doi:10.1093/brain/awp322 Brain 2010: 133; 433–447 | 433

BRAIN
A JOURNAL OF NEUROLOGY

Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment

Kevin D. Beck,[1,*] Hal X. Nguyen,[1,2,3,*] Manuel D. Galvan,[1] Desirée L. Salazar,[1,3] Trent M. Woodruff[4] and Aileen J. Anderson[1,2,5]

1 Anatomy and Neurobiology, University of California, Irvine, CA 92697-4292, USA
2 Physical Medicine and Rehabilitation, 1105 Gillespie Neuroscience Research Facility, University of California, Irvine, CA 92697-4292, USA
3 Sue and Bill Gross Stem Cell Research Centre, University of California, Irvine, CA 92697-4292, USA
4 School of Biomedical Sciences, University of Queensland, St. Lucia, Brisbane 4072, Australia
5 Reeve-Irvine Research Centre, University of California, Irvine, CA 92697-4292, USA

*These authors contributed equally to this work.

Correspondence to: Aileen J. Anderson,
Physical Medicine and Rehabilitation,
1105 Gillespie Neuroscience Research Facility,
Irvine, CA 92697-4292,
USA
E-mail: aja@uci.edu

Traumatic injury to the central nervous system results in the disruption of the blood brain/spinal barrier, followed by the invasion of cells and other components of the immune system that can aggravate injury and affect subsequent repair and regeneration. Although studies of chronic neuroinflammation in the injured spinal cord of animals are clinically relevant to most patients living with traumatic injury to the brain or spinal cord, very little is known about chronic neuroinflammation, though several studies have tested the role of neuroinflammation in the acute period after injury. The present study characterizes a novel cell preparation method that assesses, quickly and effectively, the changes in the principal immune cell types by flow cytometry in the injured spinal cord, daily for the first 10 days and periodically up to 180 days after spinal cord injury. These data quantitatively demonstrate a novel time-dependent multiphasic response of cellular inflammation in the spinal cord after spinal cord injury and are verified by quantitative stereology of immunolabelled spinal cord sections at selected time points. The early phase of cellular inflammation is comprised principally of neutrophils (peaking 1 day post-injury), macrophages/microglia (peaking 7 days post-injury) and T cells (peaking 9 days post-injury). The late phase of cellular inflammation was detected after 14 days post-injury, peaked after 60 days post-injury and remained detectable throughout 180 days post-injury for all three cell types. Furthermore, the late phase of cellular inflammation (14–180 days post-injury) did not coincide with either further improvements, or new decrements, in open-field locomotor function after spinal cord injury. However, blockade of chemoattractant C5a-mediated inflammation after 14 days post-injury reduced locomotor recovery and myelination in the injured spinal cord, suggesting that the late inflammatory response serves a reparative function. Together, these data provide new insight into cellular inflammation of spinal cord injury and identify a surprising and extended multiphasic response of cellular inflammation.

Received April 13, 2009. Revised October 14, 2009. Accepted November 12, 2009. Advance Access publication January 19, 2010

Understanding the role of this multiphasic response in the pathophysiology of spinal cord injury could be critical for the design and implementation of rational therapeutic treatment strategies, including both cell-based and pharmacological interventions.

Keywords: spinal cord injury; inflammation; flow cytometry; immunohistology; complement

Abbreviations: BBB = Basso, Beattie and Bresnahan locomotor rating scale; C5aRa = C5a receptor antagonist; dpi = days post-injury; FITC = fluorescein isothiocyanate; HBSS = Hank's buffered saline solution; Ig = immunoglobulin; PMNs = polymorphonuclear leucocytes (neutrophils)

Introduction

Traumatic injury to the central nervous system results in disruption of the blood brain/spinal barrier, facilitating the entrance of immune cells and proteins. The cellular immune response has garnered increasing attention because of accumulating evidence suggesting a contributing role for immune cells in spinal cord injury pathology and repair. The infiltration of neutrophils (polymorphonuclear leucocytes, PMNs) or macrophages/microglia exacerbates poor recovery from traumatic brain and spinal cord injury (Giulian and Robertson, 1990; Popovich *et al.*, 1999; Weaver *et al.*, 2000; Hains *et al.*, 2001; Noble *et al.*, 2002; Saville *et al.*, 2004; Stirling *et al.*, 2004). Likewise, central nervous system-reactive T cells have been suggested to exacerbate axonal injury, demyelination and functional loss (Popovich *et al.*, 1996*b*; Howe *et al.*, 2007). Conversely, macrophages/microglia and T cells can also secrete factors that promote neuroprotection and/or neuroregeneration after spinal cord injury (Guth *et al.*, 1994; Rapalino *et al.*, 1998; Moalem *et al.*, 1999; Hauben *et al.*, 2001; Li *et al.*, 2001; Crutcher *et al.*, 2006; Yin *et al.*, 2006). These contrasting immune functions may support a bimodal role for immune cells in the injured spinal cord.

Although a few studies have shed light onto the possible roles of immune cells in the injured central nervous system, the underlying mechanisms are complex and remain unclear. Growth factors and protease inhibitors released by macrophages or T cells are thought to promote neuro regeneration (Cantini and Carraro, 1995; La Fleur *et al.*, 1996; Jameson *et al.*, 2002; Yin *et al.*, 2006). Conversely, conditioned-media collected from PMNs or macrophages has been shown to promote neurotoxicity *in vitro* (Flavin *et al.*, 1997; Brana *et al.*, 1999; Nguyen *et al.*, 2007). Although specific macrophage-derived neurotoxic factors have not been identified, we have shown that matrix metalloproteinases, reactive oxygen species and tumour necrosis factor-α released by PMNs all promote neurotoxicity *in vitro* (Nguyen *et al.*, 2007). Additionally, physical PMN-to-neuron contact has been shown to enhance neurotoxicity (Dinkel *et al.*, 2004). As similar mechanisms are likely to be involved in immune cell function after spinal cord injury, understanding the kinetics of cellular inflammation after spinal cord injury is essential for the development of effective therapeutic interventions.

Previous studies on kinetics of the cellular immune response to spinal cord injury have generally focused on a subset of time points, ranging from 1 to 42 days post-injury (dpi), which represent a relatively narrow range of assessment within the lifespan of animals with spinal cord injury. These studies have shown that PMNs infiltrate the spinal cord within hours after injury and remain detectable at the injury epicentre at 42 dpi (Taoka *et al.*, 1997; Royo *et al.*, 1999; de Castro *et al.*, 2004). In contrast, macrophages/microglia and T cells infiltrate the spinal cord from 3 to 14 dpi (Saville *et al.*, 2004; Jones *et al.*, 2005; Wu *et al.*, 2005; Conta and Stelzner, 2008), remaining detectable for at least 42 dpi (Guizar-Sahagun *et al.*, 1994; Kigerl *et al.*, 2006). In parallel with data from rodent studies, Fleming *et al.* (2006) described the presence of PMNs, macrophages/microglia and T cells in the post-mortem human spinal cord up to 12 months after injury (Fleming *et al.*, 2006), although the study design prohibited quantitative comparisons. Critically, longer time points and finer quantitative assessments of neuroinflammation are needed to understand the kinetics of neuroinflammation in the injured spinal cord fully, as it relates to functions of specific cell types.

Most previous studies have characterized cellular inflammation in the injured central nervous system using histological techniques that cannot always provide true quantitative analysis. Flow cytometry is an alternative method to quantify immune cells in the injured spinal cord tissue quickly and effectively. Historically, flow cytometry has been used to quantify immune cells collected from blood or dissociated from the spleen or thymus, and only a few studies have attempted to quantify immune cells in the injured spinal cord by flow cytometry using fresh dissociated cord tissue or cells separated by the Percoll gradient system (Gonzalez *et al.*, 2003; Tjoa *et al.*, 2003; Stirling and Yong, 2008). In this study, we describe a novel and alternative cell preparation methodology using the OptiPrep gradient system (Fisher Scientific) to separate cells effectively from lipid/myelin debris and assess cellular inflammation by flow cytometry. Additionally, we demonstrate the reliability and sensitivity of the OptiPrep gradient-purified cell preparation by (i) testing the sensitivity of this gradient purified cell preparation in comparison with preparation of samples by enzymatic dissociation alone; (ii) assessing changes in PMN and macrophage/microglial infiltration in response to injury severity and (iii) comparing flow cytometric data of cellular infiltration with quantitative stereology of immunolabelled spinal cord sections at selected time points. Critically, we also report the first characterization of acute and chronic cellular inflammation after spinal cord injury to include a complete time course for PMNs, macrophages/microglia and T cells over a period ranging from 2 h to 180 dpi, identifying a novel second phase of cellular inflammation. The second phase did not coincide with changes in motor recovery after spinal cord injury. However, reduction of delayed C5a-mediated inflammation reduced locomotor recovery and myelination in the injured spinal cord, suggesting that chronic

inflammation is reparative. Findings from this study demonstrate, for the first time, a temporally distinct second phase of cellular inflammation in the injured central nervous system and reveal an important multiphasic component of neuroinflammation that may be critical for the design and implementation of rational therapeutic treatment strategies, including both cell-based and pharmacological interventions for spinal cord injury.

Methods

Additional experimental details and selected methods are given in the online supplementary material.

Spinal cord injury

Female Sprague Dawley rats received T9 spinal contusions with the Infinite Horizons Impactor (Precision Instuments, Lexington, KY) (Nguyen *et al.*, 2008). For graded contusion injury experiments, contusion injuries of 150, 200 or 250 kilodyne (kd) were administered ($n = 5$/group). A 200 kd force was used for all other experiments including the flow cytometric timecourse ($n = 3$–5/time point, Table 1), stereologic quantification timecourse (1 dpi: $n = 6$; 7 dpi: $n = 7$; 28 dpi: $n = 6$; 90 dpi: $n = 7$), 1 and 7 day C5a receptor antagonist (Ra) efficacy ($n = 5$/group/time point), C5aRa depletion ($n = 11$/group) and 91 day openfield locomotor assessment ($n = 12$). Animals used for histology were perfused with phosphate-buffered saline-buffered 4% paraformaldehyde, and tissue from spinal cord segments containing the injury epicentre were dissected from spinal roots (T6–T12) for subsequent immunolabelling. Animals used for flow cytometric analyses (three to five animals per group or time point) were sacrificed by CO_2 asphyxiation, tissue from spinal segments T8-T10 was rapidly dissected and placed in Hank's buffered saline solution (HBSS) on ice. All work was conducted with the approval of the Institutional Animal Care and Use Committee at the University of California, Irvine.

Table 1 Animal samples in timecourse experiments

	PMN	ED1	CD3
0 h	5	5	5
2 h	5	5	4
1 dpi	4	5	5
2 dpi	5	3	5
3 dpi	5	3	5
4 dpi	4	5	5
5 dpi	5	5	5
6 dpi	5	5	5
7 dpi	4	5	4
8 dpi	5	5	5
9 dpi	5	5	5
10 dpi	5	5	5
14 dpi	5	5	4
21 dpi	5	5	–
28 dpi	4	5	–
60 dpi	5	5	–
90 dpi	4	4	4
180 dpi	5	5	5

For each time point (0 h to 180 dpi), five animals received a moderate (200 kd) contusion injury at T9 and spinal cord tissue was assessed by flow cytometry for the numbers of PMNs, $ED1^+$ macrophages/microglia and $CD3^+$ T cells. However, not all animal samples were recovered successfully for PMN (4–5), ED1 (3–5) and CD3 (4–5) flow cytometric analyses.

Open-field behavioural assessment

Functional recovery was assessed using the Basso, Beattie and Bresnahan locomotor rating (BBB) scale (Basso *et al.*, 1995). Two experienced raters, blinded to experimental treatment, observed open-field locomotion in 4 min testing periods at 2 and 7 dpi, and then weekly until sacrifice.

Tissue preparation for use in flow cytometry and immunohistochemistry

For flow cytometry, spinal cord segments T8–T10 were mechanically dissociated with fine scissors in HBSS. Cells were retrieved by centrifugation and enzymatically dissociated with 2.5 mg trypsin and 5 mg collagenase in 5 ml Dulbecco's modified Eagle's medium for 20 min at 37°C before trituration with a Pasteur pipette. Ten ml Dulbecco's modified Eagle's medium plus 10% foetal bovine serum was added before filtering with a 40 μm cell strainer. The pellet was resuspended in 6 ml of HBSS and overlayed on an OptiPrep gradient (Krickhahn *et al.*, 2001; Bagamery *et al.*, 2005). The OptiPrep gradient was constructed first by diluting OptiPrep (Fisher Scientific) 1:1 with 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (0.15 M NaCl, 10 mM MOPS, pH 7.4). Four dilutions were made with 350, 250, 200 or 150 μl OptiPrep/MOPS and added to HBSS for a final volume of 1 ml. The four solutions were carefully placed in layers in a 15 ml conical tube with the least dilute at the bottom and the most dilute on the top (Fig. 1A). Dissociated spinal cells in HBSS (6 ml) were layered on top of the OptiPrep gradient before centrifugation (15 min, 1900 rpm, 20°C), separating the cell solution into distinct layers with debris on top, followed by three layers of neurons, and inflammatory cells, glia and red blood cells in the pellet. The debris layer was carefully aspirated. The remaining cells were washed and resuspended in HBSS for immunolabelling.

For immunohistochemistry, fixed T6–T12 spinal cord segments were cryoprotected in 20% sucrose in phosphate-buffered saline-buffered 4% paraformaldehyde overnight at 4°C, frozen in isopentane at −56°C and then stored at −80°C. Coronal sections (30 μm) of spinal cord tissue were cut on a sliding microtone. Serial sections were collected in phosphate-buffered saline with sodium azide (0.02%) and stored at 4°C until use.

Immunocytochemistry for flow cytometry

Cells collected from spinal cord preparations were pelleted and resuspended in 0.85% ammonium chloride for 5 min to lyse red blood cells, blocked for 30 min in normal rabbit or goat serum, and incubated for 1 h with primary antibodies or isotype immunoglobulin (Ig)Gs diluted in HBSS for 1 h [rabbit anti-PMN fluorescein isothiocyanate (FITC), Accurate Chemical and Scientific; mouse anti-rat granulocytes (RP1) RPE, BD Pharmingen; mouse anti-rat ED1, Serotec; mouse anti-rat CD11b, Serotec; mouse anti-rat CD3, Serotec; rabbit IgG1 FITC, MP Biomedicals; mouse IgG1 RPE, Serotec; mouse IgG2a, BD Pharmingen]. Non-conjugated antibodies required a 1 h incubation with conjugated secondary antibody (Alexa 488 or 555 goat anti-mouse IgG1 or IgG2a, Invitrogen) solution diluted in HBSS. Cells

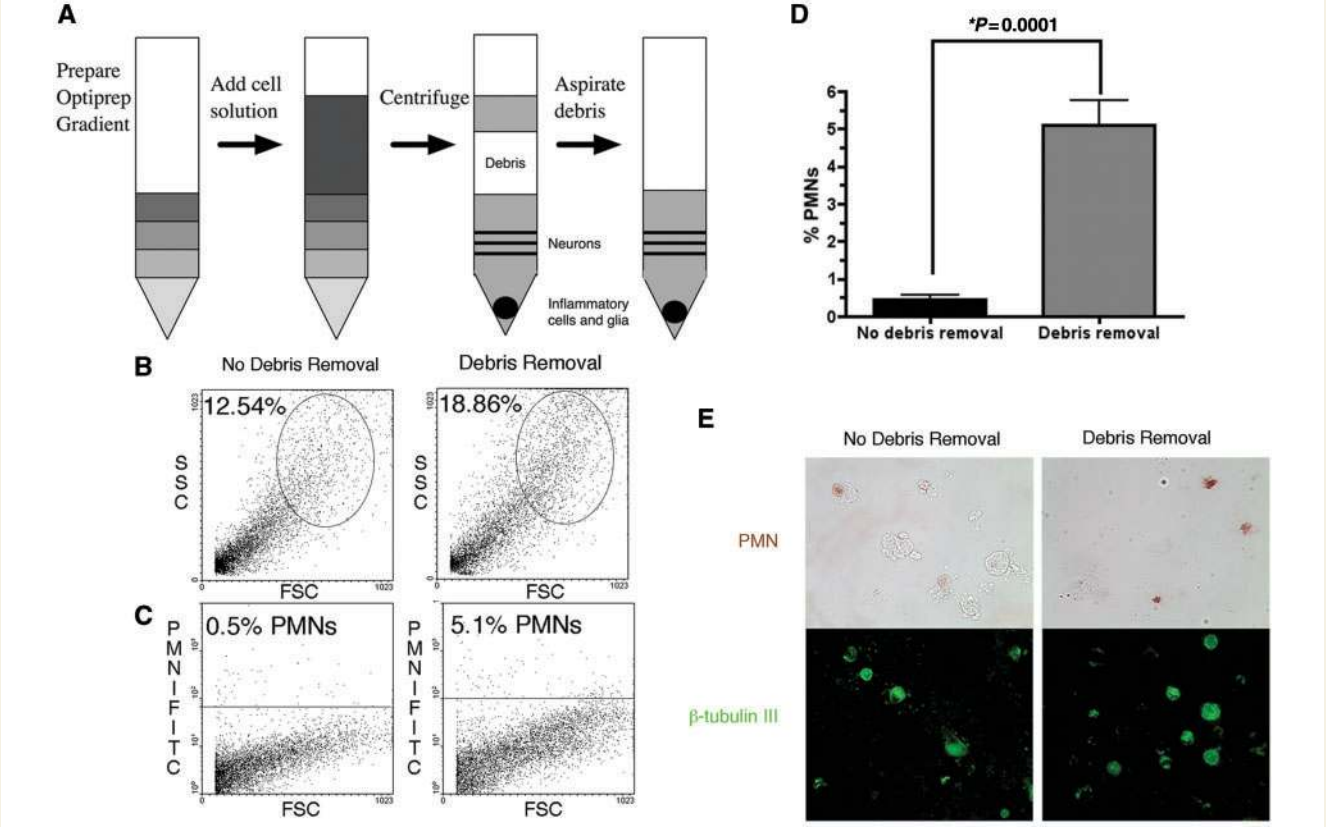

Figure 1 OptiPrep gradient densities separate most myelin and debris from injured spinal cord tissues/cells and improve immune cell assessment by flow cytometry. (**A**) Myelin and debris were separated from cells (neurons, glia and immune cells) after centrifugation of dissociated spinal cord tissue through an OptiPrep gradient, followed by aspiration of the myelin and debris layer. (**B**) Flow cytometric plots of spinal cord cells 1 dpi with or without debris removal by OptiPrep. Gradient densities show differences in FSC/SSC profile of cells. The region with FSC/SSC profiles distinctive to PMNs is indicated. (**C** and **D**) PMN number in the injured spinal cord, showing increased sensitivity in detecting PMNs after debris removal (Student's *t*-test, $^*P = 0.0001$). (**E**) However, cell samples in both preparations contain both PMNs and neurons (β-tubulin III+). All flow cytometric gates were set using labelled cells from uninjured animals; $n = 5$ per group, mean ± SEM.

were washed twice after each step and then resuspended in HBSS after the final step. Samples were analysed on a fluorescence-activated cell sorting Calibur (Becton-Dickinson) flow cytometer using Cell Quest software. Five thousand events were read for all samples. Data analysis was completed with Summit (DakoCytomation). Flow cytometric gates were set using control IgG isotype labelled cells or spinal cord cells from uninjured control animals to set baseline values for normalization across time points. Control IgG isotype-labelled cells from the injured spinal cords show very little difference in flow cytometric reading from the spinal cord cells of uninjured control animals (Nguyen *et al.*, 2008). All antibodies used for flow cytometry were diluted 1:100 prior to use. The mean values of positively labelled cells were expressed as per cent (SEM) of the entire sample.

In some conditions, cells were adhered to slides by centrifugation (Cytospin, Shandon, USA) and immunolabelled. Briefly, cells (10^6/ml) were fixed with acetone for 10 min, quenched in 0.3% hydrogen peroxide for 15 min, blocked (3% bovine serum albumin, 0.05% Tween-20), incubated in primary antibody (mouse anti-CD43, 1:500, Serotec; or rabbit anti-β-tubulin III, 1:1000, Covance), followed by secondary antibody (goat anti-rabbit IgG conjugated with Alexa Fluor 488, 1:1000, Invitrogen; or biotinylated donkey anti-mouse IgG, 1:500, Jackson Immunoresearch) followed by ABC and 3,3′-diaminobenzidine exposure as described below. Antibody control slides were processed identically, except that incubation with primary antibody was omitted.

Immunohistochemistry of spinal cord tissue sections

Immunohistochemistry of spinal cord sections was performed as previously described (Anderson *et al.*, 1996). Primary antibodies were targeted against CD43 (1:1000, Serotec), ED1 (1:1200, Serotec), CD3 (1:1200, Serotec) and myelin basic protein (1:250, Chemicon). Species-specific affinity-purified biotinylated secondary antibodies were applied (1:500, Jackson Immunoresearch), followed by incubation in ABC reagent (Vector Laboratories) and development with diaminobenzidine (Vector Laboratories) before methyl green nuclear counterstaining.

Stereological analysis of spinal cord tissue sections

Sprague Dawley rats were given 200 kd T9 contusion injuries and sacrificed 1 ($n = 6$), 7 ($n = 7$), 28 ($n = 6$) and 90 ($n = 7$) dpi. The injured spinal cord (T6–T12) was dissected and 30 μm serial spinal cord

sections (1:24 sampling) were used for immunohistochemistry as described above for PMNs, macrophages/microglia or T cells. An Olympus BX51 microscope with motorized stage and StereoInvestigator (Version 6.3 Software, Microbrightfield, Williston, VT, USA) were used to estimate stereologically the number of PMNs and T cells with the optical fractionator probe (Hooshmand *et al.*, 2009). Macrophage/microglia volume was determined with the Cavalieri estimator probe.

Inhibition of C5a-mediated inflammation

Animals received 200 kd T9 contusions, as described above, and were given either a C5a receptor (CD88) antagonist {C5aRa, PMX205, (HC)-[OP(D-Cha)WR], 10 mg/kg p.o., Promics Ltd} ($n = 5$) or water ($n = 5$) as a vehicle control twice daily for 1 or 7 days until sacrifice, for flow cytometric determination of PMN or macrophage/microglia infiltration to the injured spinal cord, respectively (Woodruff *et al.*, 2006).

A separate cohort of Sprague Dawley rats received 200 kd T9 contusions as described above. The effect of sub-acute to chronic inflammation was tested by administering the C5aRa ($n = 11$) or vehicle control ($n = 11$) twice daily, as above, for 14–28 dpi until sacrifice. Rats were assessed for locomotor recovery with the BBB scale. Following sacrifice, injured spinal cords were harvested for immunohistochemistry.

Statistical analysis

Comparisons of PMN or macrophage/microglial accumulation in the injured spinal cord in response to graded injuries were performed using one-way ANOVAs with *post hoc* Tukey's multiple comparison tests with Prism 4.0 (Graphpad, Inc.). Correlations were calculated using the actual force applied by the Infinite Horizons Impactor with the linear regression function of Prism 4.0 (Graphpad, Inc.). A *post hoc* power analysis (ANOVA, single factor design) was used to determine statistical power in the graded injury experiments ($n = 3$–5) to validate flow cytometric sample sizes (Supplementary Table S1). For the timecourse experiments, changes over time were assessed with one-way ANOVA following by *post hoc* Bonferroni's multiple comparison tests (Table 2). Statistical significance was defined as $P < 0.05$. All data are presented as mean ± standard error mean (SEM).

Table 2 Select Bonferroni's multiple comparison tests

Cell type	Comparison	Flow cytometry	Stereology
PMNs	2 h versus 1 dpi	$P < 0.001$	–
	1 dpi versus 2 dpi	$P < 0.01$	–
	1 dpi versus 28 dpi	$P < 0.01$	$P < 0.01$
	1 dpi versus 90 dpi	NS	$P < 0.01$
	2 dpi versus 180 dpi	$P < 0.001$	–
	28 dpi versus 90 dpi	NS	NS
Macrophages/ microglia	2 hr versus 7 dpi	$P < 0.001$	–
	7 dpi versus 14 dpi	$P < 0.001$	–
	7 dpi versus 28 dpi	NS	NS
	7 dpi versus 90 dpi	$P < 0.01$	$P < 0.001$
	14 dpi versus 60 dpi	$P < 0.001$	–
	28 dpi versus 90 dpi	$P < 0.05$	$P < 0.001$
	60 dpi versus 180 dpi	$P < 0.001$	–
T cells	2 h versus 9 dpi	NS	–
	7 dpi versus 28 dpi	–	$P < 0.05$
	7 dpi versus 90 dpi	NS	NS
	9 dpi versus 180 dpi	$P < 0.01$	–
	28 dpi versus 90 dpi	–	NS

One-way ANOVA was used to confirm a change in PMN, ED1$^+$ macrophages/microglia or CD3$^+$ T cells over time, while Bonferroni's multiple comparison *post hoc* tests were used to compare individual timepoints for both flow cytometric and stereological data, illustrating periods of maximum and minimum cell presence within the injured spinal cord. NS = not significant.

Results

Removing myelin debris from dissociated spinal cord tissue improves immune cell assessment by flow cytometry in the injured spinal cord

The use of flow cytometry to quantify immune cells in the central nervous system is complicated by lipid/myelin content and debris. In addition to interfering with antibody staining and specificity, myelin debris can have similar size and granulation properties to immune cells, decreasing measurement sensitivity and accuracy (Lipton *et al.*, 2005).

We have developed a novel cell preparation procedure that effectively removes myelin debris and improves the sensitivity of flow cytometry to detect changes in cellular inflammation in the injured spinal cord. The ability of OptiPrep gradient isolation to remove myelin debris and improve PMN detection by flow cytometry was tested 1 dpi. Myelin debris was removed from each sample by centrifugation through an Optiprep density gradient (Fig. 1A). Following centrifugation, sample contents separated into distinct layers, in which myelin and most debris were separated from neurons, glia, red blood cells and immune cells. Alternatively, identically dissected spinal cords were enzymatically dissociated without removal of myelin debris. There was a shift in the position of events for samples isolated with OptiPrep gradient purification compared to enzymatic dissociation alone (Fig. 1B). However, immunolabelled slide preparations of samples from both methods contained equivalent numbers of PMNs and neurons (Fig. 1E), suggesting the observed shift in the position of events following OptiPrep purification was due to a reduction in myelin debris. Together, these observations prompted the prediction that debris removal by OptiPrep purification may lead to improved flow cytometric detection of PMNs.

Accordingly, PMNs were elevated in the injured spinal cord 1 dpi for both cell preparations; however, the percentage of PMNs detected in samples with intact myelin debris was only a fraction (0.5%) of the percentage of PMNs detected (5.1%) in OptiPrep purified samples (Fig. 1C and D). These data suggest that myelin debris in tissue preparations can obscure flow cytometric readings and demonstrate that removal of myelin debris enhances the sensitivity of immune cell detection in the injured spinal cord.

Characterization of antibody specificity

Flow cytometric analysis does not permit morphological criteria to distinguish cell types further; therefore, characterization of

antibody specificity is important. Anti-CD3 is a pan-T cell marker for both flow cytometry and immunocytochemistry (Tanaka *et al.*, 1989; Kappelmayer *et al.*, 2000). Anti-PMN and anti-ED1 have been used extensively for immunocytochemistry, but have not been widely used for flow cytometry. Accordingly, the specificity of these antibodies was tested by comparing labelling of peritoneal PMNs, alveolar macrophages and cells in the injured spinal cord with antibodies used in this and previous studies. PMNs isolated from peritoneal cavities were positive for both anti-PMN FITC and an alternative PMN antibody, anti-granulocyte (RP1) RPE (Supplementary Fig. S1A and B), but were negative for anti-ED1 Alexa-555 (Supplementary Fig. S1C and D). Similarly, alveolar macrophages (ATCC) were positive for both anti-ED1 Alexa-488 and an alternative macrophage antibody, anti-CD11b Alexa-555 (Supplementary Fig. S1E and F), but were not detected by anti-PMN FITC (Supplementary Fig. S1G and H). These data demonstrate the specificity of the antibodies for PMNs and macrophages *in vitro*. Next we tested antibody specificity in the injured spinal cord, predicting minimal cross-reactivity between anti-PMN or anti-ED1 antibodies at 1 and 7 dpi. As predicted, 6.12% of cells in the injured spinal cord were positive for anti-PMN FITC but not for anti-ED1 Alexa-555 (0.20%) 1 dpi (Supplementary Fig. S2A and B). In contrast, 6.46% of cells in the injured spinal cord were positive for anti-ED1 Alexa-555 at 7 dpi, and only a small percentage of cells were positive for anti-PMN FITC (Supplementary Fig. S2C and D). Importantly, very few cells were double-positive for anti-PMN FITC and anti-ED1 Alexa 555 at 1 dpi (0.98%) and 7 dpi (0.92%) as compared to respective IgG controls (0.86% and 1.08%), suggesting that these antibodies are specific and accurate in detecting PMNs and macrophages/microglia after spinal cord injury.

Quantification of infiltrating immune cells by flow cytometry in response to graded injury severity

Injury severity is correlated with locomotor recovery (Scheff *et al.*, 2003; Nishi *et al.*, 2007) as well as PMN infiltration (Tjoa *et al.*, 2003). Therefore, we tested the ability of our flow cytometric technique to detect changes in cellular inflammation in the injured spinal cord in response to graded injury severities.

Changes in cellular inflammation in the injured spinal cord were assessed in response to graded injuries after 1 dpi (PMNs, Fig. 2A) or 7 dpi (macrophages/microglia, Fig. 2B), based on the peak infiltration of these cells (Popovich *et al.*, 1997; Carlson *et al.*, 1998). Although five animals were assigned to each injury

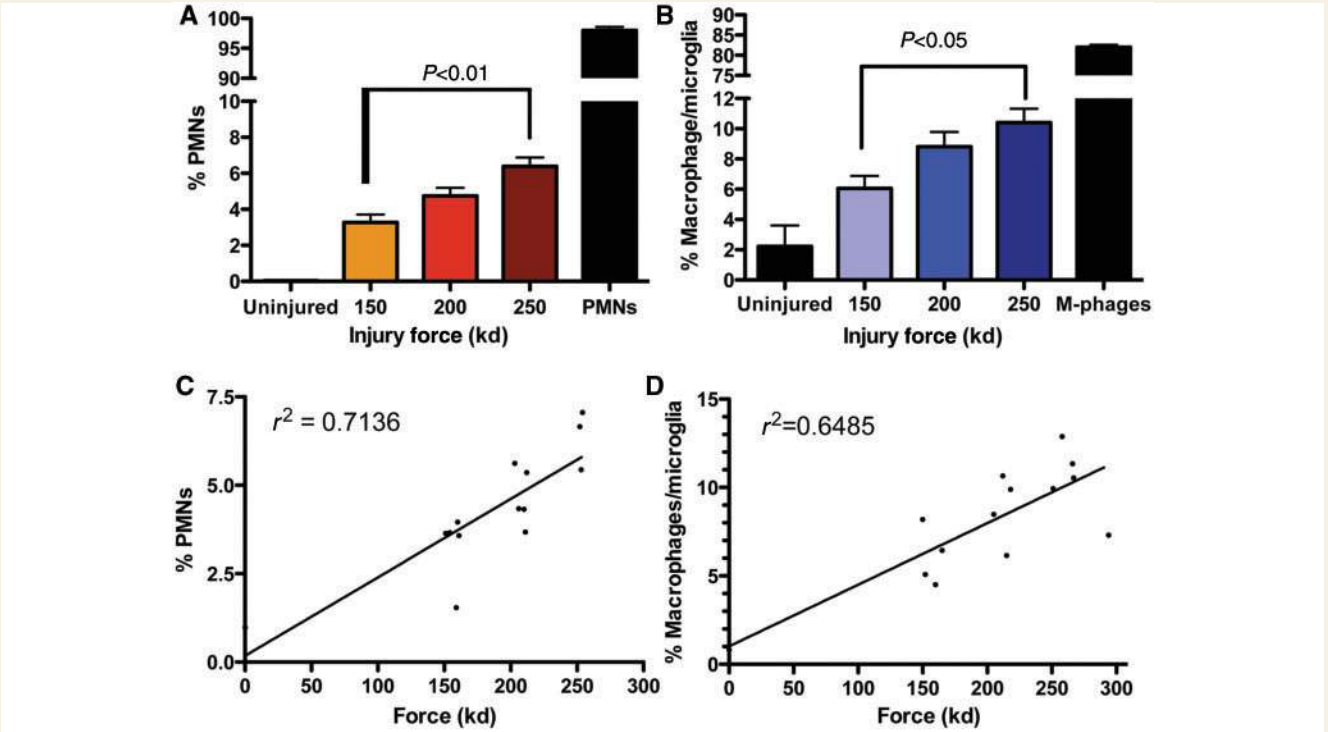

Figure 2 Debris removal procedure provides the ability to discern differences in cellular inflammation between rats that received graded injuries. The sensitivity of flow cytometry to detect differences in cellular inflammation was confirmed by giving rats mild (150 kd), moderate (200 kd) or severe (250 kd) spinal cord injury before assessing spinal cord tissue for (**A**) PMNs (1 dpi) or (**B**) ED1$^+$ macrophage/microglial (7 dpi) cell number. One-way ANOVA detected changes over time in PMN [ANOVA: $F(3,10)$: 22.90, $P<0.0001$] and ED1$^+$ macrophages/microglia [ANOVA: $F(3,11)$: 10.15, $P<0.0017$]. Tukey's *post hoc* multiple comparison tests detected differences in cell counts between mild and severe injuries for both PMNs ($P<0.01$) and ED1$^+$ macrophages/microglia ($P<0.05$). Injury force positively correlated with (**C**) PMN infiltration (all animals: $r^2=0.71$; injured animals only: $r^2=0.67$) and (**D**) ED1$^+$ macrophage/microglial infiltration (all animals: $r^2=0.65$; injured animals only: $r^2=0.40$). $n=3$–5 per group, mean ± SEM.

group, between three and five cell samples were recovered successfully for analysis. Statistical power was calculated using *post hoc* power analysis (ANOVA, single factor design) (Cohen, 1988). The effect size was large and statistical power adequate for both PMN ($f = 1.360$, power = 0.950) and macrophage/microglia ($f = 0.954$, power = 0.753) graded injury data sets (Supplementary Table S1), confirming the sample sizes were sufficient.

One-way ANOVA comparison of PMN infiltration into the spinal cord following graded contusions revealed a significant effect [ANOVA: $F(2,10) = 10.69$, $P = 0.0042$]. Following mild spinal cord injury, 3.3% of total cells in the injured spinal cord were PMNs 1 dpi, significantly less than after severe spinal cord injury (6.4% PMNs) (Tukey's multiple comparison tests: $P < 0.01$), but not moderate spinal cord injury (4.8% PMNs) (Fig. 2A). Linear regression analysis of naïve control, mild spinal cord injury, moderate spinal cord injury and severe spinal cord injury groups revealed a significant, positive correlation between injury force and PMN detection (Fig. 2C, $r^2 = 0.71$, $P = 0.0001$). Restriction of the regression analysis to injury groups, excluding naïve controls, also yielded a significant, positive correlation ($r^2 = 0.67$, $P = 0.0007$).

Similarly, one-way ANOVA comparison of macrophage/microglia accumulation after graded contusions revealed a significant effect [ANOVA: $F(2,10) = 5.752$, $P = 0.0218$] across mild, moderate and severe spinal cord injury animal groups. After mild spinal cord injury, 6.1% of cells in the injured spinal cord were ED1$^+$ macrophages/microglia at 7 dpi, significantly less than after severe spinal cord injury (10.4% ED1$^+$ cells) (Tukey's multiple comparison tests, $P < 0.05$), but not moderate spinal cord injury (8.8% ED1$^+$ cells). As with PMNs, macrophage/microglia accumulation in the injured spinal cord was significantly correlated with injury force (Fig. 2D, $r^2 = 0.65$, $P = 0.0005$). Restriction of the regression analysis to injury groups, excluding naïve controls, also yielded a significant correlation between injury force and macrophage/microglia detection ($r^2 = 0.40$, $P = 0.02$).

Assessment of acute and chronic cellular inflammation in the injured spinal cord by flow cytometry

As the first immune cells to infiltrate the injured spinal cord, PMNs are thought to affect injury acutely within hours or days following spinal cord injury. The early time course of PMN infiltration generated by flow cytometry confirmed immunohistochemical data from previous studies (Popovich *et al.*, 1997; Carlson *et al.*, 1998; Kigerl *et al.*, 2006), showing that PMN infiltration peaked 1 dpi (Fig. 3A and B). However, although PMN number decreased after 1 dpi, PMNs persisted in the cord for up to 6 months after spinal cord injury (Fig. 3B). These data suggest continuing PMN infiltration because PMNs are absent in the uninjured spinal cord, do not replicate in tissue and exhibit a tissue half-life of ~48 h (Cartwright *et al.*, 1964). Persistence of PMNs was confirmed by immunohistochemistry 1 dpi (Fig. 3C), 14 dpi (Fig. 3D) and 90 dpi (Fig. 3E). Quantitative stereologic analysis showed elevated PMN numbers at 1 dpi (536 177 ± 135 556), 28 dpi (59 906 ± 7958) and 90 dpi (64 782 ± 12 642), confirming the chronic persistence of PMNs in the injured spinal cord (Fig. 3B).

Unlike PMNs, macrophages/microglia were not detected in the injured spinal cord until 3 dpi, and peaked at 7 dpi (Fig. 4A, B and D). Interestingly, macrophages/microglia were detected at very low levels 14 dpi. The population increased after 14 dpi, peaked for the second time 60 dpi and remained elevated throughout 180 dpi (Fig. 4A and B). While not detected by flow cytometry 14 dpi, immunohistochemistry detected some macrophages/microglia localized at the injury epicentre (Fig. 4E). Macrophages/microglia were detected again by flow cytometry 21 dpi, as macrophage/microglia counts began to increase for the second time (Fig. 4B). Moreover, a striking 15.9% of all cells counted by the cytometer were macrophages/microglia at 90 dpi, as represented in parallel by immunohistochemistry in Fig. 4F. Accordingly, the biphasic response of macrophages/microglia was confirmed by quantitative stereology, which showed elevated ED1$^+$ macrophage/microglia at 7 dpi (6.270 $mm^3 \pm 0.783$), a significant decrease at 28 dpi (1.650 $mm^3 \pm 0.342$) and a significant rise in cell number at 90 dpi (20.034 $mm^3 \pm 1.649$) (Fig. 4B). These novel findings implicate macrophages/microglia as a significant part of a prolonged, multiphasic response after spinal cord injury.

Similar to macrophages/microglia, T cell infiltration was predicted to peak 7 dpi (Popovich *et al.*, 1997); however, flow cytometry did not detect any change in T cell number in the first 7 dpi, though an elevated number of T cells was detected at 9 dpi (1.6%) (Fig. 5A and B). In contrast, 40% of cells were positive for CD3 in spleen tissue (Fig. 5B). While T cell number was decreasing by 10 dpi, a persistent T cell response was observed throughout 180 dpi, at which time 4.4% of cells were labelled for CD3 (Fig. 5B). The persistence of T cells in the injured spinal cord was verified by quantitative stereology, showing elevated T cell numbers in the spinal cord at 7 dpi (4008 ± 913), 28 dpi (1252 ± 109) and 90 dpi (2243 ± 744) (Fig. 5C and E). Few T cells can be found in the parenchyma of the uninjured spinal cord, as there are no resident T cell populations, though a very low number of cells can be found in the subarachnoid space (Popovich *et al.*, 1997; Sroga *et al.*, 2003). Therefore, the detection of T cells in the injured spinal cord by both methodologies suggests an ongoing T cell response, perhaps indicative of increased immune surveillance.

Together, these data demonstrate a time-dependent multiphasic response of cellular inflammation after spinal cord injury (Fig. 6A); the initial phases of cellular inflammation were composed of the early peak of PMNs 1 dpi, followed by a peak of ED1$^+$ macrophages/microglia 7 dpi and T cells 9 dpi, while the later phases were composed of all three cellular populations rising after 14 dpi and persisting throughout 180 dpi, with a notable second peak of macrophages/microglia at 60 dpi.

Functional behavioural recovery and cellular inflammation after spinal cord injury

Acute (0–7 dpi) blockade of PMN (Taoka *et al.*, 1997; Tonai *et al.*, 2001) or monocyte/macrophage (Popovich *et al.*, 1999; Gris *et al.*, 2004) infiltration results in improvement of locomotor

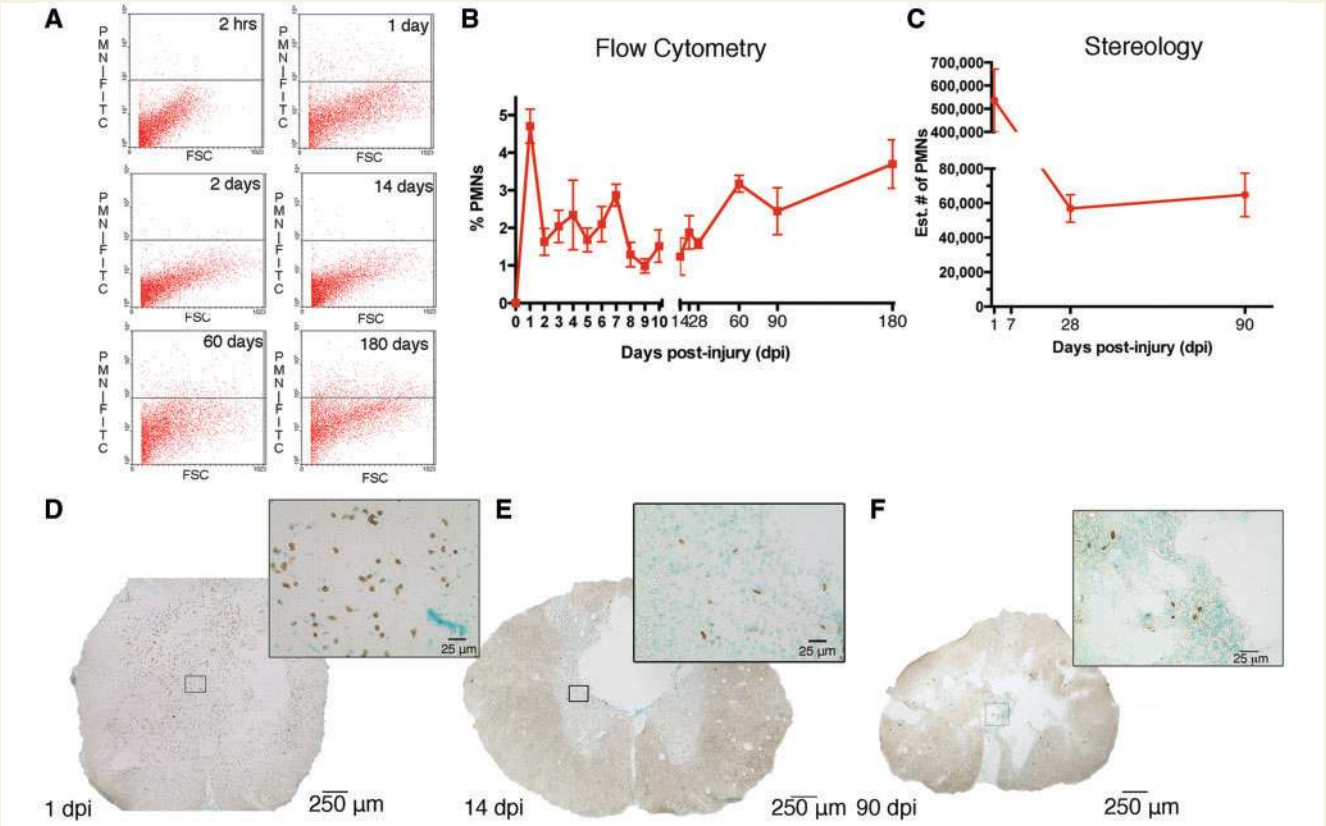

Figure 3 Assessment of PMNs in the spinal cord following a moderate (200 kd) contusion injury at T9. (**A** and **B**) PMNs quickly entered the spinal cord with an acute peak at 1 dpi, before falling to lower levels at 14 dpi, and peaked a second time at 60 dpi. One-way ANOVA confirmed differences in PMN infiltration over time [ANOVA: $F(16,62) = 6.789$, $P < 0.0001$]. (**C**) An acute peak and the chronic presence of PMNs were confirmed by quantitative stereology. Select Bonferroni's multiple comparison tests are reported in Table 1. Immunohistology confirmed PMN presence in the spinal cord at (**D**) 1 dpi, (**E**) 14 dpi and (**F**) 90 dpi. Spinal cord sections were labelled with anti-PMN antibody (brown) and methyl green nuclear counterstain. PMNs at both time points aggregate around areas of cavitation near the injury epicentre. $n = 3$–5 per group, mean ± SEM.

function. We have reported novel evidence for an extended second phase of inflammation, raising the direct question of whether the later phase of cellular inflammation is associated with a decline in locomotor function. Accordingly, we investigated the relationship between functional locomotor recovery and the kinetics of cellular inflammation after spinal cord injury using the BBB scale from 1 to 91 dpi using a separate cohort of rats that received a 200 kd contusion spinal cord injury at the T9 vertebral level.

Initially after trauma, hindlimb locomotor function was minimal (BBB score at 1 dpi = 0.5 ± 0.14) (Fig. 6B). As previously reported (Basso *et al.*, 1995), BBB performance improved steadily from 2 to 7 dpi, beginning to plateau at 14 dpi. Coincidently, PMN, macrophage/microglia and T cell numbers peaked at 1, 7 and 9 dpi (Fig. 6A), respectively, before motor recovery plateaued (Fig. 6B). In contrast, the second phase of inflammation, ranging from 14 to 180 dpi, did not coincide with changes in BBB performance, suggesting that the later phase may not have an independent effect on open-field locomotor function after spinal cord injury.

Blocking C5a-mediated inflammation 14–28 days after spinal cord injury reduces functional recovery and myelination

Previously, we have shown that the complement cascade is activated following spinal cord injury (Anderson *et al.*, 2004). Complement activation results in the cleavage of C5, releasing C5a, a potent chemoattractant for PMNs, macrophages/microglia, T cells and other immune cells. It is predicted that blocking receptor–ligand interactions with C5aRa will reduce immune cell recruitment to the site of injury (Woodruff *et al.*, 2006). To test this hypothesis, rats with spinal cord injury were given C5aRa or water twice daily for 1 or 7 dpi and sacrificed to assess PMN or macrophage/microglial infiltration to the spinal cord. While no difference in PMN presence was detected at 1 dpi (data not shown), C5aRa-treated animals (3.5% ± 0.2) had significantly fewer macrophages/microglia at 7 dpi than vehicle-treated animals (5.7% ± 0.6) (Student's *t*-test, $P = 0.05$) (Supplementary Fig. S3).

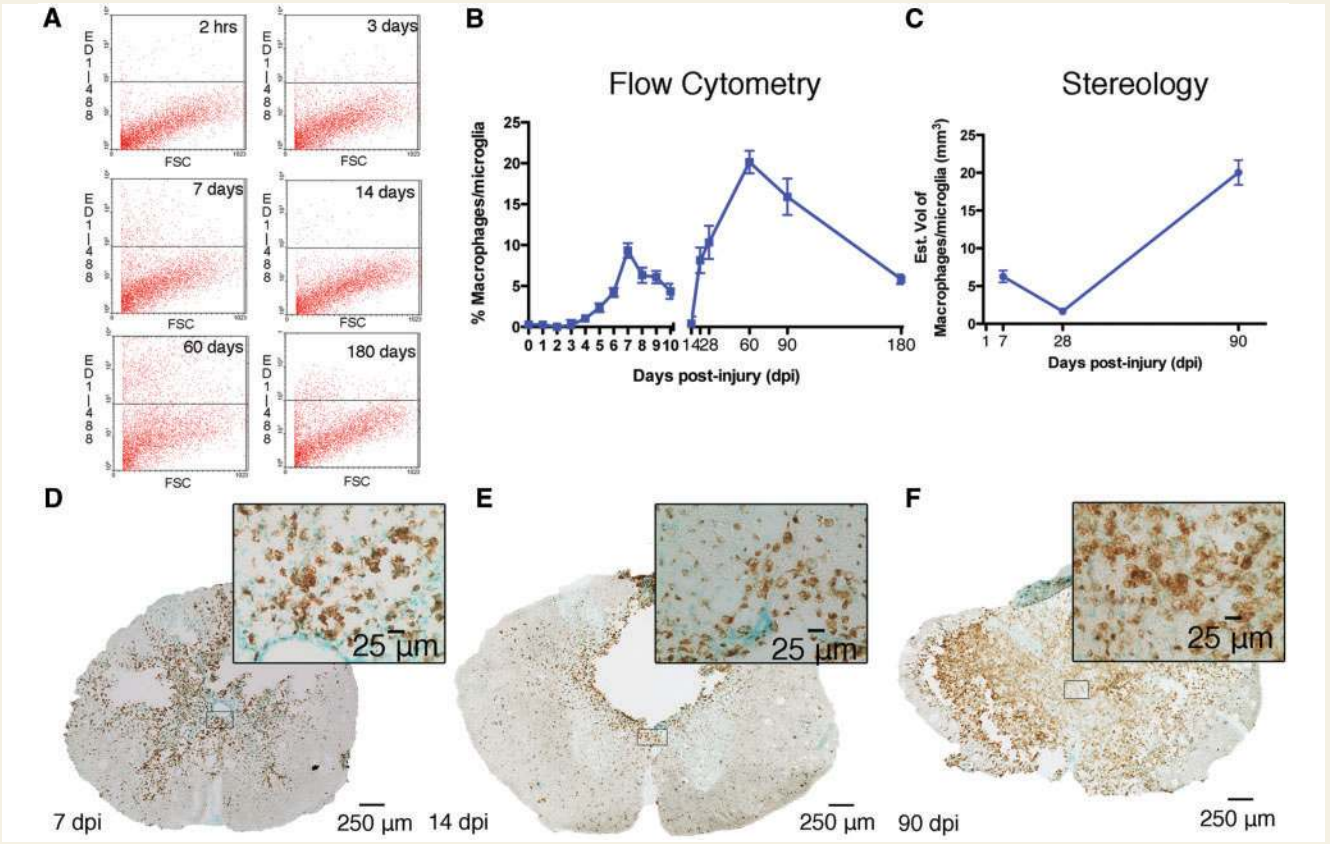

Figure 4 Assessment of ED1$^+$ macrophages/microglia in the spinal cord by flow cytometry following a moderate (200 kd) contusion injury at T9. (**A** and **B**) Number of ED1$^+$ macrophages/microglia increased in the injured spinal cord starting at 3 dpi, peaked acutely at 7 dpi, dropped to low levels at 14 dpi, before rising to a second peak 60 dpi and remained in the injured spinal cord up to 180 dpi. One-way ANOVA confirmed differences in the number of ED1$^+$ cells over time [ANOVA: $F(16,63) = 31.29$, $P < 0.0001$]. (**C**) Quantitative stereology confirmed both the acute and chronic peaks of macrophage/microglia. Select Bonferroni's multiple comparison tests are reported in Table 1. Immunohistology confirmed ED1$^+$ macrophage/microglial presence (**D**) 7 dpi, (**E**) 14 dpi and (**F**) 90 dpi in the injured spinal cord. Spinal cord sections were labelled with anti-ED1 antibody (brown) and methyl green nuclear counterstain. $n = 3$–5 per group, mean ± SEM.

Having confirmed the efficacy of C5aRa in reducing macrophage/microglia presence in the injured spinal cord, our next aim was to deplete immune cells after spinal cord injury to test their role in mediating damage and/or repair following spinal cord injury. As many studies have assessed the role of acute inflammation, generally yielding improved locomotor function and/or pathology (Popovich *et al.*, 1999; Tonai *et al.*, 2001; Gris *et al.*, 2004; Trivedi *et al.*, 2006), we focused our experiment on the sub-acute to chronic phase of inflammation, as its role has yet to be defined. This period is dominated by macrophages/microglia (Fig. 6A), whose presence we were able to reduce in the injured cord with the C5aRa. Injured rats were treated from 14 to 28 days with C5aRa or vehicle. The control animals exhibited greater locomotor recovery than C5aRa-treated animals (Fig. 7A). Repeated measures ANOVA revealed main effects of drug treatment [$F(1,20) = 5.66$, $P = 0.027$], time [$F(5,20) = 438.80$, $P < 0.0001$] and drug treatment by time interaction [$F(5,20) = 2.60$, $P = 0.030$]. Bonferroni *post hoc* *t*-tests showed control rats significantly outperformed C5aRa-treated rats at 21 (vehicle: 15.8 ± 0.9, C5aRa: 13.3 ± 1.0, $P < 0.01$) and 28 dpi (vehicle: 13.5 ± 0.8, C5aRa: 11.4 ± 0.3, $P < 0.05$). Chi-square analysis revealed that significantly more control rats achieved frequent coordination or better (BBB score > 12) at 28 dpi than C5aRa-treated rats ($P = 0.03$, $\chi^2 = 4.701$). Animals were sacrificed 28 dpi and spinal cord myelin basic protein quantified (Fig. 7C–H), revealing that C5aRa-treated rats had less myelination caudal to the injury epicentre than controls (Fig. 7B, two-tailed *t*-tests, 1.44 µm from epicentre: $P = 0.016$, 2.16 µm from epicentre: $P = 0.004$). Together, these data suggest that the late inflammatory response after 14 dpi improves functional recovery and myelination post-spinal cord injury.

Discussion

The data presented here provide new insight into the cellular immune response to spinal cord injury, including a detailed picture of the daily dynamics of cellular inflammation. We used a novel OptiPrep gradient cell preparation method combined with flow cytometry to assess the principal immune cell types present in

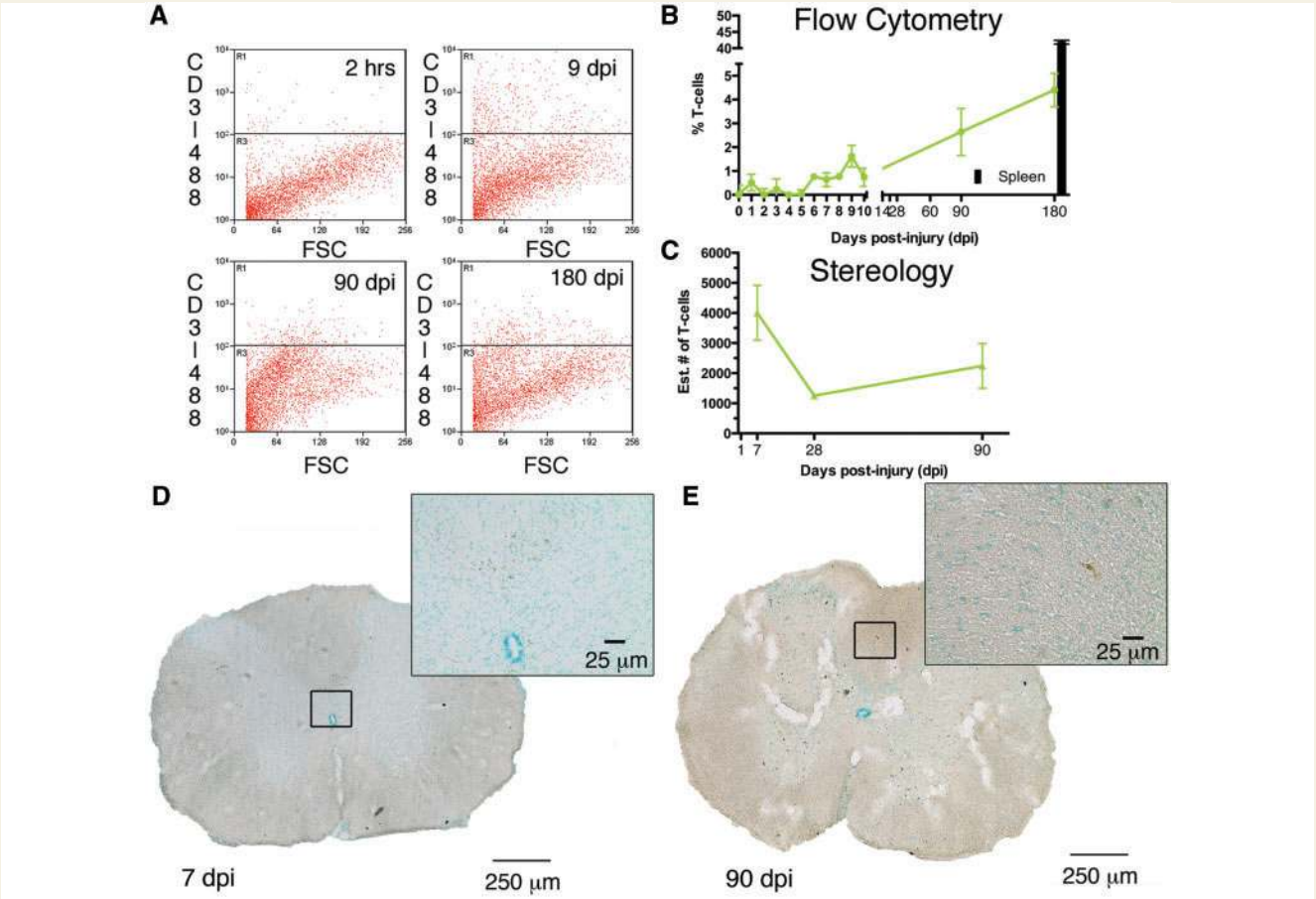

Figure 5 Assessment of T cells in the spinal cord by flow cytometry following a moderate (200 kd) contusion injury at T9. (**A**) $CD3^+$ T cells were minimal immediately following injury, reaching an acute peak 9 dpi. A prolonged T cell presence was detected up to 180 dpi. (**B**) The number of $CD3^+$ T cells in the injured spinal cord changed over time [ANOVA: $F(13,52) = 9.449$, $P < 0.0001$]. A non-significant acute peak of $CD3^+$ T cell presence was observed 9 dpi followed by a chronic T cell response persisting to at least 180 dpi. (**C**) An acute peak and the chronic presence of $CD3^+$ T cells were confirmed by quantitative stereology. Select Bonferroni's multiple comparison tests are reported in Table 1. Immunohistology with anti-CD3 antibody (brown) confirmed a limited number of T cells in the injured spinal cord at (**D**) 7 dpi and (**E**) 90 dpi; T cells were only detected in a few spinal cord sections. T cell occupation of the spinal cord was low compared to other cell types, with many T cells confined to the vasculature not far from the injury epicentre. $n = 3$–5 per group, mean ± SEM.

the injured spinal cord. Removal of myelin debris by the OptiPrep gradient method enhanced detection of PMNs compared to enzymatic dissociation alone. Additionally, the OptiPrep method allowed detection of a graded increase in both PMNs at 1 dpi and macrophages/microglia at 7 dpi, showing a quantitative linear relationship between the cellular inflammatory response and spinal cord injury severity. The persistent multiphasic inflammatory response of PMNs, macrophages/microglia and T cells observed here by flow cytometry was supported by quantitative stereologic data at a subset of time points. Each time point in the flow cytometric timecourse was normalized to uninjured animals, providing confirmation within this study that the uninjured spinal cord has minimal immune cells, as previously reported (Popovich *et al.*, 1997; Nguyen *et al.*, 2008). Some temporal differences that were statistically significant in the flow cytometric data set failed to yield significant differences in the stereologic data set (Table 2). Nonetheless, the stereologic data demonstrate the chronic presence of PMNs, macrophages/microglia and T cells. Together, the data revealed a multiphasic response of cellular inflammation after spinal cord injury.

Consistent with previous reports, the first cellular inflammation phase was comprised principally of PMNs (peaking 1 dpi), macrophages/microglia (peaking 7 dpi) and T cells (peaking 9 dpi). A novel and prolonged second phase of cellular inflammation was first observed after 14 dpi and persisted throughout 180 dpi, with a dramatic peak in the macrophage/microglial response at 60 dpi that doubled the magnitude of the earlier peak. Cellular inflammation was lowest for all three cell populations 14 dpi, suggesting a clear division between the phases of inflammation.

Later peaks of cellular inflammation after 14 dpi did not coincide with changes in locomotor function, suggesting ongoing inflammation is insufficient to affect locomotor recovery at this scale of analysis. In this regard, it is possible that more subtle changes in function occur during this timeframe but require more sensitive

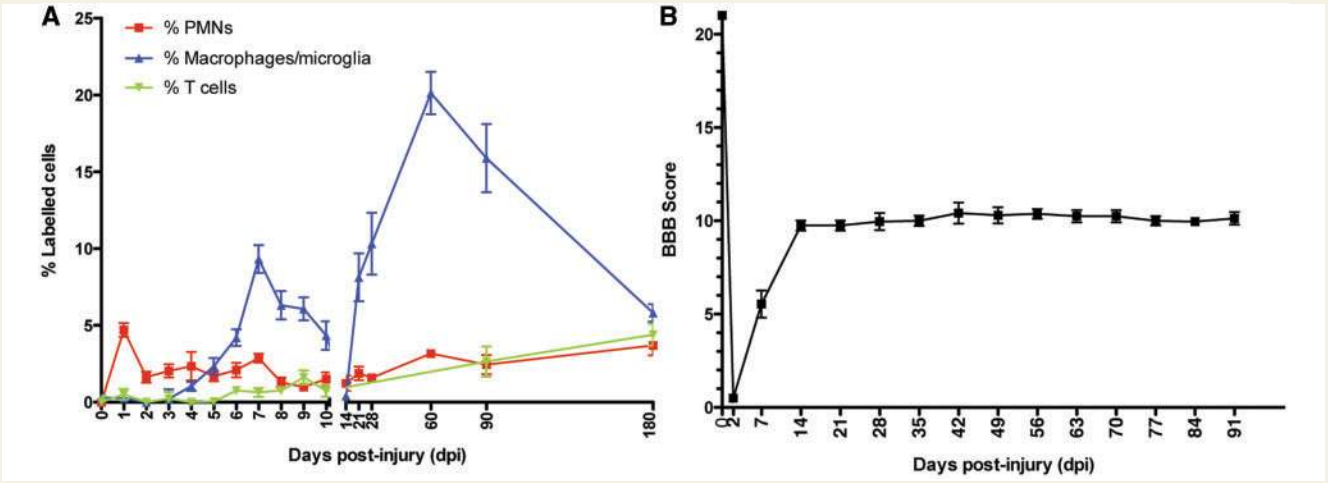

Figure 6 Chronic inflammation is not linked with changes in functional recovery. (**A**) A time course of cellular inflammation in the spinal cord following a moderate (200 kd) contusion injury at T9. As assessed by flow cytometry, numbers of PMNs, ED1$^+$ macrophages/microglia and CD3$^+$ T cells peaked acutely (1, 7 and 9 dpi, respectively) and persisted chronically in the injured spinal cord. (**B**) Locomotor performance on the BBB scale did not change during periods of chronic inflammation within the injured spinal cord. $n = 5–8$ per group, mean ± SEM.

methodology. Interestingly, the rat contusion spinal cord injury syrinx shrinks from 14 to 28 dpi (Scheff *et al.*, 2003), suggesting a relationship between the second phase of the cellular inflammatory response and tissue reorganization. Accordingly, the hypothesis that this second phase of macrophage/microglial response plays a role in preventing further loss of function must be considered. In this case, one would predict that reducing cellular inflammation during this period would result in a decline in locomotor function. Accordingly, blocking sub-acute to chronic inflammation via C5aRa resulted in reduced functional recovery and myelination in the injured spinal cord. The data presented here are unique in that this is the first study to test the role of delayed inflammation, providing novel insight into the multiphasic immune response following spinal cord injury.

Data from the current and previous studies suggest that cellular inflammation has complex, time-dependent functions. Our data show that PMNs, despite a reduction in number after peaking at 1 dpi, persisted for many months, suggesting that they may have a long-term role in promoting damage and/or repair. PMNs are thought to aggravate acute spinal cord injury within several dpi and have been previously demonstrated to promote neuronal cell death in culture through PMN-to-neuron contact or release of toxic factors (Dinkel *et al.*, 2004; Nguyen *et al.*, 2007). However, potentially PMNs have alternative roles in the long term as they secrete growth factors and cytokines that can promote scar formation/cytogenesis after spinal cord injury (Cicco *et al.*, 1990; Jablonska *et al.*, 2005; Nguyen *et al.*, 2007). Moreover, PMNs express complement proteins, such as C1q, which may play a complex role in the injured microenvironment (Hogasen *et al.*, 1995; Nguyen *et al.*, 2008). The extended presence of PMNs suggests a long-term role after spinal cord injury, which may be very different from the conventional role suggested for acute PMNs.

In parallel with these observations, the role of macrophages/microglia, detected in two distinct phases of inflammation in the present study, may also be complex and time-dependent. The first phase of macrophages/microglia, which peaked 7 dpi, exacerbated spinal cord injury (Popovich *et al.*, 1999). However, the role of the second phase, which peaked 60 dpi, is not known. The data presented here suggest that early chronic inflammation from 14 to 28 dpi modulates myelination in the injured spinal cord. The successful replacement of myelin damaged by spinal cord injury is a biphasic process of debris removal followed by remyelination that may hinge on interactions between the central nervous system and the immune system. Myelin degradation after spinal cord injury results in debris around the injury site, which may inhibit oligodendrocyte progenitor cell maturation (Robinson and Miller, 1999; Kotter *et al.*, 2006). Additionally, macrophages have also been shown to modify the microenvironment, causing an upregulation of factors that promote oligodendrocyte progenitor cell proliferation (fibroblast growth factor-2 and interleukin-1β) and maturation (transforming growth factor-β1 and insulin-like growth factor-1), although factors that may inhibit successful remyelination are also upregulated (transforming growth factor-α and Scya4) (Diemel *et al.*, 1998; Kotter *et al.*, 2005; Setzu *et al.*, 2006). The net effect of these signalling molecules probably determines how oligodendrocyte progenitor cells respond to demyelinated axons, and while there is clear evidence that inflammation aids in remyelination (Foote and Blakemore, 2005; Setzu *et al.*, 2006), the exact mechanism remains unknown.

Interestingly, infiltrated macrophages have been shown to switch from pro-inflammatory to anti-inflammatory over time (Arnold *et al.*, 2007; Villalta *et al.*, 2009). While the role of macrophages in spinal cord injury has been debated (Rapalino *et al.*, 1998; Popovich *et al.*, 2003), the data presented here suggest a functional switch over time, though we did not specifically examine macrophage phenotype. Nonetheless, a thorough understanding of the dual role of these cells in both pro- and anti-inflammatory functions is critical for a complete interpretation of the data.

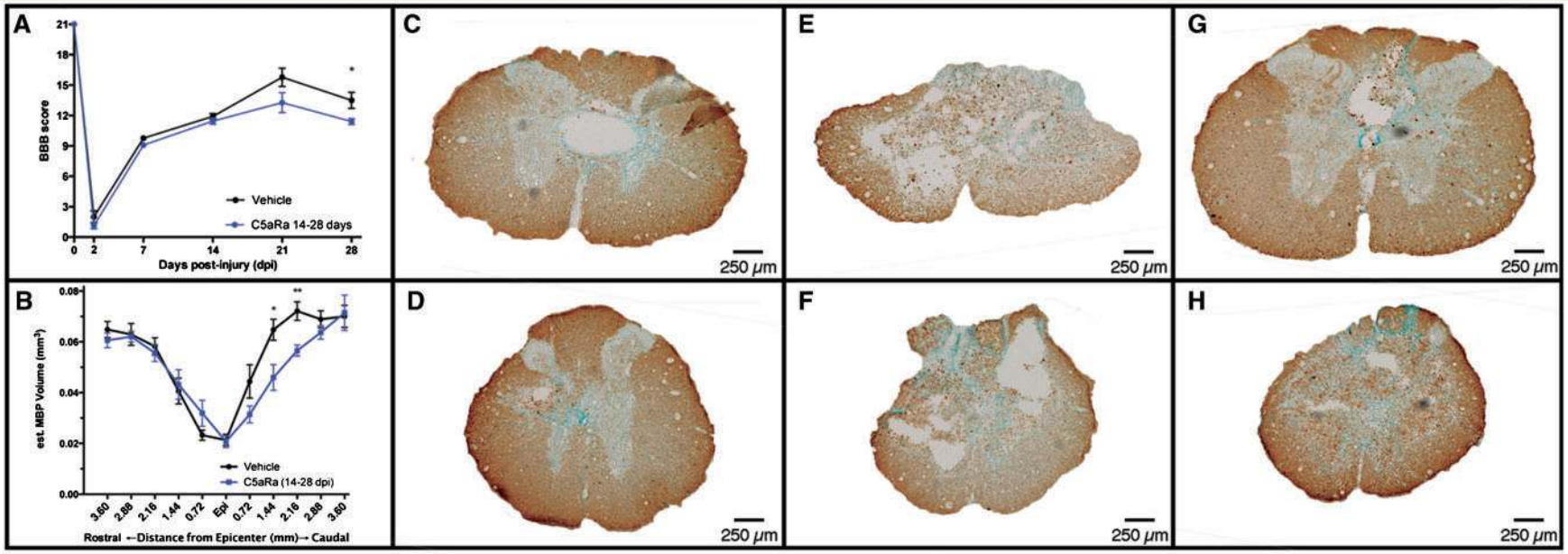

Figure 7 Rats received 200 kd T9 contusion spinal cord injury and were tested for openfield locomotor recovery. (**A**) Animals treated with C5aRa from 14 to 28 dpi showed significantly less functional recovery 28 dpi than controls. Repeated-measures ANOVA found significant main effects of drug treatment ($P = 0.027$), time ($P < 0.0001$) and drug treatment × time interaction ($P = 0.030$). Bonferroni *post hoc* tests found the C5aRa-treated group had significantly higher BBB scores at 21 and 28 dpi ($P < 0.01$ and $P < 0.05$, respectively). (**B**) C5aRa-treated rats had significantly less intact myelin in regions caudal to the injury epicentre than vehicle-treated controls (two-tailed *t*-test, $^{*}P = 0.0162$, $^{**}P = 0.0042$). Representative images of sections 1.44 mm rostral to epicentre, the injury epicentre and 1.44 mm caudal to epicentre are shown for vehicle (**C**, **E** and **G**) and C5aRa-treated rats (**D**, **F** and **H**).

Unlike PMNs and macrophages/microglia, T cells were scarcely represented in the injured spinal cord. However, the number of cells does not necessarily reflect their importance in affecting the pathophysiology of spinal cord injury. While present in lower numbers, T cells could play an important role in injury and repair by modulating the function and recruitment of both innate and adaptive immune cells after spinal cord injury (Hendrix and Nitsch, 2007; Ankeny *et al.*, 2009).

The issue of which factor is driving the multiphasic kinetics of cellular inflammation after spinal cord injury has not been resolved, although at least three possibilities have been identified. Normally, the spinal cord is a site of relative immunoprivilege; traumatic injury causes an opening of the blood–brain barrier by 3 dpi, which is mostly intact at 14 dpi, and open again at 28 dpi (Popovich *et al.*, 1996*a*). These periods coincide with our observations of increased immune cell presence acutely after injury, low levels at 14 dpi and increasing again beginning 21 dpi. An increased permeability of the blood–brain barrier would allow immune cells to easily enter the spinal cord from the circulation, although specific cues are also likely to be involved. One such cue could be the complement anaphylatoxin, C5a, a strong recruiter of PMNs, macrophages/microglia and T cells (Klos *et al.*, 2009). Local complement activation in neurons and glia, resulting in C5a production, has been demonstrated as early as 1 dpi and persists to at least 42 dpi (Anderson *et al.*, 2004). Furthermore, PMNs have been shown to express a subset of complement proteins following spinal cord injury (Nguyen *et al.*, 2008), while macrophages expressed the entire complement cascade *in vitro* (Johnson and Hetland, 1988). It is unlikely that complement proteins are the only factor to recruit immune cells to the injured spinal cord. It has been suggested that a homeostatic balance of pro- and anti-inflammatory cytokines modulates the recruitment of inflammatory cells after central nervous system injury (Lenzlinger *et al.*, 2001; Stoll *et al.*, 2002). Pro-inflammatory cytokines are expressed mostly within days after mouse spinal cord injury, though tumour necrosis factor-α has a second phase of expression beginning 14 dpi and lasting to at least 28 dpi (Pineau and Lacroix, 2007). The expression pattern of anti-inflammatory cytokines after spinal cord injury has not yet been thoroughly investigated, nor has any direct link between cytokine production and leucocyte recruitment.

The temporal and quantitative analysis of cellular inflammation presented here has provided new insight into the dynamics of the spinal cord injury microenvironment, and identified for the first time an extended multiphasic response of cellular inflammation. The development of a novel OptiPrep gradient cell preparation method combined with flow cytometry to assess the principal immune cell types present in the injured spinal cord allowed detection of a graded increase in both PMNs and macrophages/microglia, showing a quantitative linear relationship between the cellular inflammatory response and spinal cord injury severity. Flow cytometric quantification as conducted here also allowed accurate comparison of data across multiple time points, which was verified by quantitative stereology, identifying a time-dependent multiphasic response of cellular inflammation after spinal cord injury. Furthermore, reduction of delayed C5a-mediated macrophage/microglial infiltration after spinal cord injury resulted in decreased functional recovery, suggesting a reparative role for chronic inflammation in this model. Understanding the role of this multiphasic response in the pathophysiology of spinal cord injury is likely to be critical for the design and implementation of rational therapeutic treatment strategies, including both cell-based and pharmacological interventions.

Acknowledgements

The authors thank Rebecca Nishi, MS, and the technicians of the Christopher and Dana Reeve Foundation Animal Core Facility at UC Irvine for their help in animal surgery. They also thank Amir Shbeeb, Jon Steller, Viktoria Nikolova and Thaddeus J. O'Barr for general technical assistance.

Funding

National Institute of Neurological Disorders and Stroke (RO1 NS43428-01 to A.J.A.); Paralysis Project of America (PPA-32574 to H.X.N.); California Institute for Regenerative Medicine (CIRM) Stem Cell Training Award (T1-00008 to H.X.N. and D.L.S.); training program in molecular and cellular neuroscience at UC Irvine (T32 NS007444 to K.D.B.); University of California Alliance for Graduate Education and the Professoriate fellowship (NSF HRD0450366 to D.L.S.).

Supplementary material

Supplementary material is available at *Brain* online.

References

Anderson AJ, Robert S, Huang W, Young W, Cotman CW. Activation of complement pathways after contusion-induced spinal cord injury. J Neurotrauma 2004; 21: 1831–46.

Anderson AJ, Su JH, Cotman CW. DNA damage and apoptosis in Alzheimer's disease: colocalization with c-Jun immunoreactivity, relationship to brain area, and effect of postmortem delay. J Neurosci 1996; 16: 1710–9.

Ankeny DP, Guan Z, Popovich PG. B cells produce pathogenic antibodies and impair recovery after spinal cord injury in mice. J Clin Invest 2009; 119: 2990–9.

Arnold L, Henry A, Poron F, Baba-Amer Y, van Rooijen N, Plonquet A, et al. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. J Exp Med 2007; 204: 1057–69.

Bagamery K, Kvell K, Landau R, Graham J. Flow cytometric analysis of CD41-labeled platelets isolated by the rapid, one-step OptiPrep method from human blood. Cytometry A 2005; 65: 84–7.

Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. J Neurotrauma 1995; 12: 1–21.

Brana C, Biggs TE, Barton CH, Sundstrom LE, Mann DA. A soluble factor produced by macrophages mediates the neurotoxic effects of HIV-1 Tat in vitro. AIDS 1999; 13: 1443–52.

Cantini M, Carraro U. Macrophage-released factor stimulates selectively myogenic cells in primary muscle culture. J Neuropathol Exp Neurol 1995; 54: 121–8.

Carlson SL, Parrish ME, Springer JE, Doty K, Dossett L. Acute inflammatory response in spinal cord following impact injury. Exp Neurol 1998; 151: 77–88.

Cartwright GE, Athens JW, Wintrobe MM. The kinetics of granulopoiesis in normal man. Blood 1964; 24: 780–803.

Cicco NA, Lindemann A, Content J, Vandenbussche P, Lubbert M, Gauss J, et al. Inducible production of interleukin-6 by human polymorphonuclear neutrophils: role of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha. Blood 1990; 75: 2049–52.

Cohen J. Statistical power analysis for the behavioral sciences. Hillsdale, NJ: Erlbaum; 1988.

Conta AC, Stelzner DJ. Immunomodulatory effect of the purine nucleoside inosine following spinal cord contusion injury in rat. Spinal Cord 2008; 46: 39–44.

Crutcher KA, Gendelman HE, Kipnis J, Perez-Polo JR, Perry VH, Popovich PG, et al. Debate: ''is increasing neuroinflammation beneficial for neural repair?''. J Neuroimmune Pharmacol 2006; 1: 195–211.

de Castro R Jr., Hughes MG, Xu GY, Clifton C, Calingasan NY, Gelman BB, et al. Evidence that infiltrating neutrophils do not release reactive oxygen species in the site of spinal cord injury. Exp Neurol 2004; 190: 414–24.

Diemel LT, Copelman CA, Cuzner ML. Macrophages in CNS remyelination: friend or foe? Neurochem Res 1998; 23: 341–7.

Dinkel K, Dhabhar FS, Sapolsky RM. Neurotoxic effects of polymorphonuclear granulocytes on hippocampal primary cultures. Proc Natl Acad Sci USA 2004; 101: 331–6.

Flavin MP, Coughlin K, Ho LT. Soluble macrophage factors trigger apoptosis in cultured hippocampal neurons. Neuroscience 1997; 80: 437–48.

Fleming JC, Norenberg MD, Ramsay DA, Dekaban GA, Marcillo AE, Saenz AD, et al. The cellular inflammatory response in human spinal cords after injury. Brain 2006; 129: 3249–69.

Foote AK, Blakemore WF. Inflammation stimulates remyelination in areas of chronic demyelination. Brain 2005; 128: 528–39.

Giulian D, Robertson C. Inhibition of mononuclear phagocytes reduces ischemic injury in the spinal cord. Ann Neurol 1990; 27: 33–42.

Gonzalez R, Glaser J, Liu MT, Lane TE, Keirstead HS. Reducing inflammation decreases secondary degeneration and functional deficit after spinal cord injury. Exp Neurol 2003; 184: 456–63.

Gris D, Marsh DR, Oatway MA, Chen Y, Hamilton EF, Dekaban GA, et al. Transient blockade of the CD11d/CD18 integrin reduces secondary damage after spinal cord injury, improving sensory, autonomic, and motor function. J Neurosci 2004; 24: 4043–51.

Guizar-Sahagun G, Grijalva I, Madrazo I, Franco-Bourland R, Salgado H, Ibarra A, et al. Development of post-traumatic cysts in the spinal cord of rats-subjected to severe spinal cord contusion. Surg Neurol 1994; 41: 241–9.

Guth L, Zhang Z, DiProspero NA, Joubin K, Fitch MT. Spinal cord injury in the rat: treatment with bacterial lipopolysaccharide and indomethacin enhances cellular repair and locomotor function. Exp Neurol 1994; 126: 76–87.

Hains BC, Yucra JA, Hulsebosch CE. Reduction of pathological and behavioral deficits following spinal cord contusion injury with the selective cyclooxygenase-2 inhibitor NS-398. J Neurotrauma 2001; 18: 409–23.

Hauben E, Agranov E, Gothilf A, Nevo U, Cohen A, Smirnov I, et al. Posttraumatic therapeutic vaccination with modified myelin self-antigen prevents complete paralysis while avoiding autoimmune disease. J Clin Invest 2001; 108: 591–9.

Hendrix S, Nitsch R. The role of T helper cells in neuroprotection and regeneration. J Neuroimmunol 2007; 184: 100–12.

Hogasen AK, Wurzner R, Abrahamsen TG, Dierich MP. Human polymorphonuclear leukocytes store large amounts of terminal complement components C7 and C6, which may be released on stimulation. J Immunol 1995; 154: 4734–40.

Hooshmand MJ, Sontag CJ, Uchida N, Tamaki S, Anderson AJ, Cummings BJ. Analysis of host-mediated repair mechanisms after human CNS-stem cell transplantation for spinal cord injury: correlation of engraftment with recovery. PLoS One 2009; 4: e5871.

Howe CL, Ure D, Adelson JD, Lafrance-Corey R, Rodriguez M. CD8+ T cells directed against a viral peptide contribute to loss of motor function by disrupting axonal transport in a viral model of fulminant demyelination. J Neuroimmunol 2007; 188: 13–21.

Jablonska E, Puzewska W, Grabowska Z, Jablonski J, Talarek L. VEGF, IL-18 and NO production by neutrophils and their serum levels in patients with oral cavity cancer. Cytokine 2005; 30: 93–9.

Jameson J, Ugarte K, Chen N, Yachi P, Fuchs E, Boismenu R, et al. A role for skin gammadelta T cells in wound repair. Science 2002; 296: 747–9.

Johnson E, Hetland G. Mononuclear phagocytes have the potential to synthesize the complete functional complement system. Scand J Immunol 1988; 27: 489–93.

Jones TB, Hart RP, Popovich PG. Molecular control of physiological and pathological T-cell recruitment after mouse spinal cord injury. J Neurosci 2005; 25: 6576–83.

Kappelmayer J, Gratama JW, Karaszi E, Menendez P, Ciudad J, Rivas R, et al. Flow cytometric detection of intracellular myeloperoxidase, CD3 and CD79a. Interaction between monoclonal antibody clones, fluorochromes and sample preparation protocols. J Immunol Methods 2000; 242: 53–65.

Kigerl KA, McGaughy VM, Popovich PG. Comparative analysis of lesion development and intraspinal inflammation in four strains of mice following spinal contusion injury. J Comp Neurol 2006; 494: 578–94.

Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, Kohl J. The role of the anaphylatoxins in health and disease. Mol Immunol 2009; 46: 2753–66.

Kotter MR, Li WW, Zhao C, Franklin RJ. Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. J Neurosci 2006; 26: 328–32.

Kotter MR, Zhao C, van Rooijen N, Franklin RJ. Macrophage-depletion induced impairment of experimental CNS remyelination is associated with a reduced oligodendrocyte progenitor cell response and altered growth factor expression. Neurobiol Dis 2005; 18: 166–75.

Krickhahn M, Meyer T, Buhler C, Thiede A, Ulrichs K. Highly efficient isolation of porcine islets of Langerhans for xenotransplantation: numbers, purity, yield and in vitro function. Ann Transplant 2001; 6: 48–54.

La Fleur M, Underwood JL, Rappolee DA, Werb Z. Basement membrane and repair of injury to peripheral nerve: defining a potential role for macrophages, matrix metalloproteinases, and tissue inhibitor of metalloproteinases-1. J Exp Med 1996; 184: 2311–26.

Lenzlinger PM, Morganti-Kossmann MC, Laurer HL, McIntosh TK. The duality of the inflammatory response to traumatic brain injury. Mol Neurobiol 2001; 24: 169–81.

Li M, Carpio DF, Zheng Y, Bruzzo P, Singh V, Ouaaz F, et al. An essential role of the NF-kappa B/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells. J Immunol 2001; 166: 7128–35.

Lipton HL, Kallio P, Jelachich ML. Simplified quantitative analysis of spinal cord cells from Theiler's virus-infected mice without the requirement for myelin debris removal. J Immunol Methods 2005; 299: 107–15.

Moalem G, Leibowitz-Amit R, Yoles E, Mor F, Cohen IR, Schwartz M. Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. Nat Med 1999; 5: 49–55.

Nguyen HX, Galvan MD, Anderson AJ. Characterization of early and terminal complement proteins associated with polymorphonuclear leukocytes in vitro and in vivo after spinal cord injury. J Neuroinflammation 2008; 5: 26.

Nguyen HX, O'Barr TJ, Anderson AJ. Polymorphonuclear leukocytes promote neurotoxicity through release of matrix metalloproteinases, reactive oxygen species, and TNF-alpha. J Neurochem 2007; 102: 900–12.

Nishi RA, Liu H, Chu Y, Hamamura M, Su MY, Nalcioglu O, et al. Behavioral, histological, and ex vivo magnetic resonance imaging assessment of graded contusion spinal cord injury in mice. J Neurotrauma 2007; 24: 674–89.

Noble LJ, Donovan F, Igarashi T, Goussev S, Werb Z. Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. J Neurosci 2002; 22: 7526–35.

Pineau I, Lacroix S. Proinflammatory cytokine synthesis in the injured mouse spinal cord: Multiphasic expression pattern and identification of the cell types involved. J Comp Neurol 2007; 500: 267–85.

Popovich PG, Guan Z, Wei P, Huitinga I, van Rooijen N, Stokes BT. Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. Exp Neurol 1999; 158: 351–65.

Popovich PG, Horner PJ, Mullin BB, Stokes BT. A quantitative spatial analysis of the blood–spinal cord barrier: I. Permeability changes after experimental spinal contusion injury. Exp Neurol 1996a; 142: 258–75.

Popovich PG, Stokes BT, Whitacre CC. Concept of autoimmunity following spinal cord injury: possible roles for T lymphocytes in the traumatized central nervous system. J Neurosci Res 1996b; 45: 349–63.

Popovich PG, van Rooijen N, Hickey WF, Preidis G, McGaughy V. Hematogenous macrophages express CD8 and distribute to regions of lesion cavitation after spinal cord injury. Exp Neurol 2003; 182: 275–87.

Popovich PG, Wei P, Stokes BT. Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. J Comp Neurol 1997; 377: 443–64.

Rapalino O, Lazarov-Spiegler O, Agranov E, Velan GJ, Yoles E, Fraidakis M, et al. Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. Nat Med 1998; 4: 814–21.

Robinson S, Miller RH. Contact with central nervous system myelin inhibits oligodendrocyte progenitor maturation. Dev Biol 1999; 216: 359–68.

Royo NC, Wahl F, Stutzmann JM. Kinetics of polymorphonuclear neutrophil infiltration after a traumatic brain injury in rat. Neuroreport 1999; 10: 1363–7.

Saville LR, Pospisil CH, Mawhinney LA, Bao F, Simedrea FC, Peters AA, et al. A monoclonal antibody to CD11d reduces the inflammatory infiltrate into the injured spinal cord: a potential neuroprotective treatment. J Neuroimmunol 2004; 156: 42–57.

Scheff SW, Rabchevsky AG, Fugaccia I, Main JA, Lumpp JE Jr. Experimental modeling of spinal cord injury: characterization of a force-defined injury device. J Neurotrauma 2003; 20: 179–93.

Setzu A, Lathia JD, Zhao C, Wells K, Rao MS, Ffrench-Constant C, et al. Inflammation stimulates myelination by transplanted oligodendrocyte precursor cells. Glia 2006; 54: 297–303.

Sroga JM, Jones TB, Kigerl KA, McGaughy VM, Popovich PG. Rats and mice exhibit distinct inflammatory reactions after spinal cord injury. J Comp Neurol 2003; 462: 223–40.

Stirling DP, Khodarahmi K, Liu J, McPhail LT, McBride CB, Steeves JD, et al. Minocycline treatment reduces delayed oligodendrocyte death, attenuates axonal dieback, and improves functional outcome after spinal cord injury. J Neurosci 2004; 24: 2182–90.

Stirling DP, Yong VW. Dynamics of the inflammatory response after murine spinal cord injury revealed by flow cytometry. J Neurosci Res 2008; 86: 1944–58.

Stoll G, Jander S, Schroeter M. Detrimental and beneficial effects of injury-induced inflammation and cytokine expression in the nervous system. Adv Exp Med Biol 2002; 513: 87–113.

Tanaka T, Masuko T, Yagita H, Tamura T, Hashimoto Y. Characterization of a CD3-like rat T cell surface antigen recognized by a monoclonal antibody. J Immunol 1989; 142: 2791–5.

Taoka Y, Okajima K, Uchiba M, Murakami K, Kushimoto S, Johno M, et al. Role of neutrophils in spinal cord injury in the rat. Neuroscience 1997; 79: 1177–82.

Tjoa T, Strausbaugh HJ, Maida N, Dazin PF, Rosen SD, Noble-Haeusslein LJ. The use of flow cytometry to assess neutrophil infiltration in the injured murine spinal cord. J Neurosci Methods 2003; 129: 49–59.

Tonai T, Shiba K, Taketani Y, Ohmoto Y, Murata K, Muraguchi M, et al. A neutrophil elastase inhibitor (ONO-5046) reduces neurologic damage after spinal cord injury in rats. J Neurochem 2001; 78: 1064–72.

Trivedi A, Olivas AD, Noble-Haeusslein LJ. Inflammation and spinal cord injury: infiltrating leukocytes as determinants of injury and repair processes. Clin Neurosci Res 2006; 6: 283–92.

Villalta SA, Nguyen HX, Deng B, Gotoh T, Tidball JG. Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. Hum Mol Genet 2009; 18: 482–96.

Weaver KD, Branch CA, Hernandez L, Miller CH, Quattrocchi KB. Effect of leukocyte-endothelial adhesion antagonism on neutrophil migration and neurologic outcome after cortical trauma. J Trauma 2000; 48: 1081–90.

Woodruff TM, Crane JW, Proctor LM, Buller KM, Shek AB, de Vos K, et al. Therapeutic activity of C5a receptor antagonists in a rat model of neurodegeneration. Faseb J 2006; 20: 1407–17.

Wu D, Miyamoto O, Shibuya S, Okada M, Igawa H, Janjua NA, et al. Different expression of macrophages and microglia in rat spinal cord contusion injury model at morphological and regional levels. Acta Med Okayama 2005; 59: 121–7.

Yin Y, Henzl MT, Lorber B, Nakazawa T, Thomas TT, Jiang F, et al. Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells. Nat Neurosci 2006; 9: 843–52.