relative to their respective

FIG. 5. Depletion of both neutrophils and macrophages enhances sparing of white matter. **(A)** Representative transverse sections at the lesioned epicenter, stained with luxol fast blue. Scale bar indicates 500 μ m. **(B)** Spared white matter at 42 days post-injury. Data are expressed as proportional area (see Methods section for more detail on this metric). Unpaired *t*-tests; *n* = 10 per group; ****p* < 0.0005.

controls, whereas no differences were seen in the other groups (Fig. 5).

Oxidative stress reduced in animals depleted of both neutrophils and monocytes

We examined indicators of oxidative stress (HO-1, MDA, and nonheme iron) in injured mice with or without leukocyte depletion. HO-1 is upregulated by oxidative stress in a variety of pathological conditions (Cuadrado and Rojo, 2008; Lin et al., 2007). Analysis of immunoblots revealed an elevation of this protein by 4 days post-injury, which was attenuated by 60% in animals depleted of monocytes or both neutrophils and monocytes (Fig. 6). Similarly, MDA, a product of lipid peroxidation (Anderson and Means, 1985; Liu et al., 2001), was increased at 4 days post-injury and reduced in animals

depleted of both neutrophils and monocytes (Fig. 7). Accumulation of nonheme iron was reduced in the epicenter of the injured spinal cord at 14 days post-injury only in animals that were depleted of both neutrophils and monocytes (by 18.8% reduction of nonheme iron compared to control) (Fig. 8).

Depletion of neutrophils reduces expression of MMP-9

MMP-9 is a determinant of neutrophil infiltration, early disruption of the blood–spinal cord barrier, and impaired long-term functional recovery after SCI (Noble et al., 2002). We found that MMP-9 colocalized with macrophages (F4-80, CD-11b), neutrophils (Gr-1), and blood vessels (CD-31) within the cord by 2 days post-injury (data not shown).

Based upon immunoblot (Fig. 9A–D), MMP-9 was dramatically increased by 155% at 2 days post-injury (Fig. 9A and C) and was reduced in the injured cords that were depleted of neutrophils or both neutrophils and monocytes (by 43.4% and 48.3%, respectively). In contrast, no differences were seen in animals that were depleted of monocytes (Fig. 9C).

The pro- and active forms of MMP-9 were evaluated in the injured cord by gelatin zymography (Fig. 9B). Both forms were significantly reduced in the injured cords that were depleted of neutrophils (30.6% reduction for the pro form and 55.4% reduction for the active form) or of both neutrophils and monocytes (46% reduction for the pro form and 74.8% reduction for the active form) at 2 days post-injury, whereas no differences were seen in the injured cords depleted of only monocytes (Fig. 9D).

Depletion of both neutrophils and monocytes reduces disruption of the blood–spinal cord barrier

Whereas there was no Evans blue dye in the uninjured cord (Fig. 10A), marked disruption of the blood–spinal cord barrier was reduced by 55.3% only in spinal cord injured animals that had been depleted of both neutrophils and monocytes (Fig. 10B and C).

Discussion

Leukocytes infiltrate the injured spinal cord, triggering pathological responses that exacerbate tissue damage and impair neurological recovery (Beck et al., 2010; Letellier et al., 2010; Stirling and Yong, 2008). Although strategies to reduce influx of leukocytes into the injured spinal cord result in recovery of function, less is known about which subsets of leukocytes influence recovery processes. Here we show that depletion of both neutrophils and monocytes resulted in an early reduction in oxidative stress, nonheme iron, and expression of MMP-9 and stabilization of the blood–spinal cord barrier. Importantly, whereas a reduction of neutrophils or infiltrating monocytes had no effect on neurological recovery, depletion of both subsets resulted in significant long-term improvement. Together, these findings provide evidence for an additive effect between neutrophils and monocytes in modulating early secondary pathogenesis and long-term neurological recovery after SCI.

Several strategies were used to deplete animals of neutrophils and monocytes. An anti-Ly6G neutralizing antibody (RB6-8C5 clone) was used to deplete mice of neutrophils. This antibody recognizes the Ly6G/Gr-1 antigen on mature

FIG. 6. Induction of heme oxygenase-1 (HO-1) in the injured cord is attenuated in macrophage-depleted animals. HO-1 immunoblots were prepared from homogenates of uninjured (UN) and injured cords. HO-1 is induced in response to injury. (A–C) HO-1 is significantly reduced in animals treated with clodronate liposomes alone or in combination with anti-Ly6G Ab at 4 days post-injury when this protein is maximally expressed. $n=3-5$ per group; two-way ANOVA with Bonferroni post-hoc test; $**p<0.001$ and $***p<0.0001$.

neutrophils (Fleming et al., 1993). Similar to Stirling and associates (2009), we found that the anti-Ly6G antibody reduced circulating Gr-1+ neutrophils in the absence of any significant change in CD11b+ or F4-80+ monocytes. However, it is of interest that CD11b/CD45 decreased at both 2 and 7 days post-injury in the Ly6G-depleted group. We can only speculate on why this may have occurred. CD11b is expressed in a subset of granulocytes that may account for the decrease at 2 days post-injury. A previous study showed that Ly6G Ab treatment indeed reduced rolling and adhering of neutrophils in venules, and that this was accompanied by a reduction of neutrophils (CD45+/CD11b+/Gr1+ population) in the injured cord (Stirling et al., 2009).

To deplete monocytes, mice were treated systemically with clodronate liposomes. Phagocytosis of these liposomes results in clodronate-mediated apoptotic cell death (Van Rooijen and Sanders, 1994; Van Rooijen et al., 1996). Although it is possible that clodronate liposomes could cross the disrupted barrier, size restrictions probably prevent their passage into the injured cord. Liposomes are >120 nm in diameter (www.clodronate-liposomes.org), making it difficult for them to be transported in transendothelial vesicles (molecular diameter of 50–100 nm) or pass between disrupted endothelial junctions that at least in venules are estimated to be 15–30 nm in diameter (Mayhan and Heistad, 1985; Neuwelt, 1989). Flow cytometry, applied to blood and spinal cord homogenates, confirmed reduction of monocytes/macrophages. In the injured spinal cord, there was a 30–40% of reduction in these cells by 7 days post-injury (Fig. 3). This is perhaps not surprising, as the number of monocytes typically reaches peak levels between 4 and 7 days post-injury (Beck et al., 2010; Stirling et al., 2009).

Three parameters were used to assess oxidative stress: namely HO-1, MDA, and nonheme iron. Here we show for the first time that induction of HO-1 and MDA are attenuated in monocyte-depleted mice, and such findings suggest a pathologic involvement of monocytes in the acutely

FIG. 7. MDA is reduced in macrophage-depleted animals at 4 days post-injury. MDA immunoblots were prepared from homogenates of uninjured (UN) and injured cords. MDA is modulated in response to spinal cord injury. MDA is significantly reduced in animals treated with clodronate liposomes alone or in combination with anti-Ly6G Ab. $n=3-5$ per group; unpaired t -test; $*p<0.01$ and $**p<0.001$.

FIG. 8. Depletion of both neutrophils and macrophages reduces nonheme iron accumulation in the injured cord. Modified Perl's staining was used to detect nonheme iron in the injured cord at 14 days post-injury. **(A)** Perl's staining was analyzed using NIH ImageJ and expressed as integrated density measurements. Whereas nonheme iron staining was similar between the vehicle and clodronate liposome treated groups, it was significantly reduced in spinal cord injured animals treated with anti-Ly6G in combination with clodronate liposomes. Unpaired *t*-tests; *n*=4–5 per group; $^{\#}p<0.05$. **(B–E)** Representative transverse sections prepared from uninjured **(B)** animals and spinal cord injured animals, treated with either Ly6G Ab or clodronate liposomes alone, or in combination with anti-Ly6G antibody, and their respective controls **(C–E)**. Scale bar indicates 500 μ m **(B, C)**.

injured cord. HO-1 (heat shock protein 32 [hsp32]) is rapidly induced by oxidative stress (Goldbaum and Richter-Landsberg, 2001; Stahnke et al., 2007) mediated by oxidant-responsive transcription factors (Lin et al., 2007) such as AP-1 (Paine et al., 2010), NF- κ B (Paine et al., 2010), hypoxia-inducible factor (Lee and Andersen, 2006) and post-transcriptional regulation by iron regulatory proteins (Lee and Andersen, 2006). This protein catalyzes the conversion of heme to bilirubin, carbon monoxide, and ferrous iron. It is likely that the end products of heme degradation influence outcome. For example, whereas bilirubin may function as a potent antioxidant, the generation of ferrous iron may potentiate damage by reacting with hydrogen peroxide to produce highly reactive hydroxyl radicals that lead to oxidative stress and cell death (Liu et al., 2002; Mautes et al., 2000; Syapin, 2008). In SCI in which there is overt intraparenchymal hemorrhage, increased HO-1 activity may become cytotoxic as a result of the generation of excess iron that overwhelms mechanisms regulating iron homeostasis (Lee et al., 2010). MDA, a reactive aldehyde resulting from lipid peroxidation by reactive oxygen species (Anderson and Means, 1985; Azbill et al., 1997; Liu et al., 2001), was likewise reduced in monocyte-depleted mice, thus further reinforcing an adverse role for monocytes in the injured spinal cord.

Abnormal accumulation of iron is also an indicator of cellular oxidative stress (Rathore et al., 2008; Yoneyama-Sarnecky et al., 2010). Cell injury results in the release and intracellular accumulation of nonheme iron and the generation of free radicals that react with H_2O_2 to produce hydroxyl radicals and generate oxidative stress (Davalos et al., 2000; Eaton and Qian, 2002; Hua et al., 2006; Lee et al., 2010; Nakamura et al., 2006; Song et al., 2007). Whereas excess nonheme iron is sequestered by intracellular iron storage and regulatory proteins, such as ferritin, ribonucleotide reductase, transferrin, and ceruloplasmin (Ponka, 2004), excessive accumulation may overwhelm these systems. Abnormal accumulation of nonheme iron occurs in a variety of pathological conditions such as ischemia (Carbonell and Rama, 2007; Davalos et al., 2000), hemorrhage (Hua et al., 2006; Lee et al., 2010), Alzheimer's disease (Ong and Farooqui, 2005), Parkinson's disease (Crichton et al., 2010), traumatic brain injury (Yoneyama-Sarnecky et al., 2010), and SCI (Rathore et al., 2008). Moreover, antioxidants such as deferoxamine reduce nonheme iron accumulation, oxidative stress, and neuronal cell death in subarachnoid hemorrhage (Lee et al., 2010). In the current study, depletion of both neutrophils and monocytes reduced nonheme iron accumulation at the site of injury. These findings are perhaps not surprising as monocytes and neutrophils are each capable of promoting oxidative stress (Gilgun-Sherki et al., 2004; Infanger et al., 2006; Nguyen et al., 2007). Our results are compatible with those of previous studies showing that blocking leukocyte recruitment into the injured spinal cord reduces various indices of cellular oxidative stress (Bao et al., 2005; Fleming et al., 2008; Schultke et al., 2010).

MMP-9 is markedly upregulated in the acutely injured spinal cord and has been shown to contribute to early disruption of the barrier, the influx of neutrophils, and white matter damage (Noble et al., 2002; Trivedi et al., 2005). Such findings are consistent with its ability to degrade constituents of the extracellular matrix (Mun-Bryce and Rosenberg, 1998) including the basal lamina of the microvasculature, and

FIG. 9. MMP-9 is reduced in spinal cord injured animals depleted of neutrophils. (A) Immunoblots, prepared from homogenates of uninjured and injured spinal cord were used to detect MMP-9 protein. (B) Gelatin zymography, prepared from homogenates of uninjured (UN) and injured spinal cords, was used to detect MMP-9. Although MMP-9 is not apparent in the uninjured cords, both the pro- and active forms are evident in the injured cord. (C, D) Quantitative analysis of immunoblots (C) and zymography (active form, D). Two-way ANOVA with Bonferroni post-hoc test; $n=3-5$ per group; $**p<0.001$ and $***p<0.0001$.

myelin basic protein. Furthermore, MMP-9 deficient mice show improved recovery of locomotor function after SCI, a finding consistent with the pathogenic role of this enzyme in the acutely injured cord. Similar to a previous study (Romanic et al., 1998), we find that depletion of neutrophils prior to

injury results in a significant reduction of MMP-9. As granules in neutrophils contain pre-formed MMP-9 (Lindsey et al., 2001; Opdenakker et al., 2001), it is likely that they are released in response to activation of these cells in the injured tissue. MMP-9 is also expressed in other cell types in the injured cord

FIG. 10. Depletion of both neutrophils and macrophages reduces vascular leakage after spinal cord injury. Representative injured spinal cord at 2 days post-injury (**A** and **B**). Scale bar indicates 500 μ m (**A**) and 5 mm (**B**). (**C**) Quantitative measurement of Evans blue (EB) using a fluorometer with uninjured animals that received Evans blue (UN), injured animals that did not receive Evans blue (NO EB), and injured animals that received Evans blue. Whereas Evans blue signal was similar among the vehicle, anti-Ly6G, and clodronate liposome treated groups, it was significantly reduced in the spinal cord injured animals treated with anti-Ly6G in combination with clodronate liposomes. Unpaired *t*-tests; *n* = 4–5 per group; **p* < 0.001.

including macrophages and astrocytes (Noble et al., 2002). Here we find that neurological recovery is dependent upon depletion of both neutrophils and monocytes. Therefore, although there is a significant reduction of MMP-9 in neutrophil-depleted animals, it may not reach a threshold that is necessary for recovery. As monocytes are also sources of MMP-9, depletion of both leukocyte subsets may achieve this critical level.

We found that barrier disruption was reduced at 2 days post-injury in spinal cord injured mice, depleted of both neutrophils and monocytes. This 2-day time point corresponds to a second peak of barrier disruption in the contused murine spinal cord (Whetstone et al., 2003) and to the trafficking of both neutrophils and monocytes into the injured cord (Stirling and Yong, 2008). Our findings are consistent with those of a previous study that evaluated the contribution of neutrophils and monocytes to barrier disruption in a model of lymphocytic choriomeningitis virus-induced meningitis (Kim et al., 2009). In that study, the authors examined the dependency of myelomonocytic cells on leakiness of the barrier. Neither depletion of neutrophils nor of monocytes prevented disruption of the barrier, whereas depletion of both leukocyte subsets preserved the integrity of the barrier to Evans blue albumin. Such findings suggest complementary pathogenic functions of neutrophils and monocytes in promoting barrier dysfunction.

Here we report that depletion of neutrophils prior to SCI had no effect on neurological recovery, a finding that contrasts with that of Sterling and associates (2009) who reported a worsened neurological outcome. These differences may have been attributed to the timing of administration of antibodies (pre- vs. post-injection) and gender (female vs. male mice).

Although our intent with anti-Ly6G Ab was to deplete animals of circulating neutrophils, it is important to acknowledge that this antibody may also influence bone marrow inducing apoptosis of neutrophils (Ribechini et al., 2009). In addition, anti-Ly6G Ab increases macrophage suppressor cell activity (Ribechini et al., 2009). As there are many events in the acutely injured cord that can influence macrophage differentiation, a key concern will be: determining the overall importance of suppressor cell activity in defining the macrophage phenotype, because macrophage suppressor cells are known non-classical macrophages (M2).

Neurological recovery was unchanged in mice depleted of monocytes, a finding that contrasts with that of Shechter and associates (2009) who reported a marked reduction in recovery in mice null for CD11c, an integrin that is expressed on monocyte-derived macrophages. In our paradigm, absence of any detectable change in functional recovery may reflect the more generic reduction of monocytes that obscures any effects exerted by subsets of these leukocytes. There may be, for

example, divergent effects of distinctive macrophages (M1 and M2) in the injured cord (Kigerl et al., 2009). Although we did not design the study specifically to assay M1 and M2 macrophages, several of the outcomes, showing down-regulation of the oxidative stress markers heme oxygenase-1 and malondialdehyde in clodronate-liposome treated animals, suggest a dominance of the M1 phenotype in the more acutely injured cord. However, further studies will be needed to address the involvement of specific subtypes of macrophages in the injured cord.

A number of studies using antibody blockade (Fleming et al., 2008; Gris et al., 2004; Mabon et al., 2000), and pharmacological (Bao et al., 2008; Noble et al., 2002) and genetic (Letellier et al., 2010) strategies to block the early infiltration of leukocytes, report an improvement in long-term neurological outcomes. In the present study, improved neurological recovery and white matter sparing were limited to those animals that had been depleted of both neutrophils and monocytes. Such beneficial effects may be in part attributed to a reduction in the hostile environment of the acutely injured cord that is dependent upon cooperativity between these leukocytes. It is also possible that these leukocytes exert an effect on the pro-inflammatory state of the injured cord, which in turn may influence neurological recovery. For example, myeloid cells deficient in CD95L show reduced trafficking into the injured cord and are associated with a down-regulation of pro-inflammatory cytokines and chemokines, such as IL-1 β , IL-6, CXCL-10, and CCL6, and improved neurological recovery (Letellier et al., 2010).

Conclusion

In conclusion, our findings provide the first evidence for cooperativity between neutrophils and monocytes in defining a hostile environment in the acutely injured cord. Our results may serve as a foundation for developing immune-based therapeutics that take into account the complex interactions between inflammatory cells in the acutely injured spinal cord that influence long-term neurologic recovery.

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Author Disclosure Statement

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References

Anderson, D.K., and Means, E.D. (1985). Iron-induced lipid peroxidation in spinal cord: protection with mannitol and methylprednisolone. *J. Free Radic. Biol. Med.* 1, 59–64.

Azbill, R.D., Mu, X., Bruce-Keller, A.J., Mattson, M.P., and Springer, J.E. (1997). Impaired mitochondrial function, oxidative

stress and altered antioxidant enzyme activities following traumatic spinal cord injury. *Brain Res.* 765, 283–290.

Bao, F., Chen, Y., Schneider, K.A., and Weaver, L.C. (2008). An integrin inhibiting molecule decreases oxidative damage and improves neurological function after spinal cord injury. *Exp. Neurol.* 214, 160–167.

Bao, F., Dekaban, G.A., and Weaver, L.C. (2005). Anti-CD11d antibody treatment reduces free radical formation and cell death in the injured spinal cord of rats. *J. Neurochem.* 94, 1361–1373.

Basso, D.M., Fisher, L.C., Anderson, A.J., Jakeman, L.B., McTigue, D.M., and Popovich, P.G. (2006). Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. *J. Neurotrauma* 23, 635–659.

Beck, K.D., Nguyen, H.X., Galvan, M.D., Salazar, D.L., Woodruff, T.M., and Anderson, A.J. (2010). Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment. *Brain* 133, 433–447.

Carbonell, T., and Rama, R. (2007). Iron, oxidative stress and early neurological deterioration in ischemic stroke. *Curr. Med. Chem.* 14, 857–874.

Christophi, G.P., Hudson, C.A., Panos, M., Gruber, R.C., and Massa, P.T. (2009). Modulation of macrophage infiltration and inflammatory activity by the phosphatase SHP-1 in virus-induced demyelinating disease. *J. Virol.* 83, 522–539.

Crichton, R.R., Dexter, D.T., and Ward, R.J. (2010). Brain iron metabolism and its perturbation in neurological diseases. *J. Neural Transm.* 118, 301–314.

Cuadrado, A., and Rojo, A.I. (2008). Heme oxygenase-1 as a therapeutic target in neurodegenerative diseases and brain infections. *Curr. Pharm. Des.* 14, 429–442.

Davalos, A., Castillo, J., Marrugat, J., Fernandez-Real, J.M., Armengou, A., Cacabelos, P., and Rama, R. (2000). Body iron stores and early neurologic deterioration in acute cerebral infarction. *Neurology* 54, 1568–1574.

Davenpeck, K.L., Sterbinsky, S.A., and Bochner, B.S. (1998). Rat neutrophils express $\alpha 4$ and $\beta 1$ integrins and bind to vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). *Blood* 91, 2341–2346.

DiStasi, M.R., and Ley, K. (2009). Opening the flood-gates: how neutrophil-endothelial interactions regulate permeability. *Trends Immunol.* 30, 547–556.

Eaton, J.W., and Qian, M. (2002). Molecular bases of cellular iron toxicity. *Free Radic. Biol. Med.* 32, 833–840.

Fleming, J.C., Bao, F., Chen, Y., Hamilton, E.F., Relton, J.K., and Weaver, L.C. (2008). $\alpha 4 \beta 1$ integrin blockade after spinal cord injury decreases damage and improves neurological function. *Exp. Neurol.* 214, 147–159.

Fleming, T.J., Fleming, M.L., and Malek, T.R. (1993). Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J. Immunol.* 151, 2399–2408.

Gilgun-Sherki, Y., Melamed, E., and Offen, D. (2004). The role of oxidative stress in the pathogenesis of multiple sclerosis: the need for effective antioxidant therapy. *J. Neurol.* 251, 261–268.

Goldbaum, O., and Richter-Landsberg, C. (2001). Stress proteins in oligodendrocytes: differential effects of heat shock and oxidative stress. *J. Neurochem.* 78, 1233–1242.

Gris, D., Marsh, D.R., Oatway, M.A., Chen, Y., Hamilton, E.F., Dekaban, G.A., and Weaver, L.C. (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.
- Hsu, J.Y., Bourguignon, L.Y., Adams, C.M., Peyrollier, K., Zhang, H., Fandel, T., Cun, C.L., Werb, Z., and Noble-Haeusslein, L.J. (2008). Matrix metalloproteinase-9 facilitates glial scar formation in the injured spinal cord. *J. Neurosci.* 28, 13467–13477.
- Hua, Y., Nakamura, T., Keep, R.F., Wu, J., Schallert, T., Hoff, J.T., and Xi, G. (2006). Long-term effects of experimental intracerebral hemorrhage: the role of iron. *J. Neurosurg.* 104, 305–312.
- Infanger, D.W., Sharma, R.V., and Davisson, R.L. (2006). NADPH oxidases of the brain: distribution, regulation, and function. *Antioxid. Redox. Signal.* 8, 1583–1596.
- Juurlink, B.H., and Paterson, P.G. (1998). Review of oxidative stress in brain and spinal cord injury: suggestions for pharmacological and nutritional management strategies. *J. Spinal Cord Med.* 21, 309–334.
- Kigerl, K.A., Gensel, J.C., Ankeny, D.P., Alexander, J.K., Donnelly, D.J., and Popovich, P.G. (2009). Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. *J. Neurosci.* 29, 13,435–13,444.
- Kim, J.V., Kang, S.S., Dustin, M.L., and McGavern, D.B. (2009). Myelomonocytic cell recruitment causes fatal CNS vascular injury during acute viral meningitis. *Nature* 457, 191–195.
- Laskin, D.L. (2009). Macrophages and inflammatory mediators in chemical toxicity: a battle of forces. *Chem. Res. Toxicol.* 22, 1376–1385.
- Lee, D.W., and Andersen, J.K. (2006). Role of HIF-1 in iron regulation: potential therapeutic strategy for neurodegenerative disorders. *Curr. Mol. Med.* 6, 883–893.
- Lee, J.Y., Keep, R.F., He, Y., Sager, O., Hua, Y., and Xi, G. (2010). Hemoglobin and iron handling in brain after subarachnoid hemorrhage and the effect of deferoxamine on early brain injury. *J. Cereb. Blood Flow Metab.* 30, 1793–1803.
- Letellier, E., Kumar, S., Sancho-Martinez, I., Krauth, S., Funke-Kaiser, A., Laudenklos, S., Konecki, K., Klussmann, S., Corsini, N.S., Kleber, S., Drost, N., Neumann, A., Levi-Strauss, M., Brors, B., Gretz, N., Edler, L., Fischer, C., Hill, O., Thiemann, M., Biglari, B., Karray, S., and Martin-Villalba, A. (2010). CD95-ligand on peripheral myeloid cells activates Syk kinase to trigger their recruitment to the inflammatory site. *Immunity* 32, 240–252.
- Lin, Q., Weis, S., Yang, G., Weng, Y.H., Helston, R., Rish, K., Smith, A., Bordner, J., Polte, T., Gaunitz, F., and Denner, P.A. (2007). Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. *J. Biol. Chem.* 282, 20,621–20,633.
- Lindsey, M., Wedin, K., Brown, D., Keller, C., Evans, A.J., Smolen, J., Burns, A.R., Rossen, R.D., Michael, L., and Entman, M. (2001). Matrix-dependent mechanism of neutrophil-mediated release and activation of matrix metalloproteinase 9 in myocardial ischemia/reperfusion. *Circulation* 103, 2181–2187.
- Liu, D., Li, L., and Augustus, L. (2001). Prostaglandin release by spinal cord injury mediates production of hydroxyl radical, malondialdehyde and cell death: a site of the neuroprotective action of methylprednisolone. *J. Neurochem.* 77, 1036–1047.
- Liu, Y., Tachibana, T., Dai, Y., Kondo, E., Fukuoka, T., Yamana, H., and Noguchi, K. (2002). Heme oxygenase-1 expression after spinal cord injury: the induction in activated neutrophils. *J. Neurotrauma* 19, 479–490.
- Mabon, P.J., Weaver, L.C., and Dekaban, G.A. (2000). Inhibition of monocyte/macrophage migration to a spinal cord injury site by an antibody to the integrin α D: a potential new anti-inflammatory treatment. *Exp. Neurol.* 166, 52–64.
- Mautes, A.E., Bergeron, M., Sharp, F.R., Panter, S.S., Weinzierl, M., Guenther, K., and Noble, L.J. (2000). Sustained induction of heme oxygenase-1 in the traumatized spinal cord. *Exp. Neurol.* 166, 254–265.
- Mayhan, W.G., and Heistad, D.D. (1985). Permeability of blood-brain barrier to various sized molecules. *Am. J. Physiol.* 248, H712–H718.
- Mun-Bryce, S., and Rosenberg, G.A. (1998). Matrix metalloproteinases in cerebrovascular disease. *J. Cereb. Blood Flow Metab.* 18, 1163–1172.
- Nakamura, T., Keep, R.F., Hua, Y., Nagao, S., Hoff, J.T., and Xi, G. (2006). Iron-induced oxidative brain injury after experimental intracerebral hemorrhage. *Acta Neurochir. Suppl.* 96, 194–198.
- Neuwelt, E.A. (1989). *Implications of the Blood-Brain Barrier and Its Manipulation*, Vol. 2, Plenum Publishing: New York.
- Nguyen, H.X., O'Barr, T.J., and Anderson, A.J. (2007). Polymorphonuclear leukocytes promote neurotoxicity through release of matrix metalloproteinases, reactive oxygen species, and TNF- α . *J. Neurochem.* 102, 900–912.
- Noble, L.J., Donovan, F., Igarashi, T., Goussev, S., and Werb, Z. (2002). Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. *J. Neurosci.* 22, 7526–7535.
- Ong, W.Y., and Farooqui, A.A. (2005). Iron, neuroinflammation, and Alzheimer's disease. *J. Alzheimers Dis.* 8, 183–200; discussion 209–115.
- Opdenakker, G., Van den Steen, P.E., Dubois, B., Nelissen, I., Van Coillie, E., Masure, S., Proost, P., and Van Damme, J. (2001). Gelatinase B functions as regulator and effector in leukocyte biology. *J. Leukoc. Biol.* 69, 851–859.
- Paine, A., Eiz-Vesper, B., Blasczyk, R., and Immenschuh, S. (2010). Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. *Biochem. Pharmacol.* 80, 1895–1903.
- Ponka, P. (2004). Hereditary causes of disturbed iron homeostasis in the central nervous system. *Ann. N. Y. Acad. Sci.* 1012, 267–281.
- Popovich, P.G., Guan, Z., Wei, P., Huitinga, I., van Rooijen, N., and Stokes, B.T. (1999). Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. *Exp. Neurol.* 158, 351–365.
- Rathore, K.I., Kerr, B.J., Redensek, A., Lopez-Vales, R., Jeong, S.Y., Ponka, P., and David, S. (2008). Ceruloplasmin protects injured spinal cord from iron-mediated oxidative damage. *J. Neurosci.* 28, 12,736–12,747.
- Romanic, A.M., White, R.F., Arleth, A.J., Ohlstein, E.H., and Barone, F.C. (1998). Matrix metalloproteinase expression increases after cerebral focal ischemia in rats: inhibition of matrix metalloproteinase-9 reduces infarct size. *Stroke* 29, 1020–1030.
- Schultke, E., Griebel, R.W., and Juurlink, B.H. (2010). Quercetin attenuates inflammatory processes after spinal cord injury in an animal model. *Spinal Cord.* 48, 857–861.
- Shanley, T.P., Warner, R.L., Crouch, L.D., Dietsch, G.N., Clark, D.L., O'Brien, M.M., Gallatin, W.M., and Ward, P.A. (1998). Requirements for α d in IgG immune complex-induced rat lung injury. *J. Immunol.* 160, 1014–1020.
- Shechter, R., London, A., Varol, C., Raposo, C., Cusimano, M., Yovel, G., Rolls, A., Mack, M., Pluchino, S., Martino, G., Jung, S., and Schwartz, M. (2009). Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice. *PLoS Med.* 6, e1000113.

- Soehnlein, O., Zernecke, A., and Weber, C. (2009). Neutrophils launch monocyte extravasation by release of granule proteins. *Thromb. Haemost.* 102, 198–205.
- Song, S., Hua, Y., Keep, R.F., Hoff, J.T., and Xi, G. (2007). A new hippocampal model for examining intracerebral hemorrhage-related neuronal death: effects of deferoxamine on hemoglobin-induced neuronal death. *Stroke* 38, 2861–2863.
- Stahnke, T., Stadelmann, C., Netzler, A., Bruck, W., and Richter-Landsberg, C. (2007). Differential upregulation of heme oxygenase-1 (HSP32) in glial cells after oxidative stress and in demyelinating disorders. *J. Mol. Neurosci.* 32, 25–37.
- Stirling, D.P., Liu, S., Kubes, P., and Yong, V.W. (2009). Depletion of Ly6G/Gr-1 leukocytes after spinal cord injury in mice alters wound healing and worsens neurological outcome. *J. Neurosci.* 29, 753–764.
- Stirling, D.P., and Yong, V.W. (2008). Dynamics of the inflammatory response after murine spinal cord injury revealed by flow cytometry. *J. Neurosci. Res.* 86, 1944–1958.
- Syapin, P.J. (2008). Regulation of haeme oxygenase-1 for treatment of neuroinflammation and brain disorders. *Br. J. Pharmacol.* 155, 623–640.
- Tjoa, T., Strausbaugh, H.J., Maida, N., Dazin, P.F., Rosen, S.D., and Noble-Haeusslein, L.J. (2003). The use of flow cytometry to assess neutrophil infiltration in the injured murine spinal cord. *J. Neurosci. Methods.* 129, 49–59.
- Trivedi, A., Hsu, J.Y., Lin, Y., Goussev, S., Gan, J., Topp, K.S., and Noble-Haeusslein, L.J. (2005). The effects of acute and extended inhibition of matrix metalloproteinases on demyelination and functional recovery after spinal cord injury. *IJNN* 2, 30–38.
- Van Rooijen, N., and Sanders, A. (1994). Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J. Immunol. Methods* 174, 83–93.
- Van Rooijen, N., Sanders, A., and van den Berg, T.K. (1996). Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. *J. Immunol. Methods* 193, 93–99.
- Whetstone, W.D., Hsu, J.Y., Eisenberg, M., Werb, Z., and Noble-Haeusslein, L.J. (2003). Blood-spinal cord barrier after spinal cord injury: relation to revascularization and wound healing. *J. Neurosci. Res.* 74, 227–239.
- Yednock, T.A., Cannon, C., Vandever, C., Goldbach, E.G., Shaw, G., Ellis, D.K., Liaw, C., Fritz, L.C., and Tanner, L.I. (1995). Alpha 4 beta 1 integrin-dependent cell adhesion is regulated by a low affinity receptor pool that is conformationally responsive to ligand. *J. Biol. Chem.* 270, 28,740–28,750.
- Yoneyama-Sarnecky, T., Olivas, A.D., Azari, S., Ferriero, D.M., Manvelyan, H.M., and Noble-Haeusslein, L.J. (2010). Heme oxygenase-2 modulates early pathogenesis after traumatic injury to the immature brain. *Dev. Neurosci.* 32, 81–90.

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