Abstract

Oligodendrocytes are vulnerable to CNS injury and disease. Because oligodendrocytes myelinate CNS axons, their death leads to demyelination and impaired axon conductance, which in turn contribute to neurologic deficits. Replacing oligodendrocytes requires proliferation and differentiation of endogenous NG2+ progenitor cells, a process that can be potently influenced by activated macrophages, which are present in most CNS pathologies. To examine the relationship between oligodendrocyte generation and macrophage activation in vivo, we compared the extent of oligodendrocyte loss and NG2 cell proliferation and differentiation after intraspinal microinjection of lipopolysaccharide (a Toll-like receptor-4 agonist) or zymosan (Toll-like receptor-2 agonist) in rats. Controls included injecting vehicle (sterile PBS; negative control) or lysolecithin (positive control for NG2 cell proliferation and oligodendrocyte differentiation). By 14 days postinjection, lipopolysaccharide injection sites displayed a significant rise in NG2 cell proliferation and oligodendrocyte differentiation, which exceeded that in vehicle and lysolecithin injections. Additionally, upregulated ciliary neurotrophic factor expression was present in lipopolysaccharide lesions. In contrast, zymosan-activated macrophages produced complete oligodendrocyte loss without stimulating NG2 cell proliferation, oligodendrocyte replacement, or ciliary neurotrophic factor expression. Zymosan also evoked a delayed lesion expansion and primary demyelination of intact myelinated axons around the lesions. These results clearly delineate the dichotomous potential of macrophage activation for influencing NG2 cell proliferation and oligodendrocyte differentiation. Because endogenous Toll-like receptor ligands are often present in injured CNS tissue, these results shed light on possible mechanisms that restrict oligodendrocyte replacement to specific domains of CNS trauma or disease sites.

Key Words: Cell genesis, Ciliary neurotrophic factor, Inflammation, Interleukin-1β, Myelin, Oligodendrocyte progenitor.

INTRODUCTION

Oligodendrocytes (OLs), the myelinating cells of the CNS, are especially vulnerable to injury and disease. For instance, traumatic injury to the spinal cord induces secondary events including excitotoxicity, ischemia, and free radical production (1–7). OLs that survive the initial trauma are vulnerable to these events and often undergo apoptosis during a protracted period of time (8–11). Because mature OLs are postmitotic, surviving OLs cannot contribute to cell replacement (12). Fortunately, new OLs can be generated from an endogenous population of progenitor cells, which are typically identified by platelet-derived growth factor receptor α and NG2 proteoglycan expression (13–19).

Activation and proliferation of NG2+ progenitors can lead to a dramatic increase in OL numbers. For instance, using a spinal contusion model, we showed that proliferating NG2 cells accumulate preferentially at the lesion/spared tissue interface, which is followed by robust OL differentiation, leading to OL numbers exceeding those in intact white matter (19). The extent of new OL formation within lesion subdomains probably depends on interactions between NG2 cells, environmental cues, and local cell populations. For instance, infiltrating hematogenous macrophages within spinal lesions will be activated by different ligands compared with macrophages along lesion borders, which mostly arise from endogenous microglia (20). Thus, interactions between NG2 cells and differentially activated microglia and macrophages could promote or inhibit OL differentiation from progenitor cells. Indeed, altering the macrophage constituency after demyelination drastically alters NG2 cell responses and remyelination (21, 22). Therefore, in this study we tested the hypothesis that differential activation of microglia and macrophages in the normal adult spinal cord would result in varying degrees of OL loss and replacement.

Previously, we and others have used intraparenchymal microinjections of potent macrophage-activating factors to examine the effect on axon integrity (23, 24). Although inflammosomes have also been used to evaluate activated microglia/macrophage effects on OL lineage cells (25–27), the role of differential macrophage activation specifically on OL formation is not known. Therefore, we extended previous studies by investigating whether activation of microglia and macrophages with different Toll-like receptor (TLR) ligands results in divergent effects on OL lineage cells. Specifically, we examined the effect of the TLR2 agonist zymosan and the TLR4 agonist...
lipopolysaccharide (LPS) on NG2 cell proliferation, OL loss, new OL formation, and expression of ciliary neurotrophic factor (CNTF), which can promote OL survival and differentiation (28–30). Our results demonstrate that activating microglia and macrophages with distinct TLR agonists evokes contrasting outcomes on OL lineage cells and tissue integrity. These results illustrate the importance of understanding the effector potential of differentially activated microglia and macrophages in injured CNS parenchyma and provide the basis for future studies to determine the mechanisms involved in these similar but clearly distinct signaling pathways.

MATERIALS AND METHODS

Microinjections

Adult female Sprague-Dawley rats (230–250 g) were anesthetized with ketamine (80 mg/kg i.p.) and xylazine (10 mg/kg i.p.) and given prophylactic antibiotics (Gentocin, 5 mg/kg s.c.). A laminectomy was performed at the T8 vertebral level using aseptic technique. After removal of the dura covering the exposed cord, a glass micropipette (custom pulled and beveled to an external tip diameter of 30–40 μm) was inserted 0.7 mm lateral to midline and 1.1 mm ventral to the surface of the cord. A pneumatic picopump (DKI, Tujunga, CA) was used to inject 200 nL of LPS (1 mg/mL, from Escherichia coli 055:B5; Sigma-Aldrich, St. Louis, MO), zymosan (1 mg/mL; Sigma-Aldrich), vehicle (0.1 M PBS), or lyssolecithin (0.1%, Sigma-Aldrich) (n = 15/group); because zymosan appeared to be the most toxic, we also examined the effect of a lower concentration or volume of zymosan on tissue pathology using additional groups of rats injected with either 50 nL of 1 mg/mL zymosan or 200 nL of 0.1 mg/mL zymosan (n = 4–5/group). After injection, the micropipette remained in place for 5 minutes to prevent back flow; the micropipette was then slowly removed and the injection site was marked with sterile charcoal (Sigma-Aldrich). After injection, the musculature surrounding the laminectomy was sutured, the skin was closed with wound clips, and each rat was given 5 mL of saline before being placed in warmed recovery cages.

Bromodeoxyuridine Administration

To label proliferating cells, the thymidine analog 5-bromo-2-´-deoxyuridine (BrdU) (50 mg/kg in sterile saline; Sigma-Aldrich) was injected intraperitoneally 1 hour after surgery and then once a day for 7 days postinjection.
Tissue Processing

To prepare for immunohistochemical identification of cell populations within lesions over time, injected rats survived 3, 7, or 14 days (n = 5/group). At the appropriate time, rats were deeply anesthetized with ketamine and xylazine and then perfused transcardially with PBS followed by 250 mL of 4% paraformaldehyde in PBS. Spinal cords were removed, postfixed for 2 hours at 4°C, and placed in 0.2 M phosphate buffer overnight. For tissue embedding, spinal cords were frozen on dry ice and cut into 4-mm blocks centered on the injection site. After submersion in OCT compound (Tissue-Tek), blocks were frozen, and 10-μm cross-sections were cut on a cryostat and mounted onto slides. Tissue was stored at −20°C until used.

A separate group of animals was used to obtain semi-thin Epon-embedded sections of the injection sites at 3 or 14 days postinjection (n = 2/group). Rats were perfused with 2% gluteraldehyde/4% paraformaldehyde in phosphate buffer. Spinal cords were blocked into 1.5-mm segments and processed for Epon embedding. Briefly, blocks were immersed in 2% osmium tetroxide for 2 hours, dehydrated with sequential ethanol washes (25, 50, 70, 80, 95, and 100%) and rinsed twice with propylene oxide. Tissue was impregnated with Spurr’s resin by immersing in propylene oxide/resin (1:1 for 1 hour and 1:2 for 1 hour) and then in 100% resin overnight. The tissue blocks were embedded in fresh resin for 4 hours and hardened at 60°C for 24 hours. Semi-thin sections were cut at 1 μm using an ultramicrotome (Leica Ultracut UCT) and stained with filtered toluidine blue (1:1 2% toluidine blue/2% sodium borate).

Immunohistochemistry

Sections were rinsed in 0.1 M PBS and blocked for nonspecific antigen binding using either 4% bovine serum albumin/0.1% Triton X-100/PBS (BP+) or 10% normal horse serum/PBS for 1 hour. Next, sections were incubated in primary antibody overnight at 4°C. Sections were rinsed and treated with mouse biotinylated antiserum (horse anti-mouse IgG 1:800 in BP+; Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. After rinsing, endogenous peroxidase activity was quenched using a 4:1 solution of methanol/30% hydrogen peroxide for 15 minutes in the dark. Sections were then treated with Elite avidin-biotin enzyme complex (Vector Laboratories) for 1 hour. Visualization of labeling was achieved by using either SG or 3,3-diaminobenzidine (Vector Laboratories). Sections were rinsed, dehydrated, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). Antibodies used included the following: anti-NG2 (1:1000; US Biological, Swampscott, MA); CC1 (antibody clone for oligodendrocytes, also called APC, 1:500; Oncogene, Boston, MA); anti-CD11b for microglia and macrophages (1:2000, Ox42 clone; Serotec, Raleigh, NC); anti-glia...
fibrillary acidic protein for astrocytes (1:4000; Sigma-Aldrich); anti-neurofilament for axons (1:2000; Developmental Studies Hybridoma Bank, Iowa City, IA); anti-ciliary neurotrophic factor (CNTF) (1:800; R&D Systems, Minneapolis, MN); and anti-BrdU for proliferating cells (1:200, G3G4; Developmental Studies Hybridoma Bank). To double-label axons and myelin, neurofilament immunohistochemistry was combined with Eriochrome Cyanine, which labels myelin. For this procedure, sections were rinsed in distilled water and treated with acetone for 5 minutes. Next, sections were sequentially incubated in Eriochrome Cyanine solution for 30 minutes in the dark, 5% iron alum for 3 minutes, and then a borax-ferricyanide solution for 1 minute. For BrdU immunohistochemistry, sections were denatured in 2N HCl at 37°C for 25 minutes before primary antibody incubation. CC1/glial fibrillary acidic protein-labeled sections were counterstained with methyl green and NG2-labeled sections were counterstained with neutral red. Immunofluorescence was used to identify CC1/BrdU+ cells using Alexa Fluor 546 anti-mouse secondary antibody (1:1000; Molecular Probes, Eugene, OR) for CC1 cells and Alexa Fluor 488 anti-mouse secondary antibody (1:1000; Molecular Probes) for BrdU.

Quantitative Real-Time Polymerase Chain Reaction

To collect tissue for polymerase chain reaction (PCR), rats were perfused with sterile saline (n = 3/group/time point). Spinal cord segments (2 mm) centered on the injection site (T8) or at T1 (for control tissue) were suspended in ice-cold TRIzol (Invitrogen, Carlsbad, CA) and homogenized. RNA was isolated according to previously described protocols (31) and quantified by spectroscopy, with A260/A280 ratios at pH 8.0 between 1.4 and 1.8 for all samples. Interleukin (IL)-1β-specific primer pairs (forward 5′ GAAGATGGAAAGCG GTTGG 3′ and reverse 5′ AACTATGTCGGACCATTGC 3′) were used to detect mRNA expression via quantitative real-time PCR as in previous studies (31). Briefly, cDNA was prepared from RNA by reverse transcription using commercially available kits that included murine leukemia virus and random primers (Applied Biosystems, Foster City, CA). PCR reactions were carried out using 10 ng of cDNA, 500 nmol/L concentrations of each primer, and SYBR Green master mix (Applied Biosystems) in 20-μL reactions. Levels of PCR product were measured using SYBR Green fluorescence collected during RT-PCR on an Applied Biosystems 7300 system (32). Standard curves were generated for each gene using a control cDNA dilution series. Melting point analyses were performed for each reaction to confirm single amplified products. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase before being expressed as fold changes from control segments rostral to the injection site.

Quantitative Analysis

A Zeiss Axioskop 2 Plus microscope (Zeiss, Thornwood, NY) with a Sony 970 3-chip color camera was used to analyze nonfluorescent sections containing the injection site.
Cells immunoreactive for NG2, NG2/BrdU, BrdU, or CC1 throughout the lesions were manually counted at 40×. Confocal microscopy (Zeiss 510 META laser scanning confocal microscope) was used to count fluorescently labeled CC1/BrdU+ cells in optical sections (≤1 μm) spanning the lesion sites. For control injections, vehicle caused no discernable tissue disruption; therefore, an area of tissue was analyzed that was equal to the average lesion size from the other groups. The criteria to count single-labeled or double-labeled cells included each profile having a well-defined border surrounding an identifiable nucleus. A cell was only counted if both criteria were met in the same plane of focus. Cell types were verified at higher power (64×) when needed. For all cell counts, data are expressed as cells per mm². To obtain the lesion area and activated microglia/macrophage density, low power (10×) images were digitized and manually outlined using image analysis software (MCID Elite, Imaging Research Inc., Canada). Lesions were defined as the area displaying the largest region of activated microglia and macrophages. The proportion of the lesion occupied by macrophages was calculated by dividing the area immunoreactive for Ox42 by the total lesion area.

CNTF within the lesions was quantified using a standardized sampling box (0.012 mm²), which was placed in the dorsal or lateral edge of each lesion for the first sample. The box was moved sequentially throughout the extent of each lesion to obtain nonoverlapping samples. Samples for each lesion were averaged to give 1 value per animal. To evaluate differences in outcome measures between the 4 groups (lysolecithin, LPS, zymosan, and control), 1-way analysis of variance (for CNTF data) or 2-way analysis of variance was used followed by Bonferroni post-tests with significance set at p < 0.05. Prism 4.03 (GraphPad Software, San Diego, CA) was used for all statistical analyses and graphs.

RESULTS

Characterization of Lesion Sites

All injections were targeted to the lateral white matter. Vehicle injections caused no overt tissue disruption or microglial activation at any time examined (Fig. 1A, B). LPS lesions extended from white matter, which appeared to have diminished myelin, into the adjacent gray matter. Macrophages were present throughout LPS lesions and were co-localized with myelin and axon profiles within edematous regions. CNTF within the lesions was quantified using a standardized sampling box (0.012 mm²), which was placed in the dorsal or lateral edge of each lesion for the first sample. The box was moved sequentially throughout the extent of each lesion to obtain nonoverlapping samples. Samples for each lesion were averaged to give 1 value per animal. To evaluate differences in outcome measures between the 4 groups (lysolecithin, LPS, zymosan, and control), 1-way analysis of variance (for CNTF data) or 2-way analysis of variance was used followed by Bonferroni post-tests with significance set at p < 0.05. Prism 4.03 (GraphPad Software, San Diego, CA) was used for all statistical analyses and graphs.

FIGURE 5. Plastic semithin sections at 14 days postinjection reveal lesion site characteristics. (A) Lysolecithin lesions contained numerous demyelinated axons (arrow) and phagocytic macrophages (arrowhead). (B) White matter portions of LPS injection sites contained numerous oligodendrocytes (arrowheads) and decreased intraparenchymal swelling compared with Figure 4B. Scattered demyelinated axons (arrows) remained. (C) The interior region of zymosan lesions contained densely packed macrophages (arrowheads), whereas the outer rim displayed a large number of demyelinated axons (arrows); this result confirms the conclusion that demyelination was present in the 14-day Eriochrome Cyanine/neurofilament-labeled sections (see Fig. 3C). Scale bar = (A–C) 20 μm.

FIGURE 6. BrdU+ cells labeled during the first week were quantified at 7 and 14 days after intraspinal injections. Numbers of cells dividing during the first week were significantly greater than those for controls in all groups (*, p < 0.05; ***, p < 0.001). During this time, cell division in the zymosan (Zym) lesion was greater than that in lipopolysaccharide (LPS) lesions (***, p < 0.001 vs Veh; ++++, p < 0.001 vs Zym). Between 7 and 14 days after injections, BrdU+ cells were maintained at an elevated level in LPS lesions. In contrast, BrdU+ cells decreased by 97% in Zym lesions. At this time, LPS lesions contained significantly more BrdU+ cells than Zym lesions (++++, p < 0.001, LPS vs Zym).
white matter (Fig. 1C, D). As we have noted previously (24), intraspinal zymosan created focal lesions filled with densely packed macrophages. These lesions were devoid of axons and myelin; tissue adjacent to the lesions appeared normal at 3 and 7 days (Fig. 1E, F); by 14 days postinjection, lesions had expanded, and white matter adjacent to the lesions displayed prominent demyelination (see below). Zymosan lesions created with lower concentrations or volumes produced similar pathology (data not shown).

On the basis of labeling with multiple antibodies, LPS and zymosan lesion sizes remained constant between 3 and 7 days postinjection. Between 7 and 14 days, however, the area of tissue pathology in LPS- and zymosan-injected sites changed dramatically. In LPS-injected spinal cords, lesion size, including the area of macrophage activation, decreased ~66% between 7 and 14 days postinjection (Figs. 2, 3). During this same time, zymosan lesions tripled in size and incorporated previously intact tissue (Figs. 2, 3). The resulting lesion contained a larger central core devoid of axons and myelin surrounded by a thick rim of apparently intact but demyelinated axons (Fig. 3).

Semithin Epon-embedded sections confirmed tissue pathology 3 and 14 days after injections. At 3 days, no discernible tissue disruption was evident in vehicle-injected areas (Fig. 4A). LPS lesions contained mostly intact myelinated axons that displayed increased spacing indicative of edema; occasional demyelinated axons were noted (Fig. 4B). Zymosan lesions contained densely packed macrophages and a total absence of axons; tissue adjacent to the lesions displayed intact myelinated axons (Fig. 4C). As expected, lysolecithin lesions contained numerous demyelinated axons and myelin debris (Fig. 4D). At 14 days, most axons remained demyelinated in lysolecithin lesions, whereas OLs and myelinated axons were present throughout the white matter portion of LPS lesions (Fig. 5A, B). In contrast, as noted above, zymosan lesions had markedly increased in size and were clearly surrounded by a rim of demyelinated axons (Fig. 5C).

Cell Proliferation Within the Injection Sites

The purpose of characterizing these lesion models was to determine whether cellular response patterns, especially of OL lineage cells, were comparable using different ligands to activate macrophages. To that end, we first examined cell proliferation in animals receiving BrdU for 7 days after injection. At 7 days postinjection, LPS and zymosan lesions contained significantly more BrdU+ cells than vehicle lesions contained many BrdU+ cells at 3 days, none of which expressed NG2.

**FIGURE 7.** NG2 cells and NG2/BrdU cells were elevated in lipopolysaccharide (LPS) injection sites. (A) NG2 cells in each injection site were quantified. By 14 days, a protracted rise in NG2 cells was noted in LPS lesions, which were elevated, compared with vehicle (Veh) and zymosan (Zym) injection sites. (B) In LPS lesions, NG2/BrdU cells were elevated at 3 and 7 days compared with Veh and Zym groups. (C) Example of NG2/BrdU labeling from vehicle-injected white matter at 3 days postinjection; no double-labeled cells are visible. (D) Many double-labeled NG2/BrdU cells were present in LPS lesions at 3 days. (E) Zymosan lesions contained many BrdU+ cells at 3 days, none of which expressed NG2. (A, B) Data represent mean ± SEM. **, p < 0.01; ***, p < 0.001 versus Veh; +++, p < 0.001 versus Zym. Scale bar = (C–E) 50 μm.
controls; BrdU+ cell numbers in zymosan lesions were 34-fold higher compared with vehicle controls and twice those found in LPS lesions (Fig. 6).

To determine whether newly generated cells were maintained in the lesions, the 14-day group of animals also received BrdU for the first 7 days after injections. Between 7 and 14 days postinjection, BrdU+ cell number remained constant in LPS lesions (Fig. 6). In zymosan lesions, however, BrdU+ cells were virtually absent at 14 days, indicating that the numerous dividing cells in zymosan lesions at 7 days were no longer present at 14 days. Using BrdU/Ox42 immunohistochemistry, it was clear that BrdU+ cells in 7-day zymosan lesions consisted almost entirely of Ox42+ macrophages (data not shown). This result suggests that the BrdU-negative macrophages present at 14 days may be a new population that entered subsequent to 7 days after zymosan injection.

NG2 Cell Distribution and Proliferation

To determine how putative OL progenitor cells were affected by each agent, we determined the average number of NG2 cells and NG2/BrdU cells within the lesions. After vehicle injection, the number of NG2 and NG2/BrdU+ cells was low (Fig. 7A–C) and was comparable to that observed previously in naive spinal cords (33). In LPS injection sites, NG2 cell number was comparable to that for vehicle controls at 3 days (~50 cells/mm²) (Fig. 7A); however, almost all NG2 cells present appeared to be dividing (~46 cells/mm²) (Fig. 7B, D). Accordingly, at 14 days postinjection, a significant rise in total NG2 cell number was observed (Fig. 7A), which was comparable to that seen in lysolecithin lesions (data not shown). In contrast, zymosan lesions contained only rare NG2 cells and no NG2/BrdU cells at any time (Fig. 7A, B, E).

Oligodendrocyte Replacement Within Injection Sites

Our work and that of others have shown that marked OL differentiation often occurs subsequent to NG2 cell infiltration and proliferation (17, 19). In previous work using CC1 immunohistochemistry, we showed that CC1+ cells expressed Olig2 but not NG2, indicating that they were differentiated OLs (19). Therefore, we used CC1/BrdU immunofluorescence and confocal microscopy to determine whether new OLs were generated within the different injection sites. At 3 days after injection, all groups contained few to no CC1/BrdU+ cells (Fig. 8). By 7 days, a striking increase in new OLs had occurred in LPS lesions; these new cells were maintained out to 14 days postinjection (Fig. 8B, D). Surprisingly, the CC1/BrdU cells in LPS lesions outnumbered...
those in lysolecithin lesions at 7 and 14 days postinjection (data not shown). In contrast, new OLs were not detected within the zymosan injection sites at any time (Fig. 8C, D).

Because formation of new OLs should theoretically lead to a rise in total OL numbers, we quantified the total number of CC1+ cells over time in each injection site. At 3 days after injection, all lesions contained significantly fewer OLs than vehicle controls, indicating that each toxin caused an initial OL loss (Fig. 9). Subsequently, the most dramatic increase in OLs was found in LPS lesions in which OL numbers rose >3-fold by 7 days and were maintained through 14 days (Fig. 9B, D). Again, OLs in LPS lesions significantly outnumbered those in lysolecithin lesions (data not shown). OLs were not detected in zymosan lesions at any time (Fig. 9C, D).

**Ciliary Neurotrophic Factor Expression Within the Lesions**

To investigate a potential mechanism for the LPS-induced increase in numbers of OLs, we evaluated the expression of interleukin-1β (IL-1β) in LPS-injected spinal cords. Previous work has shown that intraspinal injection of LPS leads to an increase in IL-1β+ cells (25). We verified the LPS-induced IL-1β expression in our model using quantitative real-time PCR (Fig. 10A). Indeed, IL-1β mRNA was significantly increased over control tissue at 12 hours postinjection and was >30-fold higher at 3 days post-injection. IL-1β is a potent inducer of CNTF expression, which is a prosurvival and differentiation factor for OLs (28–30). Therefore, we next examined whether lesion sites contained increased CNTF immunoreactivity. Notably, elevated CNTF expression was detected only in LPS injection sites (Fig. 10B–E). CNTF was slightly higher at 3 days and significantly elevated compared with controls at 7 days postinjection (Fig. 10B); levels had declined by 14 days. In accordance with previous data (34), CNTF was not detected in lysolecithin lesions; it was also not increased in zymosan lesions and in fact showed a trend for a reduction compared with vehicle-injected spinal cords (Fig. 10B, E).

**DISCUSSION**

CNS injury and disease sites typically contain heterogeneous subdomains in which neuroinflammatory-mediated injury and repair occur concomitantly. Because of this cooccurrence, identifying cellular and environmental constituents involved in each process is challenging. To shed light

![FIGURE 9](http://jnen.oxfordjournals.org/)

Sections double-labeled for CC1 (brown) and glial fibrillary acidic protein (black) and counterstained with methyl green were used to identify oligodendrocytes (OLs) in each injection site. (A–C) CC1 immunolabeling reveals OLs (arrows) in vehicle (Veh) (A) and LPS (B) but not zymosan (Zym) (C) injection sites at 7 days postinjection; astrocyte processes (black) are also visible in Veh and LPS injections. Normal OL density is observed in Veh injection sites, whereas markedly elevated numbers of OLs are present in LPS lesions. Scale bar = (A–C) 50 μm. (D) Quantification revealed a significant reduction in OLs in LPS and Zym lesions at 3 days compared with Veh lesions; numbers of OLs in Zym lesions were also significantly lower than those in LPS injection sites. At 7 and 14 days postinjection, numbers of OLs in LPS lesions were significantly greater than those in Veh or Zym lesions. Numbers of OLs in Zym lesions remained near zero through 14 days. Data represent mean ± SEM. **, p < 0.01; ***, p < 0.001 versus Veh; ++, p < 0.01; ++++, p < 0.001 versus Zym.
on interactions between 2 cell populations that are colocalized and affected in virtually all neuropathologies (i.e. OLs and macrophages), we used simple intraspinal models of neuroinflammation to examine the fate of OL lineage cells after differential macrophage activation. Specifically, small quantities of agonists for TLR2 or TLR4 present on microglia and macrophages were microinjected into the adult rodent spinal cord. This method for activating macrophages was nontraumatic and did not disrupt the cellular environment; therefore, any changes in OL lineage cells resulted, either directly or indirectly, from activation of microglia and macrophages. Results were compared with those for lysolecithin microinjections, which elicit macrophage activation due to myelin damage (35). Lysolecithin creates reproducible demyelinating lesions that undergo OL-mediated remyelination (17, 36–40); therefore, it served as a positive control for OL generation. Our data show that activating 2 similar but distinct TLRs, both of which evoke proinflammatory reactions, induced opposite effects on the endogenous population of OL lineage cells. The TLR2 agonist zymosan evoked total OL (and axon) loss without OL replacement. The TLR4 agonist LPS, in contrast, caused an acute loss of OLs followed by a dramatic rise in overall OL numbers. Indeed, within 7 days new OLs in LPS lesions exceeded those in vehicle-injected spinal cords. Furthermore, LPS lesions contained significantly more CNTF-positive cells, exemplifying the neurotrophic nature of the LPS lesion environment. Thus, if multiple TLR ligands are present in regions of CNS pathology, it is possible that neighboring macrophages could function in disparate ways in terms of tissue repair or destruction, depending on the type and/or combination of ligands activating each particular cell.

The function of TLRs is classically described as recognizing viral and bacterial pathogens. Their ligands, however, are not restricted to exogenous infectious agents. Indeed, ligands for TLRs are commonly present in regions of CNS pathology. For instance, heat shock proteins, which activate TLR4, are present in models of Alzheimer’s disease and cause accumulation of Aβ peptides in microglia (41). Another endogenous TLR4 ligand is fibronectin, which can be synthesized by CNS glial cells (42–44) and is elevated in the cerebrospinal fluid of patients with bacterial meningitis (45). Several other endogenous TLR ligands have been detected in regions of CNS pathology, including hyaluronan and biglycan (46). Moreover, mice lacking TLR2 or TLR4 display altered anatomical and functional phenotypes after spinal contusion revealing that endogenous TLR signaling occurs in regions of spinal cord pathology (31). Thus, it is feasible that activation of TLRs on microglia and macrophages influences their responses to CNS injuries and can thereby indirectly affect the fate of OLs. To clarify the role

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**FIGURE 10.** Increased expression of interleukin-1β (IL-1β) and ciliary neurotrophic factor (CNTF) in lipopolysaccharide (LPS) injection sites. Quantitative real-time polymerase chain reaction was used to quantify IL-1β mRNA at 12 and 72 hours postinjection in LPS injection sites (IS). For control tissue, mRNA was isolated from rostral (R) sections at the T1 vertebral level of the same animals that received LPS injections. (A) During the first 12 hours after injections, IL-1β was 31-fold higher in the LPS injection site compared with rostral sections. IL-1β remained >30-fold higher 72 hours postinjection. (B) Sample boxes were used to quantify CNTF immunoreactivity in vehicle (Veh), lysolecithin (Lyso), LPS, and zymosan (Zym) injection sites at 7 days; the proportion of CNTF in LPS injection sites was significantly greater than that in lesions from all other groups. (C–E) Examples of CNTF labeling from Veh (C), LPS (D), and Zym (E). Data represent mean ± SEM. In A, ***, p < 0.001 versus rostral control. In B, **, p < 0.01; ***, p < 0.001 versus LPS. Scale bar = (C–E) 50 μm.
of CNS TLRs, most studies have injected single TLR ligands peripherally or centrally and characterized the resultant pro-inflammatory cascades and neuron loss. Only a subset of studies have examined OL responses after TLR activation in vivo, and none, to our knowledge, directly compared LPS- and zymosan-induced effects on OLs. Consistent with our data, a recent study showed that intracerebral injection of LPS induced macrophage activation followed by recruitment and mobilization of OL progenitor cells (26). In contrast, however, Lehnardt et al (47) described microglial-mediated OL loss and demyelination after LPS injection into the brain. Similarly, Felts et al (25) observed marked demyelination throughout LPS lesions in the dorsal spinal cord. Although we also saw occasional demyelinated axons in LPS lesions, overall this was rare, making the OL generation noted in LPS lesions more striking as it was driven, directly or indirectly, by macrophage activation rather than by large numbers of bare axons. The differences between our results and those of others may be due to variation in the strains of LPS used, injection locations, and/or injection volumes/concentrations. For instance, in our study we used a 5-fold lower concentration and injection volume than that of Lehnardt et al (47).

Factors that promote OL progenitor proliferation and/or OL survival have been examined predominately in demyelinating lesions. For instance, insulin-like growth factor-1, transforming growth factor-β1, platelet-derived growth factor, and basic fibroblast growth factor are upregulated after demyelination (34, 48). Interestingly, microglia and macrophages can produce many of these factors (49, 50). For example, microglia promote OL progenitor survival and OL differentiation in a platelet-derived growth factor-dependent fashion in vitro (49). In addition, LPS exposure induces macrophage expression of neurotrophin-3 and nerve growth factor, both of which enhance OL formation and survival (51). Furthermore, depletion of peripheral macrophages after chemical demyelination reduced the typical upregulation of insulin-like growth factor-1 and prevented remyelination (22). The area of reduced macrophage infiltration correlated spatially with the loss of IGF-1, suggesting that hematogenous macrophages were the source of insulin-like growth factor-1 expression in this model of demyelination.

Macrophages and microglia could also indirectly promote OL differentiation by inducing growth factor release from astrocytes. For example, macrophage-derived tumor necrosis factor-α and IL-1β promote astrocyte production of neurotrophins and CNTF (52, 53). In the current study, we noted increased IL-1β mRNA at 12 hours and 3 days after LPS injection and a subsequent rise in CNTF expression. Because astrocytes were present throughout LPS lesions, they could have responded to signals such as IL-1β derived from the TLR4-activated macrophages. It is also possible, although somewhat controversial, that astrocytes express TLR4 in vivo and may respond directly to LPS (for review, see Reference 54). Collectively, our results reveal that in vivo activation of TLR4 leads to prominent formation of new BrdU+ OLs, potentially through release of IL-1β, CNTF, and/or other neuroprotective and reparative molecules.

On the other hand, activated macrophages can kill OLs and neurons, which has been shown repeatedly in vitro and in vivo (55, 56). In our study, macrophage-mediated killing was most evident after zymosan injection, which will activate macrophages both by direct TLR2 ligation and through phagocytic zymosan uptake (57). This activation could lead to production of toxic molecules such as free radicals and nitric oxide, both of which can cause bystander damage, including death of OLs and their progenitors. Interestingly, inflammatory mediators such as tumor necrosis factor-α and IL-1β can cause OL death but also are essential for OL remyelination (58–60). This observation accentuates the need to evaluate glial interactions in the context of the entire environmental milieu. Indeed, certain molecules may only become toxic when present in combination with other substances. For instance, injections of harmless levels of tumor necrosis factor-α or kainate into spinal gray matter became toxic when combined (61). Thus, it appears that the unique combination of local factors acting on microglia and nearby cells ultimately determines whether a particular environment becomes a reparative site or one predominated by cell death.

An unexpected finding in the current study was the delayed lesion expansion and induction of primary demyelination by zymosan. These may be due to a protracted accumulation of monocyte-derived macrophages throughout the lesions (24). Indeed, there was almost complete turnover of the macrophage population within the lesions (based on BrdU labeling; see Fig. 6B) between 7 and 14 days post-injection. Whether infiltrating macrophages caused the secondary demyelination will be tested in future macrophage depletion studies.

Because endogenous NG2 cells proliferate and migrate into and around almost all CNS injuries, it is intriguing that they did not enter the boundaries of the zymosan lesions. Although Setzu et al (62) showed that zymosan-induced inflammation enhanced remyelination by transplanted oligodendrocyte progenitor cells, this phenomenon was not observed in our model. This is probably due to inherent differences between spinal cord and retina and between transplanted and endogenous progenitor cells. The lack of NG2 cell infiltration was possibly due to the local environment created by the activated macrophages and/or the absence of axons within these lesions. Indeed, axons are active players in the myelination process and release growth factors that promote NG2 cell migration and differentiation (for reviews, see References 63, 64). Still, the lack of axons cannot entirely explain this phenomenon as we have previously noted marked NG2 cell migration into spinal contusion sites containing few axons (65).

Microinjection of inflammogens provides a simplified model for analyzing the effect of activated macrophages on CNS structure and function. Here, we have shown that activating microglia and macrophages with different ligands evokes unique pathologies with correspondingly distinct effects on OLs and OL progenitors. Although LPS and zymosan both signal through TLR-mediated pathways that display significant overlap, differences do exist in inflammatory cell responses to these ligands. For instance, a recent report showed that polymorphonuclear leukocytes display a strong induction of arachidonic acid metabolism in response to...
TLR2 activation (e.g. with zymosan) but a complete lack of arachidonic acid breakdown after LPS stimulation (66). Furthermore, as stated above, zymosan particles are phagocytosed by macrophages in addition to binding to surface receptors (57). This activity is unique to zymosan compared with LPS and may initiate the secretion of detrimental cytokines not observed after LPS stimulation. By studying the subtle differences evoked in macrophage signaling and activation, clues may be obtained to clarify what makes a particular macrophage population deleterious or reparative, particularly in terms of OL lineage cells. Because microglia and macrophages are exquisitely sensitive to changes in the extracellular milieu, our results may parallel the changes that occur within distinct microdomains of CNS pathology. For instance, after spinal cord injury, we have noted the development of a potent gliogenic zone along the lesion borders, which displays a marked increase in new OL formation (19). This is in contrast to the lesion core, where minimal OL generation occurs. Although both areas contain substantial numbers of activated macrophages, it is clear that the lesion border environment is much more favorable for OL replacement, which may be due in part to the type of macrophage activation occurring therein. Collectively, our results demonstrate the extreme differences in reparative and deleterious consequences of microglia and macrophage activation within the CNS, which calls into question the use of global immunosuppression for CNS injuries or diseases. It may be more beneficial, although recognizably difficult, to identify local responses of macrophages and microglia within lesion subdomains and then target specific deleterious processes rather than overall inflammation. Still, the models described in this report reveal that by understanding the distinct signaling pathways in macrophages they may be exploited to produce drastically different effects on the CNS parenchyma.

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