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Cytokine mRNA expression in painful radiculopathy

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Abstract

Inflammatory cytokines contribute to lumbar radiculopathy. Regulation of cytokines for transient cervical injuries, with or without longer-lasting inflammation, remains to be defined. The C7 root in the rat underwent compression (*10gf*), chronic gut suture exposure (*chr*), or their combination (*10gf+chr*). Ipsilateral C7 spinal cord and dorsal root ganglia (DRG) were harvested at 1 hour after injury for real-time PCR analysis of IL-1 β , IL-6 and TNF α . Cytokine mRNA increased following all three injuries. TNF α mRNA in the DRG was significantly increased over *sham* following *10gf+chr* ($p=0.026$). Spinal IL-1 β was significantly increased over *sham* following *10gf* and *10gf+chr* ($p<0.024$); IL-6 was significantly increased after *10gf+chr* ($p<0.024$). In separate studies, the soluble TNF α receptor was administered at injury and again at 6 hours in all injury paradigms. Allodynia was assessed and tissue samples were harvested for cytokine PCR. Allodynia significantly decreased with receptor administration for *10gf* and *10gf+chr* ($p<0.005$). Treatment also significantly decreased IL-1 β and TNF α mRNA in the DRG for *10gf+chr* ($p<0.028$) at day 1. Results indicate an acute, robust cytokine response in cervical nerve root injury with varying patterns dependent on injury type, and that early increases in TNF α mRNA in the DRG may drive pain-related signaling for transient cervical injuries.

Perspective—Inflammatory cytokine mRNA in the DRG and spinal cord are defined following painful cervical nerve root injury. Studies describe a role for TNF α in mediating behavioral sensitivity and inflammatory cytokines in transient painful radiculopathy. Results outline an early response of inflammatory cytokine upregulation in cervical pain.

Keywords

cytokine; pain; radiculopathy; mRNA; PCR

Introduction

Nearly one-third of Americans suffer from pain, with as many as 13% of them experiencing neck pain.^{13,23,56} Painful cervical radiculopathy can result from nerve root compression by disc herniation or transient loading by the vertebrae.^{3,17,21,41} Inflammation and pro-inflammatory mediators contribute to painful lumbar radiculopathy.^{8,22,28,36,37,63} Although cytokine production and release have been studied extensively for *sustained* nerve

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root compression, cytokine profiles in the dorsal root ganglion (DRG) and spinal cord for *transient* painful cervical nerve root compression have not been defined.

An inflammatory response is produced in the nervous system following radiculopathy, including infiltration and activation of immune cells, cytokine upregulation and growth factor release.^{12,36,49} Spinal inflammatory cytokines also contribute to neuropathic pain.^{11,14,15,22,30,48,49,58} Cytokines, including TNF α , IL-1 β , IL-6 and IL-10, are synthesized and released by astrocytes, microglia and macrophages and act throughout the nervous system in both paracrine and autocrine signaling. Cytokine production can indirectly induce the expression of a variety of pain mediators and directly activate glial cells in the CNS, leading to spinal sensitization.^{2,12,31,52} Spinal glial activation is produced in lumbar radiculopathy and exhibits a relationship with behavioral hypersensitivity.^{5,6,10,22,36,39,60,62} Recent work from our lab demonstrates that mechanical allodynia and spinal glial activation are similarly produced after a transient nerve root compression in the cervical spine and both responses are sensitive to the severity of compression and the presence of chemical irritation.^{26,28,46,47} Yet, while relationships among injury parameters, mechanical allodynia, and spinal glial activation have been described for cervical radiculopathy, it is not known whether painful cervical injuries also modulate inflammatory cytokine expression in the DRG and/or spinal cord, and if these responses are specific to the type of injury.

Cytokines are differentially regulated in the DRG and spinal cord in radiculopathy.^{18,22,33,42,49,58,63} Inflammatory cytokine genes including TNF α , IL-1 β and IL-6 increase in the DRG following root injury; while mRNA for these cytokines increases in both the DRG and spinal cord, it peaks earlier (day 1) in the the DRG.³³ Spinal TNF α mRNA and protein also increase immediately following painful trauma to the nerve roots.^{63,66} TNF α provides positive-feedback for its own production, and initiates the production and release of other cytokines.^{20,61} Despite the known early upregulation of TNF α in pain and its direct involvement in the cytokine cascade, it is not known if, and how, TNF α mRNA is modulated immediately after insult to the cervical nerve root and whether inflammatory cytokine upregulation in the DRG and spinal cord are mediated by TNF α .

While many studies have quantified inflammation in the DRG and spinal cord in neuropathic pain, there is no characterization for cervical radiculopathy. Similarly, it remains to be seen if pro-inflammatory cytokines are increased following *transient* compression of the nerve root and whether these cytokines are influenced by a chemical insult. A goal of this study is to quantify changes in inflammatory cytokine mRNA in the DRG and spinal cord following two cervical nerve root compression injuries. A separate investigation tests whether allodynia produced by these injuries can be ameliorated by immediate antagonism of TNF α , and if it also modulates cytokine mRNA.

Materials and Methods

Two studies were performed to define cytokine mRNA expression immediately following painful insults to the cervical nerve root and to utilize a cytokine antagonist to evaluate if TNF α modulates behavioral hypersensitivity. In the first study, cytokine (IL-1 β , IL-6, TNF α) mRNA in the DRG and spinal cord was characterized at an early time point (1 hour) following cervical nerve root injury:⁴⁷ either applied compression, chronic gut suture exposure, or their combination, in separate investigations. Based on those findings, a second study was performed using the TNF receptor-1 to neutralize TNF α and evaluate its role in modulating allodynia in those three models. Experiments were performed using male Holtzman rats (Harlan Sprague-Dawley; Indianapolis, ID), weighing 250-350 grams at the start of the study. All experimental procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and carried out according to the guidelines of the Committee for Research and

Ethical Issues of the IASP.⁶⁷ Animals were housed with a 12-12 hour light-dark cycle and free access to food and water.

Cytokine Characterization Study

Rats underwent one of four previously reported surgical procedures:^{28,46,47} (1) 10gf compression of the right C7 dorsal nerve root proximal to the DRG for 15 minutes (*10gf*), (2) exposure of the nerve root to four pieces of 3-0 chromic gut suture material (*chr*), (3) 10gf compression of the dorsal nerve root in addition to placement of four pieces of 3-0 chromic gut suture (*10gf+chr*), or (4) exposure of the nerve root with no additional insult (*sham*). At 1 hour after the surgical procedure, the C7 spinal cord on the side ipsilateral to the compressed nerve root was harvested (n=6 *10gf*, n=6 *chr*, n=7 *10gf+chr*) for analysis of cytokine mRNA (IL-1 β , IL-6, TNF α) using real-time reverse transcriptase polymerase chain reaction (RT-PCR). For some rats, the C7 ipsilateral and contralateral DRGs (n=6 each group) were also harvested. Tissue from normal, unoperated rats (n=5), and from rats receiving *sham* surgery (n=5 spinal cord samples; n=6 DRG samples) were also used for comparison for both DRG and spinal cord assays.

TNF α Receptor Treatment Study

In a separate study, soluble TNF receptor 1 (sTNFR1-PEG; gift from Amgen, Inc) was administered at the site of the compressed nerve root in the *10gf*, *chr* and *10gf+chr* injury models. For this study, 200 μ g sTNFR1 in 40 μ L was administered directly to the injured nerve root at the time of injury, according to other neuropathic pain studies.^{58,63} In addition, at 6 hours following the initial surgery, the C7 nerve root was again exposed and a second, identical dose of sTNFR1 was administered directly to the injured nerve root. Spinal cord and DRG samples were harvested at day 1 following either surgery alone (*10gf*, *chr*, *10gf+chr*, *sham*) or surgery with sTNFR1 treatment (*10gf+sTNFR1*, *chr+sTNFR1*, or *10gf+chr+sTNFR1*). Each rat was tested for bilateral mechanical allodynia at day 1 following injury to assess behavioral hypersensitivity. In this study, C7 spinal cord on the side ipsilateral to the nerve root injury was harvested after behavior testing was performed (n=4 *sham*, n=4 *10gf*, n=5 *10gf+sTNFR1*, n=6 *chr*, n=6 *chr+sTNFR1*, n=4 *10gf+chr*, n=4 *10gf+chr+sTNFR1*). In addition, for a subset of rats, the ipsilateral and contralateral C7 DRGs were harvested at day 1 from each group (n=4 *sham*, n=3 *10gf*, n=3 *10gf+sTNFR1*, n=6 *chr*, n=6 *chr+sTNFR1*, n=3 *10gf+chr*, n=4 *10gf+chr+sTNFR1*). DRG and spinal cord tissue from normal rats (n=5) were used as controls for the cytokines probed (IL-1 β , IL-6, TNF α).

Specific Methods

Surgical Procedures

All procedures were performed under inhalation anesthesia (4% halothane for induction, 2% for maintenance). Surgical procedures have been detailed previously.^{28,46,47} Briefly, rats were placed in a prone position, and an incision was made in the skin from the base of the skull to the second thoracic vertebra. A C6/C7 hemilaminectomy and partial facetectomy were performed on the right side to expose the C7 dorsal root. Procedures for nerve root compression (*10gf*) involved transient compression of the C7 dorsal nerve root for 15 minutes using a 10gf microvascular clip (World Precision Instruments, Inc; Sarasota, FL). Procedures for chromic gut exposure (*chr*) involved placing four pieces of 3-0 chromic gut suture (2mm in length) (Surgical Specialties; Reading, PA) on the right C7 dorsal nerve root proximal to the dorsal root ganglia. Procedures for the compression with chromic gut material (*10gf+chr*) involved those described for *10gf* in addition to placement of four pieces of 3-0 chromic gut suture on the right C7 dorsal nerve root proximal to the DRG. Following *chr* and *10gf+chr*, chromic suture pieces were left in place for the duration of the study. Procedures for *sham* involved the same protocol but had only exposure of the C7 dorsal root. All wounds were closed after surgery.

using 3-0 polyester suture and surgical staples. Rats were recovered in room air and monitored continuously.

Behavioral Assessment

Each rat was evaluated for bilateral forepaw mechanical allodynia postoperatively until the time of tissue harvest, as previously described.^{28,46} Prior to surgery, rats were acclimated to the tester and environment. Allodynia was measured for each unoperated rat before the surgical procedure to provide an assessment of baseline and unoperated control values. A single tester performed all testing and was blinded to the surgical procedures. For each testing session, after 20 minutes of acclimation, rats were stimulated on the plantar surface of each forepaw using 3 von Frey filaments (1.4, 2, 4g; Stoelting). Each testing session had 3 rounds of 10 stimulations, separated by 10 minutes. Total withdrawals were recorded for each forepaw of each rat and averaged for each group.

Tissue Processing of Cytokine mRNA

For tissue harvest, rats were deeply anesthetized with sodium pentobarbital (65 mg/kg) followed by transcardiac perfusion with 200ml of phosphate buffered saline (PBS). Following perfusion, the ipsilateral and contralateral C7 DRGs were identified, harvested and immediately frozen. The cervical spinal cord was exposed by laminectomy, the C7 segment of the cervical spinal cord was harvested, separated into ipsilateral and contralateral sections, and frozen immediately on dry ice. Total RNA was then isolated using Trizol reagent (Life Technologies, Rockville, MD) according to the manufacturer's instructions. Bilateral DRGs were assayed as well as the ipsilateral spinal cord sample. Tissue was homogenized thoroughly in 1ml Trizol reagent using a pellet pestle motor. After phase separation with 1-bromo-3-chloropropane, the lysate was centrifuged at 12,000g for 15 minutes at 4°C. RNA was precipitated with isopropanol for 10 minutes at room temperature. The pellet was centrifuged at 12,000g for 10 minutes at 4°C and washed with 75% ethanol, and then dissolved in RNase free water and stored at -80°C until use.

RNA concentration and quality were measured by a NanoDrop spectrometer (NanoDrop Technologies, Wilmington, DE). For reverse transcription, 1-2µg of total RNA was treated with 1 unit of amplification grade DNase I (Invitrogen; Carlsbad, CA). The DNase I treated total RNA was subject to reverse transcription according to the manufacturer's instructions using SuperScript III reverse transcriptase (Invitrogen; Carlsbad, CA), random hexamer primer (Invitrogen; Carlsbad, CA) and RNase inhibitor RNaseOUT (Invitrogen; Carlsbad, CA) at 50°C for 1 hour. Single strand synthesized cDNA was used for real-time PCR. Specific primer sequences are listed in Table 1. Sybr-green real-time PCR was performed in an ABI-7300 system (Applied Biosystems; Foster City, CA). Each reaction contained a total of 25µl of Sybr-green master reagent (Applied Biosystems; Foster City, CA), cDNA, and 900nM of each primer. The PCR conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A no cDNA template control was included in each run. All samples and no-template controls were assayed in duplicate. The target gene expression was normalized against the cyclophilin-A gene. Samples were normalized to results from normal tissue. The relative target gene expression was analyzed using the $-\Delta\Delta C_t$ method.³⁴ Cytokine mRNA values were recorded for each protein for each sample and averaged for each group.

Statistical Analysis

For the cytokine characterization study, a one-way ANOVA was performed to compare differences in mRNA between groups (*10gf*, *chr*, *10gf+chr*, *sham*, *normal*) for each cytokine (IL-1β, IL-6, TNFα) for the spinal cord and DRG samples, separately. For the sTNFR1 treatment study, a one-way ANOVA tested for differences in mechanical allodynia between

sham, treated and untreated groups for each injury paradigm. T-tests were used to detect differences in cytokine mRNA between injury and treatment (*10gf*, *10gf+sTNFR1*), (*chr*, *chr+sTNFR1*) and (*10gf+chr*, *10gf+chr+sTNFR1*), with separate tests for the spinal cord and DRG. For all tests, significance was defined at $p < 0.05$ (SYSTAT; Richmond, CA).

Results

Cytokine Characterization Study

All pro-inflammatory cytokines probed in this study were detected in the ipsilateral DRG of all groups at 1 hour. Sham procedures increased all three cytokines in the ipsilateral DRG over normal levels, but none of these changes were significant. IL-1 β mRNA was significantly increased compared to normal for all injury groups ($p < 0.003$) (Figure 1A). All three types of injuries nearly doubled IL-6 mRNA in the DRG relative to *sham*, but only *10gf* displayed significant increases over normal ($p = 0.005$) (Figure 1B). Lastly, TNF α mRNA expression in the ipsilateral DRG was only increased following injury (Figure 1C). This increase was significant compared to normal for all types of nerve root insult ($p < 0.042$). In addition, following *10gf+chr* this increase was significant over *sham* ($p = 0.026$). Expression of each of these cytokines in the contralateral DRG was unchanged compared to normal (data not shown).

As with the DRG response, *sham* increased spinal cytokine mRNA at 1 hour for all cytokines probed, but not significantly over normal (Figure 2). IL-1 β was significantly elevated over *sham* for the *10gf* and *10gf+chr* groups ($p < 0.024$) and over normal for all three injuries ($p < 0.005$) (Figure 2A). The trend was similar for IL-6 with all three injuries producing elevated mRNA over *sham* and normal; yet increases over *sham* and normal were only significant for *10gf* ($p < 0.024$) (Figure 2B). Spinal TNF α mRNA levels were significantly greater than normal after both *10gf* and *10gf+chr* ($p < 0.019$), but the increase was not significant for *chr* (Figure 2C).

TNF α Receptor Treatment Study

Mechanical allodynia was produced in the forepaws following all injuries (Figure 3). However, although ipsilateral allodynia was increased over *sham* following chronic exposure, this increase was only significant in the ipsilateral forepaw for testing with the 4g von Frey filament ($p = 0.046$) (Figure 3). In addition, no significant decreases were measured for treatment with sTNFR1-PEG in the *chr* injury paradigm (Figure 3). Ipsilateral allodynia at day 1 following *10gf* was significantly greater than *sham* for stimulation using all von Frey filaments ($p < 0.007$) (Figure 3). In general, treatment with the sTNFR1 attenuated allodynia for the compression alone insult (*10gf*), but this was only significant for testing using the 1.4g von Frey filament ($p < 0.005$) (Figure 3). Administration of the sTNFR1-PEG significantly reduced ipsilateral mechanical allodynia when given with the *10gf+chr* injury ($p < 0.001$, for all filaments). For that injury, treatment reduced allodynia to *sham* levels (Figure 3). Allodynia in the contralateral forepaw was also produced at day 1 but was lower than ipsilateral allodynia (Figure 3). Contralateral allodynia increases were significant over *sham* only for the *10gf+chr* injury ($p < 0.014$). The *10gf* injury also displayed contralateral increases in allodynia compared to *sham*, but this was only significant for testing with the 4g von Frey filament ($p = 0.011$). Treatment with sTNFR1 significantly reduced contralateral allodynia following *10gf+chr* for all three filaments ($p < 0.019$) (Figure 3).

IL-1 β and TNF α mRNA levels were elevated over normal and *sham* at day 1 in the ipsilateral DRG (Figure 4). This increase was significant over *sham* only for the *10gf+chr* injury for IL-1 β ($p = 0.021$). Application of sTNFR1 in the compression only injury (*10gf*) did not alter the expression of the genes for IL-1 β , IL-6 or TNF α in the DRG. In contrast, application of the TNF receptor in the *10gf+chr* group significantly reduced cytokine mRNA in the ipsilateral

DRG for both IL-1 β (p=0.025) and TNF α (p=0.028) (Figure 4). Chronic exposure (*chr*) did not induce an increase in the expression of any cytokine probed for either the DRG or spinal cord (data not shown). Probed cytokines (IL-1 β , IL-6, TNF α) were unchanged in the contralateral DRG relative to normal levels for all groups (data not shown).

Spinal cytokine mRNA at day 1 was variable across all injury models and cytokines probed (Figure 5). There was no significant difference in any cytokine relative to normal or *sham* levels, for any group (Figure 5A, B, C). Moreover, administration of the TNF receptor reduced spinal TNF α mRNA in the combined (*10gf+chr*) insult injury, yet this decrease was not significant (Figure 5C).

Discussion

A transient nerve root compression (*10gf*) alone is sufficient to produce immediate (1 hour) significant elevations in gene transcription over *sham* of inflammatory cytokines IL-1 β and IL-6 in the ipsilateral spinal cord (p<0.024) (Figure 2). With the addition of longer lasting inflammation (*10gf+chr*), TNF α is significantly elevated over *sham* in the DRG at this same time point (p=0.026); TNF α mRNA remains elevated at 1 day in the DRG (Figures 1 & 4). Because TNF α exhibited the most consistent and significant changes, especially following a combined compression and inflammatory injury (*10gf+chr*), the soluble TNF receptor-1 was administered at the injury site to saturate and competitively block TNF α *in vivo*. Our results show that blocking the TNF α pathway by local administration of sTNFR1 significantly reduces mechanical allodynia and mRNA for TNF α and IL-1 β in the ipsilateral DRG (Figures 3 & 4). In addition, TNF α in the ipsilateral spinal cord at day 1 in the *10gf+chr* group is also reduced, albeit not significantly (Figure 5). However, while administration of the receptor is able to decrease allodynia produced by compression alone (Figure 3), cytokine mRNA in the DRG and spinal cord is unaltered in that model (Figures 4 & 5). These results suggest that the initiation of persistent allodynia which is produced by a transient mechanical trauma to the nerve root in the cervical spine may be regulated by the early response of pro-inflammatory cytokines in the DRG and spinal cord.^{36,50,58}

Although many cytokines have been investigated in several rat models of neuropathy, their interactions and signaling in pain are still poorly defined, especially for transient injuries. The present findings suggest that early upregulation of TNF α transcription in the DRG, together with upregulation of genes for TNF α , IL-1 β , and IL-6 in the spinal cord, may trigger downstream pain-related signaling cascades. Indeed, although no significant differences were noted between mRNA levels of different injury groups, the pro-inflammatory cytokine responses were most robust following the combination of both compression and application of inflammatory material (*10gf+chr*) (Figures 4 & 5). This is consistent with other reports that this combined insult model has both greater allodynia and more robust spinal glial activation than that produced for compression alone.⁴⁷ For example, following either *10gf* or *10gf+chr*, TNF α is increased in the DRG at 1 day; however for IL-1 β this increase is present after *10gf+chr* only (Figures 1 & 4). Further, while it seems the increase in IL-1 β in the DRG after *10gf+chr* is due to TNF α signaling (Figure 4A), this corresponding decrease is not noted for *10gf* alone, implying varying responses to pharmacologic treatment for different injury paradigms.

In the current study, only a slight increase in cytokine mRNA was noted following *chr* injury alone. This is in contrast to previous studies that show cytokine protein and mRNA increases in models of inflammatory injury to the DRG or nerve root.^{38,57,65} However, those models of inflammatory pain utilize compounds such as Complete Freund's Adjuvant or zymosan which exhibit immediate effects, whereas chronic gut suture resorbs slower and over a longer timeframe.³⁵ It is likely that the 1 hour and 1 day time points studied here may be too early to detect the inflammatory effects produced from the chronic gut suture alone. Indeed, previously

published work from our lab indicates that astrocytic activation is present by day 7, but not day 1, following chronic exposure,⁴⁷ further implying that inflammation from chronic gut sutures increases over time.

While allodynia can persist for 14 days after *10gf* or *10gf+chr*,⁴⁷ it is necessary to probe early cytokine responses (both mRNA and protein) to fully define the temporal and spatial relationship of these cytokines, as they relate to pain initiation and maintenance in cervical radiculopathy. For example, mRNA levels in the spinal cord for all of these cytokines exhibit positive, significant correlations with imposed injury strain for ligation with silk suture, indicating their spinal responses are sensitive to the severity and type of injury.⁶³ Further, inflammatory cytokines are also upregulated locally in neuropathic pain.³³ TNF α , along with IL-1 β , actively modulates afferent activity of DRG neurons following inflammation; application of either of these cytokines to the DRG can increase the basal discharge, mechanosensitivity, and peripheral receptive fields of DRG A δ fibers in rats in a dose-dependent manner.⁴⁴ Nerve root injuries involving both mechanical and chemical components initiate greater and longer-lasting symptoms of behavioral sensitivity.^{24,25,43,47} In these transient cervical nerve root injury paradigms it is possible that pro-inflammatory cytokines have the similar effect in modulating afferent activity of DRG neurons and enhancing mechanical allodynia; further studies are necessary to explore the mechanism through which these inflammatory cytokines specifically relate to behavioral hypersensitivity.

Competitively blocking TNF α signaling in models of lumbar nerve and nerve root injury attenuates behavioral hypersensitivity indicating a direct role for TNF α in mediating neuropathic pain.^{50,51,58} In the present study, we tested the hypothesis that TNF α mediates behavioral sensitivity in cervical radiculopathy using administration of sTNFR1. The soluble TNFR1 competitively blocks soluble TNF α preventing it from binding to its receptor. The sTNFR1 was chosen as it has a higher affinity and specificity to TNF α than the sTNFR2 (p75).⁵⁹ Application of sTNFR1 following either *10gf* or *10gf+chr* significantly decreased ipsilateral forepaw withdrawals compared to matched untreated groups for testing with the 1.4g filament (Figure 3). These results are in agreement with previous studies attenuating mechanical allodynia after spinal nerve ligation when sTNFR1 treatment was given preemptively.^{50,51,58} Our results imply that TNF α signaling in the acute period immediately following injury plays a crucial role in the onset of pain. Previous studies have also demonstrated that sTNFR1 treatment is ineffective in abolishing existing allodynia.^{50,51,58} Taken together, these data indicate that TNF α signaling is active immediately after injury and causes the initiation of a pain cascade. This is corroborated by studies reporting increases in TNF α protein in the spinal cord and DRG very early following nerve root injury.^{8,63} While the present study did not utilize a post-injury treatment paradigm for sTNFR1, such a treatment following injury may not attenuate allodynia since TNF α increases early and other inflammatory cytokines and signaling molecules increase at later time points.^{1,33,63}

The TNF α signaling pathway is activated early in pain and plays a pivotal role in initiation of the pro-inflammatory cytokine cascade in both the spinal cord and DRG. TNF α , and other pro-inflammatory cytokines such as the interleukins, are upregulated at the site of trauma and in the spinal cord in persistent pain.^{8,22,30,63} Data from our own lab indicate a significant increase in TNF α protein in the spinal cord at 1 hour following the *10gf+chr* injury.²⁷ Cytokines directly sensitize neurons by binding to their receptors, which are expressed on neurons.^{36,37,40} Inflammation and cytokine production can indirectly induce the expression of many potent pain mediators such as glutamate, nitric oxide, and prostaglandins in the CNS, further leading to spinal sensitization.^{4,12,31} TNF α and IL-1 β also act synergistically, producing greater inflammation than either cytokine acting alone.^{36,55,61} In addition to directly sensitizing neurons, cytokines can have indirect effects on neurons by stimulating glial cells. Both TNF α and IL-1 β induce microglial and astrocytic reactivity both in vivo and in

vitro.^{2,52} TNF α is produced by infiltrating and resident activated macrophages and Schwann cells at the early stage of nerve and nerve root injury, as well as by astrocytes and microglia at different stages after injury.^{29,36,53} Glial cells are a likely source of TNF α in neuropathic pain, based on a transgenic mouse model of conditional expression of TNF α in astrocytes under the GFAP promoter.⁹ Results from our lab also show that spinal glial activation and mechanical allodynia are increased when mechanical and chemical factors are combined at injury.⁴⁷ Taken with results presented here, this could indicate a specific role for glial cells in upregulating cytokine mRNA after mechanical and chemical nerve root injury.

Only TNF α mRNA was significantly increased in the ipsilateral DRG at 1 hour after any of the injuries studied. This may suggest that the insults of mechanical injury and chronic gut suture elicit an early response of TNF α in the DRG as compared with IL-1 β and IL-6. On the other hand, mRNA results in the ipsilateral spinal cord demonstrate different cytokines were increased significantly at this same time point in either *10gf* or *10gf+chr* group, suggesting that the type of injury may encode a specific profile for gene expression of pro-inflammatory cytokines in the spinal cord. Other aspects of the inflammatory response, such as glial activation and macrophage infiltration, are also variably activated for different types of injuries. Upon spinal nerve injury, macrophages infiltrate the endoneurial blood vessels adjacent to the DRG, and can produce and release TNF α and other pro-inflammatory cytokines.⁵⁴ However, data from our lab demonstrate macrophage infiltration in the dorsal root and dorsal root ganglia at day 7, but not day 1, following injury.²⁹ Further, macrophage infiltration is common in other models which demonstrate axonal degeneration and release of inflammatory cytokines.^{7,16,19,32} TNF α and IL-1 β mRNA induction have been reported to peak earlier (at day 3) than IL-6 mRNA (at day 7) in the DRG and spinal cord following a painful chronic nerve constriction model, implying that TNF α and IL-1 β have roles in initiating pain, while IL-6 may be responsible for its maintenance.³³

Studies quantifying cytokine protein and release after injury are important for defining a role for inflammatory cytokines in mediating nerve root pain. Assays at later time points would provide further insight into mechanisms of persistent pain in this transient injury. In addition, assays probing dorsal and ventral horn inflammation separately would offer information on regional patterns of cytokine changes. Further, defining the mechanical allodynia responses at the 1 hour time point would be valuable to place the cytokine mRNA changes in the context of behavioral hypersensitivity. Yet, in the current study such testing was not possible owing to the need to preserve the time period of 1 hour for tissue harvest following injury. Although mechanical allodynia was the only behavioral outcome probed for this injury model it provides a clinically relevant measure of behavioral hypersensitivity. Sustained sensitivity in the forepaw has been previously reported for this nerve root compression model;¹⁸ patients with cervical nerve injury can present with increased sensitivity to touch and heat stimuli extending from the spine into the forearms and fingers in some cases.²¹

The present study indicates that mechanical compression of the cervical nerve root, both with and without chemical irritation, initiates very early increases in local and central cytokine mRNA. Administration of sTNFR1 at the site and time of injury decreased cytokine mRNA and attenuated mechanical allodynia. These findings suggest that TNF α signaling mediates both downstream cytokine increases and behavioral hypersensitivity in cervical radiculopathy. While inflammatory cytokine mRNA at day 1 was not decreased following *10gf+sTNFR1*, this could be due to the fact that these cytokines demonstrate only a transient increase in that model. Conversely, following *10gf+chr*, inflammatory cytokine mRNA remains elevated at day 1. It may be possible that the inflammatory component of the chronic gut material introduced in the *10gf+chr* injury dominates over the mechanical one present in either injury and that the sTNFR1 was more active against the elements of the nociceptive cascade which are inflammatory-based than those from the mechanical component. Further studies outlining

changes in cytokine protein and their specific cellular sources are required to further elucidate inflammatory signaling in cervical radiculopathy. Nonetheless, results presented here demonstrate both local and central inflammation following nerve root injury and provide evidence for a role for TNF α in cervical radiculopathy.

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References

1. Arruda J, Colburn R, Rickman A, Rutkowski M, DeLeo J. Increase of interleukin-6 mRNA in the spinal cord following peripheral nerve injury in the rat: potential role of IL-6 in neuropathic pain. *Mol Brain Res* 1998;62:228–235. [PubMed: 9813345]
2. Aschner M. Astrocytes as mediators of immune and inflammatory responses in the CNS. *Neurotoxicology* 1998;19:269–281. [PubMed: 9553964]
3. Atlas S, Keller R, Wu Y, Deyo R, Singer D. Long-term outcomes of surgical and nonsurgical management of sciatica secondary to a lumbar disc herniation: 10 year results from the maine lumbar spine study. *Spine* 2005;30:927–935. [PubMed: 15834338]
4. Cavanaugh, J. Neurophysiology and neuroanatomy of neck pain. Pintar, FA.; Yoganandan, N., editors. IOS Press; Amsterdam: 2000. p. 79-96.
5. Clark A, Gentry C, Bradbury E, McMahon S, Malcangio M. Role of spinal microglia in rat models of peripheral nerve injury and inflammation. *Eur J Pain* 2007;11:223–230. [PubMed: 16545974]
6. Colburn R, Rickman A, DeLeo J. The effect of site and type of nerve injury on spinal glial activation and neuropathic pain behavior. *Exp Neurol* 1999;157:289–304. [PubMed: 10364441]
7. Cui J, Holmin S, Mathiesen T, Meyerson B, Linderöth B. Possible role of inflammatory mediators in tactile hypersensitivity in rat models of mononeuropathy. *Pain* 2000;88(3):239–48. [PubMed: 11068111]
8. DeLeo J, Colburn R, Nichols M, Malhotra A. Interleukin-6-mediated hyperalgesia/allodynia and increased spinal IL-6 expression in a rat mononeuropathy model. *J Interferon Cytokine Res* 1996;16:695–700. [PubMed: 8887053]
9. DeLeo J, Rutkowski M, Stalder A, Campbell I. Transgenic expression of TNF by astrocytes increases mechanical allodynia in a mouse neuropathy model. *Neuroreport* 2000;11(3):599–602. [PubMed: 10718321]
10. DeLeo J, Tawfik V, LaCroix-Fralish M. The tetrapartite synapse: path to CNS sensitization and chronic pain. *Pain* 2006;122:17–21. [PubMed: 16564626]
11. DeLeo J, Winkelstein B. Physiology of chronic spinal pain syndromes. *Spine* 2002;27:2526–2537. [PubMed: 12435988]
12. DeLeo J, Yeziarski R. The role of neuroinflammation and neuroimmune activation in persistent pain. *Pain* 2001;90:1–6. [PubMed: 11166964]
13. Elliott A, Smith B, Penny K, Smith W, Chambers W. The epidemiology of chronic pain in the community. *The Lancet* 1999;354:1248–1252.
14. Eskes C, Juillerat-Jeanneret L, Leuba G, Honegger P, Monnet-Tschudi F. Involvement of microglia–neuron interactions in the tumor necrosis factor release, microglial activation, and neurodegeneration induced by trimethyltin. *J Neurosci Res* 2003;71:583–590. [PubMed: 12548715]
15. Flatters S, Fox A, Dickenson A. Spinal interleukin-6 (IL-6) inhibits nociceptive transmission following neuropathy. *Brain Res* 2003;984:54–62. [PubMed: 12932839]
16. Fry E, Ho C, David S. A role for Nogo receptor in macrophage clearance from injured peripheral nerve. *Neuron* 2007;53(5):649–62. [PubMed: 17329206]
17. Frymoyer J. Back pain and sciatica. *N Engl J Med* 1988;318:291–300. [PubMed: 2961994]
18. George A, Marziniak M, Schäfers M, Toyka K, Sommer C. Thalidomide treatment in chronic constrictive neuropathy decreases endoneurial tumor necrosis factor-alpha, increases interleukin-10

and has long-term effects on spinal cord dorsal horn met-enkephalin. *Pain* 2000;88(3):267–75. [PubMed: 11068114]

19. Gupta R, Channal J. Spatiotemporal pattern of macrophage recruitment after chronic nerve compression injury. *J Neurotrauma* 2006;23(2):216–26. [PubMed: 16503805]
20. Hanisch U. Microglia as a source and target of cytokines. *Glia* 2002;40:140–155. [PubMed: 12379902]
21. Hart L, Deyo R, Cherkin D. Physician office visits for low back pain. Frequency, clinical evaluation, and treatment patterns from a U.S. national survey. *Spine* 1995;20:11–19. [PubMed: 7709270]
22. Hashizume H, DeLeo J, Colburn R, Weinstein J. Spinal glial activation and cytokine expression after lumbar root injury in the rat. *Spine* 2000;25:1206–1217. [PubMed: 10806496]
23. Hasselstrom J, Liu-Palmgren J, Rasjo-Wraak G. Prevalence of pain in general practice. *Eur J Pain* 2002;6:375–385. [PubMed: 12160512]
24. Homma Y, Brull S, Zhang J. A comparison of chronic pain behavior following local application of tumor necrosis factor α to the normal and mechanically compressed lumbar ganglia in the rat. *Pain* 2002;95:239–246. [PubMed: 11839423]
25. Hou S, Tang J, Chen H, Chen J. Chronic inflammation and compression of the dorsal root contribute to sciatica induced by the intervertebral disc herniation in rats. *Pain* 2003;105:255–64. [PubMed: 14499443]
26. Hubbard R, Chen Z, Winkelstein B. Transient cervical nerve root compression modulates pain: Load thresholds for allodynia and sustained changes in spinal neuropeptide expression. *J Biomech* 2008;41:677–85. [PubMed: 17976629]
27. Hubbard, R.; Rothman, S.; Winkelstein, B. Biomechanical effects of neural tissue loading and pain. In: Zhang, J.; Dobsretsov, M., editors. *Mechanics of Pain in Peripheral Neuropathy*. Chapter 9. 2008.
28. Hubbard R, Winkelstein B. Transient cervical nerve root compression in the rat induces bilateral forepaw allodynia and spinal glial activation: Mechanical factors in painful neck injuries. *Spine* 2005;30:1924–32. [PubMed: 16135981]
29. Hubbard R, Winkelstein B. Nerve root compression produces local axonal pathology and inflammation dependent upon mechanics and directly related to mechanical allodynia. *Exp Neurol*. 2008epub May 17PMID 18572166
30. Hunt J, Winkelstein B, Rutkowski M, Weinstein J, DeLeo J. Repeated injury to the lumbar nerve roots produces enhanced mechanical allodynia and persistent spinal neuroinflammation. *Spine* 2001;26:2073–2079. [PubMed: 11698881]
31. Kawakami, M.; Weinstein, J. Associated neurogenic and nonneurogenic pain mediators that probably are activated and responsible for nociceptive input. Weinstein, J.; Gordon, S., editors. *AAOS*; Rosemont, IL: 1986. p. 265-273.
32. Kobayashi S, Yoshizawa H, Yamada S. Pathology of lumbar nerve root compression. Part 2: morphological and immunohistochemical changes of dorsal root ganglion. *J Orthop Res* 2004;22(1): 180–8. [PubMed: 14656678]
33. Lee H, Lee K, Son S, Hwang S, Cho H. Temporal expression of cytokines and their receptor mRNAs in a neuropathic pain model. *Neuroreport* 2004;15(18):2807–2811. [PubMed: 15597059]
34. Livak K, Schmittgen T. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 2001;25(4):402–408. [PubMed: 11846609]
35. Maves T, Pechman P, Gebhart G, Meller S. Possible chemical contribution from chronic gut sutures produces disorders of pain sensation like those seen in man. *Pain* 1993;54:57–69. [PubMed: 8378104]
36. McMahon S, Cafferty W, Marchand F. Immune and glial cell factors as pain mediators and modulators. *Exp Neurol* 2005;192:444–462. [PubMed: 15755561]
37. Millan M. The Induction of Pain: An Integrative Review. *Prog Neurobiol* 1999;57:1–164. [PubMed: 9987804]
38. Narita M, Shimamura M, Imai S, Kubota C, Yajima Y, Takagi T, Shiokawa M, Inoue T, Suzuki M, Suzuki T. Role of Interleukin-1 β and tumor necrosis factor- α -dependent expression of cyclooxygenase-2 mRNA in thermal hyperalgesia induced by chronic inflammation in mice. *Neurosci* 2008;152:477–486.

39. Narita M, Yoshida T, Nakajima M, Narita M, Miyatake M, Takagi T, Yajima Y, Suzuki T. Direct evidence for spinal cord microglia in the development of a neuropathic pain-like state in mice. *J Neurochem* 2006;97:1337–1348. [PubMed: 16606373]
40. Newman E. New roles for astrocytes: regulation of synaptic transmission. *Trends Neurosci* 2003;26:536–542. [PubMed: 14522146]
41. Ohnmeiss D, Vanharanta H, Ekholm J. Degree of disc disruption and lower extremity pain. *Spine* 1997;22:1600–1605. [PubMed: 9253095]
42. Ohtori S, Takahashi K, Moriya H, Myers R. TNF-alpha and TNF-alpha receptor type 1 upregulation in glia and neurons after peripheral nerve injury: studies in murine DRG and spinal cord. *Spine* 2004;29(10):1082–8. [PubMed: 15131433]
43. Olmarker K, Myers R. Pathogenesis of sciatic pain: role of herniated nucleus pulposus and deformation of spinal nerve root and dorsal root ganglion. *Pain* 1998;78:99–105. [PubMed: 9839819]
44. Ozaktay A, Kallakuri S, Takebayashi T, Cavanaugh J, Asik I, DeLeo J, Weinstein J. Effects of interleukin-1 beta, interleukin-6, and tumor necrosis factor on sensitivity of dorsal root ganglion and peripheral receptive fields in rats. *Eur Spine J* 2006;15:1529–37. [PubMed: 16474945]
45. Pineau I, Lacroix S. Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved. *J Comp Neurol* 2007;500(2):267–85. [PubMed: 17111361]
46. Rothman S, Kreider R, Winkelstein B. Spinal neuropeptide responses in persistent and transient pain following cervical nerve root injury. *Spine* 2005;30:2491–2496. [PubMed: 16284585]
47. Rothman S, Winkelstein B. Chemical and mechanical nerve root insults induce differential behavioral sensitivity and glial activation that are enhanced in combination. *Brain Res* 2007;1181:30–43. [PubMed: 17920051]
48. Ruohonen S, Jagodi M, Khademi M, Taskinen H, Ojala P, Olsson T, Roytta M. Contralateral non-operated nerve to transected rat sciatic nerve shows increased expression of IL-1beta, TGF-beta1, TNF-alpha, and IL-10. *J Neuroimmunol* 2002;132:11–17. [PubMed: 12417428]
49. Rutkowski M, DeLeo J. The role of cytokines in the initiation and maintenance of chronic Pain. *Drug News Perspect* 2002;15(10):626–632. [PubMed: 12677247]
50. Schafer M, Lee D, Brors D, Yaksh T, Sorkin L. Increased sensitivity of injured and adjacent uninjured rat primary sensory neurons to exogenous tumor necrosis factor after spinal nerve ligation. *J Neurosci* 2003;23:3028–3038. [PubMed: 12684490]
51. Schafer M, Svensson C, Sommer C, Sorkin L. Tumor necrosis factor- α induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. *J Neurosci* 2003;23(7):2517–2521. [PubMed: 12684435]
52. Schubert P, Morino T, Miyazaki H, Ogata T, Nakamura Y, Marchini C, Ferroni S. Cascading glia reactions: a common pathomechanism and its differentiated control by cyclic nucleotide signaling. *Ann NY Acad Sci* 2000;903:24–33. [PubMed: 10818485]
53. Shamash S, Reichert F, Rotshenker S. The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. *J Neurosci* 2002;22(8):3052–60. [PubMed: 11943808]
54. Sommer C. Painful neuropathies. *Curr Opin Neurol* 2003;16(5):623–8. [PubMed: 14501847]
55. Sommer C, Kress M. Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. *Neurosci Lett* 2004;361:184–187. [PubMed: 15135924]
56. Strine T, Hootman J. US national prevalence and correlates of low back and neck pain among adults. *Arthritis Rheum* 2007;57(4):656–65. [PubMed: 17471542]
57. Sweitzer S, Colburn R, Rutkowski M, DeLeo J. Acute peripheral inflammation induces moderate glial activation and spinal IL-1 β expression that correlates with pain behavior in the rat. *Brain Res* 2009;1209:209–221. [PubMed: 19350552]
58. Sweitzer S, Martin D, DeLeo J. Intrathecal interleukin-1 receptor antagonist in combination with soluble tumor necrosis factor receptor exhibits an anti-allodynic action in a rat model of neuropathic pain. *J Neurosci* 2001;21:529–539.
59. Vandenabeele P, Declercq W, Beyaert R, Fiers W. Two tumor necrosis factor receptors: structure and function. *Trends Cell Biol* 1995;5(10):392–399. [PubMed: 14732063]

60. Watkins L, Maier S. Glia: a novel drug discovery target for clinical pain. *Nat Rev Drug Discov* 2003;2:973–985. [PubMed: 14654796]
61. Watkins L, Maier S, Goehler L. Immune Activation: The role of pro-inflammatory cytokines in inflammation, illness responses and pathological pain states. *Pain* 1995;63:289–302. [PubMed: 8719529]
62. Winkelstein B, DeLeo J. Nerve root injury severity differentially modulates spinal glial activation in a rat lumbar radiculopathy model: considerations for persistent pain. *Brain Res* 2002;956:294–301. [PubMed: 12445698]
63. Winkelstein B, Rutkowski M, Sweitzer S, Pahl J, DeLeo J. Nerve injury proximal or distal to the DRG induces similar spinal glial activation and selective cytokine expression but differential behavioral responses to pharmacologic treatment. *J Comp Neurol* 2001;439:127–139. [PubMed: 11596043]
64. Wolf G, Yirmiya R, Goshen I, Iverfeldt K, Holmlund L, Takeda K, Shavit Y. Impairment of interleukin-1 (IL-1) signaling reduces basal pain sensitivity in mice: genetic, pharmacological and developmental aspects. *Pain* 2003;104:471–480. [PubMed: 12927619]
65. Xie W, Deng H, Li H, Bowen T, Strong J, Zhang J. Robust increase of cutaneous sensitivity, cytokine production and sympathetic sprouting in rats with localized inflammatory irritation of the spinal ganglia. *Neuroscience* 2006;142:809–822. [PubMed: 16887276]
66. Xu J, Xin W, Zang Y, Wu C, Liu X. The role of tumor necrosis factor-alpha in the neuropathic pain induced by Lumbar 5 ventral root transection in rat. *Pain* 2006;123(3):306–21. [PubMed: 16675114]
67. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983;16:109–110. [PubMed: 6877845]

Fig 1.

Quantification of IL-1 β (A), IL-6 (B), TNF α (C) mRNA expression in the ipsilateral DRG at 1 hour following *sham*, *10gf*, *chr*, or *10gf+chr*. Asterisk (*) indicates a significant difference from *sham* and the pound sign (#) indicates a significant difference from normal. At 1 hour after *10gf*, *chr*, or *10gf+chr*, IL-1 β and TNF α mRNA were both significantly increased compared to normal (#, $p < 0.042$). This increase was also significant compared to *sham* for TNF α following *10gf+chr* (*, # $p < 0.026$). Error bars indicate +/- standard deviation (SD).

Fig 2.

Quantification of spinal cord IL-1 β (A), IL-6 (B), TNF α (C) mRNA expression at 1 hour after *sham*, *10gf*, *chr*, or *10gf+chr*. At 1 hour following *10gf*, *chr*, or *10gf+chr*, IL-1 β mRNA was significantly increased compared to normal (# $p < 0.005$); this increase was also significant over *sham* for *10gf* and *10gf+chr* (*, # $p < 0.001$). TNF α was also significantly increased compared to normal for *10gf* and *10gf+chr* ($p < 0.019$). IL-6 mRNA were significantly increased after *10gf* compared to normal and *sham* (*, # $p < 0.024$). Error bars indicate $\pm$ standard deviation (SD).

Fig 3.

Average mechanical allodynia in the ipsilateral and contralateral forepaws at day 1 for *sham*, *10gf*, *10gf+sTNFR1*, *chr*, *chr+sTNFR1*, *10gf+chr*, or *10gf+chr+sTNFR1*. Allodynia is measured by the number of paw withdrawals for stimulation with each von Frey filament: (A) 1.4g, (B) 2g, and (C) 4g. Asterisk (*) indicates significant difference from *sham* values. Treatment with sTNFR1 significantly decreased allodynia compared to non-treatment responses for *10gf* and *10gf+chr* for testing with the 1.4g (A) filament. This trend was conserved across filaments for *10gf+chr* injury (A,B,C). Error bars indicate $\pm$ standard deviation (SD).

Fig 4.

Quantification of cytokine gene expression in the ipsilateral DRG at 1 day after injury either with or without sTNFR1 treatment: IL-1 β (A), IL-6 (B), TNF α (C). Treatment with sTNFR1 significantly decreased IL-1 β and TNF α mRNA compared to their matched un-treated groups ($p < 0.028$). Error bars indicate +/- standard deviation (SD).

Fig 5. Quantification of relative levels of IL-1 β (A), IL-6 (B), TNF α (C) mRNA expression in the ipsilateral spinal cord at 1 day following 10gf or 10gf+chr, either with or without sTNFR1 treatment. Treatment with sTNFR1 decreased TNF α mRNA compared to un-treated rats. Error bars indicate +/- standard deviation (SD).

Table 1

<u>Gene</u>	<u>Primer</u>	<u>Sequence</u>
IL-1 β	forward	5'-CACCTCTCAAGCAGAGCACAG-3'
	reverse	5'-GGGTTCATGGTGAAGTCAAC-3'
IL-6	forward	5'-AAGTCGGAGGCTTAATTACATATGTTTC-3'
	reverse	5'-TGCCATTGCACAACTCTTTTCT-3'
TNF α	forward	5'-ATCATCTTCTCAAACTCGAGTGACAA-3'
	reverse	5'-CTGCTCCTCTGCTTGGT-3'
Cyclophilin A	forward	5'-TATCTGCACTGCCAAGACTGAGTG-3'
	reverse	5'-CTTCTTGCTGGTCTTGCCATTCC-3'