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Research Paper

The effectiveness of the anti-CD11d treatment is reduced in rat models of spinal cord injury that produce significant levels of intraspinal hemorrhage

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ABSTRACT

We have previously reported that administration of a CD11d monoclonal antibody (mAb) improves recovery in a clip-compression model of SCI. In this model the CD11d mAb reduces the infiltration of activated leukocytes into the injured spinal cord (as indicated by reduced intraspinal MPO). However not all anti-inflammatory strategies have reported beneficial results, suggesting that success of the CD11d mAb treatment may depend on the type or severity of the injury. We therefore tested the CD11d mAb treatment in a rat hemi-contusion model of cervical SCI. In contrast to its effects in the clip-compression model, the CD11d mAb treatment did not improve forelimb function nor did it significantly reduce MPO levels in the hemi-contused cord. To determine if the disparate results using the CD11d mAb were due to the biomechanical nature of the cord injury (compression SCI versus contusion SCI) or to the spinal level of the injury (12th thoracic level versus cervical) we further evaluated the CD11d mAb treatment after a T12 contusion SCI. In contrast to the T12 clip compression SCI, the CD11d mAb treatment did not improve locomotor recovery or significantly reduce MPO levels after T12 contusion SCI. Lesion analyses revealed increased levels of hemorrhage after contusion SCI compared to clip-compression SCI. SCI that is accompanied by increased intraspinal hemorrhage would be predicted to be refractory to the CD11d mAb therapy as this approach targets leukocyte diapedesis through the intact vasculature. These results suggest that the disparate results of the anti-CD11d treatment in contusion and clip-compression models of SCI are due to the different pathophysiological mechanisms that dominate these two types of spinal cord injuries.

1. Introduction

Intraspinal inflammation is a major component of secondary damage following spinal cord injury (SCI) (Bartholdi and Schwab, 1995; Blight, 1992; Lee et al., 2000; Popovich et al., 2002; Taoka and Okajima, 1998). Neutrophils infiltrate the injured cord, peaking at 12 h in the rat (Donnelly and Popovich, 2008), releasing cytokines, and free radicals that further damage the spinal cord (Popovich et al., 1999; Taoka and Okajima, 1998). Strategies that reduce the early neutrophil and monocyte/macrophage infiltration are effective in promoting neuroprotection (Bao et al., 2004a,b; Gorio et al., 2007; Hamada et al., 1996; Nguyen et al., 2007; Saville et al., 2004; Sroga et al., 2003; Taoka et al., 1997; Tonai et al., 2001).

Preclinical rodent SCI studies have demonstrated the promising effects of a monoclonal antibody (mAb) 217 L that is directed against the CD11d subunit of the CD11/CD18 integrin found on the surface of leukocytes and binds to vascular cell adhesion molecule-1 (VCAM-1, rat and human) and intercellular adhesion molecule-3 (human only). Blocking this integrin prevents the migration of leukocytes into the damaged tissue after injury (Grayson et al., 1999; Schnell et al., 1999b; Van der Vieren et al., 1995). Anti-CD11d mAb treatment has been shown to reduce secondary damage by decreasing leukocyte infiltration (Bao et al., 2004a,b; Bao et al., 2005). The anti-CD11d mAb treatment results in improved locomotor and autonomic recovery and reduced neuropathic pain in a clip-compression model of spinal cord injury at the 4th or 12th thoracic (T4 or T12) levels in both rats and mice (Ditor et al., 2006; Geremia et al., 2012; Gris et al., 2004; Oatway et al., 2005). These results support the anti-integrin treatment as a potentially viable therapy for SCI. However, not all anti-inflammatory therapies are beneficial, especially when used in different injury models (Stirling et al., 2009). A recent study using anti-CD11d mAb suggests that differences in the severity or type of injury may be an important determinant in the efficacy of the treatment (Hurtado et al., 2012; Weaver et al., 2012). To investigate this idea, we tested the anti-CD11d treatment in

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two models of blunt, non-penetrating SCI to permit comparisons of treatment effects after contusion versus clip-compression SCI. One study, executed at the International Collaboration on Repair Discoveries in Vancouver, BC tested the mAb in a cervical hemi-contusion model of rat SCI and the other, conducted at the Robarts Research Institute in London ON, tested the mAb in contusion models of SCI at the 12th (T12) thoracic spinal segment of rats. Our analyses of locomotor recovery, intraspinal oxidative damage, inflammation and hemorrhage in anti-CD11d-treated and control rats provides evidence that the anti-CD11d strategy is most effective in instances where injury severity is modest, non-pentrating and frank hemorrhage into the injured spinal cord is minimal.

2. Materials and methods

All animal procedures using the cervical hemi-contusion model of SCI were performed in accordance with the guidelines of the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee. All animal procedures using the thoracic contusion model and clip-compression model of SCI were conducted in accordance with Western University Animal Care Committee Guidelines, adhering to the Canadian Guide to the Care and Use of Experimental Animals. All surgeries, behavioral testing and data analysis were performed by experimenters blinded to the treatment group of the animals. Animals were housed individually in standard cages with food and water ad libitum and on a 12 h light and dark cycle (See Table 1).

2.1. Cervical hemi-contusion injury

Thirty-three male Sprague-Dawley rats (Charles River Breeding Laboratories) weighing between 300 g to 350 g were used in this study. Hemi-contusion SCI at the 5th cervical spinal segment (C5) was induced using the Infinite Horizon (IH) Spinal Cord Impactor as described previously (Lee et al., 2012). Briefly, the rats were anesthetized with 4% isoflurane and placed in a stereotaxic frame in a prone position (maintenance 1.5–2.5% isoflurane). A left C5 hemi-laminectomy was performed and the spinal column was stabilized with a custom-made clamp designed to wedge under the cervical transverse processes of C4 to C6 (Choo et al., 2009). The clamp was then positioned in a frame, which was tilted at a 22.5 degree angle off the horizontal plane. Following positioning of the impactor tip, the IH impactor was then triggered to deliver a set force of 150 kdyne. Animals were excluded from further study if the measured peak force exceeded 165 kdyne. Animals were euthanized at 24 h, or 6 weeks after the SCI.

Post-operative care after the hemi-contusion SCI consisted of subcutaneous buprenorphine injections (0.02 mg/kg Temgesic®, Reckitt Benkiser Healthcare Ltd., UK) just prior to and at 1 and 2 days after the surgery and hydration was supplemented with subcutaneous injections of saline (10 ml) for 3 days post injury. These protocols were abbreviated for the rats studied at 24 h after injury. Temperature was monitored with a rectal thermometer (TCAT-2LV Temperature Controller, Kopf, Tujunga, CA) during the operation and an incubator was used to maintain a body temperature of 37 °C prior to and after the surgery until the animals were fully awake and moving about.

2.2. T12 contusion injury

Forty-four Wistar rats (17 male rats for behavior study, 18 female rats for myeloperoxidase (MPO) assay and 9 female rats for histology studies; Charles River) weighing 225–250 g were pre-medicated with diazepam (3.5 mg/kg, intraperitoneally; Sabex International Ltd., Boucherville, Quebec, Canada) and atropine (0.05 mg/kg, subcutaneously; Sigma Chemical, St. Louis, MO). Anesthesia in the rats was induced with 4% isoflurane and maintained with 1.5% isoflurane. A laminectomy was performed to expose the T12 spinal segment and the animal was mounted into the Infinite Horizon spinal cord impactor frame (Precision Systems and Instrumentation, Fairfax, VA). Modified Allis tissue forceps mounted in a custom frame were used to stabilize the animal by holding the dorsal processes on the rostral (T9) and caudal (T11) vertebral segments. After positioning the impactor tip, the Infinite Horizon impactor was then triggered to deliver a 200 kdyne injury with a 30 s dwell time. Animals were excluded from the study if the force delivered exceeded the desired force by 10%. Rats were given 10 ml of saline s.c., 5 mg/kg of Baytril s.c. (Bayer) and buprenophine (0.01 mg/kg s.c.) twice daily for three days following surgery. The experiments were completed at 24 h, or 8 weeks after the contusion injuries. In the chronic studies, urinary bladders were manually emptied twice daily until bladder function was regained.

2.3. Clip-compression injury

Twenty-eight female Wistar rats (Charles River) weighing 200–250 g were premedicated and anesthetized as described above for contusion injury. The T12 spinal cord segment was exposed by dorsal laminectomy and injured by a 60 s, 35-g clip-compression, as described previously (Oatway et al., 2005). After clip-compression SCI, the rats received the same care as those after contusion SCI described above. Spinal cords of these rats were examined at 24 h after SCI.

Table 1
Summary of experimental groups.

Injury	Treatment group	Duration	Assessment
Cervical Hemi-contusion (ICORD, UBC)	Anti-CD11d: $n = 12$, male 1B7: $n = 11$, male	6 weeks	Behavioral: Montoya staircase Horizontal ladder test Cylinder rearing test
	Anti-CD11d: $n = 12$, male 1B7: $n = 11$, male	6 weeks	Histology: sparing (eriochrome Cyanine)
	Anti-CD11d: $n = 5$, male 1B7: $n = 5$, male	24 h	MPO assay
T12 contusion (RRI, Western Univ)	Anti-CD11d: $n = 8$, male 1B7: $n = 9$ males	8 weeks	Behavioral: locomotor testing (BBB)
	Anti-CD11d: $n = 9$, female 1B7: $n = 9$, female	24 h	MPO assay
	$n = 4$, female	24 h	Gross anatomy
	$n = 5$, female	24 h	Histology: H&E Lesion size and hemorrhage analysis
T12 clip compression (RRI, Western Univ)	Anti-CD11d: $n = 9$, female 1B7: $n = 9$, female	24 h	MPO assay
	$n = 4$, female	24 h	Gross anatomy
	$n = 6$ female	24 h	Histology: H&E Lesion size and hemorrhage analysis

2.4. Antibody treatment

Following unilateral cervical contusion SCI at C5 the animals were randomized to the following treatment groups with each group comprising five animals. Rats to have a 24 h survival time were given one treatment with the anti-CD11d mAb (217 L; 1.0 mg/kg i.v. by tail vein injection) at 2 h after injury. Rats having a 6–8 week survival time were given three treatments with the anti-CD11d mAb (1.0 mg/kg, i.v.) at 2, 24 and 48 h after the injury. Control rats were given an isotype-matched mAb against dinitrophenol (1B7, 1.0 mg/kg, i.v.). The rats with thoracic contusion or clip-compression SCI were administered the mAb following the same protocol. The dose employed in all the current studies was chosen as it was optimized in previous studies of SCI in rats (Gris et al., 2004; Saville et al., 2004). The CD11d and 1B7 mAb were generously provided by Eli Lilly & Company (Indianapolis, IN). The specific number of rats per treatment group in different experiments is stated in Results.

2.5. Behavioral and histological assessments cervical hemi-contusion model

After one week of acclimatization, all rats were individually trained for 15 days prior to SCI for the horizontal ladder test, cylinder rearing test, and modified Montoya staircase. Behavioral recovery was assessed biweekly until week 6 post-injury.

2.5.1. Montoya staircase test

On five consecutive days, animals were acclimatized to the staircase apparatus by placing the apparatus in their home cages with the lid open so animals could voluntarily enter the narrow corridor with a removable baited double staircase. Food pellets were placed on the staircase and presented bilaterally at 7 graded 'stages' (stairs) at varying depths with increasing reaching difficulty (Montoya et al., 1991). Before testing commenced, the animals were food-restricted for a total 20 h. On testing days, rats were tested in the staircase box for a period of 15 min each and the number of successfully grasped food pellets and eaten using each forepaw was counted and recorded; each well was baited with four food pellets. Because animals are able to use their tongue to retrieve pellets from the top two steps of the staircase, reaching scores considered only the bottom five steps, from which forelimb use was necessary for successful retrieval. Additionally, the maximum distance reached (well #1–7) was determined.

2.5.2. Cylinder rearing test

Animals were individually placed in a topless clear Plexiglas cylinder measuring 20 cm in diameter and 30 cm in height until 20 incidences of rearing-explorations were recorded with a HD camcorder (Sony HDR-SR12). To determine forelimb usage, independent use of the left, right and the simultaneous use of both forepaws were assessed for the initial contact of the cylinder wall and all lateral movements along the wall during each individual rear. Percent ipsilateral usage was calculated by adding the number of ipsilateral forelimb placements and the number of simultaneous forelimb placements divided by the total number of forelimb placements [percent ipsilateral usage = (ipsilateral forelimb placements + simultaneous forelimb placements) / total forelimb placements) × 100].

2.5.3. Horizontal ladder test

Animals were acclimatized for a total of 5 days to run across an elevated horizontal ladder with a length of 70 cm and a width of 15 cm with irregularly spaced rungs. After acclimatization, baseline performance was assessed with five horizontal ladder crosses per rat. Performance was captured using a high definition (HD) camcorder (Sony HDR-SR12) as the rats traversed across the ladder. The footages were later replayed in slow motion to analyze the number of forelimb steps and forelimb errors (slips and falls). The percent ipsilateral forelimb error was then calculated as number of slips and falls divided by total number of steps taken by that paw [percent ipsilateral forelimb error = (number of error / total number of steps) × 100].

2.5.4. Histological examination

Six weeks after injury, animals were euthanized with an overdose of sodium pentobarbital as described above and transcardially perfused with 150 mL 1× PBS followed by 300 ml of 4% paraformaldehyde (PF) in 0.1 M phosphate buffer. A 5 mm segment of the cervical spinal cord centered on the injury site was removed, post-fixed for 24 h in 4% PF and then cryoprotected in 12, 20 and 28% sucrose. The spinal cord segments were frozen on dry ice and stored at −80 °C until further use. A 5 mm block of spinal cord was cut into 10 serial sets of 20 μm thick sections (collecting every other section) using a cryostat and mounted on glass slides. To quantify tissue sparing, sections were stained with Eriochrome Cyanine.

Sections were stained with Eriochrome Cyanine (EC) as described by (Rabchevsky et al., 2001) and counter-stained with Neutral Red. Images were then captured using a Leica DM5000B microscope with a 2.5× objective. The area of spared white and gray matter were manually traced and quantified using Sigma Scan Pro version 5.0.0 (Systat Software Inc.). White matter was considered spared if the deep blue EC stain on the myelin was dense and contained profiles resembling myelin rings; spared gray matter was defined as areas in which light EC staining could be discerned, and the parenchyma was relatively free of macrophages and cavitation. The lesion epicenter was defined as the cross-section with the smallest amount of white matter sparing. Tissue sparing was assessed at 400 μm intervals centered on the lesion epicenter.

2.6. Myeloperoxidase activity assay

The neutrophil and, to a lesser extent, macrophage presence in the injured cord was measured using a myeloperoxidase (MPO) activity assay (Bao et al., 2004b). At 24 h post- injury, hemi-contused rats were euthanized with an overdose of sodium pentobarbital (107 mg/kg, Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada) and T12 contusion and clip-compression SCI rats were euthanized with a ketamine (100 mg/ml) and xylazine (20 mg/ml) mix (2:1; 0.1 ml/100 g, i.p.). After an intracardiac injection of heparin (0.5 ml), the rats were perfused transcardially with 250 ml cold 0.9% NaCl. In the hemi-contused rats, a 1-cm segment of the injured cervical spinal cord centered on the C5 lesion was removed and divided longitudinally in half along the central line. The injured side and the contralateral side were collected separately in the cold vials, frozen immediately in dry ice, and stored at –80 °C. In T12 contusion and clip compression rats, a 1-cm segment of spinal cord centered on the thoracic lesion site was removed and immediately homogenized. The samples were homogenized on ice with 5 volumes (w/v) of 50 mm potassium phosphate buffer (lysis buffer) and 5 volumes (w/v) of 0.5% hexadecyltrimethylammonium bromide. The inclusion of hexadecyltrimethylammonium bromide was used to extract MPO from the leukocyte granules. Following centrifugation at 10,000 ×*g* for 20 min, MPO activity in the supernatant fluid was determined with 0.3 ml potassium phosphate buffer solution containing 1.23 mg/ml δ-dianisidine dihydorchloride and 0.05% hydrogen peroxide. The reaction was started by adding 0.1 ml of sample and was stopped 5 min later by adding 0.1 ml 0.1% sodium azide. Absorbance was measured at 460 nm, MPO activity was calculated using a standard curve prepared with purified MPO and expressed as units/mg protein.

2.7. Locomotor testing in thoracic contusion model

The locomotor recovery of 17 cord-injured rats was assessed using the 21 point Basso, Beattie and Bresnahan (BBB) open field locomoter scale (Basso et al., 1995). Male rats were used for behavior testing, in case there was an effect of the female estrus cycle. The animals were assessed by two or three observers. Individual scores for each hindlimb were averaged for each animal. Locomotor testing was conducted

on day 3 post-injury and subsequently twice per week for the duration of the study.

2.8. Examination of the cervical and thoracic lesions at 24 h after injury

Rats without mAb treatment were anesthetized (as above) at 24 h after T12 contusion or compression SCI and perfused with cold 0.9% NaCl. The injury site was exposed by dissection and examined in situ to view the extent of hemorrhage apparent on the cord surface. The lesion sites were photographed. In addition, the injury at 24 h after cervical hemi-contusion or T12 thoracic contusion or compression was examined for characteristics of the lesion, including the amount of hemorrhage within the cord. Rats with these injuries were anesthetized and perfused with fixative as described above and 5 mm samples centered on the respective lesions were embedded in OCT (Tissue Tek, Sakura Finetek U.S.A. Inc., Torrance, CA), sectioned in the longitudinal plane at 16 μm and stained with hematoxylin and eosin. The slides were viewed using an Olympus microscope (BX50, Olympus America Inc., Center Valley, PA) and photomicrographs were acquired using a digital camera (Retiga, Quantitative Imaging Corporation, Burnaby, BC, Canada) and Image Pro software version 5.1 (Media Cybernetics, Silver Spring, MD). Images of the longitudinal sections were viewed beginning with the dorsal surface of the cords and were captured when the section of cord was complete from side to side. Volumes of the lesions and of blood within the lesion were determined using the Image Pro quantitative software. First, the area of the lesion was determined for each of the serial sections, and then, knowing the thickness of the sections, mean volumes of all adjacent sections were summed to provide an estimate of the total volume of the lesion and blood.

2.9. Statistical analyses

Group data are expressed as mean ± SEM. Outcomes were only compared between animals of the same gender. Statistical analysis was performed using one-way or two-way repeated measures Analysis of Variance (ANOVA). Upon finding significant group variance differences in one-way ANOVA analysis, differences were tested by a Holm-Sidak's multiple comparison test post-hoc analysis. When only two groups were compared a Student's *t*-test was used. Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Effect of CD11d mAb on neurological recovery after C5 hemi-contusion SCI

We previously demonstrated that intravenous delivery of a mAb directed against the CD11d integrin improves locomotor and autonomic recovery and reduces neuropathic pain in a clip-compression model of SCI at the 4th or 12th thoracic (T4 or T12) levels in both rats and mice (Geremia et al., 2012; Gris et al., 2004; Oatway et al., 2005). To test this therapeutic strategy in a second model of SCI and in an independent laboratory, we evaluated the anti-CD11d treatment in a rat cervical (C5) hemi-contusion model (Lee et al., 2012). After hemi-contusion injury rats were administered either the anti-CD11d mAb 217 L or the isotype matched control mAb, 1B7 (1.0 mg/kg, i.v. by tail vein injection) at 2, 24 and 48 h after the injury. Behavioral analyses ($n = 12$ for CD11d treated rats and $n = 11$ for 1B7-treated controls) were carried out over a 6 week survival time.

3.1.1. Montoya's staircase test

The number of pellets retrieved by the forepaw ipsilateral to the injury was video recorded (Fig. 1A). After hemi-contusion SCI, all animals demonstrated a marked decrease in the number of food pellets retrieved and eaten; there was no significant difference between anti-CD11d mAb-treated animals and 1B7 controls. At 6 weeks after injury, the anti-CD11d mAb group retrieved 2.75 ± 1.1 pellets out of 20, whereas the 1B7 controls retrieved and consumed 6.1 ± 1.7 pellets. The maximum distance reached did not differ between the two groups.

3.1.2. Cylinder rearing test

Prior to SCI, paw usage was similar between both groups (Fig. 1B) in terms of calculated percent paw placements using the cylinder-rearing test. No significant differences were observed between the anti-CD11d mAb group and 1B7 controls at either 2, 4 or 6 weeks after C5 hemi-contusion. At the end of the experiment (6 weeks post-injury), ipsilateral usage of anti-CD11d mAb treated animals was 13.6 ± 5.6% compared to 8.5 ± 3.0% in the 1B7 controls.

3.1.3. Horizontal ladder

Using the horizontal ladder test (Fig. 1C,D), we analyzed foot placement accuracy (percentage of errors). Cervical hemi-contusive SCI led to increases in errors (slips + falls) for both groups at weeks 2–6 compared to pre-injury performance. No differences were observed between the anti-CD11d mAb and 1B7 control animals at any given time point. Six weeks after injury, the percent error in the anti-CD11d mAb group was 33.8 ± 4.1% and the 1B7 control group was 27.0 ± 3.9%.

3.2. Effect of the CD11d mAb on tissue sparing after C5 hemi-contusion SCI

Rats that underwent C5 hemi-contusion SCI were euthanized for histology upon completion of 6 weeks of behavioral testing. The extent of gray and white matter sparing was measured in EC-stained serial cross sections 400 μm apart ($n = 12$ for CD11d treated rats and $n = 11$ for 1B7-treated controls). Extensive white and gray matter damage occurred in the cervical spinal cords of all rats after hemi-contusion SCI (Fig. 2). Six weeks after injury, the gross tissue damage extended from the lesion epicenter by ~2800 μm both rostrally and caudally. There were no differences between the groups in area of spared white matter or in area of spared gray matter.

3.3. Effect of CD11d mAb on MPO levels in the spinal cord after C5 hemi-contusion SCI

MPO activity in cord homogenates sampled 24 h after SCI is proportional to the presence of neutrophils and, to a lesser extent, phagocytic macrophages and in our previous studies, anti-CD11d treatment always reduced this activity significantly by approximately 30–40% (Bao et al., 2004b; Geremia et al., 2012). Spinal cord samples ipsilateral and contralateral to the lesion were taken 24 h after SCI and studied for MPO activity as a marker for neutrophil accumulation/activation ($n = 5$ per group). MPO enzymatic activity was increased at 24 h within the injured ipsilateral spinal cord compared to the uninjured contralateral side (Fig. 3). Anti-CD11d mAb treatment did not reduce MPO activity at 24 h in the injured ipsilateral or uninjured contralateral spinal cord compared to 1B7 control values (Fig. 3).

3.4. Effect of the anti-CD11d mAb treatment on locomotor recovery after a T12 contusion SCI

The study of cervical hemi-contusion demonstrated that the CD11d mAb did not influence intra-parenchymal MPO activity, tissue sparing, or behavioral recovery – all outcome measures that significantly improved in our studies of thoracic clip-compression SCI (Bao et al., 2004a,b; Bao et al., 2005; Gris et al., 2004; Oatway et al., 2005). We questioned whether the anti-CD11d treatment might be efficacious in a model of contusion SCI more closely related to our own previous clip-compression studies. Therefore, we set out to produce a contusion injury similar to that caused by 35 g clip-compression (duration 60 s) at the lower thoracic spinal segments. We designed a study of contusion SCI at the T12 spinal segment, replicating the location of our published T12 clip-compression studies (Gris et al., 2004; Oatway et al., 2005).

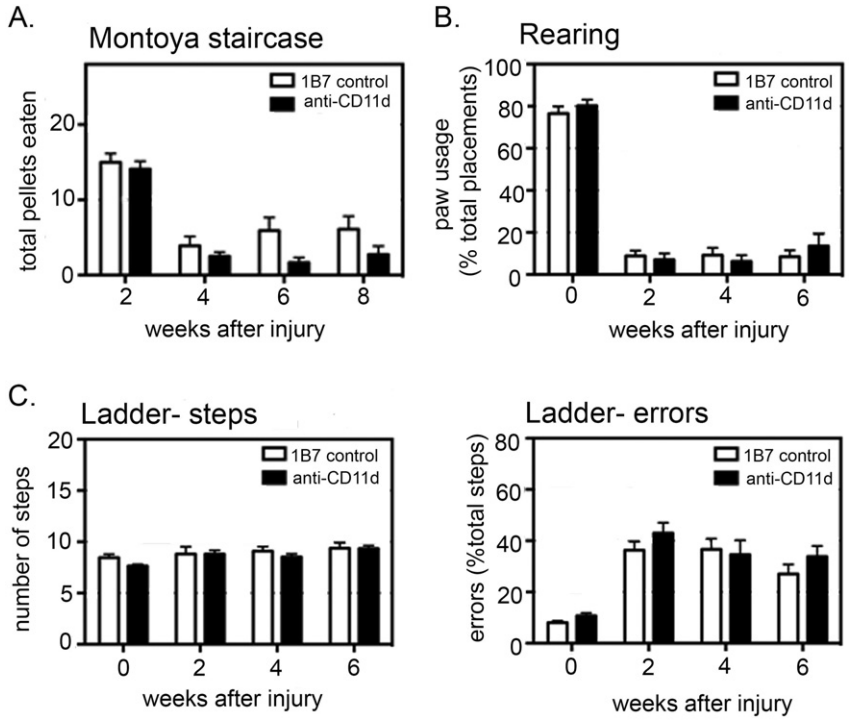

Fig. 1. Anti-CD11d treatment does not improve behavioral recovery in after a C5 hemi-contusion SCI. (A) Effect of the anti-integrin treatment on Montoya staircase reaching performance. The average number of pellets reached and eaten at baseline and 2, 4, 6 and 8 weeks post-SCI was recorded. Recovery of skilled limb use, indicated by the number of pellets successfully retrieved and eaten, was not affected by the anti-CD11d treatment. (B) Effect of the anti-integrin treatment on use of the fore limb ipsilateral to the hemi-contusion SCI. No differences in the use of the affected forelimb were detected between mAb-treated and control rats. (C, D) Effect of the anti-CD11d mAb on ipsilateral forelimb placement during horizontal ladder crossing. The average number of steps (C) and the percentage of errors (D, slips and falls) in 1B7 controls and anti-CD11d mAb-treated rats at baseline and post-injury weeks 2, 4 and 6 are indicated. No differences were observed between both groups ($n = 12$/anti-CD11d; $n = 11$/1B7) at any time point after injury.

Delivering a contusion injury of 200 kdyne with a dwell time of 30 s generated BBB scores quite similar to those after 35 g clip-compression for the first 2 weeks after the injury (Fig. 4). At 1 week and 2 weeks after contusion injury the mean scores were approximately 3 and 5 and we reported mean scores of approximately 2 and 6 at these times after clip-compression injury (Oatway et al., 2005). Anti-CD11d treatment did not improve the BBB scores in the T12 contusion SCI model (Fig. 4). At 4 weeks after contusion injury, rats treated with CD11d mAb ($n = 8$) had average BBB scores of 5.7 ± 0.5 and rats treated with the control 1B7 mAb had almost identical BBB scores of 5.7 ± 0.6 ($n = 9$). We continued to test these rats for 8 weeks after injury and mean BBB scores in the two groups never diverged (two-way repeated measures ANOVA: time $P < 0.0001$, treatment $P = 0.6157$, time/treatment interaction $P = 0.539$).

3.5. Anti-CD11d treatment reduces intraspinal MPO activity after T12 clip-compression but not after T12 contusion SCI

The proposed mechanism of anti-CD11d action is to reduce activated neutrophil and macrophage infiltration of the injured spinal cord (Mabon et al., 2000; Saville et al., 2004). We considered the possibility that the contusion injury may produce a smaller inflammatory response than clip-compression injury, limiting the target for the CD11d mAb treatment. We compared MPO activity in homogenates of the T12 lesion and adjacent area at 24 h after 35 g clip-compression injury or 200 kdyne (30 s dwell) contusion injury (Fig. 5). Significant differences were detected between the anti-CD11d and 1B7 treatment groups after the SCI (one-way ANOVA: $P = 0.0216$). After clip-compression at T12, MPO activity was 29% lower in the anti-CD11d group than in the control 1B7 group ($n = 9$, Holm-Sidak's multiple comparison test, $P = 0.0424$). In contrast, treatment with the CD11d mAb after contusion SCI did not reduce MPO activity significantly in the treated group in comparison to the 1B7 control group ($n = 9$, Holm-Sidak's multiple comparison test, $P = 0.1830$). Although the mean MPO activity in the 1B7 control group after T12 contusion injury was lower than the mean MPO activity in the 1B7 control group after T12 clip injury, they were not significantly different from one another (Holm-Sidak's multiple comparison test, $P = 0.2067$).

3.6. Contusion SCI causes more hemorrhage than clip-compression SCI

The spinal cords of rats were compared at 24 h after T12 injury by 35 g clip-compression ($n = 4$) or a 200 kdyne contusion with a 30 s dwell time ($n = 4$). When viewed in situ, both resulted in significant hemorrhage on the cord surface. The clip-compression injury produced a hematoma that was confined to a ~ 2 mm wide line at the site of injury that approximated the width of the aneurysm clip (Fig. 6, top row). The contusion injury produced a hematoma much wider than after clip-compression that extended 4 mm or more in the rostral and caudal directions from the centre of the injury (Fig. 6, bottom row).

A histological study of the T12 thoracic lesions of rats 24 h after injury by contusion ($n = 5$) and by clip-compression ($n = 6$) was undertaken to examine this apparently greater hemorrhage after contusion than clip-compression SCI. Sections stained with hematoxylin and eosin revealed lesions with pallor, necrosis and areas of cavitation (Fig. 7A). Upon inspection, the lesions after contusion appeared to be more extensive and to contain greater amounts of hemorrhage within the cord in contrast to the lesions after clip-compression that appeared smaller and less hemorrhagic. We compared these photomicrographs to those of the C5 hemi-contusion lesion in sections stained by hematoxylin and eosin. These lesions were confined to half the cervical cord, and accordingly were smaller than those we produced in the thoracic cord. They too contained areas of pallor and necrosis. The outstanding observation from these cervical sections was the extensive hemorrhage found at the lesion sites (Fig. 7A).

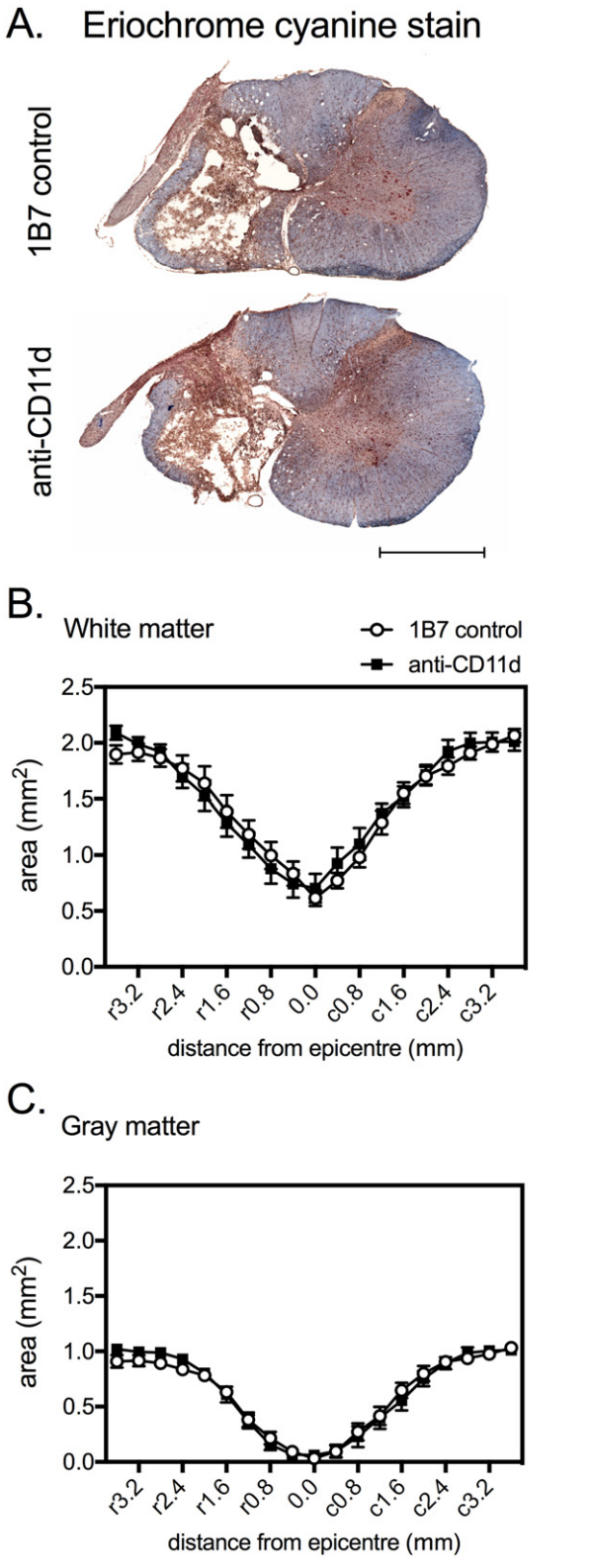

Fig. 2. Anti-CD11d treatment does not increase white or gray matter sparing after a C5 hemi-contusion SCI. (A) Photomicrographs of eriochrome cyanine-stained coronal sections at the injury epicenter of 1B7 control (top) and CD11d mAb-treated (bottom) rats after C5 hemi-contusion. Bar = 1 mm. The average areas of spared white (B) and gray (C) matter measured in EC-stained serial sections from 3.6 mm rostral to 3.6 mm caudal of the center of the injury site are not significantly different between 1B7 controls and Cd11d mAb-treated rats ($n = 12$/anti-CD11d; $n = 11$/1B7).

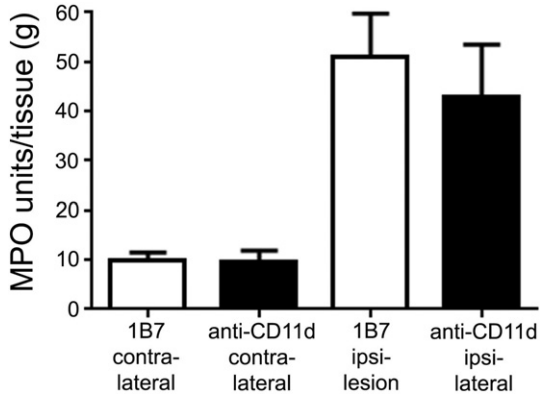

Fig. 3. Effect of the anti-CD11d treatment on MPO activity in the lesion epicenter in the C5 hemi-contusion model. Average MPO activity as measured in the ipsilateral anti-CD11d and 1B7-treated control injured spinal cords, 24 h post-injury. The mean levels of MPO activity were increased in the ipsilateral spinal cord compared to the contralateral side. No significant differences were observed between 1B7 controls and anti-CD11d mAb-treated animals ($n = 5$/anti-CD11d; $n = 5$/1B7).

The volumes of lesion and of hemorrhage within the thoracic lesions were measured by a colour-threshold detecting function of the Image Pro software. The volume of lesion was first calculated as described in methods, and then the volume of hemorrhage was calculated. The mean lesion volume after contusion injury was larger but only tended to be different from the mean lesion volume after clip-compression injury (Fig. 7B, 1-tailed Student's *t*-test $P = 0.06$), whereas the volume of hemorrhage in the sections after contusion injury were significantly greater than after compression injury (Fig. 7C, Student's *t*-test 2-tailed $P = 0.01$).

4. Discussion

Acute intravenous delivery of the CD11d mAb improves locomotor recovery and reduces pain in rats after a clip-compression SCI. We initially set out to determine whether this therapy would also promote improved functional recovery following C5 hemi-contusion SCI in a laboratory independent to that where much of the promising work on CD11d mAb had previously occurred. Using an array of behavioral tests, no significant differences were found between treated and control groups in the cervical SCI model. Since we had also previously shown that the CD11d mAb increases white matter sparing (Gris et al., 2004; Oatway et al., 2005) in the rat clip-compressed spinal cord, we also assayed white matter and gray matter sparing in the C5 hemi-contused rat. Again, we found no significant difference between treated and control groups on these measures. To evaluate whether the absence of a

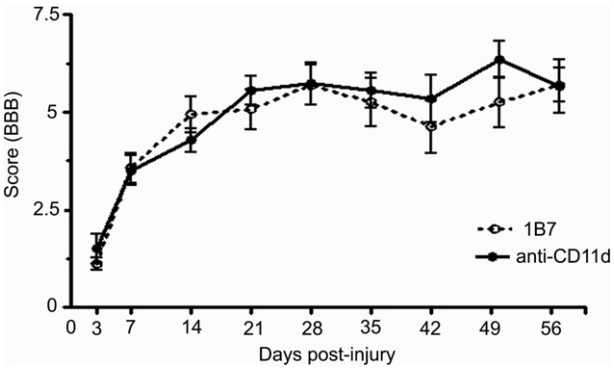

Fig. 4. The effect of the CD11d mAb treatment on locomotor recovery following a T12 contusion injury. The CD11d mAb treatment did not improve locomotor recovery after T12 contusion SCI. There was no difference in BBB scores in the anti-CD11d treated group (black circles) when compared to 1B7 control treated group (open circles) over the period of 56 days ($n = 8$/anti-CD11d; $n = 9$/1B7).

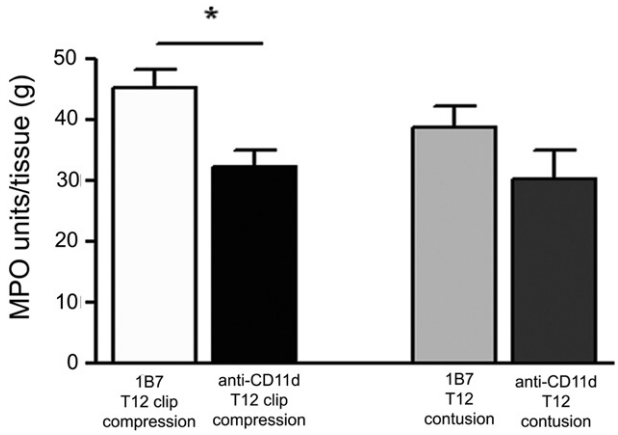

Fig. 5. The Anti-CD11d treatment reduces intraspinal myeloperoxidase activity after T12 clip (35 g) compression SCI but not after T12 contusion SCI. MPO activity was measured 24 h post-injury. Treatment with the CD11d mAb significantly reduced the amount of MPO in the T12 clip-compression SCI when compared to isotype control ($n = 9$/group;) but not in the T12 contusion SCI ($n = 9$/group).

treatment effect in the C5 hemi-contusion model might apply to contusion SCI in general, we evaluated the CD11d mAb treatment in a T12 contusion injury. As in the case of the C5 hemi-contusion, the anti-CD11d therapy failed to improve locomotor recovery in the cord-contused rats.

To investigate why the CD11d mAb improves recovery in T12 clip-compressed rats but not in T12 contused rats, we evaluated MPO levels in treated and untreated rats after both types of SCI at 24 h post-injury. In keeping with our previous reports, the CD11d mAb reduced MPO levels after T12 clip-compression SCI, however, the treatment did not reduce MPO levels after T12 contusion SCI. Upon examination of the gross in situ spinal cords, we observed a striking difference in the surface appearance of the cords between the compression and contusion injury groups: the clip-compression injury produced a small hematoma across the cord corresponding to the placement of the clip, whereas the contusion injury produced a large hemorrhage that appeared to spread in the rostral-caudal axis from the point of impact. We therefore hypothesized that neutrophils were entering the cord through different mechanisms in the two different injury models: CD11d-dependent diapedesis in the case of compression, and intraparenchymal hemorrhage in the case of contusion. Histological investigation and quantification of blood at the lesion site 24 h after injury confirmed that, whereas the lesion volumes were not significantly different between clip-compressed and contused rats, the lesion site of rats contained significantly more blood after contusion SCI. A qualitative evaluation of histological sections from rats after C5 hemi-contusion also showed a great deal of intraspinal hemorrhage. The increased frank bleeding into the spinal cord after contusion SCI compared to clip-compression SCI may explain why the two injury models respond differently to the CD11d mAb. This treatment targets the interaction between activated white blood cells and the intact vascular endothelium to reduce neutrophil and monocyte infiltration of the injury site.

Within the injured spinal cord, inflammatory chemoattractant signals recruit leukocytes preferentially via an abundant venous system within the gray matter. This process is further enhanced as spinal venules, unlike arterioles, constitutively express intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and platelet-endothelial cell adhesion molecule (PECAM) (Schnell et al., 1999a) that mediate the extravasation of leukocytes into the lesion. More recently, it has been proposed that an additional source of inflammatory leukocytes, particularly monocyte/macrophages, is the blood-cerebrospinal fluid interface at the brain choroid plexus that delivers leukocytes to the lesion via the central canal and CSF (Shechter et al., 2013). A one minute clip-compression SCI leaves the cord tissue locally bruised (size defined by width of the compression clip), but also affected by ischemia-reperfusion injury. Thus, despite the local hemorrhage

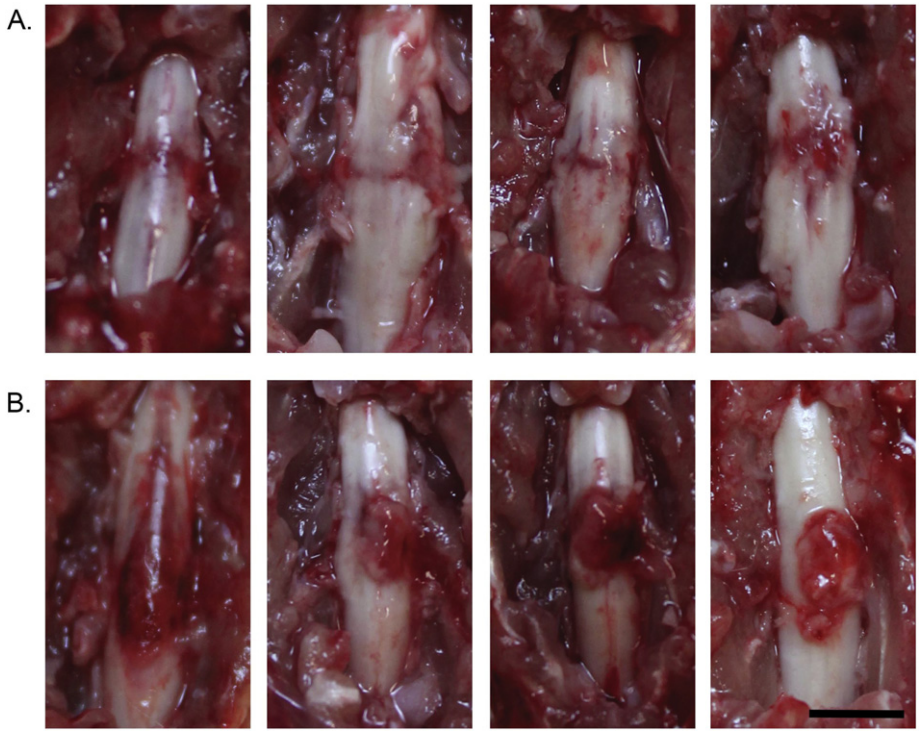

Fig. 6. Gross inspection of the lesion sites in T12 clip-compressed and T12 contused spinal cords reveal different pathologies. Injured spinal cords were compared at 24 h after (A)T12 injury by 35 g clip-compression ($n = 4$, top row) or (B) a 200 kdyne contusion SCI with a 30 s dwell time ($n = 4$, bottom row). Both resulted in significant hemorrhage on the cord surface but the clip-compression injury produced a hematoma that was confined to a ~2 mm wide line at the site of injury that approximated the width of the aneurysm clip while the contusion injury produced a much larger hematoma that extended 4 mm or more in the rostral and caudal directions from the centre of the injury. Scale bar = 5 mm.

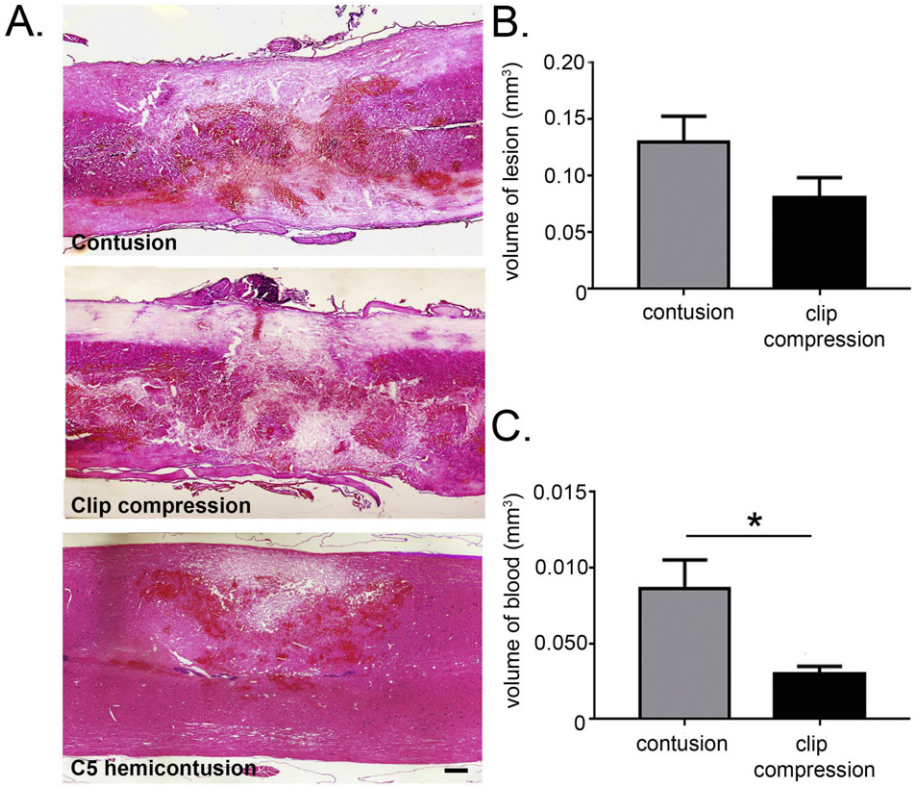

Fig. 7. Lesion assessment reveals greater amounts of intraspinal blood after T12 contusion SCI compared to T12 clip-compression SCI. (A) Representative photomicrographs of hematoxylin and eosin stained sections after T12 contusion, T12 clip-compression, and a C5 hemi-contusion SCI, ($n = 5$/contusion group; $n = 6$/clip compression, $n = 4$/C5 hemi-contusion group). Bar = 200 μm. (B) There was no significant difference in lesion volume between the clip-compression and contusion SCI rats at 24 h post-injury but the amount of intraspinal blood in the T12 contusion SCI rats was significantly greater than that in the T12 clip-compression SCI rats.

in the bruised area of the cord, the surrounding vasculature, central canal and meningeal membrane are left intact. The vasculature will, however, become more permeable due the presence of ischemia-reperfusion induced reactive oxygen species, complement and the release of proteases by the ever increasing number of neutrophils, all of which loosen intracellular adhesions that normally maintain the spinal cord blood barrier (Anwar et al., 2016; Takigawa et al., 2010). The constitutively expressed adhesion molecules on venules will immediately facilitate the early entry of the arriving neutrophils and later monocyte/macrophages into the injured spinal cord via adhesion molecule-integrin mediated processes in response to pro-inflammatory signals (Anwar et al., 2016; Fleming et al., 2006; Saville et al., 2004). Similarly, the migration of leukocytes from the CSF and meningeal membranes will also require adhesion molecule-integrin interactions in response to the same pro-inflammatory signals. Hence, therapies directed at blocking leukocyte migration by preventing adhesion molecule-integrin interactions into the lesion can have impact in preclinical models, as we have previously demonstrated (Fleming et al., 2008; Gris et al., 2004; Saville et al., 2004). However, in SCI models such as that generated by the piston-driven contusion impactor, significant intraparenchymal hemorrhage extends both rostrally and caudally from the epicenter (Anwar et al., 2016) and the structures that constrain CSF are mechanically disrupted. Therefore, the adhesion molecule integrin-mediated process of leukocyte recruitment is no longer the dominant means by which leukocytes gain access to the lesion. Blood leukocytes will enter freely into the lesion area until thrombosis, and initial vascular repair take over in the first 24 to 48 h (Bartanusz et al., 2011; Fehlings et al., 1989). Under these circumstances, early anti-adhesion molecule-integrin strategies to block leukocyte extravasation will not be effective, as we have demonstrated here.

Therapeutic strategies for the treatment of SCI are typically developed to interfere with specific molecular and/or cellular events. Thus, it stands to reason that their effectiveness will rely upon the extent to which the pathophysiology triggered by an injury depends upon the targeted molecular or cellular event. The CD11d mAb targets leukocyte infiltration of the injured spinal cord by disrupting the CD11d-VCAM-1 interaction necessary for diapedesis. We suggest that the CD11d mAb was not effective in the contusion SCI models tested because the pathology post-contusion is dominated by the severe primary injury and accompanying increased intraspinal hemorrhage. Secondary damage and inflammation, that are driven by leukocyte diapedesis and CD11d-VCAM-1 interactions are less important in this model of SCI compared to clip-compression SCI. In addition a greater primary injury after contusion SCI could have reduced the amount of tissue available as a substrate for neuroprotection. Our results highlight the importance of characterizing the pathophysiological mechanisms at work in various models of SCI or of the same model in different hands when interpreting the results of therapeutic studies. Negative results of preclinical therapeutic studies are important to steer the research field toward developing beneficial therapies but we must be mindful that inconsistent results between models may simply result from the fact that the pathophysiological mechanism targeted by the therapeutic is less relevant in one model than another.

This foregoing argument suggests that the usefulness of the CD11d mAb therapy in human SCI will depend on whether the pathology in a particular case of SCI is driven by CD11d-VCAM interactions and consequent secondary injury or by other mechanisms. Neutrophil and macrophage infiltration of the injured human spinal cord has been previously described (Fleming et al., 2006). The extent to which neutrophil and macrophage levels in the injured human spinal cord may be attributed to hemorrhage or to CD11d-VCAM interactions is difficult to assess. Human pathological studies indicate that while hemorrhage may be a typical feature of SCI (Miyanji et al., 2007; Tator, 1995; Witiw and Fehlings, 2015) it is variable (Fleming et al., 2006) and may not be

present even when predicted to be a prominent feature of the disease (Quencer et al., 1992). The heterogeneity in the SCI patient population suggests that each case will have its own constellation of pathophysiological mechanisms at play after injury reflective of the mechanism of injury, the patient's genetics, epigenetics and pre-existing conditions. While the clip compression model of injury may replicate many of the pathological features of human SCI, it should be acknowledged that most human injuries occur due to high energy trauma in which the spinal cord is "contused" with velocities that even exceed what is achieved with typical contusion SCI models. Our results argue that a therapy's efficacy in one model of SCI should prompt investigation in other SCI models to address how it would work in different patient populations.

The extent to which secondary injury caused by infiltrating pro-inflammatory leukocytes contributes to the pathology of human SCI injury most likely will vary from patient to patient and thus the potential therapeutic benefit of the CD11d mAb to human SCI cannot be readily determined. The fact that hemorrhage is a typical feature of acute traumatic human SCI certainly points to the importance of replicating this pathologic feature in animal models. As shown in our paper, such hemorrhage is a prominent feature of the acute contusive mechanism of injury, and not the compressive mechanism of injury. This heterogeneity of human SCI is important to acknowledge when considering the efficacy of a particular preclinical therapy that is tested in only a single injury paradigm. Our study uniquely demonstrates that differences in injury mechanism in the laboratory influences therapeutic efficacy. This provides a strong argument to investigate a novel treatment in more than one preclinical injury model, which is a form of "replication" that is somewhat distinct from, but arguably as clinically relevant as, doing a formal replication. In this regard it is interesting to note that we (Bao et al., 2012; Shultz et al., 2013) and others (Utagawa et al., 2008) have demonstrated a neuroprotective effect of the CD11d mAb in models of traumatic brain injury.

Our work indicates that the best candidates for the CD11d mAb may be those with imaging studies suggesting limited bleeding into the injured cord or those for whom the effect of the CD11d mAb on co-morbid conditions may justify its use. For example, the systemic inflammatory response syndrome (SIRS) is a significant contributor to morbidity and mortality in trauma patients (Acosta et al., 1998) and frequently occurs after SCI (Kesani et al., 2014). We have shown that the CD11d mAb treatment reduces SIRS in spinal cord-injured rats (Bao et al., 2011; Weaver et al., 2015). If the CD11d mAb proves efficacious in the treatment of SIRS then spinal cord-injured people that meet the criteria for SIRS (Kesani et al., 2014) may be the ideal candidates for evaluating the effects of the anti-CD11d treatment on recovery from SCI.

Declaration of interest

A.B., G.A.D. and L.C.W. are named as co-inventors on a patent application related to CD11d antibodies.

Acknowledgments

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