The Journal of Neuroscience, July 1, 2001, 21(13):4649–4656

MIP-1α, MCP-1, GM-CSF, and TNF-α Control the Immune Cell Response That Mediates Rapid Phagocytosis of Myelin from the Adult Mouse Spinal Cord

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The slow immune response in the adult mammalian CNS results in slow myelin phagocytosis along degenerating white matter after injury. This has important consequences for axon regeneration because of the presence of axon growth inhibitors in myelin. In addition, abnormal immune cell responses in the CNS lead to demyelinating disease. Lysophosphatidylcholine (LPC) can induce an inflammatory response in the CNS, producing rapid demyelination without much damage to adjacent cells. In this study, we searched for the molecular switches that turn on this immune cell response. Using reverse transcription PCR analysis, we show that mRNA expression of macrophage inflammatory protein-1α (MIP-1α), macrophage chemotactic protein-1 (MCP-1), granulocyte macrophage-colony stimulating factor (GM-CSF), and tumor necrosis factor-α (TNF-α) in the spinal cord is rapidly and transiently upregulated after intraspinal injection of LPC. Neutralizing these signaling molecules with function-blocking antibodies suppresses recruitment of T-cells, neutrophils, and monocytes into the spinal cord, as well as significantly reduces the number of phagocytic macrophages and the demyelination induced by LPC. These findings will have important implications for CNS regeneration and demyelinating disease.

Key words: lysophosphatidylcholine; myelin; cytokine; chemokine; macrophage; T-cells

The immune cell response in the adult mammalian CNS generally occurs at a slower rate than in non-CNS tissues (Perry et al., 1987; Stoll et al., 1989a,b). This inadequate immune reaction underlies the slow removal of myelin and axonal debris distal to the site of injury during Wallerian degeneration in the CNS. Wallerian degeneration takes several weeks to months to complete in the CNS (Bignami and Ralston, 1969; Perry et al., 1987; George and Griffin, 1994) but occurs within 7–14 d in injured peripheral nerves (Griffin et al., 1992; George and Griffin, 1994). Slow myelin clearance has important implications for axon regeneration in the injured CNS because of the presence of axon growth inhibitors in myelin (David, 1998; Bandtlow and Schwab, 2000). The robust regeneration of peripheral nerves, despite the presence of inhibitors in peripheral nerve myelin (Bahr and Przyrembel, 1995; David et al., 1995; Shen et al., 1998), is likely attributable to the rapid clearance of myelin debris by immune cells after injury (Beuche and Friede, 1984; Griffin et al., 1992; George and Griffin, 1994). However, a rapid immune cell response leading to myelin phagocytosis can be provoked in the CNS under certain experimental conditions, such as after lysophosphatidylcholine (LPC) injections (Ousman and David, 2000).

LPC triggers a rapid and highly reproducible form of demyelination in the CNS without producing much damage to adjacent cells and axons (Hall, 1972; Jeffrey and Blakemore, 1993). It is therefore an ideal model to study the control of the immune response underlying rapid myelin phagocytosis. We have shown previously that LPC induces rapid macrophage recruitment (6–12 hr) and activation in the adult mouse spinal cord (Ousman and David, 2000). These macrophage responses were preceded and accompanied by recruitment of T-cells and neutrophils. Furthermore, the rapid activation of macrophages to the phagocytic state led to the removal of myelin debris within 4 d of LPC injection into the spinal cord. We have now performed experiments to identify the molecular triggers, i.e., chemokines and cytokines, that signal these immune cell responses.

Chemokines mediate chemotaxis, extravasation, and activation of leukocytes (Asensio and Campbell, 1999). These molecules induce leukocytes to migrate along concentration gradients and are cell type-selective chemoattractants, e.g., macrophage inflammatory protein-1α (MIP-1α) and macrophage chemotactic protein-1 (MCP-1) promote chemotaxis of monocytes and T-cells (Karpus and Ransohoff, 1998). In addition to chemokines, an immune response represents the differential actions of multiple cytokines. Besides immune cells, astrocytes and microglia are capable of producing many proinflammatory [e.g., interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α)], immunosuppressive [e.g., IL-10 and transforming growth factor-β (TGF-β)], and hematopoetic [e.g., macrophage-colony stimulating factor (M-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF)] (Campbell, 1998) cytokines. Regulation of cytokine and chemokine expression is complex, and understanding their interactions that ensue in an immune response is important, particularly in terms of enhancing myelin clearance during Wallerian degeneration in the injured CNS.

In this study, the expression and role of 11 chemokines and cytokines was examined after intraspinal injections of LPC. We provide evidence for the involvement of MIP-1α, MCP-1, GM-CSF, and TNF-α in the rapid recruitment of immune cells,

Received Dec. 8, 2000; revised March 30, 2001; accepted April 5, 2001.

This work was funded by Canadian Institutes of Health Research Grant 14828. S.S.O. was supported by a studentship from the Multiple Sclerosis Society of Canada.

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Table 1. PCR primers, annealing temperatures, and amplification product sizes

Cytokine–chemokine	Annealing temperature (°C)	5′ Primer (5′–3′)	3′ Primer (5′–3′)	PCR product (bp)
GAPDH	60	TGAAGGTCGGTGTGAACGGATTTGGC	CATGTAGGCCATGAGGTCCACCAC	982
IFN-γ	50	ATGAACGCTACACACTGCATC	AACAGCTGGTGGACCACTC	424
RANTES	55	CCTCACATCATCCTCAC	GCTCATCTCCAAATAGTTG	252
IL-10	55	CTGCTATGCTGCCTGCTCTTAC	CATTACTGGCCTTGTAGA	462
MIP-1α	60	ATGAAGGTCTCCACCACTG	GCATTCAGTTCCAGGTCA	268
IL-1α	55	TGTTCCTGAACTCAACTGTG	AGACAGGCTTGTGCTCTG	734
TNF-α	45	ATGAGCACAGAAAGCATG	GAAGACTCCTCCCAGGTA	604
MCP-1	65	ATGCAGGTCCCTGTCATG	GCTTGAGGTGGTTGTGGA	411
IL-1β	55	AAGTTTGTCATGAATGATTCCCTC	GTCTCACTACCTGTGATGAGT	263
TGF-β	55	TGGCTTCTAGTGCTGACG	ATCATGTTGGACAACTGC	1099
IL-4	60	AGCTAGTTGTCATCCTGC	GATGCTCTTTAGGCTTTCC	486
GM-CSF	55	TGTGGCTGCAGAATTTAC	GCTGTCTATGAAATCCGC	374

activation of macrophages, and clearance of myelin from the adult mouse spinal cord.

MATERIALS AND METHODS

Microinjections and tissue preparation for reverse transcription-PCR

Female BALB/c mice (8–12 weeks old) were deeply anesthetized with ketamine–xylazine (150 and 10 mg/kg, respectively), and spinal cord segments T12–L1 were exposed. One microliter of LPC (L1381; Sigma, St. Louis, MO) at a concentration of 2 μg/μl or 1 μl of sterile PBS was injected into the left half of the spinal cord immediately lateral to the midline dorsal artery using a 50- to 75-μm-diameter-tipped glass micropipette. Animals were killed after survival times of 0.5, 1, 3, 6, 12, 24, 48, and 96 hr by decapitation. A 5-mm-long portion of the spinal cord containing the injection site was immediately removed and placed in 1 ml of TRIZOL reagent (Life Technologies, Frederick, MD) at 4°C. For each experiment at every time point, three mice were injected with either LPC or PBS. Experiments were repeated with another set of mice. The tissues from each time point were pooled, and RNA was isolated according to the protocol of the manufacturer (Life Technologies).

Reverse transcription-PCR and Southern blotting

The appropriate 5′ and 3′ primers for PCR and the internal probes for Southern blotting were designed using Gene Runner, and the complete cDNA sequences were obtained from the NIH GenBank Entrez program. For reverse transcription, 1 μg/μl RNA from each sample was transcribed at 37°C using murine Moloney leukemia virus reverse transcriptase. For PCR, 10 μl of cDNA from each sample was amplified for various cytokines and chemokines using ampliTaq polymerase (N801–0060; PerkinElmer Life Sciences, Branchburg, NJ). The appropriate conditions [e.g., annealing temperatures ($MgCl_2$)] for each cytokine and chemokine were first established (Table 1) using a positive control consisting of lung tissue of BALB/c mice infected with *Pseudomonas* or spleen from malaria-infected mice before their expression in the experimental samples was determined. PCR for each cytokine and chemokine, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was performed within the linear range of amplification. GAPDH was amplified at 25 cycles and the chemokines and cytokines at 30 cycles. For Southern blotting, internal probes were labeled with [^{32}P]dCTP and hybridized with the appropriate cDNA oligonucleotide for 18 hr at 42°C. The results are expressed as a proportion of the optical density of GAPDH as scanned from the autoradiographs after Southern blotting. For each experiment, reverse transcription (RT)-PCR was performed two to three times with the same RNA sample.

Neutralizing antibody experiments

Microinjection and tissue preparation. Female BALB/c mice were anesthetized as described above. On the basis of the RT-PCR results, neutralizing antibodies against MCP-1 (18240D, hamster monoclonal; PharMingen, San Diego, CA), MIP-1α (AB-450-NA, goat polyclonal; R & D Systems, Minneapolis, MN), GM-CSF (1723–01, rat monoclonal; Genzyme, Cambridge, MA), and TNF-α (IP-400, rabbit polyclonal; Genzyme) were used for microinjections into the spinal cord. One microliter of a cocktail containing LPC (2 μg/μl) and the neutralizing antibodies individually or together (0.4 μg/μl each) was injected into the left side of the mouse cord between T12 and L1. Control animals received a 1 μl injection containing LPC (2 μg/μl) and the appropriate species and isotype-specific control Ig: hamster IgG (HM00; Cedarlane, Burlingame, CA), rat IgG (sc-2026; Santa Cruz Biotechnology, Santa Cruz, CA), goat IgG (sc-2028; Santa Cruz Biotechnology), and rabbit IgG (sc-2027; Santa Cruz Biotechnology). Six hours and 4 d after injection, the mice were killed by perfusion with 0.1 M phosphate buffer, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.5. Longitudinal cryostat sections of the spinal cord containing the injection site were used for immunohistochemistry.

Immunohistochemistry. This was performed to detect the following cell types: monocytes and microglia (rat monoclonal antibody, Mac-1); CD4+ T-cells (goat polyclonal; Santa Cruz Biotechnology); CD8+ T-cells (goat polyclonal; Santa Cruz Biotechnology), and neutrophils (rat monoclonal, Clone 7/4; Serotec, Oxford, UK). Immunohistochemistry was performed as described previously. Binding of the primary antibodies was revealed using the chromogen diaminobenzidine (D5905; Sigma) enhanced with nickel ammonium sulfate (Ousman and David, 2000). Sections were counterstained with 1% Neutral Red.

Quantification

The RT-PCR results for the cytokines and chemokines at each time point are expressed as a proportion of the corresponding optical density value of GAPDH as scanned from the Southern blot autoradiographs. As a consequence, the scales of the *y*-axes in Figure 1 are different because the intensity of the autoradiographic bands for each cytokine–chemokine varied. A total of 48 mice injected with LPC and 48 mice injected with PBS were used for RT-PCR. RT-PCR for MIP-1α, MCP-1, TNF-α, GM-CSF, RANTES (regulated upon activation, normal T expressed, and secreted), IL-1β, and TGF-β was performed two to three times with each of the two batches of mice, i.e., a total of four to five times. For each batch of mice, RNA from three mice were pooled for each of the eight survival times. RT-PCR for IL-4, IL-10, IL-1α, and interferon-γ (IFN-γ) were done two or more times using RNA from one batch of mice.

Counts of the various cell types in the white and gray matter were made from longitudinal sections of the spinal cord at 25× magnification using an ocular grid. Only cells containing a cell nucleus were counted. These estimates were obtained from three tissue sections, which were 45 μm apart and contained the injection site. Cell counts were obtained from regions that extended for 500 μm on either side of the injection site. Graphs depict the number of cells per square millimeter. Statistically significant differences between various experimental and control groups was determined using the Student's *t test*.

Epon embedding

Some of the mice used for the antibody blocking experiments were perfused with 0.5% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.5. One-millimeter-thick cross-sections of the

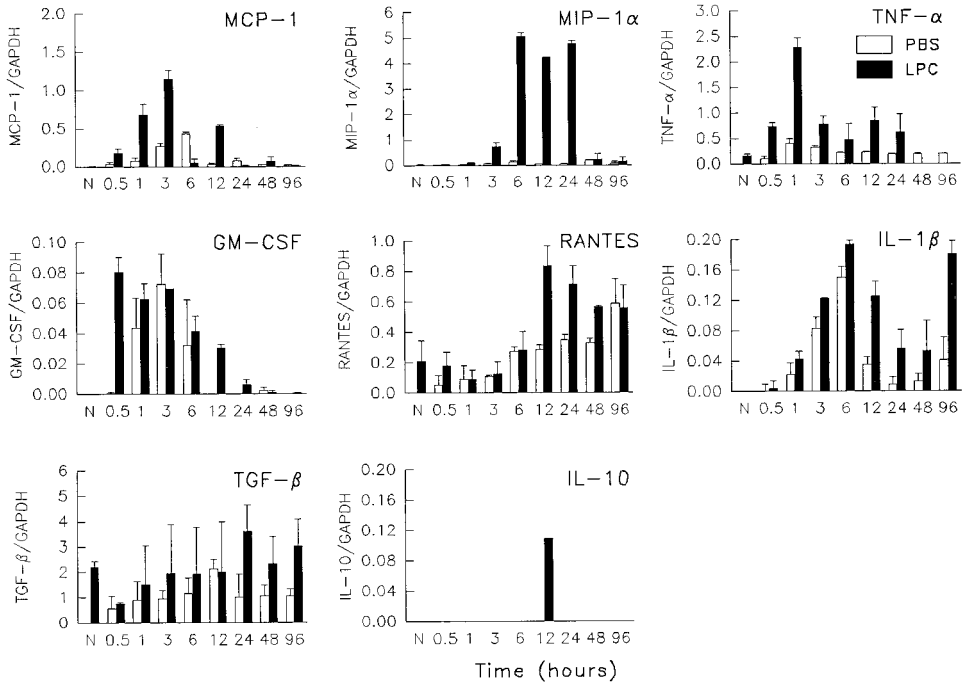

Figure 1. Time course of changes in cytokine and chemokine mRNA levels in the adult mouse spinal cord after injection of LPC and PBS. MCP-1, MIP-1α, TNF-α, and GM-CSF mRNA levels increased above that in PBS controls within 0.5–3 hr after LPC (■) injection compared with animals injected with PBS (□). Levels reached a peak between 0.5 and 6 hr. Upregulation of RANTES, IL-1β, and TGF-β mRNA was seen later between 12 and 96 hr. An upregulation of IL-10 was seen only in the LPC group at one time point, 12 hr. IL-1α, IFN-γ, and IL-4 mRNA was not detected at any time point in either the LPC- or PBS-injected mice. Graphs represent densitometric values that were normalized to GAPDH. *N* indicates normal uninjured spinal cord. Mean ± SEM.

spinal cord containing the injection site were post-fixed in 2% osmium tetroxide for 2 hr at room temperature and then processed for embedding in Epon. One-μm-thick cross-sections of the spinal cord were stained with 1% toluidine blue for light microscopy.

RESULTS

MIP-1α, MCP-1, GM-CSF, and TNF-α are expressed rapidly after intraspinal injection of LPC

We have shown previously that rapid demyelination in the adult mouse spinal cord induced by LPC is accompanied by recruitment of monocytes, T-cells, and neutrophils and activation of macrophages. These immune cell changes lead to myelin phagocytosis and demyelination. To identify the molecules that mediate these immune cell responses, we examined by RT-PCR the mRNA expression of 10 chemokines and cytokines after LPC or PBS injections into the adult mouse spinal cord. Of the 11 chemokines and cytokines examined, LPC induced rapid expression of MIP-1α, MCP-1, GM-CSF, and TNF-α in the spinal cord compared with PBS-injected controls (Fig. 1). In the normal, uninjured spinal cord, the mRNA of three of these molecules (MIP-1α, MCP-1, and GM-CSF) was not detectable by RT-PCR, whereas TNF-α was expressed at very low levels (Fig. 1).

The expression of MCP-1, GM-CSF, and TNF-α increased as early as 30 min after LPC injection compared with mice injected with PBS, whereas the level of MIP-1α mRNA increased by 3 hr (Fig. 1). Interestingly, the peak level of expression of GM-CSF was reached at 30 min, TNF-α at 1 hr, MCP-1 at 3 hr, and MIP-1α at 6–24 hr after LPC injection (Fig. 1), which precedes the LPC-induced recruitment of monocytes, neutrophils, and T-cells (6 hr) and activation of macrophages (12–96 hr) into mouse spinal cord (Ousman and David, 2000). At these times, MCP-1 and TNF-α levels were increased approximately fivefold after LPC compared with PBS injections, whereas MIP-1α mRNA was ~40-fold greater in the LPC versus the PBS group. At the 30 min time point and at 12 and 24 hr, GM-CSF mRNA expression was only detected in the LPC group. However, at 1–6 hr, GM-CSF mRNA level in the LPC group was not significantly different from PBS-injected controls (Fig. 1). The high expression of this cytokine at 1–6 hr in both groups of mice is possibly attributable to the mechanical injury induced by the microinjection pipette.

A delayed increase in mRNA expression was observed for other cytokines and chemokines. RANTES mRNA levels increased between 12 and 96 hr after injection of LPC and were at least twofold higher than in mice injected with PBS between 12 and 48 hr (Fig. 1). A threefold to fourfold higher expression of IL-1β was also detected in the LPC group between 12 and 96 hr. High levels of IL-1β mRNA were detected early after both LPC and PBS injections, at 3 and 6 hr, arguing for a mechanical injury-induced response at these time points. Increased expression of TGF-β mRNA was evident in the LPC group between 24 and 96 hr. Expression of IL-10 mRNA was detected only at 12 hr after LPC injection but not at any other times points or in the PBS group (Fig. 1). The mRNA for IFN-γ, IL-4, and IL-1α was not detected at any time points in either group of mice, although strong expression was detected in the positive control consisting of lung tissue infected with *Pseudomonas*. These data indicate that upregulation of the mRNA for MCP-1, MIP-1α, GM-CSF, and TNF-α after LPC injection occurs before and during the period of immune cell recruitment and activation (Ousman and David, 2000). These molecules are, therefore, good candidates to be involved in triggering the immune cell responses induced by LPC.

Blocking the activity of MCP-1, MIP-1α, GM-CSF, or TNF-α decreases LPC-induced activation of macrophages and immune cell recruitment

Effects on macrophage activation

We next used function-blocking antibodies to obtain direct evidence whether MCP-1, MIP-1α, GM-CSF, and TNF-α, which are rapidly expressed after intraspinal LPC injection, mediate the activation of macrophages in the spinal cord induced by LPC. We have shown previously that large, rounded, Mac-1$^+$ cells with

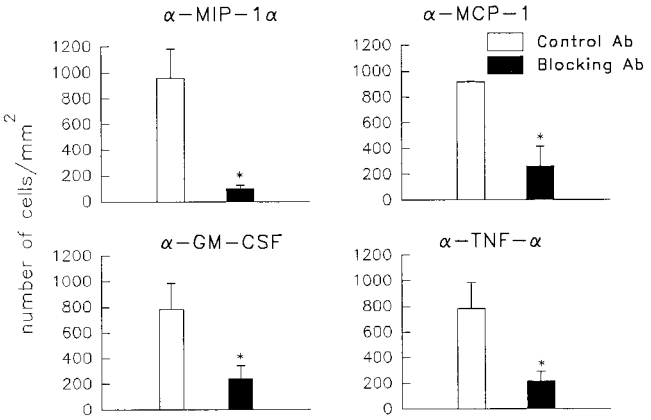

Figure 2. Neutralizing MIP-1α, MCP-1, GM-CSF, and TNF-α individually with function-blocking antibodies reduces the number of LPC-induced phagocytic macrophages. Graphs show the number of Mac-1$^+$ phagocytic macrophages in the white matter 4 d after injection of LPC along with blocking antibodies (■) or LPC plus control antibodies (□). Blocking each of these molecules resulted in a statistically significant reduction in the number of phagocytic macrophages compared with controls. Anti-MIP-1α injection produced the greatest reduction, ~10-fold. Mean ± SEM (*$p < 0.003$, MCP-1; $p < 0.003$, MIP-1α; $p < 0.02$, TNF-α; $p < 0.01$, GM-CSF); $n = 4$ animals.

clear unstained areas in the cytoplasm are activated macrophages that have phagocytosed myelin debris (Ousman and David, 2000). These Mac-1$^+$ cells are seen in the spinal cord within 4 d after LPC injection. Neutralizing antibodies against each of the four chemokines and cytokines were tested individually by injecting 1 μl of solution containing the antibody and LPC into the spinal cord. All four antibodies significantly decreased the number of large, round Mac-1$^+$ macrophages at 4 d after LPC injection compared with the LPC plus control Ig injections (Fig. 2). The largest effect was seen after blocking MIP-1α, which showed a 10-fold reduction of the number of activated Mac-1$^+$ macrophages (Fig. 2). Inhibition of MCP-1, GM-CSF, or TNF-α also induced a threefold to fourfold decrease in the number of activated macrophages (Fig. 2).

Because blocking the activity of MCP-1, MIP-1α, GM-CSF, and TNF-α individually resulted in only a partial reduction in the number of activated macrophages induced by LPC, we assessed whether inhibiting all four molecules together would lead to a more pronounced decrease. Few if any Mac-1$^+$-activated macrophages were detected in the spinal cord after neutralizing all four molecules together (Fig. 3*A–C*). The Mac-1 staining of the few large, round cells that were present was weaker than that seen in mice injected with LPC plus control Ig.

Effects on recruitment of monocytes, T-cells, and neutrophils

We have shown previously that activated macrophages seen after LPC injection into the spinal cord are likely to arise in part from monocytes recruited to the area within 6 hr (Ousman and David, 2000). Monocytes were identified on the basis of their size, shape, and Mac-1 immunoreactivity. We therefore examined whether the absence of LPC-induced Mac-1$^+$ phagocytic macrophages 4 d after neutralizing MCP-1, MIP-1α, GM-CSF, and TNF-α may be attributable to a lack of recruitment of monocytes from the peripheral circulation. Neutralizing all four molecules with blocking antibodies almost completely inhibited LPC-induced recruitment of monocytes into the spinal cord at 6 hr (Table 2). In contrast, the spinal cord of control mice injected with LPC plus

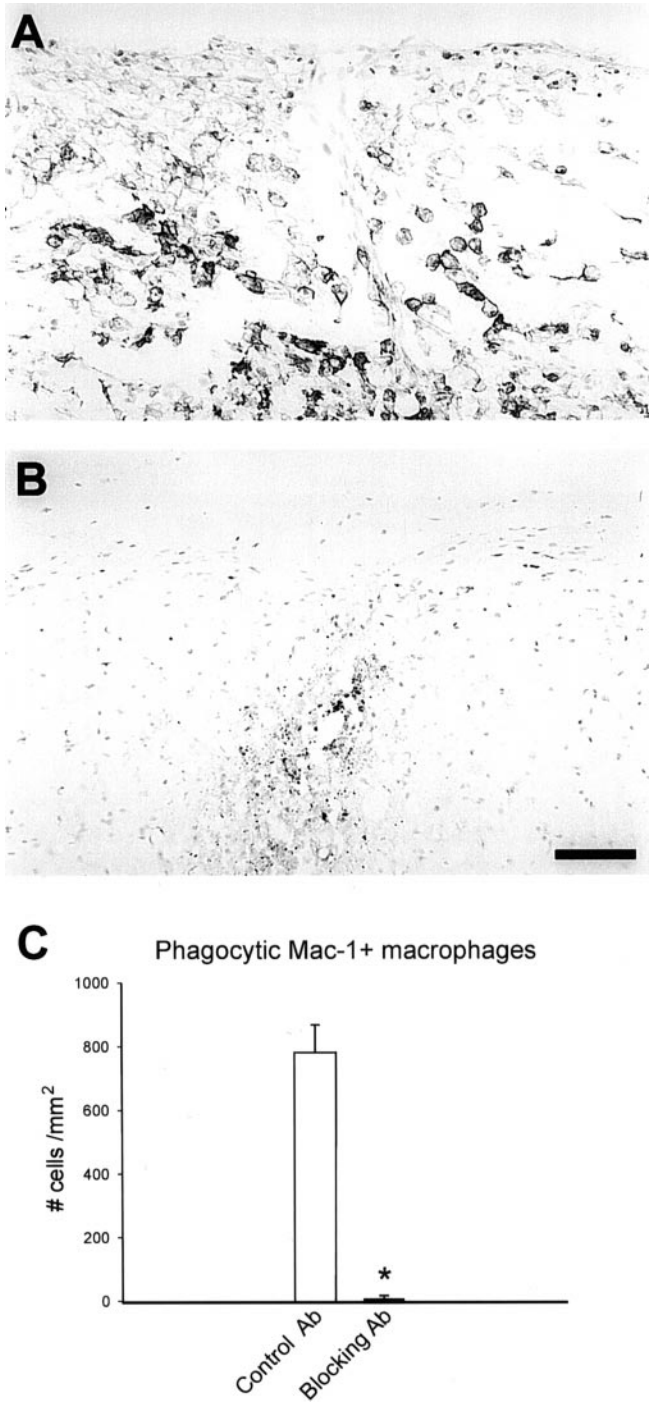

Figure 3. Neutralizing MIP-1α, MCP-1, GM-CSF, and TNF-α together results in marked reduction in the number of LPC-induced phagocytic macrophages. *A*, *B*, Mac-1 immunohistochemistry of longitudinal sections of the adult mouse spinal cord 4 d after injection of LPC along with either control antibodies (*A*) or all four neutralizing antibodies (*B*). In control animals, large, round Mac-1$^+$ macrophages that have clear areas in the cytoplasm are seen (*A*). In contrast, very few of these Mac-1$^+$ cells are seen in animals injected with LPC plus blocking antibodies (*B*). Sections were counterstained with Neutral Red. Scale bar, 80 μm. *C*, Quantification of Mac-1$^+$ macrophages in the spinal cord 4 d after injections of LPC along with either control antibodies (□) or blocking antibodies (■). Blocking MCP-1, MIP-1α, TNF-α, and GM-CSF together almost completely reduced the number of Mac-1$^+$ macrophages induced by LPC. Mean ± SEM (*$p < 0.001$); $n = 4$ animals. *Ab*, Antibodies.

Table 2. Immune cells in spinal cord 6 hr after injection of blocking anitbodies plus LPC

	Blocking Ab (# cells/mm^2)	Control Ab (# cells/mm^2)
Monocytes	8.4 ± 7.0	781.1 ± 87.7
CD4+ T-cells	0	326.7 ± 62.3
CD8+ T-cells	0	393.0 ± 27.7
Neutrophils	0	205.9 ± 37.6

Ab, Antibody.

control Ig contained a large number of monocytes. In addition to this effect on macrophage recruitment, only a few, weakly labeled Mac-1$^+$ ramified microglia were seen ~500 μm away from the injection site compared with controls (data not shown).

In addition to monocytes, LPC also induces an early and transient recruitment (6–12 hr) of T-cells and neutrophils into the white and gray matter of the adult mouse spinal cord (Ousman and David, 2000). Neutralizing MCP-1, MIP-1α, GM-CSF, and TNF-α together completely blocked the LPC-induced recruitment of CD4+ T-cells, CD8+ T-cells, and neutrophils into the spinal cord (Table 2).

Neutralizing MCP-1, MIP-1α, GM-CSF, and TNF-α prevents LPC-induced myelin phagocytosis and demyelination

To further assess whether neutralizing the activity of MCP-1, MIP-1α, GM-CSF, and TNF-α also results in impairment of myelin clearance, i.e., demyelination, we examined cross-sections of Epon-embedded spinal cord sections 4 d after intraspinal injection of all four blocking antibodies together with LPC. The area of demyelination in function-blocking antibody-injected animals was limited to a very narrow region immediately adjacent to the needle tract (Fig. 4*B*). In contrast, sections from mice injected with LPC and control Ig displayed a large area of demyelination (Fig. 4*A*). Neutralizing antibody treatment reduced the area of demyelination approximately eightfold (Fig. 4*C*). Furthermore, a threefold greater amount of myelin was preserved within areas showing demyelination in neutralizing antibody-treated mice compared with controls (Fig. 4*D*). These results clearly show that the MCP-1, MIP-1α, GM-CSF, and TNF-α induce immune cell responses that control myelin phagocytosis leading to demyelination.

DISCUSSION

In this study, we provide evidence that MCP-1, MIP-1α, GM-CSF, and TNF-α mediate rapid recruitment of monocytes, T-cells and neutrophils, and activation of macrophages in the adult mammalian CNS in response to the demyelinating agent LPC. In addition, we show that blocking the activity of all four of these molecules suppresses the rapid demyelination characteristically induced by LPC. We therefore present the first clear evidence that these four chemokines and cytokines play a key role in initiating the immune cell responses that lead to rapid phagocytosis and clearance of myelin from the adult mammalian CNS. These findings have important implications for stimulating rapid myelin clearance during Wallerian degeneration in the CNS, as well as provide additional insights into the role of chemokines and cytokines in the pathogenesis of demyelinating diseases, such as multiple sclerosis (MS) and experimental allergic encephalomyelitis (EAE).

Chemokines and cytokines that mediate immune cell responses leading to rapid clearance of CNS myelin

Immune cell responses generally occur very slowly in the CNS. However, during LPC-induced demyelination T-cells, neutrophils and macrophages are recruited into the CNS within hours (Ousman and David, 2000). We now show that these cellular responses are accompanied by a rapid upregulation in the expression of MCP-1, MIP-1α, GM-CSF, and TNF-α mRNA as early as 30 min to 3 hr after injection of LPC into the adult mouse spinal cord. In addition, this high level of expression is maintained for 12–24 hr.

MCP-1 is a potent chemoattractant for monocytes, whereas MIP-1α induces chemotaxis of both T-cells and monocytes (Karpus and Ransohoff, 1998). These chemokines are therefore likely to be involved in promoting the migration of T-cells and monocytes into the CNS after LPC injection (Ousman and David, 2000). Activated T-cells, astrocytes, microglia, and monocytes secrete MCP-1 and MIP-1α (Ransohoff and Tani, 1998; Asensio and Campbell, 1999), and all are possible sources of these two chemokines after LPC injection. Furthermore, TNF-α can induce MCP-1 expression in astrocytes *in vitro* (Hurwitz et al., 1995; Guo et al., 1998), which hints at a complex interplay between cell types and their secreted molecular signals in generating the cytokine–chemokine profile seen after LPC injection.

The activation of macrophages to phagocytose the damaged myelin in the LPC–demyelination model may be facilitated by the increased presence of TNF-α and GM-CSF. GM-CSF stimulates proliferation of microglial cells *in vitro* and the phagocytic activity of CNS macrophages *in vivo* (Giulian and Ingeman, 1988). In degenerating peripheral nerves, this cytokine induces activation of peripheral macrophages (Saada et al., 1996). The rapid upregulation of GM-CSF in the LPC-injected mice argues for a local CNS source, likely astrocytes. These glial cells are capable of expressing GM-CSF *in vitro* (Ohno et al., 1990) and are suggested to secrete a GM-CSF-like activity that was detected in injured CNS (Giulian et al., 1990). In addition to its macrophage activation role (Philip and Epstein, 1986), TNF-α is implicated in promoting the demyelination seen in MS and EAE because of its damaging effects on myelin and oligodendrocytes *in vitro* (Robbins et al., 1987; Selmaj and Raine, 1988). However, its brief upregulation in the spinal cord after LPC injection likely prevents any substantial injury to adjacent cells. In fact, axons that are stripped of their myelin sheaths after injection of LPC appear morphologically intact (Hall, 1972; Jeffrey and Blakemore, 1993).

With respect to the other cytokines and chemokines we investigated, several interesting observations were noted. IL-1β expression displayed a biphasic response (at 6 and 96 hr) after LPC and PBS injections. This response was much more pronounced and significantly higher in the LPC group at 96 hr. A biphasic (1 and 6 hr) increase in IL-1β mRNA has also been reported after spinal cord hemisection (Bartholdi and Schwab, 1997). The initial expression was attributed to a CNS cellular source, likely microglia, and resurged by the later inflammatory cell influx. Streit et al. (1998) have also suggested microglia to be the source of rapidly expressed IL-1β (within 1 hr) in contused rat spinal cord. The functional importance for the higher levels in the LPC group at the later time points is not known at present.

Interestingly, the temporal pattern of upregulation of MCP-1, MIP-1α, GM-CSF, and TNF-α mRNA precedes and/or accompanies LPC-induced recruitment of immune cells (6–12 hr) into the spinal cord and activation of macrophages (12–48 hr) (Ous-

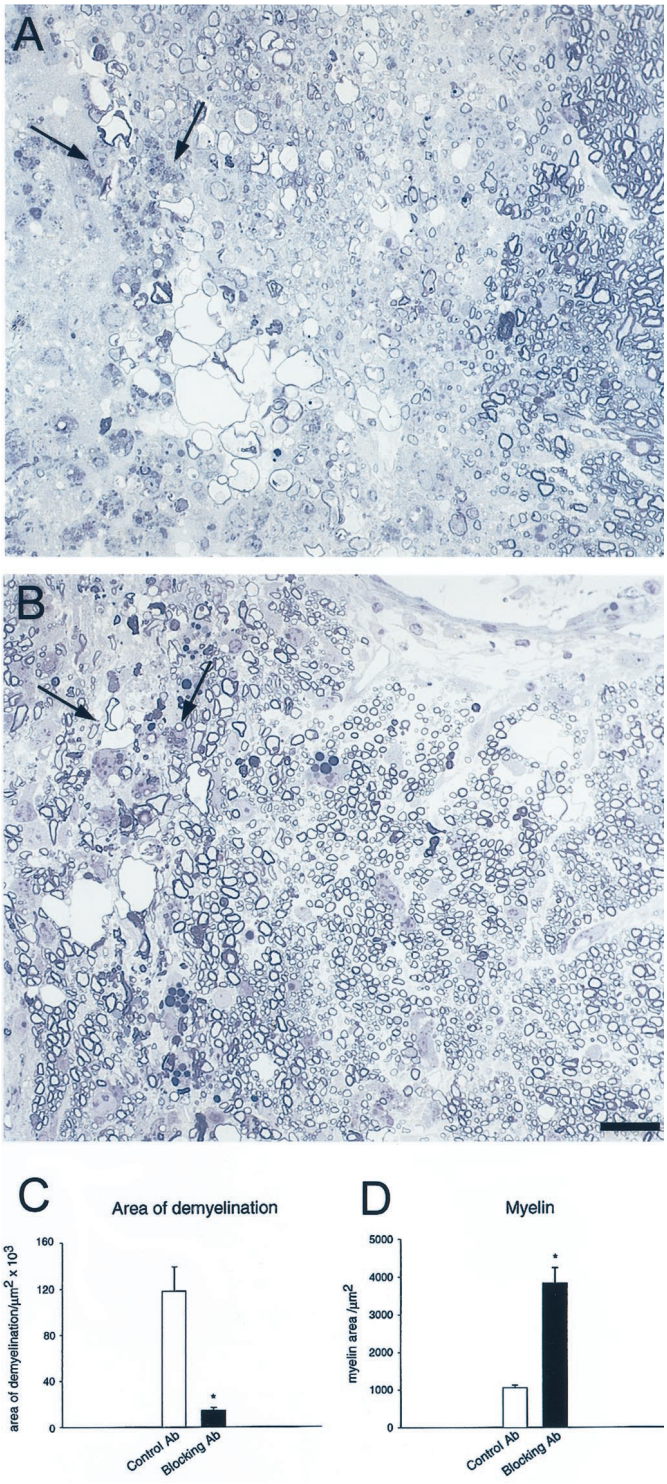

Figure 4. Neutralizing MCP-1, MIP-1α, TNF-α, and GM-CSF together reduces LPC-induced demyelination in the mouse spinal cord. *A*, *B*, Light micrographs of toluidine blue-stained Epon-embedded cross-sections of the spinal cord through the region of the dorsal columns, 4 d after injection of LPC plus either control antibodies (*A*) or all four blocking antibodies (*B*). In the control antibody-treated animal, LPC produces a wide area of demyelination (*A*). The size of this area is substantially reduced in mice treated with blocking antibodies (*B*). *Arrows* point to the injection sites. Scale bar, 25 μm. *C*, *D*, Quantification of the area of demyelination (*C*) and the amount of myelin present in a 15,000 μm^2 area on either side of the injection site (*D*) 4 d after injections of LPC plus either neutralizing antibodies (■) or control antibodies (□). Measurements were obtained from Epon-embedded sections of the mouse dorsal column. Neutralizing antibodies reduced the area of demyelination induced by LPC by approximately sixfold (*C*). In addition, myelin clearance within this area was reduced approximately four times in the antibody-treated mice compared with controls (*D*). Mean ± SEM (*$p < 0.008$, *C*; $p < 0.002$, *D*); $n = 3$ animals. *Ab*, Antibodies.

man and David, 2000). Antibody neutralization of each of these four molecules significantly reduced macrophage activation. Furthermore, neutralizing all four together led to almost complete blocking of T-cells, neutrophils and monocytes, and activation of macrophages, suggesting that two or more of these four molecules are involved in or sufficient to promote the immune response seen after LPC injection. Blocking the function of these molecules with antibodies also ensued in a marked reduction in LPC-induced demyelination. These data therefore provide strong evidence for the contribution of these immunoregulatory molecules in inducing rapid immune cell changes and myelin phagocytosis in the adult mammalian CNS.

Can chemokines stimulate a safe and effective immune cell response?

Increased expression of several chemokines and cytokines, including MCP-1, MIP-1α, TNF-α, and IL-1β, is also seen at the immediate site of CNS injury (Bartholdi and Schwab, 1997; McTigue et al., 1998; Streit et al., 1998; Lee et al., 2000) and have been implicated in the immune cell response seen at this site (Perry et al., 1987; Dusart and Schwab, 1994; Popovich et al., 1997; Schnell et al., 1999). Infiltrating immune cells are also implicated in mediating oligodendrocyte and myelin damage, leading to demyelination after CNS trauma (Blight, 1994; Popovich et al., 1999), and in MS and EAE (Huitinga et al., 1990; Tran et al., 1998). These immune cells, as well as CNS resident cells, can release toxic molecules that can cause tissue damage (Selmaj and Raine, 1988; Merrill et al., 1993). However, the LPC-induced immune response produces demyelination without much damage to axons or cells. What then may account for the rapid and safe immune response seen in the LPC model compared with the tissue damage seen in EAE or after spinal cord injury? The time course of expression of cytokines and chemokines may provide some answers. MCP-1, MIP-1α, TNF-α, and GM-CSF were expressed within 0.5–3 hr after LPC injection and returned to control levels by 24 hr. In EAE, however, MIP-1α, MCP-1, and TNF-α expression is seen later, between 10 and 18 d (Hulkower et al., 1993; Godiska et al., 1995; Karpus et al., 1995; Glabinski et al., 1997). This may account for the later and prolonged appearance of immune cells in EAE (Fritz et al., 1983; Hickey et al., 1983). Therefore, after LPC injection, any damaging effects that these chemokines and cytokines could mediate would be aborted rapidly because of the limited period during which they are expressed. Although proinflammatory cytokines (IL-1β and TNF-α) and chemokines (MCP-1 and MIP-1α) are upregulated rapidly (within 1–6 hr) after spinal cord injury (Bartholdi and Schwab, 1997; McTigue et al., 1998; Streit et al., 1998; Lee et al., 2000), massive tissue damage still ensues compared with the LPC-induced demyelination model. One possible explanation for this difference is the slightly later appearance (72 hr) of immunosuppressive TGF-β expression in contused spinal cord (Streit et al., 1998; McTigue et al., 2000) and the expression of IL-10 after LPC injection. Second, a contusion or hemisection injury creates a very large lesion, leading to widespread breakdown of the blood–brain barrier and massive influx of inflammatory cells

(Popovich et al., 1997; Schnell et al., 1999) secreting a plethora of cytotoxic mediators that can gain wider access to the CNS parenchyma. In this study, the mechanical injury to the cord was minimal, being sustained with a single injection with a 50-μm-diameter glass micropipette. It is also possible that the histological differences in the two models may be attributable to differences in the levels of these chemokines–cytokines, lower levels of which may serve to sculpt a more subtle and controlled response that limits tissue damage.

In addition to the downregulation of the proinflammatory chemokines and cytokines, TGF-β and IL-10 mRNA are upregulated at later times after LPC injection. Because these two cytokines are known to be capable of reducing the immune response (Tsunawaki et al., 1988; Lodge and Sriram, 1996), they can serve to control and dampen the continued progression of the inflammatory response after LPC injection. The overlapping expression of IL-10 with the appearance of "irregular-shaped" macrophages at 12 hr after LPC injection (Ousman and David, 2000) suggests that these cells and/or astrocytes (Cannella and Raine, 1995; Renno et al., 1995) are the most likely source of this immunosuppressive cytokine during LPC-induced demyelination. The profile of different chemokines and cytokines expressed in different experimental models are thus likely to influence whether one type of response is cytotoxic, whereas another can safely and effectively remove myelin or other debris from the adult mammalian CNS.

Possible chemokine–cytokine network involved in rapid myelin phagocytosis in the CNS

In our previous work, we showed that monocytes are recruited to the CNS at the site of LPC injection within 6–12 hr. T-cells are also seen at this time, which coincides with the expression of MIP-1α that has been shown to mediate T-cell recruitment via a very late antigen-1 (VLA-1)-mediated adhesion mechanism (Carr et al., 1996). VLA-1 is expressed within 3–6 hr of LPC injection (Ousman and David, 2000). The recruited T-cells may then be activated by perivascular macrophages to secrete TNF-α that could induce astroglial expression of MCP-1 (Hurwitz et al., 1995; Guo et al., 1998), a potent chemoattractant of monocytes. LPC has been shown *in vitro* to have chemotactic effects on monocytes (Quinn et al., 1988) and may also contribute to influx of monocytes after LPC injection (Ousman and David, 2000). Cytokines, such as TNF-α and GM-CSF, released by T-cells, astrocytes, or other cells could then lead to the activation of macrophages of both monocytic and microglial origin. Although we do not yet know which cell types are expressing which chemokine or cytokine, we know from our blocking experiments that MCP-1, MIP-1α, GM-CSF, and TNF-α are indeed involved in mediating rapid phagocytosis of CNS myelin damaged by LPC.

This work will have important implications for developing strategies to speed the rate of Wallerian degeneration in the CNS after trauma, as well as provide additional insights into our understanding of the pathogenesis of autoimmune demyelinating diseases, such as EAE and MS.

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