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Spinal Toll-like receptor signaling and nociceptive processing: Regulatory balance between TIRAP and TRIF cascades mediated by TNF and IFNβ

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Abstract

Toll-like Receptors (TLRs) play a pivotal role in inflammatory processes and individual TLRs have been investigated in nociception. Here, we examine overlapping and diverging roles of spinal TLRs and their associated adaptor proteins in nociceptive processing. Intrathecal (IT) TLR2, TLR3, or TLR4 ligands (-L) evoked persistent (7 day) tactile allodynia (TA) that was abolished in respective TLR deficient mice. Using *Tnf*$^{-/-}$ mice, we found that IT TLR2 and TLR4 TA was TNF-dependent, while TLR3 was TNF-independent. In toll-interleukin 1 receptor (TIR) domain containing adaptor protein (*Tirap*$^{-/-}$) mice (downstream to TLR2 and TLR4), allodynia after IT TLR2-L and TLR4-L was abolished. Unexpectedly, in TIR-domain-containing adapter-inducing interferon-β (*Trif*^{lps2}) mice (downstream of TLR3 and TLR4), TLR3-L allodynia was abrogated, but intrathecal TLR4-L produced a persistent *increase* (>21 days) in TA. Consistent with a role for interferon (IFN)β (downstream to TRIF) in regulating recovery after IT TLR4-L, prolonged allodynia was noted in *Ifnar1*$^{-/-}$ mice. Further, IT IFNβ given to *Trif*^{lps2} mice reduced TLR4 allodynia. Hence, spinal TIRAP and TRIF cascades differentially lead to robust TA by TNF dependent and independent pathways, while activation of TRIF modulated processing through type I IFN receptors. Based on these results, we believe that processes leading to the activation of these spinal TLRs initiate TNF-dependent and -independent cascades, which contribute to the associated persistent pain state. In addition, TRIF pathways are able to modulate the TNF-dependent pain state through IFNβ.

INTRODUCTION

Innate immunity is involved in tissue-level responses to infection and injury. Elements of this process, notably the Toll-like receptors (TLRs) are expressed by glia [3,4,24], and neurons [33,47]. TLRs have been implicated in the nociceptive processing initiated by inflammation and peripheral nerve injury [5,9,12,32,41,55]. These observations, indicating a

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Conflicts of Interest Statement
The authors claim no conflicts of interest.

role for TLRs in these pain models in the absence of an infectious process, are in accord with observations that a variety of endogenous ligands known to activate TLRs have been identified in biologic systems and may serve to act though these constitutively expressed receptors [6,13,25,35]. Several approaches have provided direct support for a role of *spinal* TLRs in pain processing. Thus, spinal (intrathecal: IT) delivery of TLR4 agonists yields nociception and allodynia [9,10,38,51,54]. Conversely, pharmacological blockade of spinal TLR4 attenuates evolution of a persistent pain state [9]. Additionally, delivery of agents, which reduce glial activation, can inhibit the facilitatory effects of IT TLR agonists [22,39,49,51].

There are thirteen identified TLRs some localized to the cell surface and others on endosomes, which signal through a limited number of adaptor proteins (Figure 1A). The Toll-interleukin 1 receptor (TIR) domain containing adaptor protein, TIRAP, is exclusive to TLR2 and TLR4, and facilitates myeloid differentiation factor 88 (MyD88) activation [20,21]. The MyD88 activation pathway, common to all TLRs except TLR3, leads to activation of NF-κB, yielding production of pro-inflammatory cytokines such as TNF and IL-1 [27]. In contrast, the TIR-domain-containing adapter-inducing interferon-β (TRIF) is shared by TLR3 and TLR4 signaling, and skews to type I interferon production [16,42]. Thus, TLR activation, through either the MyD88 or the TRIF pathways, can lead to a wide-range of effects. Given this complex organization and the expression of TLRs by glia and neurons, the net effect of activating any one of the multiple spinal TLRs cannot be predicted in the absence of specific data on outcomes associated with defined spinal TLR activation.

Here we investigate the role of spinal TLRs and their associated adaptor proteins in spinal nociceptive processing using both *in vitro* and *in vivo* techniques. With primary spinal cell cultures of microglia and astrocytes, we determined the expression levels of TNF and IFNβ following TLR activation. To assess roles of the respective spinal TLRs in initiating a hyperpathic state, eponymous TLR ligands were intrathecally administered and IT TLR2-L (HKLM), TLR3-L (Poly(I:C)) and TLR4-L (LPS) were found to initiate long lasting allodynic states. Using genetically modified mice, we found that TLR2 and TLR4 ligands acted through TNF (as defined by a diminished effect in $Tnf^{-/-}$ mice) while TLR3-L did not. Unexpectedly, in mice that lacked TRIF or type I IFN receptor signaling, a markedly prolonged and enhanced allodynia was noted. Allodynia induced by IT TLR2 or TLR4 ligand was transiently relieved by IT IFNβ. These studies revealed TNF-dependent and -independent spinal pro-allodynic cascades are differentially activated by TRIF and TIRAP signaling, and a potential suppressive role of TRIF signaling through IFNβ.

METHODS

Animals

All animal experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego (under the Guide for Care and Use of Laboratory Animals, National Institutes of Health publication 85-23, Bethesda, MD, USA). Mice were housed up to four per standard cage at room temperature and maintained on a 12-hour light/dark cycle (light on at 07:00h). Testing was performed during the light cycle. Food and water were available *ad libitum*. C57BL/6 mice (male, 25–30g) were purchased from Harlan (Indianapolis, IN). $Tlr2^{-/-}$, $Tlr3^{-/-}$, $Tlr4^{-/-}$, and $Tirap^{-/-}$ mice were a gift from Dr. S. Akira (Osaka University, Japan) and were bred for 10 generations onto the C57Bl/6 background. $Trif^{lps2}$ mice were a gift from Dr. B. Beutler (UT Southwestern, Texas) and were directly generated on the C57Bl/6 background. $Tlr5^{-/-}$ and $Tnf^{-/-}$ mice were purchased from The Jackson Laboratory. $Ifnar1^{-/-}$ mice were originally obtained from B&K Universal Limited (Hull, United Kingdom) and backcrossed over 10

generations onto the C57Bl/6 background. Figure 1B lists the murine strains used and the standard nomenclature to be used throughout the paper.

Rat Microglia and Astrocyte Primary Cell Culture

Purified cultures of rat spinal microglia and astrocytes were prepared as previously described with some modifications [22]. One- to three-day-old Holtzman Sprague–Dawley rat pups were anesthetized, and the spinal cords were ejected, mechanically triturated, then centrifuged at 215 g for 5 min and re-suspended in DMEM containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 1% penicillin/streptomycin (P/S; Gibco, Carlsbad, CA, USA), and plated in a flask previously coated with poly l-lysine (Sigma, St. Louis, MO, USA). Flasks were maintained at 37°C in a humidified 5% CO_2 incubator for 2 weeks until 80–90% confluent, with media changes every other day. On day 14, microglia were removed by shaking for 2 hours at 37 °C, centrifuged at 215 g for 5 min, and plated onto 24-well plates at 80,000 cells/mL and allowed to adhere for 4 hours. For astrocyte cultures, on day 15 mother cultures were shaken a second time, media discarded and replaced, trypsinized, centrifuged at 215×*g* for 5 min, re-suspended in DMEM with 10% FBS and 1% P/S, and plated on to 24-well plates at 100,000 cells/mL until they reached 70–80% confluence (2 days). Individual wells were stimulated with 5μL of specific murine TLR agonists available in a complete kit (Invivogen: tlrl-kit1mw; Supplementary Table 1). The doses chosen were within the range recommended by the manufacturer.

TNF ELISA

TNF in the culture supernatant was assayed at both 6- and 20-hours following TLR agonist administration, by ELISA kits per the manufacturers instructions (R&D Biosystems). TNF is expressed as pg/mL of culture media sample.

Quantitative real time-PCR

At 20-hours post TLR agonist addition to the culture media, the media was removed and immediately replaced with 0.5mL of Trizol (Invitrogen), allowed to sit for 2 minutes then flash frozen. The mRNA was then isolated using RNeasy columns (Qiagen). Complementary DNA was prepared using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR was performed with pre-developed specific primers and probes (Taqman Gene Expression Assay, Applied Biosystems) were used to detect mouse Interferon-beta 1 (IFNβ1) (Assay ID Rn00569434_s1) and GAPDH (Assay ID Rn01775763_g1) (Applied Biosystems). The relative abundance was calculated by comparing delta-CT values [44] and the data were then normalized to GAPDH gene expression and presented as relative gene expression.

Primary Cell Culture Staining

Primary microglia and astrocytes cells in cDMEM were aliquoted (200μL) in to coated 8-chamber cell culture slides at the same density as stated previously and allowed to proliferate for 24-hours (microglia) or 36-hours (astrocytes). Cells were fixed for 5 minutes in 4% PFA then washed with PBS and stained overnight in primary antibodies at 4°C. Astrocytes and microglia were incubated with anti-Vimentin (Zymed, 1:500) and anti-Iba-1 (Abcam, 1:250) antibodies. Secondary antibodies conjugated to Alexa-488 and 594 were used at 1:300. Slides were visualized and images captured on a Leica confocal microscope.

LDH Cytotoxicity Assay

Cytotoxicity following the TLR ligands was assessed by Lactate dehydrogenase (LDH) release. LDH was measured in media samples of microglia and astrocytes following each

TLR ligand treatment group at the 20-hour time point using a CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) following the manufacturer's directions.

Intrathecal (IT) Injection and Drug Administration

Intrathecal needle placement procedure for the IT saline and IT sham treatment is performed as previously described [23,55]. Briefly, mice were induced with 3% isoflurane (with 2% oxygen and 2% room air) in a chamber until a loss of the righting reflex was observed. A 1″ 30-gauge needle attached to a 50μL Hamilton syringe was inserted between the L5 and L6 vertebrae, evoking a tail flick reflex. The following TLR agonists were administered in 5μL diluted in 0.9% sterile saline: HKLM (1×10^8cells/5μL), Poly(I:C) (1μg/5μL), LPS-EK Ultrapure (1μg/5μL), and FLA-ST (1μg/5μL) (Supplementary Table 2). The doses were chosen from the Invivogen product protocol, as well as the results of the cell culture studies outlined above. Doses ranging from 0.1μg/5μL to 10μg/μL were first tested, and 1μg/μL produced the maximal effect at the lowest dose for all ligands. For the TLR5 ligand (FLA-ST) the 10μg/5μL dose showed the same minimal TA as the 1μg/5μL dose (Supplemental Figure 2A). Thus, the 1μg/5μL dose was chosen to correspond to the other TLR ligand doses. All ligands were diluted in 0.9% sterile saline to a stock solution and then aliquoted to avoid repeated freeze-thaw cycles. IT administration doses were then diluted to the specified concentration from a frozen aliquot.

Following recovery from anesthesia, as evidenced by a vigorous righting reflex and spontaneous ambulation, typically around 1–2 minutes, mice were evaluated for motor coordination and muscle tone. Tactile thresholds were measured using the up-down application of von Frey hairs along the following time course: Baseline (P=Pre-injection), 30-, 60-, 90-, 120-, 180-, and 240-minutes, 24-hours and 7-days after treatment. We previously noted the effects of the use of isoflurane in this procedure and TA [55], thus, while all the above time points were recorded, only the baseline, 4-hour, 24-hour, and 7-day time points are presented here. IFNβ (Chemicon, 100ng/5μL in 0.1% BSA) was administered intrathecally either 1 hour before IT LPS (1μg/5μL) or as a post-treatment, 7 days after IT LPS induced TA. Gabapentin (Toronto Research Chemicals) was administered (100mg/kg) i.p. diluted in 0.9% sterile saline.

Behavioral Tests

Mechanical sensitivity was assessed using the von Frey up-down method. Filaments with values ranging from 2.44 to 4.31 (0.03g to 2.00g) were applied to the paw as previously described [7]. The 50% probability withdrawal threshold (in principle, the calculated force to which an animal reacts to 50% of the presentations) was recorded. Mechanical values for both the paws were measured and averaged to produce a single data point per day of measurement.

Western Blot

Mice were deeply anaesthetized and spinal cords were ejected from the vertebral column using a saline-filled syringe. The lumbar part of the spinal cord was immediately homogenized in extraction buffer [50 mm Tris buffer, pH 8.0, containing 0.5% Triton X-100, 150 mm NaCl, 1 mm EDTA, protease inhibitor cocktail (P-8340; Sigma, 1:100), phosphatase inhibitor cocktail I and II (Sigma, 1:100)] by sonication. The tissue extracts were subjected to denaturing NuPAGE 4–12% Bis-Tris gel electrophoresis and then transferred to nitrocellulose membranes (Micronic Separation Inc. Westborough, MA, USA). Membrane was first blocked with 5% non-fat milk in Tris-buffer (50 mm Tris-HCl, 6 mm NaCl) containing 0.1% Tween 20 for 1 hour at room temperature. The membranes were incubated with antibodies overnight at 4°C (IFNβ 1:1000; Chemicon and β-actin 1: 10,000). After washing, the antibody–protein complexes were probed with appropriate secondary

antibodies labeled with horseradish peroxidase for 1 hour at room temperature and detected with chemiluminescent reagents (SuperSignal; Pierce, Rockford, IL, USA). Intensity of immunoreactive bands was quantified using Image Quant software (Molecular Dynamics, Sunnyvale, CA, USA). The intensity of the IFNβ immunopositive bands was normalized relative to that of β-actin. Two exposures for anti-IFNβ of the same blot are shown. The longer exposure is presented to demonstrate that all lanes had a band present. Quantification was performed on the shorter exposure, since it provided a more accurate differential lane expression.

Statistics

Data are presented as group mean ± SEM. Tactile threshold time course curves (plotted as the mean ± SEM vs. time after treatment) were analyzed with a 2-way analysis of variance (ANOVA) with repeated measures over time, followed by Bonferroni *post hoc* test. The allodynic index is the area under the time course curve after treatment, in which the percentage change from baseline threshold is plotted against time: 100 × ((baseline threshold treatment threshold)/(baseline threshold)). Multiple comparison tests were performed by two-way ANOVA with Bonferonni *post hoc* tests. Statistical analyses utilized Prism software (GraphPad Software, Inc., San Diego, CA).

RESULTS

Primary astrocyte and microglia cultures

Rat spinal primary microglia and astrocyte cell cultures were generated from neonatal rats and stained with anti-vimentin and anti-Iba-1 to assess purity (Supplementary Figure 1C and D). These primary microglia and astrocyte cultures were stimulated with TLR ligands specific to individual receptors (Supplementary Table 1), supernatants collected at both 6-hours and 20-hours, and the levels of TNF release in the media was assayed by ELISA. Primary microglia cultures (Figure 2B) showed a robust TNF release at the 6-hour time point following the addition of TLR2, 4, and 5 ligands, and at the 20-hour time point following TLR1/2, 2, 4, 5, and 2/6 ligands. Primary astrocyte cultures (Figure 2A) showed a significant level of TNF release at the 6-hour time point following the addition of TLR2, and 5 ligands, and at the 20-hour time point following TLR2, 4, and 5 ligands. The TLR3-L (Poly(I:C)) produced a minimal response as expected since TNF release has been reported to have slower kinetics and to be at a lower level. However, both astrocyte (Figure 2C) and microglia (Figure 2D) showed an increase in IFNβ following TLR3-L (Poly(I:C)) and TLR7/8 (ssRNA40) agonist treatment, which attained statistical significance for (Poly(I:C)) treatment. To control for cytotoxicity, LDH release was measured in the primary cell culture media samples at 20-hours post TLR agonist addition. In both the microglia and astrocyte cultures, only Poly(I:C) and LPS administration resulted in minimal cell death signal (5–10%), while the other TLRs had no apparent effect (Supplementary Figure 1A–B).

Spinal TLR activation and nociceptive thresholds

The following *in vivo* studies address the role of spinal activation of TLRs on tactile thresholds. TLR2-L (HKLM), TLR4-L (LPS) and TLR5-L (FLA-ST) were selected for further study *in vivo* based on TNF release and TLR3-L (Poly(I:C)) based on IFNβ mRNA induction in the primary microglia and astrocyte cell cultures (Figure 2). TLR ligands were spinally delivered via IT injection at the L5 level in C57Bl/6 mice (Figure 3). The effect of IT administration of these selected TLR ligands on tactile thresholds was measured by von Frey filament testing using the up-down method. Mice were tested before IT administration (P) and at 0.5, 1, 1.5, 2, 3, 4, 24 hours, and 7 days post injection. We previously noted the effects of the use of isoflurane in this procedure and TA [55] and, thus, while all the above time points were recorded, only the, baseline (P), 4-hour, 24-hour, and 7-day time points are

presented here. TLR2-L (HKLM), TLR3-L (Poly(I:C)) and TLR4-L (LPS) produced a robust TA, lasting longer than 7 days (Figure 3A–C). Alternatively, TLR5-L (FLA-ST) produced a short-lived 3-hour TA that was resolved by the 4-hour time point (Figure 3D and Supplementary Figure 2B).

TLR deficient mice and spinal TLR ligands

To assess the specificity of the administered TLR ligands to their receptor, TLR deficient mice were used. This method was employed since there are no specific antagonists available for these receptors, with the exception of TLR4. Each TLR null mouse received the corresponding TLR ligand IT, as well as a different TLR ligand to show that other TLR signaling pathways were not impaired. As expected IT administration of the corresponding TLR ligand did not produce a TA in the respective TLR strain (Figure 4A–D). IT TLR4-L (LPS) was used as a control in *Tlr2*$^{-/-}$, *Tlr3*$^{-/-}$, and *Tlr5*$^{-/-}$ mice, as it is specific to TLR4, and produced a robust TA in these mice (Figure 4A–B, D, Supplementary Figure 2C). IT TLR2-L (HKLM) was used as a control for *Tlr4*$^{-/-}$ mice, as it is specific to TLR2, and produced a robust TA in the *Tlr4*$^{-/-}$ mice (Figure 4C). These results not only confirm the specificity of the selected TLR ligands, but also the integrity of the gene targeted strain and its signaling pathways.

TLR adapter protein deficient mice and spinal TLR ligands

We next assessed the role of signaling intermediates in the TLR pathways: TIRAP, which leads to proinflammatory cytokine release, and TRIF, which leads to type I interferon production (Figure 1A). To investigate signaling mediators, mice deficient in TIRAP and TRIF signaling were subject to IT TLR ligands (Figure 1B). TIRAP is specific to TLR2 and TLR4 signaling, while TRIF is specific to TLR4 and TLR3 signaling. IT LPS had no effect in *Tirap*$^{-/-}$ mice (Figure 5A), while IT TLR4-L (LPS) in *Trif*^{lps2} mice produced a robust, long-lasting TA (Figure 5C). This finding suggests a role for the TRIF pathway, and possibly interferon release, in the resolution phase following injury. Additionally, as expected, IT TLR2-L (HKLM) had no effect in *Tirap*$^{-/-}$ mice (Figure 5A) and IT TLR3-L (Poly(I:C)) had no effect in *Trif*^{lps2} mice (Figure 5B), but IT TLR2-L (HKLM) did have a robust effect in *Trif*^{lps2} mice (Figure 5D). This confirms the roles of TRIF and TIRAP as specific adaptor proteins to TLR3 and TLR2, respectively.

Role of TNF in nociceptive processing

Considerable work with genetically engineered animals and spinally derived anti-TNF agents has pointed to a pervasive role of this cytokine in mediating neuraxial events underlying pain processing [40,49,59]. To assess the role of TNF in the TA elicited by IT TLR ligands administration, *Tnf*$^{-/-}$ mice were used. *Tnf*$^{-/-}$ mice received IT injections of the same TLR ligands used previously. Following IT TLR2-L (HKLM) only a modest TA was observed (Figure 6A), while the IT TLR4-L (LPS) produced only a brief TA similar to that of IT saline (Figure 6C). This result suggested that the TLR4 allodynia-inducing pathway is absolutely TNF-dependent, while TLR2 induced TA was only partially dependent on TNF. In contrast, IT TLR3-L (Poly(I:C)) in the *Tnf*$^{-/-}$ mice continued to produce a robust TA (Figure 6B), suggesting that the pronociceptive effects of the TLR3/TRIF pathway was independent of TNF in producing pain. The brief 3-hour TA effect of the TLR5 agonist was also almost completely abolished in the TNF deficient mice (Figure 6D). Accordingly, when the allodynic indices were calculated, *Tnf*$^{-/-}$ mice were shown to be less responsive to the TLR2-L (HKLM), TLR4-L (LPS), and TLR5-L (FLA-ST) (Figure 6E), while the TLR3-L (Poly(I:C)) produced a robust effect.

Role of IFN in nociceptive processing

Type I interferons (IFNs) can have both proinflammatory actions (as in the response to viral infections *via* TLR3, 7, 8 and 9 activation) primarily through macrophage stimulation and can serve to *suppress* the inflammatory cascade as in models of tumor growth [30]. To assess the role of interferon on TLR induced TA, *Ifnar1*$^{-/-}$ mice were used. Similar to that of the *Trif*^{lps2} mice, IT TLR4-L (LPS) produced a robust, long-lasting TA in the *Ifnar1*$^{-/-}$ mice (Figure 7A). This TA was transiently reversed by 100mg/kg i.p. gabapentin (Supplementary Figure 3). The anti-allodynic effects observed in these studies after systemic gabapentin is consistent with the presence of a facilitated state. In contrast to the *Trif*^{lps2} mice, the *Ifnar1*$^{-/-}$ mice developed prolonged allodynia following IT TLR3-L (Poly(I:C)) resembling the pattern of the IT TLR4-L (LPS) treated mice (Figure 7B). Together these results suggested that the TA following TLR3 activation was independent of TNF, but the rapid resolution of the facilitated TA required type I IFN signaling. Confirming the increased presence of interferons following TLR3 signaling, an increase in spinal IFNβ was indeed detected by western blot following TLR3-L (Poly(I:C)) (Supplementary Figure 4).

Roles of spinal IFN in regulating spinal TLR mediated TA

To test if type I IFN treatment could relieve pain after IT TLR4-L (LPS) treatment we selected *Trif*^{lps2} mice, which develop a protracted course of mechanical allodynia after IT LPS treatment (Figure 5C), and have intact type I IFN receptor signaling. In *Trif*^{lps2} mice at 7-days post IT TLR4-L (LPS) or TLR2-L (HKLM), a post-treatment with IT IFNβ (100ng/5μL) reversed the TA (Figure 8). In two separate TLR-induced TA pathways IFNβ treatment temporarily alleviated the observed allodynia. Additionally, *Trif*^{lps2} mice were pre-treated with IT IFNβ (100ng/5μL) one hour before IT LPS (Supplemental Figure 5). Pre-treatment with IFNβ transiently blocked the LPS-induced TA. IT vehicle plus IT LPS still produced a robust TA in the *Trif*^{lps2} mice, and IFNβ plus IT saline had no effect on tactile thresholds. This confirmed the importance of IFNβ on resolving the central pain state following TLR activation.

DISCUSSION

In models of persistent inflammation, and mono-/poly-neuropathy, mice with defects in TLR expression show a prominent attenuation of behaviorally defined hyperpathy [4,8,11,28,36,49]. Although those studies employed globally deficient mice, expression of TLRs on spinal glia [3,4,24] and neurons [33,47] and the ability of TLR agonists to evoke release of cytokines, including TNF, has emphasized the role of spinal TLRs in spinally mediated facilitated pain states. In the face of tissue injury and inflammation there can be a transition from an acute to a persistent facilitated state [2,8]; and spinal TLRs may particularly function in this transition [9]. One characteristic of the “painful” phenotype after tissue and nerve injury is the response to anti-hyperpathic agents such as gabapentin, which we show here to antagonize the TA observed after IT TLR4 agonist. Collectively these data suggest complex roles of TLRs in both the induction and the recovery of facilitated pain states.

Prior reports of the contributions TLRs to spinal facilitated pain states focused on individual receptors, However, there are 13 TLR family members, which signal through a more restricted number of adaptor proteins resulting in the release of neurohumoral factors (Figure 1). We undertook to characterize the signaling cascades in the spinal cord initiated by local TLR activation of membrane bound (TLR2, TLR4, TLR5) and endosomal (TLR3) receptors though the use of intrathecal TLR ligands (Supplemental Table 2). Here, we show that activation of spinal TLR2, TLR3, and TLR4 evokes a long lasting (up to 7 days) decrease in touch evoked hind paw withdrawal thresholds (tactile allodynia). The TLR5

ligand, though also a membrane TLR, produced only a short-lived (<4hr) TA, regardless of dose. These results are consistent with previously reported results in mice [9] and rats [51]. In contrast to the morbidity observed with peripheral TLR agonists [18,29,58], IT TLR agonists did not elicit detectable changes in general behavior, body weight, or motor function.

TLR coupling

As the TLR2, TLR3 and TLR4 deficient mice prevented TA produced only by IT HKLM, Poly(I:C), and LPS, respectively, these effects reflect a specific action mediated by the eponymous spinal receptor and confirm the lack of cross talk between the respective spinal receptors. In particular, the specificity of Poly(I:C) for TLR3 was confirmed in the *Tlr3*$^{-/-}$ mice. There are other intracellular RNA sensors, such as RIGI and MDA5 that would be present in the *Tlr3*$^{-/-}$ mice, yet the TA was absent in *Tlr3*$^{-/-}$ mice. To characterize the signaling cascades initiated by spinal membrane and endosomal TLR activation leading to TA, we examined the role of associated adaptor proteins for the TLRs selected for *in vivo* studies. As schematically summarized in Figure 9, IT TLR agonists in these mutant mice revealed several novel characteristics of these spinal TLR-effector cascades.

TIRAP signaling in spinal TLR initiated allodynia

Consistent with coupling of TLR2 and TLR4, but not TLR3, through TIRAP, IT TLR2 and TLR4 agonists initiated TA was absent in *Tirap*$^{-/-}$ mice, while TLR3-L effects were unaltered. TIRAP signals through MAPKs and NF-κB, leading to inflammatory cytokine release [20]. The development of TA by IT TLR4-L injection was absent in the *Tirap*$^{-/-}$ mice indicating that the TIRAP-MyD88 pathway predominantly elicits pain after IT TLR4-L (LPS) injection. Hence, the TRIF pathway might have slower activation kinetics and, thus, might not be associated with pain induction, but rather pain resolution. The *Tlr3*$^{-/-}$ mice injected with IT TLR4-L (LPS) had a complete resolution of pain by 7 days, suggesting that the absence of TLR3, may have facilitated TLR4 signaling toward TRIF activation.

Activation of TLR2/TLR4 in a variety of cell systems, including glia in the present study, increases TNF synthesis and release [27]. In the present work, TA after IT TLR2-L and TLR4-L was reduced in *Tnf*$^{-/-}$ mice, indicating that TLR2/TLR4 allodynia is TNF-dependent (Figure 9). These results are consistent with previous work in which IT TNF inhibitors block effects of IT TLR4 agonists [51]. Further, TNF is upregulated in various chronic inflammatory and nerve injury models [14,50,52,61], but other inflammatory cytokines such as IL-1 and IL-6 have also been associated with spinal pain states. In contrast to effects observed with TLR2 and TLR4 ligands, TLR3 activation in *Tnf*$^{-/-}$ mice produced a TA, suggesting that the TLR3/TRIF pathway leading to TA is TNF *independent*. This lack of effect of TNF on TLR3 allodynia was unexpected as the TRIF pathway (downstream to both TLR3 and TLR4), can induce NF-κB activation and cytokine production [43,60]. *Myd88*$^{-/-}$ mice retain TLR4-L activated phosphorylation of mitogen-activated protein kinase family members (ERK1/2, p38 kinase and Jun kinase), and NF-κB, albeit with delayed kinetics [26]. Conversely, in the absence of TRIF signaling (e.g. *Trif*^{lps2}), TLR4 stimulation continues to activate ERK and IκBα degradation and produce TNF [19].

TRIF signaling in spinal TLR initiated allodynia

The TRIF adapter protein is common to TLR3 and TLR4 cascades, but not TLR2, and functions through IRF3 to induce type I interferon production, specifically IFNβ [16,20,21,42,57]. Consistent with this cascade, IT TLR3-L, but not TLR2-L initiated TA was abolished in *Trif*^{lps2} mice. However IT TLR3-L initiated TA was unaltered in *Tnf*$^{-/-}$ mice. Other proinflammatory cytokines, such as IL-6, or IL-1β, down stream to TRIF, likely provided parallel signaling in the *Tnf*$^{-/-}$ mice that received IT TLR3-L.

An unexpected finding was the exaggerated effect of IT TLR4 ligand signaling in the *Trif*^{lps2} and *Ifnar1*$^{-/-}$ mice. IT TLR4 agonism resulted in a robust, long-lasting (>21 days) TA, which was transiently reversed by gabapentin. These results suggested that the 7-day course of TLR4 mediated TA in C57/Bl6 mice was, in part, dependent upon TRIF signaling. *Trif*^{lps2} mice do not produce IFNβ after TLR stimulation, while *Ifnar1*$^{-/-}$ mice produce IFNβ, but the IFN receptor is unable to signal. Since *Trif*^{lps2} mice had functional IFN receptors, we showed the importance of IFNβ in regulating spinal TLR4 initiated TA by reintroducing it in the *Trif*^{lps2} mouse by IT delivery. While *Trif*^{lps2} mice continued to respond to IT TLR2-L, IT IFNβ also antagonized that TA. Accordingly, given the TLR4 coupling though *Tirap*$^{-/-}$ and *Trif*^{lps2}, we believe that unlike TLR2, which activates only the TIRAP-TNF cascade, TLR4 initiates an allodynic state though the TIRAP-TNF pathway, and concurrently activates the TRIF-IFN-β pathway which counter-modulates TIRAP-TNF cascade (Figure 9).

With regard to spinal IFN, the effects of type I interferons are complex. Both IT IFNα and IFNβ inhibit CFA-hypersensitivity [56] and proinflammatory cytokine upregulation [28]. Conversely, IFNα has been reported to enhance excitatory transmission [48]. Consistent with present spinal results, IFNβ suppression of proinflammatory cytokines has been shown in a variety of peripheral and neuraxial inflammatory states [11,36,37,53]. Mechanisms of IFNβ action can include an increase in anti-inflammatory IL-10, reduction of proinflammatory IL-17 and modulation of matrix metalloproteinase [31]. Also, IFNβ stimulates the production of IL-1 receptor antagonist (IL1Ra), which directly binds to the IL-1 receptor as an endogenous regulator. An imbalance between IL-1β and IL-1Ra has been reported in the spinal fluid of patients with rheumatoid arthritis, a condition with chronic inflammation and pain [34].

TLRs and glial activation

Microglia show constitutive expression of virtually all TLRs, while astrocytes predominately express TLR3 and sometimes TLR2 [3,4,24]. We show that with primary microglia and astrocyte cultures, ligands activating a TIRAP cascade released TNF. Conversely, TLR3 coupled though TRIF displayed IFNβ release. *In vivo,* spinal glial cells play a role in nociceptive processing. Intrathecal inhibitors of glial activation suppress injury-evoked hyperpathias [22,39,49,51]. Upon activation, glia release proinflammatory mediators, including cytokines such as TNF, which activate neighboring glial cells and neurons leading to a facilitation of their response to subsequent afferent traffic and an increase in IFNβ. While we specifically assessed TLR activation on primary glial cells, TLR3 expression by brain neurons [33,46], and TLRs 3, 4, 7 and 9 in dorsal root ganglia (DRG) [1,47] has been reported, suggesting that neurons can directly respond to TLR ligands. Cultured mouse DRGs stimulated with TLR ligands augment expression of proinflammatory chemokines and cytokines, and repression of the TRPV1 receptor [47], demonstrating the complexity of TLR responses in several molecular mechanisms associated with pain responses.

In conclusion, IT TLR agonists show the robust effects of spinal TLR activation on nociceptive processing and the complexity of the downstream signaling initiated by the direct activation of these spinal TLRs. The present work reveals TNF-dependent and -independent facilitatory signaling, as well as an unexpected modulatory feedback through the TRIF pathway of TNF-dependent signaling. While we emphasize here the effects of TLR activation on spinal cytokines, processes leading to activation of NF-κB are also associated with the upregulation of a variety of channels and transcription factors such as ATF3 [17]. TLR4 null mice have indeed been shown to prevent such changes in ATF3 expression [9]. Finally, these effects reflect the downstream coupling initiated by tissue and nerve injury states in the absence of an infectious process, which can lead to the release of

molecules shown to activate spinal TLRs [6,13,15,25,35,45]. Elucidation of the events leading to such release, and the subsequent activation of the downstream facilitatory and inhibitory cascades noted here is an important target for future research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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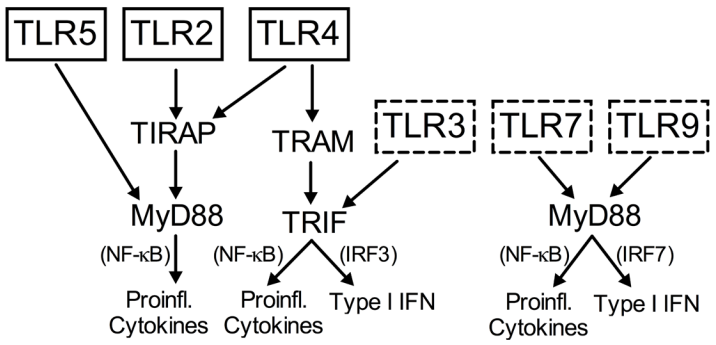
Summary

Spinal TLR activation mediates persistent change leading to robust TA by TNF-dependent and -independent pathways. TRIF leads to modulation of TLR-evoked TA mediated by IFNβ.

A



B

Target Protein	Genotype Nomenclature	Type of Protein Deficiency
TLR2	*Tlr2*$^{-/-}$	TLR2 whole-body knock-out
TLR3	*Tlr3*$^{-/-}$	TLR3 whole-body knock-out
TLR4	*Tlr4*$^{-/-}$	TLR4 whole-body knock-out
TLR5	*Tlr5*$^{-/-}$	TLR5 whole-body knock-out
TIRAP	*Tirap*$^{-/-}$	TIRAP adaptor protein whole-body knock-out
TRIF	*Trif*^{lps2}	TRIF adaptor protein point-mutation on the *ticam1* gene
TNF	*Tnf*$^{-/-}$	TNF whole-body knock-out
IFNAR	*Ifnar*$^{-/-}$	Alpha chain of the IFN receptor whole body knock-out, which binds both IFNα and IFNβ

Figure 1. Schematic of the TLR pathways

(A) This figure highlights the key TLRs and their relevant pathways in this paper. TLR2, 4, and 5 are found on the cell surface, while TLR3, 7, and 9 are in the cell endosomes. MyD88 is a key adaptor protein for all TLRs except TLR3. TIRAP is exclusive to TLR2 and TLR4 leading to proinflammatory cytokine release. TRIF is restricted to TLR3 and TLR4, resulting predominantly in type I interferon production. (B) This table summarizes the knock-out mice used in the presented studies and the nomenclature used throughout the paper.

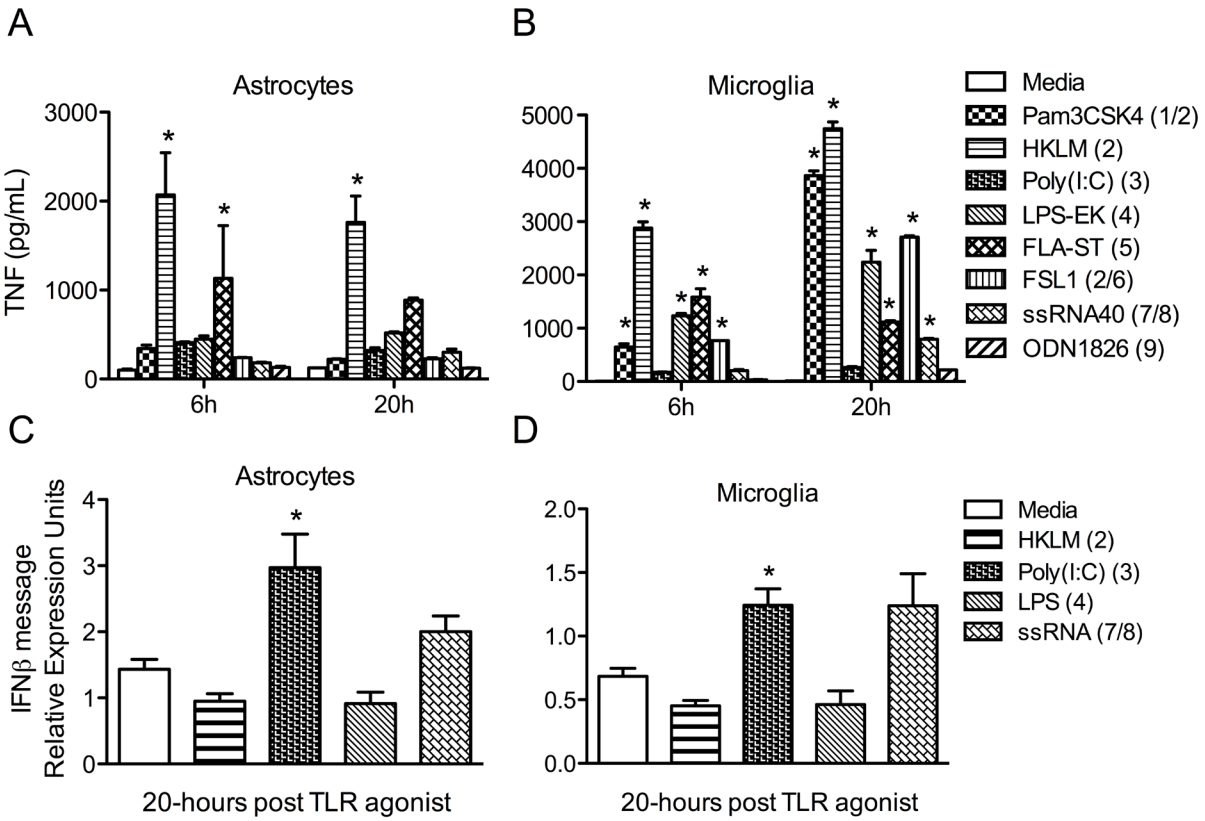


Figure 2. TLR activation of rat primary cultured astrocyte and microglia

Specific ligands of TLRs were added to primary spinal astrocyte (A, C) and microglia (B, D) cell cultures (Supplementary Table 1). Media samples were harvested, and evidence of cell activation was assessed by measurement of TNF in the astrocyte (A) and microglia (B) media samples at 6-hours and 20-hours post TLR agonist addition. IFNβ mRNA was detected using quantitative RT-PCR for astrocyte (C) and microglia (D) cultures 20-hours post TLR agonist addition. Data are expressed as mean ± SEM (n=3 samples/group) and analyzed by 1-way ANOVA, followed by Dunnett's *post hoc* test to compare each treatment to the media control (*$p<0.05$).

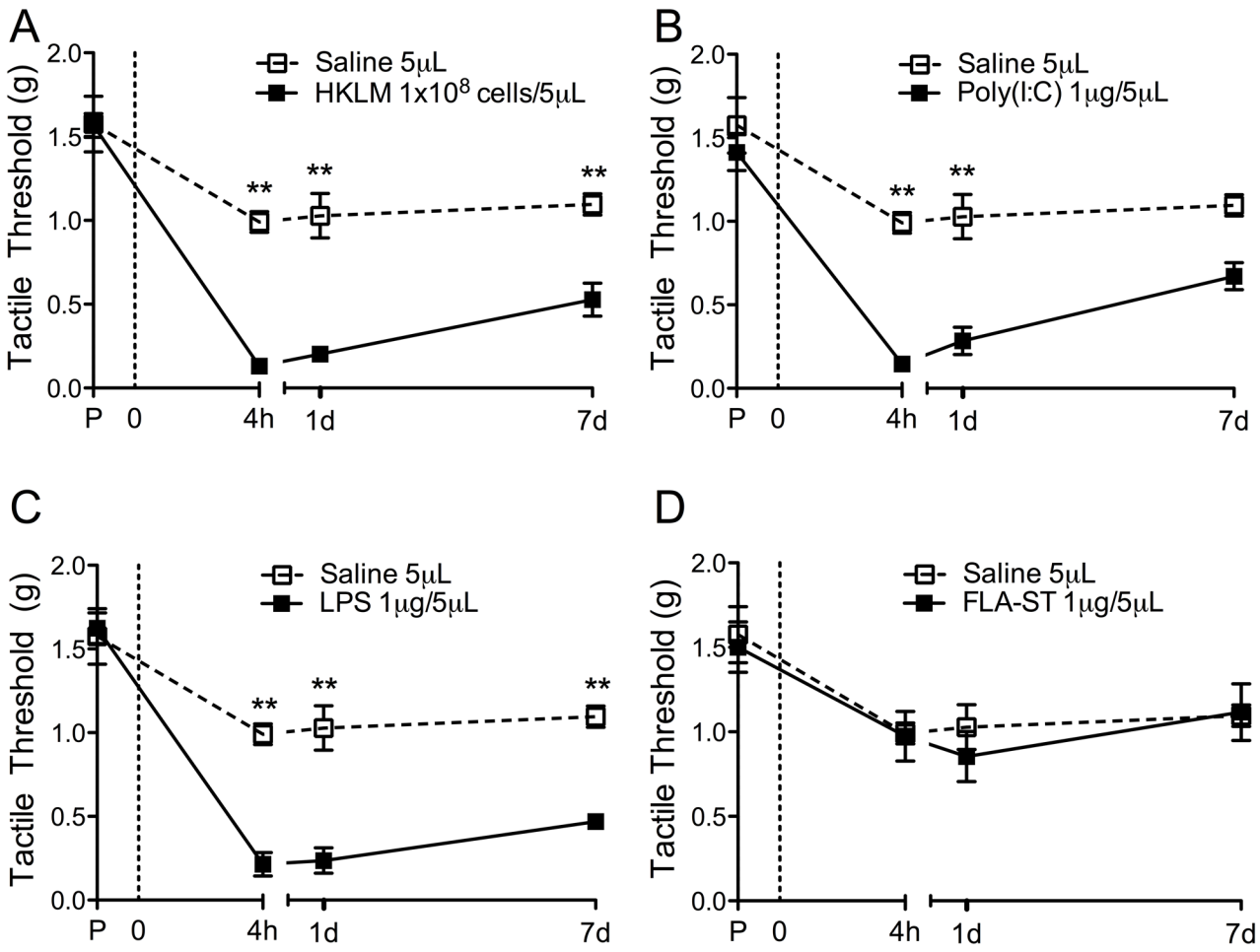


Figure 3. IT administration of TLR ligands in C57Bl/6 mice produced robust TA

TLR ligands were spinally delivered via IT injection at the L5 level in C57Bl/6 mice. The effect of intrathecal (IT) administration of TLR ligands (Supplemetary Figure 2) on tactile thresholds was measured by von Frey filament testing, using the up-down method (A–D). Data expressed as mean ± SEM (n=5–8 mice/group) and analyzed via 2-way ANOVA, followed by Bonferroni *post hoc* test to compare each time point to the IT saline group (*p<0.05; **p<0.01). The same IT saline group is represented in all four graphs.

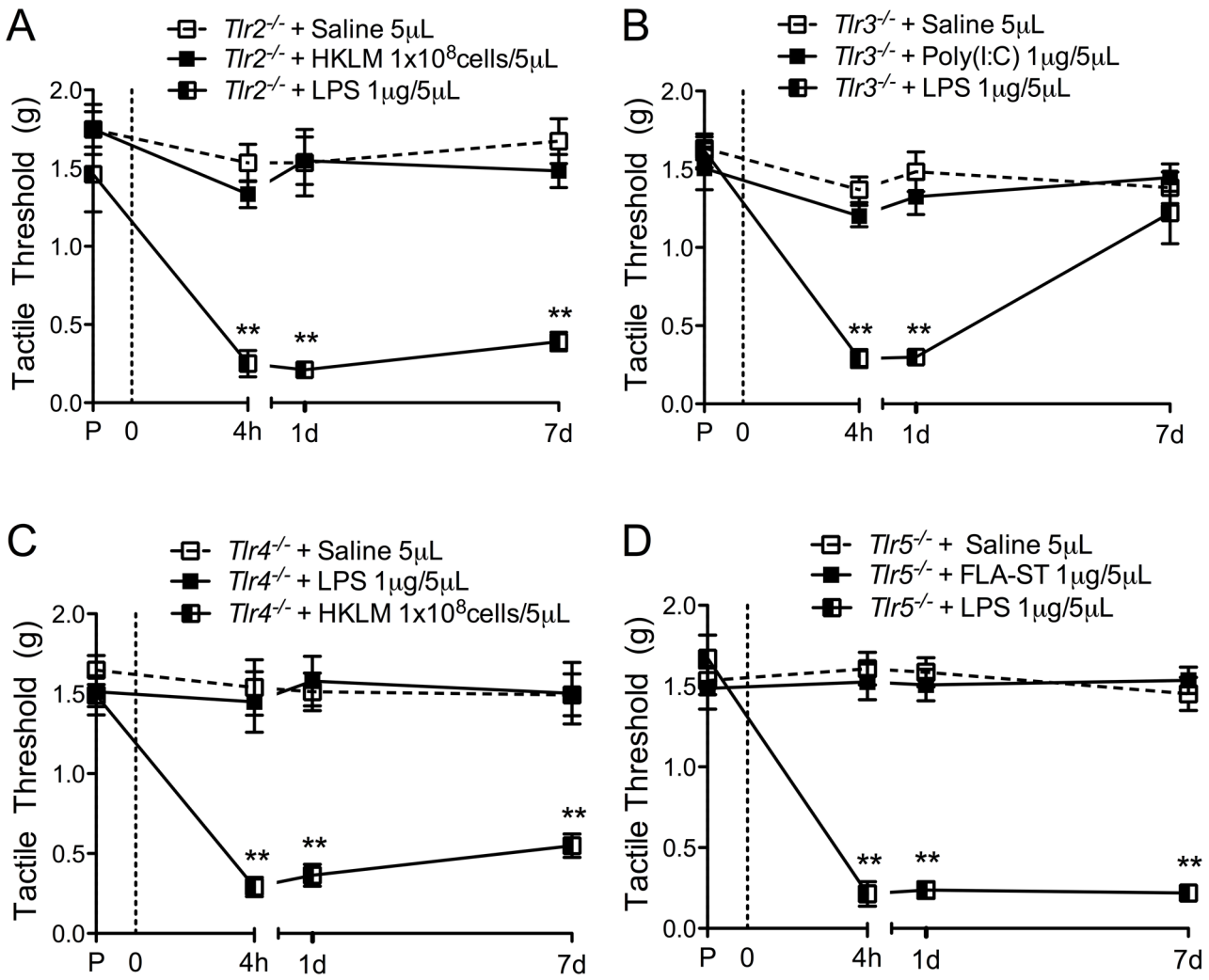


Figure 4. Specific TLR null mice confirm TLR ligand specificity

TLR ligands were spinally delivered via IT injection at the L5 level in *Tlr2*$^{-/-}$ (A), *Tlr3*$^{-/-}$ (B), *Tlr4*$^{-/-}$ (C), and *Tlr5*$^{-/-}$ (D) mice. Saline and TLR ligand matching that of the TLR null mouse were first used to confirm agonist specificity. A different TLR ligand was then administered to show that the each mouse strain was still able to produce a robust TA following TLR activation. The effect of intrathecal (IT) administration of TLR ligands on tactile thresholds was measured by von Frey filament testing, using the up-down method. Data expressed as mean ± SEM (n=5–8 mice/group) and analyzed via 2-way ANOVA, followed by Bonferroni *post hoc* test to compare the groups over the entire time course (*p<0.05; **p<0.01). Asterisks indicate comparison of corresponding TLR ligand group vs. different TLR ligand group. All comparisons of the IT saline vs. corresponding TLR ligand group, were not significant in these panels.

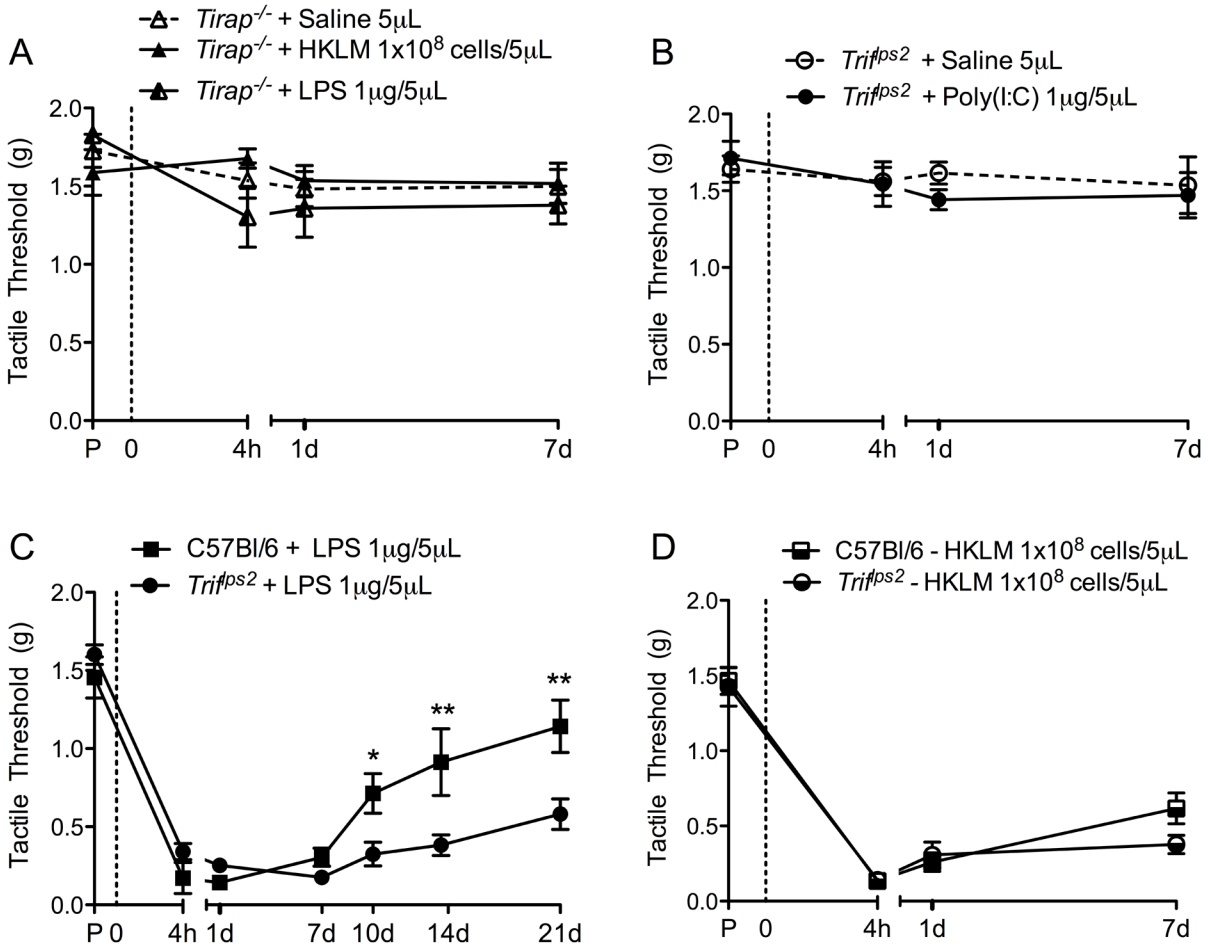


Figure 5. Functional loss of specific TLR adaptor proteins suggest TRIF-mediated resolution pathway

TLR ligands were spinally delivered via IT injection at the L5 level in *Tirap*$^{-/-}$ (A) and *Trif*^{lps2} mice (B, C, D). In *Tirap*$^{-/-}$ mice there was no effect on tactile thresholds of IT HKLM or LPS when compared to IT saline (A). *Trif*^{lps2} mice showed a robust long-lasting TA following IT LPS (B). IT Poly(I:C) had no effect on tactile thresholds in the *Trif*^{lps2} mice (C), but IT HKLM produced a robust TA in *Trif*^{lps2} mice (D). Data expressed as mean ± SEM (n=4–7 mice/group) and analyzed via 2-way ANOVA, followed by Bonferroni post hoc test to compare the *Trif*^{lps2} group to the C57Bl/6 group (*p<0.05; **p<0.01).

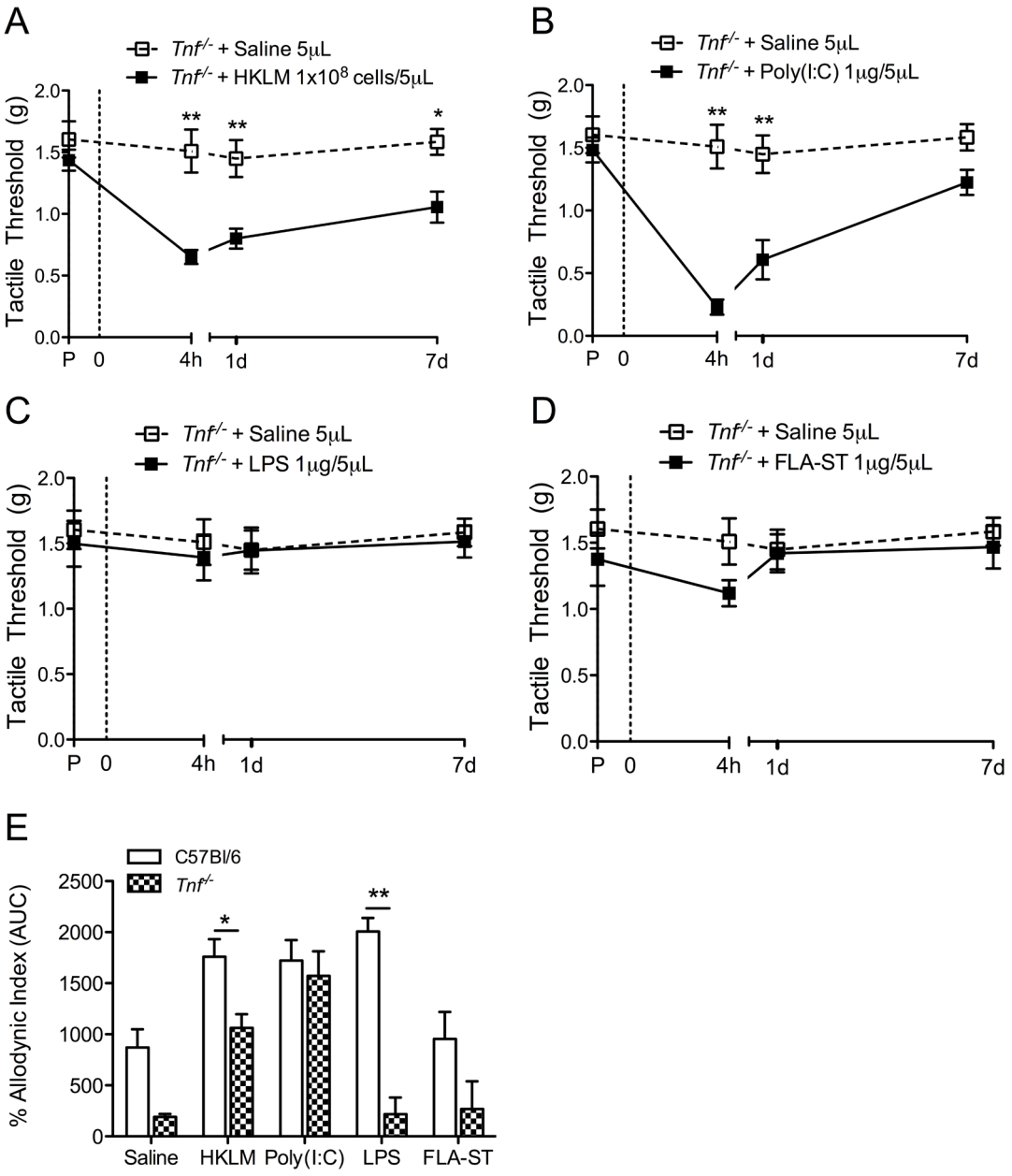


Figure 6. TLR activation in Tnf$^{-/-}$ mice

TLR ligands were spinally delivered via IT injection at the L5 level in *Tnf*$^{-/-}$ mice. The effect of intrathecal (IT) administration of TLR ligands on tactile thresholds was measured by von Frey filament testing, using the up-down method. (A–D) Data expressed as mean ± SEM (n=5–8 mice/group) and analyzed via 2-way ANOVA, followed by Bonferroni *post hoc* test to compare each time point to the IT saline group (*p<0.05; **p<0.01). The same IT saline group is represented in all four graphs. (E) Hyperalgesic indices were calculated for each mouse using their individual baseline threshold and calculating the area under the curve. Data expressed as mean ± SEM. Hyperalgesic index was analyzed via 2-way ANOVA followed by Bonferroni *post hoc* test to compare each C57Bl/6 treatment group to the same *Tnf*$^{-/-}$ treatment group (*p<0.05; **p<0.01).

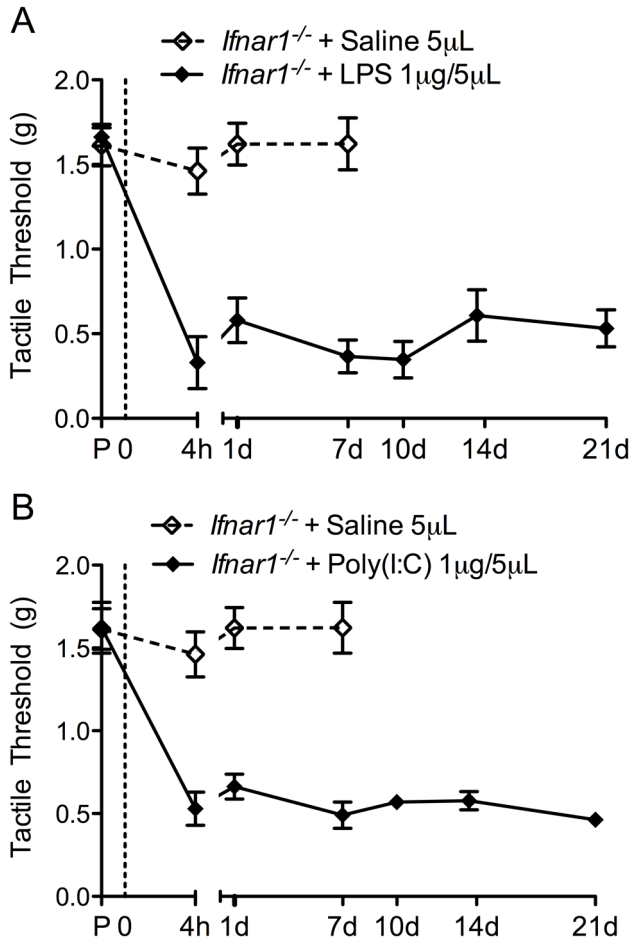


Figure 7. IT TLR3 and TLR4 agonist produce TA in Ifnar1$^{-/-}$ mice

Ifnar1$^{-/-}$ mice were treated with IT TLR3-L (Poly(I:C)) and TLR4-L (LPS). (A) IT LPS produced a robust long-lasting TA, very similar to that observed in the *Trif*^{lps2} mice. (B) IT Poly(I:C) agonist also produced a robust TA, but was slower onset when compared to IT LPS. The same IT saline group is represented in both graphs. Data expressed as mean ± SEM (n=6–7 mice/group). The same C57Bl/6 saline group is represented in this figure. Data analyzed via 1-way ANOVA followed by Bonferroni *post hoc* test to compare IT saline treatment group to IT LPS and IT Poly(I:C) treatment group. For IT saline vs. IT LPS and IT Saline vs. IT Poly(I:C) $p<0.01$.

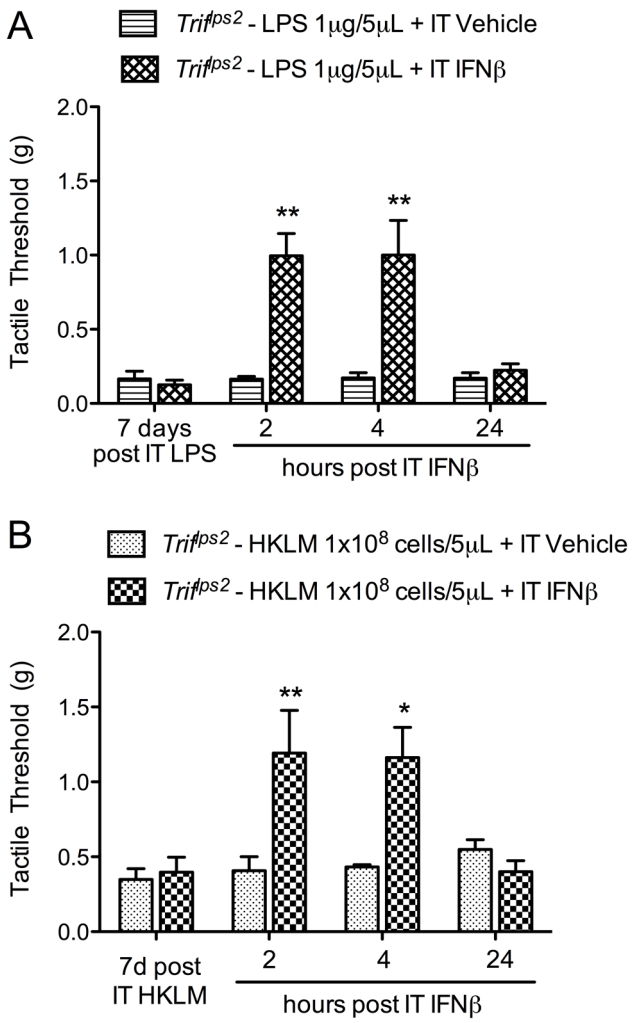


Figure 8. Post-treatment with IFNβ blocks LPS and HKLM-induced TA in Trif^{lps2} mice

Trif^{lps2} mice were treated with either IT LPS (1μg/5μL) or IT HKLM (1×10^8 cells/5μL) 7 days before IT IFNβ (100ng/5μL). Post-treatment with IFNβ 7-days post IT LPS (1μg/5μL) transiently reversed the LPS-induced TA (A) and HKLM-induced TA (B). Data expressed as mean ± SEM (n=3–4 mice/group) and analyzed via 2-way ANOVA, followed by Bonferroni *post hoc* test to compare each time point after IFNβ post-treatment to the respective 7-day post IT LPS or HKLM group (*p<0.05; **p<0.01).

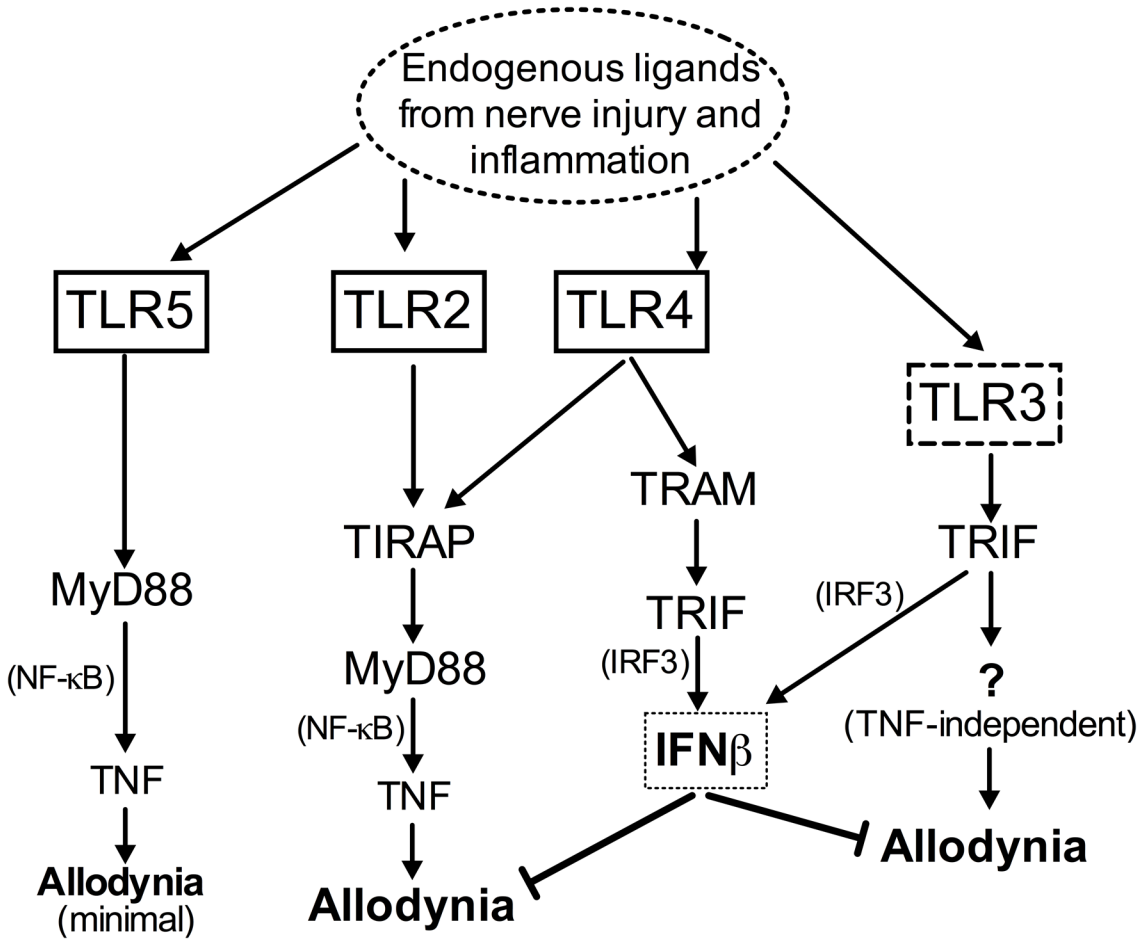


Figure 9. Schematic of the TLR cascades emphasized in the present work

Endogenous ligands present in the injured and inflamed system activate resident TLRs, localized to glia and/or neurons. Based on the effects of IT TLR ligands, it is hypothesized that TLRs signaling through TIRAP and MyD88 lead to NFκB mediated cytokine (TNF) release and to a TNF dependent allodynia (e.g. TLR2 and TLR4). However, TLR3 leads to a TNF independent allodynia and IFNβ production. Based on the effects of *Ifnar*$^{-/-}$ mice, the increased IFNβ production regulates the allodynic actions mediated by TIRAP (TLR2/TLR4 activation) and induced by IT TLR3-L. As TLR4 activated both TRIF and TIRAP signaling, the net allodynic effect reflects the facilitation mediated by spinal TNF release and the inhibition initiated by TRIF mediated IFNβ production.