Preconditioning with lipopolysaccharide activates spinal cord microglia without causing neuropathology

Honors Research Thesis

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Introduction

1.1 Spinal Cord Injury

Spinal cord injury (SCI) is devastating event resulting in vast physiological and financial loss. In the United States alone, an estimated 300,000 people are living with SCI with lifetime financial burden reaching upwards of $4.5 million (NSCISC, 2013). Damage from SCI arises in a phasic manner, with the acute mechanical damage leading to a more chronic secondary damage mechanism (Oyinbo, 2011). Secondary damage occurs largely through an inflammatory cascade mediated by resident and infiltrating immune cells of the central nervous system (CNS). While there are a multitude of therapies currently being studied for SCI, one promising target is altering the inflammatory phenotype of macrophage populations inside of the cord (Gensel, Donnelly, & Popovich, 2011).

1.2 Macrophage Biology

Following spinal cord injury, macrophage/microglia populations activate within a spectrum of inflammation ranging between two extreme poles described *in vitro* (Kigerl et al., 2009). On one end are the classically activated (M1) macrophages, which secrete an assortment of inflammatory cytokines and release reactive oxidative species (Ding, Nathan, & Stuehr, 1988). These cells have proficient antimicrobial activity, while exacerbating damage in inflammatory states (Murray & Wynn, 2011). On the other end of the spectrum are alternatively activated (M2) macrophages, which are characterized by a reparative phenotype consisting of tissue remodeling and neurite growth in the spinal cord (Kigerl et al., 2009; Nahrendorf et al., 2007). After SCI, the M1-like phenotype is maintained chronically, potentially causing further damage to the injury environment, while the M2-like phenotype is down regulated after as little as a week (Kigerl et al., 2009). Shifting the macrophage inflammatory milieu from a predominantly M1 to a more M2 phenotype, therefore, may decrease secondary damage after SCI. Although this dichotomy is an oversimplified description of polarization, describing such macrophage populations has aided in explaining the beneficial and damaging effects of macrophages.

1.3 Microglia

Microglia, the resident immune cell of the CNS, are also major effectors of secondary injury after SCI that are thought to follow similar polarization patterns. In the normally functioning CNS, microglia are responsible for maintaining equilibrium through a variety of mechanisms. Synaptic remodeling, phagocytosis of debris, and the secretion of neurotrophins are only a few of the processes in which microglia ensure proper neuronal function in the CNS (Ousman & Kubes, 2012; Popovich & Longbrake, 2008). Upon activation by injury or infection, however, microglia release cytotoxic substances and inflammatory cytokines which further contribute to neuroinflammation (Czeh, Gressens, & Kaindl, 2011). Microglia are involved in many neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, and Multiple Sclerosis (Friese & Fugger, 2007;

More et al., 2013; Solito & Sastre, 2012). In SCI, microglia contribute to acute injury through early release of inflammatory cytokines interleukins 1β and 6, and tumor necrosis factor-alpha (TNF-α) (Yang et al., 2004). Activation of microglia also results in the release of reactive oxygen and nitrogen species which leads to neuronal death (Boekhoff et al., 2012; Kaushal et al., 2007). Targeting the detrimental effects of microglia while maintaining the synaptic reorganizing and neurotrophic properties is, therefore, a potential strategy for reducing inflammatory damage.

1.4 Background on Preconditioning

Preconditioning offers one possible route of utilizing microglial activation to be beneficial in neuroinflammatory events. By exposing an inflammatory system to a mild noxious stimulus, the system can be primed to be more anti-inflammatory when exposed to a subsequent serious stimulus. Possible preconditioning stimuli include ischemia, physical trauma, as well as various biochemical additives. This phenomenon has been extensively studied in models of stroke, traumatic brain injury (TBI), and myocardial protection (Monson et al., 2014; Yamakawa et al., 2014; Yokobori et al., 2013). By briefly occluding the carotid artery prior to middle cerebral artery occlusion, investigators are able to decrease infarct volume while also decreasing levels of inflammatory cytokine IL-1β (Shin et al., 2013). In TBI, cell death and cognitive deficit are decreased in animals preconditioned with N-methyl-d-aspartate (NMDA) when compared to controls (Moojen et al., 2012).

1.5 Endotoxin Preconditioning

Lipopolysaccharide (LPS) is a component of gram-negative bacterial cell membrane that normally elicits inflammatory cascades in immune cells (Beutler & Rietschel, 2003). While normally inducing an M1 macrophage activation phenotype (Kigerl et al., 2009), LPS also has implications in preconditioning inflammatory systems. In stroke, LPS-preconditioning decreases the damage done by TNF-α signaling pathways by priming the system with initial production of TNF-α (Rosenzweig et al., 2007). It is currently believed, therefore, that LPS-preconditioning occurs as a result of regulating cytokine signaling through negative feedback mechanisms, as well as through latent expression of anti-inflammatory cytokines in response to the initial stimulus (Shpargel et al., 2008). Specifically in CNS trauma, LPS-preconditioning has been used as a method of shifting the inflammatory phenotype of microglia. After a 4 day injection regimen of LPS prior to TBI, microglia express higher levels of M2 genes as well as exhibiting increased synaptic "pruning". This effect resulted in less neuronal cell death and a smaller overall lesion volume (Chen et al., 2012).

While preconditioning microglia in the brain has been shown to be beneficial, no such work has currently been done in the spinal cord. LPS does not actively cross the blood-brain barrier (Singh & Jiang, 2004), but instead is thought the signal via circumventricular organs (CVOs) to the brain (Rivest, 2003). Under this assumption, however, the spinal cord would only exhibit LPS-mediated activation via a trickle down from the brain as the spinal cord does not contain CVOs. To examine the potential of

microglial preconditioning in the spinal cord, we have characterized the spatial orientation and activation patterns after peripheral LPS injection. Here, we show that systemic LPS activates spinal cord microglia without inducing neuropathology. Our results also indicate that LPS may signal directly across spinal cord vasculature to microglia, thereby providing an alternative hypothesis to the CVO theory.

Materials and Methods

Animals and lipopolysaccharide injection
Male mice, 8-10 weeks of age, from the C57bl/6 strain were used in these experiments (The Jackson laboratories). All experiments were performed in accordance with the Institutional Animal Care and Use Committee of The Ohio State University. For four days, animals received either 0.1M phosphate buffer saline (PBS) or 1mg/kg LPS (*E. coli*, serotype 055:B5, Sigma) injection intraperitoneally. Prior to each injection, body weights were recorded.

Activity Data
On a subset of animals, open field activity analysis was performed using activity box (Animal Activty Meter: Opto M3). Sensors measured vertical and horizontal activity for 30 min and data were compiled using Accuscan software. Measurements included movement time, rest time, vertical activity time, and horizontal movement count. These data were analyzed using two-way repeated measures ANOVA on Prism (GraphPad Software, Inc; $p < 0.05$).

Tissue preparation and immunohistochemistry

Twenty-four hours after the final injection, mice were anesthetized with ketamine (27.5 mg/mL)/xylazine (5 mg/mL) and transcardially perfused with 4% paraformaldehyde/0.1 M PBS. Spinal cords were removed and fixed for an additional 24 h, then transferred to 30% sucrose for three days. Tissue was blocked in Tissue-Tek OCT (Sakura) and sectioned using a cryostat. Sections were either cut at 10μm or 30μm, depending on the staining protocol. **For Iba-1 floating sections**: 30μm thick sections were floated in 0.1M PBS. Sections were then blocked in 4%BSA/0.3%Triton X-100/PBS for 1 h prior to primary antibody incubation. Sections were incubated in rabbit anti-Iba-1 (1:1000; Wako; Richmond, VA) diluted in blocking reagent overnight at room temperature while agitated by orbital shaker. The next day, sections were washed in PBS and then incubated with biotinylated goat anti-rabbit secondary antibody (1:1000; Vector Laboratories, Burlingame, CA) for 1 h. Sections were then treated with 30% hydrogen peroxide in methanol (1:5), stained by avidin-biotin complex (Vector), and developed using diaminobenzidine (Vector). Sections were then mounted onto glass slides, dehydrated, and coverslipped with Permount (Thermo Fisher Scientific; Waltham, MA). **For all other stains**: 10μm thick sections were mounted onto glass slides and blocked as earlier stated. Slides were incubated with primary antibodies overnight. Primary antibodies used include: rabbit anti-Iba-1 (1:1000; Wako), rat anti-CD16/32 (1:800; BD Biosciences; San

Jose, CA), mouse anti-iNOS (1:1000; BD Biosciences), rat anti-CD31 (PECAM-1, 1:5000; BD Biosciences), mouse anti-NeuN (1:1000; Chemicon, Millipore; Billerica, MA). The next day, slides were washed three times in PBS, and then incubated in secondary antibodies for 2 h. Secondary antibodies used are: Alexa-488 conjugated goat anti-rabbit (1:1000; Life Technologies; Grand Island, NY), Alexa-546 conj. goat anti-rat (1:1000; Life Technologies), Alexa-546 conj. goat anti-mouse (1:1000; Life Technologies), and Alexa-488 conj. goat anti-mouse (1:1000; Life Technologies). Slides were then coverslipped with Immu-mount (Fisher). For cresyl violet staining, slides were rinsed in distilled water and then submersed in 0.1% Cresyl Echt Violet (ScyTek Laboratories; West Logan, UT) for 20 min. Slides were then rinsed, dehydrated, and coverslipped with Permount (Fisher). Histological data were analyzed using Student's t-test and two-way ANOVA (gray matter/white matter stain).

Image analysis

Images were captured using a Zeiss Axioplan2 microscope, AxioCam MRm camera, and AxioVision imaging software (Carl Zeiss Microscopy; Peabody, MA). All images for a given marker were captured using the same exposure settings. Fluorescent images were analyzed using MetaMorph (Molecular Devices; Sunnyvale, CA) and all other images were analyzed using MCID (Imaging Research; St. Catharine's, Canada) and ImageJ (US NIH; Bethesda, MD). For Iba-1 density, threshold intensities were applied based on Iba-1 staining (thresholds were consistent throughout all images) and proportional area of Iba-1 staining was measured. For colocalization expression studies, images were thresholded for Iba-1 and the percent area of the second marker was analyzed on Iba-1+ cells. For PECAM analysis, images were taken in the ventral horn based on the PECAM channel (so as not to be biased by microglia location). Number of microglia in each image were counted, and then a proportion of those microglia that associated with PECAM+ vessels was calculated. For neuron pathology analysis, images were taken within the ventral horn and thresholded based on NeuN intensity. NeuN+ cells were then counted and the total area of NeuN was divided by the number of cells in each image to obtain an average somal area. Data were analyzed using unpaired, two-tailed t-test in Prism (GraphPad; $p < 0.05$).

RNA isolation, conversion, and qPCR

Animals used for RNA isolation were perfused using PBS in DEPC water. Spinal cords were removed and homogenized in Trizol (Life Techonologies). After isolation, RNA concentration purity and concentration were measured via spectrophotometer (NanoDrop, ThermoFisher Scientific). 1 μg of RNA was converted to cDNA using M-MLV (Life Technologies) reverse transcriptase in a thermal cycler (Eppendorf NA; Hauppauge, NY). Quantitative polymerase chain reaction (qPCR) was run on the cDNA through The Ohio State University's Nucleic Acid Share Resource to determine changes in gene expression. Readouts were compared to a control curve from pooled cDNA from all samples. Data are presented as fold changes compared to the control curve.

Results

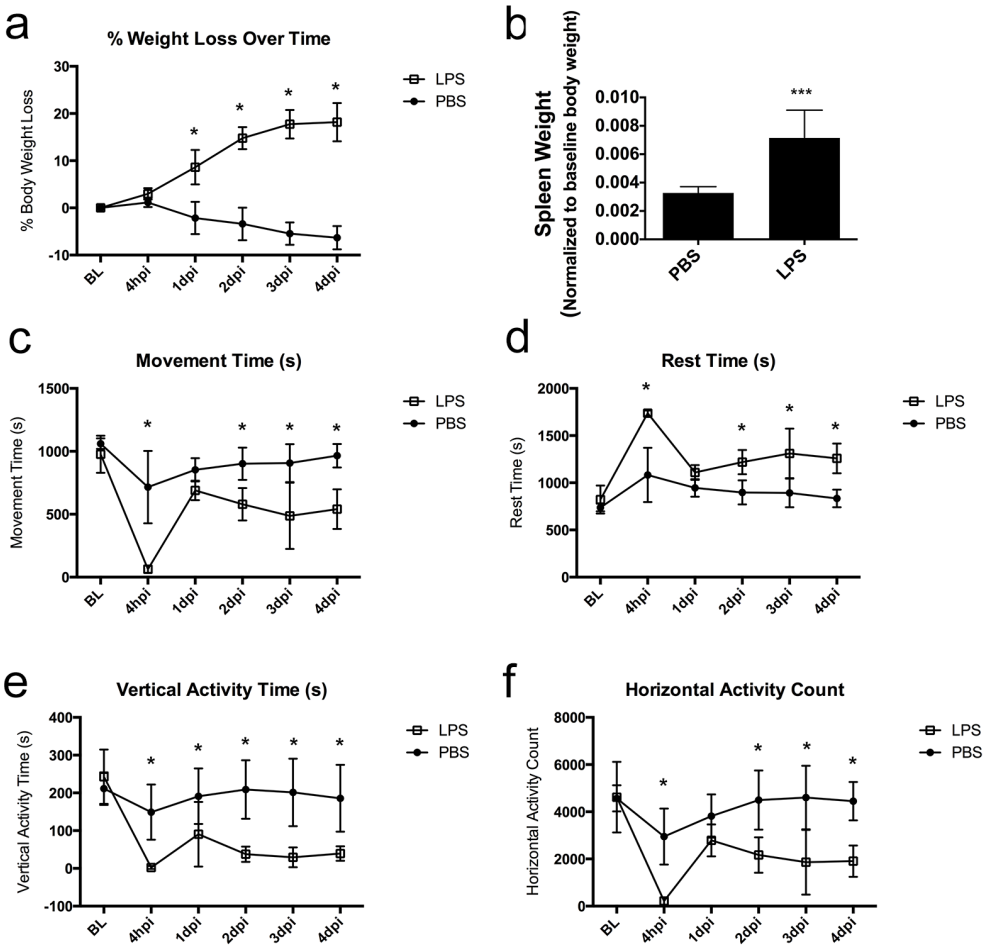
LPS injection paradigm induces sickness behavior

To ensure that our LPS was active and triggering the appropriate peripheral immune response, we used sickness behavior in the mice as a quantifiable outcome measurement. Body weights of all animals were recorded prior to each injection time, as well as just before sacrifice. As expected, animals treated with LPS showed a consistent increase in percent body mass lost throughout all time points (Fig 1, a). We measured spleen weight at time of dissection as another measurement of peripheral immune response. Compared to PBS controls, LPS animals had significantly larger spleens, indicating increased lymphocyte activity (Fig 1, b). We also measured activity in an open field test to further demonstrate sickness behavior. In each parameter measured, LPS treated animals displayed increased lethargy and inactivity. As anticipated LPS mice spent significantly less time moving (Fig 1, c), rested longer (Fig 1, d), spent less time "rearing" as indicated by vertical movement time (Fig 1, e), and had less horizontal movement events (Fig 1, f). This data allows us to conclude that our LPS injection paradigm generates the expected immune response.

Systemic LPS injection causes widespread microglial activation in the spinal cord

To test our hypothesis that peripherally injected LPS could activate microglia in the spinal cord, we used an LPS preconditioning model shown to be beneficial in brain injury (Chen et al., 2012). Mice were subjected to daily LPS injections intraperitoneally for four days as outlined in the Methods section. To maintain consistency between studies, we began with a floating section peroxidase staining method, using Iba-1 to visualize microglial activation. At the cervical level, microglia exhibited significantly increased proportional area of Iba-1 after LPS injection (Fig 2, a-c). Similar trends in activation were seen at both the thoracic (Fig 2, d-f) and lumbar (Fig 2, g-i) levels, with lumbar activation reaching statistically significance. High-powered images of microglia show differences in cell morphology after LPS treatment. In control animals, microglia exhibited the "ramified" morphology normally seen in surveying microglia (Kreutzberg, 1996) with long, branched processes (Fig 2, a,d,g insets). Conversely, LPS-activated microglia retracted their processes and maintained a "bushy" morphology (Fig 2, b,e,h insets). These results indicate that repeated doses of LPS, when administered peripherally, are able to successfully reach the spinal cord and activate microglia.

Figure 1



LPS injection paradigm induces sickness behavior. (a) LPS-treated animals lost body weight throughout the injection period. PBS animals gained weight throughout. (b) After dissection, spleen weights were measured and normalized to baseline body weight. LPS-treated animals had significantly larger spleens (***$p < 0.001$). Activity box measurements were taken to observe behavioral differences. (c) LPS treated animals moved for significantly less time when compared to PBS controls. (d) LPS animals rested for longer periods of time (e) LPS animals vertically "reared" for less time compared to controls. (f) LPS animals had less total horizontal movement (* $p < 0.05$)

Microglial activation is more widespread in gray matter

To assess whether LPS-mediated microglial activation is region specific in the spinal cord, we analyzed the extent of activation in the gray matter versus the white matter. As with previous data, total microglia activation increased in LPS-treated animals at all levels, although only reaching significance in the cervical cord (Fig 3, g). When gray and white matter activation is parsed apart, however, it is evident that microglia activation occurs more extensively in the gray matter. Quantification of Iba-1 proportional area in PBS treated animals showed no significant differences in activation between gray matter and white matter at baseline (Fig 3, h,j,l). This indicates, therefore, that the differences seen in LPS treated animals are due to a change in the amount of microglial activation and not simply differences in the amount of microglia originally present in those areas. Differences in microglia subpopulations within the spinal cord activate in functionally distinct ways as shown in a model of spinal cord injury (McKay, 2007). We hypothesize, therefore, that the differences in activated microglia density from endotoxin exposure are due to these distinct populations. Generally, there was noticeably more activation in the ventral horns of the spinal cord compared to other areas of gray matter. While these specific differences were not quantified, it provided us a target area for further analysis on microglial function.

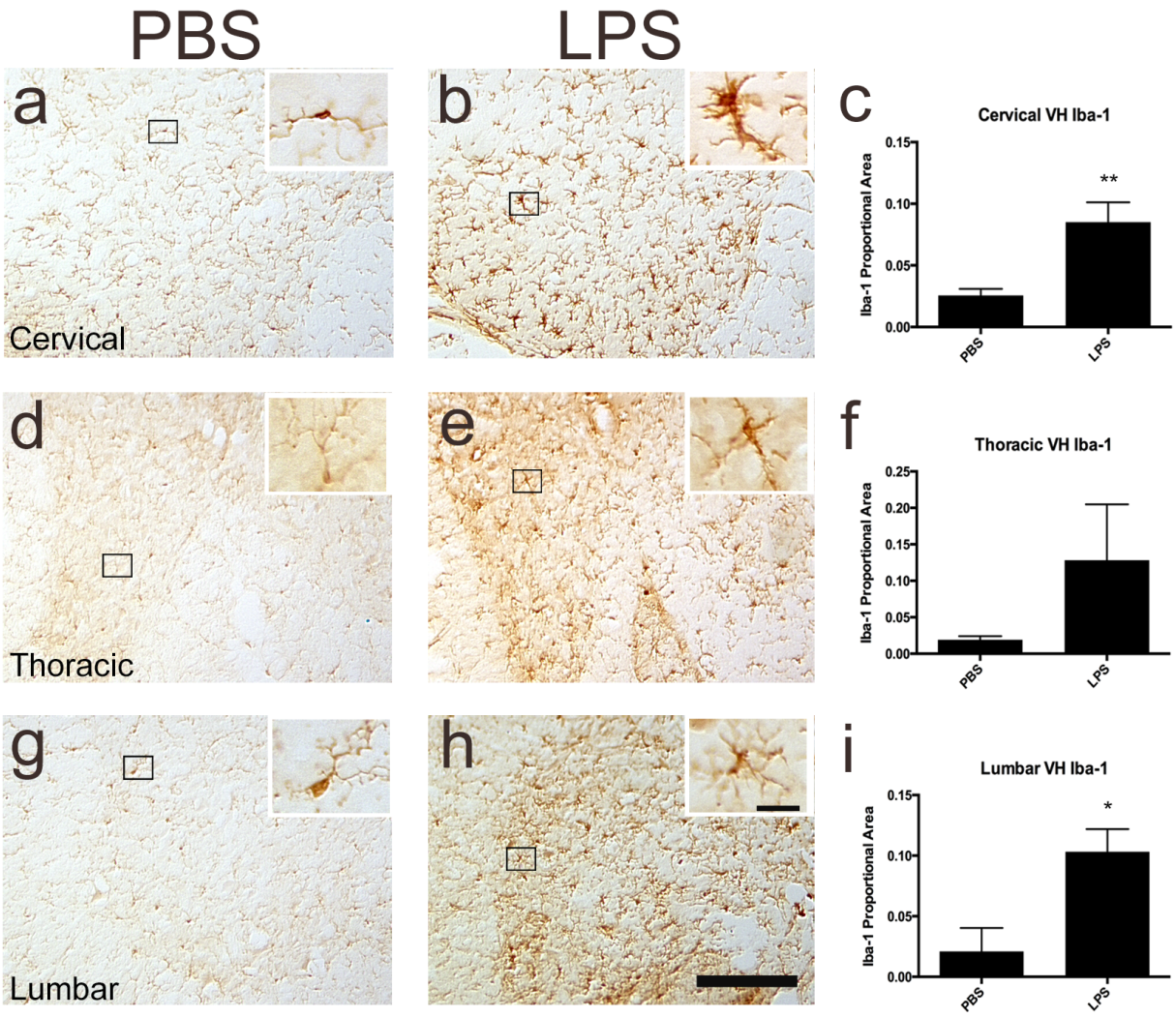
Extent of microglial activation is not altered by varying staining protocols

In order to test the functional implications of microglial activation later on, we first wanted to verify that we would observe similar activation patterns when using fluorescently stained sections that were pre-mounted on slides. Similar to the floating section peroxidase method, microglia fluorescently labeled with Iba-1 exhibited significant activation after LPS. Interestingly, thoracic microglia were significantly activated in slide-mounted sections (Fig 4, f) while failing to reach significance in the floating sections (Fig 2, f). Also, microglial activation of LPS-treated sections at the lumbar level reached nearly three times that of controls (Fig 4, g-i). This data provides evidence that fluorescently stained slide-mounted sections are more sensitive to LPS-induced changes in microglial reactivity, allowing us to further explore immunostaining using this method.

Primed microglia interact uniquely with spinal cord anatomy

To further characterize the implications of LPS-primed microglia in the spinal cord, we sought to observe how these cells were spatially associating with various components of the spinal cord, specifically within the gray matter. Similar to activation patterns seen in the ventral horn, microglia in the dorsal horn exhibited increased activation in animals

Figure 2



Microglia morphology indicates increased activation in the ventral horns of LPS treated animals when compared to PBS controls. (a,d,g) PBS treated animals exhibit microglia with long processes and round body (see insets). (b,e,h) LPS-treated microglia have a more robust and amorphous morphology. (c,f,i) Quantification of proportional area of Iba-1 in the ventral horn indicates significant differences between group in the cervical and lumbar spinal cord. Images taken at 10x. Scale bar = 200um. Insets taken at 40X. Scale bar = 20um. *$p<0.05$ **$p<0.01$

treated with LPS (Fig 5, a,d). As expected, dorsal horn microglia were more "bushy" in appearance with drawn in processes. The central canal of the spinal cord was lined with proliferative cells called ependyma which, when activated, can rescue neurological function in the diseased and injured cord (Johansson et al., 1999; Moreno-Manzano et al., 2009). We wanted to see if primed microglia interacted with the central canal differently compared to saline treated cells. In general, LPS-treated microglia surrounded the central canal in a more robust fashion, interacting extensively with the presumed ependyma. Control microglia, while located around the central canal, sent only a few processes in to survey the ependymal cell layer (Fig 5, b,e).

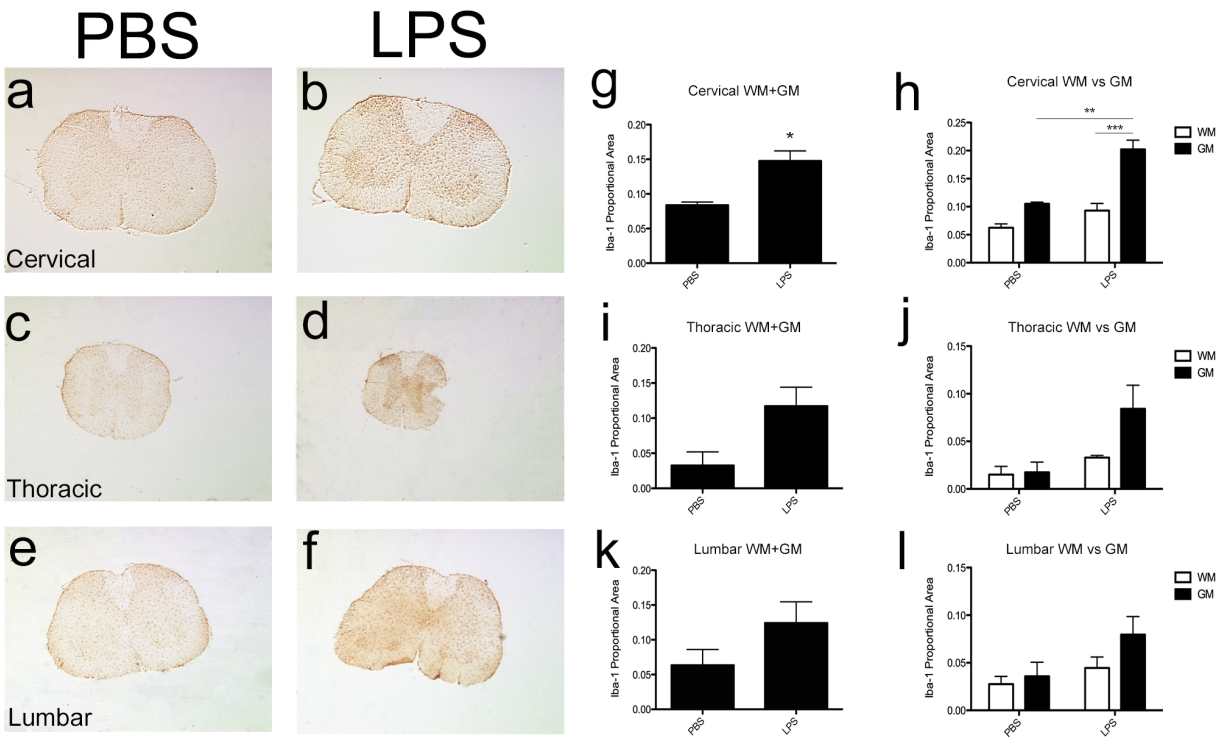
Differences in microglial involvement with spinal cord blood vessels were also seen. Blood vessel lumen were largely surrounded by LPS-activated microglia, while control microglia were not associated with vessels as frequently (Fig 5, c,f). In all of the sections analyzed, nearly every noticeable vessel in LPS-treated animals includeed a microglia surrounding it. This phenomenon suggests a possible route of signaling for the LPS. There is evidence, however, that LPS does not cross the blood-brain barrier directly, and may instead signal via receptors on endothelium (Banks & Robinson, 2010). The numerous associations of microglia with vasculature seen with routine histology provide evidence that the activation of microglia is through LPS signaling across blood vessels.

Together, these data suggest that LPS-primed microglia have unique interactions within the gray matter of the spinal cord, while also providing a hypothesis to how LPS is signaling to spinal cord microglia.

Primed microglia exhibit increased association with blood vessels in the spinal cord

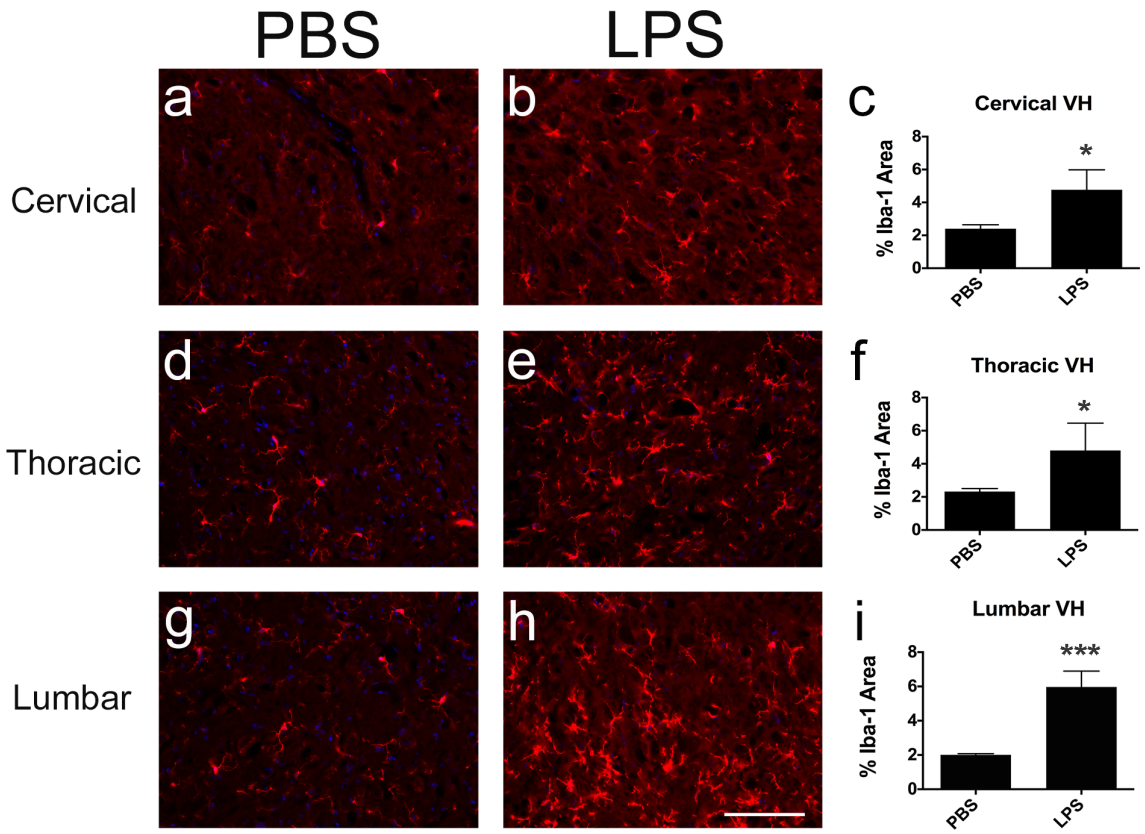
In order to further explore the hypothesis that LPS is activating microglia through signaling across spinal cord vasculature, we stained specifically for platelet endothelial cell adhesion molecule-1 (PECAM-1). This allowed us to visualize blood vessels that were traversing in multiple different planes as opposed to cross-sectional lumen seen in figure #. We could also document colocalization of Iba-1 and PECAM-1, allowing us to further examine how microglia are interacting with vasculature. There was a striking difference in the amount of microglia involvement with vasculature after LPS injection when compared to saline controls. PBS treated animals exhibited only slight interactions with blood vessels at all levels of the spinal cord (Fig 6, a,d,g). When interactions did exist, they are usually due to microglia extending out processes to survey the vessels. Conversely, LPS treated microglia were often found in close contact with vessels, interacting by wrapping themselves around the vasculature (Fig 6, b,e,h). Instead of merely reaching out processes, LPS-treated microglia encompassed the vessel using the entire cell body and collection of processes. Interestingly, in LPS treated animals, microglia that are associated with vessels seemed to be more robustly activated morphologically than those that were not interacting with vessels. This could indicate further necessity of trans-endothelial signaling to activate spinal cord microglia. Quantification of the proportion of microglia associated with vessels shows statistical significance at all levels of the spinal cord. (Fig 6, c,f,i). Because there is widespread

Figure 3



LPS activates microglia differently depending on location in the spinal cord. LPS treated animals (b,d,f) exhibited higher microglial activation at all levels compared to PBS (a,c,e) controls. (g,i,k) Quantification of proportional area of Iba-1 staining for the entire spinal cord section showed significant differences at the cervical level (g) but not at thoracic or lumbar (i,k). Within the cervical level, microglia occupied more than 2x the surface area in gray matter compared with white matter (h). No such spatial differences were seen in thoracic and lumbar quantification (j,l) Images taken at 2.5x. *p<0.05,**p<0.01,***p<0.001

Figure 4



Slide mounted, fluorescently stained Iba-1+ microglia exhibit similar activation patterns as floating section stain. Microglia display similar morphological differences as seen in peroxidase staining. This activation is seen at cervical (a,b), thoracic (d,e), and lumbar (g,h) levels. (c,f,i) Quantification of Iba-1 % area shows significant differences between groups at all levels. (i) The lumbar cord has the highest differences between groups with the LPS group demonstrating 3x the amount of microglial activation. Images taken at 20x. Scale bar, 20μm.

activation throughout the spinal cord as well as similar patterns of vasculature association, it seems that LPS is not reaching the spinal cord via trickling down from the brainstem CVOs. This data indicates, instead, that LPS is likely signaling across vasculature endothelium.

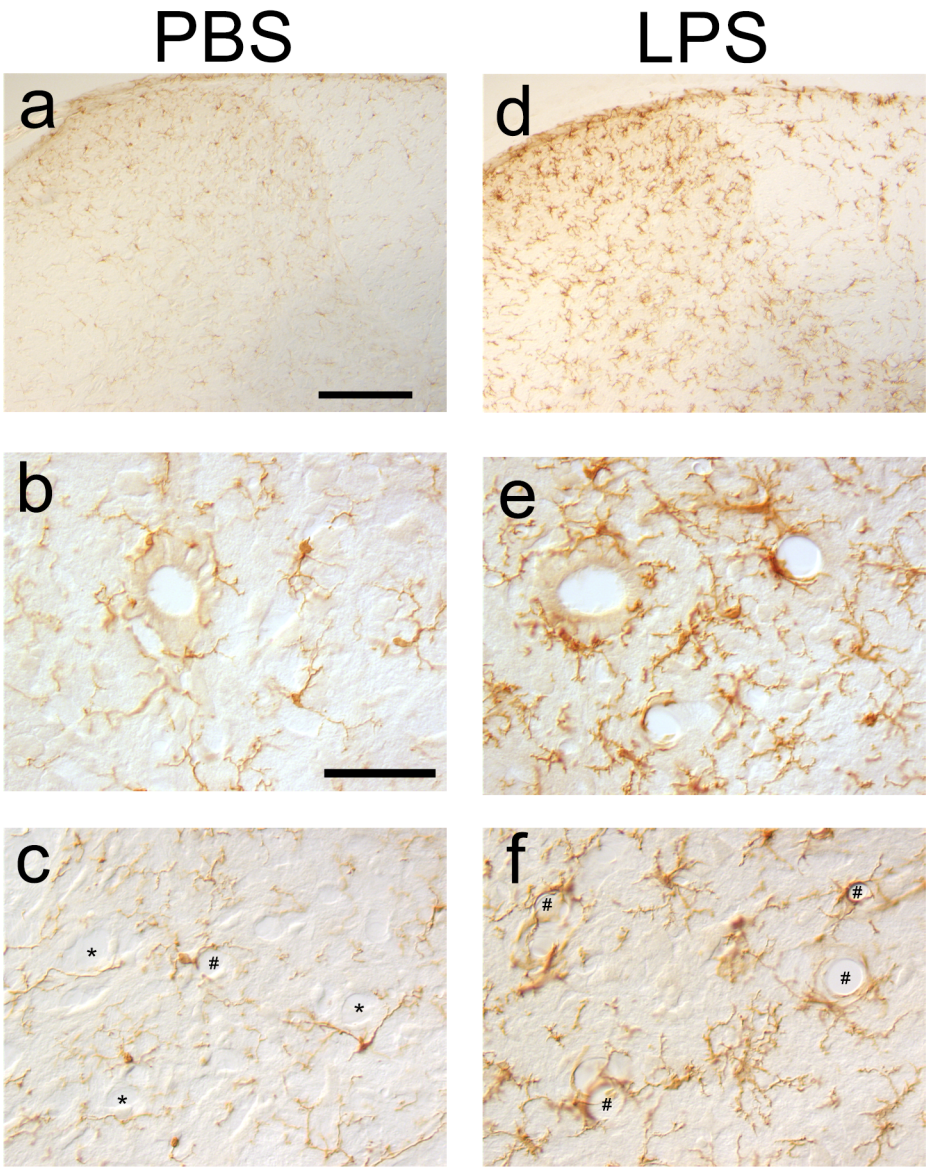
Activated microglia do not induce noticeable neuropathology

In order to determine whether or not preconditioned microglia cause neuronal death or pathology in the spinal cord, we first looked to routine histology. Using cresyl violet staining, we were able to visualize neuron morphology as well as changes in extraneuronal cells. While pathological neurons often exhibit an "eccentric nucleolus" which is indicative of chromatolysis (Rees, 1971), neurons treated with LPS showed normal neuronal morphology (Fig 7, a,d). This analysis was performed within the cervical ventral horn because morphological differences would be more evident in these large cells. When we looked at the association of extraneuronal cells around neurons, we noticed some slight differences. In control animals, when we noticed cells surrounding neurons, there was usually only one cell associated with the neuron (Fig 7, b). In LPS treated cells, however, there were usually two to three cells per neuron (Fig 7, e). Although we are not certain of the identity of these cells based on this analysis, we presume that these cells are likely microglia interacting with neurons uniquely between groups.

To determine if this LPS preconditioning paradigm affects morphology of other cells within the spinal cord, we examined ependymal cells surrounding the central canal. Ependymal cells are neural stem cells capable of proliferating into a wide variety of cell types including neurons and astrocytes (Johansson et al., 1999). In our model, we saw markedly different morphologies in ependyma. In saline treated animals, ependyma lined the central canal in a single cell layer with the cells achieving a columnar shape (Fig 7, c). LPS-treated ependymal cells, however, looked to be more rounded in shape and occur often in multiple cell layers (Fig 7, f). This phenomenon can be explained by activation and proliferation of ependyma. Should this be the case, this injection model may also serve as a tool for inducing proliferation in ependyma.

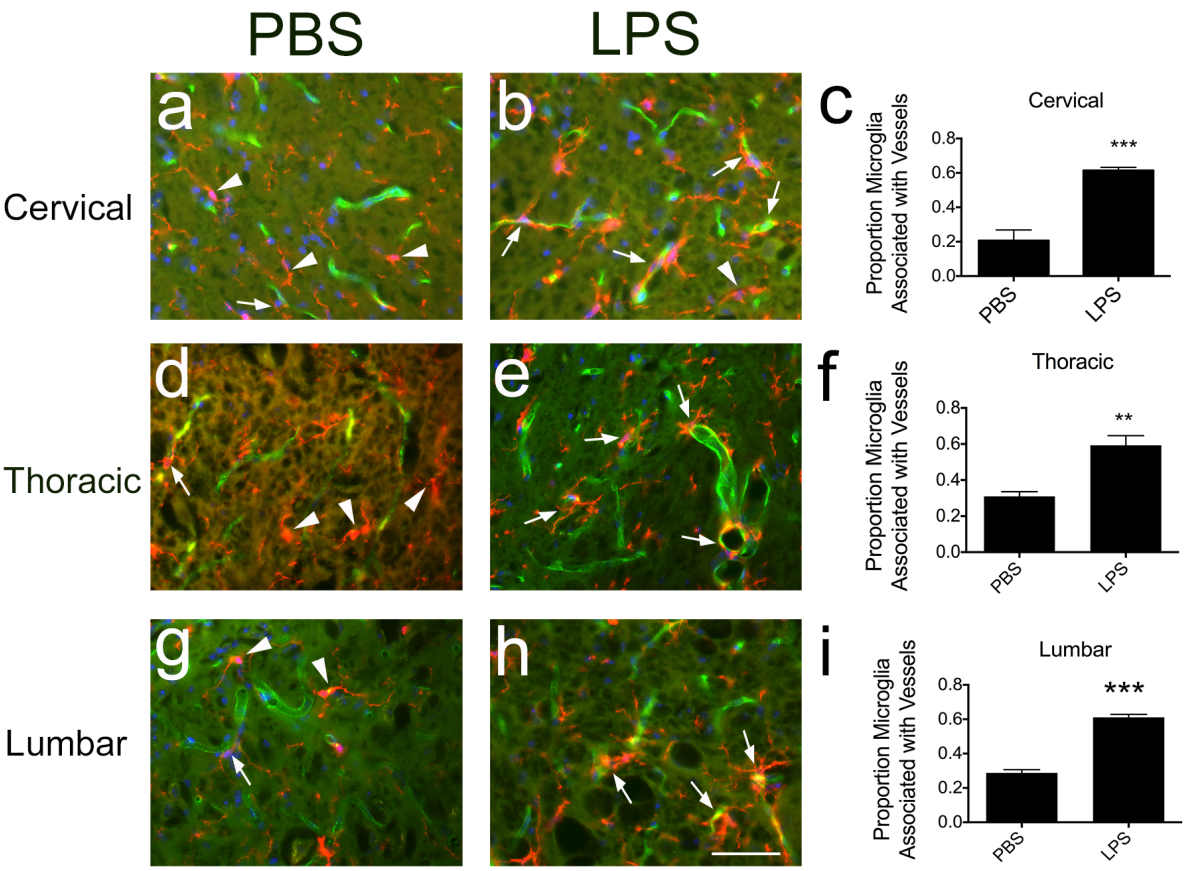
In order to further explore possible neuropathology, we analyzed the amount and size of neurons in the ventral horn using NeuN staining. We saw similar morphology of neurons in both control and LPS-treated spinal cords (Fig 8, a-b). To compare the number of neurons in the ventral horn, we counted NeuN+ cells in multiple 1500μm^2 sample areas and found that the average number of cells within this area were not different between groups (Fig 8, c). Similarly, the average somal areas of these cells were not appreciably different between groups (Fig 8, d). These data suggest that microglial activation via LPS preconditioning does not induce neuropathology.

Figure 5



Activated microglia exhibit unique associations with spinal cord gray matter. (a,d) Microglial activation at the dorsal horn shares similar patterns with the ventral horn. LPS microglia, again, have a more amorphous, bushy morphology. Images at 10x. Scale bar, 200μm (b,e) Generally, more microglia are associated around ependymal cells of the central canal after LPS treatment. (c,f) LPS treated microglia are found associated with vessels in large numbers (#) compared to PBS controls where many vessels do not have any interactions with microglia (*) Images (b,c,e,f) taken at 40x. Scale bar, 60μm.

Figure 6



LPS-activated microglia associate extensively with spinal cord vasculature. Staining for platelet endothelial cell adhesion molecule-1 (PECAM-1, vessels) and Iba-1 (microglia) shows changes in microglia association with vessels. PBS-treated microglia (a,d,g) show very little association with vasculature as indicated by arrow heads. LPS cells, however, (b,e,h) wrap around PECAM-1+ vessels (arrows). Quantification (c,f,i) demonstrates that LPS animals have higher proportion of microglia which associate with vessels. This trend is significant at all levels of the spinal cord. Images taken at 40x. Scale bar, 10μm. **$p < 0.01$, *** $p < 0.001$

LPS preconditioning induces an inflammatory expression profile in the spinal cord

In order to determine the functional capabilities of preconditioned microglia, we examined a wide array of known pro- and anti-inflammatory surface markers. Based on previous work, we expected primed microglia to express anti-inflammatory markers. Instead, however, neither LPS nor PBS showed any discernible expression of anti-inflammatory markers Arginase-1 and CD206 (Data not shown). Antibodies against these markers were shown to be effective using test tissue known to positively express the markers. Differences in inflammatory markers were seen between groups, however. CD16/32 are a part of the Fc gamma receptor family, which are involved in acute and chronic inflammation (van Lent et al., 2001). In LPS treated microglia, there were significant increases in CD16/32 expression (Fig 9). Inducible nitric oxide synthase (iNOS) is an enzyme that is induced by LPS (Chan & Riches, 2001). Here, microglia exposed to repeated doses of LPS expressed higher levels of iNOS than PBS controls as demonstrated by immunohistochemical analysis (Fig 10). While these results were not significant, the quantification exhibits a noticeable trend (Fig 10, g). These data indicate a inflammatory phenotype for preconditioned microglia based on expression patterns.

In order to determine if genetic expression in the spinal cord follows the inflammatory patterns seen specifically in microglia protein expression, we performed qPCR on spinal cord homogenate. We targeted mainly inflammatory genes due to trends discussed earlier. Expression of major inflammatory mediator, IL-1β, was increased in LPS treated animals (Fig 11, a). Interestingly, we saw no increase in expression of other inflammatory genes we targeted (Fig 11, b-c). This suggests a specific inflammatory mechanism, possibly mediated by IL-1β. As expected, we saw no changes in expression of Arg1, a major marker of anti-inflammatory response (Fig 11, d).

Discussion

In the present study, we have elucidated a novel tool in studying microglial effects in spinal cord inflammation. Basing our model off of endotoxin preconditioning, we are able to activate centrally located microglia through peripheral administration of LPS. These cells exhibit distinct morphological changes after exposure to LPS while demonstrating unique interactions with gray matter landmarks. Through the use of routine histology and specific markers for vasculature, we now hypothesize that LPS signals across endothelium of blood vessels as a mechanism of activating spinal cord microglia. Although robustly activated, these microglia are not inducing noticeable neuropathology as demonstrated through neuron size, morphology, and quantity. Ependymal cells, however, display an altered morphology that may be a possible indicator of activation. While morphologically active, we also found that LPS-primed microglia are functionally activated, expressing increased surface inflammatory markers and mRNA. These data suggest that microglia are capable of being activated via peripheral mechanisms without damaging the spinal cord.

Preconditioning inflammatory systems to decrease damage

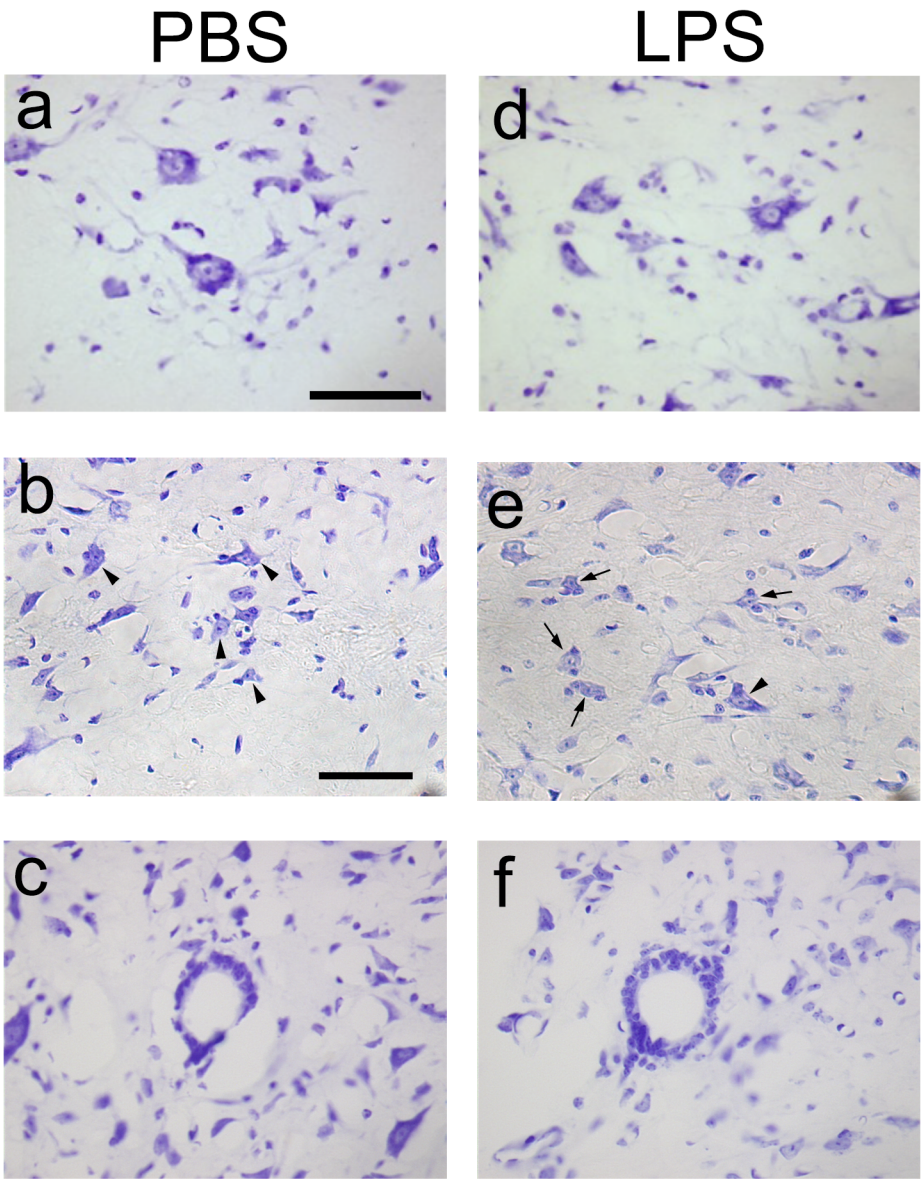
The concept of preconditioning has been extensively studied in various models of inflammation. The administration of a normally detrimental stimulus can induce protection to subsequent stimuli through a multitude of various mechanisms. In the heart, ischemic preconditioning can lead to myocardial protection through various mechanisms including circulating factors, anti-inflammatory transcription changes, and neural responses (Hausenloy & Yellon, 2008). Ischemic preconditioning also has clinical implications in both cardiac surgery and patients who have experienced hemorrhage of the brain (Ali et al., 2007; Hausenloy et al., 2007; Koch et al., 2011). The therapeutic potential of ischemic preconditioning is vast, however the cellular and molecular mediators of this mechanism are poorly understood.

Similarly, endotoxin preconditioning (through LPS administration) has shown great potential in neuroprotection. After a low dose of LPS, rodent brains are protected against a subsequent larger dose of LPS, cerebral ischemia, and traumatic brain injury (Kumral et al., 2012; Tasaki et al., 1997; Longhi et al., 2011). The mechanism of this counterintuitive phenomenon is thought to be through Toll-like receptor (TLR-) 4 induction of inflammatory genes. Vartanian et al. (2011) showed that mice preconditioned with LPS prior to middle cerebral artery occlusion (MCAO) exhibited a shift to Myd88-independent Toll/IL-1R domain-containing adaptor inducing IFN (TRIF) signaling which leads to increased expression of anti-inflammatory genes. Furthermore, there is evidence that inflammatory cytokine tumor necrosis factor (TNF-) alpha may be necessary in inducing neuroprotective preconditioning (Rosenzweig et al., 2007). Basal levels of inflammatory marker expression, therefore, seem to be critical in preconditioning. Currently, endotoxin preconditioning for neuroinflammation is studied almost exclusively in the brain. There is a draught in the literature regarding preconditioning in the spinal cord, especially that involving LPS, save a few studies done in rat SCI (Li et al., 2013). This study, however, has characterized a tool of studying preconditioning in the spinal cord through the manipulation of microglia.

Microglia as targets of neuroprotection through preconditioning

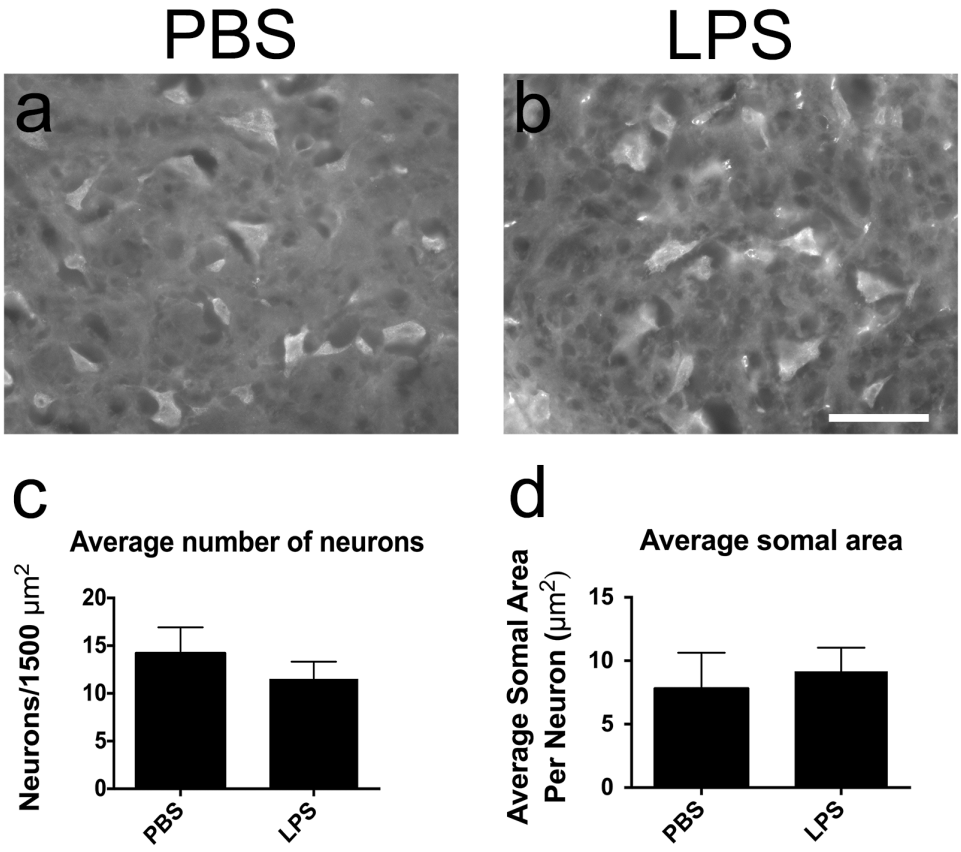
Because of the implications of LPS receptor TLR-4 in preconditioning, a logical cellular target for preconditioning in the CNS are microglia. Microglia are capable of a wide range of functions depending on their activation pattern, making them especially interesting and complicated targets for preconditioning. The responses of microglia to endotoxin preconditioning vary greatly depending on the injection protocol. With lower doses (0.2 mg/kg) or single injections, microglia do not exhibit the robust activation patterns that we observed (Chen et al., 2012; Rosenzweig et al., 2004). This study and similar ones performed in the brain suggest that microglial activation occurs due to repeated exposure to LPS. Despite early opinions of microglia being solely detrimental cells in models of CNS injury or disease, studies now show the neuroprotective potential of microglia. Cultured microglia, when stimulated with nerve fragments to simulate CNS

Figure 7



Cresyl violet staining demonstrates cellular changes in the spinal cord.
(a,d) Demonstrations of "normal" neurons. Neurons have centralized nucleoli and regular shape. (b,e) Neurons in contact with extraneuronal cells, presumably microglia. PBS treated animals exhibit neurons in contact with only 1 cell (arrowheads) while LPS treated animals typically have 2-3 associated cells (arrows) (c,f) Ependymal cells around the central canal in LPS treated animals are more rounded and numerous, forming multiple layers around the central canal. (a,c,d,f) Taken at 40x. Scale bar, 60μm. (b,e) Taken at 20x. Scale bar, 100μm

Figure 8



Preconditioned microglia do not exhibit neuropathology. Neurons stained with NeuN were analyzed for number and size. (a,b) Representative images of ventral horn neurons. (c) There are no discernible differences in neuron number in the ventral horn between groups. (d) Neurons in both groups had similar neuron size with no noticeable differences in shape throughout the gray matter. Images at 40x. Scale bar, 10µm.

injury, secrete neurotrophic factors that are beneficial to neuron growth and survival (Glanzer et al., 2007). Studies *in vivo* have also shown neurogenic and neurotrophic properties of microglia in the context of stroke and excitotoxicity (Liang et al., 2010; Thored et al., 2009).

While these reparative functions are the long-term goal in endotoxin preconditioning, we also needed to characterize possible detrimental effects known to be associated with activated microglia. Interestingly, many of the damaging inflammatory functions of microglia can be a result of TLR4 signaling, the same receptor implicated in neuroprotective endotoxin preconditioning (Lehnardt, 2010). Our histological analysis (Fig 7,8) suggests that the LPS injection paradigm used in this study does not induce neuropathology normally seen with LPS stimulated microglia (Chao, 1992). This phenomenon may be due to a variety of factors. Due to the repeated nature of the injection protocol, neuronal pathology may be occurring previous to the date of perfusion. Should this be the case, however, we would expect to see an overall decrease in neurons within the gray matter, which is not the case (Fig 8, c). We may also be seeing a shift in TLR4 signaling cascades as discussed previously. This occurrence should be accompanied by an increase in anti-inflammatory genes via IRF3 signaling. Our genetic analyses, although on total spinal cord homogenate, demonstrated an increase in IL-1β, a prominent inflammatory factor. The specific mechanism of preconditioning in this model, therefore, needs to be uncovered.

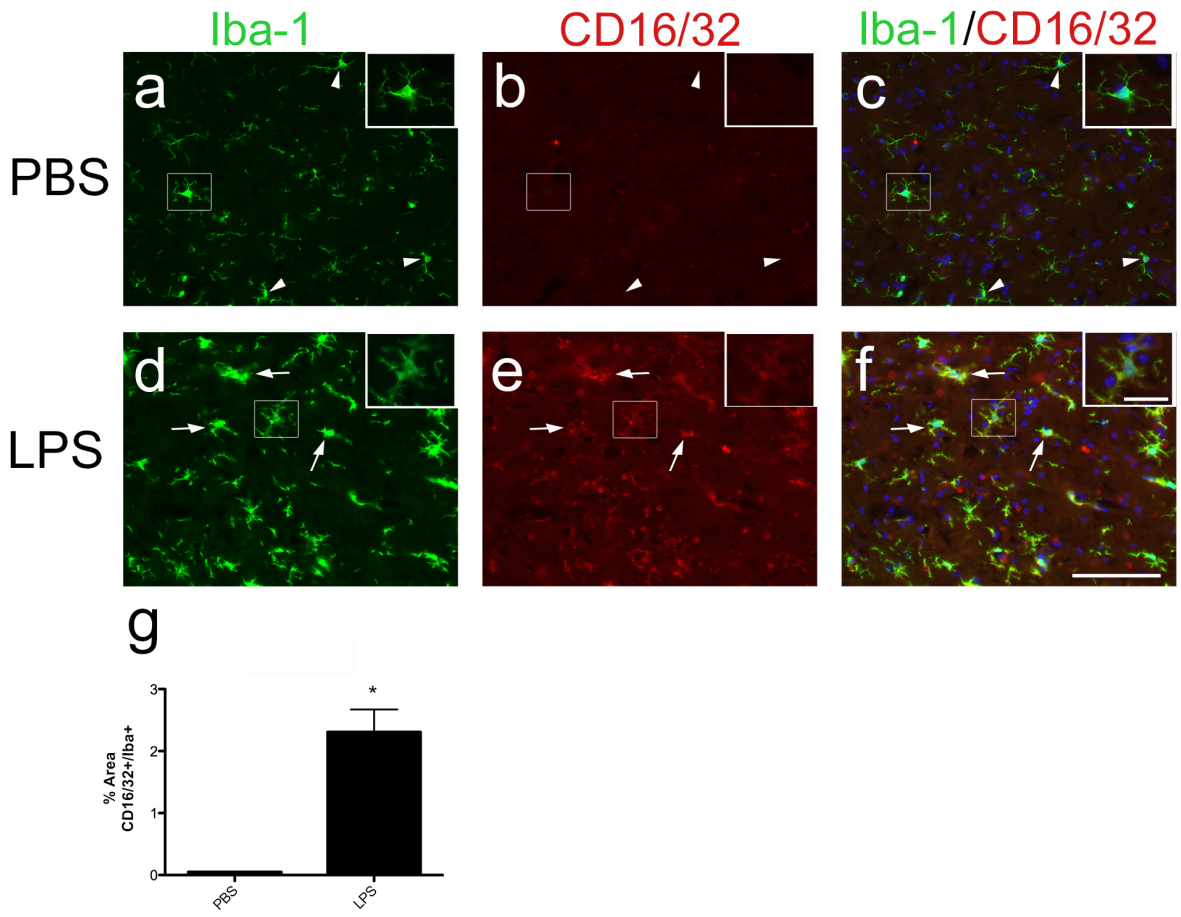
Vasculature endothelium as a possible signaling intermediate for LPS-preconditioning

In a similar model of microglia preconditioning in the brain, investigators discovered that non-hematogenous TLR4 signaling is necessary for LPS-preconditioning (Chen et al., 2012). This suggests that microglial and/or endothelial TLR4 signaling is responsible for the LPS signaling across the blood-brain barrier. Our findings of microglial interactions with blood vessels further strengthen this hypothesis. We now believe that circulating LPS is docking to TLR4 receptors on the endothelium on spinal cord vasculature is responsible for signaling into the centrally located microglia. This interaction may be through cytokine secretion by activated endothelium (Dauphinee & Karsan, 2006), which attracts microglia to the vessels, leading to activation. This hypothesis, however, will require further investigation.

Inflammatory characterization of endotoxin conditioned microglia

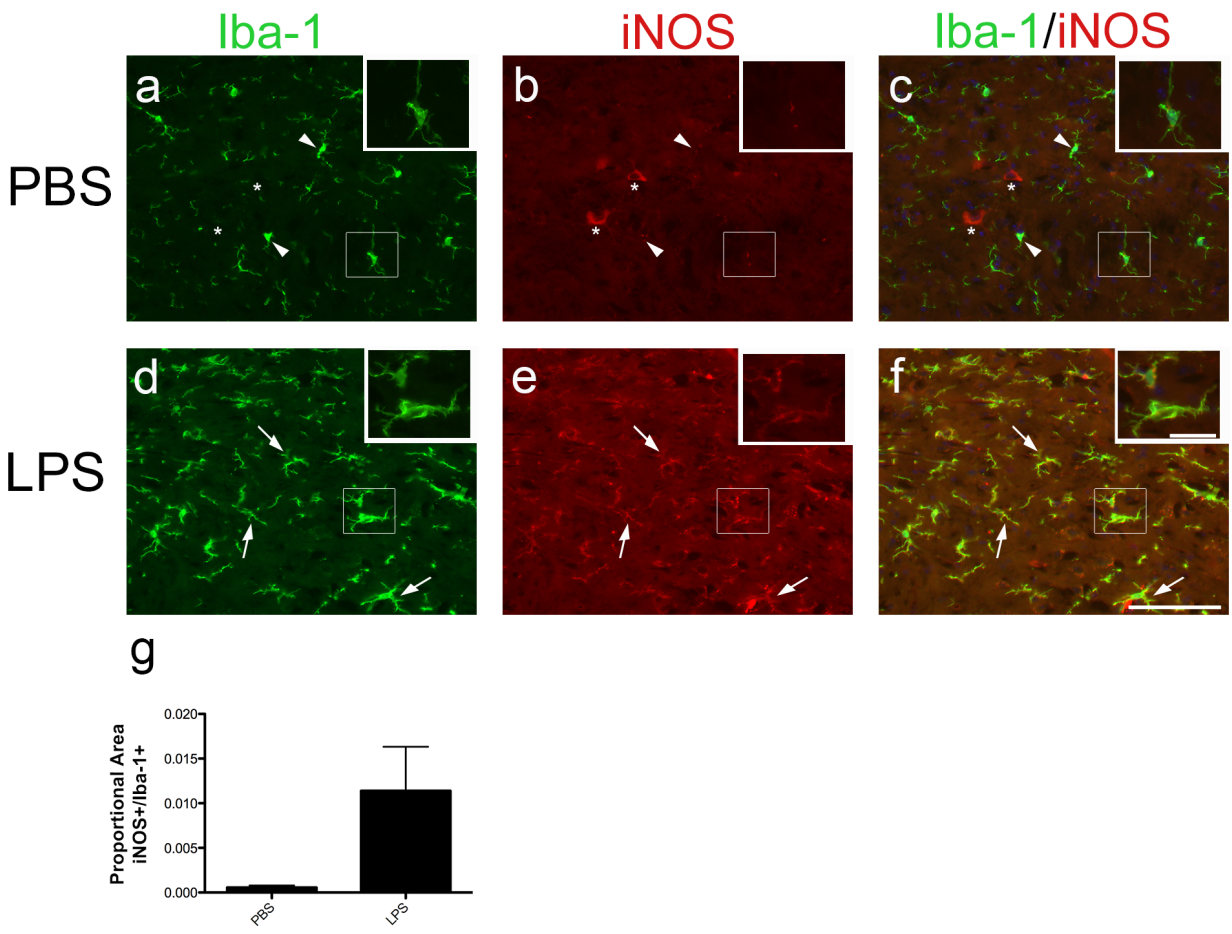
We also saw an increase in inflammatory markers and gene expression after preconditioning. In contrast to Chen et al. (2012), who showed that microglia in their model expressed increased anti-inflammatory genes after brain injury, we seem to have induced a inflammatory polarized microglial population. Despite following the injection protocol described in their study, we see appreciable differences in microglia phenotype. This difference in inflammatory phenotype may be due to when the preconditioned microglia are analyzed. Prior to the major stimulus against which we are attempting to protect, microglia may exhibit a more inflammatory expression pattern. Once exposed to

Figure 9



Immunostaining for CD16/32 is increased in cells treated with LPS. (a-c) PBS animals exhibit cells positive for microglial marker Iba-1 (green, arrowheads), but not also positive for CD16/32 (red). (d-f) LPS treated animals exhibit cells dually positive for Iba-1 and CD16/32 (arrows), indicating microglia expressing CD16/32. (g) Quantification of the % area of Iba-1+ cells also positive for CD16/32 indicates significant differences between groups. Images taken at 20x with insets at 40x. 20x scale bar, 20um. 40x scale bar, 5μm. *$p<0.005$

Figure 10



LPS activation causes microglia to increase expression of iNOS (a-c) PBS treated animals did not have noticeable amounts of iNOS expressed in Iba-1+ cells. There were, however, cells resembling neurons that expressed the marker (*). Microglia that do not have expression of iNOS are denoted by arrowheads. (d-f) LPS treated animals have increased numbers of microglia expressing iNOS (arrows) as seen through immunostaining. (g) Although not reaching significant levels, there is a trend of increased iNOS expression in Iba-1+ cells in LPS treated animals. Images taken at 20X with insets at 40X. 20x scale bar, 20μm. 40x scale bar, 5μm.

a large inflammatory stimulus such as injury, however, microglia may shift to an anti-inflammatory state. Because brain and spinal cord inflammatory physiology differ (Schnell et al., 1999), the microglial response to LPS-preconditioning may very well be different as well.

A particularly interesting finding of this study is the increase in IL-1β gene expression without increases in other inflammatory genes. Studies in the brain have shown that LPS induced activation of the CNS may be due to IL-1β acting on endothelial cells (Quan, He, & Lai, 2003). Because the increase in IL-1β expression is measured from spinal cord homogenate, it cannot be determined specifically which cells are increasing the expression. Also, it should be noted that mRNA expression does not directly correlate with physiological production of IL-1β. A study *in vitro* shows that endothelial cells, when activated by LPS, express IL-1β (Wang et al., 2011). The same study also showed that endothelial cells do not exhibit endotoxin tolerance, whereas glial cells do (as indicated by downregulated cytokine expression). These endothelial cells also exhibit increased expression of MyD88 and TRIF, which offers a possible mechanism of endothelial involvement in LPS preconditioning. As mentioned earlier, TRIF signaling has been implicated in the benefits of LPS preconditioning. One possible mechanism of preconditioning in this model, therefore, is that circulating LPS activates endothelial cells causing them to express IL-1β. This IL-1 expression leads to activation of TRIF pathways within the endothelium, leading to further endothelial activation or eventual expression of anti-inflammatory genes. The details of how LPS, IL-1β, and endothelium interact to precondition microglia are not fully understood, but this study sheds light on how these might interact to activate, and eventually precondition, microglia within the spinal cord.

While the increase in inflammatory surface markers in our model of LPS-preconditioning may seem detrimental to future inflammatory stimuli, low-levels of inflammation have been shown to have beneficial properties. TNF-α is implicated with remyelination and neuroprotection (Arnett et al., 2001; Turrin & Rivest, 2006), while IL-1β aids in the production of nerve growth factor CNTF (Herx, Rivest, & Yong, 2000). Because we saw no noticeable damage to nervous tissue, we believe that preconditioned microglia are not expressing dangerous levels of inflammatory signals due to LPS administration. In order to verify this hypothesis, however, *ex vivo* co-cultures of treated microglia with neurons may be beneficial.

Concluding remarks and significance

Within the central nervous system, a crucial homeostasis must be kept in both naïve and damaged states. After a major insult to the CNS, neuroinflammation can cause an acute occurrence to shift to a more chronic condition, exacerbating damage to nervous tissue. There is a need, therefore, to identify cellular and molecular targets of intervention. Due to their early involvement in major inflammatory events in the CNS and their documented beneficial properties, microglia have the potential to act as cellular targets.

Here, we have characterized a model of preconditioning in the spinal cord that is capable of activating microglia without invasive injection protocols. We hypothesize that these morphologically and functionally active cells will be neuroprotective when exposed to a more severe inflammatory stimulus based on literature referenced above. This study, therefore, will act as a useful tool in translating endotoxin preconditioning into many models of spinal cord disease and injury. This will lead to a more expansive knowledge on how microglia affect the compromised spinal cord, and how they can be manipulated to be advantageous in pathological states.

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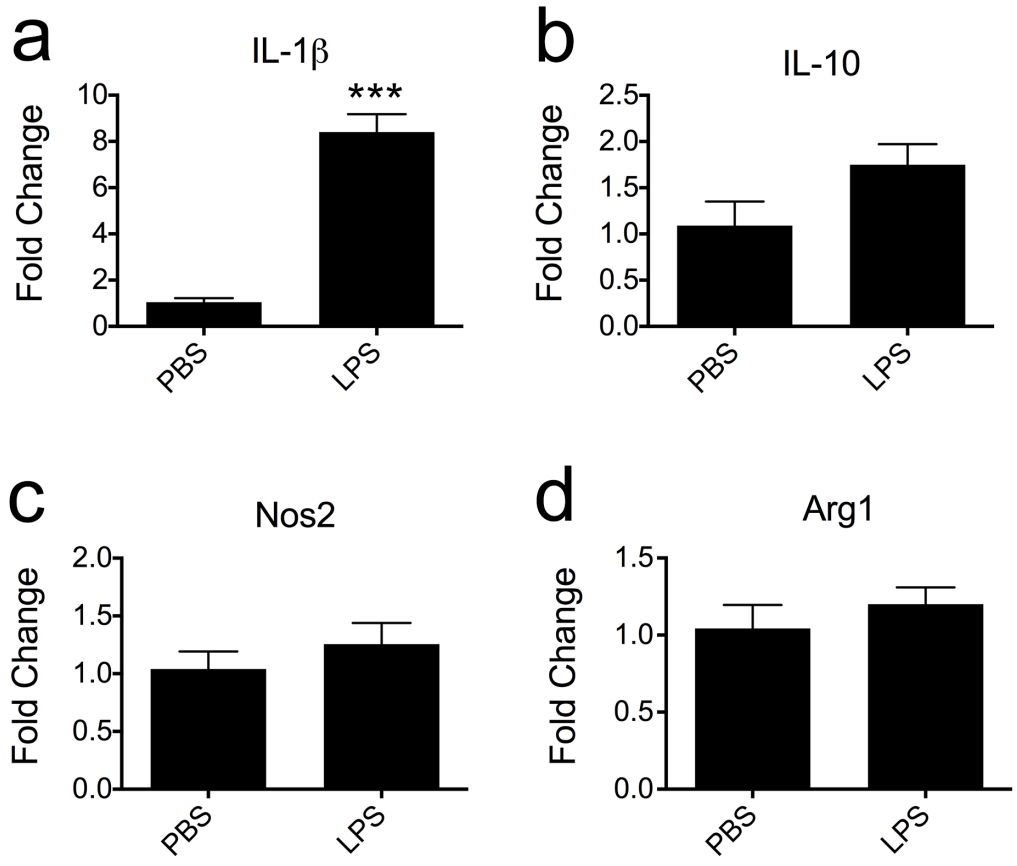
Figure 11



LPS-treated spinal cords express increased levels of IL-1β mRNA but not other targeted genes. qPCR was run on spinal cord homogenate to determine expression levels of inflammatory genes. (a) LPS-treated animals have significantly increased expression of IL-1β mRNA. (b,c) Other inflammatory genes, IL-10 and Nos2, exhibit no changes in expression. (d) Anti-inflammatory gene Arg1 also shows no differences in expression. *** $p < 0.0001$.

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