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CD47 Knockout Mice Exhibit Improved Recovery from Spinal Cord Injury

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Abstract

Recent data have implicated thrombospondin-1 (TSP-1) signaling in the acute neuropathological events that occur in microvascular endothelial cells (ECs) following spinal cord injury (SCI) (Benton et al., 2008b). We hypothesized that deletion of TSP-1 or its receptor CD47 would reduce these pathological events following SCI. CD47 is expressed in a variety of tissues, including vascular ECs and neutrophils. CD47 binds to TSP-1 and inhibits angiogenesis. CD47 also binds to the signal regulatory protein (SIRP) α and facilitates neutrophil diapedesis across ECs to sites of injury. After contusive SCI, TSP-1^{-/-} mice did not show functional improvement compared to wildtype (WT) mice. CD47^{-/-} mice, however, exhibited functional locomotor improvements and greater white matter sparing. Whereas targeted deletion of either CD47 or TSP-1 improved acute epicenter vascularity in contused mice, only CD47 deletion reduced neutrophil diapedesis and increased microvascular perfusion. An *ex vivo* model of the CNS microvasculature revealed that CD47^{-/-}-derived microvessels (MVs) prominently exhibit adherent WT or CD47^{-/-} neutrophils on the endothelial lumen, whereas WT-derived MVs do not. This implicates a defect in diapedesis mediated by the loss of CD47 expression on ECs. *In vitro* transmigration assays confirmed the role of SIRP α in neutrophil diapedesis through EC monolayers. We conclude that CD47 deletion modestly, but significantly, improves functional recovery from SCI via an increase in vascular patency and a reduction of SIRP α -mediated neutrophil diapedesis, rather than the abrogation of TSP-1-mediated anti-angiogenic signaling.

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Keywords

CD47; thrombospondin-1; spinal cord injury; microvasculature; angiogenesis; inflammation; neutrophils

Introduction

Contusive SCI leads to a cascade of secondary complications including loss of spinal microvasculature and innate inflammatory responses. These secondary responses contribute to cell death, axonal degeneration, and demyelination within the contusion and injury penumbra (Alexander and Popovich, 2009; Mautes et al., 2000). The onset of vascular pathology following injury is rapid and restricted to the injury epicenter (Benton et al., 2008a; Casella et al., 2006; Goodman et al., 1979; Griffiths et al., 1978; Whetstone et al., 2003). Early vessel dysfunction and increased permeability of the blood-spinal cord-barrier (BSCB) induces edema and contributes to the initiation of inflammation (Amar and Levy, 1999; Mautes et al., 2000; Popovich and Longbrake, 2008). Delayed pathological transformation of the spinal microvasculature at penumbral sites occurs following a loss of astrocytic investment (Whetstone et al., 2003), regression of pericytes (Benton et al., 2008a) and the delayed perivascular localization of progressive waves of infiltrating inflammatory cells (Fleming et al., 2006; Popovich and Jones, 2003). Infiltrating leukocytes mediate tissue damage via the production of oxygen radicals, cytokines and chemokines (Tonnesen, 1988). Blocking neutrophil and macrophage influx into the SCI leads to neuroprotection and improved locomotor function (Giulian and Robertson, 1990; Gris et al., 2004; Popovich and Jones, 2003). An initial loss of nearly all epicenter vasculature is followed by a prolific angiogenic response from day 3–7 post-SCI (Benton et al., 2008a; Casella et al., 2002; Loy et al., 2002; Wagner et al., 1977; Whetstone et al., 2003). These new vessels are immature and leaky. In the mouse, the epicenter eventually evolves into a fibrotic, aglial heterodomain. This second phase of microvascular instability has been hypothesized to contribute to chronic histopathology following traumatic SCI (Casella et al., 2002; Loy et al., 2002; Wagner et al., 1977). Indeed, rescue of ECs and stabilization of the microvasculature within the contusion epicenter and penumbra facilitates tissue sparing and functional recovery following SCI (Han et al., 2010).

Using a highly purified preparation of spinal MVs, Benton et al. (2008b) identified *TSP-1*, a potent anti-angiogenic factor, as the most significantly upregulated mRNA (58-fold) 24 hours after contusive thoracic SCI. TSP-1 activation of CD47 induces apoptosis via caspase-dependent and independent mechanisms in cultured cerebral cortical neurons and NB4 cells, respectively (Koshimizu et al., 2002; Saumet et al., 2005). Consistently, TSP-1 through CD47 increases cytotoxicity in CNS-derived EC cultures (Xing et al., 2009), though changes in proliferation were not assessed. Increases in retinal vascular density in TSP-1^{-/-} mice were identified (Wang et al., 2003). Similarly, TSP-1^{-/-} mice exhibit an increased density of capillaries in cardiac and skeletal muscle (Malek and Olfert, 2009). We hypothesized that TSP-1/CD47 signaling reduces spinal vascularity and contributes to functional deficits after SCI. This hypothesis was tested in a contusive model of SCI using mice with targeted deletions in either *TSP-1* or *CD47*.

Materials and methods

Reagents

The recombinant C-terminal TSP-1 peptide, E3T3C1 (Adams and Lawler, 1994), was a kind gift from Mark Duquette and Jack Lawler (Beth Israel Deaconess Medical Center, Boston, MA). FITC-LEA (*Lycopersicon esculentum* agglutinin lectin; FL-1171), TR-LEA

(TL-1176) and polyclonal rabbit anti-Ki67 [VP-K451; 1:1000 dilution] were purchased from Vector Labs (Burlingame, CA). Monoclonal rat anti-PECAM-1 [CD31; 550274; clone MEC13.3; 1:50 dilution], monoclonal hamster anti-ICAM-1 [550287; clone 3E2; 1:50 dilution], and monoclonal rat anti-CD47 [clone MIAP301; without sodium azide; 1:50 dilution] were purchased from BD Pharmingen (San Diego, CA). Polyclonal sheep anti-Von Willebrand factor [NB100-62174; 1:100 dilution] was purchased from Novus Biologicals (Littleton, CO). Polyclonal rabbit anti-ZO-1 [clone ZR1; 1:50 dilution] was purchased from Zymed (Invitrogen, Carlsbad, CA). Monoclonal mouse anti-TSP-1, targeted to the C-terminus [MS-420-PABX; without sodium azide; clone C6.7], and BCA Protein Assay Kit (#23225) was purchased from Thermo Scientific (Waltham, MA). Monoclonal mouse anti-alpha smooth muscle actin [A2547; clone 1A4; 1:100 dilution] and polyclonal rabbit anti-laminin [L9393; 1:100 dilution] were purchased from Sigma (St. Louis, MO). Polyclonal goat anti-SIRP [sc-6921; clone N-19; 1:50 dilution] was purchased from Santa Cruz (Santa Cruz, CA). Monoclonal mouse anti-CD47 [ab3283; clone B6H12.2; 1:50 dilution] was purchased from Abcam (Cambridge, MA). Monoclonal rat anti-PMN [MCA7716A; clone 7/4; 1:150 dilution] was purchased from AbD Serotec (Raleigh, NC). Polyclonal rabbit anti-GFAP [Z0334; 1:500 dilution] was purchased from Dako (Carpinteria, CA). Mouse IgG₁ Isotype control [MAB002; clone 11711] was purchased from R&D Systems (Minneapolis, MN). Texas red (1:200)-, FITC (1:200)-, or AMCA (1:100)-conjugated secondary antibody F(ab') fragments (all from donkey) and normal donkey serum (017-000-121) were purchased from Jackson ImmunoResearch (West Grove, PA). BD Matrigel Basement Membrane Matrix [#354234] and mouse collagen IV [#354233] were purchased from BD Biosciences (San Jose, CA). Plasma-derived serum (BT-214) was purchased from Biomedical Technologies Inc. (Stoughton, MA). EC growth supplement (#02-102) was purchased from Millipore (Billerica, MA). Collagenase Type 1 [lot# X7H9763A, 235 U/ml] was purchased from Worthington Biochemical (Lakewood, NJ). Twenty μ m (CMN-0020-D) and 500 μ m (CMN-0500-D) mesh filters were purchased from smallparts.com. Transwell inserts (#3421) were purchased from Corning (Lowell, MA). The CD47-derived FG loop, H-cyclo[Cys-Glu-Val-Thr-Glu-Leu-Ser-Arg-Glu-Gly-Lys-Cys]-OH, and scrambled, H-cyclo[Cys-Val-Glu-Arg-Leu-Glu-Gly-Ser-Lys-Thr-Glu-Cys]-OH, peptides were synthesized and cyclized by Peptides International (Louisville, KY). These peptides were fully soluble at 20 mg/ml in 1 $\times$ PBS, pH 7.4. Recombinant mouse TNF- α (#14-8321) was purchased from eBioscience (San Diego, CA). Pertussis toxin (#516560) was purchased from Calbiochem (San Diego, CA). Luciferase (#L9506), heparin (H4784), percoll (P1644), fMLP (F3506), puromycin (P8833), DNase I (DN25), genistein (G6649) and dextran [D4876] were purchased from Sigma (St. Louis, MO). Luciferase Assay System (#E4030) was purchased from Promega (Madison, WI). Recombinant human TSP-1 was purchased from EMP Genetech (Ingolstadt, Germany). Myeloperoxidase (MPO) Fluorometric Detection Kit (#907-029) was purchased from StressGen (Assay Designs, Ann Arbor, MI).

Surgical procedures

All surgical intervention, care, and treatment of animals were in strict accordance with the PHS Policy on Humane Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, 1996) and University of Louisville IACUC guidelines. The genetic backgrounds of all strains used in this study were C57BL/6. WT mice were obtained from Harlan (Indianapolis, IN). TSP-1^{-/-} [#006141; B6.129S2-Thbs^{tm1Hyn}/J], CD47^{-/-} [#003173; B6.129-Cd47^{tm1Fpl}/J], VECAD-CRE transgenic [#006137; B6.Cg-Tg(Cdh5-cre)7Mlia/J], and ROSA-YFP transgenic [#006148; B6.129X1-Gt(ROSA)26Sor^{tm1(EYFP)Cos}/J] mice were purchased from Jackson Laboratories (Bar Harbor, ME). Adult (6–8 weeks old, 18–21 g) female C57BL/6 mice were used in this study. Mice received sham T10 laminectomies or 50 kdyn contusions using the IH impactor (Infinite Horizons Inc., Lexington, KY) as described (Benton et al., 2008a; Mahoney et al.,

2009; Scheff et al., 2003). Between all groups, no statistically significant differences existed between mean displacements (530–580 μm). For functional assessment, mice were scored by the Basso Mouse Scale (BMS) (Basso et al., 2006) one week prior to surgery and weekly after SCI. All raters were trained and certified at OSU by Dr. Basso and were blinded to genotype/treatment group.

Treadscan analysis

Recording and analysis of mice placed on a motor-driven treadmill were performed as described (Beare et al., 2009), except the blinded scorer set the treadmill at the best walking speed for each untrained mouse, as determined by slowly increasing the treadmill speed until the animal was consistently walking with minimal lateral/longitudinal movement (Beare et al., 2010). Locomotion was recorded at 100 frames/sec, and TreadScan software (CleverSys, Reston, VA) was used to track and quantify overall run speed.

Tissue processing, immunohistochemistry, and immunocytochemistry

At the indicated time points, mice were anesthetized and 100 μg of FITC-LEA was delivered systemically by i.v. injection into the surgically exposed right external jugular vein and allowed to circulate for 15 minutes prior to transcardial perfusion with 20 ml PBS alone or followed by 15 ml of 4% paraformaldehyde (PFA) in PBS, pH 7.4. Spinal cords were dissected, cryopreserved in 30% sucrose, and longitudinally sectioned at 20 μm on a cryostat. Sections were thaw mounted on microscope slides (Fisher Scientific, Pittsburgh, PA) and stored at -80°C . Slides were warmed at 37°C for 20 minutes. Lightly fixed sections were postfixed in ice-cold methanol for 20 min. Isolated ECs or MVs were washed $3\times$ with PBS and likewise fixed in 4% PFA for 1 hour at room temperature, or with methanol for 20 min at -20°C for the detection of PFA-sensitive antigens. Cords or cells were blocked in TBS + 0.1% Triton X-100, 0.5% BSA, and 10% normal donkey serum for 1 hour at room temperature and then incubated overnight at 4°C with primary antibodies in blocking buffer, followed by incubation in secondary antibodies at room temperature for 1 hour. Negative controls included appropriate species-specific non-immune IgGs instead of primary antibodies. All images were captured with a Nikon TE 300 inverted microscope equipped with a Spot CCD camera using identical exposure settings. Elements software (Nikon) was used to threshold baseline brightness and contrast identically for each image for both object and field quantitative measurements. For all assessments, the mean areas of the regions of interest (ROI) quantified, which were centered on the contusion epicenters, were not statistically different between groups. The mean areas quantified from the injured cords were used to define the ROIs used to quantify sham cords at roughly T10 (Benton et al., 2008a). For assessment of vascular patency, every fourth longitudinal section (14–15 20 μm sections) from each cord was stained and photographed with a $10\times$ objective and stitched with Elements software during acquisition. The injury epicenter was defined as the domain exhibiting significant extravascular deposition and disorganization of vascular laminin immunoreactivity (7 days post injury) or GFAP immunoreactivity (1 day post injury). The injury penumbra was defined as 500 μm rostral and caudal to the epicenter. A scorer blinded to treatment quantified LEA area intensity using Elements software thresholding to normalize baseline brightness and contrast identically for each image. For assessment of penumbral PECAM-positive MVs, every fifth longitudinal section (11–12 20 μm thick sections) from each group was stained under identical conditions. Images were acquired with a $10\times$ objective and stitched with Elements software during acquisition. A scorer blinded to treatment counted the number of PECAM-positive MVs within the contusion epicenter and penumbra that crossed the axes of a 100 μm grid using Elements software. For assessment of neutrophil extravasation, every fourth longitudinal section (14–15 20 μm sections) from each group was stained, photographed and stitched using a $4\times$ objective, and Elements software thresholding was performed using identical parameters within the

'Object Count' feature to define neutrophil counting criteria based on size, circularity, and intensity. For assessment of white matter sparing, spinal cords were dissected after transcardial perfusion of PBS and 4% PFA. Cords were submerged in 4% PFA overnight at 4°C, stored in a 30% sucrose solution for 72 hr at 4°C, and cut serially in 20 µm coronal sections on a cryostat 1 cm rostral and caudal to the injury epicenter. Sections were thaw mounted on microscope slides and stored at -80°C. Eriochrome cyanine staining was performed to delineate spared myelin (Magnuson et al., 2005; Scheff et al., 2003). The total cross-sectional area of the spinal cord and the lesion boundary were captured with an Olympus BX60 microscope and measured and analyzed using NeuroLucida (Micro-brightfield, Colchester, VT). The epicenter of each injury was determined based on the section with the least amount of spared white matter. A code was used to randomize the epicenter sections, allowing for an unbiased, blinded quantification. The data were normalized to spared white matter in corresponding sham sections.

Transendothelial cell permeability

For the assessment of transendothelial permeability 3 days post-injury, 40 µg luciferase (in 100 µl PBS + 0.001% BSA, pH 7.4) was delivered systemically by i.v. injection into the surgically exposed right external jugular vein and allowed to circulate for 45 minutes prior to transcardial perfusion with cold saline. Five mm of cord (~2.5 mm rostral/caudal from the epicenter) were dissected and homogenized in 150 µl Promega lysis buffer and snap frozen on dry ice. Luciferase activity was determined according to Promega's (#E4030) protocol. Total protein concentrations were determined by BCA assay according to the manufacturer's instructions. For the assessment of myeloperoxidase activity 3 days post-injury, mice were transcardially perfused with cold saline and 5 mm of spinal cord (~2.5 mm rostral/caudal from the epicenter) were dissected and processed according to the manufacturer's instructions (StressGen). Total protein concentrations were determined by BCA assay according to the manufacturer's instructions.

Statistical analyses

For functional assessments after injury, a repeated measures analyses of variance (ANOVA) with fixed effects and Bonferroni post-hoc *t* tests were performed to detect differences in BMS and subscores between the injury groups over the entire 6 week testing period. For analyzing spared white matter, mean values of percent spared white matter area were compared using repeated measures ANOVA followed by Tukey HSD post hoc *t* tests. For all other analyses, two-tailed Student's *t* tests assuming equal variance were performed.

Cell culture

Primary murine cortex EC isolation was adapted from protocols as described (Hoying et al., 1996). Briefly, murine cortices were removed and aseptically dissected in L15 media/2% FBS. Cortices were minced and incubated for 20–40 min at 37°C in 2 mg/ml collagenase/1 mg/ml DNase I. Homogenates were washed in PBS, resuspended in 15% dextran, and spun at 4000 × *g* for 20 min. The microvascular pellet was resuspended in PBS, passed through a 500 µm screen, and collected on a 20 µm screen. MVs were washed in PBS, pelleted, and resuspended in media.

For *in vitro* EC monolayers, MVs were resuspended in DMEM/F12 with 20% platelet-derived serum (PDS), 100 µg/ml EC growth supplement, 80 µg/ml heparin, and 4 µg/ml puromycin. MVs were plated into 24 wells precoated with 5 µg/cm² murine collagen IV at a density of 100,000 MV/ml. The puromycin was removed after 2–2.5 days, leaving 95–99% pure EC cultures as assessed by CD31 and α -actin (smooth muscle cell) immunocytochemistry. Eight days after isolation, antibodies (without sodium azide)/

peptides were added to ECs in DMEM/F12 + 1% platelet-derived serum for 24 hours at 37°C/5% CO₂. All experiments were performed using passage 0–1 ECs.

For *in vitro* MV cultures, MVs were resuspended in 4 mg/ml matrigel + 50 µg/ml murine collagen IV at 75,000 MVs/ml after final pelleting, seeded in 96 wells, and allowed to solidify at 37°C for 30 min. Media consisting of DMEM/F12 + 20% FBS, 100 µg/ml EC growth supplement, and 80 µg/ml heparin was added on top of the solidified Matrigel and changed every other day. Six days after isolation, antibodies (without sodium azide)/peptides were added to MVs in DMEM/F12 + 1% FBS for 48 hours at 37°C/5% CO₂. All experiments were performed using passage 0 MVs.

Ex vivo EC grafting

Subcutaneous implantation of isolated MV constructs was adapted from protocols as described (Hoying et al., 1996). MVs were resuspended in 4 mg/ml matrigel (+ 0.2 µM BSA or 0.2 µM TSP-1) at 90,000–100,000 MVs/ml after final pelleting, seeded in 48 well (1 cm²) culture plates, and allowed to solidify at 37°C for 30 min. Matrigel plugs were then implanted dorsally above each flank in mice of the indicated genotype. *Ex vivo* MVs were allowed to anastomose and inosculate with host vasculature for 30 days. LEA vasculature labeling, tissue processing, and immunohistochemical staining of matrigel plug sections were performed as with spinal cords.

Diapedesis assays

Passage 0–1 ECs were plated onto murine collagen IV-coated transwell inserts (5 µm pores) at 20,000 cells/well and grown to confluency. ECs were treated with 10 ng/ml TNF-α without serum and when indicated, 1 µg/ml pertussis toxin or 50 µg/ml MIAP301 (without sodium azide) overnight and then washed prior to the experiment. Primary murine polymorphonuclear leukocytes (PMNs) were isolated from peritoneal fluid as described (Watt et al., 1979). Isolates were 95–98% neutrophils as assessed by counting 7/4-immunopositive cells/total Hoechst-stained nuclei. After isolation, neutrophils were added in the upper transwell chambers at 6×10⁵ cells/well after a 15 minute incubation at room temperature with either vehicle, 40 µg/ml anti-SIRPα, 200 µM genistein, or the indicated concentrations of either the CD47 FG loop or scrambled control peptides. One µM fMLP was added to bottom wells, and PMNs were allowed to migrate for 1.5–2 hr. Neutrophils in the lower chamber were fixed in 4% PFA for 1 hour at room temperature, stained, and counted.

Results

Knockout of CD47 improves functional recovery after SCI

We hypothesized that TSP-1/CD47 signaling contributes to functional deficits after contusive injury. Therefore, TSP-1^{-/-} and CD47^{-/-} mice were examined after SCI using the BMS. TSP-1^{-/-} mice (n=11) exhibited no functional difference after injury relative to WT mice (n=11) (data not shown). In contrast, assessment of locomotor function after moderate contusion of the T10 spinal cord revealed that CD47^{-/-} mice (n=18) exhibited a statistically significant improvement in recovery relative to WT (n=19) as early as one week after injury (Fig. 1A). This improvement was sustained up to six weeks after injury (the latest time point examined). The increase in motor function above a score of 5 is significant in this nonlinear scale as it reflects the restoration of the capacity for plantar stepping and weight support. BMS subscore analysis revealed a significant increase in motor function in CD47^{-/-} mice, with primarily an improvement in hindlimb coordination relative to WT mice (Fig. 1B). TreadScan analysis (Beare et al., 2009; Beare et al., 2010) revealed that the overall speed CD47^{-/-} mice ran on a treadmill was significantly higher (a 21% increase) than WT mice

six weeks after injury, despite having no significant difference in baseline speeds before injury (Fig. 1C). Though these evaluation methods have limitations, these data suggest that CD47 ablation modestly improves recovery from SCI.

Knockout of CD47 improves spared white matter after SCI

A correlation between increased BMS scores and epicenter white matter sparing after contusive injury in C57BL/6 mice has been established (Basso et al., 2006). We hypothesized that the functional improvements after CD47 deletion may partially be due to improved white matter sparing. Therefore, the degree of spared white matter throughout the contused cords six weeks after injury was assessed (Fig. 1D,E). Eriochrome cyanine staining revealed that CD47^{-/-} mice exhibited significantly more white matter at the injury epicenter (37.6% ± 1.4) relative to WT animals (31.7% ± 1.5), and up to 100 µm caudal to epicenter (Fig. 1E). This 6% increase in spared white matter is consistent with the observed 1 point increase in the BMS score (Basso et al., 2006). These data indicate that the increased white matter at the contusion epicenter likely contributes to the enhanced behavioral recovery from SCI in animals with targeted deletion of CD47.

CD47 localizes to hypoperfused MVs within the contusion epicenter after SCI

An acute two-fold upregulation of *CD47* mRNA was identified within the contusion epicenter 24 hours after murine moderate T8 SCI, which subsequently returned to baseline by 3 days post injury [GSE5296 (Edgar et al., 2002)]. We therefore investigated the protein localization of CD47 post-injury to test the hypothesis that CD47 is expressed in the spinal microvasculature after contusion. Whereas no detectable expression was found in sham uninjured cords (Fig. 2A), CD47 protein was localized within the contusion epicenter microvasculature 24 hours after injury, often in MVs that appear to be underperfused as assessed by intravenous administration of the perfusion marker LEA (Fig. 2B,C). No detectable CD47 expression was found in contused cords rostral and caudal to the epicenter (data not shown). By 14 days post-injury, CD47 expression was significantly reduced (data not shown). Though CD47 is known to be expressed on inflammatory cells, no detectable CD47 expression co-localized with either the neutrophil-specific marker 7/4 or the general inflammatory cell marker CD45 one day post-injury (data not shown).

CD47 ablation rescues epicenter and penumbral MVs after SCI

We hypothesized that ablation of CD47 signaling would lead to improvements in microvasculature post-contusion. We therefore quantified PECAM immunohistochemical stained microvessels in WT (Fig. 3A) or CD47^{-/-} mice (Fig. 3B) after SCI. Three days after injury, a time point in the mouse during which epicenter vascularity begins to increase after initial trauma (Benton et al., 2008a; Whetstone et al., 2003), WT mice retained about half of total MVs (52% ± 2.0) within the injury epicenter and penumbra relative to sham uninjured WT animals (Fig. 3C). Both CD47^{-/-} (64% ± 2.0) and TSP-1^{-/-} (65% ± 2.3) injured animals exhibited a significantly higher number of total epicenter and penumbral MVs relative to WT when normalized to sham uninjured animals of their respective genotypes. This increase in penumbral MVs was even greater in the white matter of CD47^{-/-} mice (a 20% increase in white matter MVs alone vs. a 12% increase in total MVs; Fig. 3C), raising the possibility that the increase in spared white matter in these mice is subsequent to the improvements in vascularity. By 7 days post injury, CD47^{-/-} and TSP-1^{-/-} mice both maintain this increase in total epicenter vascularity, though this increase did not reach statistical significance in the white matter of TSP-1^{-/-} mice (Fig. 3D).

Deletion of CD47 increases perfusion of epicenter and penumbral vasculature after SCI

To address the potential mechanism by which loss of CD47 enhances white matter sparing and functional recovery, changes in the status of microvascular perfusion after contusive SCI were assessed in WT (Fig. 4A) and CD47^{-/-} mice (Fig. 4B). We hypothesized that deletion of CD47 signaling would improve the function and perfusion status of the spinal microvasculature after injury. Genetic and pharmacologic inhibition of TSP-1/CD47 signaling enhances acute nitric oxide (NO)- and cGMP-mediated vasodilation and tissue survival after ischemic injury in several models (Isenberg et al., 2008a; Isenberg et al., 2008c). Quantitation of LEA-bound MVs revealed that deletion of CD47 led to significant improvements in perfusion in the injury epicenter and penumbra both 1 and 7 days post injury (Fig. 4E). Targeted knockout of TSP-1 failed to affect vascular patency 7 days post injury (Fig. 4E).

The C-terminal domain of TSP-1, via CD47, decreases CNS-derived EC proliferation in vitro

To elucidate the mechanism of increased vascularity in mice with disrupted TSP-1/CD47 signaling after contusion, ECs isolated from murine cortex or spinal cord were cultured to assess the effects of TSP-1/CD47 inhibition on EC proliferation. We hypothesized that inhibition of CD47 signaling improves CNS-derived EC proliferation. Incubation of passage 1 (P1) ECs with functional blocking antibodies to either the C-terminus of TSP-1 (α -TSP-1 C-terminus; clone C6.7) or the extracellular IgV domain of CD47 (α -CD47; clone MIAP301) led to a roughly two-fold increase in the percentage of total nuclei expressing the proliferation marker Ki67 (Fig. 5A–C). Consistently, addition of the peptide E3T3C1, which includes the CD47-interacting C-terminus of TSP-1, reduced the percent of proliferating cells by roughly half of control (Fig. 5C).

The C-terminal domain of TSP-1, via CD47, decreases CNS-derived MV formation and proliferation in vitro

We repeated the experiments performed on purified CNS ECs using isolated, intact CNS MVs (ECs with supporting pericytes/smooth muscle cells (SMCs)) suspended in a three-dimensional matrigel plug in order to assess capillary-like tube formation and MV proliferation in a more biologically relevant model. Addition of the α -TSP-1 C-terminus antibody to intact WT MVs led to an increase in the area of von Willebrand factor-expressing MVs (Fig. 6A–C), but not in TSP-1^{-/-}-derived MVs. Relative to WT MVs, however, TSP-1^{-/-} MVs exhibited a roughly two-fold increase in EC area (Fig. 6C). No significant difference was observed in mean MV length or number of tip cell sprouts between groups (data not shown).

We assessed the degree of proliferation within these MV cultures by quantifying Ki67 immunocytochemical staining (Fig. 7A–C). Both TSP-1^{-/-} and CD47^{-/-}-derived MVs exhibited a roughly 10-fold increase in Ki67-positive nuclei per field (Fig. 7C). Co-localization of Ki67 with α -actin indicates that many of the proliferating cells in these MVs are pericytes/SMCs. Consistently, incubation of WT MVs with the TSP-1 and CD47 blocking antibodies both significantly increased proliferating cells 9- and 3-fold, respectively (Fig. 7C). While addition of the TSP-1 C-terminal agonist peptide (E3T3C1) reduced the number of proliferating cells in WT MVs, addition of this peptide to CD47^{-/-} MVs surprisingly led to a significant increase in proliferating MVs over CD47^{-/-} MVs alone. Since this TSP-1 peptide retains the $\alpha_v\beta_3$ -binding domain N-terminal to the CD47-binding domain (Saumet et al., 2005), this increase may reflect the pro-angiogenic effects of $\alpha_v\beta_3$ activation in the absence of anti-angiogenic CD47 signaling.

TSP-1 exhibits anti-angiogenic effects on inosculated, patent MVs in *ex vivo* cortical MV implants

We hypothesized that the pro-angiogenic effects of inhibiting TSP-1/CD47 signaling observed *in vitro* might translate to functional improvements in angiogenesis within a more physiologically relevant model. The effects of TSP-1 signaling on the functional angiogenesis of purified MVs were therefore assessed within an *ex vivo* model of vascularity (Hoying et al., 1996). In this model, the sub-cutaneous implantation of matrigel plugs containing isolated CNS MVs leads to the anastomosis and inosculature of grafted MVs with the host vasculature after 30 days. This allows assessment of MV patency subsequent to graft inosculature via jugular infusion of the isolectin perfusion marker LEA. Addition of 0.2 μ M TSP-1 (a concentration consistent with that found in ischemic tissue levels (Isenberg et al., 2008b)), to MVs isolated from mice expressing YFP driven by a VE-cadherin promoter (Alva et al., 2006), and subsequently implanted into TSP-1^{-/-} hosts, led to a pronounced reduction in MV density (Fig. 8A,B). Consistently, TSP-1^{-/-} MVs exhibited a higher density within the graft relative to WT MVs, each implanted into hosts of their respective genotype (Fig. 8C,D). The mean MV diameter was significantly lower when TSP-1 was increased either genetically or pharmacologically (Fig. 8E), consistent with the increase in *in vitro* MV area measurements after TSP-1 inhibition. These results corroborate the hypothesis that abrogation of TSP-1/CD47 signaling can, at least acutely, improve vascularity in injured CNS tissue.

CD47 ablation decreases neutrophil extravasation and myeloperoxidase activity in the contusion epicenter

To further address the potential mechanism by which loss of CD47 enhances white matter sparing and functional recovery, we investigated acute inflammatory responses. CD47^{-/-} mice were originally identified as having deficits in granulocyte migration to sites of peritoneal infection despite normal circulating leukocyte levels (Lindberg et al., 1996). We hypothesized that CD47^{-/-} mice would exhibit deficits in the inflammatory response to traumatic SCI. The early inflammatory response to SCI in both TSP-1^{-/-} and CD47^{-/-} mice was therefore assessed. CD47^{-/-} mice exhibited significantly fewer neutrophils than WT mice within the injury epicenter and 1 mm rostral and caudal to the injury at 1 day post injury (Fig. 9A–C). By 3 days post injury, CD47^{-/-} mice exhibited a roughly 50% decrease in neutrophils in the injury site relative to WT mice (Fig. 9C). Consistently, CD47^{-/-} mice exhibited a 3-fold decrease in myeloperoxidase activity (primarily a neutrophil-mediated oxidative event) relative to WT within the contusion epicenter 3 days post-injury (Fig. 9D). Sham injured mice exhibited few invading neutrophils and little myeloperoxidase activity, and no significant differences existed between groups. Ablation of TSP-1 had no significant effect on neutrophil extravasation three days post-injury (Fig. 9C).

Endothelial, not neutrophil, expression of CD47 primarily mediates extravasation from CNS-derived MVs

We hypothesized that CD47 expression on ECs is primarily responsible for neutrophil diapedesis, not CD47 expression on neutrophils. The effects of CD47 on neutrophil diapedesis from purified MVs were therefore assessed using the *ex vivo* model. WT-derived MVs exhibited no luminal adhesion of LEA-stained hematopoietic cells, whether implanted into WT (Fig. 10A) or CD47^{-/-} (Fig. 10C) hosts. CD47^{-/-}-derived MVs exhibited prominent luminal adhesion of LEA-stained hematopoietic cells when implanted into either WT (Fig. 10B) or CD47^{-/-} (Fig. 10D) hosts. Many of these hematopoietic cells were identified as neutrophils (Fig. 10B,D, red). Only sparse and immature host MVs are recruited to the edges of negative control matrigel plugs (Fig. 10E – inset in A), suggesting the MVs observed in Fig. 10A–D are grafted, and not host, MVs. These data support the hypothesis that EC expression of CD47, not CD47 expression on neutrophils, mediates

neutrophil diapedesis through CNS MVs. Deletion of EC expression of CD47 would lead to deficits in polymorphonuclear (PMN) leukocyte extravasation and, presumably, a subsequent increase in the number of neutrophils that had successfully rolled and adhered to the MV lumen.

Disruption of CD47/SIRP α signaling decreases neutrophil extravasation through ECs in vitro

As TSP-1 ablation had no effect on neutrophil extravasation into the injury penumbra, we tested the role of another CD47 agonist, SIRP α , on neutrophil diapedesis through CNS-derived ECs. We hypothesized that inhibition of CD47/SIRP α signaling would reduce transendothelial diapedesis of neutrophils through a monolayer of ECs (Fig. 11A–C). Incubation of primary murine neutrophils with a SIRP α functional blocking antibody for 15 min reduced the number of neutrophils transmigrating through a monolayer of primary cortex-derived ECs in response to 1 μ M fMLP (Fig. 11C). SIRP α possesses four tyrosine phosphorylation sites within its cytoplasmic C-terminal tail known to be required for activation of downstream signaling via SHP-1, which is believed to regulate activity of the small GTP-binding protein Rho during cell migration (Matozaki, 2008). Incubation of isolated neutrophils with the tyrosine kinase blocker genistein inhibited EC transmigration (Fig. 11C). Additionally, incubation of ECs with the CD47 functional blocking antibody MIAP301 reduced the number of transmigrated neutrophils (Fig. 11C), consistent with the *in vivo* knockout data. As CD47 is believed to initiate downstream signal transduction via the heterotrimeric G protein G $_i$, EC monolayers were incubated with 1 μ g/ml pertussis toxin. Pertussis toxin reduced the number of transmigrated neutrophils to the same extent as the CD47 functional blocking antibody (Fig. 11C). These pharmacologic inhibitors did not significantly affect passive diffusion of 70 kDa dextran through the EC monolayers at 30 minutes ($p > 0.1$) and up to two hours ($p > 0.08$) after addition to the transwell chambers.

The CD47/SIRP α co-crystal structure was recently solved (Hatherley et al., 2008). Consistent with previous site-directed mutagenesis data (Hatherley et al., 2007; Liu et al., 2007), these structural data confirmed the importance of the CD47 FG β -loop in its interaction with SIRP α . A novel peptide based on this β -loop was synthesized and found to significantly antagonize neutrophil extravasation with a high potency ($IC_{50} = 5.8$ nM) relative to scrambled control peptide (Fig. 11D). These data suggest that the CD47 interaction with SIRP α , not TSP-1, facilitates neutrophil diapedesis through the CNS microvasculature endothelium and extravasation into the contusion penumbra, where neutrophil-mediated inflammatory responses exacerbate secondary damage to the spinal cord (Jones et al., 2005).

Targeted knockout of CD47 or TSP-1 had no effect on passive transendothelial permeability after SCI

Deletion of CD47 could lead to a general increase in passive MV permeability within the contusion and/or a decrease in tight junction proteins localized to the EC plasma membrane necessary for maintenance of the BSCB, rather than a specific inhibition of neutrophil diapedesis. Three days post-injury, a time point at which disruption of the BSCB remains elevated (Whetstone et al., 2003), mice were administered jugular infusions of luciferase to assess the degree of transendothelial permeability within the epicenter and injury penumbra. No significant difference in MV leakage within the contusion was observed in either CD47 $^{-/-}$ or TSP-1 $^{-/-}$ mice relative to WT (Fig. 12A). Consistently, plasma membrane localization of the tight junction protein ZO-1, used here as a marker for BSCB stability, was undisturbed after incubation with functional blocking antibodies to either CD47 (Fig. 12C,E) or the TSP-1 C-terminus (Fig. 12D). These data corroborate the specific functional

role of CD47 in mediating active neutrophil diapedesis, rather than passive microvascular permeability.

Discussion

Targeted deletion of CD47 leads to increased white matter sparing, decreased neutrophil extravasation and modest, but significantly improved functional recovery after SCI. Knockout of CD47 also leads to an increase in MV perfusion within the epicenter both 1 and 7 days post-injury. The early 24 hour time point may reflect the capacity of TSP-1/CD47 signaling to regulate acute hemodynamic control of the vasculature following hemorrhage (Isenberg et al., 2008b; Isenberg et al., 2008c). CD47 deletion would be expected to abrogate negative regulation of cGMP signaling, thus leading to an increase in NO-mediated vasodilation and, subsequently, vascular patency. The 24 hour peaks in epicenter mRNA expression each for *TSP-1*, *CD47*, and *endothelial nitric oxide synthase (eNOS)* after moderate contusion in mice [(Benton et al., 2008b), GSE5296(Edgar et al., 2002)], and the localization of CD47 protein to hypoperfused MVs within the epicenter, are consistent with this interpretation. However, the lack of functional improvement in TSP-1^{-/-} mice suggests that this acute hemodynamic regulation may do little to affect behavioral outcome after SCI.

The increase in LEA-stained MVs within the epicenter at 7 days post-injury in CD47^{-/-} mice may reflect the increase in the overall number of functional MVs in these mice. An increase in the number of patent MVs within the contusion epicenter would be expected to contribute to the increase in spared white matter and subsequent behavioral improvements seen in these mice. No improvements in MV patency were observed in TSP-1^{-/-} mice at this time point, consistent with *TSP-1* mRNA expression returning to baseline by 3 days post-injury [GSE5296 (Edgar et al., 2002)] and protein expression decreasing after 3 days post-injury (Benton et al., 2008b). The mRNA expression profile for *TSP-2*, however, peaks at 3 days and remains elevated past 7 days post-injury [GSE5296 (Edgar et al., 2002)]. TSP-2 also possesses a conserved C-terminal domain known to interact with CD47, although the TSP-2 C-terminus exhibits a significantly lower affinity for CD47 relative to the TSP-1 C-terminus (Isenberg et al., 2009). The possibility of a role for TSP-2 in microvascular patency after SCI cannot be excluded.

CD47 appears to negatively regulate the spinal microvasculature after contusive SCI, and this regulation appears to be mediated in part by TSP-1. Although the *in vitro* data suggests that these effects are due to a decrease in EC proliferation, technical considerations did not allow conclusive quantification of EC-specific proliferation within the injury penumbra. *In vivo*, CD47 activation by TSP-1 might enhance acute EC cytotoxicity (Xing et al., 2009) or apoptosis after injury in addition or as opposed to negatively regulating proliferation.

The observed changes in MV stability induced by TSP-1 activation of CD47 after contusion may only negligibly affect recovery from SCI, as TSP-1^{-/-} mice are not significantly different in functional recovery from WT mice. These data do not exclude the possibility that this lack of significant functional difference could result from an opposition between benefits of MV sparing with deficits in axonal sprouting and synaptic density formation observed in TSP-1^{-/-} mice, as found in a post-ischemic murine stroke model (Liauw et al., 2008). Indeed, the behavioral deficits subsequent to a thoracic contusive injury primarily correlate with white, not gray matter loss (Magnuson et al., 2005). These data also do not exclude the possibility of a role for TSP-2 in MV loss after contusive injury.

Sprouting angiogenesis involves a complex program including growth factor-induced activation of ECs, protease degradation of extracellular matrix and basement membrane, and EC proliferation and migration through the matrix. As many proliferating, Ki67-positive

nuclei were localized to the sides of parent MVs *in vitro* after antibody blockade of TSP-1/CD47 signaling, an increase in the number of sprouting tip cells (devoid of supporting smooth muscle cells) might be expected. The lack of a significant difference in either the number or lengths of these sprouts, combined with the increase in parent MV area *in vitro* and the mean diameter of mature, inoscultated vessels *ex vivo*, suggests that TSP-1/CD47 signaling may regulate CNS MV diameter and wall thickness rather than *de novo* proliferative sprouting angiogenesis. TSP-1/CD47 signaling could thus be seen as a positive regulator of angiostasis, rather than a negative regulator of sprouting angiogenesis.

Three distinct vascular phenotypes, sprouting angiogenesis, neovascular remodeling, and subsequent network maturation have been identified in this model of tissue neovascularization (Au et al., 2007; Jain et al., 2007; Nunes et al., 2010). The intermediate remodeling stage is characterized by moderate cell proliferation, high apoptosis and significant vascular pruning. As both a genetic and pharmacological increase in TSP-1 led to qualitatively fewer MVs after 30 days *ex vivo* (Fig. 6A, D), it is possible that the angiostatic effects of TSP-1/CD47 signaling lead to thinner, less robust MVs during the initial angiogenic phase that subsequently are more susceptible to vascular pruning during the intermediate remodeling stage, though further substantiation is required.

Secondary inflammatory reactions consequent to contusive SCI include a rapid recruitment of neutrophils and monocytes from the blood to the injured extravascular parenchyma (Alexander and Popovich, 2009; Mautes et al., 2000). Using *in vitro* models of transmigration, neutrophil and monocyte chemotaxis through epithelial or EC monolayers have been shown to be dependent upon CD47 (Cooper et al., 1995; Liu et al., 2001; Rosseau et al., 2000) and SIRP α (de Vries et al., 2002; Liu et al., 2002). *In vivo*, a delay in the influx of neutrophils in response to intra-peritoneal *E. coli* infection was observed in CD47^{-/-} mice despite normal levels of circulating intravenous leukocytes, supporting a role for CD47 in PMN cell migration across vascular ECs (Lindberg et al., 1996). Consistently, in a *Staphylococcus aureus*-induced model of arthritis, in which recruited neutrophils are essential in the initial stages of cartilage and bone destruction, CD47^{-/-} mice exhibited reduced clinical and histological signs of disease (Verdrengh et al., 1999). Collagen-induced arthritis similarly led to a reduced inflammatory response in a transgenic mouse mutant lacking the cytoplasmic signaling domain of SIRP α (Okuzawa et al., 2008). These studies support the hypothesis that CD47/SIRP α signaling might therefore regulate neutrophil diapedesis after SCI.

We conclude that CD47 facilitates neutrophil diapedesis into the spinal cord lesion parenchyma. Neutrophils induce secondary damage to healthy tissue by the production of high levels of proinflammatory cytokines and oxidative metabolites such as the highly destructive HOCl, a product of myeloperoxidase activity (Hampton et al., 1998). Our data indicates that reduction of extravasated neutrophils and parenchymal myeloperoxidase activity correlate with improvements in white matter sparing and behavioral recovery from SCI in CD47^{-/-} mice. In our CNS model, neutrophil diapedesis appears to be dependent on EC, not neutrophil expression of CD47. Based upon the presence of lumenally adherent neutrophils in CD47^{-/-} MVs, both rolling and adhesion phases of neutrophil migration appear intact in the absence of CD47.

Our *in vivo* data suggest that the differences in parenchymal neutrophil levels are not due to changes in passive transendothelial diffusion (Fig. 12A), and our *in vitro* data suggest that TSP-1/CD47 signaling does not regulate maintenance of the BSCB (Fig. 12B-E). Consistently, the overall numbers of extravasated, parenchymal neutrophils were higher at 1 relative to 3 days post-injury, reflective more of the 24 hour peak of neutrophil infiltration (Mautes et al., 2000) than the peak of BSCB disruption post-injury. The downstream signal

transduction pathways involving the small heterotrimeric G protein, G_i , and tyrosine phosphorylation of CD47 and SIRP α , respectively, were implicated in mediating neutrophil diapedesis, at least *in vitro*.

In conclusion, targeted knockout of CD47 signaling leads to improved white matter sparing, decreased inflammation, improved penumbral vascularity and modest but significant functional recovery after SCI. The CD47 agonist TSP-1 may mediate the angiostatic effects observed after injury, but these effects are insufficient to lead to behavioral changes post-injury. CD47 deletion leads to a decrease in secondary inflammatory responses, specifically neutrophil extravasation and subsequent myeloperoxidase activity, and these effects may be mediated by the CD47 agonist SIRP. We conclude that the anti-inflammatory effects of CD47 deletion alone, or in combination with the observed improvements in penumbral microvasculature and patency, lead to the functional improvements observed after contusive SCI.

Research Highlights

- CD47 inhibition improves functional recovery from contusive thoracic SCI
- CD47 inhibition increases vascular patency after SCI
- CD47 inhibition reduces neutrophil extravasation into the contusion
- CD47 is a potential novel therapeutic target for the treatment of SCI

Acknowledgments

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Figure 1.

CD47 ablation improves functional outcome after moderate contusion. (A) Repeated measures ANOVA with fixed effects and Bonferroni posthoc t tests revealed a significant improvement in locomotor recovery by day 7 in CD47^{-/-} mice (n=18, ■) relative to wildtype (n=19, ♦) as assessed by the BMS (**p < 0.001). (B) Subscore analysis revealed a similar improvement in hindlimb coordination after day 14. Means ± standard error include data from two independent experiments. (C) Six weeks post-SCI, CD47^{-/-} (n=18) mice walk at higher overall speeds than WT (n=19) as assessed by TreadScan analysis (* p < 0.05). (D) The epicenters of contused CD47^{-/-} mice exhibit more white matter than WT as determined via Eriochrome cyanine staining (images converted to monochrome) (E) Eriochrome cyanine staining of serial coronal sections throughout the contusion revealed significantly more white matter at the epicenters of CD47^{-/-} cords (n=18) relative to WT (n=19) six weeks post injury. Data are means ± SEM. (** p < 0.01).

Figure 2.

CD47 localizes to hypoperfused MVs within the contusion epicenter after SCI. CD47 expression is absent in sham uninjured cords (A), or rostral and caudal to the contusion epicenter (data not shown), but is increased 24 hours after injury within the epicenter (blue in B; red in C). This CD47 MV staining colocalizes with PECAM (blue in C), especially in spinal MVs that appear hypoperfused (arrows) due to the absence of LEA (green). XZ and YZ images are shown in (C).

Figure 3.

CD47 ablation increases PECAM-positive MVs at the injury epicenter. (A,B,D) Both total (black bars) and white matter-localized MVs (white bars) were counted from longitudinal sections of 7 d.p.i. spinal cords. Data are means $\pm$ SEM (WT, n=4; CD47^{-/-}, n=4; TSP-1^{-/-}, n=3). * p < 0.05. (C) MVs counts from 3 d.p.i. cords. Data are means $\pm$ SEM (WT, n=4; CD47^{-/-}, n=5; TSP-1^{-/-}, n=5). *** p < 0.001.

Figure 4.

Deletion of CD47 increases perfusion of epicenter and penumbral vasculature after SCI. FITC-LEA-stained area (green) was quantified 7 days post-injury in WT (A,C) and CD47^{-/-} (B,D) longitudinal sections within the total laminin-defined (blue) heterodomain areas (black bars) and white matter penumbral areas alone (white bars) and normalized to sham uninjured cords of their respective genotypes (E). As no significant behavioral or perfusion changes were observed at 7 d.p.i., TSP-1^{-/-} mice were not assessed at 1 d.p.i. Data are means ± SEM (WT, n=4; CD47^{-/-}, n=4; TSP-1^{-/-}, n=3). * p < 0.05; *** p < 0.001.

Figure 5.

The C-terminus of TSP-1, through CD47, decreases CNS EC proliferation. (A–C) Percent of Hoechst-stained nuclei (blue) of PECAM-expressing (green) WT EC monolayers positive for the proliferative marker Ki67 (red) were counted from at least six 20 $\times$ images each from three independent experiments (cortex ECs, black bars; spinal cord ECs, white bars). A purified peptide of the CD47-binding TSP-1 C-terminal domain (E3T3C1) or functional blocking antibodies to TSP-1 (α -TSP-1 C-terminus) or CD47 (α -CD47) were incubated with ECs for 24 hours. Buffer control for spinal cord ECs was not determined (n.d.). Data are the means $\pm$ SEM (* $p < 0.05$).

Figure 6.

The C-terminus of TSP-1, through CD47, decreases CNS MV capillary-like tube formation.

(A–C) Elements software was used to measure the area of vWF-expressing (red) MVs normalized to WT MVs + media alone (control) from five 4× images each from at least 3 independent experiments. Data are the means ± SEM (* $p < 0.05$). Smooth muscle cells/pericytes were stained with α -actin (blue).

Figure 7.

The C-terminus of TSP-1, through CD47, decreases CNS MV proliferation. (A–C) Ki67-positive nuclei (green) from WT, CD47^{-/-}, and TSP-1^{-/-} MV cultures were counted from at least six 20× images per drug treatment per experiment and normalized to WT MVs + media alone (control). A purified peptide of the CD47-binding TSP-1 C-terminal domain (E3T3C1) or functional blocking antibodies to TSP-1 (α-TSP-1 C-terminus) or CD47 (α-CD47) were incubated with MVs for 48 hours. Effects of antibody treatment of knockout-derived MVs were not determined (n.d.). Data are means ± SEM (* p < 0.05). WT MVs (n=4), CD47^{-/-} MVs (n=3), TSP-1^{-/-} (n=3). vWF (red), α-actin (blue).

Figure 8.

TSP-1 decreases the number of inosculated, patent MVs in *ex vivo* implants. Isolated MVs from VE-cadherin-YFP mice were resuspended in matrigel + BSA (A) or recombinant TSP-1 (B), implanted subcutaneously in TSP-1^{-/-} mice, and allowed to inosculate with host vasculature for 30 days. Images are representative of three independent experiments. Texas-Red-LEA is red, YFP-expressing ECs are yellow. Isolated MVs from WT (C) or TSP-1^{-/-} (D) mice were implanted into hosts with their identical, respective genotypes. Images are representative of three independent experiments; FITC-LEA is green and Hoechst is blue. (E) The absence of TSP-1 increases MV diameter, and the addition of exogenous TSP-1 reduces MV diameter. Data are means from at least seven 20× images per condition per experiment ± SEM (** p < 0.01).

Figure 9.

CD47 ablation decreases neutrophil extravasation and myeloperoxidase activity in the contusion epicenter. (A–C) Knockout of CD47 (n=5) leads to significantly fewer neutrophils within the injury epicenter and penumbra both 1 day (* $p < 0.05$) and 3 days (***) $p < 0.001$) post injury (d.p.i.) relative to WT (n=4) [PMN clone 7/4 (red), LEA (green), GFAP (blue)]. Ablation of the CD47 ligand TSP-1 (n=5) failed to change levels of neutrophil extravasation 3 d.p.i. As no significant behavioral or extravasation effects were observed at 3 d.p.i., TSP-1^{-/-} mice were not assessed at 1 d.p.i. All images were taken with a 4× objective. Data are means $\pm$ SEM. (D) CD47 ablation (n=5) reduces epicenter myeloperoxidase activity (mU MPO activity/mg total protein) 3 d.p.i. relative to WT (n=4). Data are means $\pm$ SEM (** $p < 0.01$).

Figure 10.

CD47 expression on CNS-derived ECs, but not on host neutrophils, appears to facilitate neutrophil diapedesis. MVs isolated from CD47^{-/-} cortex and implanted into either WT (B) or CD47^{-/-} (D) hosts inosculate with host vasculature and exhibit pronounced luminal adhesion of LEA-stained (green) hematopoietic cells, suggesting a defect in MV extravasation of neutrophils (B,D; red, arrows). WT MVs exhibit no luminal adhesion of neutrophils from either host (A,C; arrows). Under these conditions, matrigel alone did not significantly promote mature WT host MV invasion into the subcutaneously engrafted plug (E, inset in A); LEA (green), PMN clone 7/4 (red), Hoechst (blue). Images are representative of 3 independent experiments.

Figure 11.

Inhibition of CD47/SIRP α signal transduction decreases neutrophil diapedesis through CNS-derived EC monolayers. (A–C) Primary murine neutrophils were isolated, incubated for 15 min with either vehicle, an N-terminal SIRP α -blocking antibody, or the protein tyrosine kinase inhibitor genistein, and added to the upper chambers of a 5 μ m-pore transwell filter containing a confluent monolayer of primary (P0-1) murine cortex-derived ECs previously incubated overnight with either vehicle (A), a CD47 blocking antibody (B), or the G_i heterotrimeric protein-blocking *Pertussis* toxin. (D) Incubation of neutrophils with the CD47-derived FG loop peptide (red squares) dose-dependently inhibited extravasation relative to control scrambled peptide (blue circles). After 1.5 hr, neutrophils in the lower

chamber were fixed, stained with the PMN specific clone 7/4 (red) and Hoechst (blue), and eight 4× images from each treatment were counted. (B, inset) Hoechst stain reveals polymorphonuclear morphology of neutrophils (20×). Data are means $\pm$ SEM (n=3, ** p < 0.01).

Figure 12.

Ablation of either CD47 or TSP-1 has no effect on either *in vivo* transendothelial permeability or tight junction cortical localization *in vitro*. (A) Jugular-infused luciferase (70 kDa) leakage into the contusion was assessed 3 days post-injury in WT (n=13), CD47^{-/-} (n=6) and TSP-1^{-/-} (n=7) mice. Data are means $\pm$ SEM. (B–E) The tight junction protein ZO-1 (green) exhibited cortical EC localization after 24 hr incubation with either 50 μ g/ml IgG control (B), 20 μ g/ml anti-CD47 Ab B6H12 (C), 50 μ g/ml -TSP-1 C-terminus (D), or 50 μ g/ml anti-CD47 Ab MIAP301 (E). PECAM (blue), ICAM-1 (red). Photomicrographs are representative of three 96-well replicates each from two independent experiments.