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Mobilisation of the splenic monocyte reservoir and peripheral CX_3CR1 deficiency adversely affects recovery from spinal cord injury

Linda V. Blomster [a,b], Faith H. Brennan [a], Hong W. Lao [a], David W. Harle [a], Alan R. Harvey [b], Marc J. Ruitenberg [a,c,*]

[a] The University of Queensland, School of Biomedical Sciences, QLD 4072, Australia
[b] The University of Western Australia, School of Anatomy, Physiology and Human Biology, WA 6009, Australia
[c] The University of Queensland, Queensland Brain Institute, QLD 4072, Australia

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ABSTRACT

Macrophages in the injured spinal cord originate from resident microglia and blood monocytes. Whether this diversity in origins contributes to their seemingly dual role in immunopathology and repair processes has remained poorly understood. Here we took advantage of Cx_3cr1^{gfp} mice to visualise monocyte-derived macrophages in the injured spinal cord via adoptive cell transfer and bone marrow (BM) chimera approaches. We show that the majority of infiltrating monocytes at 7 days post-injury originate from the spleen and only to a lesser extent from the BM. Prevention of early monocyte infiltration via splenectomy was associated with improved recovery at 42 days post-SCI. In addition, an increased early presence of infiltrating monocytes/macrophages, as a result of CX_3CR1 deficiency within the peripheral immune compartment, correlated with worsened injury outcomes. Adoptive transfer of identified $Cx_3cr1^{gfp/+}$ monocytes confirmed peak infiltration at 7 days post-injury, with inflammatory ($Ly6C^{high}$) monocytes being most efficiently recruited. Focal SCI also changed the composition of the two major monocyte subsets in the blood, with more $Ly6C^{high}$ cells present during peak recruitment. Adoptive transfer experiments further suggested high turnover of inflammatory monocytes in the spinal cord at 7 days post-injury. Consistent with this, only a small proportion of infiltrating cells unequivocally expressed polarisation markers for pro-inflammatory (M1) or alternatively activated (M2) macrophages at this time point. Our findings offer new insights into the origins of monocyte-derived macrophages after SCI and their contribution to functional recovery, providing a basis for further scrutiny and selective targeting of $Ly6C^{high}$ monocytes to improve outcomes from neurotraumatic events.

Introduction

The presence of activated macrophages is a prominent neuropathological feature in both acute and post-acute phases of spinal cord injury (SCI) (Beck et al., 2010; Fleming et al., 2006; Kigerl et al., 2006). Because of this sustained presence and the ability of macrophages to cause secondary tissue damage (Kigerl et al., 2009; Popovich et al., 1999), these cells have long been viewed as therapeutic targets in SCI (for review, see David and Kroner (2011)). However, pro-regenerative and reparative roles have also been proposed for activated macrophages in the injured central nervous system (CNS) (Kigerl et al., 2009; Kotter et al., 2005; Rapalino et al., 1998; Shechter et al., 2009; Yin et al., 2003), prompting the need for a better understanding of their functional diversity.

Activated macrophages in SCI comprise a mixed population of cells with diverse phenotypes and origins. They include macrophage-like cells derived from resident CNS microglia as well as blood monocytes that infiltrate the compromised area following blood–spinal cord barrier (BSB) breakdown (Popovich et al., 1996). No definitive markers exist at present to distinguish between the aforementioned cells in vivo, particularly when in an activated state. This has significantly hindered understanding whether SCI leads to activated macrophages with reparative ability and 'concurrent neurotoxicity' (Gensel et al., 2009), or if their seemingly dual role can be attributed to functional heterogeneity based on different (precursor) origins, in which case indiscriminate targeting of resident and blood-derived macrophages could adversely affect wound-healing processes.

Blood-derived macrophages arise from monocytes that are mobilised into the circulation from the bone marrow (BM; van Furth and Cohn, 1968) and the more recently identified splenic reservoir (Swirski et al., 2009). In mice, circulating monocytes consist of two principal subsets that differentially express the chemokine receptor CX_3CR1 in addition to other phenotypic surface markers. The so-called inflammatory

* Corresponding author at: The University of Queensland, School of Biomedical Sciences, St Lucia, QLD 4072, Australia. Fax: +61 7 3346 7602 (3365 1766).
E-mail address: m.ruitenberg@uq.edu.au (M.J. Ruitenberg).

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monocytes (hereafter referred to as 'monocyte subset 1') are defined as $CX_3CR1^{low}Ly6C^{high}$ cells while the second major monocyte subset (hereafter referred to as 'monocyte subset 2') consists of $CX_3CR1^{high}Ly6C^{neg/low}$ cells (Geissmann et al., 2003). The relative contributions of these monocyte subsets to the overall pool of macrophages at sites of SCI have not been studied to date and the functional implications of their putative presence thus remain unclear. Other outstanding questions include whether mobilisation of the splenic monocyte reservoir occurs in response to SCI, and if polarisation of blood-derived macrophages in the injured spinal cord towards a pro-inflammatory (M1) or alternatively activated (M2) phenotype relates back to their precursor origins. A possible relationship between monocyte subset recruitment and macrophage polarisation may have important implications for SCI repair strategies as M1 macrophages are associated with tissue destruction and regenerative failure while M2 phenotypes link to repair (Kigerl et al., 2009).

In the present study, we used BM chimeric mice in combination with splenectomy and adoptive cell transfer of identified monocyte subsets to advance our understanding of the origins, distribution and immunophenotype of blood-derived macrophages in the injured spinal cord.

Material and methods

Animals

In total, 102 wild-type (WT), 57 $Cx_3cr1^{gfp/+}$ and 3 $Cx_3cr1^{gfp/gfp}$ mice, in which the sequence for green fluorescent protein (GFP) was inserted into the mutant Cx_3cr1 locus (Jung et al., 2000), were used for this study. All mice were females on a C57BL6/J genetic background. Animals were housed in clean facilities under conventional conditions on a 12 h light–dark cycle and with unlimited access to food and water. All experiments were approved by The University of Queensland's Animal Ethics Committee and conducted in accordance with the relevant National Health and Medical Research Council of Australia policies.

Generation of bone marrow chimeric mice

[$Cx_3cr1^{gfp/+}$ > WT], [$Cx_3cr1^{gfp/gfp}$ > WT] and [WT > WT] BM chimeras were generated as described previously (Vukovic et al., 2010). In brief, donor BM was isolated from 6–8 week old WT, $Cx_3cr1^{gfp/+}$ or $Cx_3cr1^{gfp/gfp}$ mice and injected (5×10^6 cells) into the lateral tail vein of recipient mice, 3–4 h after the second dose of irradiation. BM chimeric mice were allowed to recover for 8 weeks before being subjected to SCI. Blood analysis of congenic [CD45.1 > CD45.2] WT chimeras confirmed the efficiency of this protocol; 93.2 ± 0.5% ($n = 10$) of all circulating leukocytes were of donor origin at the time of SCI.

Surgical procedures

Mice were anaesthetised with a mixture of xylazine (20 mg/kg; Troy Laboratories) and tiletamine/zolazepam (50 mg/kg; Virbac Animal Health) and subjected to severe force-controlled (70 kdyn) contusive SCI at the level of the thoracic vertebra 9 (T9), which corresponds to spinal level T12, as detailed elsewhere (Blomster et al., 2013; Harrison et al., 2013). Experimental mice received a single dose of 0.05 mg/kg buprenorphine in Hartmann's solution, in addition to gentamycin (1 mg/kg), which was administered once daily for the first 5 days post-SCI. Bladders were voided manually twice daily for the duration of the experiment. Where applicable, splenectomy was performed prior to SCI as described by Nagy et al. (2006). Sham-operated animals were subjected to the same surgical procedure except for those aspects that involved the actual removal of the spleen.

Behavioural analysis

Open field locomotor scoring was used to assess the impact of splenectomy and select CX_3CR1 deficiency within the peripheral immune compartment on recovery from SCI. The performance of individual animals was rated according to the Basso Mouse Scale (Basso et al., 2006) at 1, 3 and 7 days post-injury and weekly thereafter until the experimental endpoint. Investigators that assigned neurological scores to mice were blinded to the experimental conditions for the duration of these studies. For splenectomy experiments, the actual applied force was 73 ± 0.5 kdyn (mean ± SEM) for splenectomised mice as compared 73 ± 1 kdyn for sham-operated animals, with an average tissue displacement of 541 ± 11 μm and 526 ± 14 μm, respectively. For experiments that examined the impact of select CX3CR1 deficiency within the peripheral immune compartment on recovery from SCI, the applied force was 74 ± 0.7 kdyn (mean ±SEM) for [WT > WT] BM chimeras as compared 73 ± 0.6 kdyn for [$Cx_3cr1^{gfp/gfp}$ > WT] BM chimeras, with an associated tissue displacement of 548 ± 15 μm and 567 ± 11 μm, respectively. There were no significant differences in injury parameters between relevant experimental groups ($p > 0.05$). Lastly, a direct comparison of injured WT mice and [WT > WT] BM chimeras revealed no differences in recovery of locomotor function in open field (BMS; $F_{(1,13)} = 0.82$, $p = 0.38$), indicating that the chimerisation procedure itself did not change SCI outcomes.

Adoptive transfer of identified monocyte subsets

To obtain subset 1 monocytes, BM was isolated from $Cx_3cr1^{gfp/+}$ donors and mononuclear cells separated from other BM cells via Ficoll-Paque (GE Healthcare) density gradient centrifugation for 40 min at 400×*g*. After red blood cell lysis, the remaining mononuclear cells were collected via low-speed centrifugation (300×*g*, 5–10 min) and carefully resuspended in MACS buffer (DPBS containing 1.5 mM KH_2PO_4, 8.1 mM NaH_2PO_4, 2.7 mM KCl, 0.14 M NaCl, 2 mM EDTA and 0.5% (w/v) bovine serum albumin (BSA; Sigma)). The cells were then incubated with rat anti-CD16/CD32 (1:200; BD Biosciences) for 5 min at 4 °C to block Fc receptors, followed by incubation with hamster anti-CD11c (1:5; Miltenyi Biotech), rat anti-MHCII (1:10; Miltenyi Biotech), both conjugated to magnetic beads, and biotinylated rat anti-Ly6G (1:5; Miltenyi Biotech) for 15 min at 4 °C to allow for depletion of dendritic cells and granulocytes, respectively. Cells were collected via low-speed centrifugation and further incubated with a magnetic bead-conjugated mouse anti-biotin antibody (1:5; Miltenyi Biotech) for 15 min at 4 °C. Cells were then collected, resuspended in MACS buffer and separated on a MiniMACS system according to manufacturer's instructions (Miltenyi Biotech). The negative fraction, now containing $CD11c^{neg}MHCII^{neg}Ly6G^{neg}$ mononuclear cells, was collected and incubated for 15 min at 4 °C with biotinylated rat anti-Gr1 (Ly6C/G) antibody (1:400, BD Biosciences) to allow for positive selection of subset 1 monocytes. In the final step, cells were incubated with anti-biotin magnetic beads and separated based on Ly6C expression using the MiniMACS system according to manufacturer's instructions. The $Gr1^{pos}$ fraction was resuspended in RPMI medium, after which a small sample was incubated with toluidine blue stain to assess viability (>90%) and yield. For adoptive cell transfer, 7–8 × 10^5 $Ly6C^{pos}$ monocytes from $Cx_3cr1^{gfp/+}$ donors were injected in ~150 μl via the lateral tail vein into WT recipients at 3, 6 or 27 days post-SCI. These mice were killed 24 h after adoptive cell transfer to study recruitment of identified $Cx_3cr1^{gfp/+}$ monocytes. An additional cohort of animals was injected with a similar number of $Ly6C^{pos}$ cells at 6 days post-SCI but sacrificed 72 h later to gain an insight into the dynamics of monocyte turnover within the injured tissue. Results are representative of three independent experiments (i.e. including monocyte isolation procedures).