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Cytokine Antagonism Reduces Pain and Modulates Spinal Astrocytic Reactivity After Cervical Nerve Root Compression

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Abstract—Relationships between nerve root compression, behavioral sensitivity, spinal cytokines, and glial reactivity are not fully defined for painful cervical nerve root compression. Spinal cytokines were quantified after mechanical root compression (*10gf*), root exposure to inflammatory chromic gut material (*chr*), the combination of both insults together (*10gf* + *chr*) or *sham*. TNFα and IL-1β significantly increased at 1 h ($p < 0.029$). IL-1α was significantly increased over normal, *sham* and *chr* at 1 h following *10gf* and over normal and *sham* after *10gf* + *chr* ($p < 0.048$). By day 1, only IL-1β after *10gf* remained elevated over normal ($p = 0.038$). Accordingly, the soluble TNF receptor-1 (sTNFR1) and the IL-1 receptor antagonist (IL-1ra) were separately administered at early time points after each injury. With sTNFR1, behavioral sensitivity was significantly decreased for 7 days after both *10gf* and *10gf* + *chr* ($p < 0.005$). Treatment with IL-1ra significantly reduced sensitivity for *10gf* + *chr* ($p < 0.034$) but not for *10gf*. Sensitivity remained significantly elevated over *sham* at all time points ($p < 0.044$). Spinal astrocytic reactivity significantly decreased for both treatments after *10gf* ($p < 0.002$); but, only IL-1ra following *10gf* + *chr* significantly reduced astrocytic reactivity ($p < 0.001$). Early increases in spinal TNFα, IL-1β, and IL-1α may induce pain, affect spinal astrocytic responses, and appear to have differential effects in mediating the behavioral hypersensitivity produced by different types of painful cervical radicular injuries.

Keywords—Cytokine, Pain, Radiculopathy, Astrocytes, Nerve root, Compression.

INTRODUCTION

Both the patterns and extent of pain that develop as a result of a nerve root injury depend on the type of injury that is imposed.[4,16,33,36,54] Nerve root compression can occur clinically in the cervical spine via traumatic injury, disc herniation, and/or slower onset stenosis, with disc herniation being the most common cause of nerve root-mediated pain.[2,11,14,31] Vertebral loading imposes only compression to the nerve root, but both inflammatory-based chemical and mechanical factors contribute to nerve root-mediated pain from disc herniation when the inflammatory material of the disc comes in contact with the nerve root, causing tissue impingement and compression.[33,42,51] Models of experimental nerve root compression induce sustained behavioral hypersensitivity, with pain symptoms that are greater for combined mechanical and inflammatory insults compared to nerve root compression alone.[4,33,41,54] Despite the fact that both types of nerve root injuries are observed clinically in the cervical spine, treatments for effective management of the associated pain symptoms remain lacking.

Endogenous inflammatory cytokines contribute to pain that is induced following spinal cord injury, neuropathy, and radiculopathy.[5,6,15,24,26,47,48,52–54] Cytokines are synthesized and released by astrocytes, microglia, and macrophages and act throughout the nervous system via paracrine and autocrine signaling.[10,13,26] Pro-inflammatory cytokine mRNA and protein profiles in the DRG differ according to the type of nerve root injury.[38–40] Differences in pain responses, spinal glial reactivity, and inflammatory cytokine mRNA upregulation between cervical nerve root compression with and without a chemical insult imply that the underlying biochemical mechanisms driving the differences in pain in these radicular injuries vary, and accordingly, that their responses to pharmacologic intervention may be different. However, while pharmacologic blocks of cytokine signaling

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have been used in peripheral nerve injuries,[47,49,56,57] there is no evidence of the efficacy of these agents in painful cervical radicular injury, and no indication of how different types of nerve root injuries respond to pharmacologic intervention.

Pharmacologic modulation of the cellular cascades following painful nerve root injury has proven to be promising for novel biomaterials approaches for controlled release of growth factors and other molecules involved in neural repair and pain alleviation.[18] As such, determining whether common cytokines are key regulators in these types of painful injuries would provide potentially valuable avenues for drug development in treating chronic pain. TNFα antagonists, such as infliximab and the soluble TNF receptor-1 (sTNFR1), reduce behavioral hypersensitivity when given in pretreatment or perioperative paradigms, with less robust effects when given after the onset of symptoms.[24,28,30,45,47,57] We have shown a reduction in mechanical allodynia (a measure of behavioral sensitivity) as early as 1 day after painful cervical radiculopathy when sTNFR1 was administered at the time of injury.[39] However, it is unknown if sTNFR1 has any effect when administered *after* cervical nerve root injury, whether pharmacologic block of IL-1 signaling modulates mechanical allodynia, and whether different painful cervical nerve root injuries (mechanical, chemical, or their combination) respond differently to cytokine antagonism.

These studies test the hypothesis that spinal proinflammatory cytokines mediate behavioral hypersensitivity following cervical nerve root injuries and begins to evaluate potential pharmacologic treatments for pain from this class of injury. As such, TNFα, IL-1β, and IL-1α were quantified in the spinal cord after nerve root injury modeling separately mechanical compressive trauma alone and a cervical disc herniation, and immunohistochemistry was used to identify the cellular source of IL-1α in these models. Further, two cytokine treatments, the soluble TNF receptor-1 (sTNFR1) and the interleukin-1 receptor antagonist (IL-1ra), were used in separate studies to determine if blocking either TNFα or IL-1β/IL-1α signaling attenuates the mechanical allodynia that results from these nerve root injuries and if there is a difference in responses between the two injuries. Astrocytic activation was quantified in the spinal cord after treatment to determine if and how those cytokines mediate that marker of spinal glial activation. Collectively, this work expands our previous studies characterizing cytokine responses in the DRG in these models and mRNA and behavior on day 1 following a single sTNFR1 treatment given at the time of injury.[39,40]

MATERIALS AND METHODS

Two separate studies were performed: (1) to define cytokine levels and cellular sources in the spinal cord immediately following two different painful insults to the C7 cervical nerve root and (2) to utilize antagonists to evaluate how two cytokines modulate behavioral hypersensitivity and astrocytic reactivity in the spinal cord following these two different nerve root injuries. In the first study, protein levels of TNFα, IL-1β, and IL-1α were quantified in the C7 spinal cord on the side ipsilateral to the nerve root injury. These proteins were characterized at two early time points (1 h, 1 day) following cervical nerve root injury: either chromic gut suture exposure (*chr*), applied compression with a 10 gram-force microvascular clip (*10gf*), or their combination (*10gf* + *chr*), in separate investigations. The cellular source of the membrane bound IL-1α was identified using immunohistochemical techniques to analyze spinal cord tissue at 1 h following injury. Based on the cytokine findings, a second study was performed using the soluble TNF receptor-1 to neutralize TNFα and eliminate the binding of TNFα to its receptor, and the interleukin-1 receptor antagonist to bind to the IL-1 receptor and prohibit IL-1 signaling. Each cytokine antagonist was evaluated separately for its respective effects on modulating allodynia and on expression of glial fibrillary astrocytic protein (GFAP), as a marker of astrocytic reactivity, in the spinal cord at day 7. Experiments were performed using male Holtzman rats (Harlan Sprague–Dawley; Indianapolis, ID), weighing 250–350 g 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.[59] Animals were housed with a 12–12 h light–dark cycle and free access to food and water.

Cytokine Characterization Study

Rats underwent one of four previously reported surgical procedures[19,41]: (1) nerve root exposure to chromic gut suture material (*chr*), (2) transient nerve root compression (*10gf*), (3) chromic gut exposure with supplemental cervical nerve root compression (*10gf* + *chr*), or (4) sham exposure (*sham*). At either 1 h or 1 day after the surgical procedure, the C7 spinal cord on the side ipsilateral to the exposed nerve root was harvested. Cytokine (TNFα, IL-1β, IL-1α) levels were analyzed using enzyme-linked immunosorbent assays (ELISA). Tissue from *sham* and *chr* subjects were harvested only at 1 h in order to provide the most robust estimate of cytokine upregulation in the most

acute period following surgery. Tissue from normal, unoperated rats ($n = 2$) was also used for comparison. Tissue samples were harvested at 1 h ($n = 4$ *chr*; $n = 12$ *10gf*; $n = 12$ *10gf + chr*; $n = 4$ *sham*) and at 1 day ($n = 6$ *10gf*; $n = 6$ *10gf + chr*) following injury. For each protein probed, the samples were processed for responses at 1 h ($n = 6$ *10gf*; $n = 6$ *10gf + ch*; $n = 4$ *chr*; $n = 4$ *sham*) and at 1 day ($n = 6$ *10gf*; $n = 6$ *10gf + chr*). Fluorescent immunohistochemistry was used to co-label IL-1α with phenotypic markers for astrocytes (GFAP), microglia (Iba1), and neurons (NeuN) in order to identify which cell type expresses spinal IL-1α at 1 h in response to these nerve root injuries. Spinal cord tissue was harvested from the C7 cervical spinal cord following the two injuries ($n = 4$ *10gf*; $n = 6$ *10gf + ch*) or *sham* ($n = 4$).

Cytokine Antagonism Study

To test for a link between cytokines and mechanical allodynia, the soluble TNF receptor-1 (sTNFR1) and the IL-1 receptor antagonist (IL-1ra) were used separately. Both treatments (sTNFR1, IL-1ra; gifts from Amgen, Inc., Thousand Oaks, CA) were administered separately for both the *10gf*, and *10gf + chr* injury models. Half of the rats for each injury (*10gf*; *10gf + chr*) received sTNFR1 (200 μg in 40 μL saline) ($n = 8$/each) via lumbar puncture at 6 h and 1 day after injury and half received IL-1ra (100 μg in 40 μL saline) ($n = 8$/each) via lumbar puncture, also at 6 h and 1 day after injury. An additional set of rats ($n = 8$) received *sham* procedures and dosing with either sTNFR1 ($n = 4$) or IL-1ra ($n = 4$). A separate set of rats was used as a vehicle control group for these studies. Those rats received doses of saline as the vehicle via the same dosing paradigm. This group consisted of *10gf* ($n = 6$), *10gf + chr* ($n = 4$), and *sham* ($n = 4$). Rats were tested for bilateral mechanical allodynia on days 1, 3, 5, and 7 to assess behavioral hypersensitivity. Spinal cord at C7 level was harvested on day 7 for immunohistochemical analysis of GFAP.

SPECIFIC METHODS

Surgical Procedures

All procedures were performed under inhalation anesthesia (4% isoflurane for induction, 2% for maintenance). Surgical procedures have been detailed previously.[19,41] 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 chromic exposure (*chr*) involved placing four pieces of 3-0 chromic gut suture (Surgical Specialties, Reading, PA) (2 mm) on the right C7 dorsal nerve root proximal to the DRG (*chr*). Nerve root compression (*10gf*) involved transient compression of the C7 dorsal nerve root for 15 min using a 10gf microvascular clip (World Precision Instruments, Inc., Sarasota, FL); compression with chromic gut material (*10gf + chr*) was the same as *10gf* but with the additional placement of four pieces of chromic gut suture on the right C7 dorsal nerve root proximal to the DRG. Following *chr* and *10gf + chr*, the chromic suture was left in place for the duration of the study. Procedures for *sham* used the same surgical protocol but only had exposure of the C7 dorsal root with no additional manipulation. 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 behavioral sensitivity by measuring bilateral forepaw mechanical allodynia postoperatively until the time of tissue harvest, as previously described.[17,19,41] Prior to surgery, rats were acclimated to the tester and environment. Allodynia was measured for each unoperated rat before the surgical procedure to assess baseline unoperated control allodynia responses. A single tester performed all testing and was blinded to the surgical procedures. For each testing session, after 20 min of acclimation, rats were stimulated on the plantar surface of each forepaw using two von Frey filaments (2, 4 g; Stoelting, Wood Dale, IL). Each testing session consisted of three rounds of 10 stimulations, separated by 10 min. Total withdrawals were recorded for each forepaw of each rat and averaged for each group.

Enzyme-Linked Immunosorbent Assay (ELISA)

For all of the rats in the cytokine characterization study, rats were deeply anesthetized with sodium pentobarbital (65 mg/kg) followed by transcardiac perfusion with 200 mL of phosphate buffered saline (PBS) for tissue harvest. Following perfusion, 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 immediately frozen on dry ice. A protease inhibitor tablet (Boehringer Mannheim, Germany) was added to 50 mL of homogenization buffer (50 mM NaCl, 10 mM tris, 2.5 mM $MgCl_2$) and each tissue sample was added to 325 μL of this solution.[49] Samples were then sonicated and homogenized for 30 s followed

by centrifugation at 20,000 rpm for 30 min at 4 °C. Total protein concentration was determined using a DCA protein assay (Pierce, Rockford, IL), according to manufacturer's instructions. Commercially available ELISA kits (R&D Systems, Minneapolis, MN) were used to determine concentrations of TNFα, IL-1β, and IL-1α, according to manufacturer's instructions. A standard curve was generated for each ELISA and all standards and samples were run in duplicate. Sensitivities for detecting each cytokine with each kit were: TNFα (>5 pg/mL), IL-1β (>5 pg/mL), and IL-1α (>1.43 pg/mL). Concentrations for each sample were expressed as pg/mg total protein.

Immunohistochemistry Procedures

In order to determine which cell type in the dorsal horn expresses IL-1α following treatment, C7 spinal cord was assayed using fluorescent co-labeling with an antibody to IL-1α and either to glial fibrillary acidic protein (GFAP) to label astrocytes, ionized calcium-binding adapter molecule 1 (Iba1) to label microglia or neuronal nucleus (NeuN) to label neurons. Rats were deeply anesthetized followed by transcardiac perfusion with 200 mL of phosphate buffered saline (PBS) and 300 mL of 4% paraformaldehyde in PBS (pH 7.4). Following perfusion, the cervical spinal cord was exposed by laminectomy, the C7 segment of the cervical spinal cord was harvested, and tissue was post-fixed in the 4% paraformaldehyde solution for 20 min.[19] For each co-labeling paradigm, four serial C7 spinal cord sections (20 μm) were prepared for free-floating staining. Sections were blocked with 5% normal donkey serum (Invitrogen, Carlsbad, CA) for 60 min followed by incubation in a primary antibody solution containing goat anti-IL-1α (1:100; Santa Cruz Biotech, Santa Cruz, CA) and rabbit anti-GFAP (1:20,000; Dako, Carpinteria, CA), rabbit anti-Iba1 (1:1000; Wako, Richmond, VA), or rabbit anti-NeuN (1:500; Millipore, Billerica, MA) overnight at 4°. Sections were then treated with an Alexa594 conjugated donkey anti-rabbit secondary antibody to label GFAP, Iba1, or NeuN (1:250; Invitrogen, Carlsbad, CA) and Alexa488 conjugated donkey anti-goat secondary antibody to label IL-1α (1:250; Invitrogen, Carlsbad, CA) for 60 min. Sections were photographed at 100× magnification using a digital camera and stereomicroscope system and Axiovision software (Zeiss, Thornwood, NY). Images were uniformly adjusted to maximize contrast, and visually inspected for co-localization of the two fluorophores (594/488) in order to identify which phenotypic markers co-localized with IL-1α in the spinal cord.

In order to assess glial activation in the dorsal horn following treatment, C7 spinal cord was assayed for astrocytic activation using antibodies to GFAP. Immunohistochemical analysis was performed on spinal cord tissue from all rats from the cytokine antagonism study. Cervical spinal cord tissue was harvested on day 7 after injury. Tissue was harvested as described above and transferred to 30% sucrose/PBS and stored for 3 days at 4 °C. Samples were freeze-mounted with OCT medium (Triangle Biomedical Sciences, Durham, NC) for axial cryosectioning. Matched spinal cord tissue from a naïve (unoperated) rat was also processed for comparison. Four serial C7 spinal cord sections (20 μm) from each rat were prepared for free-floating immunohistochemical staining. A polyclonal antibody to GFAP (Dako, Carpinteria, CA) was used as a marker of reactive astrocytes. Slices were blocked with normal goat serum (Vector Labs, Burlingame, CA) for 60 min followed by incubation in a primary antibody solution directed against GFAP (1:20,000) overnight. Tissue sections were then treated with a secondary antibody solution containing an Alexa546 conjugated goat anti-rabbit secondary antibody (1:500; Invitrogen, Carlsbad, CA). Previous studies were performed to determine optimal antibody dilutions. A negative control with no primary antibody was always included for verification of specificity of immunohistochemical techniques.

GFAP staining in the spinal cord was analyzed using a quantitative densitometry method as previously reported.[41] The ipsilateral and contralateral dorsal and ventral horns were photographed at 100× magnification using a digital camera and stereomicroscope system and Axiovision software (Zeiss, Thornwood, NY). Pictures were cropped to include the region of interest in either the dorsal (1000 × 200 pixels) or ventral (500 × 500 pixels) horns. Pictures were then inverted and analyzed for a percentage of pixels above a defined threshold; the threshold pixel intensity was chosen based on staining in normal tissue. This approach gives a quantitative measure of the degree and amount of staining above what is present in normal naïve tissue. Values were normalized, averaged, and compared.

Statistical Analysis

For the cytokine characterization study, a one-way ANOVA with post hoc Bonferroni correction was performed to compare differences in protein levels for each cytokine (TNFα, IL-1β, IL-1α) between groups (*chr*, *10gf*, *10gf* + *chr*, *sham*, normal) at 1 h and 1 day, separately. For the cytokine antagonism study, mechanical allodynia was compared using two separate two-way ANOVAs with repeated measures to test for the effect of injury (*10gf*, *10gf* + *chr*, *sham*) or treatment (vehicle, antagonist), for each of sTNFR1 and IL-1ra separately. A one-way ANOVA with post

hoc Bonferroni correction tested for differences at each day (injury + treatment, injury + saline, *sham*) for each injury and each treatment separately. Lastly, a one-way ANOVA with post hoc Bonferroni correction tested for differences in GFAP expression between treatment and vehicle, for each injury and each cytokine antagonist in separate tests.

RESULTS

Cytokine Characterization Study

All pro-inflammatory cytokines probed in this study were detected in the ipsilateral C7 spinal cord of all groups at 1 h to varying degrees (Fig. 1). *Sham* procedures produced only slight increases over normal levels in all three cytokines in the ipsilateral spinal cord at 1 h, and none of these changes was significant (Fig. 1). In addition, for the *chr* exposure control group there were slight increases in each of the three cytokines relative to normal and *sham*, but these were also not significant for any protein probed (Fig. 1). At 1 h, TNFα was increased in the ipsilateral C7 spinal cord following both *10gf* and *10gf* + *chr*; these increases were significant over normal, *sham* and *chr* for both types of injuries ($p < 0.029$) and were also not different from each other (Fig. 1a). Similarly, IL-1β was also increased at 1 h following both *10gf* and *10gf* + *chr*, but this increase was only significant over normal ($p < 0.003$) (Fig. 1b). Further, IL-1α spinal levels at 1 h following *10gf* and *10gf* + *chr* followed the same trend, with a significant increase for *10gf* over normal, *sham* and *chr* ($p < 0.048$), and for *10gf* + *chr* over normal and *sham* ($p < 0.016$) (Fig. 1c). In contrast to the early responses at 1 h, by day 1, all of these cytokines had substantially decreased and returned to *sham* levels. No significant differences from *sham* were noted for any injury at day 1. The only significant increase was for IL-1β following *10gf*, which was significantly elevated over normal at day 1 ($p = 0.038$) (Fig. 1b).

Fluorescent immunohistochemistry demonstrated the presence of IL-1α in the ipsilateral dorsal horn at 1 h following both of the *10gf* and *10gf* + *chr* injuries (Fig. 2). Tissue from the *sham* procedures did not display any elevation in IL-1α expression above normal. IL-1α co-localized only with GFAP after *10gf* and *10gf* + *chr*, but there was no co-localization of IL-1α with either Iba1 or NeuN after *10gf* (Fig. 2).

Cytokine Antagonism Study

Mechanical Allodynia

Mechanical allodynia was similar in trends for testing with both the 2 and 4 g von Frey filaments. As such, only the 2 g testing results are presented in the figures, while results from both filaments are described here. Treatment with sTNFR1 reduced ipsilateral mechanical allodynia compared to vehicle treatment for both of the injury models (*10gf*, *10gf* + *chr*) (Fig. 3). Significant differences in mechanical allodynia were detected between injury type (*10gf*, *10gf* + *chr*, *sham*) and treatment (vehicle, sTNFR1), for testing with both filaments ($p < 0.001$). Further, for the 2 g filament, but not the 4 g filament, the effect of treatment on mechanical allodynia was significantly dependent on the type of injury imposed ($p = 0.017$).

At day 1 following *10gf*, sTNFR1 treatment significantly reduced ipsilateral allodynia compared to vehicle treatment, for testing with the 2 g filament ($p < 0.002$) (Fig. 3). By day 3 after *10gf* and sTNFR1 treatment, allodynia was significantly lower than for vehicle treatment, for testing with both filaments ($p < 0.016$). This trend continued over time. At day 5 after *10gf* and sTNFR1, allodynia was significantly lower than vehicle treatment for testing with the 4 g filament (data not shown) and by day 7 allodynia after sTNFR1 was significantly lower than vehicle for testing with both filaments ($p < 0.001$) (Fig. 3). However, despite being reduced compared to vehicle treatment, ipsilateral allodynia following *10gf* and sTNFR1 treatment was still significantly elevated over *sham* for all time points for testing with both filaments ($p < 0.021$) (Fig. 3). For the *10gf* + *chr* injury model, in which compression was supplemented with chromic gut suture exposure, sTNFR1 treatment significantly reduced allodynia at day 1 compared to the corresponding model with vehicle treatment, for testing with both filaments ($p < 0.027$) (Fig. 3). Yet, on day 3 after *10gf* + *chr*, ipsilateral allodynia after sTNFR1 treatment was not significantly different than vehicle treatment for testing with either filament, and on day 5, sTNFR1 reduced allodynia for testing with the 2 g filament only (Fig. 3) ($p = 0.031$). On day 7 after *10gf* + *chr* and sTNFR1 treatment, ipsilateral allodynia was significantly reduced compared to vehicle treatment for testing with both filaments ($p < 0.005$) (Fig. 3). However, although treatment with sTNFR1 reduced allodynia, ipsilateral allodynia was significantly different from *sham* responses at all days after sTNFR1 treatment following *10gf* + *chr* for testing with both filaments ($p < 0.044$).

There was no effect of treatment over time for contralateral allodynia. No significant differences in contralateral allodynia were measured between treatment and vehicle after the *10gf* injury at any time point probed (data not shown). Contralateral allodynia after *10gf* and vehicle treatment was significantly elevated over *sham* at day 3 for testing with the 2 g filament and at day 7 for testing with both filaments ($p < 0.044$).

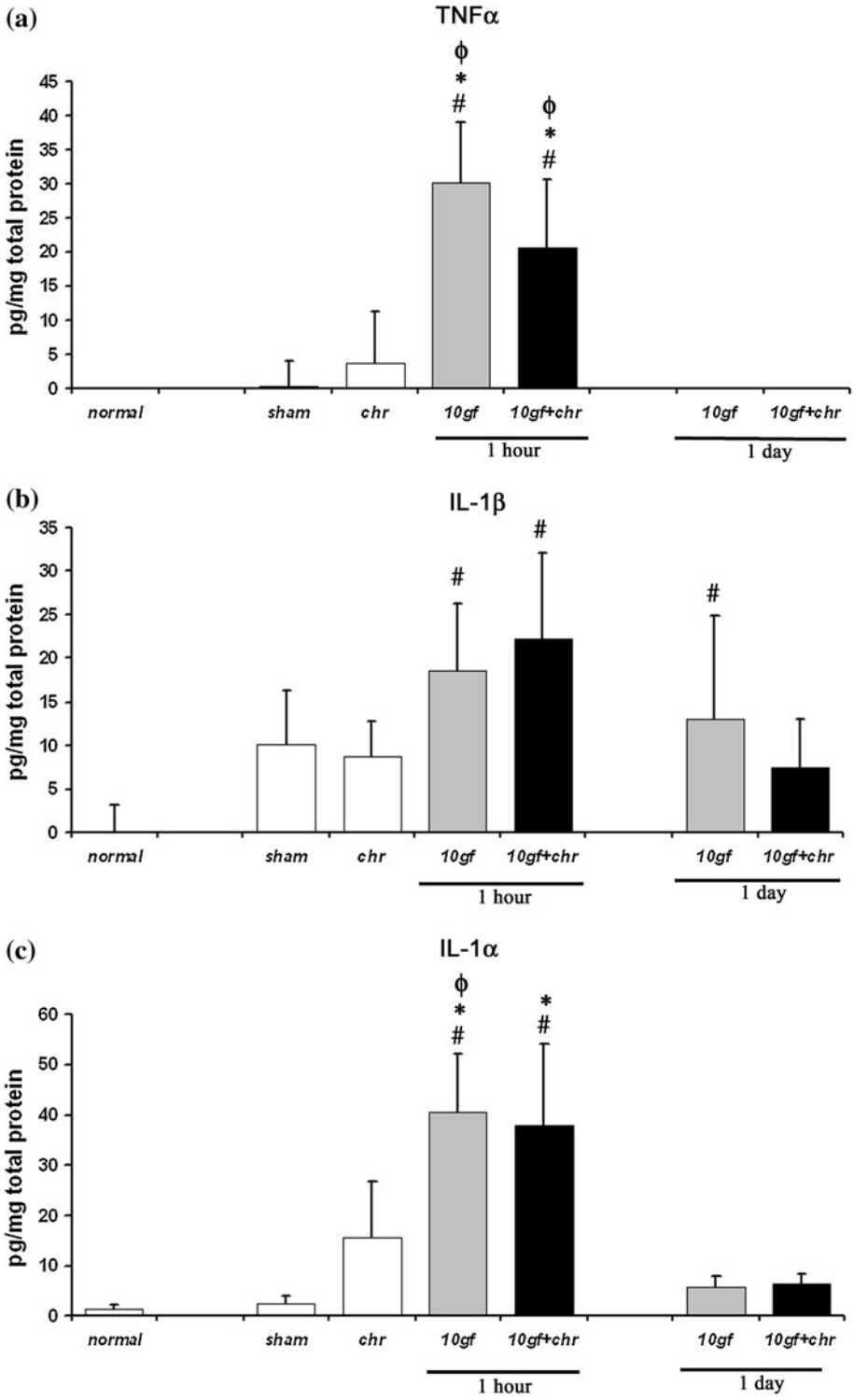

FIGURE 1. Quantification of TNFα (a), IL-1β (b), and IL-1α (c) protein in the ipsilateral C7 spinal cord at 1 h and 1 day following *sham*, *chr*, *10gf*, and *10gf + chr*, as well as normal naive levels. At 1 h after *10gf* and *10gf + chr*, all three cytokines were significantly increased over normal ($p < 0.016$). (a) TNFα, was also increased significantly over *sham* and *chr* for both *10gf* and *10gf + chr* ($p < 0.029$) and returned to *sham* levels for both injuries at day 1. (b) IL-1β remained significantly elevated compared to normal at 1 day after *10gf* ($p = 0.038$). (c) IL-1α protein levels were increased over normal, *sham* and *chr* at 1 h following *10gf* ($p < 0.048$) and elevated over normal and *sham* at 1 h after *10gf + chr* ($p < 0.002$). By day 1, IL-1α had returned to *sham* levels. Pound sign (#) indicates a significant increase over normal. Asterisk (*) indicates a significant increase over *sham* and phi (ϕ) indicates a significant increase over *chr*.

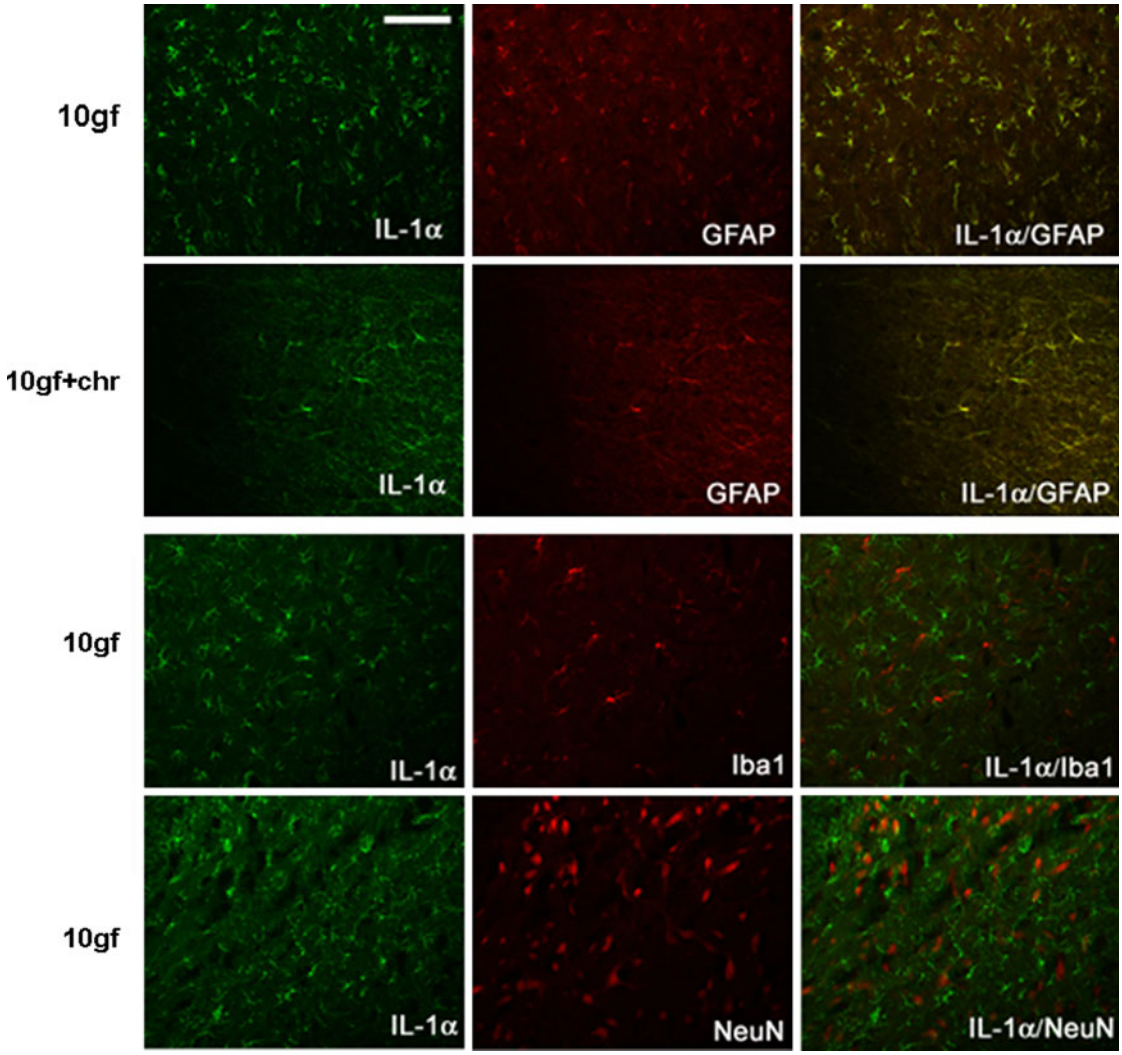

FIGURE 2. Representative images demonstrating co-labeling of IL-1α with GFAP at 1 h after *10gf* and *10gf* + *chr*, and lack of co-localization of IL-1α with Iba1 and NeuN for *10gf*. Scale bar is 200 μm and applies to all images.

For the combined injury (*10gf* + *chr*), treatment with sTNFR1 was unable to reduce contralateral allodynia compared to vehicle controls for any timepoint. Further, contralateral allodynia after *10gf* + *chr* and sTNFR1 treatment was significantly elevated over *sham* at day 1 for testing with the 4 g filament and at days 3 and 7 for testing with both filaments ($p < 0.043$) (data not shown).

Although IL-1ra treatment reduced allodynia compared to vehicle, this reduction did not follow the same trends as treatment with sTNFR1 (Figs. 3 and 4). Significant differences in mechanical allodynia were detected between injury type (*10gf*, *10gf* + *chr*, *sham*) and treatment (vehicle, IL-1ra) for testing with both filaments ($p < 0.029$). IL-1ra treatment did not significantly reduce ipsilateral allodynia at days 1, 3, and 5 following *10gf*, compared to vehicle treatment (Fig. 4), although the reduction in ipsilateral allodynia was significant at day 7 after that compression injury, for testing with both filaments ($p < 0.044$) (Fig. 4). Further ipsilateral allodynia after *10gf* and IL-1ra treatment was significantly different ($p < 0.001$) from *sham* at all time points for testing with both filaments ($p < 0.003$) (Fig. 4). In contrast, a reduction in ipsilateral allodynia was observed for IL-1ra treatment compared to vehicle treatment in the *10gf* + *chr* model (Fig. 4). Ipsilateral allodynia after *10gf* + *chr* was significantly reduced after IL-1ra treatment compared to vehicle controls for all days probed for testing with the 2 g filament, and for days 1, 3, and 7 for testing with the 4 g filament ($p < 0.034$). However, as observed with the *10gf* model, ipsilateral allodynia following *10gf* + *chr* and IL-1ra treatment was also significantly different from *sham* for all time points

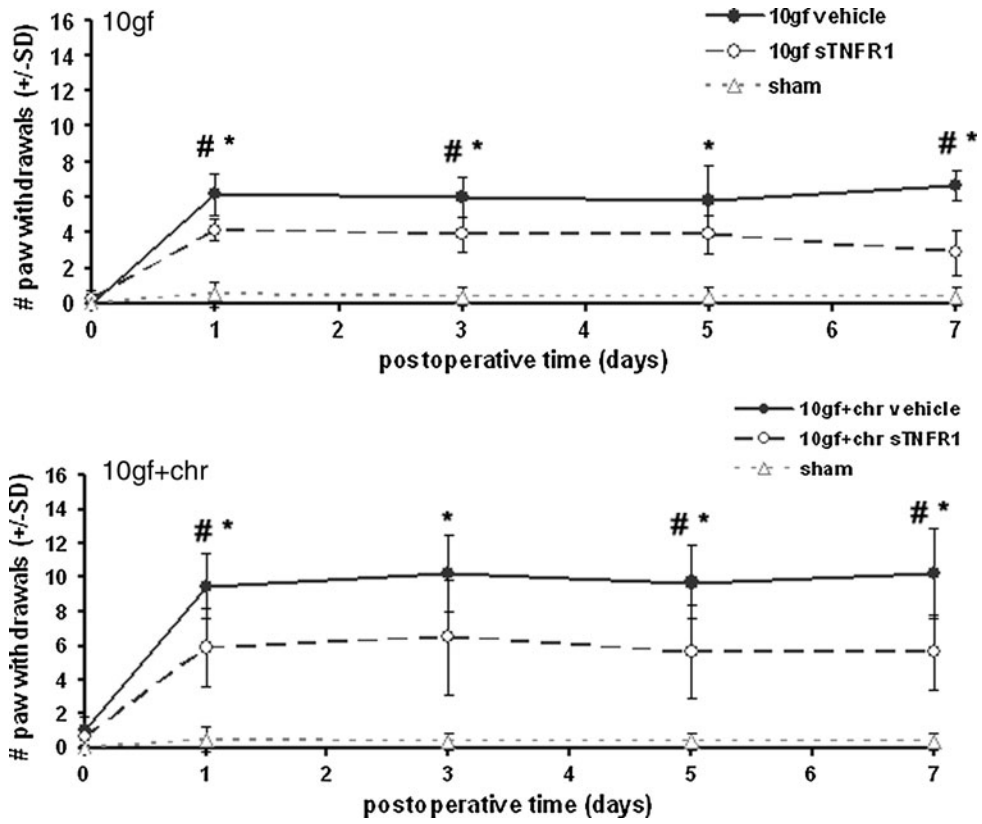

FIGURE 3. Average mechanical allodynia in the ipsilateral forepaw following sTNFR1 treatment for *sham*, *10gf*, and *10gf + chr*, for testing with the 2 g von Frey filament. Treatment with sTNFR1 significantly reduced allodynia compared to vehicle controls at days 1, 3, and 7 after *10gf* and at days 1, 5, and 7 after *10gf + chr* ($p < 0.031$). Trends for testing with the 4 g filament were similar and are described in the Results section. Pound sign (#) indicates differences between treatment and corresponding vehicle groups. Asterisk (*) indicates significant differences between each respective treated group and *sham*.

probed for testing with both filaments ($p < 0.021$) (Fig. 4).

There was no effect of treatment over time for contralateral allodynia following treatment with IL-1ra. No significant differences in contralateral allodynia were measured between treatment and vehicle after the *10gf* injury at any timepoint probed (data not shown). In addition, no significant differences in contralateral allodynia were measured between treatment and vehicle after the *10gf + chr* injury at any timepoint probed (data not shown). After *10gf + chr* and either IL-1ra or vehicle administration, contralateral allodynia was significantly elevated over *sham* at days 5 and 7 for testing with the 2 g filament, and at day 7 for testing with the 4 g filament ($p < 0.043$).

Spinal GFAP Reactivity

Both vehicle treated groups displayed dorsal GFAP reactivity that was significantly elevated over both normal and *sham* ($p < 0.001$) (Figs. 5 and 6). In the *10gf* compression model, treatment with sTNFR1 significantly reduced GFAP reactivity in the ipsilateral dorsal horn compared to reactivity observed in that model with vehicle treatment ($p < 0.001$) (Figs. 5 and 6). In contrast, in the model of combined injury (*10gf + chr*), treatment with sTNFR1 did not reduce GFAP reactivity in the ipsilateral dorsal horn relative to vehicle treatment. In fact, following *10gf + chr* and sTNFR1, GFAP reactivity was significantly elevated over normal and *sham* ($p < 0.016$) (Fig. 6).

Treatment with IL-1ra was similarly effective in reducing ipsilateral GFAP expression after *10gf*. Following *10gf*, treatment with IL-1ra significantly reduced GFAP reactivity in the ipsilateral dorsal horn compared to the vehicle group for that model ($p = 0.002$) (Figs. 5 and 6). Dorsal horn GFAP reactivity was also significantly decreased following IL-1ra treatment compared to vehicle in the *10gf + chr* model ($p = 0.002$) (Fig. 6). Yet, after *10gf + chr* and IL-1ra, GFAP reactivity also remained significantly elevated over *sham* ($p = 0.022$). GFAP reactivity in the contralateral dorsal horn was unchanged by either of the treatments in both types of injuries (data not shown).

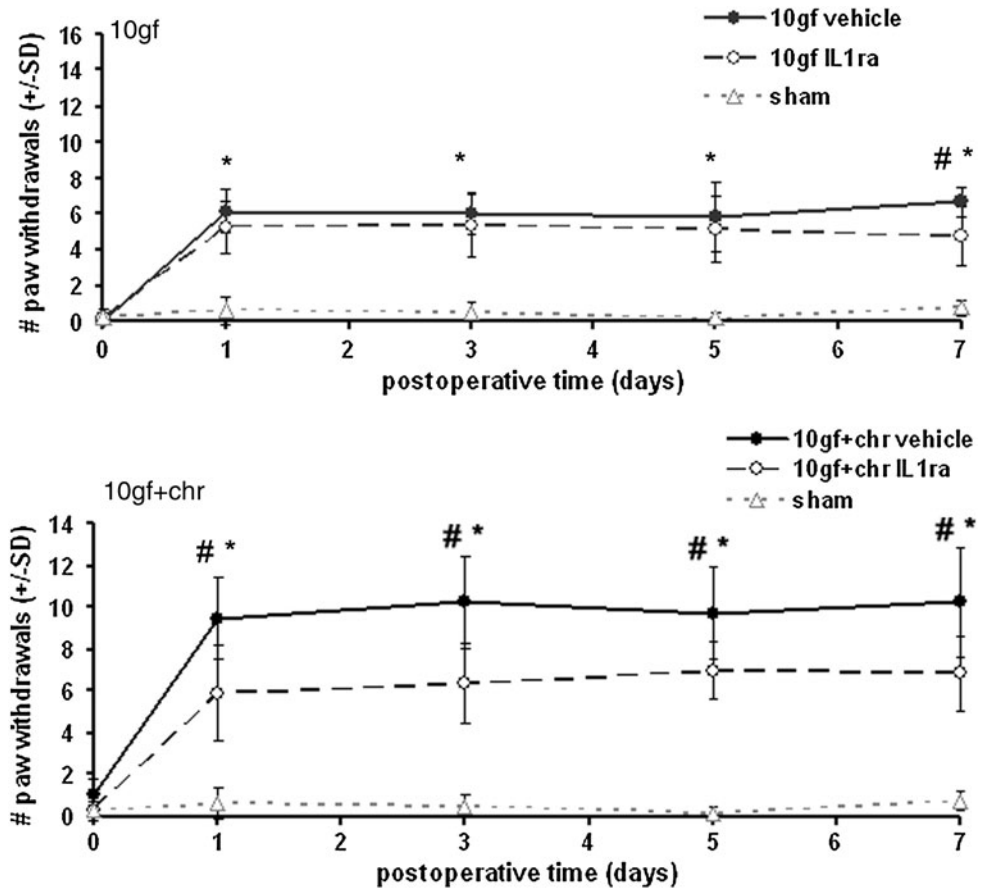

FIGURE 4. Average mechanical allodynia in the ipsilateral forepaw following IL-1ra treatment, for *sham*, *10gf*, and *10gf + chr*, for testing with the 2 g von Frey filament. After *10gf*, administration of IL-1ra significantly reduced allodynia only at day 7; whereas IL-1ra treatment did significantly reduce allodynia at all time points after *10gf + chr* ($p < 0.044$). Trends for testing with the 4 g filament were similar and are described in the Results section. Pound sign (#) indicates differences between treatment and corresponding vehicle groups; asterisk (*) indicates significant differences between each respective treated group and *sham*.

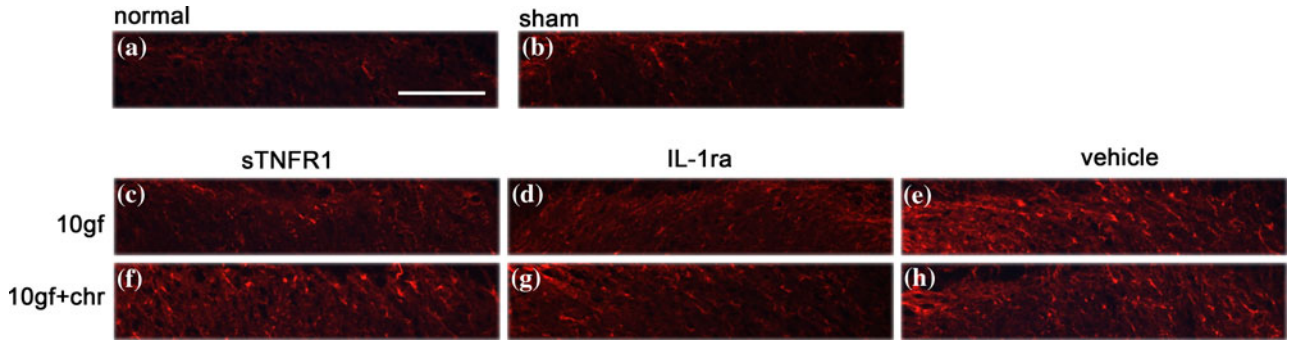

FIGURE 5. Representative ipsilateral C7 dorsal horn sections at day 7 stained against GFAP. Normal (a) and *sham* (b) are shown for comparison. GFAP reactivity after sTNFR1 (c, f), IL-1ra (d, g), or vehicle (e, h) treatment, following either *10gf* (c–e) or *10gf + chr* (f–h) injury are also shown. The scale bar in (a) is 100 μm and applies to all panels.

DISCUSSION

The pro-inflammatory cytokines probed in this study, TNFα, IL-1β, and IL-1α, were significantly increased early (1 h) in the cervical spinal cord following painful cervical radiculopathy (Figs. 1 and 2), and pharmacologic treatment blocking the signaling of these cytokines significantly decreased both mechanical allodynia and GFAP reactivity in the spinal cord (Figs. 3–6; Table 1). Although pharmacologic block of either TNFα or IL-1 signaling reduced mechanical

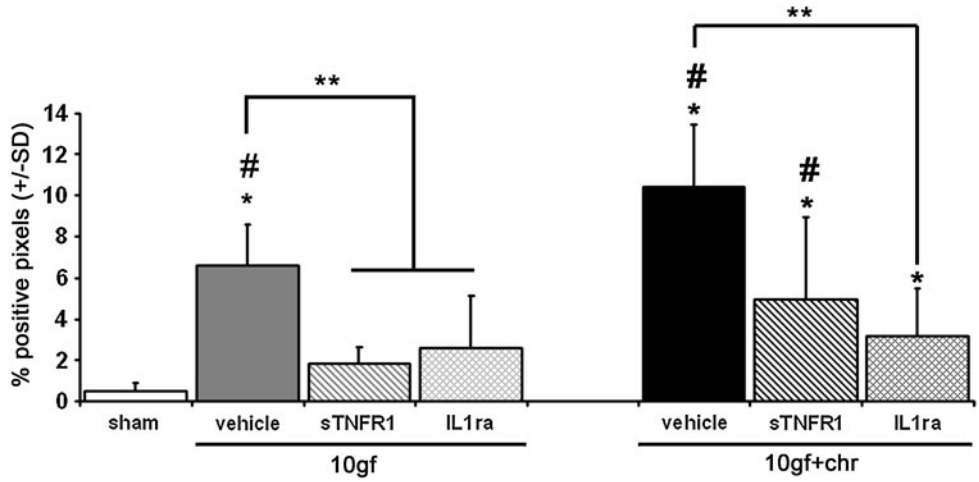

FIGURE 6. Automated densitometry results quantifying the percentage of pixels identified as positive for GFAP staining in the ipsilateral dorsal horn of the spinal cord following either sTNFR1, IL-1ra, or vehicle treatment. The two injury types (*10gf*, *10gf + chr*) are separated and the corresponding vehicle groups are also shown, as well as *sham* levels. Pound sign (#) indicates a significant increase over normal. Asterisk (*) indicates significant increases over *sham* and the double asterisks (**) indicates significant differences between groups.

TABLE 1. Comparison of differences in cytokines & pharmacology responses in the two injury models.

	Compression insult	Combined compression & chemical insult
Cytokine expression		
mRNA		
DRG[a]	TNFα, IL-1β, and IL-6 $\gg$ normal at 1 h	TNFα and IL-1β $\gg$ normal at 1 h TNFα $\gg$ *sham* at 1 h
Spinal cord[a]	TNFα, IL-1β, and IL-6 $\gg$ normal at 1 h IL-1β and IL-6 $\gg$ *sham* at 1 h	TNFα and IL-1β $\gg$ over normal at 1 h IL-1β $\gg$ over sham at 1 h
Protein		
DRG[b]	IL-1β $\gg$ normal at 1 h IL-1β, IL-6, TNFα, MIP3 not elevated at 1 day	IL-1β, IL-6 $\gg$ normal at 1 h IL-1β, IL-6, TNFα, MIP3 are all elevated at 1 day
Spinal cord	TNFα, IL-1β, and IL-1α $\gg$ normal at 1 h TNFα, IL-1β, and IL-1α $\gg$ *sham* at 1 h Only IL-1β $\gg$ normal at 1 day	TNFα, IL-1β, and IL-1α $\gg$ normal at 1 h TNFα, IL-1β, and IL-1α $\gg$ *sham* at 1 h No cytokines elevated at 1 day
Cytokine pharmacology		
sTNFR1	Significant reduction in allodynia at day 1 Allodynia remains reduced up to day 7 Allodynia NOT abolished	Significant reduction in allodynia at day 1 Allodynia remains reduced up to day 7 Allodynia NOT abolished
IL-1ra	No reduction in allodynia compared to vehicle	Significant reduction in allodynia at day 1 Allodynia remains reduced up to day 7 Allodynia NOT abolished
Spinal GFAP	sTNFR1 $\ll$ GFAP compared to vehicle at day 7 IL-1ra $\ll$ GFAP compared to vehicle at day 7	sTNFR1 NOT alter GFAP at day 7 IL-1ra $\ll$ GFAP compared to vehicle at day 7

[a]From Rothman *et al.*[39]
[b]From Rothman *et al.*[40]

allodynia compared to vehicle treatment after a combined injury modeling a disc herniation (*10gf + chr*), only sTNFR1 reduced allodynia after the compression alone (*10gf*). This suggests that IL-1 may selectively mediate the behavioral hypersensitivity that develops after an insult with chemical mediators (Figs. 2–4). Although there is a decrease in cytokines in the spinal cord at 1 day after injury (Fig. 1), previous work with these models has shown that a subset of these cytokines is upregulated in the DRG at 1 day after injury, which could indicate that variable inflammatory responses in the DRG between the two injury types may be responsible for the difference in outcomes.[40] GFAP reactivity in the ipsilateral spinal cord was also significantly reduced after either sTNFR1 and IL-1ra treatment (Figs. 5 and 6), suggesting a direct link between these cytokines and astrocytic reactivity in these painful injuries that was also observed via

co-labeling studies at 1 h (Fig. 2). However, reductions in spinal GFAP reactivity after cytokine antagonist administration depended on the injury type, suggesting that the relationship between pro-inflammatory cytokines and spinal astrocytic reactivity also depend on the type of injury imposed (Table 1).

This study shows significant increases in spinal IL-1α after nerve root injury (Fig. 1), implying a role for this cytokine in painful radiculopathy. Both IL-1α and IL-1β bind to the same receptors and share biologic activities[9]; yet, intrathecal injection of IL-1β, but not IL-1α, induces hyperalgesia in a naïve rat, suggesting that these cytokines may not have the same spinal effects.[23,27] IL-1α is mainly membrane-bound,[8,43] but can exert activity on neighboring cells and is capable of inducing the production and release of other cytokines, particularly when acting synergistically with TNFα.[8,35] In the current study, IL-1ra, which blocks the effect of both IL-1α and IL-1β, reduced mechanical allodynia compared to vehicle treatment (Figs. 3 and 4).

Administration of sTNFR1 was equally effective in reducing allodynia compared to vehicle for both types of painful the compression injuries (*10gf*, *10gf* + chr) (Fig. 3). This similarity in effectiveness could be due to the comparable expression of TNFα that was detected in the spinal cord for both of those injuries. Administration of sTNFR1 via lumbar puncture targets the spinal cord but could have also contacted the DRG. Treatment with sTNFR1 likely prevents TNFα from binding to its receptor, which is widely expressed on the surface of neurons, microglia, and astrocytes in the spinal cord.[1,32] When TNFα binds to its receptor on the surface of neurons, it can increase excitatory synaptic transmission, and an increase in synaptic transmission in the dorsal horn is hypothesized to be responsible for the development of persistent pain.[20,21,55] Therefore, a pharmacologic block that prevents TNFα from binding to its receptor could reduce the increase in excitatory neurotransmission that is induced in the dorsal horn after nerve root compression, and thus reduce behavioral hypersensitivity. In fact, sTNFR1 reduced mRNA levels for TNFα,[39] implying that decreased production of TNFα, which could also potentially lower excitatory transmission in the dorsal horn and attenuate pain.

IL-1ra significantly reduced ipsilateral allodynia after the combined mechanical and chemical injury (*10gf* + chr) compared to vehicle treatment, but that same IL-1ra treatment was ineffective in significantly reducing allodynia in the model of mechanical injury alone (*10gf*) (Fig. 4). The current study did not probe cytokine responses in the DRG; however, IL-1β protein in the DRG increases more at day 1 after the combined injury than for compression alone (Table 1).[40] The dosing paradigm used in the current study did not specifically target either the DRG or spinal cord; IL-1ra was delivered intrathecally via lumbar puncture that could allow IL-1ra to bind to cells in the DRG that contain the IL-1 receptor. In fact, since DRG neurons express the IL-1 receptor,[3] this is a likely possibility. Further, application of IL-1β increases the discharge rate of DRG neurons *in vitro* and induces behavioral hypersensitivity *in vivo*, further implicating the IL-1 receptors on the surface of DRG neurons as mediating electrophysiological and behavioral changes.[34,58] IL-1β does not increase in the DRG at day 1 after a mechanical injury alone[40]; therefore, the finding that administration of IL-1ra after a mechanical injury alone (*10gf*) was less effective in reducing ipsilateral mechanical allodynia is reasonable, since a blockade of IL-1 receptors will have little effect if there is no IL-1β present.

Although either antagonist reduced allodynia compared to controls, allodynia still remained elevated over *sham* after both types of compression injuries (Figs. 3 and 4). Both TNFα and IL-1β regulate synaptic activity in the dorsal horn; however, the mechanisms are not necessarily distinct from each other. TNFα induces an increase in excitatory neurotransmission, whereas IL-1β suppresses inhibitory neurotransmission.[21] These two effects may be hard to distinguish without electrophysiological data. In fact, the overlapping roles of TNFα and IL-1β could explain why an increase in the effectiveness of reducing mechanical allodynia has been reported when sTNFR1 and IL-1ra were combined for nerve transection.[49] It is possible that complete abolishment of allodynia may require blocking both the increase in excitatory transmission mediated by TNFα and the decrease in inhibitory transmission mediated by IL-1β.

Contralateral allodynia was significantly reduced after administration of either sTNFR1 or IL-1ra only in the combined nerve root injury. In contrast, contralateral allodynia was not significantly different between treatment and vehicle after compression alone. However, this apparent discrepancy in effectiveness between nerve root compression with (*10gf* + *chr*) and without (*10gf*) a chemical insult may be due to the disparity in the contralateral allodynia responses between these two types of injuries.[41] The mechanism inducing contralateral sensitivity after the combined injury is unclear; however, it is hypothesized that this phenomenon may be due, at least in part, to the diffusion of cytokines to the contralateral side of the spinal cord or to plasticity of the inter-neurons that form the commissural connections with the contralateral side of the spinal cord.[46] Contralateral behavioral hypersensitivity does not appear until day 3 after the combined injury (*10gf* + *chr*)[41] and pharmacologic blocks in the current study were given only at 6 h and

1 day after injury. It is possible that neither antagonist had direct influence on the contralateral spinal neurons. It is more likely that the reduction in contralateral allodynia observed for the combined injury may be due to the same mechanisms that reduce ipsilateral hypersensitivity. Either pharmacologic block did reduce ipsilateral allodynia, implying that sTNFR1 and/or IL-1ra may also attenuate the increase in excitatory synaptic transmission that is measured in the dorsal horn after nerve injury. It is possible that in the absence of any increase in excitatory transmission on the ipsilateral spinal cord, the inter-neurons projecting to the contralateral side remain desensitized as well. The lack of onset of contralateral hypersensitivity observed following administration of either sTNFR1 or IL-1ra after the combined mechanical and chemical (*10gf* + *chr*) injury suggests that early and transient administration of these antagonists is sufficient to induce downstream effects that can cause pain, such as the production of additional inflammatory cytokines and the sensitization of interneurons that project to the contralateral side of the spinal cord.

Although application of cytokine antagonists was completed by day 1, the decreases in mechanical allodynia were sustained until day 7 (Figs. 2 and 3). Day 7 is also a time point at which spinal ipsilateral GFAP reactivity decreased with antagonists (Figs. 5 and 6). Pro-inflammatory cytokines can induce reactivity in both microglia and astrocytes.[1,44,45,50] Microglia produce IL-1β and TNFα in response to stimulation by IL-1β *in vitro*.[23] Data from our lab show a reduction in TNFα mRNA in the DRG at day 1 after administration of sTNFR1.[39] It is hypothesized that in the current study the administration of the cytokine antagonists only twice and soon after injury had long-lasting effects on allodynia because antagonism of TNFα or IL-1β inhibits the further production of other pro-inflammatory cytokines.

Production and release of inflammatory cytokines can directly and indirectly affect glial reactivity. GFAP reactivity in the ipsilateral dorsal horn decreased at day 7 after either nerve root compression when either treatment was administered (Figs. 5 and 6). Although little is known about the specific triggers of spinal glial reactivity in models of pain or neural trauma, cytokines directly induce certain aspects of glial reactivity. Indeed, TNFα increases excitatory neurotransmission in the dorsal horn, which implies that it can also increase the release of glutamate.[21] A pharmacologic block of TNFα may reduce the release of glutamate in the spinal cord that occurs after painful nerve root injury, and since astrocytes regulate glutamate levels at neuronal synapses[7,29] they may react by upregulating GFAP. Treatment with either cytokine intervention decreased spinal GFAP reactivity compared to vehicle after a compression alone (Fig. 6). Yet, sTNFR1 did not reduce GFAP expression in the ipsilateral dorsal horn after the combined nerve root injury (Fig. 6). Interestingly, contralateral GFAP responses were not modified by either treatment approach in either injury. Of note, the current study did not probe for microglial reactivity in the spinal cord after administration of the cytokine antagonists. It is possible that after the *10gf* + *chr* injury, the actions of reactive microglia, such as production and release of nitric oxide and phagocytosis, induce astrocytic reactivity, and that the application of sTNFR1 may be ineffective in reducing these aspects of microglial reactivity.[37] A study of nerve transection showed no effect of either sTNFR1 or IL-1ra or a combination of the two treatments on microglial reactivity in the spinal cord.[49] Although microglia contain cytokine receptors and respond to pro-inflammatory cytokines, those cells are sensitive to a wide range of insults and a pharmacologic block of pro-inflammatory cytokines may not necessarily modulate other signaling molecules that induce reactivity in microglia.

Further studies of other markers of glial reactivity at earlier time points in these models are necessary to fully define relationships between cytokines and glial reactivity since the effects of sTNFR1 and IL-1ra may be manifested earlier than 7 days. Since previous work demonstrates a decrease in TNFα mRNA in the DRG at day 1 after *10gf* + *chr* and sTNFR1 treatment,[39] further investigations of cytokine upregulation and release after the administration of antagonists are necessary. A dose–response curve was not performed for this study, but dosing was chosen based on studies of low back pain[49] and the dosing paradigm was chosen to begin after the transient increase in spinal cytokines (Fig. 1). It should be noted that there were 18 h between the first and second doses; since the half-life of sTNFR1 is between 20 and 70 h and the half-life of IL-1ra is 4–6 h, biologically active levels of sTNFR1 were likely maintained between dosing, whereas biologically active levels of IL-1ra may not have been.[12,22,25] This discrepancy hampers direct comparison of the efficacy of the two antagonists.

Results presented here begin to define relationships between inflammatory cytokines, spinal astrocytic reactivity, and behavioral outcomes in two painful nerve root injuries (Table 1). Spinal cytokine levels were similar for the injuries; however, pharmacologic block of IL-1 reduced allodynia after the combined injury, and not after a compression alone, suggesting that IL-1 may only mediate behavioral hypersensitivity after injuries with chemical components. Further, although two different types of blockades of cytokine signaling decreased spinal GFAP reactivity, the extent of this response depended on the type of the treatment

and the injury imposed. Further data on how spinal inflammation is affected by pharmacologic block of early cytokine signaling in the spinal cord will help develop strategies for treating chronic pain.

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