TLR4 Deficiency Impairs Oligodendrocyte Formation in the Injured Spinal Cord

Jamie S. Church,^{1,2} Kristina A. Kigerl,^{2,3} Jessica K. Lerch,^{2,3} Phillip G. Popovich,^{2,3} and Dana M. McTigue^{2,3}

¹Neuroscience Graduate Program, ²Center for Brain and Spinal Cord Repair, and ³Department of Neuroscience, Ohio State University, Columbus, Ohio 43210

Acute oligodendrocyte (OL) death after traumatic spinal cord injury (SCI) is followed by robust neuron–glial antigen 2 (NG2)-positive OL progenitor proliferation and differentiation into new OLs. Inflammatory mediators are prevalent during both phases and can influence the fate of NG2 cells and OLs. Specifically, toll-like receptor (TLR) 4 signaling induces OL genesis in the naive spinal cord, and lack of TLR4 signaling impairs white matter sparing and functional recovery after SCI. Therefore, we hypothesized that TLR4 signaling may regulate oligodendrogenesis after SCI. C3H/HeJ (TLR4-deficient) and control (C3H/HeOuJ) mice received a moderate midthoracic spinal contusion. TLR4-deficient mice showed worse functional recovery and reduced OL numbers compared with controls at 24 h after injury through chronic time points. Acute OL loss was accompanied by reduced ferritin expression, which is regulated by TLR4 and needed for effective iron storage. TLR4-deficient injured spinal cords also displayed features consistent with reduced OL genesis, including reduced NG2 expression, fewer BrdU-positive OLs, altered BMP4 signaling and inhibitor of differentiation 4 (ID4) expression, and delayed myelin phagocytosis. Expression of several factors, including IGF-1, FGF2, IL-1 β , and PDGF-A, was altered in TLR4-deficient injured spinal cords compared with wild types. Together, these data show that TLR4 signaling after SCI is important for OL lineage cell sparing and replacement, as well as in regulating cytokine and growth factor expression. These results highlight new roles for TLR4 in endogenous SCI repair and emphasize that altering the function of a single immune-related receptor can dramatically change the reparative responses of multiple cellular constituents in the injured CNS milieu.

Key words: growth factor; inflammation; macrophage; microglia; myelin; phagocytosis

Significance Statement

Myelinating cells of the CNS [oligodendrocytes (OLs)] are killed for several weeks after traumatic spinal cord injury (SCI), but they are replaced by resident progenitor cells. How the concurrent inflammatory signaling affects this endogenous reparative response is unclear. Here, we provide evidence that immune receptor toll-like receptor 4 (TLR4) supports OL lineage cell sparing, long-term OL and OL progenitor replacement, and chronic functional recovery. We show that TLR4 signaling is essential for acute iron storage, regulating cytokine and growth factor expression, and efficient myelin debris clearance, all of which influence OL replacement. Importantly, the current study reveals that a single immune receptor is essential for repair responses after SCI, and the potential mechanisms of this beneficial effect likely change over time after injury.

Introduction

Oligodendrocytes (OLs), the myelinating cells of the CNS, die after spinal cord injury (SCI) at the time of impact and for several weeks thereafter (Crowe et al., 1997; Grossman et al., 2001). OL

loss contributes to demyelination, which impairs axon conduction and neurological function. New OLs are produced after SCI and arise from neuron–glial antigen 2-positive (NG2⁺) oligodendrocyte progenitor cell (OPC) proliferation and differentiation. This reparative response restores OL numbers to baseline in spared tissue and results in significantly greater OLs along the lesion border (Tripathi and McTigue, 2007; Hesp et al., 2015).

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Correspondence should be addressed to Dr. Dana McTigue, Department of Neuroscience, Ohio State University, 692 Biomedical Research Tower, 460 West Twelfth Avenue, Columbus, OH 43210. E-mail: Dana.McTigue@osumc.edu.

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New OLs myelinate spinal axons for at least 3 months after injury and thereby contribute to spontaneous endogenous repair (Hesp et al., 2015). Although developmental oligodendrogenesis is guided by known intrinsic (transcriptional changes) and extrinsic (growth factors, cytokines) cues, the signals directing this endogenous repair response after adult SCI are unknown. Determining the mechanisms controlling OL replacement after SCI will provide insight into glial replacement in the injured adult CNS and may guide therapeutic development for other forms of neurological disease involving OL loss or demyelination.

Key players in post-SCI OL replacement and remyelination likely include inflammation and activated macrophages. For instance, activated macrophages express pro-oligogenic growth factors and clear lipid debris, which was shown to be essential for OL genesis and remyelination after toxin-induced demyelination (Kotter et al., 2005, 2006). Activated macrophages can also shuttle iron-containing ferritin to OPCs *in vivo*, which is needed for differentiation of OPCs into myelinating OLs (Schonberg et al., 2012). A key inflammatory signaling pathway is mediated by the toll-like receptor 4 (TLR4), which can influence all these features, including iron regulation (Carraway et al., 1998; Yang et al., 2002; Theurl et al., 2008; Recalcati et al., 2010; Layoun and Santos, 2012; Urrutia et al., 2013), cytokine secretion (Aderem and Ulevitch, 2000; Wells et al., 2003a,b), phagocytosis (Vallières et al., 2006; Boivin et al., 2007; Li et al., 2014), and oligodendrogenesis (Schonberg et al., 2007; Schonberg and McTigue, 2009). Our previous work showed that TLR4 is essential after SCI, because mice with deficient TLR4 signaling displayed exacerbated white matter pathology and impaired functional recovery (Kigerl et al., 2007). In other work, Schonberg et al. (2007) demonstrated that activating intraspinal TLR4 signaling induced OL progenitor proliferation and new OL genesis (Schonberg and McTigue, 2009). Because microglia and macrophages express by far the greatest amount of TLR4 in the spinal cord (and OLs do not express TLR4), they most likely are responsible for the beneficial effects of intraspinal TLR4 activation (Lehnardt et al., 2002; Kigerl et al., 2007).

The link between TLR4 activation and OL genesis in the normal spinal cord and increased white matter loss after SCI in the absence of TLR4 suggest that post-SCI TLR4 signaling may induce a favorable post-SCI environment for OL survival and formation. This is especially true when considering that TLR4 ligands, such as heme, fibronectin, and high-mobility group protein 1 (HMGB1), are abundant in the injured spinal cord (Kigerl et al., 2009). Thus, we used mice deficient in TLR4 signaling to test the hypothesis that TLR4 activation regulates OL survival and replacement after SCI. The data reveal that TLR4 signaling is protective for OLs and OPCs acutely after SCI and is important for the normal timing and response of OL lineage cells; it also facilitates myelin debris clearance and regulates post-SCI growth factor expression. Thus, TLR4 appears central to many reparative endogenous responses in the injured adult spinal cord and may represent an attractive therapeutic target to enhance recovery from injury.

Materials and Methods

Animals. All surgical and postoperative care procedures were performed in accordance with the Ohio State University Institutional Animal Care and Use Committee. Twelve-week-old male C3H/HeJ ($n = 47$) and C3H/HeOuJ ($n = 44$) mice were obtained from The Jackson Laboratory. The C3H/HeJ mice have a point mutation at a single residue in the cytoplasmic tail of the TLR4 molecule; although there may be compensatory changes developed in response to the mutations, TLR4 signaling in

C3H/HeJ mice has been verified to be deficient (Poltorak et al., 1998). C3H/HeOuJ mice have functional TLR4 signaling, and, although they are not littermates, they are the line typically used as wild-type (WT) controls for C3H/HeJ [TLR4-deficient (TLR4d)] mice, including in previous work from our laboratory (Kigerl et al., 2007). In total, five WT and eight TLR4d mice were lost as a result of problems with anesthesia, surgeon error, or bladder expression as detailed below.

SCI. During initial studies with these mice, difficulties with anesthesia led to a loss of six mice (mice were not reaching adequate levels of unconsciousness and were given multiple anesthesia injections). After determining a higher than usual initial dose of ketamine was needed, all mice were anesthetized with an intraperitoneal mixture of ketamine (180 mg/kg)/xylazine (10 mg/kg) after which a partial laminectomy was performed at vertebral level T9. All mice received a moderate spinal contusion injury (75 kDyn) using the Infinite Horizons device (Precision Systems and Instrumentation). An additional six mice were lost as a result of surgical complications. Postoperatively, animals were hydrated with 2 ml of saline (subcutaneously) and were given prophylactic antibiotics (0.1 ml of gentamicin, s.c.) for 5 d. Bladders were voided manually twice daily for duration of the study. One mouse was lost during bladder expression.

5-Bromo-2'-deoxyuridine administration. To label proliferating cells, the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) (50 mg/kg in sterile saline; Sigma-Aldrich) was injected intraperitoneally once a day on 1–7 d post-injury (dpi).

Behavioral analysis. Hindlimb locomotor function was assessed using the Basso Mouse Scale (BMS; Basso et al., 2006) and automated horizontal ladder. Mice were tested using the BMS at −1, 1, 7, 14, 21, 28, 35, and 42 dpi by evaluators blinded to groups. Individual hindlimb scores were averaged for each animal at each time point. Once mice regained plantar stepping ability (BMS score of 4), they were tested using the automated horizontal ladder on −1, 13, 20, 27, 34, and 41 dpi. The horizontal ladder has rungs spaced 0.85 cm apart for 86.25 cm. Each foot slip made contact with a metal pan 0.5 cm below the rungs and was counted by the apparatus as a misstep. During baseline acclimation to the ladder, the optimal distance of the pan below the rungs was determined based on its reliability to register a misstep while not providing a convenient alternative to place the paws while walking. Mice were motivated to walk along the ladder by placing a dark box at the far end. Scores for each animal were the average of three trials at each time point. Locomotor function in both tasks was normal before injury in both genotypes.

Tissue processing. Mice for immunohistochemical analyses were killed at 1, 7, 14, or 21 dpi; a group of naive mice was included as non-injury controls ($n = 3–5$ per genotype per time point). All mice were anesthetized with a lethal mixture of ketamine/xylazine (1.5× the surgical dose) and then transcardially perfused with 0.1 M PBS, followed by 4% paraformaldehyde (PFA). Fixed animals were dissected for spinal cord tissue centered on the lesion site. Spinal cords were postfixed in 4% PFA for 2 h, transferred to 0.2 M phosphate buffer overnight, and then cryopreserved in 30% sucrose solution in water for 3 d. Frozen spinal cords were blocked into 8 mm segments centered on the impact site and then were embedded in Tissue-Tek optimum cutting temperature medium (VWR International). Serial cross-sections (10 μ m) were cut through each block using a Microm cryostat (HM 505 E), collected on SuperFrost Plus slides (Thermo Fisher Scientific), and stored at −20°C.

Injured and naive mice for Real Time qRT-PCR analyses were transcardially perfused at 7 or 21 dpi ($n = 3$ per group) with DEPC PBS, and 5 mm of tissue centered on the injury site was rapidly dissected, snap frozen in liquid nitrogen, and stored at −80°C until processed for cDNA.

An additional set of mice was used for spinal cord Epon embedding. Naive or mice injured as above were perfused with PBS, followed by 4% PFA/2% glutaraldehyde solution at 21 or 42 dpi ($n = 3–4$ per group). Spinal cords were removed, and a 2 mm segment of tissue centered on the lesion epicenter was blocked, which was then bisected to produce two 1 mm blocks of tissue containing the rostral and caudal portions of the lesion. Tissue was processed for Epon embedding and semithin sections cut at 1 μ m in a transverse orientation on an ultramicrotome (Ultracut MZ6; Leica). Sections were stained with 1% toluidine blue, dehydrated through increasing ethanol, and coverslipped.

Table 1. Primer sequences for real-time RT-PCR

Mus gene	Forward primer (5'-3')	Reverse primer (5'-3')
18S	TTCGGAAGTGGCCATGAT	TTTCGCTCTGGTCCGCTCTTG
BMP2	CGTGGCAGCTTCATCAG	GAAGAAGCGCCGGCCGTTT
BMP4	GCATCCGAGCTGAGAGACCCCA	ATCCCATCAGGGACGGAGACCA
FGF2	GGCTGCTGGCTTCTAAGTGT	ACTGGAGTATTTCCGTGACCG
ID2	GCTCTACAACATGAACGACTGCTACT	TGCAGGTCCAAGATGAATCGA
ID4	GAGACTCACCTGCTTTGCT	AGAATGCTGTCAACCTGCTT
IGF-1	TGACATGCCCAAGACTCAGAAG	GCGGTGATGTGGCATTTC
IL-1 β	CAGGCTCCGAGATGAACAAC	GGTGGAGAGCTTTCAGCTCATAT
PDGF-A	ACCCACATCGGCAACT	CCGTGAAGGTGGGCACTT
TGF β	TGAGTGGCTGCTTTTGACGTC	TTCATGTCATGGATGGTGCC

qRT-PCR. Frozen spinal cord segments were suspended in 1 ml of ice-cold TRIzol (Invitrogen) and homogenized with a tissue grinder. Gene-specific primer pairs (Table 1) were used to detect mRNA expression in uninjured and SCI samples by qRT-PCR. Primer sequence specificity was confirmed by BLAST analysis for highly similar sequences against known sequence databases. Briefly, total RNA was purified using TRIzol (Invitrogen) and quantified by spectrophotometry. cDNA was prepared from RNA by RT with SuperScript II and random primers (Invitrogen). PCR reactions were performed using 10 ng of cDNA, 500 nM each primer, and SYBR Green master mix (Applied Biosystems) in 10 μ l reactions. Levels of PCR product were measured using SYBR Green fluorescence collected on a 7900HT Real-Time PCR System (Applied Biosystems). 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 are calculated using the $\Delta\Delta C_t$ method (Schmittgen and Livak, 2008) and expressed as fold change from uninjured spinal cord samples (gene/18S ratio for uninjured samples equals one). Forward and reverse primer sequences for each gene are listed in Table 1.

Immunohistochemistry. For tissue analysis, the following targets were visualized using immunohistochemistry: OPCs (rabbit anti-NG2, 1:600, catalog #C5067-70D; US Biological), OLs (rabbit anti-GST π , 1:2000, catalog #Orb18037; Biorbyt), ferritin (rabbit anti-H-ferritin, 1:10,000, catalog #65080; Abcam; plus rabbit anti-L-ferritin, 1:2000, catalog #69090; Abcam), macrophages [rat anti-cluster of differentiation (CD) 68, 1:1000, catalog #MCA1957; Serotec and rat anti-CD11b, 1:800, catalog #MCA-74G; Serotec], proliferating cells (biotinylated sheep anti-BrdU, 1:200, catalog # ab2284). Sections were rinsed in 0.1 M PBS and blocked for nonspecific antigen binding using 4% BSA/0.1% Triton X-100/PBS (BP⁺) for 1 h. Next, sections were incubated in primary antibody overnight at 4°C. Sections were rinsed and treated with rat or rabbit biotinylated antiserum (rabbit anti-rat IgG at 1:800 or goat anti-rabbit IgG at 1:2000 in BP⁺; Vector Laboratories) for 1 h at room temperature. After rinsing, endogenous peroxidase activity was quenched using a 4:1 solution of methanol/30% hydrogen peroxide for 15 min in the dark. Sections were treated with Elite avidin–biotin enzyme complex (ABC; Vector Laboratories) for 1 h. Visualization of labeling was achieved using DAB substrates (Vector Laboratories). Sections were rinsed, dehydrated, and coverslipped with Permount (Thermo Fisher Scientific). For GST π (DAB) immunohistochemistry, sections underwent antigen retrieval using high pH. For GST π /TUNEL immunofluorescence, sections were immunolabeled for GST π (1:200, with goat anti-rabbit 546 at 1:500), followed by TUNEL staining according to the instructions of the manufacturer (catalog #4810-30-K; Trevigen) tagged with Streptavidin 488 (1:500; Invitrogen). For GST π /BrdU immunohistochemistry, sections were immunolabeled for GST π (1:200, with goat anti-rabbit 546 at 1:500), treated with 2N HCl at 37°C for 25 min to denature DNA before primary antibody incubation, and then tagged with Streptavidin 488 (1:500; Invitrogen). Sections labeled with GST π antibodies (DAB) to identify OLs were counterstained with methyl green. Sections labeled with NG2 antibody were counterstained with neutral red. Endogenous peroxidase activity was used to visualize red blood cells (RBCs) through reaction with DAB (Vector Laboratories) without previous treatment with hydrogen peroxide or ABC solution.

Tissue staining. Eriochrome cyanine (EC) labeling of myelin was used to determine the lesion epicenter (cross-section with least amount of spared white matter).

Oil Red O stain was used to measure lipid accumulation. Sections were rinsed in dH₂O and 70% ethanol each for 2 min before being placed in a saturated solution of Oil Red O (catalog # O0625-25G; Sigma-Aldrich) dissolved in 70% ethanol at 60°C for 30 min. The tissue was differentiated in ethanol, washed in distilled water, and then coverslipped with Immumount (Thermo Fisher Scientific).

Perls Prussian Blue stain (catalog #24199-1; Polysciences) was used to visualize iron. Tissue sections were rinsed in 0.1 M PBS and 0.1% Triton X-100/PBS for 10 min each, followed by incubation in a 1:1 mixture of HCl plus potassium ferrocyanide solution for 30 min and DAB plus nickel intensification (Vector Laboratories). Sections were rinsed, dehydrated, and coverslipped with Permount (Thermo Fisher Scientific).

Proportional area analysis. To quantify NG2, Perls, DAB, CD11b, and CD68 immunoreactivity, low power (5 $\times$) images located 0.6 mm rostral or caudal to the lesion epicenter were digitized and manually outlined using image analysis software (MCID Elite; Imaging Research). The proportion of the cross-section occupied by positive immunoreactivity was calculated by dividing the area immunoreactive by the total cross-sectional area.

Intraspinal lipid accumulation was analyzed using the proportional area of Oil Red O staining normalized to the lesion scan area outlined using EC/neurofilament staining. The trace of the core lesion area outlined at low power (4 $\times$) was overlaid on Oil Red O-stained cross-sections, and positive staining was analyzed within that area. Proportional area analysis of H- and L-ferritin was conducted in the same way.

Axon counts. Axons were counted in Epon-embedded sections stained with 1% toluidine blue. Two representative images per animal were taken at 100 $\times$ magnification (5951 μ m²) in the ventromedial white matter 0.5 mm caudal to the lesion epicenter (AxioVision; Zeiss). All visible axons within the sampled area were counted. Counts from both images were averaged to obtain one value per animal.

Cell density quantification. OLs (GST π ⁺ cells) were counted in tissue cross-sections at 5 $\times$ using image analysis software (MCID Elite; Imaging Research). First, the average positive cell size was determined, and manual immunoreactivity thresholding was used to detect and automatically count all positively labeled cells in the sample area. Naive or epicenter sections were analyzed at 1 dpi, and sections 0.6 mm rostral and caudal to the epicenter were analyzed at 1, 7, 14, and 21 dpi.

Cells immunoreactive for NG2 in the epicenter at 1 dpi were manually counted at 40 $\times$. The criteria to count a cell 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 type was verified at higher power (64 $\times$) when needed. NG2⁺ macrophages or pericytes were excluded based on morphological criteria (McTigue et al., 2001).

Cells immunofluorescent for both GST π /TUNEL or GST π /BrdU were counted at 20 $\times$ with 3 $\times$ zoom using confocal microscopy (510 META laser scanning confocal microscope; Zeiss) in optical sections (~1 μ m). Bilateral sample boxes (0.0441 mm²) were placed in the ventromedial white matter 0.6 mm rostral and caudal to the lesion epicenter. All cell count data are expressed as cells per cubic millimeter.

Bone marrow-derived macrophage cell culture. Bone marrow-derived macrophage (BMDM) cultures were generated as described previously (Longbrake et al., 2007) from adult male C3H/HeJ and C3H/HeOuJ mice (The Jackson Laboratory). Briefly, BMDMs were obtained from bilateral femurs and tibias using aseptic techniques. Marrow cores were flushed into sterile tubes using syringes fit with 26 gauge needles and filled with DMEM/10% FBS. Cells were triturated three to five times, and RBCs were lysed in lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA, pH 7.4). Cells were washed once in media and then plated and cultured in DMEM supplemented with 0.5% gentamicin, 1% glutaMAX, 1% HEPES, 0.001% β -mercaptoethanol, 10% FBS, and 20% supernatant from sL929 cells. The sL929 (which contains macrophage colony-stimulating factor) is needed to promote differentiation of bone marrow cells into macro-

phages (7–10 d; Burgess et al., 1985). Cells were replated at 5000 cells per well on day 7 in DMEM supplemented with 10% FBS, 1% glutamax, and 0.5% gentamicin. Cells were treated with pHrodo Red-labeled myelin at 1 μ g per 80,000 cells (see below) and replating media or LPS (0.1 μ g/ml; Sigma-Aldrich) made in replating media for 24 h. All treatment groups were run in triplicate.

Myelin isolation and pHrodo Red dye labeling. Myelin from the brain of an adult male C3H/HeOuJ (WT) mouse was isolated using a modified version of the methods outlined previously (Hendrickx et al., 2014). The whole brain was mechanically dissociated through a 70 μ m mesh filter using cold 0.1 M PBS, followed by density gradient separation using Percoll (catalog #GE17-0891-01; Sigma) of 70, 35, and 0% Percoll in PBS. Myelin was collected from the 0–35% interface, resuspended in 0.32 M sucrose, and further purified using a sucrose gradient (Norton and Poduslo, 1973) of 0.85, 0.32, and 0 M sucrose. Liquid was aspirated, and myelin was washed in dH₂O and suspended in 0.1 M PBS, pH 7.4.

Myelin concentration was measured using the Pierce BCA protein assay kit (catalog #23225; Thermo Fisher Scientific) and labeled with pH-sensitive dye pHrodo Red, succinimidyl ester (catalog #P36600; Thermo Fisher Scientific) dissolved in DMSO. Myelin was suspended in 0.1 M PBS, pH 8.4, and incubated with 0.1 mM dye at room temperature for 45 min. Myelin + dye was spun and resuspended in 0.1 M PBS, pH 7.4, and stored at -80°C until used.

Phalloidin immunofluorescence. BMDM morphology was visualized using phalloidin staining with Hoechst nuclear stain. Fixed cells were washed in 5% FBS/PBS, permeabilized with 0.2% Triton X-100, and incubated with Alexa Fluor Phalloidin 488 (1:1000, catalog #A12379; Thermo Fisher Scientific) for 3 h at room temperature. Cells were washed and treated with Hoechst (1:50,000) for 5 min and left in PBS.

Myelin phagocytosis analysis of BMDMs. Cells were identified as BMDMs if they were Hoechst⁺ and phalloidin⁺. Phalloidin⁺ BMDMs were determined by thresholding against the background intensity in non-stained wells. The average phalloidin intensity was at least three times the background intensity. Hoechst⁺ cells that were phalloidin negative were excluded from analysis. BMDM myelin uptake was determined by thresholding the myelin intensity against the background intensity in non-treated BMDM wells. A BMDM that had taken up myelin was at least three times the maximum background intensity in the nontreated wells. The presence of myelin was quantified using the ArrayScanXTI Spot Detector Algorithm (Thermo Fisher Scientific). Two hundred fifty Hoechst/phalloidin⁺ cells were analyzed per well for their myelin content. The proportion of cells containing myelin was determined for WT and TLR4d-derived BMDMs that received either media or LPS (0.1 μ g/ml) treatment. The fold change was determined by comparing the proportion of myelin⁺ BMDMs in each condition to the proportion in the media-treated wells. Three wells per treatment were analyzed and averaged.

Data analysis. All data were collected in a blinded manner. Data were analyzed using GraphPad Prism 5.0c and are presented as mean $\pm$ SEM. Behavioral data and GSTpi/BrdU counts at 14 dpi were analyzed by two-way repeated-measures ANOVA with Bonferroni's *post hoc* test. Ferritin, Perls and DAB proportional area, OL counts, and NG2 cell counts at 1 dpi were analyzed by Student's two-tailed *t* test. OL counts over time, NG2 proportional area, and Oil Red O proportional area were analyzed by two-way ANOVA at each distance with Bonferroni's *post hoc* test. All other data (including cell culture) were analyzed by two-way ANOVA with Bonferroni's *post hoc* test.

Results

Functional recovery is reduced in TLR4d mice after SCI

Previous data from our group showed that, after SCI, female TLR4d (C3H/HeJ) and WT (C3H/HeOuJ) mice had similar degrees of gross hindlimb locomotor recovery; however, female TLR4d mice displayed significant deficits in fine details of locomotion, most notably interlimb coordination (Kigerl et al., 2007). Here, male TLR4d mice were used to evaluate recovery after SCI. Both genotypes had normal locomotion before SCI. At

Figure 1. TLR4d mice displayed significantly worse hindlimb recovery after moderate SCI. **A**, Open-field locomotion was analyzed by two investigators in a blinded manner using the BMS. This revealed that WT and TLR4d mice displayed significant hindlimb paralysis at 1 dpi, followed by recovery plantar stepping by 7 dpi. At 14 dpi, the locomotor function of WT mice was significantly greater than in TLR4d mice, which was maintained through 42 dpi. **B**, Automated horizontal ladder analysis beginning once stepping was regained confirmed that TLR4d mice displayed worse hindlimb function compared with WT. $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < .001$ vs WT. Data are mean $\pm$ SEM.

1 dpi, all mice displayed significant hindlimb paralysis (BMS score < 2) and then recovered the ability to plantar step over the first 7 dpi (score ≥ 4 ; Fig. 1A). WT mice progressed to coordinated and consistent plantar stepping by 14 dpi (mean score of 6.6), whereas TLR4d mice failed to achieve forelimb–hindlimb coordination, and BMS scores remained significantly impaired through 42 dpi (mean score of 4.8; $p = 0.0001$).

Once mice regained plantar stepping, locomotor ability was tested using the automated horizontal ladder test (Fig. 1B). Although both genotypes navigated the ladder with few errors before SCI, TLR4d mice had significantly more missteps after SCI than WT mice ($p < 0.0001$). WT mice walked steadily across the ladder, whereas TLR4d mice were shaky, slow, and relied on the apparatus walls for support. These data confirm that TLR4 signaling is important for spontaneous recovery of locomotion after SCI, especially more advanced recovery milestones such as coordination, paw position, and balance.

Acute OL and NG2 cell loss is exacerbated after SCI in TLR4d mice

Our previous work showed that mice deficient in TLR4 signaling have significantly less white matter sparing after SCI (Kigerl et al., 2007). Because previous work also linked TLR4 signaling and OL replacement (Schonberg et al., 2007), we hypothesized that TLR4d spinal cords would have greater OL lineage cell loss and/or impaired OL replacement. Thus, OLs and NG2⁺ cells (putative OL progenitors) were quantified in WT and TLR4d epicenters at 1 dpi. Significant OL and NG2 cell loss occurred in both genotypes; however, TLR4d mice had a greater loss of OLs

Figure 2. OL lineage cell sparing is reduced concurrent with less ferritin in TLR4d mice after SCI. GST π^{+} OLs (**A**) and NG2 $^{+}$ cells (**B**) were significantly reduced at 1 dpi in the lesion epicenter compared with naive (---). TLR4d tissue had significantly fewer OLs and NG2 $^{+}$ cells compared with WT. **C**, **D**, Both genotypes show comparable levels of intraspinal iron and RBCs in the lesion epicenter at 1 dpi. **E**, Proportional area of immunoreactivity for the iron storage molecule ferritin is reduced in TLR4d mice at 1 dpi in the lesion epicenter. **F**, **G**, Representative images of WT and TLR4d ferritin labeling in epicenter lesions 1 dpi. Map in **F** shows region of image collection. Inset shows confocal image of macrophages (CD11b, red), one of which expresses ferritin (green, arrow). **H**, Proportional area of CD11b immunoreactivity in the epicenter after SCI. **A**, **B**, --- indicates naive level. Scale bars: **F**, **G**, 100 μ m; inset, 25 μ m. *** p < 0.001 vs naive; $^{\wedge}$ p < 0.05 and $^{\wedge\wedge}$ p < 0.01 versus WT. Data are mean $\pm$ SEM.

(p < 0.05) and NG2 cells (p < 0.01) compared with WT mice at 1 dpi (Fig. 2*A,B*). These data suggest that TLR4 signaling confers protection for OL lineage cells within the acute SCI lesion environment.

Ferritin expression is reduced in TLR4d lesion epicenters 1 d after injury

Iron-mediated damage can contribute to acute glial cell loss after SCI. Intraparenchymal hemorrhage causes iron-rich RBCs to accumulate rapidly within the injured spinal cord (Noble and Wrathall, 1989; Sauerbeck et al., 2013). Macrophages are adept at removing extracellular iron, and TLR4 activation promotes their iron sequestration by stimulating iron uptake and storage in intracellular ferritin (Recalcati et al., 2010; Urrutia et al., 2013). Because iron is toxic to OLs (for review, see McTigue and Tripathi, 2008; Almad et al., 2011), excess iron in TLR4d spinal cords could exacerbate acute post-SCI OL loss.

Epicenter sections from TLR4d and WT mice were analyzed at 1 dpi, which is the time of peak intraspinal blood and iron accumulation (Sauerbeck et al., 2013). Iron and RBCs

were analyzed using Perls stain (non-heme iron) and DAB stain (RBCs). We found comparable levels of intraspinal iron and RBCs in both genotypes (Fig. 2*C,D*), which indicates that TLR4d and WT mice had similar magnitudes of primary trauma, bleeding, and breakdown of blood-derived hemoglobin. Although ferritin levels increased rapidly in both TLR4d and WT spinal cords (undetectable in naive tissue with immunohistochemistry), ferritin was significantly lower in TLR4d lesion sites (p < 0.01 vs WT; Fig. 2*E–G*). Our previous work showed that the vast majority of ferritin $^{+}$ cells in the SCI lesion site were macrophages (Sauerbeck et al., 2013), which was confirmed here (Fig. 2*F*). Therefore, reduced ferritin in TLR4d lesions could indicate an attenuated inflammatory response (i.e., fewer macrophages) or impaired TLR4-mediated ferritin expression by macrophages. To assess this, CD11b immunoreactivity as a function of time after injury was analyzed. There was no difference in the magnitude of the microglia or macrophage response between genotypes over the first week after injury and up to 21 dpi (Fig. 2*H*). CD68 immunohistochemistry to label activated microglia/macrophages showed similar results (data not shown). Together, these data

Figure 3. OLs are reduced chronically in TLR4d mice after SCI. **A**, Counts of GST π^+ OLs at 0.6 mm rostral and caudal to the lesion epicenter at 1, 7, 14, and 21 dpi compared with naive (—). **B**, **C**, Representative images of GST π^+ OLs in the ventral spinal cord 0.6 mm caudal to the epicenter at 21 dpi in WT and TLR4d mice. Inset shows representative GST π^+ cells (arrows). Map in **C** shows region of image collection for **B** and **C**. **D**, Percentage of GST π^+ /TUNEL $^+$ apoptotic OLs normalized to overall GST π^+ OLs in ventromedial white matter at 0.6 mm rostral and caudal to the lesion epicenter at 14 and 21 dpi. Scale bar: **B**, **C**, 100 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus naive. Data are mean $\pm$ SEM.

indicate that, although the overall number of macrophages is comparable between genotypes, TLR4 deficiency impairs iron sequestration and storage. This is significant because extracellular iron could exacerbate OL (and other cell) loss.

TLR4d mice have reduced OLs chronically after SCI

Previously, we showed that SCI induces robust OL genesis, producing more OLs in spared tissue than in naive, especially in regions distal to the epicenter where chronic remyelination is greatest (Tripathi and McTigue, 2007; Hesp et al., 2015). We also showed that intraspinal TLR4 activation stimulates OL genesis (Schonberg et al., 2007), raising the possibility that TLR4 signaling contributes to spontaneous OL replacement after SCI. Here, we quantified OLs distal to epicenter in TLR4d and WT spinal cords (Fig. 3A). In both genotypes, OLs were significantly reduced at 1 dpi ($p < 0.001$ vs naive) and increased to naive levels by 7–14 dpi. In WT mice, OL numbers continued to rise through 21 dpi, with OL numbers exceeding those in naive tissue ($p < 0.05$). In contrast, OL numbers did not change after 14 dpi in TLR4d tissue, resulting in significantly fewer OLs compared with WT (genotype effect for caudal tissue $p = 0.033$; Fig. 3A–C). Notably, this is also the time when functional recovery diverges between the two genotypes (Fig. 1).

OL apoptosis was not different between WT and TLR4d mice

TLR4 deficiency results in enhanced OL loss acutely after SCI (see above). To determine whether the lack of TLR4 signaling increases chronic OL apoptosis, cross-sections were double-labeled for GST π and TUNEL to label apoptotic OLs in sections from 14 and 21 dpi, times when post-SCI OL apoptosis in descending tracts is prevalent (Warden et al., 2001). Double-labeled cell counts in ventromedial white matter rostral and caudal to the epicenter revealed comparable numbers of apoptotic OLs at both times in all animals (Fig. 3D). Thus, lower OL numbers in TLR4d tissue was not attributable to significantly enhanced OL apoptosis.

TLR4d mice have reduced axon sparing chronically after SCI

To determine whether fewer OLs in TLR4d mice led to reduced axon myelination chronically, semi-thin Epon-embedded sections from uninjured, 21 dpi, and 42 dpi spinal cords were prepared. Given their pivotal role in locomotor function, axons in the caudal ventromedial white matter were examined (Rosenberg and Wrathall, 1997). As expected, naive spinal cords were intact and indistinguishable between genotypes (Fig. 4A,B). At 21 and 42 dpi, few bare axons were present in either genotype, indicating that TLR4 deficiency did not exacerbate demyelination in this white matter region. However, significantly fewer total axons were present in the ventral funiculus in TLR4d mice compared

with WT at both time points (Fig. 4A,B), which likely contributed to the reduced locomotor recovery in the TLR4d group. Tissue disruption was more extensive in TLR4d tissue, including increased pathological axon profiles and more myelin debris, suggesting reduced debris clearance and/or greater active chronic degeneration.

NG2 expression was reduced in TLR4d mice after injury

The fact that chronic OL apoptosis was comparable between genotypes but TLR4d tissue had lower OL numbers 2–3 weeks after injury suggests that fewer new OLs were produced. New OLs arise mainly from NG2 $^+$ OPCs after spinal contusion. To determine whether TLR4 altered NG2 cells after SCI, NG2 immunoreactivity was analyzed 0.6 mm rostral and caudal to the epicenter. These are the same distances at which OL numbers were quantified (Fig. 3) and are known regions of NG2 cell proliferation after SCI (McTigue et al., 2006; Tripathi and McTigue, 2007). Because counting individual NG2 cells was not feasible due to dense NG2 accumulation along the lesion and adjacent spared tissue (Fig. 5A–D), the overall area of NG2 immunoreactivity was quantified. Rostrally, NG2 immunoreactivity robustly increased in both genotypes by 7 dpi and then declined slightly by 14 dpi, after which it stabilized in WT

tissue ($p < 0.001$ vs naive). This decline in NG2 continued in the TLR4d cords and became significantly reduced by 21 dpi ($p < 0.05$; Fig. 5E).

Caudally, NG2 immunoreactivity increased in both groups by 7 dpi ($p < 0.001$ vs naive). However, the NG2 cell response was muted in TLR4d cords and was significantly lower than WT at 7–21 dpi ($p < 0.0001$; Fig. 5E). These results are consistent with the OL number changes, which showed a more drastic deficit caudal to the epicenter.

In addition to an overall reduction in NG2, the morphology of NG2⁺ profiles differed between TLR4d and WT spinal cords. In WT, NG2⁺ cells formed thick interwoven processes along and within the lesion border (Fig. 5A,C). In contrast, in TLR4d spinal cords, NG2⁺ cells and processes were shorter and did not form a boundary or scar (Fig. 5B,D), similar to what Kigerl et al. (2007) noted for astrocytes after SCI in TLR4d mice.

TLR4 deficiency impairs differentiation into OLs of acutely proliferating OL progenitors

NG2⁺ OL progenitors divide during the first week after SCI, and a portion differentiate into new OLs. To determine whether the generation of new OLs was affected by TLR4 deficiency, mice were injected with BrdU from 1 to 7 dpi to label dividing OPCs, and the tissue was examined at 14pi for the percentage of new OLs (BrdU⁺/GST π ⁺). This analysis revealed a significant effect of distance (rostral vs caudal, $p = 0.0456$) and significant interaction between genotype and distance ($p = 0.0371$; Table 2).

In rostral WT sections, ~48% of OLs were BrdU⁺, indicating that they were formed after SCI from OPCs dividing during the first 7 dpi (Table 2). In contrast, the percentage of new OLs in TLR4d sections was half that of WT (~24%). Caudal to the epicenter, WT mice had a trend for a greater percentage of new OLs compared with TLR4d (~24 vs ~17%; Table 2). These data indicate that TLR4 signaling contributes to the post-SCI differentiation of proliferating OPCs into OLs.

TLR4d mice had reduced lipid debris clearance after SCI

To gain insight into possible mechanisms contributing to reduced NG2 and OLs in injured TLR4d spinal cords, several parameters were examined. First was myelin debris clearance because myelin debris potentially inhibits OL differentiation (Robinson and Miller, 1999; Kotter et al., 2006; Plemel et al., 2013), and TLR4 activation facilitates phagocyte recruitment and myelin debris clearance after SCI and peripheral nerve injury (Valières et al., 2006; Boivin et al., 2007; Wu et al., 2013; Li et al., 2014; Rajbhandari et al., 2014). To visualize myelin debris within phagocytes, sections were stained with Oil Red O (Fig. 6A,B). From 7 to 21 dpi, TLR4d mice had significantly less phagocytosed myelin debris in the epicenter ($p = 0.006$) and caudal to the lesion epicenter ($p = 0.0359$; Fig. 6C). Because the number of

Figure 4. Axon numbers are reduced chronically in TLR4d mice after SCI. **A**, Semithin Epon embedded sections stained with toluidine blue in uninjured, 21 dpi, and 42 dpi WT and TLR4d mice in the ventromedial white matter 0.5 mm caudal to the epicenter. Map shows region of image collection. **B**, Total intact axon counts in uninjured, 21 dpi, and 42 dpi tissue 0.5 mm caudal to the epicenter in the ventromedial white matter. Scale bar (in **A**), 10 μ m. * $p < 0.05$ versus naive. Data are mean $\pm$ SEM.

macrophages was comparable between genotypes (see above), these results suggest that macrophage lipid phagocytosis was impaired by the lack of TLR4 signaling.

To confirm this finding, we used BMDMs from TLR4d and WT mice in an *in vitro* myelin phagocytosis assay. BMDMs were treated for 24 h with media alone or LPS (a TLR4 agonist) and pHrodo Red-labeled WT myelin, which fluoresces red in acidic environments, such as inside a phagosome. TLR4 activation of WT BMDMs doubled the number of cells phagocytosing myelin compared with baseline ($p < 0.01$ vs media; Fig. 6D,F). In contrast, myelin debris phagocytosis by TLR4d BMDMs was unchanged by LPS treatment ($p < 0.05$ vs WT; Fig. 6E,F). This confirms that TLR4 signaling stimulates macrophage myelin debris phagocytosis and that lack of this pathway *in vivo* likely hampered myelin debris clearance in response to endogenous TLR4 ligands.

Post-SCI expression of growth factors and cytokines related to OL genesis are altered in TLR4d mice

Many growth factors and cytokines with potent effects on OL lineage cells are altered in the SCI environment. Thus, real-time RT-PCR was used to determine whether impaired TLR4 signaling altered expression of factors that influence the proliferation and differentiation of OL lineage cells and subsequent myelination (for review, see McMorris and McKinnon, 1996; Rosenberg et al., 2007).

Figure 5. Overall NG2 expression is reduced in TLR4d mice after SCI. *A, B*, NG2 immunoreactivity increased after SCI at 0.6 mm caudal to the lesion epicenter at 21 dpi in both WT and TLR4d mice. *C, D*, Higher-power images of the lesion border from WT (*C*) and TLR4d (*D*) taken from area of red boxes in *A* and *B* illustrate differences in NG2 cell morphology. *E*, NG2 immunoreactivity at 0.6 mm rostral and caudal to the epicenter was increased in all animals compared with naive (----). NG2 reactivity in TLR4d mice compared with WT was significantly reduced at 21 dpi rostral and 7–21 dpi caudal to the epicenter. Scale bars: *A, B*, 500 μ m; *C, D*, 100 μ m. ** p < 0.01, *** p < 0.001 versus naive; ^ p < 0.05, ^^ p < 0.001 versus WT. Data are mean $\pm$ SEM.

Table 2. Percentage of new oligodendrocytes (BrdU⁺/GST π ⁺) at 14 dpi

	Rostral (%)	Caudal (%)
WT	48.10 $\pm$ 16.48	23.67 $\pm$ 11.58
TLR4d	16.60 $\pm$ 5.68	17.38 $\pm$ 4.95

TLR4 deficiency impairs differentiation into OLs of acutely proliferating OL progenitors. The percentage of new OLs (BrdU⁺/GST π ⁺) at 14 dpi 0.6 mm rostral and caudal to the lesion epicenter was quantified in animals receiving BrdU 1–7 dpi. This analysis revealed a significant effect of distance (rostral vs caudal, p = 0.0456) and a significant interaction between genotype and distance (p = 0.0371). Data are mean $\pm$ SEM.

Fibroblast growth factor 2 (FGF2) alone (McKinnon et al., 1990) or in combination with insulin-like growth factor-1 (IGF-1; Jiang et al., 2001; Frederick and Wood, 2004; Frederick et al., 2007) can maintain OPCs in a progenitor state and prevent

their differentiation. At 7 dpi, TLR4d tissue had significantly greater IGF-1 and FGF2 mRNA compared with WT and naive tissue (Fig. 7*A, B*). At 21 dpi, IGF-1 mRNA was significantly increased in TLR4d spinal cords compared with WT and naive (Fig. 7*A*), whereas FGF2 mRNA had increased in both genotypes (Fig. 7*B*). Platelet-derived growth factor-A (PDGF-A), a growth factor that promotes OPC survival and proliferation, increased significantly in WT at 21 dpi (p < 0.05) but not TLR4d mice (Fig. 7*C*).

Given the potent actions of TLR4 signaling on cytokine expression, levels of the proinflammatory cytokine interleukin-1 β (IL-1 β) was examined, because it is necessary for OL remyelination in mice (Mason et al., 2001) and was noted previously to be lower in TLR4d SCI mice at 3 and 7 dpi (Kigerl et al., 2007). Here, IL-1 β RNA

Figure 6. Intraspinal lipid debris is increased in TLR4d mice after SCI. **A, B**, High-power representative images of Oil Red O stain 0.6 mm caudal to the lesion epicenter at 7 dpi demonstrate increased lipid debris phagocytosis in WT (**A**) versus TLR4d (**B**) mice. Map in **A** shows the region of image collection for **A** and **B**. **A**, Inset, DIC image of phagocytic cell containing lipid debris. **C**, Quantification of the proportional area of Oil Red O stain normalized to lesion size at 7, 14, and 21 dpi at the epicenter and 0.6 mm rostral and caudal. **D, E**, High-power representative images of WT (**D**) and TLR4d (**E**) phalloidin⁺ BMDMs containing myelin (arrowheads) 24 h after treatment with LPS. Cells in the bottom portion indicated by arrowheads are shown as phalloidin and myelin single channels on the right. **F**, Quantification of myelin containing BMDMs treated with pHrodo Red tagged WT myelin and media or LPS for 24 h *in vitro*. $^{\wedge}p < 0.05$, $^{**}p < 0.001$. Scale bars: **A, B**, 100 μ m; **D, E**, 50 μ m. Data are mean $\pm$ SEM.

levels were similar at 7 dpi and elevated in WT at 21 dpi ($p < 0.001$ vs naive) but not in TLR4d mice ($p < 0.01$ vs WT; Fig. 7D). This reveals that TLR4 signaling is likely required for chronic IL-1 β expression after SCI.

Inhibitors of OL differentiation are elevated in TLR4d mice after SCI

Another family of molecules that regulate glial responses are the bone morphogenetic proteins (BMPs), part of the transforming growth factor- β (TGF β) superfamily. BMPs rise over time after SCI (Chen et al., 2005; Hampton et al., 2007; Sahni et al., 2010; Hesp et al., 2015) and are affected by TLR signaling and nuclear factor- κ B activation (Huang et al., 2014; Yang et al., 2014). Therefore, mRNA for BMP2, BMP4, and TGF β were compared in TLR4d and WT SCI sites.

BMP2 RNA showed no significant difference in either genotype (data not shown). At 7 dpi, BMP4 mRNA had doubled in TLR4d tissue but was unchanged in WT (Fig. 8A). By 21 dpi, BMP4 mRNA in WT tissue was variable and had risen on average 2.5 times over naive; in TLR4d tissue, BMP4 mRNA was three times higher than naive ($p < 0.05$ vs naive). TGF β mRNA was two-fold lower in TLR4d tissue at 7 dpi but was significantly increased more than fourfold at 21 dpi in TLR4d compared with WT tissue and naive ($p < 0.001$ vs WT and naive; Fig. 8B).

Figure 7. Gene expression of factors known to influence OL lineage cell survival or fate are altered after injury in TLR4d mice during peak oligodendrogenesis. qRT-PCR on a 5 mm section of injured spinal cord centered on the lesion epicenter measured mRNA expression of IGF (**A**), FGF2 (**B**), PDGF-A (**C**), and IL-1 β (**D**). ---- indicates naive. $^{*}p < 0.05$, $^{***}p < 0.001$ versus naive; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ versus WT. Data are mean $\pm$ SEM.

BMPs and TGF β signaling can induce expression of inhibitor of differentiation (ID) molecules (Samanta and Kessler, 2004; Cheng et al., 2007; Lasorella et al., 2014). Both ID2 and ID4 are cell-intrinsic inhibitors of OL differentiation, and both have been

Figure 8. Inhibitors of OL differentiation are increased after SCI in TLR4d mice. qRT-PCR on a 5 mm section of injured spinal cord centered on the lesion epicenter measured mRNA expression of BMP4 (**A**), TGFβ (**B**), ID2 (**C**), and ID4 (**D**). ---- indicates naive. * $p < 0.05$, *** $p < 0.001$ versus naive; ^ $p < 0.05$, ^^ $p < 0.001$ versus WT. Data are mean $\pm$ SEM.

shown to rise chronically after SCI (Hesp et al., 2015). ID2 mRNA was twice as high in TLR4d tissue at 7 dpi; by 21 dpi, WT ID2 had increased comparably (Fig. 8C). ID4 RNA expression was significantly greater in TLR4d spinal cords at 7 and 21 dpi compared with WT and uninjured controls ($p < 0.05$ vs WT and naive; Fig. 8D). Collectively, these data show elevated BMP4, TGFβ, and ID4 mRNA in the TLR4d spinal cords after injury. Thus, TLR4 may function normally to keep levels of these inhibitors of OL differentiation low after SCI.

Discussion

This work demonstrates that TLR4 signaling is essential for maximal sparing and replacement of OL lineage cells after SCI. Acutely, TLR4d tissue lost significantly more OLs and NG2 cells compared with WT. This was despite comparable injury severities, lesion sizes, and intraspinal bleeding. However, other notable differences were detected between TLR4d and WT injured spinal cords that might be related to greater acute cell loss. One example was significantly lower ferritin expression 1 dpi in TLR4d cords, which is required for safe iron storage. Because overall iron levels were comparable in both groups, the blunted ferritin response in TLR4d lesions would reduce the typically robust post-SCI iron sequestration by macrophages (Sauerbeck et al., 2013). Iron is highly reactive, and iron sequestration by macrophages protects nearby cells from iron-induced oxidative damage (Olakanmi et al., 1993). Therefore, it is possible that excess free iron in TLR4d cords could lead to a more toxic injury milieu that would exacerbate loss of vulnerable cells such as OLs. TLR4 activation can stimulate ferritin gene expression (Torti and Torti, 2002; Zhang et al., 2005). Thus, it is likely that TLR4 activation is an early post-SCI trigger for stimulating ferritin expression by microglia and macrophages.

OL numbers rebound after SCI, rising from a significant loss at 1 dpi back to baseline within 1–2 weeks, which was confirmed

here in WT and TLR4d animals. This occurred despite the lower number of NG2⁺ OPCs in acute TLR4d spinal cords, suggesting that the remaining cells overcame this deficit and differentiated into OLs over the first week after injury. In WT mice at 7–21 dpi, the number of OLs rose beyond naive levels, confirming previous studies (Tripathi and McTigue, 2007; Hesp et al., 2015). However, this chronic increase in OLs was absent in TLR4d injured spinal cords, which is consistent with their lower number of new (BrdU⁺) OLs at 14 dpi. Previous work showed that new OLs generated 2–8 weeks after SCI myelinate spinal axons for at least 3 months after injury, revealing an important contribution to endogenous repair (Hesp et al., 2015). The lack of chronic rise in OLs in TLR4d spinal cords implicates TLR4 signaling in promoting or sustaining chronic oligodendrogenesis after SCI.

The hampered oligogenic response after SCI may be the result of a combination of differences between TLR4d and WT spinal cords. First, we demonstrate that TLR4 activation stimulates myelin debris phagocytosis *in vitro* and that a lack of TLR4 hampered lipid debris clearance from the injury site *in vivo*. This fits with previous observations demonstrating that TLR4 activation accelerates macrophage debris phagocytosis (Vallières et al., 2006; Boivin et al., 2007; Li et al., 2014; Rajbhandari et al., 2014) and indicates that TLR4 is necessary to facilitate phagocytic removal of myelin and other debris from the injured area. Myelin debris inhibits OL differentiation, making debris removal essential for optimal post-SCI OL replacement (Robinson and Miller, 1999; Kotter et al., 2006; Plemel et al., 2013). Furthermore, myelin-laden macrophages have an anti-inflammatory phenotype (Boven et al., 2006; van Rossum et al., 2008; Bogie et al., 2013; Kroner et al., 2014; Wang et al., 2015). Thus, in addition to reduced debris clearance in TLR4d mice, reduced macrophage phagocytosis may impair a phenotypic switch in macrophages that supports chronic oligodendrogenesis and tissue repair.

Lack of TLR4 signaling after SCI also markedly altered mRNA expression of several factors known to affect OL lineage cell fate and survival. At 7 dpi, IGF-1, FGF-2, and ID4 mRNA were all significantly higher in TLR4d spinal cords. Collectively, this combination would hamper OPC cell cycle exit and differentiation, which is consistent with the reduced number of BrdU⁺ OLs derived from OPCs proliferating during the first week after injury in TLR4d tissue. The marked increase in IGF-1 in TLR4d tissue is in accord with work showing that TLR4 activation decreases IGF-1 expression by microglia (Pang et al., 2010; Suh et al., 2013). After SCI in WT mice, endogenous TLR4 ligands may similarly suppress IGF-1 expression in the injured spinal cord. As stated above, the combination of IGF-1 and FGF-2 promotes cell cycle entry by OPCs (Jiang et al., 2001; Frederick and Wood, 2004; Frederick et al., 2007), and FGF-2 alone can prevent OL differentiation (McKinnon et al., 1990).

By 21 dpi, IGF-1, TGFβ, BMP4, and ID4 expression was increased whereas PDGF-A and IL-1β expression was decreased in

TLR4d spinal cords. Although factors such as IGF-1 and TGF β can enhance OL survival and differentiation, the balance of the environment, with lower PDGF-A and IL-1 β and elevated BMP4 and ID4 (and likely additional changes not tested), must have been such that the long-term “oligogenic” nature of the TLR4d injured spinal cords was reduced. BMP signaling can induce expression of IDs, which in turn inhibit OL genesis. As their name suggests, ID proteins in general reduce differentiation of various progenitor cells (Ling et al., 2014). ID4 in particular inhibits OL differentiation by sequestering Olig transcription factors in the cytoplasm, thereby preventing transcription of genes needed for OL maturation (Samanta and Kessler, 2004; See and Grinspan, 2009). Interestingly, TLR4 signaling can reduce BMP2 and ID4 expression in bone (Yang et al., 2014). Our results suggest that it may be an endogenous regulator of IDs and BMPs in the CNS as well.

In addition to lower OL numbers, the normally robust increase in NG2 expression after SCI was tempered in TLR4d tissue. NG2 proteoglycan is expressed on OPCs and pericytes (for review, see Stallcup, 2002) and also by infiltrating nonmyelinating Schwann cells and macrophages within the lesions (Jones et al., 2002; McTigue et al., 2006). In addition to being expressed on the cell surface, NG2 can be cleaved and accumulate extracellularly (Nishiyama et al., 1995; Sakry et al., 2014) and may contribute to glial scarring along lesion borders. Here we noted reduced overall NG2 expression and a marked difference in NG2 cell morphology around the lesions. Because oligodendrogenesis is most prominent along lesion borders (Tripathi and McTigue, 2007), reduced overall NG2 reactivity could signify a reduced or dystrophic population of OPCs available for OL production in TLR4d spinal cords. In addition, NG2 facilitates signaling of several growth factors in OPCs (Nishiyama et al., 1996; Goretzki et al., 1999; Grako et al., 1999). Therefore, growth factor effects on OPCs may have been impaired as a result of insufficient NG2, potentially explaining why enhanced expression of some growth factors was not associated with increased OL genesis.

A final potential contributor to reduced OL generation is the lower axon number detected at 21 and 42 dpi in TLR4d tissue. This extends the work by Kigerl et al. (2007) who detected reduced white matter sparing in TLR4d SCI tissue; the current data show the loss is not only to myelin but extends to axons as well. Axons can regulate OPC responses, and the lower number of surviving or sprouting axons in TLR4d tissue may have provided a reduced drive for chronic OPC proliferation and OL differentiation, at least in that region. This highlights the need for understanding the role of TLR4 in acute neuroprotection and in debris removal because myelin debris not only reduces OL genesis but also inhibits axon growth.

These results detailing reduced cell replacement and hampered debris cleanup indicate that TLR4 signaling plays an important and ongoing role in CNS lesion resolution. TLR4 signaling after SCI is most likely mediated through microglia and macrophages as they maintain high TLR4 expression (Kigerl et al., 2007). Despite being a “sterile” lesion, many ligands are present in the injury environment that can activate TLR4, such as heme, HMGB1, and fibronectin (Kigerl and Popovich, 2009). Absence of TLR4 signaling in microglia and macrophages after SCI likely altered their functions, which in turn may have affected the function of other cellular constituents, such as astrocytes, which produce many of the growth factors measured here. Glial scar formation by astrocytes is markedly defective in TLR4d spinal cords after SCI, and this in turn is thought to have allowed entry of activated macrophages into spared white matter (Kigerl

et al., 2007). Results here show that the typically robust NG2 cell response within the glial scar was also abrogated, which could have contributed to faulty lesion containment. This collectively confirms that TLR4 signaling positively regulates glial scar formation after SCI.

In summary, this work expands the roles of TLR4 signaling after SCI to include regulating survival of OLs, OPCs, and axons, promoting OL replacement, facilitating debris clearance, and regulating growth factor mRNA and ferritin expression. This highlights the integral role of inflammatory cells in an array of CNS injury processes essential for lesion stabilization and tissue repair. It also shows the complex and interrelated roles of the cellular constituents within the injured spinal cord, in that preventing signaling from a single receptor on one cell type dramatically altered the lesion microenvironment and cellular responses of multiple other cells. Last, it adds to our knowledge of regulatory mechanisms important for reducing death of OL lineage cells and enhancing their replacement in the injured adult CNS.

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