Tumor Necrosis Factor–α Contributes to Below-Level Neuropathic Pain after Spinal Cord Injury

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Objective: Our objective was to elucidate the mechanisms responsible for below-level pain after partial spinal cord injury (SCI).
Methods: We used lateral hemisection to model central neuropathic pain and herpes simplex viral (HSV) vector–mediated transfer of the cleaved soluble receptor for tumor necrosis factor–α (TNF-α) to evaluate the role of TNF-α in the pathogenesis of below-level pain.
Results: We found activation of microglia and increased expression of TNF-α below the level of the lesion in the lumbar spinal cord after T13 lateral hemisection that correlated with emergence of mechanical allodynia in the hind limbs of rats. Lumbar TNF-α had an apparent molecular weight of 27kDa, consistent with the full-length transmembrane form of the protein (mTNF-α). Expression of the p55 TNF soluble receptor (sTNFRs) by HSV-mediated gene transfer resulted in reduced pain behavior and a decreased number of ED1-positive cells, as well as decreased phosphorylation of the p38 MAP kinase (p-p38) and diminished expression of mTNF-α in the dorsal horn.
Interpretation: These results suggest that expression of mTNF-α after injury is related to development of pain, and that reverse signaling through mTNF-α by sTNFR at that level reduces cellular markers of inflammatory response and pain-related behavior.

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Pain below the level of spinal cord injury (SCI) represents a clinically important form of central neuropathic pain that is particularly difficult to treat effectively.[1] In rodent models of peripheral neuropathic pain caused by chronic constriction injury,[2] spinal nerve ligation,[3,4] inflammatory nerve lesion,[5] or axotomy,[2] nerve injury is accompanied by activation of microglia in the ipsilateral dorsal horn of the spinal cord. The role of microglial activation in central neuropathic pain after SCI has not been established.

Tumor necrosis factor (TNF)–α, a prototypical proinflammatory cytokine, plays a central role in initiating inflammatory reactions of the innate immune system through induction of the expression and release of other cytokines,[6] among other actions. TNF-α is synthesized as a type II transmembrane protein (mTNF-α) that is cleaved by the TNF-converting enzyme, a member of the disintegrin and metalloproteinase family, to release the 17kDa soluble cytokine sTNF-α.[7] Both mTNF-α and sTNF-α are bioactive although their effects are distinct. sTNF-α is a diffusible peptide with a half-life of approximately 30 minutes,[8] and its activity is neutralized in part by binding to a cleaved soluble form of the TNF receptor (p55 or p75 sTNFR). mTNF-α mediates bidirectional cell–cell interactions through binding to the membrane receptor complex (p55 or P75 TNFR) on neighboring cells; binding of p55 or p75 sTNFR to mTNFR may prevent such interactions and/or activate intracellular signaling pathways in the mTNF-α–expressing cells.[9]

TNF-α appears to play an important role in the development of chronic peripheral neuropathic pain. After nerve crush, the amount of TNF-α detected by immunocytochemistry increases in astrocytes and microglia in the dorsal horn.[10] In the chronic constriction injury model of peripheral neuropathic pain, administration of neutralizing antibodies against TNF-α or the p55 TNFR reduces thermal hyperalgesia and mechanical allodynia.[11] In the spinal nerve ligation model of peripheral neuropathic pain, intrathecal ad-

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ministration recombinant p75 sTNFR (etanercept) before nerve injury reduces mechanical allodynia.[12,13] In this study, we investigated the role of lumbar TNF-α in the development of pain below the level of thoracic spinal hemisection, using herpes simplex virus (HSV) vector–mediated release of the soluble cleaved product of p55 TNFR to modulate mTNFα effects.

Materials and Methods

Vectors

Construction of p55 sTNFR-expressing HSV vector, T0TNFSR, has been described previously.[14] This vector contains the coding sequence for amino acids 1 to 211 of the human p55 sTNFR under regulatory control of the HSV ICP0 immediate early promoter in the UL41 locus of an HSV recombinant defective for HSV genes ICP4, ICP22, and ICP27. Control vector T0Z.1 is defective in the same genes but contains the *Escherichia coli lacZ* coding sequence under the control of ICP0 promoter in the UL41 locus.

Experimental Animals and Surgical Procedures

Female Sprague-Dawley rats weighing 200 to 250gm were used in all experiments. Housing conditions and experimental procedures were approved by the University of Michigan Committee on Use and Care of Animals.

Isoflurane gas was used to anesthetize each rat, and then the T11-T12 spinal laminae were located by palpating the last rib. A longitudinal incision was made exposing several segments, and a laminectomy was performed at T11-T12. The lumbar enlargement was identified with accompanying dorsal vessels, and the spinal cord was hemisected at T13 using a scalpel blade. Extra care was taken not to damage the major dorsal vessels or vascular branches. A tuberculin syringe with a 28-gauge needle was placed dorsoventrally at the midline of the cord and pulled laterally to ensure that hemisection was complete. Muscle and fascia were sutured closed and the skin closed with autoclips. After surgery, all animals were maintained under preoperative conditions and were eating and drinking within 3 hours after surgery. Locomotor function was examined and recorded using the BBB Locomotor Rating Scale[15] to ensure that motor recovery of the limb ipsilateral to the hemisection was sufficient to allow somatosensory behavioral testing. Animals meeting behavioral criteria then were inoculated in the plantar surface of both hind paws with 30μl of vector containing 1.5×10^7 plaque-forming units of T0TNFSR or T0Z.1.

Behavioral Analyses

Behavioral testing for mechanical allodynia was performed during the day portion of the circadian cycle (8:00 AM to 4:00 PM). Mechanical allodynia was assessed by measuring the threshold of paw-withdrawal response to mechanical stimuli using an electronic pressure algometer (Somedic Sales, Horby, Sweden).[16] Rats were placed inside acrylic cages turned upside down on top of a wire grid allowing access to the paws and were left to acclimate for a period of at least 30 minutes, after which the probe of the electronic pressure algometer was applied to the midplantar surface of the left and right hind paws. A brisk paw withdrawal after probe application was regarded as a positive response, and the force was recorded automatically by the device.

Cell Culture

Dorsal root ganglia (DRG) from 17-day-old rat embryos were treated with 0.25% trypsin-EDTA (Gibco-BRL, Carlsbad, CA) for 45 minutes at 37°C, dissociated and plated on poly-D-lysine–coated coverslips at 1.5×10^5 cells per well in a 24-well plate with 500μl of defined Neurobasal Media containing B27, Glutamax I, Albumax I, and penicillin/streptomycin according to manufacturer's recommendation (Gibco-BRL) and supplemented with 100ng/ml of 7.0S nerve growth factor (Sigma-Aldrich, St. Louis, MO). After 17 days in culture, the cells were transfected with either T0TNFSR or T0Z.1 at a multiplicity of infection (MOI) of 1 for 2 hours. Fresh medium was replaced and collected 48 hours later for determination of sTNFR by enzyme-linked immunosorbent assay (ELISA) and Western blot. DRG neurons were also examined for expression of sTNFR protein by immunocytochemistry and Western blot.

Enzyme-Linked Immunosorbent Assay

The amount of p55 sTNFR released from the transduced cells was determined using a commercial p55 sTNFR ELISA kit (TNFR1; R&D Systems, Minneapolis, MN).

Western Blot

The dorsal quadrant was dissected from L4-L6 spinal cord and homogenized in lysis buffer containing 50mM Tris, 10mM NaCl, 1% NP40, 0.02% NaAzide, and protease inhibitor cocktail (Sigma-Aldrich) at pH 7.4. Cultured DRG cells were collected in similar lysis buffer after being dislodged from culture plates with a cell scraper. Cell lysate and tissue homogenates were sonicated and then centrifuged at 15,000*g* for 10 minutes at 4°C. Medium from DRG cell cultures was centrifuged at 1,000*g* for 10 minutes at 4°C. Ammonium sulfate was added to the supernatant to 35% saturation and centrifuged at 10,000*g* for 15 minutes at 4°C, and the pellet was collected for analysis.

Protein concentration in tissue homogenates and cell lysates was determined using the BCA assay (Pierce Biotechnology, Rockford, IL) and spectrophotometry (AD340; Beckman Coulter, Fullerton, CA).

Aliquots containing 20μg of protein were dissolved in Laemmli buffer and boiled at 95°C for 5 minutes, and the proteins separated by 12% Tris-Glycine SDS-PAGE (BioRad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes. Prestained protein standards (RPN800; Amersham Biosciences, Piscataway, NJ) were used to determine relative molecular weights of proteins. Immunoblots were blocked and incubated with primary antibodies at 4°C. Primary antibodies included an antibody against the p55 sTNFR (anti-TNF R1, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA; directed against amino acids 30–301 of the receptor), anti–TNF-α (1:1000; R&D Systems), and anti-phospho-p38 and anti-p38 (1:500; Cell Signaling Technology, Beverly, MA). Peroxidase-coupled secondary antibodies (Calbiochem, La Jolla, CA) were used for amplification. Protein bands were visualized using X-OMAT AR film (Kodak, Rochester, NY) after chemiluminescence (DuPont NEN,

Boston, MA). The membranes were stripped and reprobed with mouse anti–β-actin (1:5000; Santa Cruz Biotechnology) as a loading control. The intensity of each band was determined by quantitative densitometry using a PC-based image analysis system (ChemiDoc XRS System, BioRad Laboratories).

Immunochemistry

Rats were perfused transcardially with 0.9% NaCl followed by freshly made 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The DRG, L4-L6 segment of spinal cord, and attached roots were removed, postfixed in the same solution for 8 hours, and cryoprotected with 30% sucrose in 0.1M phosphate buffer for 2 days at 4°C. Cryostat sections (15μm) of DRG were thaw-mounted onto cold Superfrost microscope slides (Fisher Scientific, Pittsburgh, PA), blocked with 5% normal goat serum in PBS-T, and incubated with rabbit anti-TNF R1 (1:200; Santa Cruz Biotechnology) overnight at 4°C followed by fluorescent anti-rabbit IgG Alexa Fluor 594 (1:2,000; Molecular Probes, Eugene, OR) for 1 hour at room temperature. Floating sections (35μm) of spinal cord were blocked and incubated overnight at 4°C with rabbit polyclonal anti-phospho p38 (1:200; Cell Signaling Technology), mouse monoclonal anti-OX42 (1:200; Chemicon International, Temecula, CA), mouse anti-ED1 (1:200, Chemicon International), mouse anti-NeuN (1:500; Chemicon International), or mouse anti-GFAP (1:500; Sigma-Aldrich) followed by fluorescent anti–rabbit IgG Alexa Fluor 594 or fluorescent anti–mouse IgG Alexa Fluor 488 (1:2,000; Molecular Probes) for 1 hour at room temperature. All sections were mounted in water-based Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA), and the fluorescent images were captured using a Nikon Eclipse E1000 microscope.

The expression of T0TNFSR in DRG neurons in vitro was determined by immunocytochemistry. Forty-eight hours after transfection, the cells were fixed, blocked, and probed with anti-sTNFR1 (1:500; Santa Cruz Biotechnology) or anti-Tuj1 (1:8000; Covance Research Products, Berkeley, CA) overnight. The secondary antibodies utilized were fluorescent anti-rabbit IgG Alexa Fluor 594 or anti-mouse IgG Alexa Fluor 488 (1:2,000; Molecular Probes).

Data Analysis

Statistical significance of the difference between vector-treated and control animals was determined by one-way analysis of variance with post hoc comparisons where appropriate. Parametric statistics, using the general linear model for repeated measures, were used to identify significant effects of treatment condition on the behavioral measure of neuropathic pain. The results were examined using the software package SPSS 12.0 for Windows (SPSS, Chicago, IL). Data are expressed as mean ± standard error of mean (SEM), with p value less than 0.05 considered significant.

Results

Lateral hemisection at T13 resulted in striking inflammatory changes in the lumbar spinal cord below the level of the cord lesion. The number of ramified microglia in the lumbar spinal cord, detected by OX42 immunoreactivity, was markedly increased compared with control spinal cord (Fig 1A, B), and there was an associated increase in the amount of phosphorylated p38 MAP kinase (p-p38) localized predominantly in the nucleus and cytoplasm of the activated microglia (see Fig 1C, D, F). Neurons stained with NeuN and astrocytes stained with GFAP showed no p-p38 immunoreactivity (data not shown). In addition, infiltrating ED1-positive cells were found in the white matter tracts including the dorsal columns and in the dorsal horn bilaterally in the lumbar cord below the level of the lesion (see Fig 1G, H). Although ED1 may label both macrophages and microglia, these cells had the rounded shape characteristic of macrophages, and the OX42-positive microglia in the gray matter did not colabel with ED1 in these sections.

In the same region of the lumbar cord, there was a substantial increase in immunoreactive TNF-α that persisted 3 days to 2 weeks after T13 hemisection (Fig 2). The immunoreactive peptide had an apparent molecular weight of 27kDa, consistent with the full-length membrane form of TNF-α (mTNF-α). We were unable to detect the presence of soluble cleaved TNF-α (sTNF-α, 17kDa) in a cytosolic fraction prepared from lumbar spinal cord using either ELISA or Western blot at those time points. Our inability to detect sTNF-α was not a technical artefact, because in other studies we were able to detect a transient increase in sTNF-α by ELISA at the site of blunt trauma to the spinal cord (data not shown).

To examine the role of TNF-α in the inflammatory process, we used a nonreplicating HSV gene transfer vector (T0TNFSR) to express sTNFR (see supplementary materials; Fig 1A). DRG neurons in culture transduced with T0TNFSR at an MOI of 1 expressed sTNFR detected by immunocytochemistry and Western blot (see supplementary materials; Fig 1B, C), and released sTNFR into the medium as detected by Western blot and ELISA (see supplementary materials; Fig 1D, E). One week after subcutaneous inoculation of T0TNFSR into the plantar surface of the hind paws, sTNFR could be detected in neurons of the L4-L6 DRG ipsilateral to the inoculation site using immunocytochemistry and Western blot (see supplementary materials; Fig 2A, B). Using Western blot, we found that the ipsilateral dorsal horn of spinal cord containing the nerve terminals of transduced cells showed substantial amounts of sTNFR in T0TNFSR-inoculated animals but not in control or TOZ.1-inoculated animals (see supplementary materials; Fig 2C).

Animals with pain below the level of lateral hemisection developed mechanical allodynia, as measured by response to von Frey filaments (Fig 3). Subcutaneous inoculation of vector T0TNFSR into the plantar surface of both hind paws 1 week after hemisection resulted in a significant reduction in pain-related re-

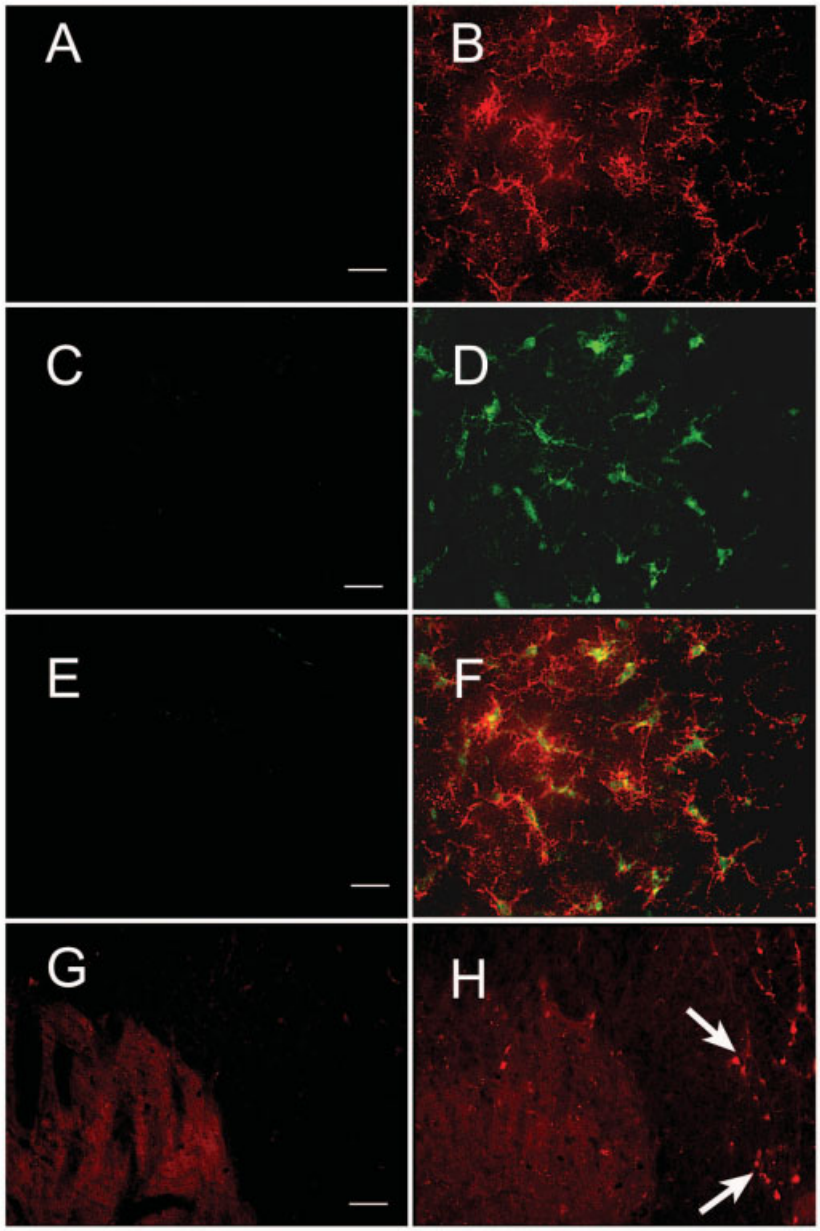

Fig 1. Immunocytochemical staining of lumbar spinal cord from control (A, C, E, G) and rats 2 weeks after T13 hemisection (B, D, F, H) show substantial increases in OX42-positive microglia (A, B), p-p38 (C, D; merged images E, F; scale bar = 10μm), and ED1-positive cells in the white matter tracts (dorsal column, arrows*) in the lumbar spinal cord (G, H; scale bar = 20μm).*

sponse (see Fig 3). The vector-mediated effect was present by 1 week after inoculation (2 weeks after injury) and peaked 2 to 3 weeks after inoculation (see Fig 3).

In conjunction with the reduction in mechanical hyperalgesia, subcutaneous inoculation of T0TNFSR 1 week after T13 hemisection also resulted in a significant reduction in mTNF-α (Fig 4A, B), p-p38 (see Fig 4 C–E), and the number of ED1-positive cells in the dorsal column and dorsal horn of the lumbar spinal cord (Fig 5), similar to levels seen in uninjured animals.

Discussion

In this study, we report that expression of mTNF-α is increased drastically in the lumbar spinal cord several segments below the level of a lateral hemisection injury: HSV-mediated expression of p55 sTNFR in DRG neurons projecting the lumbar spinal cord (1) reversed the activation of microglial p38; (2) reduced mTNF-α protein; and (3) decreased the number of invading ED1-positive macrophages in the dorsal columns and dorsal horn of lumbar spinal cord below the level of the lesion, resulting in reduced mechanical al-

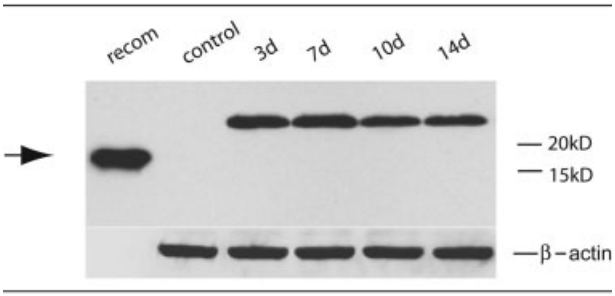

*Fig 2. mTNF-α in lumbar spinal cord increased 3 days to 2 weeks after T13 spinal hemisection. Immunoreactive TNF-α had an apparent molecular weight of 27kDa, consistent with full-length mTNF-α compared with sTNFα (*arrow*, recombinant sTNF-α) that has an apparent molecular weight of 17kDa.*

lodynia in this model of central neuropathic pain below the level of SCI.

Chronic neuropathic pain represents a major complication of spinal cord injury. The current taxonomy proposed by the SCI Pain Task Force of the International Association for the Study of Pain recognizes three types of neuropathic pain after SCI: above-level pain, at-level pain, and below-level pain.[1] Several animal models have been established that exhibit behavioral manifestations that are interpreted to reflect essential features for each of the different types of post-SCI pain.[17] This study focused on below-level pain that develops after SCI,[1] utilizing the widely accepted lateral hemisection model of below-level pain in the rat.[18]

Central immune activation plays an important role in the development of chronic pathological pain after damage to the nervous system.[19] In rodent models of peripheral neuropathic pain, partial peripheral nerve damage results in activation of glia in the spinal cord.[20–22] Treatments that reduce pain also block glial activation,[23] and disruption of spinal glial activation reduces nocisponsive behaviors.[24–26] Activated glia synthesize several potentially algesic substances including TNF-α, interleukin-1, and the prostanoid PGE2.[27] Intrathecal administration of inflammatory cytokines has a proalgesic effect in normal[28] and nerve-injured animals,[29] and inhibition of spinal TNF-α activity reduces pain behavior in several models.[5,24] Transgenic mice that overexpress TNF-α in glia show increased mechanical allodynia after peripheral nerve damage compared with control animals.[30]

Microglial activation caused by peripheral nerve injury results in increased phosphorylation of p38 MAP kinase in spinal cord.[13,31] A role for TNF-α is suggested by the observations that direct injection of TNF-α into peripheral nerve induces thermal hyperalgesia and mechanical allodynia, and damaged nerves are hypersensitive to the proalgesic effects of TNF-α.[12,32,33] Interference with TNF-α effects by intrathecal administration of the soluble form of p75 TNFR (etanercept) blocks the increase of p-p38 in spinal cord after spinal nerve ligation[13] but only if the antibody is administered before the injury. Intrathecal administration of neutralizing antibodies against the p55 TNFR (but not antibodies against the p75 TNFR) reduce pain in the chronic constriction injury model of peripheral neuropathic pain.[11]

We found substantial amounts of full-length mTNF-α, identified by its molecular weight on Western blot, in the lumbar spinal cord at a time when we were unable to detect soluble cleaved TNF-α in a supernatant from the same sample either by ELISA or Western blot. Our inability to detect soluble TNF-α below the level of hemisection was not the result of a technical problem, because we were able to detect soluble TNF-α transiently at the site of blunt trauma to

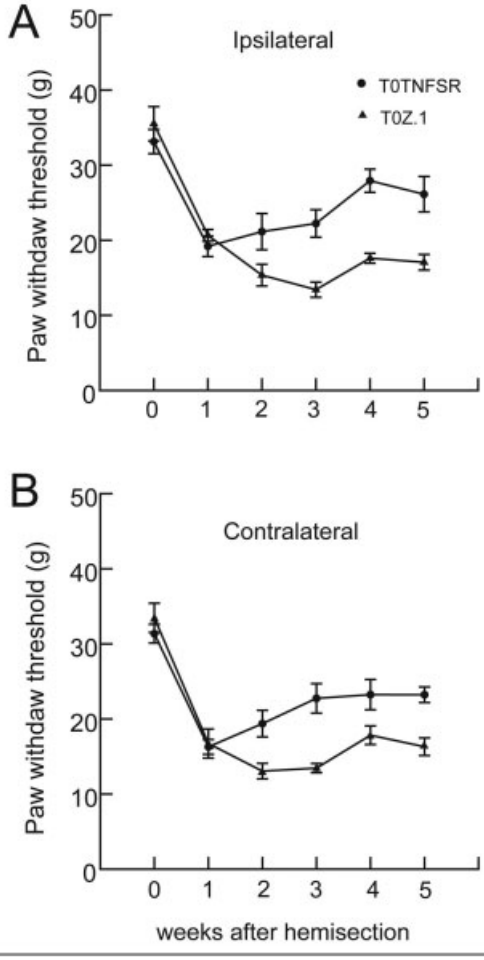

Fig 3. T0TNFSR reduces mechanical allodynia. T13 hemisection resulted in a decrease in paw withdrawal threshold (mechanical allodynia), which persisted for 5 weeks as shown in T0Z.1-treated animals (triangles). *Animals inoculated with T0TNFSR showed an increase in threshold value* (circles) *both ipsilateral (A) and contralateral (B) to the hemisection.* $p < 0.01$ *by repeated measures;* $N = 6$ *animals per group.*

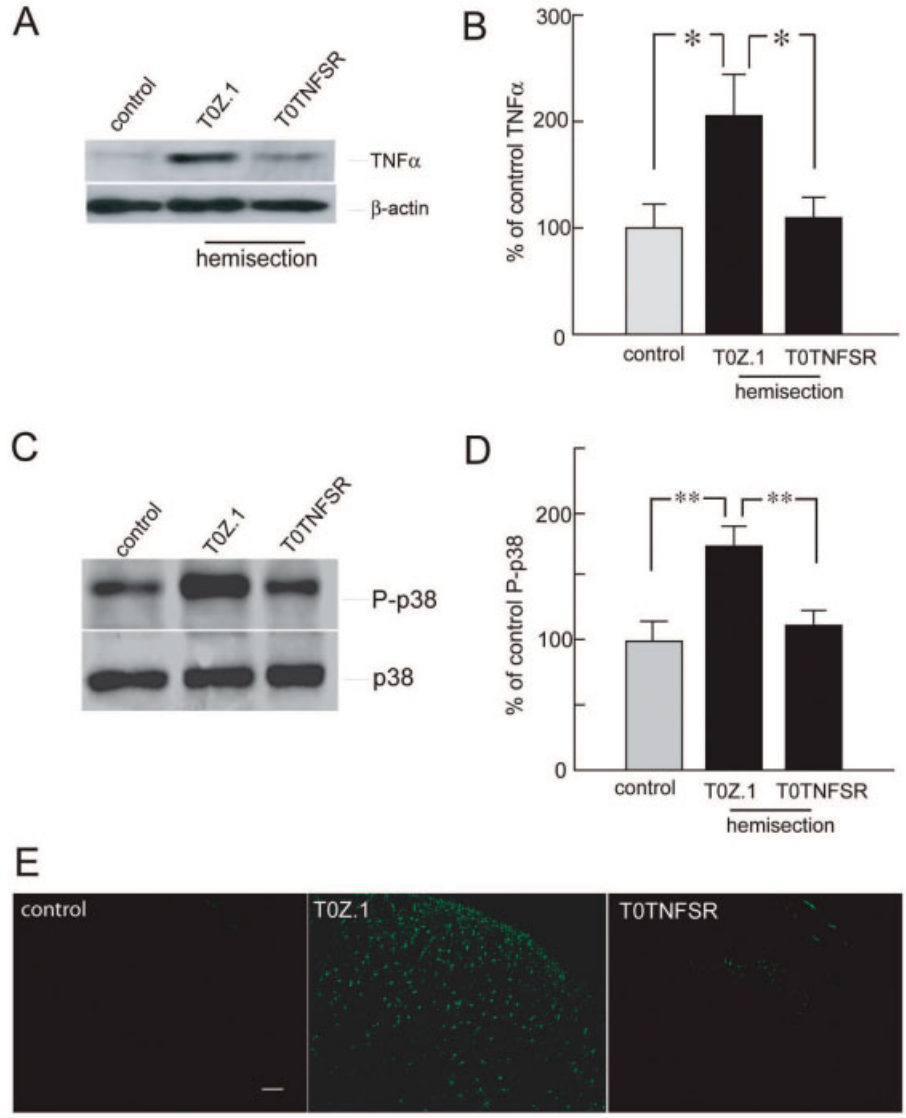

Fig 4. T13 hemisection resulted in an increased in mTNF-α in lumbar spinal cord. Animals inoculated with T0TNFSR, but not animals inoculated with T0Z.1, showed a reduction in TNF-α 1 week after injection, 2 weeks after hemisection. (A) Western blot. (B) Relative optical density × area expressed to relative control. Mean ± SEM. N = 5, (asterisks) $p < 0.05$. *The level of p-p38 in L4-L6 lumbar spinal cord also increased after T13 hemisection, but this increase was blocked in animals transduced with T0TNFSR. (C) Western blot. (D) Level of p-p38 was determined by densitometry, normalized to total p38, and expressed as percentage-change relative to control. Mean ± SEM, N = 5, (double asterisks)* $p < 0.01$. *(E) Anti–p-p38 immunostained section showed a marked increase in cells of the dorsal horn that were reduced in animals inoculated with T0TNFSR. Scale bar = 40μm.*

the spinal cord in the first few hours after injury using the same methods.

In the immune system, an emerging body of literature suggests that "reverse" signaling through mTNF may serve to regulate the immune response.[9] Stimulation of mTNF in monocytes confers resistance to bacterial endotoxin to reduce the release of proinflammatory cytokines[34] and resistance to the influence of inflammatory agents, while in other settings reverse signaling through mTNF may induce apoptosis in inflammatory cells.[35] In monocytes, p55 sTNFR is a potent activator of reverse signaling through mTNF-α,[36,37] and it seems likely that release of sTNFR from the terminals of transduced DRG neurons reduces mTNF-α expression in microglia and macrophages through a reverse signaling mechanism. Although we cannot exclude the possibility that biologically relevant but undetectable amounts of soluble TNF-α were present, this would be a less likely mechanism to account for the observed effects of sTNFR.

The studies reported do not establish that block of p38 phosphorylation is required to downregulate

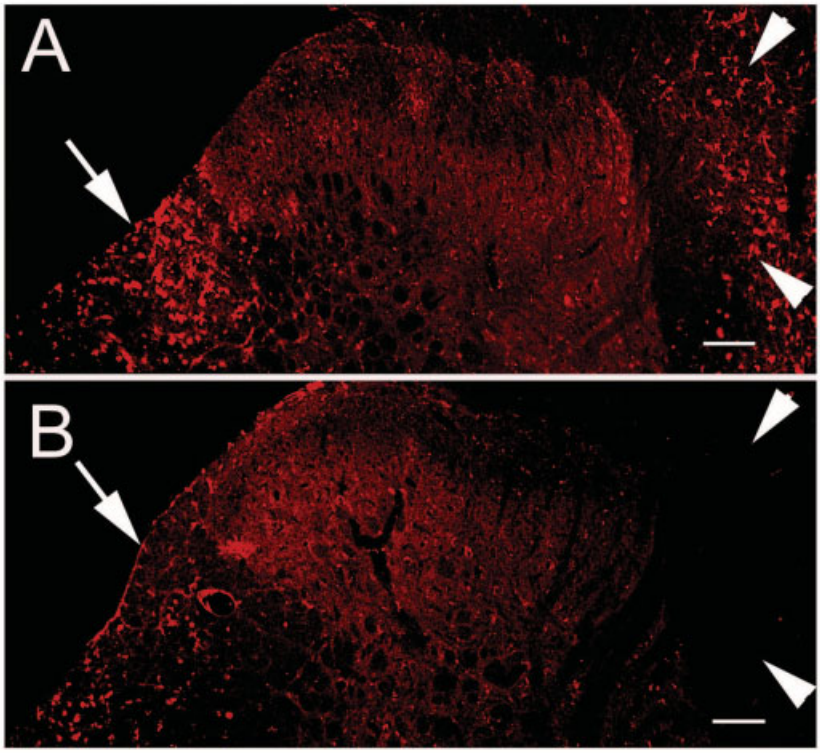

Fig 5. The number of ED1-positive cells in the dorsal column (arrowheads) *and lateral white matter tracts* (arrows) *in the lumbar spinal cord were substantially reduced in animals inoculated with T0TNFSR (A) compared with animals inoculated with T0Z.1 (B) 1 week after T13 hemisection and killed 2 weeks after hemisection. Scale bar = 20μm.*

mTNF expression, but expression of proinflammatory cytokines including TNF, IL-1, IL-6, and IL-8 as well as the enzymes cyclooxygenase II, collagenase, and iNOS occur downstream of p38 activation,[38] and this expression can be blocked by p38 inhibitors.[39] In addition, TNF-α acting through the p38 MAPK pathway is a potent activator of NF-kB which in turn is an inducer of TNF-α expression, a positive feedback loop that contributes to the development of chronic inflammatory conditions and possibly chronic pain states.[40,41] Pretreatment prior to onset of pain is required for etanercept (p75 sTNFR) to provide an antiallodynic effect in the spinal nerve ligation model of peripheral neuropathic pain.[13,42] However, we found that transgene-mediated expression of the p55 form of sTNFR from the vector 1 week after SCI and after a time of established pain behavior was nonetheless effective in reducing the phosphorylation of p38 MAP kinase and reducing nocisponsive behavior.

What accounts for the increase in mTNF-α levels at L5 after a lateral hemisection at T13? SCI induces a rapid upregulation in expression of TNF-α and TNFR within hours in the region of injury that resolves rapidly[43,44] and may play a role in cell death in that area.[45, 46] After dorsal hemisection, remote activation of several chemokines including monocyte chemoattractant protein-1 and macrophage inflammatory protein 1α has been reported, presumably related to distal fiber degeneration after injury.[47]

In summary, T13 hemisection resulted in invasion of inflammatory cells and activation of microglia in the lumbar spinal cord with concurrent expression of mTNFα below the level of the spinal cord lesion. Downregulation of mTNFα through reverse signaling by sTNFR through mTNFα correlated with a reduction in p38 MAP kinase and mechanical allodynia in this model.

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