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Intraspinal TLR4 activation promotes iron storage but does not protect neurons or oligodendrocytes from progressive iron-mediated damage

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Abstract

Iron is essential for basic cellular functions but in excess is highly toxic. For this reason, free iron and iron storage are controlled in the periphery by elaborate regulatory mechanisms. In contrast, iron regulation in the central nervous system (CNS) is not well defined. Given that excess iron is present after trauma, hemorrhagic stroke and neurodegeneration, understanding normal iron regulation and promoting iron uptake in CNS pathology is crucial. Peripherally, toll-like receptor 4 (TLR4) activation promotes iron sequestration by macrophages. Notably, iron-rich sites of CNS pathology typically contain TLR4 agonists, which may promote iron uptake. Indeed, our recent work showed impaired iron storage after acute spinal cord injury in mice with TLR4 deficiency. Here we used a reductionist model to ask if TLR4 activation in the CNS stimulates iron uptake and promotes neuroprotection from iron-induced toxicity. For this, we measured the ability of microglia/macrophages to sequester exogenous iron and prevent pathology with and without concomitant intraspinal TLR4 activation. Results show that, similar to the periphery, activating intraspinal TLR4 via focal LPS injection increased mRNA encoding iron uptake and storage proteins and promoted iron sequestration into ferritin-expressing macrophages. However, this did not prevent oligodendrocyte and neuron loss. Moreover, replacement of oligodendrocytes by progenitor cells – a normally robust response to *in vivo* macrophage TLR4 activation – was significantly reduced if iron was present concomitant with TLR4 activation. Thus, while TLR4 signaling promotes CNS iron uptake, future work needs to determine ways to enhance iron removal without blocking the reparative effects of innate immune receptor signaling.

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Keywords

progenitor; myelin; cytokine; ferritin; microglia; spinal cord injury; NG2

Introduction

Iron is essential for virtually all cellular processes, including energy production, proliferation and protein synthesis. Iron is highly reactive, however, and in excess will induce oxidative radicals that directly damage proteins, DNA and lipids. Thus, excess iron in the central nervous system (CNS) following injuries such as hemorrhagic stroke or neurotrauma can exacerbate neuropathology. Iron also accumulates in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (Zecca, 2004). Thus, excess iron within the pathological CNS may be a common mechanism for progressive neuron and glial loss (Armstrong et al., 2001; Uttara et al., 2009; Caliaperumal et al., 2012). How iron is handled in the intact and injured CNS, however, is not yet well understood.

In addition to iron, sites of CNS disease and trauma commonly contain ligands that activate toll-like receptor 4 (TLR4) (Kigerl & Popovich, 2009). This is relevant as systemic TLR4 activation promotes iron uptake and sequestration by macrophages. Indeed, iron chelation by innate immune cells (e.g., macrophages) is a key mechanism for controlling infection. When pathogens bind TLR4, signaling pathways are initiated that promote iron sequestration, thereby restricting iron from microbes, which need it for proliferation and infiltration (Nairz et al, 2010). Given that TLR4-expressing microglia and macrophages accumulate at CNS injury sites, central TLR4 signaling may be an endogenous repair mechanism that limits the toxicity of excess iron at sites of neuropathology. In support of this, our prior work showed that functional recovery and intraspinal iron storage were impaired after spinal cord injury in mice with deficient TLR4 signaling (Kigerl et al., 2007; Church et al., 2016a). Also, activation of intraspinal TLR4 by a non-pathogenic TLR4 agonist promotes myelin repair in a model of CNS demyelination (Church et al, 2016b).

To test the hypothesis that TLR4 activation stimulates iron uptake in the CNS, we measured the ability of microglia/macrophages to sequester exogenous iron and prevent iron-induced pathology with and without concomitant TLR4 activation. A reductionist non-traumatic microinjection model was used to allow focusing on the specific interaction between TLR4 activation and iron, without the confounds of other pathological mechanisms present in CNS disease or trauma. Results show that TLR4 activation increases intraspinal mRNA for iron storage proteins and enhances iron uptake and storage by microglia and ferritin-positive macrophages. However, enhanced TLR4 signaling did not prevent iron-induced neurotoxicity or oligodendrocyte loss. Moreover, replacement of oligodendrocytes by progenitor cells – a normally robust response to intraspinal TLR4 activation – was significantly impaired when iron was present at the time of TLR4 activation.

Collectively, these data indicate that it is possible to co-opt intraspinal inflammation to enhance iron sequestration. However, alone, this is not a mechanism that can overcome the pathology caused by excessive inflammation and/or iron accumulation. For neurological diseases associated with intraparenchymal iron overload (e.g., brain/spinal cord injury,

stroke), additional strategies are needed to enhance iron uptake without also eliciting inflammatory-mediated cytotoxicity.

Material and methods

Neonatal spinal cord microglia

Neonatal spinal cord microglia were cultured as previously described with minor modifications (McCarthy & De Vellis 1980; Kerstetter and Miller 2012). Briefly, 2-day-old Sprague Dawley rat pups were decapitated and their vertebrae were removed by cutting bilaterally along the length of their spinal columns. After the removal of meninges, the spinal cords were isolated and cut into small pieces, digested in TrypLE express (Thermo Fisher) for 30 min and neutralized with cell culture media containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% glutamax, 100IU/ml penicillin and 100µg/ml streptomycin. After brief centrifugation, the spinal cord tissue was resuspended and triturated in cell culture media and the resulting cell suspension was plated into 75cm² tissue culture flasks coated with 10µg/ml poly-L-lysine. The mixed glial cultures were maintained for 10d in cell culture media with media changes on days 3 and 6. On day 10, the mixed glial cultures were vigorously shaken for 18h on a rotary shaker incubator (240rpm) and the detached cells were collected. Oligodendrocyte progenitors and microglia in the cell suspension were separated by plating the cells in uncoated plastic wells, to which only microglia adhere. The following day, microglia were stimulated with FeCl₃ (1mM), LPS (100ng/ml; Sigma 0111:B4), and a combination of both with or without TAK-242 (0.5 µM; Millipore) for 24h. Microglia were then either fixed in 4% PFA for 30min or homogenized in TRIzol reagent (Life Technologies).

In vitro iron uptake

Fixed microglia were stained for non-heme bound iron using a modified Perls Prussian blue staining protocol. Briefly, endogenous peroxidase activity was quenched using a 4:1 mixture of methanol and 30% hydrogen peroxide for 15 min followed by an incubation of the cells in a 1:1 mixture of 4% HCl and 4% potassium ferrocyanide solution (Polysciences, Inc) for 30 min. Finally, after 10min incubation with 0.1% Triton X-100 in PBS, the Prussian blue signal was amplified using DAB with nickel (Vector). DAPI counterstain was used to identify the nuclei. Then, images from 50 arbitrary viewfields were taken from each well ($n = 4/\text{group}$) using an ArrayScanXTI High Content Analysis Reader (Thermo Fisher) and the images were analyzed using the associated HCS Studio Cell Analysis Software to identify the number of cells positive for Perls stain per viewfield. The percentage of iron positive cells per well was calculated and reported.

Glutamate and Griess assay

Adult rat bone marrow-derived macrophages (BMDMs) were generated using a modified protocol described previously (Longbrake et al., 2007). Briefly, bilateral femurs and tibias were dissected from adult Sprague Dawley rats. Using a 23 gauge needle, bone marrow was flushed into sterile conical tubes using a syringe filled with ice cold DMEM. Cells were triturated into a single cell solution, and red blood cells were lysed in lysis buffer (0.15M NH₄Cl, 10mM KHCO₃, 0.1mM Na₂EDTA, pH 7.4). After washing, cells were plated at

1x10⁶ cells per ml of DMEM supplemented with 10% FBS, 20% L929 supernatant, 0.5% gentamicin, 1% HEPES, 1% glutamax, and 0.001% β -mercaptoethanol. Supernatant from L929 cells contains macrophage colony stimulating factor (CSF1) required to drive bone marrow cells to differentiate into macrophages (Burgess et al., 1985). After 7d *in vitro*, BMDMs were re-plated into wells with DMEM, supplemented with 10% FBS, 1% glutamax, and 0.25% gentamicin. The following day, BMDMs were stimulated with the either FeCl₃ (1mM), LPS (100ng/ml, Sigma; 0111:B4) or both LPS and FeCl₃ for 24h. Supernatants were collected and spun at 13,000 x g to remove insoluble material. Colorimetric glutamate (Sigma) and Griess (ThermoFisher Scientific) assays were conducted according to manufacturer's instructions.

Solution preparation for *in vivo* microinjections

Vehicle and isosmotic ferric-citrate solutions were prepared as described by McDonald *et al.* (2002). First, the vehicle solution was made using sodium citrate-dihydrate (180mM), sodium bicarbonate (11.4mM), and tris base (0.455M), in 0.1M sterile PBS. A 0.75nM ferric citrate solution was made with ferric chloride hexahydrate (100mM) diluted with vehicle. LPS (1mg/ml, Sigma; 0111:B4) was similarly diluted in the vehicle solution. LPS + iron solution consisted of 2x concentrated LPS and ferric-citrate solution mixed 1:1. All solutions were filter sterilized using a 0.22 μ m filter, and iron+LPS solutions were mixed immediately before use.

Intraspinal microinjections

All surgical and postoperative care procedures were performed in accordance with The Ohio State University Institutional Animal Care and Use Committee. Adult female Sprague-Dawley rats (~250 grams; $n = 56$) were randomly assigned to treatment groups (vehicle control, iron, iron+LPS, LPS; $n = 10$ /group), then anesthetized with an intra-peritoneal injection of ketamine (80mg/kg) and xylazine (10mg/kg). Using aseptic technique, a laminectomy was performed at the T8 vertebral level. Custom pulled UV-sterilized glass micropipettes beveled to an outer tip diameter of 25–40 μ m were loaded with the proper solution and positioned 0.7mm lateral to the dorsal spinal cord midline. Using a hydraulic micropositioner (David Kopf Instruments, Tujunga, CA), pipettes were lowered 1.1mm into the spinal cord. For histological experiments, a 500nl bolus injection was administered to the lateral gray-white matter border using a PicoPump (World Precision Instruments). For tissue RNA experiments, a 200nl bolus injection was administered bilaterally in the lateral gray-white matter border. Injection sites were labeled with sterile charcoal (Sigma), muscles surrounding the laminectomy were sutured, skin was stapled with wound clips, and rats were given 5cc sterile saline (subcutaneous) before being placed into a warmed recovery cage. Two rats died due to complications with anesthesia.

Bromodeoxyuridine administration

The thymidine analog 5-bromo-2-deoxyuridine (BrdU) (50mg/kg, in sterile saline; Roche) was used to label proliferating cells. Rats were given an i.p. injection of BrdU (20mg/ml) at 2 and 4h post-microinjection and once a day until they were euthanized.

Tissue processing: immunohistochemistry

At 1d or 7d post-injection, rats ($n = 4-5$ per group) were deeply anesthetized and perfused transcardially with 4% paraformaldehyde (PFA) in PBS. Spinal cords were carefully removed and post-fixed for an additional 2h. Following overnight incubation in 0.2M PB, spinal cords were cryoprotected in a 30% sucrose solution for 48–72h. 8 mm segments of spinal cord centered on the injection site were then blocked and frozen on dry ice. Once frozen, cords were embedded in OCT (Electron Microscopy Sciences) and the blocks were frozen on dry ice. Spinal cord cross-sections were cut at 10 μ m using a cryostat, and mounted sequentially onto SuperFrost Plus slides (Thermo Fisher Scientific). Tissue was stored at -20°C until use.

Tissue processing: mRNA extractions

Rats ($n = 4$ per group) were deeply anesthetized and transcardially perfused using ice-cold 0.1M DEPC-PBS. A 2mm segment of thoracic spinal cord centered on the injection site was dissected, homogenized in TRIzol (Life Technologies) and frozen at -80°C until processed for RNA isolation.

Immunohistochemistry

Briefly, slides were rinsed with 0.1M PBS followed by blocking of nonspecific antigens with 4% BSA/0.1% Triton-100/PBS (BP^+) for 1h. Primary antibodies were then applied overnight at 4°C . After rinsing, biotinylated secondary antibodies were applied for 1h at room temperature. Endogenous peroxidase activity was quenched using a 4:1 mixture of methanol and 30% hydrogen peroxide. Secondary antibodies were visualized using Elite-ABC (Vector Laboratories) with DAB or SG as substrates (Vector). In some instances, tissue was counter-stained with methyl green or neutral red. Last, slides were dehydrated in ethanol and coverslipped with Permount (Fisher). Sections labeled for BrdU were incubated in 2N HCl at 37°C for 25min prior to primary antibody incubations. Primary antibodies include: mouse Ox42 (CD11b on CNS macrophages – 1:2000; Serotec), mouse ED1 (CD68 on CNS macrophages – 1:2000; Serotec), rabbit NG2 (NG2 cells - 1:200; Millipore), mouse CC1 (OLs - 1:500; Abcam), rabbit GFAP (astrocytes - 1:4000; Sigma), mouse BrdU (proliferating cells, 1:200; DSHB), and mouse NeuN (neurons - 1:50,000; Chemicon). Non-heme bound iron was labeled using a modified Perls Prussian blue staining protocol. Briefly, endogenous peroxidase activity was quenched using a 4:1 mixture of methanol and 30% hydrogen peroxide. Then, slides were incubated in a 1:1 mixture of 4% HCL and 4% potassium ferrocyanide solution (Polysciences, Inc) for 30min. Finally, after a 10min incubation with 0.1% tritonX-100 in PBS, the Prussian blue signal was amplified using DAB with nickel (Vector).

Immunofluorescence

Briefly, slides were rinsed with 0.1M PBS followed by blocking nonspecific antigens with BP^+ for 1h. Primary antibodies were applied overnight at 4°C . Following rinses, secondary Alexa Fluor antibodies (1:500–1000; Invitrogen) were applied for 1h at room temperature. Slides were incubated in DAPI (1:50,000; Invitrogen) for 15min at room temperature to label nuclei. After PBS and dH_2O rinses, slides were coverslipped in Immu-Mount (Thermo

Scientific). Primary antibodies: mouse Ox42 (CD11b on CNS macrophages – 1:2000; Serotec), rabbit H-Ferritin (1:5000; Abcam), and rabbit L-Ferritin (1:2000; Abcam).

Microscopy and quantitative analysis

All data are reported as mean $\pm$ SEM and all analyses were performed in a blinded manner. Fluorescent images of double-labeled ferritin/CD11b cells were captured using an Olympus FV1000 laser scanning confocal microscope, and processed with the corresponding Fluoview software. A Zeiss Axioskop 2 Imaging microscope with a Sony 970 three-chip color camera was used to capture and analyze bright-field images. To quantify macrophage density in the ipsilateral cord, low power images were digitized and manually outlined using the MCID Elite imaging software (Imaging Research Inc., Canada). For Perls iron and GFAP quantification, a 0.1mm² box was centered on the injection site, overlapping gray and white matter. Proportional area was calculated as the area of CD11b, CD68, GFAP, or Perls immunoreactivity, divided by sample area.

Cell counts for oligodendrocytes (CC1), NG2 cells, NG2/BrdU double-labeled cells and neurons (NeuN) were manually collected at high magnification using 0.02mm² reticule boxes. For all quantifications, counts from 3 serial sections centered on the injection site were averaged for each animal. For OL and NG2 cell counts, one 0.02mm² reticule box in the ventral/intermediate GM and three non-overlapping 0.02mm² reticule boxes in the WM directly adjacent to GM were reported as “injection site.” NeuN counts consisted of one 0.02mm² reticule box in the ventral/intermediate GM. For all analyses, data are expressed as the number of cells per cubed millimeter.

Because NG2 is also expressed on other cell types, conservative NG2 cell counts were conducted based on morphological criteria (Tripathi & McTigue, 2007). A single- or double-labeled NG2 cell was only counted if the NG2 immunoreactivity surrounded an identifiable nucleus in a single plane of focus and possessed multiple NG2-expressing processes (to rule out counting pericytes or macrophages). Because astrocytes can also express CC1, OLs were only counted if the CC1 expressing profile did not co-express GFAP. Occasionally, NG2+ and CC1+ profiles were verified at higher magnification (64x).

Statistical analyses and graphs were generated in Prism 5.0 (GraphPad Software Inc.). Quantitative differences between groups were analyzed using a 1-way ANOVA followed by Tukey's *post hoc* test or a 2-way ANOVA followed by Bonferroni's *post hoc* test. Significance was set at $p < 0.05$.

Quantitative real-time PCR

TRIzol reagent was used to extract RNA from tissue or neonatal microglia following the manufacturers protocol (Life Technologies). RNA was reverse transcribed using SuperScript II (Invitrogen) to generate cDNA. Quantitative real-time PCR was performed using gene-specific primers to measure mRNA levels in the microinjected tissue ($n = 4$ per group) or stimulated microglia ($n = 3$ per group). PCR product was measure using SYBR Green fluorescence using an Applied Biosystems 7900HT Fast Real-Time PCR System. The sequences for primers used were: 18S(F) TTCGGAAGTGGAGCCATGAT, (R) TTTTCGCTCTGGTCCGCTTGT; H-Ferritin (F) TTGCAACTTCGTCGCTCCGCC, (R)

TGGCGCACTTGCGAGGGAGA; L-Ferritin (F) GCAGCGCTTTGGAGATCCCG, (R) AGTCCCCGGGTCTGTTCCGT; transferrin receptor (F) GGCTATGAGGAACCAGACCGCTACA, (R) TGGACTTCGCAACACCAGGGC; DMT-1 (F) TGTCGCCTGTCCATTTGGCCG, (R) TGGCGTGGCGGGGTTGAAAT; hepcidin (F) TATCTCCGGCAACAGACGAG, (R) AGCGCACTGTCATCAGTCTT; ceruloplasmin (F) TGCAACAAGCCCTCACCGGA, (R) TGGTCTCCTCGGCAGCGATGTA; iNOS (F) TTGGTGAGGGGACTGGACTTT, (R) CCGTGGGGCTTGTAGTTGA; IL-1 α (F) TCACTCGCATGGCATGTGCTGA, (R) TCGGGCTGGTTCCACTAGGCT; TNF α (F) TGATCCGAGATGTGGAAGTGG, (R) CGATCACCCCGAAGTTCAGTAG. Gene expression relative to the housekeeping gene 18S was analyzed using the comparative C_T method (Schmittgen & Livak, 2008). Significance was reported when $p < 0.05$ after running a one-way ANOVA with Tukey *post hoc* test.

Results

Activation of TLR4 in spinal cord microglia promotes iron uptake in vitro

Microglia are the primary TLR4-expressing cells in the CNS (Lehnardt et al., 2002; Kigerl et al., 2007; Trotta et al., 2014). *In vitro* data indicate that activating TLR4 on brain-derived microglia upregulates the free iron importer divalent metal transporter 1 (DMT1) and increases intracellular iron (Urrutia et al., 2013). However, spinal cord microglia do not consistently mimic brain microglial responses (de Haas et al., 2008; Zhang & Gensel, 2014) and internalization of iron following TLR4 stimulation of spinal cord microglia has not been examined. Thus, cultured spinal cord microglia were treated with iron (1mM Ferric chloride), LPS (100ng/ml), iron+LPS, or iron+LPS+TLR4-inhibitor TAK-242 (0.5 μ M; Matsunaga et al., 2011) for 24h and intracellular iron identified with Perls stain to detect iron uptake. Treating microglia with media or LPS had no effect on iron uptake, likely because little iron was present in the culture medium (Fig. 1A). When iron was added to the microglial cultures, the number of iron-containing microglia increased significantly (Fig. 1A, B). When the same amount of iron was added concomitant with LPS, iron uptake was even greater, with twice as many iron-containing microglia present compared to treating with iron alone ($p < 0.001$; Fig 1A, B). When the selective TLR4-inhibitor TAK-242 was added concomitant with iron+LPS, the number of iron-containing microglia returned to iron alone levels (Fig. 1A, B), verifying LPS acted via TLR4 signaling. Overall, these data confirm that TLR4 activation promotes iron uptake by spinal cord microglia.

Intraspinal TLR4 activation promotes iron sequestration and ferritin expression

To gain insight into CNS iron handling and toxicity, we tested whether TLR4 activation in the spinal cord increased local iron uptake. For this, iron was microinjected with or without LPS into the spinal cord gray matter/white matter (GM/WM) border.

At 1d post-injection, sections were labeled with Perls stain to visualize iron. As expected, vehicle-injected spinal cords had no demonstrable iron at 24h. Sections from the iron group displayed dense Perls stain at the GM/WM border, suggesting that most iron remained deposited at the injection site for 24h (Fig. 2A, B). In contrast, iron co-injected with LPS had a completely different pattern; it was reduced and more diffusely distributed throughout

the tissue within cells resembling macrophages (confirmed below) (Fig. 2C & I). In sections treated with LPS alone, iron labeling was not different from controls.

By 7d post-injection, iron staining in the iron-injected tissue was less dense than at 1d but was still 8-fold greater than controls (Fig. 2F, I). In the iron+LPS sections, iron mostly persisted in gray matter cells resembling macrophages (Fig. 2G). LPS injected tissue had a modest increase in iron compared with 1d (Fig. 2H, I), consistent with previous results from our group (Schonberg & McTigue, 2009).

These data demonstrate that when a bolus of iron is introduced into the CNS, concomitant TLR4 activation shifts its distribution from dense local accumulation to a pattern indicative of diffuse macrophage uptake within 24h. To verify this, 1d post-injection tissue was double-labeled for CD11b and ferritin, an iron storage protein upregulated by increased intracellular iron and by TLR4 activation (Carraway et al., 1998; Rouault, 2006; Piccinelli & Samuelsson, 2007). In the iron injected group, ferritin expression remained at baseline, verifying that local cells did not internalize the extracellular iron (Fig. 3A, B). In contrast, when iron was co-injected with LPS, ferritin-expressing macrophages were abundant (Fig. 3C), matching the distribution of iron in adjacent sections (see Fig. 2C). LPS injected alone induced a similar distribution of macrophages with fewer co-expressing ferritin, again consistent with low iron levels in the tissue at 24h (Fig. 3D).

To examine astrocyte responses to the exogenous iron, 1d post-injection tissue was double-labeled for GFAP and ferritin. A significant reduction of astrocytes in the injection site of both iron conditions suggests that the concentration of iron used here is cytotoxic to astrocytes (Fig. 4). Additionally, astrocytes did not appear to sequester iron in this paradigm, based on a lack of ferritin-positive astrocytes (not shown). Collectively, these data indicate that within the CNS, spontaneous uptake of extracellular iron by microglia and macrophages is minimal but can be increased by concomitant activation of TLR4.

TLR4 activation overrides the CNS macrophage response to iron in vivo

To compare how iron and/or LPS affected microglia or macrophage accumulation, their overall response was characterized at 1d and 7d post-injection by quantifying CD11b immunoreactivity. Vehicle injection had no effect beyond minor microglial activation in the pipette track (Fig. 5A). In iron injected tissue, a ring of activated microglia surrounded iron injection sites in gray matter with minimal white matter activation at 1d (Fig. 4B). When iron was co-injected with LPS, the pattern was similar to iron and ferritin distribution above; small round CD11b+ cells were spread throughout the ipsilateral gray and white matter at 1d (Fig. 5C), a response similar to that following LPS alone (Fig. 5D). Quantification of CD11b + immunoreactive area revealed that LPS and iron+LPS comparably and significantly increased CD11b expression compared with vehicle or iron injection (Fig. 6). Thus, TLR4 activation overrides the effect of iron on acute macrophage activation and distribution.

Between 1 – 7d post-injection, microglia and macrophage accumulation and/or size had changed in all groups except control. By 7d after iron treatment, CD11b had significantly increased as activated microglia and macrophages filled the local gray matter injection site (Fig. 5F; Fig. 6). At 7d after LPS, overall levels of CD11b were comparable to 1d, although

most cells displayed a larger phenotype (Fig. 5H; Fig 6). In the iron+LPS group, CNS macrophage accumulation was markedly denser in the gray matter, again with most cells displaying a larger phenotype compared with 1d (Fig. 5G–H'). At this time, the iron+LPS group had significantly more CD11b immunoreactivity compared with iron or LPS groups (Fig. 6). Thus, exposing CNS macrophages to iron+LPS enhanced their reactivity by 7d compared with either given alone.

The differences in CD11b+ cell morphology suggest injecting iron alone did not induce robust phagocytosis whereas injecting iron with LPS did (i.e., small round cells). Indeed, TLR4-activation increases the number and phagocytic activity of CNS macrophages (Vallières et al., 2005). To verify this, adjacent sections were immunolabeled for CD68, an indicator of phagocytic cells. At 1d, CD68 was almost completely absent in regions of increased microglial CD11b reactivity in iron-injected tissue (Fig. 7A, E). In contrast, CD68+ cells were 4-fold denser throughout the region of microglia/macrophage activation in the iron+LPS group (Fig. 7B, E). By 7d, CD68 had increased in both iron groups suggesting increased phagocytosis over time (Fig. 7C–E). However, CD68+ cells in iron-injected tissue were smaller and less dense than when iron was combined with TLR4 activation (Fig. 7E). Thus, exposure to iron alone induced delayed and lower phagocytosis than iron+LPS despite inducing significant cell death within 24h (see below).

Intraspinal delivery of a TLR4 agonist regulates transcription of iron sequestration genes

Because cellular iron is vital but toxic in excess, an elaborate program of evolutionarily conserved proteins regulates its import, export, storage, and oxidation status (Hentze et al., 2004). To characterize the *in vivo* response to iron and TLR4 activation, we isolated mRNA from spinal cords 1d post-injection then used real-time PCR to quantify transcriptional changes.

The two main iron import proteins are transferrin receptor and divalent metal transporter 1 (DMT1). Neither were affected by intraspinal iron injection, which is consistent with the lack of iron uptake at this time. LPS and iron+LPS slightly but significantly decreased transferrin receptor expression ~14% and significantly increased DMT1 expression ~3-fold (Fig. 8A, B), suggesting that TLR4 activation primes the intraspinal microenvironment to take up transferrin-free iron. This is consistent with increased iron uptake and phagocytosis as DMT1 is expressed on the plasma membrane and by phagosomes within macrophages (Picard et al., 2000; Forbes & Gros, 2001; Jabado et al., 2002).

The iron storage protein ferritin consists of two subunits, heavy (H) and light (L) chain. Iron alone moderately but significantly increased H-ferritin mRNA but had no effect on L-ferritin (Fig. 8C, D). Consistent with the findings above, both LPS and iron+LPS significantly increased L- and H-ferritin mRNA compared with vehicle or iron alone (Fig. 8C, D). Interestingly, despite having no effect on its own, iron potentiated LPS-induced upregulation of L-ferritin transcripts (Fig. 8C). Collectively, these data indicate that TLR4 activation promotes an iron storage response.

Finally, transcription of iron export-related molecules was examined. The only mechanism for iron efflux from cells is through the exporter ferroportin, which works in conjunction

with the ferroxidase ceruloplasmin; iron export is blocked by hepcidin, which binds to ferroportin causing its internalization and degradation (Rossi, 2005). Since ferroportin is regulated at the protein level, its mRNA was not examined. Ceruloplasmin mRNA, however, significantly increased two-fold by TLR4 activation (+/- iron) compared with iron or vehicle (Fig. 8E). Ceruloplasmin alone cannot export iron without ferroportin. To get an indication of ferroportin status, hepcidin mRNA was examined, revealing that TLR4 activation (+/- iron) increased hepcidin mRNA >20-fold compared with vehicle or iron alone, suggesting iron export would be reduced by LPS (Fig. 8F). Taken together, data in Figures 2–8 support the hypothesis that activating TLR4 in the spinal cord enhances iron sequestration, likely within intracellular ferritin in macrophages; without TLR4 activation, intraspinal iron is not taken up and fails to elicit transcription of iron storage genes.

TLR4 activation does not rescue progressive iron-induced neuron loss

To determine if the enhanced iron uptake and sequestration “program” described above reduces iron-induced neurotoxicity, neurons were counted at the injection site. By 1d post-injection, neurons were significantly reduced 53–64% in iron and iron+LPS groups; LPS alone caused a non-significant 42% reduction in neurons (Fig. 9A, B). Neuron loss progressed over time such that by 7d, 85–95% of neurons at the injection sites were lost in all groups except controls (Fig. 9A, B). Thus, TLR4 activation and/or iron exposure both cause a delayed loss of neurons, and delayed iron-induced neurotoxicity was not reduced by TLR4 activation.

TLR4 activation does not rescue iron-induced oligodendrocyte loss and excess iron may impair oligodendrocyte progenitor differentiation

Oligodendrocytes are the highest iron-containing cells in the CNS and are exquisitely sensitive to iron overload. To determine if TLR4-induced iron sequestration protected OLs from iron toxicity, OLs were counted in injection sites at 1d post-injection (Fig. 10A). As expected, iron was toxic and killed >85% of OLs by 1d. Concomitant injection of LPS with iron or LPS alone killed a similar number of OLs at 1d, revealing that TLR4 signaling and excess iron both kill OLs in the intact CNS.

Previously, we found that intraspinal LPS elicited robust iron-dependent OL genesis by 7d (Schonberg et al., 2007, 2009). Thus, we examined 7d tissue to determine if iron altered this reparative response. While OL numbers increased in all three groups, they were significantly lower in iron+LPS injection sites compared with vehicle or LPS alone (Fig 10A). Because all groups began with similar OL numbers at 1d, these data suggest that the oligodendrogenic response was impaired when TLR4-activated microglia/macrophages were iron-loaded.

OL replacement in the adult CNS involves survival, proliferation and differentiation of NG2+ glial progenitor cells. The lower OL generation in the iron+LPS group suggests at least one of these steps was negatively affected. To examine survival, NG2 cells were counted in injection sites at 1d post-injection (Fig. 10B). LPS and iron+LPS both significantly reduced NG2 cells >50%; however, unlike OLs, NG2 cells were not killed by iron exposure alone.

To determine if NG2 cell numbers recovered over time, NG2 cells were counted in the 7d post-injection tissue. Between 1d and 7d, NG2 cells rebounded to baseline levels the LPS +iron and LPS groups (Fig. 10B). Interestingly, they also rose in the iron group (which had not lost NG2 cells at 1d); at 7d, the iron group had significantly more NG2 cells than control, LPS and LPS+iron groups. This suggests iron and LPS both stimulated NG2 cell proliferation (directly or indirectly), which was verified with BrdU immunolabeling (Fig. 10C). Thus, reduced OL replacement in the iron+LPS group was not due to impaired NG2 cell proliferation, suggesting that progenitor differentiation into OLs instead was likely blocked by the presence of excess iron.

Reduced ferritin mobilization may impair NG2 cell differentiation

Prior work by our group and others documented a link between iron, TLR4 signaling, ferritin transfer to NG2 cells, and OL genesis (Schonberg et al., 2007, 2009, 2012; Todorich et al., 2011). To determine if ferritin expression in CNS macrophages was altered by concomitant treatment with iron and LPS, ferritin-expressing macrophages were quantified at 7d post-injection (Fig. 11). At this time, iron+LPS tissue contained twice as many ferritin-positive macrophages compared with iron or LPS alone (Fig. 11A–D). Again, there was no evidence of ferritin-expressing astrocytes in any group (not shown). Thus, the increased number of ferritin-expressing macrophages after iron+LPS injection suggests that TLR4 activation promoted prolonged ferritin expression and iron retention, meaning that less iron and/or ferritin transfer to NG2 cells likely occurred, which in turn may impair differentiation into new OLs.

Cytokines, nitrites and glutamate may mediate cytotoxicity in LPS but not iron alone treated tissue

Since all treatment groups caused acute neuron and OL loss, potential mediators of cell death were examined to determine if LPS and iron induced common mechanisms or if iron altered the response to LPS. Using mRNA isolated from 1d post-injection spinal cords, quantitative real-time PCR revealed that LPS with or without iron significantly increased transcription of inducible nitric oxide synthase (iNOS, ~400-fold; Fig 12A), tumor necrosis factor alpha (TNF α , ~6-fold; Fig 11B), interleukin-1 alpha (IL-1 α , ~20-fold; Fig 12C) and interleukin-1 β (~5-fold; not shown), all factors that contribute to neuron and/or OL toxicity (Fig. 12A–C; Li et al., 2005 & 2008; Bastien et al., 2015). Cultured microglia were similarly treated to examine direct effects of LPS and iron. Results demonstrated the same pattern of upregulated iNOS, TNF α and IL-1 α mRNA after LPS+iron or LPS, suggesting that microglia likely contribute to *in vivo* transcriptional changes (Fig. 12D–F). Interestingly, despite showing above that microglia internalize iron (Fig. 1), exposure to iron alone did not alter transcription of any of these factors *in vivo* or *in vitro*.

iNOS produces superoxide and nitric oxide, which react to form peroxynitrite, a strong oxidant (Xia & Zweier et al., 1997). To determine if enhanced iNOS mRNA corresponded to increased nitrite production, the Griess assay was performed 24h after stimulation of bone marrow-derived macrophages (BMDMs; large cell numbers are needed for this assay). Nitrites in supernatants from iron+LPS and LPS-treated macrophages increased 30–40-fold compared with control or iron-treated macrophages, verifying that TLR4 activation induced

nitrite production (Fig. 12G). Because glutamate excitotoxicity is another mechanism of OL and neuron death (McDonald et al., 1998; Pitt et al., 2003), glutamate levels were quantified from the same supernatants. Iron+LPS and LPS treatment induced a significant two-fold increase in glutamate levels in supernatants; in contrast, glutamate remained at control levels in iron-treated BMDMs (Fig. 12H). Collectively, these data suggest that LPS toxicity may be mediated by multiple factors examined here (cytokines, nitrites and/or glutamate) but that iron toxicity is likely due to separate mechanisms.

Discussion

CNS injury and disease sites often contain supra-physiological levels of iron, which is highly reactive and toxic to neurons and glia. It is unlikely, however, that excess iron would be deposited in the absence of other injury-related factors, such as ligands that activate TLR4 signaling (Kigerl and Popovich, 2009). TLR4 activation regulates iron sequestration peripherally but, since CNS iron signaling is mostly independent from the periphery, the effect of TLR4 signaling on iron regulation within the CNS is not clear. Here we tested if intraparenchymal TLR4 activation increased iron uptake by CNS cells and altered iron-induced pathology. The goal was to focus specifically on the interaction of TLR4 signaling and iron regulation. Therefore, a non-traumatic microinjection model of an isosmotic ferric citrate solution and LPS was used to avoid complicating factors such as blood and serum proteins that would be present in hemorrhage or trauma models (Sauerbeck et al., 2013; Sahinkaya et al., 2014).

Multiple outcome measures indicate that intraspinal TLR4 activation concurrent with iron exposure prevented a prolonged deposition of extracellular iron and instead stimulated iron uptake and storage in CNS macrophages. First, *in vitro* studies confirmed that TLR4-activated spinal cord microglia internalize twice as much iron compared with microglia treated with iron alone. This is consistent with prior work showing that microglia can take up iron (Widmer & Grune, 2005), and that TLR4-activated microglia shift protein expression towards an iron sequestering phenotype (Urrutia et al., 2013). When iron was microinjected alone *in vivo*, a dense deposit of iron remained at the injection site at 24h. It was surrounded by activated ferritin-negative microglia, suggesting that most iron was extracellular, although H-ferritin mRNA was slightly but significantly increased at 24h. In contrast, co-injecting iron with a TLR4 agonist drastically altered iron distribution pattern to one that appeared intracellular and mirrored that of TLR4-activated macrophages at 24h. Macrophages throughout the iron+LPS tissue had robust ferritin expression at 24h indicative of high intracellular iron. Meanwhile, astrocytes were largely absent from injection sites, and did not appear to participate in iron sequestration. Thus, TLR4 activation converted the fate of iron from being a dense bolus to robust uptake and storage in ferritin within macrophages by 24h. This is consistent with mRNA changes noted at 24h in both LPS groups (but not iron alone), including increased ferritin, DMT-1 and hepcidin mRNA, all of which would promote iron uptake and retention if translated to protein.

To determine if injected iron was eventually internalized, sections were examined 7d post-injection. The gray matter region containing the iron bolus at 24h was filled with macrophages and activated microglia by 7d, a subset of which expressed ferritin indicating

iron uptake; however, the number of ferritin-positive macrophages was twice as high in the iron+LPS group. This suggests that while 7d intraspinal iron exposure caused prolonged microglial activation (perhaps due to iron-induced cell death), iron uptake and storage was minimal. In contrast, intraspinal exposure to iron plus a TLR4 agonist promoted robust iron uptake and storage in ferritin-positive macrophages within 24h that was maintained for at least 7d. Notably, LPS alone also induced ferritin+ macrophages by 7d, as we have noted before (Schonberg et al., 2009), but the numbers were also only half that seen in the iron +LPS group.

This response to iron+LPS harkens to what occurs peripherally after TLR4 activation. There, TLR4 signaling limits iron accessibility to invading pathogens by stimulating immune cell expression of iron sequestration proteins (Nairz et al., 2010). For example, TLR4-activated macrophages increase DMT1 but decrease transferrin receptor, which enhances free iron uptake (Kim & Ponka, 2000; Ludwiczek et al., 2003). Additionally, monocytes and neutrophils produce hepcidin after TLR4 activation, which causes ferroportin degradation and reduced iron release (Peyssonnaud et al., 2006; Theurl et al., 2008). Here, intraspinal TLR4 activation via LPS reproduced *all* of these RNA changes, suggesting that CNS parenchyma respond to TLR4 activation similar to peripheral immune cells in terms of producing an iron sequestration and storage phenotype.

The *in vivo* mRNA changes noted here were obtained from homogenized spinal cords and therefore the cellular sources cannot be definitively identified. Astrocytes, neurons, and OL lineage cells express iron-related proteins, and may express TLR4 (although to a lesser extent than microglia) and therefore might contribute to TLR4-induced transcriptional changes (Trotta et al., 2014; Vaure & Liu, 2014). For example, TLR4 activation of astrocytes and neurons stimulates DMT1 expression (Urrutia et al., 2013). However, given the significant loss of neurons, OLs and astrocytes in the injection site by 24h (the time of RNA collection), the contribution by these cells was likely minimal compared to microglia and macrophages, which mimicked the *in vivo* transcription changes when treated in culture.

Collectively, the data suggest that TLR4 activation concomitant with iron exposure stimulated significantly more iron uptake and storage in CNS macrophages compared with iron exposure alone. Despite this, neuron and OL loss was not reduced, likely because any protective effects of enhanced iron sequestration were negated by TLR4-induced toxicity. Here, LPS (+/- iron) injection stimulated transcription of iNOS, TNF α and IL-1 α , in contrast to iron and control groups, which did not. Furthermore, macrophage supernatants from LPS +/- iron groups had significantly more nitrites and glutamate than iron or control supernatants, suggesting these factors may have contributed to TLR4-induced cell loss *in vivo*. Notably, prior *in vitro* work showed that TLR4-activated microglia kill OL lineage cells and neurons by releasing cytokines and reactive oxygen/nitrogen species (Lehnardt et al., 2002 & 2003; Li et al., 2005; Li et al., 2008; Pang et al., 2010), which could have occurred *in vivo* in our study. Although previous descriptions of TLR4 effects on NG2 cells *in vivo* did not describe acute loss, this may be because cells were quantified 3–11d after TLR4 activation (Schonberg et al., 2007; Miron et al., 2013; Shigemoto-Mogami et al., 2014) compared to 24h here.

Another possible reason for lack of cytoprotection in iron+LPS-injected tissue is that iron may have had cytotoxic effects prior to internalization by TLR4-activated cells. *In vitro* and *in vivo* treatment with iron alone did not elevate cytokines, iNOS, or glutamate release; therefore rapid iron-induced cell death was likely mediated by different mechanisms. Known methods of iron entry into neurons and NG2 cells include transferrin receptors and DMT1, while OLs can also take in iron-containing ferritin via the TIM2 ferritin receptor (Todorich et al., 2008; Rouault 2013). Once inside cells, iron can catalyze production of highly reactive hydroxyl radicals via the Fenton reaction, which directly damage proteins, DNA and lipids (Winterbourn, 1995). Iron can also induce endoplasmic reticulum stress and apoptosis in cultured hypoxic OLs (Rathnasamy et al., 2016). Thus, assuming the injected iron was initially internalized, direct iron-induced cytotoxicity may have killed local neurons and glia (Bao & Liu, 2004; Liu et al., 2004). Since extracellular iron appeared to still be present at 24h, it is possible that uptake by remaining neurons continued over the next several days leading to their protracted loss. It is interesting to note that while neurons continued dying over 7d, new oligodendrocytes were produced and survived during this same time.

The finding that TLR4 activation alone caused neurotoxicity adds to some inconsistencies in the literature. For example, in a prior study by our group, injection of LPS into the mouse spinal cord was only neurotoxic when combined with cystine (Kigerl et al., 2012). Similarly, intrastriatal LPS injection did not cause neuron loss (Nadeau & Rivest, 2002; Glezer et al., 2003). However, intracerebral injection of LPS killed dopaminergic neurons, indicating that neuronal heterogeneity may explain varying degrees of vulnerability to TLR4 activation (Herrera et al., 2000; Fan et al., 2005; Hoban et al., 2013; Zhang et al., 2014). Another explanation is heterogeneity in commercially available LPS. For instance, endotoxin units (EU, a measure of LPS potency) between lots of LPS can differ by an order of 10. Furthermore, personal observations in our lab noted that LPS isolated from different lines of *E. coli* induce varying levels of systemic immune activation. Additionally, TLRs exhibit species-specific ligand recognition and downstream gene expression (Schroder et al., 2012; Vaure & Liu 2014), which may explain why CNS macrophage and oligodendrogenic responses vary between rats and mice in our hands (unpublished observation). Regardless, the current work shows clear evidence of rapid intraspinal neuron death induced by iron and LPS that expands over 7d.

As noted before (Schonberg et al., 2007), the initial OL loss after LPS injection was followed by robust OL replacement that returned OL numbers to baseline within 7d. Interestingly, co-injecting iron with LPS impaired OL replacement. Since iron did not hinder TLR4-induced NG2 cell proliferation, impaired NG2 cell differentiation is a plausible cause for reduced OL genesis in iron+LPS-treated tissue. LPS-induced NG2 cell differentiation depends upon available iron (Schonberg et al., 2009) and is stimulated by ferritin, which can be transferred from macrophages to NG2 cells *in vivo* (Morath & Mayer-Pröschel, 2001; Todorich et al., 2011; Schonberg et al., 2012). The excess iron present in iron+LPS tissue may have promoted iron retention, thereby preventing iron or ferritin release from macrophages. Additionally, an increase in peripheral macrophage infiltration into the injection site might contribute to the prolonged iron retention as iron+LPS tissue has higher CD11b and CD68 expression at 7d. This is consistent with the significantly greater number of ferritin-positive macrophages in the iron+LPS tissue at 7d compared to LPS-treated

tissue. Indeed, in cases of iron overload, ferritin mRNA is more readily translated via release of iron responsive element (IRE)-mediated inhibition (Piccinelli & Samuelsson, 2007). Thus, an increased drive for iron storage might have reduced the available iron needed for NG2 cell differentiation in this group and thereby restrained the reparative response evoked by TLR4 activation. This could suggest that regions of CNS pathology containing both iron and TLR4 agonists such as fibronectin or HMGB1 would have reduced ability to repair oligodendrocyte and myelin loss compared to those with lower levels of iron.

Conclusions

Overall, this work demonstrates that in cases of extracellular CNS iron overload, TLR4-activation promotes iron uptake and prolongs storage by macrophages, which would in theory be beneficial. However, potentially rapid iron-mediated toxicity and/or damage induced by TLR4 activation itself negated any cytoprotection. Further, activating TLR4 in the presence of excess iron impaired OL replacement, perhaps due to a strong drive for iron retention in iron-loaded CNS macrophages. At the surface, sequestering iron within the CNS does not appear to be beneficial. However, methods to sequester or chelate iron that occur more rapidly, are less “inflammatory” and allow safe release iron when needed for repair could have therapeutic impact due to the high number of situations exhibiting iron-induced toxicity. For instance, a non-pathologic activation of TLR4 prior to surgeries inducing CNS bleeding may be neuroprotective. Thus, future studies should examine such strategies as well as test them in more “complicated” models such as CNS trauma or hemorrhage.

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Highlights

- Intrapinal TLR4 activation induces iron sequestration by CNS macrophages
- TLR4 activation itself contributes to acute cytotoxicity
- TLR4-induced iron sequestration slows oligodendrogenesis (potentially via reduced ferritin mobilization)

Figure 1.

TLR4 activation promotes iron uptake by spinal cord microglia *in vitro*. **A)** Low power images of microglia labeled with Perls stain for iron 24h after treatment with vehicle (Control), iron (1mM Ferric chloride), LPS (100ng/ml), iron+LPS, or iron+LPS+TAK-242 (0.5 μ M). **B)** Percent of microglia treated with iron+LPS internalized iron was significantly greater than microglia treated with iron alone. TLR4 antagonist TAK-242 treatment prevented the LPS-induced increase. Bars represent mean $\pm$ SEM. One-way ANOVA with Tukey's *post hoc* test: *** p <0.001 vs. control unless otherwise noted. Scale bar: (A) 200 μ m.

Figure 2.

Intraspinal TLR4 activation alters iron distribution *in vivo*. **A–D**) Perl's stain of ipsilateral microinjection sites from vehicle (Veh), iron, iron+LPS or LPS groups at 24h post-injection. Iron injection resulted in dense Perl's staining at the injection site, while iron+LPS injection resulted in small round iron+ profiles resembling macrophages in the ipsilateral gray and white matter. Insets show high-power views of boxes. **E–H**) Injection sites at 7d post-injection stained for iron with Perl's staining show reduced iron compared to 1d in iron-injected tissue but increased iron in iron+LPS-injected sections. **I**) Densitometric quantification of Perl's labeling in the ipsilateral spinal cord reveals that iron-injected tissue had significantly more Perl's labeling than Veh and LPS +/- iron at 1d. Bars represent mean $\pm$ SEM. Two-way ANOVA with Bonferroni *post hoc* test: *** $p < 0.001$ vs. Veh unless otherwise noted. Dotted lines indicate the outline of gray matter. Insets show high-power views of boxes. Scale bars: (**A–H**) 150 μ m; (**A–H inset**) 20 μ m.

Figure 3.

Iron+LPS injection induces robust ferritin expression in macrophages by 24h post-injection. Single-label and merged confocal images from the injection site immunolabeled for Cd11b (green), Ferritin (Heavy+Light; red), and DAPI (blue). **(A)** Vehicle-injected tissue had negligible ferritin expression, as expected. **(B)** Iron-injected alone induced microglial reactivity but minimal ferritin expression over baseline. **(C)** The combination of iron+LPS injection induced robust ferritin expression by macrophages (arrows). **(D)** Macrophages were equally prevalent in LPS-injected tissue but few co-expressed ferritin (arrow). Scale bar: **(A–D'')** 50 μ m.

Figure 4.

Intraspinal iron injection induces a significant reduction of astrocytes in the injection site by 1d. **A–D**) Confocal images from the injection site immunolabeled for GFAP (green). **E**) Iron and iron+LPS injections had significantly reduced GFAP staining compared with vehicle and LPS injections at 1d. Bars represent mean $\pm$ SEM. One-way ANOVA with Tukey's *post hoc* test: ** $p < 0.01$ vs. Veh, unless otherwise noted. Scale bar: (**A–D**) 75 μ m.

Figure 5.

TLR4 activation overrides the CNS macrophage response to iron *in vivo*. Injection sites from cross-sections immunolabeled for CD11b are shown. **A–D)** Low-power images from 1d post-injection. Dotted lines outline gray matter. **A'–D')** High-power views of boxed areas in A–D. **(A,A')** Vehicle injection (Veh) did not cause microglial reactivity except in the pipette track. **(B,B')** A ring of activated microglia surrounded the iron injection site, while small round CD11b+ cells were prevalent throughout the ipsilateral gray and white matter after iron+LPS and LPS injections **(C,D)**. **E–H)** Low-power images from injection sites at 7d post-injection. High-power views of boxes are shown in **(E'–H')**. No microglial reactivity was detected in Veh injection sites at 7d **(E,E')**. **(F,F')** Activated microglia and macrophages filled in the ipsilateral grey matter iron injection site by 7d. **(G,G',H,H')** In 7d iron+LPS and LPS injection sites, white matter CD11b was reduced while larger macrophages filled the grey matter. Scale bars: **(A–H)** 200µm; **(A'–H')** 50µm.

Figure 6.

Densitometric quantification of CD11b immunoreactive area in ipsilateral spinal cords revealed that LPS alone and LPS+iron (L+I) significantly increased CD11b expression compared with Veh or iron alone at 1d. By 7d, the LPS+iron group had significantly more CD11b immunoreactivity compared with iron or LPS groups, both of which had increased CD11b compared to Veh. Bars represent mean $\pm$ SEM. Two-way ANOVA with Bonferroni *post hoc* test: * p <0.05, *** p <0.001 vs. control unless otherwise noted.

Figure 7.

Differential expression of the phagocytic marker CD68 between iron and iron+LPS groups.

A–D) CD11b of injection sites at 1d (A,B) and 7d post-injection (C,D). **A'–D')** CD68 immunolabeling in adjacent sections at 1d and 7d. At 1d, CD68 was expressed by a small subset of macrophages in the iron+LPS (B') but not iron group (A'). By 7d, CD68 had increased in both iron groups suggesting increased phagocytosis over time. **E)** Densitometric quantification of ipsilateral CD68 immunoreactivity revealed that iron+LPS tissue had significantly more CD68 expression than iron or LPS alone. Bars represent mean $\pm$ SEM. Two-way ANOVA with Bonferroni *post hoc* test: ** $p < 0.01$, *** $p < 0.001$ vs. Veh unless otherwise noted. Dotted lines indicate the gray matter border and insets show high-power views of boxes. Scale bars: (A–D') 200 μ m; (A–D'inset) 20 μ m.

Figure 8.

Intraspinal delivery of the TLR4 agonist LPS regulates transcription of iron sequestration genes. Spinal cords were injected with vehicle (Veh), iron, iron+LPS (I+L) or LPS alone. Real time qRT-PCR on spinal cord homogenate from injection sites 1d after injection measured the relative mRNA expression of **A)** transferrin receptor, **B)** divalent metal transporter 1 (DMT1), **C)** L-ferritin, **D)** H-ferritin, **E)** ceruloplasmin, and **F)** hepcidin. Bars represent mean $\pm$ SEM. One-way ANOVA with Tukey's *post hoc* test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. Veh, unless otherwise noted.

Figure 9.

TLR4 activation does not rescue progressive iron-induced neuron loss. **A,B)** Low-power images of 1d and 7d injection sites immunolabeled for NeuN reveal significant loss of ~50% of neurons in cords injected with iron, LPS+iron (L+I) or LPS compared to vehicle (Veh) at 1d. Neuron loss was greater at 7d post-injection, when NeuN counts were reduced ~90% compared to Veh in iron +/- LPS injection sites. Bars represent mean $\pm$ SEM. Two-way ANOVA with Bonferroni *post hoc* test: ** $p < 0.01$; *** $p < 0.001$ vs. Veh. Scale bar: **(A)** 200 μ m.

Figure 10.

TLR4 activation did not rescue iron-induced oligodendrocyte (OL) loss and iron may impair NG2+ oligodendrocyte progenitor differentiation. **A)** CC1+ OL counts in injection sites from Veh, iron, LPS+iron (L+I) or LPS alone revealed that all treatments killed comparable numbers of OLs by 1d compared to Veh. By 7d, OL numbers returned to baseline in iron alone and LPS groups; they had risen but remained significantly lower than Veh in LPS+iron tissue. **B)** NG2+ cell counts at 1d in injection sites revealed no change in NG2+ cell numbers in the iron group but significant NG2 cell loss in LPS+iron and LPS groups. By 7d, NG2 cells had increased in all groups (except Veh) and were significantly greater in the iron group compared to all others. **C)** NG2+/BrdU+ cell counts revealed that NG2 cell proliferation was stimulated in iron, LPS+iron, and LPS injection sites between 1d – 7d post-injection. Bars represent mean $\pm$ SEM. Two-way ANOVA with Bonferroni *post hoc* test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. Veh, unless otherwise noted.

Figure 11.

LPS+iron injection promoted a significant increase in ferritin+ macrophages 7d post-injection compared with iron or LPS alone. **A–C''**) Single-label and merged confocal images from 7d post-injection tissue immunolabeled for CD11b (green) H+L-Ferritin (red), and DAPI (blue). Inset shows high-power view of lowest arrow in B. **(D)** Iron+LPS injection sites had twice as many ferritin-expressing macrophages than iron or LPS alone. Bars represent mean $\pm$ SEM. One-way ANOVA with Tukey's *post hoc* test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. Veh, unless otherwise noted. Scale bar: (A–C'') 75 μ m.

Figure 12.

Intraspinal or *in vitro* treatment with iron+LPS (I+L) or LPS but not iron significantly increased cytokine mRNA, nitrites and glutamate release. (A–C) Real-time qRT-PCR on spinal cord homogenate 1d after microinjection showed a significant increase in the relative mRNA expression of inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF α), and interleukin-1 alpha (IL-1 α) in tissue treated with iron+LPS or LPS compared to iron or vehicle (Veh) microinjection. (D–F) Real-time qRT-PCR on cultured microglia RNA revealed no effect of control (Cont) or iron and differential effects of iron+LPS (I+L) and LPS on iNOS, TNF α , and IL-1 α , with iron+LPS inducing the most robust changes. (G,H) Supernatant from bone marrow-derived macrophages stimulated for 24h revealed that iron+LPS and LPS-treated macrophages produced significantly more nitrites (G) and glutamate (H) compared with control and iron-treated macrophages. Bars represent mean $\pm$ SEM. A–H) One-way ANOVA with Tukey's *post hoc* test: * p <0.05; ** p <0.01; *** p <0.001 vs. Veh or control, unless otherwise noted.