Astrocytes initiate inflammation in the injured mouse spinal cord by promoting the entry of neutrophils and inflammatory monocytes in an IL-1 receptor/MyD88-dependent fashion

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A B S T R A C T

CNS injury stimulates the expression of several proinflammatory cytokines and chemokines, some of which including MCP-1 (also known as CCL2), KC (CXCL1), and MIP-2 (CXCL2) act to recruit Gr-1+ leukocytes at lesion sites. While earlier studies have reported that neutrophils and monocytes/macrophages contribute to secondary tissue loss after spinal cord injury (SCI), recent work has shown that depletion of Gr-1+ leukocytes compromised tissue healing and worsened functional recovery. Here, we demonstrate that astrocytes distributed throughout the spinal cord initially contribute to early neuroinflammation by rapidly synthesizing MCP-1, KC, and MIP-2, from 3 up to 12 h post-SCI. Chemokine expression by astrocytes was followed by the infiltration of blood-derived immune cells, such as type I “inflammatory” monocytes and neutrophils, into the lesion site and nearby damaged areas. Interestingly, astrocytes from mice deficient in MyD88 signaling produced significantly less MCP-1 and MIP-2 and were unable to synthesize KC. Analysis of the contribution of MyD88-dependent receptors revealed that the astrocytic expression of MCP-1, KC, and MIP-2 was mediated by the IL-1 receptor (IL-1R1), and not by TLR2 or TLR4. Flow cytometry analysis of cells recovered from the spinal cord of MyD88- and IL-1R1-knockout mice confirmed the presence of significantly fewer type I “inflammatory” monocytes and the almost complete absence of neutrophils at 12 h and 4 days post-SCI. Together, these results indicate that MyD88/IL-1R1 signals regulate the entry of neutrophils and, to a lesser extent, type I “inflammatory” monocytes at sites of SCI.

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1. Introduction

Damage to the central nervous system (CNS) induces an almost immediate reaction from microglia and astrocytes (Davalos et al., 2005; Kim and Dustin, 2006; Nimmerjahn et al., 2005). This reaction, referred to as gliosis, is characterized by different changes at the molecular level that can result in activation, proliferation, and migration of glial cells. Gliosis in the injured CNS is thought to lead to the recruitment of blood-derived immune cells to the site of lesion. In the context of a spinal cord injury (SCI), whether recruited immune cells exacerbate tissue damage or participate in CNS repair remains an open question (David and Lacroix, 2005; Donnelly and Popovich, 2008; Schwartz and Yoles, 2006). Recent evidence suggests, however, that immune cells may play a role in both neural damage and repair, depending on the cellular population involved and their state of activation (Barrette et al., 2008; Gensel et al., 2009; Stirling et al., 2009).

The proinflammatory cytokine interleukin (IL-1) is thought to play a key role in gliosis in the injured CNS (Busu et al., 2002). Supporting a role for this cytokine in the early events leading to gliosis is a study from our laboratory which has demonstrated that IL-1β is expressed within minutes by glial cells after SCI in mice (Pineau and Lacroix, 2007). Recent work by de Rivero Vaccari et al. has revealed the existence of a molecular platform, the NACHT leucine-rich-repeat protein-1 (NALP1) inflammasome, consisting of the NOD-like receptor (NLR) NALP1, caspase-1, caspase-11, ASC, and XIAP, in neurons of the normal rat spinal cord (de Rivero Vaccari et al., 2008). In these animals, SCI in the absence of pathogens triggered the activation of this multiprotein complex. Activation of the NALP1 inflammasome resulted in cleavage of caspase-1 and XIAP and upregulation of caspase-11 and ASC, leading to maturation of bioactive IL-1β. Notably, de Rivero Vaccari et al. have also reported the expression of ASC, an adaptor protein essential for caspase-1 recruitment and known to interact with several inflammasomes of the NALP family (Tschopp et al., 2003), in microglia and oligodendrocytes (de Rivero Vaccari et al., 2008). Thus, IL-1 appears to
be in an ideal position to initiate gliosis and immune cell recruitment into the injured spinal cord. In the peritoneal cavity, the recruitment of neutrophils and, to a much lesser degree, monocytes is compromised in MyD88- and IL-1R1-knockout mice in response to sterile inflammation induced by the injection of dead cells (Chen et al., 2007). In spite of this, the role of IL-1 signaling in the expression of the chemokines involved in the recruitment of blood-derived immune cells after CNS injury remains poorly investigated.

Chemokines are a subclass of chemotactic cytokines classified into two large families, on the basis of the number and location of cysteine residues near the N-terminus, i.e., CC ligands (CCLs) and CXC ligands (CXCLs) (Ransohoff, 2002). One chemokine of particular interest is the monocyte chemoattractant protein-1 (MCP-1), also referred to as CCL2 and best known for its role as a chemoattractant for type I “inflammatory” monocytes (Gr-1⁺CCR2⁺CX3CR1⁺) (Auffray et al., 2007; Henderson et al., 2003; Nahrendorf et al., 2007). Evidence accumulated to date indicates that type I “inflammatory” monocytes are recruited to sites of inflammation/injury via the MCP-1 receptor CCR2, and are primarily involved in inflammation, proteolysis, and phagocytosis. Another subset of monocytes that has received significant attention lately are the so-called type II “resident” monocytes (Gr-1⁺CCR2⁻CX3CR1⁺). Resident monocytes are apparently implicated in both immune surveillance and the healing process (Nahrendorf et al., 2007). Like monocytes, neutrophils accumulate in the first few hours to days after SCI, and are primarily involved in inflammation, proteolysis, and phagocytosis. Although neutrophils have long been suspected to contribute to secondary tissue loss in the context of SCI (Farooque et al., 1999; Hamada et al., 1996; Taoka et al., 1997), a recent study has shown that depletion of Gr-1⁺ neutrophils by the administration of anti-Gr-1 antibody reduced wound healing and worsened functional recovery (Stirling et al., 2009).

However, because the cell depletion strategy used by Stirling et al. targeted all leukocytes expressing the Gr-1 antigen (Ly6C/G), it is possible that the treatment might have depleted immune cells other than neutrophils, including monocytes and lymphocytes. Together, these results emphasize the need to better define the roles of the various subsets of immune cells involved in innate immunity after CNS injury and molecular events regulating their recruitment.

Given that glial cell responses that rapidly develop after SCI may lead to the recruitment of blood-derived immune cells and cause exacerbation of tissue damage, it is important to define the exact role(s) of individual immune molecules during the course of pathological conditions and the mechanisms by which their production is regulated. A better knowledge of the functions of these molecules could allow the identification of new potential targets to treat CNS injuries by either promoting CNS repair or reducing tissue loss. In this study, we present the complete spatial distribution and temporal expression patterns of MCP-1, MIP-2, and KC at sites of SCI. Furthermore, we have determined the cellular sources of these chemokines after SCI. Finally, we identified a key signaling pathway regulating MCP-1, MIP-2, and KC synthesis and the recruitment of neutrophils and type I “inflammatory” monocytes in the injured mouse spinal cord.

2. Experimental methods

2.1. Animals

A total of 188 mice (8–12 weeks old) were used in this study. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MyD88-/- mice in the C57BL/6 background were generously provided by Dr. S. Akira (Department of Host Defense, Osaka, Japan). IL-1R1-knockout (ko) and TLR2-ko (backcrossed with C57BL/6 mice for at least nine generations) were purchased from The Jackson Laboratory. C57BL/6 mice were used as controls for MyD88-ko, IL-1R1-ko, and TLR2-ko mice. C3H/HeJ (TLR4-/- mouse mutant; TLR4d) and their wild-type counterparts, C3H/HeOuJ, were also obtained from the The Jackson Laboratory. Mice had ad libitum access to food and water.

2.2. Surgical procedures

All surgical procedures were approved by the Laval University Animal Care Committee and followed Canadian Council on Animal Care guidelines.

2.2.1. SCI

C57BL/6 (n = 123), MyD88-ko (n = 32), IL-1R1-ko (n = 27), TLR2-ko (n = 3), and TLR4d (n = 3) female mice were anesthetized with isoflurane and underwent a laminectomy at vertebral level T9–10, which corresponds to spinal segment T10–11. Briefly, the vertebral column was stabilized and a contusion of 70 kdyn was performed using the Infinite Horizon (IH) SCI device (Precision Systems & Instrumentation, Lexington, KY). For the sham-operated mice, the exposed spinal cord was left untouched. Over-lying muscular layers were then sutured and cutaneous layers stapled. Post-operatively, animals received manual bladder evacuation twice daily to prevent urinary tract infections. Depending on the experiment performed, spinal cord injured and sham-operated mice were sacrificed by perfusion at 5, 15, 30, and 45 min, 1, 3, 6, 12, and 24 h, and 2, 4, 7, 14, 28, and 35 days post-contusion.

2.3. Tissue processing, histology, and 3D spinal cord reconstruction

Spinal cords were collected and prepared as previously described (Pineau and Lacroix, 2007). Briefly, mice were overdosed with a mixture of ketamine–xylazine and transcardially perfused with 4% paraformaldehyde (PFA), pH 9.5, in borax buffer. Spinal cords were dissected out, post-fixed for 2 days, and placed overnight in a 4% PFA-borax/10% sucrose solution. For each animal, a spinal cord segment of 12 mm centered over the lesion site was cut in several series of 30-μm-thick coronal sections using a cryo-stat. Sections were collected directly onto slides having a permanent positive charged surface (Surigapha Canada Inc., Winnipeg, MB, Canada) and stored at −20 °C until used.

To identify the lesion epicenter and for the three-dimensional (3D) reconstruction of the lesion, one series of adjacent sections was stained with luxol fast blue (LFB) and then counterstained with cresyl violet (CV), as described before (Pineau and Lacroix, 2007). Three-dimensional spinal cord reconstructions were performed using the Bioquant Nova Prime computerized image analysis system (Bioquant Image Analysis Corporation, Nashville, TN), as described in Pineau and Lacroix (2007). Briefly, the outline of 1 out of 14 coronal sections within a pre-determined spinal cord segment, including the lesion epicenter and sections located up to 5 mm distal to the center of the lesion in both directions (i.e., rostral and caudal), were reconstructed.

2.4. In situ hybridization (ISH)

ISH was carried out to detect mRNAs coding for chemokines MIP-1, KC, and MIP-2 and the proinflammatory cytokine IL-1β. Full-length cDNA cloned into expression vectors pGEM-1 (MCP-1) and pCR® II (IL-1β) were obtained from Dr. Serge Rivest (Laval University, QC, Canada). cDNAs for KC and MIP-2 were amplified from a C57BL/6j mouse brain cDNA library. The following primers
were used for PCR amplification: primers complementary to nucleotides 333–352 (forward: 5'-gaagcttgctgttcgagac-3') and 804–823 (reverse: 5'-gagagctcagagggagga-3') for KC and primers complementary to nucleotides 429–448 (forward: 5'-gctgctgttcgagtagt-3') and 964–982 (reverse: 5'-gacgtctgttcagaggga-3') for MIP-2. PCR products were subcloned into pCR®-Blunt II (Invitrogen Canada, Burlington, ON, Canada). Sequences chosen for probe synthesis were selected to match only the intended genes, as verified by BLAST analysis in Genbank. Radiolabeled cRNA probes were synthesized using the Riboprobe Combination System SP6/T7 (Promega, Madison, WI). ISH was performed according to a previously described method (Lacroix et al., 1998, 2002; Pineau et al., 2006). All sections were prehybridized, hybridized and posthybridized in parallel to equalize background intensity.

2.5. Combination of ISH with multiple immunofluorescence labeling

To identify the populations of cells expressing IL-1β, MCP-1, MIP-2, and KC mRNAs, ISH was combined with immunofluorescence detection of the following cellular markers: the ionized calcium-binding adaptor molecule 1 (iba1), the galactose-specific lectin-3 (Gal-3), the glial fibrillary acid protein (GFAP), the carbonic anhydrase II (CAII), and the HuC/HuD neuronal antigen (HuC/HuD), which are specific for macrophages/microglia, activated macrophages/microglia, astrocytes, oligodendrocytes, and neurons, respectively. Secondary antibodies conjugated with either the fluorophore Alexa-488 or Alexa-568 (dilution 1:200; Invitrogen Canada) were used. Secondary antibodies conjugated with either the fluorophore Alexa-488 or Alexa-568 (dilution 1:200; Invitrogen Canada) were used. Cells were considered positive when the signal represented more than 4× the background value. Double-labeled cells were quantified at 20× magnification and counted only if their nucleus was visible and cell body isolated from neighboring cells. Results were expressed as total number of cells expressing mRNA signal per cross-section at each level analyzed. Meningeal layers were excluded from the quantitative analyses.

2.7.2. Immunohistochemistry

Cells expressing the 7/4 antigen were counted at the lesion epicenter and from evenly spaced coronal sections located on both sides of the lesion epicenter. The outline of the cross-section was first traced at 4× magnification, a grid positioned over the spinal cord using the Bioquant Nova Prime image analysis system, and all 7/4+ cells counted at 20× magnification. Results were expressed as total number of 7/4+ cells per cross-section at each level analyzed. Cells fluorescently labeled for 7/4 and counterstained with DAPI were visualized using a Fluoview confocal microscope system (Olympus America Inc., Melville, NY).

2.8. Flow cytometry analysis of spinal cord samples

To address the question of whether MyD88–IL-1R1 signaling deficiency altered the entry of neutrophils, monocytes, and other immune cells after SCI, cells freshly isolated from the spinal cord of injured mice were analyzed using flow cytometry, following a protocol adapted from Stirling et al. (Stirling and Yong, 2008). Briefly, animals were transcardially perfused with cold DPBS to remove immune cells from the vasculature, their spinal cords dissected out, and a 2–cm segment centered at the site of the lesion isolated and immediately placed into a 1.5-mL microtube (one spinal cord per tube) containing cold DPBS. Spinal cords were then cut into multiple small segments, mechanically homogenized with a small tissue grinder, and filtered through a 40-μm nylon mesh cell strainer (BD Bioscience, Mississauga, ON, Canada) to obtain a single-cell suspension. Cells were centrifuged at 200g for 10 min, washed once with PBS, and resuspended with PBS containing 20% fetal bovine serum (FBS; Sigma–Aldrich Canada Ltd., Oakville, ON, Canada). Cell concentration and percentage viability were estimated using a hemocytometer and the Trypan blue exclusion method.

For multicolor immunofluorescent labeling, cells were incubated with Mouse Fc Block (i.e., purified anti-mouse CD16/CD32; BD Bioscience) for 5 min in order to prevent nonspecific binding, followed by labeling for 30 min at room temperature with the following fluorescently conjugated primary antibodies: PerCP-conjugated anti-CD45, PE-conjugated anti-CD11b, FITC-conjugated anti-Gr-1 (Ly6C/G), and APC-conjugated anti-F4/80. The complete description of the primary antibodies and the dilution used for flow cytometry are given in Supplementary Table 1 available online. For negative controls, primary antibodies were omitted. Finally, cells were washed again and fixed with 1% PFA (pH 7.4, in PBS). A total of 250,000 events were analyzed in duplicate for each spinal cord sample using CellQuest Pro software on a FACSCalibur flow cytometer (BD Biosciences). Relative CD45 levels (high vs. intermediate) were used to distinguish recruited CD11b+ myeloid cells from CD11b+ resident microglia (CD45hi CD11b+), respectively (Babcock et al., 2006; Bedard et al., 2007; Sedgwick et al., 1991). Neutrophils were identified based on their coexpression of CD45, CD11b, and Gr-1 and lack of expression of F4/80 (i.e., CD45hi CD11b+ Gr-1hi F4/80−) (Auffray et al., 2007; Henderson et al., 2003; Stirling and Yong, 2008). Monocyte/macrophage subsets (CD45hi CD11b+ F4/80−) were further differentiated based on Gr-1 expression (Auffray et al., 2007; Henderson et al., 2003; Nahren-dorf et al., 2007).
2.9. Statistical analysis

In this study, results were compared among all subjects to generate mean ± standard error of the mean (SEM). All statistical evaluations were performed with two-way ANOVA. Post-ANOVA comparisons were made using the Bonferroni test. Statistical analyses were performed using the GraphPad Prism software (version 4.03; GraphPad Software, San Diego, CA). A p value < 0.05 was considered as statistically significant. Data in graphs are presented as mean ± SEM.

3. Results

3.1. SCI induces multiphasic expression of MCP-1 mRNA over time

MCP-1 mRNA levels increased rapidly (< 5 min) after SCI (data not shown). On average, we counted as many as 60 MCP-1+ cells at the lesion epicenter at 3 h post-injury (Fig. 1A). This number remained stable up to 24 h post-SCI, and the number of MCP-1+ cells steadily increased thereafter from 57 ± 7 at 24 h to 87 ± 7 at 2 days and 115 ± 20 at 4 days. Then, the number of MCP-1+ cells at the lesion site progressively decreased to 80 ± 8 at 7 days, 24 ± 12 at 14 days, and 22 ± 8 at 28 days. Quantitative analyses performed at a distance of 1680 μm caudal to the lesion epicenter revealed that the number of MCP-1+ cells also remained stable from 3 to 24 h post-SCI (Fig. 1B). Intriguingly, while the number of MCP-1+ cells at the lesion epicenter increased from 24 h up to 4 days, this number decreased by 12-fold at 1680 μm caudal to the epicenter during the same period of time (Fig. 1A and B). The peak of MCP-1+ cells was seen during the first 24 h post-injury.

At early time points post-SCI, the number of MCP-1+ cells was sometimes difficult to count from 2 days up to 28 days, because MCP-1+ cells were often clustered together, levels of MCP-1 mRNA were also quantified under darkfield illumination using proportional area measurements, as described before (Vallières et al., 2006). Proportional area measurements for MCP-1 mRNA levels paralleled the actual numbers of MCP-1+ cells counted for each of the distances and time points analyzed (data not shown). In the laminectomy control group, only few MCP-1+ cells were seen during the first 24 h post-injury.

3.2. Neutrophil chemoattractant genes KC and MIP-2 are primarily expressed by astrocytes after SCI

In the mouse CNS, KC and MIP-2 have been shown to be the main chemokines responsible for neutrophil recruitment in the context of infection (Kielian et al., 2001). Using ISH, we therefore examined mRNA expression levels for these two genes at various time points post-SCI. In contrast to MCP-1, only few KC+ and MIP-2+ cells could be detected at the 3-h time point, and the vast majority of these cells were confined within damaged areas (Fig. 2A and B). Although the number of cells expressing KC mRNA remained relatively small at all time points analyzed, the production of MIP-2 increased progressively and reached a peak at 12 h, with an average number of 184 ± 27 positive cells at the lesion epicenter (Fig. 2C–E). To further define the cellular source(s) of the two CXC chemokines after SCI, we next assessed the in vivo phenotype of the CNS resident cells expressing KC and MIP-2 mRNA transcripts by combining multiple immunofluorescence labeling with ISH on the same spinal cord tissue sections. As shown in Fig. 2F, MIP-2 expression was primarily upregulated in astrocytes. The same was true for KC (data not shown).

3.3. MyD88/IL-1R1 signaling regulates the expression of MCP-1 by astrocytes after SCI

Receptors of the IL-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily are thought to play a crucial role in the orchestration and maintenance of the inflammatory response during infection (O’Neill and Dinarello, 2000). To activate key regulators of the transcription of proinflammatory cytokines and chemokines (e.g., NF-κB and MAP kinases), IL-1R1 and TLRs depend on the presence of the adaptor protein myeloid differentiation primary response gene 88 (MyD88). Thus, to identify signals that may regulate early and late expression of MCP-1 after SCI, we examined the contribution of MyD88-dependent receptors to these responses using various strains of deficient mice. By combining multiple immunofluores-
Fig. 1. Multiphasic expression of MCP-1 mRNA in the injured mouse spinal cord. (A and B) Time course of MCP-1 mRNA expression at the lesion epicenter (A) and at a distance of 1680 μm caudal to the lesion site (B) after spinal cord contusion in C57BL/6 mice (for each time point: n = 2 laminectomized mice; n = 4 SCI mice). (C–E) Darkfield photomicrographs showing expression of MCP-1 mRNA at the lesion epicenter (C and E) and at 1050 μm caudal to the lesion epicenter (D) at 6 h (C and D) and 4 days (E) post-SCI. (F) Three-dimensional reconstructions of injured spinal cords showing damaged areas and the spatial distribution of MCP-1 mRNA positive cells at 3 h, 4 days, and 28 days post-SCI. Note that MCP-1+ cells are widely distributed throughout the entire spinal cord segment analyzed during the acute phase, whereas they are restricted to damaged areas during the subacute and chronic phases. (G–L) Colocalization of MCP-1 mRNA within astrocytes (G–I; green), microglia (J; red, astrocytes are shown in green), oligodendrocytes (K; red), and neurons (L; red) at 6 h (G–I) and 12 h (J–L) post-SCI. (M–N) High-power view with Nomarski optics showing MCP-1+ cells (endothelial cells and/or perivascular macrophages) surrounding the lumen of several blood vessels found within the injured mouse spinal cord at 6 h post-SCI. (O and P) Colocalization of MCP-1 mRNA within activated microglia/macrophages (red) at 4 days post-SCI. Scale bar: (C–E) 100 μm; (G–I) 10 μm; (J) 14.5 μm; (K) 8.5 μm; (L) 13 μm; (M and N) 25 μm; (O and P) 10 μm. (For interpretation of color mentioned in this figure, the reader is referred to the web version of this article.)
cene labeling with ISH, we were able to examine MCP-1 expression in vivo at the single-cell level.

As described earlier, our results show that MCP-1 mRNA was expressed as early as 5 min post-SCI and was maintained at least up to Day 35. Of particular interest was the fact that distinct populations of neural cells were found to participate in the expression of MCP-1 during the acute phase of SCI. Thus, we next compared the number of MCP-1+ cells in sections prepared from the spinal cords of C57BL/6, MyD88-ko, and IL-1R1-ko mice killed at 3 and 12 h post-SCI. As observed before at 3 h, we detected the presence of many MCP-1+ cells dispersed throughout nearly the entire spinal cord segment analyzed in C57BL/6 mice (Fig. 3A). In contrast, MCP-1 expression was considerably reduced in MyD88-ko and IL-1R1-ko mice. When compared with control mice, statistical significance in the number of MCP-1+ cells in MyD88-ko and IL-1R1-ko mice was reached at several different distances both rostral and caudal to the lesion epicenter. For example, the number of MCP-1+ cells was reduced by 4- and 3-fold at 840 μm rostral and by 3- and 2.4-fold at 1680 μm caudal to the epicenter in MyD88-ko and IL-1R1-ko, respectively, compared with control SCI animals at 3 h. No significant differences in the number of MCP-1+ cells were observed between MyD88-ko and IL-1R1-ko mice at any of the distances analyzed. Results of quantitative analysis performed on spinal cord tissue harvested 12 h after injury paralleled the results obtained at 3 h (data not shown). As shown in Supplementary Fig. 1 (available online), the number of MCP-1+ cells was slightly

![KC](image1)

![MIP-2](image2)

**Fig. 2.** Neutrophil chemoattractant genes KC and MIP-2 are primarily expressed by astrocytes at sites of SCI. (A–D) Number of cells expressing KC (A and C) and MIP-2 (B and D) mRNAs at various rostral (R) and caudal (C) distances from the lesion epicenter at 3 (A and B) and 12 (C and D) hours post-SCI (n = 4–8 mice per time point). (E) Darkfield photomicrograph showing MIP-2 mRNA expression at the lesion epicenter at 12 h post-SCI. (F) Colocalization of MIP-2 mRNA within astrocytes (green) at 12 h post-SCI. Scale bars: (E) 100 μm; (F) 7.5 μm. (For interpretation of color mentioned in this figure, the reader is referred to the web version of this article.)
increased in TLR2-ko compared with C57BL/6 mice at 3 h post-injury, whereas no change was observed between C3H/HeJ (TLR4d) and their wild-type counterparts, C3H/HeOUJ mice. Together, these results indicate that during the acute phase of SCI, the MyD88/IL-1R1 signaling pathway regulates MCP-1 expression.

To identify the neural cell type(s) that express MCP-1 in an IL-1R1/MyD88-dependent fashion, quantitative analysis was performed on sections obtained from the spinal cords of C57BL/6, MyD88-ko, and IL-1R1-ko mice after immunofluorescence labeling for GFAP, iba1, CAII, and HuC/HuD and ISH for MCP-1. In addition
to confirming that astrocytes were the principal cellular source of MCP-1 among the main populations of CNS resident cells at 3–12 h after injury (Fig. 3B), quantification revealed that only astrocytes appeared to depend on MyD88/IL-1R1 signals to express MCP-1 (Fig. 3C and D). Although microglia and neurons appeared to compensate for the reduced expression of astrocytic MCP-1 at 12 h, these results did not reach statistical significance. In light of these results, we propose that astrocytes found in proximity of a spinal cord trauma rapidly express MCP-1 in response to IL-1.

As opposed to the results obtained at 3 h post-SCI, deficiency in MyD88 and IL-1R1 signaling only had a small effect on MCP-1 mRNA expression at 4 days (Fig. 3E). A significant decrease in the number of MCP-1+ cells in MyD88- and IL-1R1-ko mice compared with the control group was detected at a single distance of 840 μm caudal to the lesion. Our interpretation of this finding is that MCP-1 expression at 4 days after injury is primarily mediated by the activation of MyD88-independent signaling pathway(s). As mentioned earlier, although we found that astrocytes were mainly responsible for the early production of MCP-1 at 3–12 h, activated macrophages and microglia appeared to be the main producers of the chemokine at Day 4.

Interestingly, the situation turned around once again at 28 days post-SCI. Like at 3 and 12 h, MCP-1 expression was severely reduced in MyD88-ko and IL-1R1-ko animals compared with control mice at 28 days, decreasing from 54 ± 9 MCP-1+ cells in C57BL/6 to 15 ± 3 and 17 ± 3 cells in MyD88-ko and IL-1R1-ko mice, respectively (Fig. 3F). When compared to the wild-type strain, this corresponded to a 72% and 68% drop in the number of MCP-1+ cells in MyD88-ko and IL-1R1-ko mice, respectively. Still, it must be pointed out that MCP-1 expression was not completely abolished in MyD88-ko and IL-1R1-ko mice at 3 and 12 h and 28 days. This suggests that in addition to the MyD88/IL-1R1 signaling pathway, other alternative pathway(s) regulate astrocytic MCP-1 expression and/or that cells other than astrocytes contribute to the expression of the chemokine in a MyD88/IL-1R1-independent fashion.

Taken together, our results suggest that MCP-1 expression during the early acute (3–12 h) phase of SCI largely depends on activation of IL-1R1 signaling in astrocytes. However, the small effect of MyD88/IL-1R1 deficiency on MCP-1 expression at Day 4 combined with our multiple-labeling analyses suggest that recruited immune cells are mainly responsible for the production of the chemokine during the subacute phase of SCI, most likely via activation of a MyD88/IL-1R1-independent pathway. Interestingly, upregulation of MCP-1 expression during the chronic phase of SCI (28 days) is once again dependent on a functional MyD88/IL-1R1 signaling. Thus, the signaling pathways involved in the regulation of MCP-1 expression seem to be linked to the types of cells activated and their regional distribution in relation to the site of injury. Based on our experimental results, the time after injury also appears to be an important factor in the regulation of MCP-1 expression.

3.4. MyD88/IL-1R1 signaling is critical for the expression of neutrophil chemomtactant genes KC and MIP-2 at sites of SCI

To determine whether MyD88/IL-1R1 signaling is important for the synthesis of KC and MIP-2 in the injured mouse spinal cord, we quantified the number of cells expressing KC and MIP-2 transcripts in C57BL/6, MyD88-ko, and IL-1R1-ko mice at 12 h post-injury, the time at which mRNA expression is maximal for these two genes (see Fig. 2). As demonstrated in Fig. 4A, very few, if any, cells expressing KC mRNA were detected in the spinal cords of MyD88-ko and IL-1R1-ko mice at 12 h. In SCI control mice, 14 ± 4 KC+ cells were observed on average at the lesion epicenter. MIP-2, on the other hand, was much more abundantly expressed than KC in C57BL/6 mice with an average number of 184 ± 27 positive cells at the lesion epicenter at 12 h. This number was reduced by 3.5- and 2.2-fold in MyD88-ko and IL-1R1-ko mice, respectively (Fig. 4B). As for MCP-1, multiple-labeling analyses confirmed that astrocytes from MyD88- and IL-1R1-ko mice synthesized significantly less KC and MIP-2 than astrocytes from wild-type animals (Fig. 4C and D).

3.5. Deficiency in MyD88/IL-1R1 signaling compromised the entry of neutrophils and reduced the recruitment of type I “inflammatory” monocytes to sites of SCI

The significant reduction in MCP-1 and MIP-2 synthesis combined with the absence of KC expression by astrocytes in MyD88- and IL-1R1-ko mice led us to examine whether the recruitment of Gr-1+ leukocytes was compromised in these animals after SCI. To this end, the use of a four-color flow cytometry approach was necessary, mainly because the distinction between different populations of leukocytes requires the simultaneous examination of multiple immune cell markers. Here, the combination of antibodies directed against CD45, CD11b, Gr-1, and F4/80 allowed us to discriminate between microglia (CD45hi CD11b+ Gr-1+ F4/80+), type I “inflammatory” monocytes (CD45hi CD11bhi Gr-1+ F4/80+), type II “resident” monocytes (CD45hi CD11bhi Gr-1+ F4/80+), and neutrophils (CD45hi CD11bhi Gr-1hi F4/80+). Analysis of the presence of these four populations of leukocytes at sites of SCI revealed that neutrophil entry was reduced by approximately 85% in the absence of MyD88/IL-1R1 signaling at 12 h (Fig. 5). The recruitment of type I “inflammatory” monocytes was also significantly reduced by about 55% in SCI MyD88- and IL-1R1-ko mice compared with injured control mice. No significant differences were found between the three groups with regard to proportions of type II “resident” monocytes at 12 h. Analyses performed at Day 4 confirmed that the neutrophilic recruitment response to SCI was mitigated (as opposed to delayed) in both MyD88- and IL-1R1-ko mice (Fig. 5). Although the proportions of type I “inflammatory” monocytes that infiltrated the injured spinal cord of C57BL/6 mice at 4 days were nearly twice as elevated as those seen in MyD88- and IL-1R1-ko, significant differences between groups were no longer detected at this time point. Like at 12 h, the proportions of “resident monocytes” remained similar between all groups at 4 days. These data suggest that type I “inflammatory” monocyte influx into the injured mouse spinal cord is reduced in the absence of MyD88/IL-1R1 signaling, whereas type II “resident” monocyte influx is unaffected. Thus, deficiency in MyD88/IL-1R1 signaling in the context of SCI results in compromised neutrophil and reduced type I “inflammatory” monocyte recruitment responses. We note that other chemokines besides the ones analyzed in this study (MCP-1, KC, MIP-2) may have been partially responsible for the reduced infiltration of neutrophils and type I “inflammatory” monocytes in the injured spinal cord of MyD88- and IL-1R1-ko mice.

To further confirm the reduction in the number of neutrophils and type I “inflammatory” monocytes present at sites of SCI in MyD88- and IL-1R1-deficient mice, immunohistochemistry was used to visualize cells expressing the 7/4 antigen (Fig. 6A–D). Although the anti-7/4 antibody has been commercially sold as a neutrophil-specific antibody, numerous studies have demonstrated that the 7/4 antigen is also present on the cell surface of type I “inflammatory” monocytes (but not type II “resident” monocytes) (Gordon and Taylor, 2005; Henderson et al., 2003; Tsou et al., 2007). At 12 h after injury, as many as 1533 ± 92 cells expressing the 7/4 marker were counted at the lesion epicenter in C57BL/6 mice (Fig. 6E). This number was reduced by approximately 5-fold in MyD88- and IL-1R1-ko mice, with an average number of 7/4+ cells on the order of 300 in both groups. At 4 days after SCI, 7/4+ cell numbers in MyD88- and IL-1R1-ko mice were reduced by about 48% and 41%, respectively, compared with con-
trol mice (Fig. 6F). As shown in Fig. 6G, a fairly significant number (435 ± 58) of 7/4+ cells were still present at the lesion epicenter in C57BL/6 mice at 28 days post-SCI. In the absence of MyD88/IL-1R1 signaling, this number dropped to less than 15 positive cells, a nearly 30-fold reduction. We noted that the shape of the graph presented in Fig. 6G looks almost identical to the one presented in Fig. 3F, which reported MCP-1 expression in C57BL/6, MyD88-, and IL-1R1-ko mice. This observation combined with the fact that neutrophil chemoattractant genes KC and MIP-2 could barely be detected at 28 days (data not shown) suggests that the 7/4+ cells present during the chronic phase of SCI were type I "inflammatory" monocytes. Confocal microscopy confirmed that the majority (but not all) of the 7/4+ cells seen at 28 days had ovoid- or kidney-shape nuclei, rather than multilobed as in neutrophils. From these results, we conclude that MyD88/IL-1R1 signals regulate the entry of neutrophils and, to a lesser extent, type I "inflammatory" monocytes at sites of SCI.

4. Discussion

A current dogma in the SCI field is that mobilization of blood-derived immune cells at sites of injury propagates tissue damage. However, recent evidence suggests that protection and repair of injured tissue requires the coordinated recruitment of several immune cell subsets, including type I "inflammatory" and type II "resident" monocytes (Nahrendorf et al., 2007; Swirski et al., 2009). In the injured peripheral nervous system (PNS), depletion of CD11b+ myeloid cells prevented the clearance of inhibitory myelin debris, neutrophil synthesis, blood vessel formation/maintenance, axonal regeneration, and recovery of sciatic nerve function (Barrette et al., 2008). Likewise, depletion of Gr-1+ neutrophils and monocytes after SCI compromised wound healing and worsened neurological outcome (Stirling et al., 2009). We believe that immune cells play a role in both tissue damage and repair after neural injury, depending on the cellular population involved and their state of activation, and that more efforts should be made to identify the signals that regulate the infiltration of the various immune cell subsets into the injured nervous system.

Here, we report a detailed analysis of the spatio-temporal distribution and cellular sources of the key chemokines involved in the recruitment of neutrophils and type I "inflammatory" monocytes, i.e., MCP-1, KC, and MIP-2, in the injured mouse spinal cord. Using combined histochemical methods, we were able to demonstrate that astrocytes are primarily responsible for chemokine synthesis during the acute phase of SCI. We also conducted experiments on SCI MyD88-, IL-1R1-, TLR2-, and TLR4-deficient mice and their respective wild-type counterparts to quantify, in situ, MCP-1, KC, and MIP-2 expression at the single-cell level. Results showed that MyD88/IL-1R1 signals are critical for the astrocytic expression of MCP-1, KC, and MIP-2 mRNAs. Finally, we found that deficiency in MyD88/IL-1R1 signaling compromised the entry of neutrophils and reduced the recruitment of type I "inflammatory" monocytes to sites of SCI.

Our findings showed that neural cells of mice subjected to SCI produce MCP-1 within minutes after injury. Colocalization studies
revealed that astrocytes and cells lining the spinal cord vasculature are the main sources of MCP-1 at early time points (3–12 h) post-SCI. In agreement with this finding is a recent study from Brambilla et al. which reported that MCP-1 immunoreactivity colocalized almost perfectly with the widespread expression of GFAP after SCI in mice (Brambilla et al., 2005). Earlier studies of other animal models of CNS injury had previously suggested that astrocytes can synthesize MCP-1 mRNA (Glabinski et al., 1996; Gourmala et al., 1997). In the injured mouse spinal cord, inhibition of astroglial NF-κB signaling was found to reduce MCP-1 expression, lesion volume, and glial scar formation and to increase axonal sparing/sprouting and functional recovery (Brambilla et al., 2005, 2009). In addition,
inhibition of astroglial NF-κB signaling reduced MCP-1 expression and leukocyte recruitment after entorhinal cortex lesion (Khoroo-shi et al., 2008). Of particular interest to the present discussion is the finding that stimulation of the IL-1R1 in astrocytes leads to activation of NF-κB transcription factors (Srinivasan et al., 2004). The activation of the classical NF-κB pathway is associated with increased transcription of multiple inflammation-associated genes, including CXC chemokines (Bonizzi and Karin, 2004). In the present study, we demonstrate that MCP-1, KC, and MIP-2 expression by astrocytes is severely reduced in MyD88- and IL-1R1-ko mice compared with control animals after SCI. Together, these results suggest that astrocytes that become reactive in response to IL-

![Immunolabeling of 7/4+ cells](image)

**Fig. 6.** Immunolabeling of 7/4+ cells confirms that significantly fewer neutrophils and type I “inflammatory” monocytes are recruited to sites of SCI in the absence of MyD88/IL-1R1 signaling. (A–D) Representative bright-field photomicrographs showing 7/4 immunolabeling in the spinal cord of C57BL/6 (A and D), MyD88-ko (B), and IL-1R1-ko (C) mice at 12 h after SCI. (E–G) Quantification of the number of 7/4+ cells at various rostral (R) and caudal (C) distances from the lesion epicenter at 12 h (E), 4 days (F), and 28 days (G) post-SCI. **p < 0.001, *p < 0.01, and *p < 0.05 compared with MyD88-ko mice; †††p < 0.001, ††p < 0.01, and †p < 0.05 compared with IL-1R1-ko mice. Scale bar: (A–C) 420 µm; (D) 10 µm.
1R1 activation may play an important role in secondary tissue damage and spinal cord repair after injury, most likely through the recruitment of blood-derived immune cells and regulation of glial scar formation. Whether the observed reductions in chemokine expression in MyDB8- and IL-1R1-ko mice resulted from alterations in astrocyte proliferation and/or survival was not investigated in the present study and therefore remains a possibility.

We noted that the spatial distribution of the MCP-1 transcript at 3–12 h almost perfectly mimics the rapid and widespread expression of IL-1β mRNA at 45–60 min post-SCI (Pineau and Lacroix, 2007). Recent multiple-labeling studies performed by us and others have revealed that microglia are predominantly responsible for IL-1β synthesis during the early acute phase of SCI, although some IL-1β-expressing astrocytes were also observed (Pineau and Lacroix, 2007; Rice et al., 2007). As the widespread expression of IL-1β mRNA (<45–60 min) precedes that of MCP-1 mRNA (from 45 min to 2 days), we speculate that IL-1β derived from microglia may have stimulated astrocytes and induced MCP-1 expression. However, a recent study by the Keane laboratory has shown that neurons could also be accountable for the rapid release of the mature and biologically active form of IL-1β, which has been detected as early as 15 min after SCI (de Rivero Vaccari et al., 2008). This particular study yielded several important discoveries, among them the finding that NALP1 inflammasome proteins are expressed by spinal cord neurons and that SCI triggers rapid changes in their expression patterns. Thus, the source of IL-1β after SCI may come from different types of neural cells including microglia, astrocytes, and neurons. One way to study the contribution of each of these cell types to the production of IL-1β will be to generate conditional knockout mice.

IL-1β is considered one of the most important ligands associated with CNS damage (Allan et al., 2005). To become biologically active, IL-1β has to be cleaved by caspase-1, and many studies have reported that this processing is ATP-dependent (for reviews (Francchi et al., 2009; Mariathasan and Monack, 2007; Martinon et al., 2009)). Growing evidence suggests that ATP is an important signaling transmitter released by stressed and injured cells. In vivo bioluminescence imaging of SCI rats has recently revealed the presence of high levels of ATP during the first few hours post-injury (Wang et al., 2004). This rapid release of ATP at 2 h after SCI (the earliest time point analyzed in the Wang study) is very similar to the spatial distribution of IL-1β and MCP-1 mRNAs at 45 min and 3 h, respectively. Like IL-1β and MCP-1 mRNA expression, fewer ATP molecules were detected at the lesion site compared to the peri-traumatic zone during the early acute phase of SCI. However, the situation changed drastically at 24 h after injury, at which point ATP levels had returned to baseline in the peritraumatic zone but increased significantly at the site of impact. In agreement with this observation, we found that IL-1β- and MCP-1-expressing cells were mostly restricted to sites of trauma starting at 12 h and 2–4 days, respectively (this study & (Pineau and Lacroix, 2007)). Based on these results, we hypothesize that ATP released in the pericellular space could be the signal that induces astrocytic MCP-1 expression, through the release of IL-1β by nearby glial cells and neurons. Supporting this hypothesis are studies which showed that ATP mediates the rapid chemotactic response of parenchymal microglia after brain trauma (Davalos et al., 2005). Interestingly, Panenka et al. have provided in vivo evidence that MCP-1 expression by astrocytes is significantly attenuated in the presence of Suramin, an inhibitor of most P2X nucleotide receptors (Panenka et al., 2001).

Although it has been shown that IL-1β is the main IL-1 ligand expressed in the injured spinal cord (Bartholdi and Schwab, 1997), it is well documented that IL-1α can also bind to IL-1R1 and that this interaction results in the expression of multiple inflammatory genes. A characteristic that is unique to IL-1α and not seen with IL-1β, however, is that its precursor form, pro-IL-1α, is biologically active and constitutively expressed either inside the nucleus or bound to the plasma membrane of many different cell types (Arend et al., 2008; Dinarello, 2009). This raises the possibility that pro-IL-1α might be released from dying cells upon injury, placing this cytokine in an ideal position to initiate inflammation. Along these lines, work done by the Rock laboratory has recently shown that activation of the IL-1R1 by IL-1α is a key pathway required for neutrophil recruitment to injured cells in peripheral tissues (Chen et al., 2007). In agreement with this, we have found that MyDB8/IL-1R1 signaling is critical for the expression of neutrophil chemotactic genes KC and MIP-2 at sites of SCI. Using flow cytometry and immunohistochemical methods, we have further established that neutrophil recruitment is severely compromised in the spinal cord of injured MyDB8- and IL-1R1-ko mice. However, we do not know at this point whether IL-1α and/or IL-1β were involved in these responses. Future experiments performed using IL-1α-, IL-1β-, and IL-1α/β-ko mice will help provide useful information regarding the IL-1 ligand regulating neutrophil influx after neural injury.

As demonstrated by the present study, the spatio-temporal expression pattern of MCP-1 is noticeably different than those of the two main neutrophil chemotactic chemokines, i.e., KC and MIP-2. In contrast to KC and MIP-2, MCP-1 expression persisted for a prolonged period of time after injury. The MCP-1 response observed at the lesion site between Day 1 and Day 28 correlates well with the time course and distribution of monocyte recruitment into the injured mouse spinal cord, which typically starts at about 24 h before reaching a peak at 4–7 days and then slowly decreasing over the next few weeks to months (Sroga et al., 2003). Not surprisingly, monocyte recruitment and myelin debris clearance are significantly delayed in the injured spinal cord of mice deficient in CCR2 (Ma et al., 2002). It should be pointed out that our colocalization studies showed that monocytes and/or activated microglia recruited at the lesion site are mainly responsible for the production of MCP-1 during the subacute phase of SCI, in agreement with results obtained with another model of CNS insult, the ischemia model (Gourmala et al., 1997). Another key finding of our study is that regulation of MCP-1 expression seems to be linked to the cell type involved. Unlike astrocytes, monocytes and neurons, for example, appear to produce MCP-1 via mechanisms that are less dependent on IL-1R1 or the adaptor protein MyD88. Although found in much reduced numbers in MyDB8- and IL-1R1-ko mice at 4 days post-SCI, monocytes that succeeded in infiltrating the injured spinal cord of these animals were visibly capable of synthesizing the chemokine. Furthermore, our results failed to demonstrate any significant effect of MyDB8/IL-1R1 deficiency on neuronal MCP-1 expression. In the sciatic nerve lesion model, lumbar spinal cord motoneurons express MCP-1 in a MyDB8-independent fashion (S. Lacroix, unpublished observation). This indicates that among all CNS cells with neuroimmune function, astrocytes are probably those that depend the most on the MyDB8/IL-1R1 signaling pathway to recruit leukocytes, in particular neutrophils and “inflammatory” monocytes. Perhaps the most important finding of our study is that neutralization of the MyDB8/IL-1R1 signaling pathway almost completely prevented neutrophil recruitment in the injured mouse spinal cord. Although neutrophils recruited via MyDB8 signals were previously shown to mediate host defense in experimental brain abscesses caused by infection with bacteria (Kielland et al., 2007), the importance of these cells in the traumatized nervous system in the absence of pathogenic conditions remains the object of intense debate. Earlier studies have consistently reported that the attenuation of neutrophil infiltration in the injured spinal cord is associated with improved sensory, autonomic, and locomotor function.
functions (Ditor et al., 2006; Farooque et al., 1999; Gris et al., 2004; Hamada et al., 1996; Taoka et al., 1997). More recently, de Rivero Vaccari et al. found that immunoneutralization of the inflammatory protein ASK reduced activation and processing of caspase-1 and IL-1β, decreased spinal cord lesion volume, and improved functional recovery (de Rivero Vaccari et al., 2008). These results contrast, however, with those of Stirling et al., who found that at least one population of Gr-1+ leukocytes is required for tissue healing after SCI (Stirling et al., 2009). As neutrophils and type I "inflammatory" monocytes express the Ly6C/G (Gr-1) antigen, although to different levels, it will be of interest in future studies to investigate the individual contribution of each cell subset to damage and repair mechanisms. In summary, we have established that astrocytes are mainly responsible for the rapid synthesis of MCP-1, KC, and MIP-2 in the injured mouse spinal cord, suggesting an important role for these cells in leukocyte recruitment and gliosis. We also provided in vivo evidence that the proinflammatory cytokine IL-1 is a key regulator of the astroglial expression of these chemokines after SCI. Finally, we have demonstrated that the infiltration of neutrophils and, to a lesser degree, type I "inflammatory" monocytes is compromised in the absence of MyD88/IL-1R1 signaling. Such knowledge will be important to further define and understand the molecular and cellular events regulating inflammation in the injured nervous system. Understanding the biology behind immune cell recruitment and gliosis will be critical for developing new therapeutic strategies to prevent tissue damage and promote neural repair and regeneration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbi.2009.11.007.

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