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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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ABSTRACT

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.

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 (Basu 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

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be in an ideal position to initiate gliosis and immune cell recruitment in 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^+ $CCR2^+$ CX_3CR1^{lo}) (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^- $CCR2^-$ CX_3CR1^{hi}). 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 (Blight, 1992; Carlson et al., 1998; Dusart and Schwab, 1994; Stirling and Yong, 2008). 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-ko 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 mutant mice; $TLR4^d$) 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 $TLR4^d$ ($n = 3$) 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. Overlying 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 cryostat. Sections were collected directly onto slides having a permanent positive charged surface (Surgipath 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 MCP-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