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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. We examined 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 tumor necrosis factor (TNF) dependent, whereas TLR3 was TNF-independent. In Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP; *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 TIR-domain-containing adapter-inducing IFNβ [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 TIR domain-containing adaptor protein (TIRAP) and TRIF cascades differentially lead to robust TA by TNF-dependent and independent pathways, whereas 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.

1. 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 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 through 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 that reduce glial activation can inhibit the facilitatory effects of IT TLR agonists [22,39,49,51].

There are 13 identified TLRs, some localized to the cell surface and others on endosomes, which signal through a limited number of adaptor proteins (Fig. 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 Nuclear Factor kappaB (NF-κB), yielding production of proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (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 1 of the multiple spinal TLRs cannot be predicted in the absence of specific data on outcomes associated with defined spinal TLR activation.

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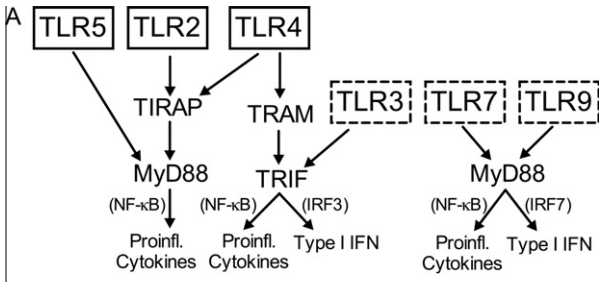

B

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

Fig. 1. Schematic of the Toll-like receptor (TLR) pathways. (A) This figure highlights the key TLRs and their relevant pathways in this report. TLR2, 4, and 5 are found on the cell surface, whereas TLR3, 7, and 9 are in the cell endosomes. MyD88 is a key adaptor protein for all TLRs except TLR3. Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein is exclusive to TLR2 and TLR4, leading to proinflammatory cytokine release. TIR-domain-containing adapter-inducing interferon-β is restricted to TLR3 and TLR4, resulting predominantly in type I interferon production. (B) This table summarizes the knockout mice used in the presented studies and the nomenclature used throughout the report.

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 interferon (IFN) β after TLR activation. To assess the 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) whereas 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 proallodynic cascades are differentially activated by TRIF and TIRAP signaling, and a potential suppressive role of TRIF signaling through IFNβ.

2. Methods

2.1. 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). Mice were housed up to 4 per standard cage at room temperature and maintained on a 12-hour light/dark cycle (light on at 07:00 AM). Testing was performed during the light cycle. Food and water were available ad libitum. C57BL/6 mice (male, 25 to 30 g) were purchased from Harlan (Indianapolis, IN). *Tlr2*$^{-/-}$, *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, and *Tirap*$^{-/-}$ mice were a gift from Dr. S. Akira (Osaka University, Osaka, Japan) and were bred for 10 generations onto the C57Bl/6 background. *Trif*^{lps2} mice were a gift from Dr. B. Beutler (UT Southwestern, Dallas, Texas) and were directly generated on the C57Bl/6 background. *Tlr5*$^{-/-}$ and *Tnf*$^{-/-}$ mice were purchased from 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. Fig. 1B lists the murine strains used and the standard nomenclature to be used throughout the report.

2.2. Rat microglia and astrocyte primary cell culture

Purified cultures of rat spinal microglia and astrocytes were prepared as previously described with some modifications [22]. The 1- to 3-day-old Holtzman Sprague-Dawley rat pups were anesthetized, and the spinal cords were ejected, mechanically triturated, and then centrifuged at 215*g* for 5 minutes and resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Gibco, Carlsbad, CA), 1% penicillin/streptomycin (Gibco), and plated in a flask previously coated with poly L-lysine (Sigma, St. Louis, MO). Flasks were maintained at 37°C in a humidified 5% CO_2 incubator for 2 weeks until 80% to 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 minutes, 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 minutes, resuspended in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin, and plated on to 24-well plates at 100,000 cells/mL until they reached 70% to 80% confluence (2 days). Individual wells were stimulated with 5 μL of specific murine TLR agonists available in a complete kit (Invivogen, San Diego, CA: tlrl-kit1mw; Supplementary Table 1). The doses chosen were within the range recommended by the manufacturer.

2.3. TNF enzyme-linked immunosorbent assay

TNF in the culture supernatant was assayed at both 6 and 20 hours after TLR agonist administration by enzyme-linked immunosorbent assay kits per the manufacturer instructions (R&D Biosystems, Minneapolis, MN). TNF is expressed as pg/mL of culture media sample.

2.4. Quantitative real-time polymerase chain reaction (PCR)

At 20 hours after TLR agonist addition to the culture media, the media was removed and immediately replaced with 0.5 mL of Trizol (Invitrogen, Carlsbad, CA), allowed to sit for 2 minutes, then flash frozen. The mRNA was then isolated using RNeasy columns (Qiagen, Hilden, Germany). Complementary DNA was prepared using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real-time PCR was performed with predeveloped specific primers and probes (Taqman Gene Expression Assay, Applied Biosystems, Foster City, CA) were used to detect mouse IFNβ1 (Assay ID Rn00569434_s1) and glyceraldehyde 3-phosphate dehydrogenase (Assay ID Rn01775763_g1) (Applied Biosystems). The relative abundance was calculated by comparing delta-Ct values [44], and the data were then normalized to glyceraldehyde 3-phosphate dehydrogenase gene expression and presented as relative gene expression.

2.5. Primary cell culture staining

Primary microglia and astrocytes cells in complete DMEM (cDMEM) were aliquoted (200 μL) into coated 8-chamber cell cul-

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ture 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% paraformaldehyde (PFA), then washed with phosphate-buffered saline and stained overnight in primary antibodies at 4°C. Astrocytes and microglia were incubated with anti-Vimentin (Invitrogen (Carlsbad, CA) 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 were captured on a Leica confocal microscope.

2.6. Lactate dehydrogenase (LDH) cytotoxicity assay

Cytotoxicity after the TLR ligands was assessed by LDH release. LDH was measured in media samples of microglia and astrocytes after each TLR ligand treatment group at the 20-hour time point using a CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) following the manufacturer's directions.

2.7. IT injection and drug administration

IT needle placement procedure for the IT saline and IT sham treatment was 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-inch, 30-gauge needle attached to a 50-μL Hamilton syringe was inserted between the L5 and L6 vertebrae, evoking a tail flick reflex. The following TLR agonists were administered in 5 μL diluted in 0.9% sterile saline: HKLM (1×10^8 cells/5 μL), Poly(I:C) (1 μg/5 μL), LPS-EK Ultrapure (1 μg/5 μL), and FLA-ST (1 μg/5 μL) (Supplementary Table 2). The doses were chosen from the Invivogen product protocol, as well as the results of the cell culture studies outlined earlier. Doses ranging from 0.1 μg/5 μL to 10 μg/μL were first tested, and 1 μg/μL produced the maximal effect at the lowest dose for all ligands. For the TLR5 ligand (FLA-ST) the 10 μg/5 μL dose showed the same minimal tactile allodynia (TA) as the 1 μg/5 μL dose (Supplementary Fig. 2A). Thus, the 1 μg/5 μL 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.

After recovery from anesthesia, as evidenced by a vigorous righting reflex and spontaneous ambulation, typically around 1 to 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 = preinjection), 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, although all the above time points were recorded, only the baseline, 4-hour, 24-hour, and 7-day time points are presented here. IFNβ (Chemicon, 100 ng/5 μL in 0.1% BSA (Bovine Serum Albumin)) was administered intrathecally either 1 hour before IT LPS (1 μg/5 μL) or as a posttreatment, 7 days after IT LPS induced TA. Gabapentin (Toronto Research Chemicals, Toronto, Ontario, Canada) was administered (100 mg/kg) intraperitoneally diluted in 0.9% sterile saline.

2.8. Behavioral tests

Mechanical sensitivity was assessed using the von Frey up-down method. Filaments with values ranging from 2.44 to 4.31 (0.03*g* to 2.00*g*) 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.

2.9. Western blot

Mice were deeply anesthetized, 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 (Ethylenediaminetetraacetic acid), 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% to 12% Bis-Tris gel electrophoresis and then transferred to nitrocellulose membranes (Micronic Separation, Westborough, MA). Membrane was first blocked with 5% nonfat 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). Intensity of immunoreactive bands was quantified using Image Quant software (Molecular Dynamics, Sunnyvale, CA). 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 because it provided a more accurate differential lane expression.

2.10. 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 a 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: $100 \times ((\text{baseline threshold} - \text{treatment threshold})/(\text{baseline threshold}))$. Multiple comparison tests were performed by 2-way ANOVA with Bonferonni post hoc tests. Statistical analyses used Prism software (GraphPad Software, San Diego, CA).

3. Results

3.1. Primary astrocyte and microglia cultures

Rat spinal primary microglia and astrocyte cell cultures were generated from neonatal rats and stained with antivimentin and anti-Iba-1 to assess purity (Supplementary Fig. 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 were assayed by enzyme-linked immunosorbent assay. Primary microglia cultures (Fig. 2B) showed a robust TNF release at the 6-hour time point after the addition of TLR2, 4, and 5 ligands, and at the 20-hour time point after TLR1/2, 2, 4, 5, and 2/6 ligands. Primary astrocyte cultures (Fig. 2A) showed a significant level of TNF release at the 6-hour time point after the addition of TLR2 and 5 ligands, and at the 20-hour time point after TLR2, 4, and 5 ligands. The TLR3-L (Poly(I:C)) produced a minimal response, as expected because TNF release has been reported to have slower kinetics and to be at a lower level. However, both astrocyte (Fig. 2C) and microglia (Fig. 2D) showed an increase in IFNβ after TLR3-L (Poly(I:C)) and TLR7/8 (ssRNA40) agonist treatment, which attained statistical sig-

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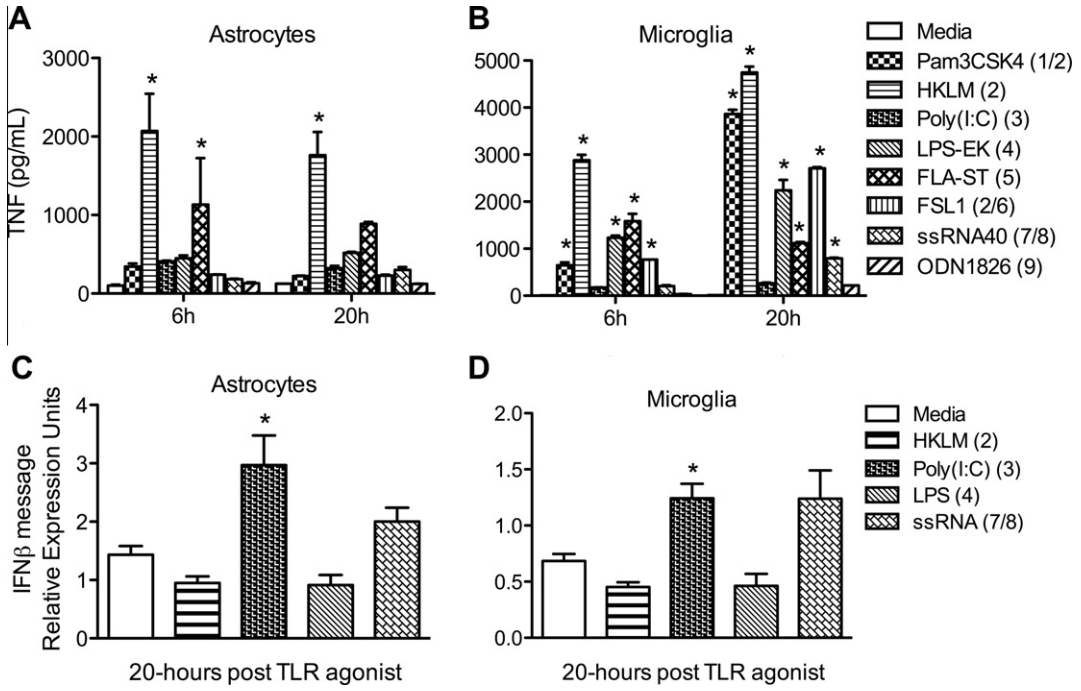

Fig. 2. Toll-like receptor (TLR) activation of rat primary cultured astrocyte and microglia. Specific ligands of TLRs were added to primary spinal astrocyte (A and C) and microglia (B and D) cell cultures (Supplementary Table 1). Media samples were harvested, and evidence of cell activation was assessed by measurement of tumor necrosis factor in the astrocyte (A) and microglia (B) media samples at 6 and 20 hours after TLR agonist addition. Interferon β mRNA was detected using quantitative real-time polymerase chain reaction for astrocyte (C) and microglia (D) cultures 20 hours after TLR agonist addition. Data are expressed as mean ± SEM (n = 3 samples/group) and analyzed by 1-way analysis of variance, followed by the Dunnett post hoc test to compare each treatment to the media control (*$P < .05$).

nificance for Poly(I:C) treatment. To control for cytotoxicity, LDH release was measured in the primary cell culture media samples at 20 hours after TLR agonist addition. In both the microglia and the astrocyte cultures, only Poly(I:C) and LPS administration resulted in minimal cell death signal (5% to 10%), whereas the other TLRs had no apparent effect (Supplementary Fig. 1A and B).

3.2. 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 (Fig. 2). TLR ligands were spinally delivered via IT injection at the L5 level in C57Bl/6 mice (Fig. 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 after injection. We previously noted the effects of the use of isoflurane in this procedure and TA [55], and thus although all of 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 (Fig. 3A–C). Alternatively, TLR5-L (FLA-ST) produced a short-lived 3-hour TA that was resolved by the 4-hour time point (Fig. 3D and Supplementary Fig. 2B).

3.3. 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 used because 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 (Fig. 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 (Fig. 4A, B and D, and Supplementary Fig. 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 (Fig. 4C). These results confirm not only the specificity of the selected TLR ligands, but also the integrity of the gene-targeted strain and its signaling pathways.

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

3.5. 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

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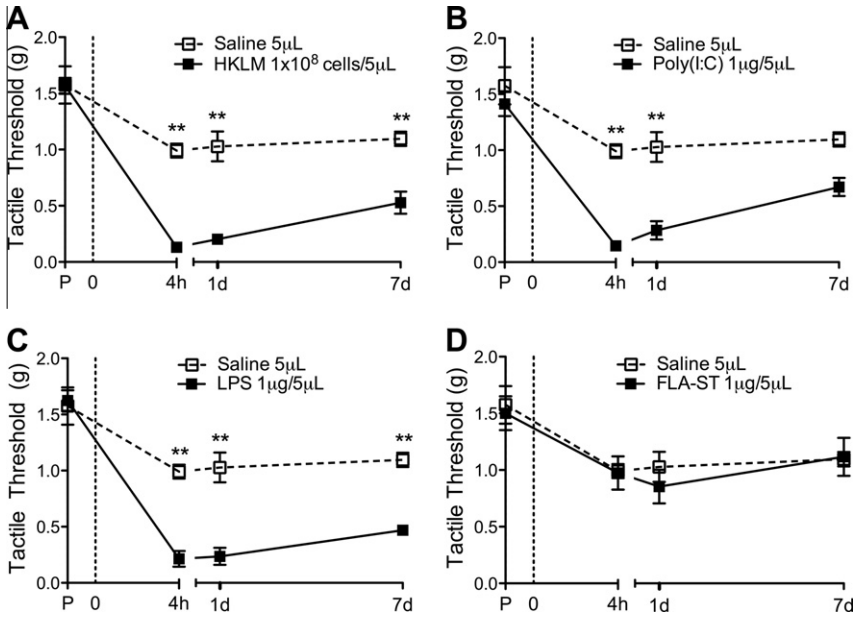

Fig. 3. Intrathecal (IT) administration of Toll-like receptor (TLR) ligands in C57Bl/6 mice produced robust tactile allodynia. TLR ligands were spinally delivered via IT injection at the L5 level in C57Bl/6 mice. The effect of IT administration of TLR ligands (Supplementary Fig. 2) on tactile thresholds was measured by von Frey filament testing using the up-down method (A to D). Data were expressed as mean ± SEM (n = 5 to 8 mice/group) and analyzed via 2-way analysis of variance, followed by a Bonferroni post hoc test to compare each time point to the IT saline group (*$P < .05$; **$P < .01$). The same IT saline group is represented in all 4 graphs.

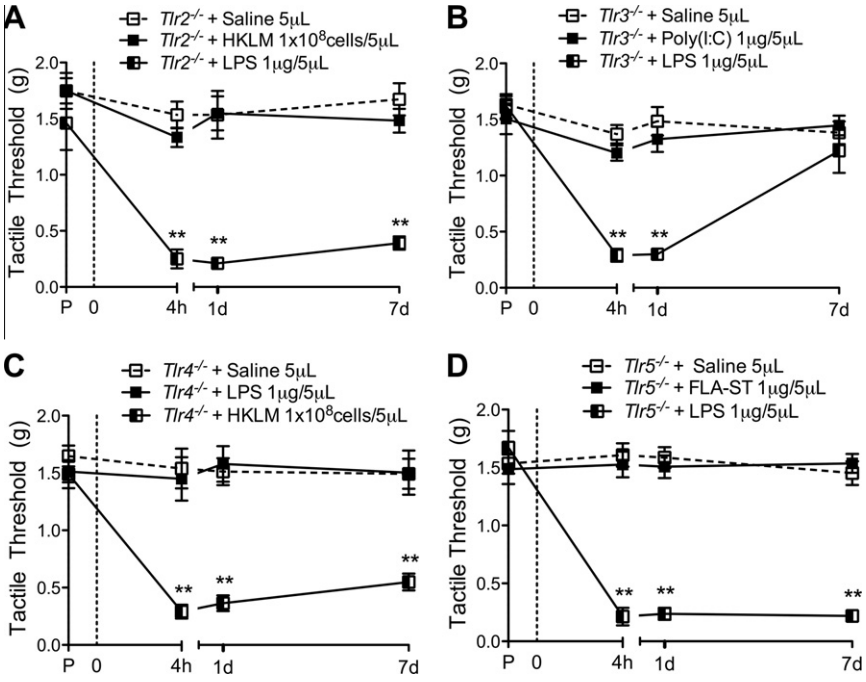

Fig. 4. Specific Toll-like receptor (TLR) null mice confirm TLR ligand specificity. TLR ligands were spinally delivered via intrathecal (IT) injection at the L5 level in *Tlr2*$^{-/-}$ (A), *Tlr3*$^{-/-}$ (B), *Tlr4*$^{-/-}$ (C), and *Tlr5*$^{-/-}$ (D) mice. Saline and TLR ligand matching that of the TLR null mouse were first used to confirm agonist specificity. A different TLR ligand was then administered to show that each mouse strain was still able to produce a robust tactile allodynia after TLR activation. The effect of 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 to 8 mice/group) and analyzed via 2-way analysis of variance, followed by a Bonferroni post hoc test to compare the groups over the entire time course (*$P < .05$; **$P < .01$). 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.

TLR ligands administration, *Tnf*$^{-/-}$ mice were used. *Tnf*$^{-/-}$ mice received IT injections of the same TLR ligands used previously. After IT TLR2-L (HKLM), only a modest TA was observed (Fig. 6A), whereas the IT TLR4-L (LPS) produced only a brief TA similar to that of IT saline (Fig. 6C). This result suggested that the TLR4 allodynia-inducing pathway is absolutely TNF dependent, whereas 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 (Fig. 6B), suggesting that the pronociceptive effects of the TLR3/TRIF pathway were 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 (Fig. 6D). Accordingly,

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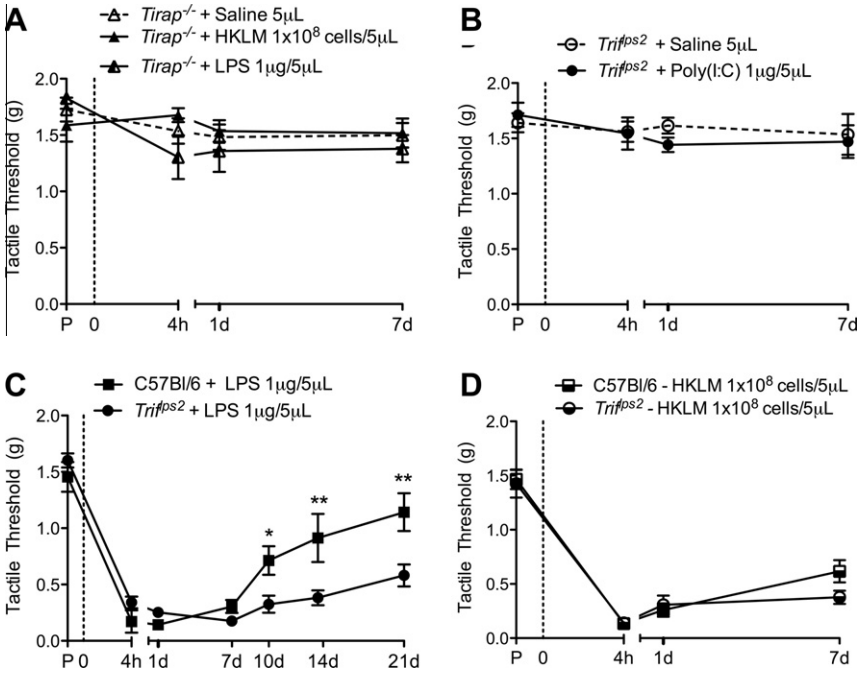

Fig. 5. Functional loss of specific Toll-like receptor (TLR) adaptor proteins suggests a Toll-interleukin 1 receptor domain-containing adapter-inducing interferon-β–mediated resolution pathway. Toll-like receptor ligands were spinally delivered via intrathecal (IT) injection at the L5 level in *Tirap*$^{-/-}$ (A) and *Trif*^{lps2} mice (B–D). In *Tirap*$^{-/-}$ mice, there was no effect on tactile thresholds of IT HKLM or LPS when compared with IT saline (A). *Trif*^{lps2} mice showed a robust long-lasting tactile allodynia after 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 tactile allodynia in *Trif*^{lps2} mice (D). Data are expressed as mean ± SEM (n = 4 to 7 mice/group) and analyzed via 2-way analysis of variance, followed by a Bonferroni post hoc test to compare the *Trif*^{lps2} group with the C57Bl/6 group (*$P < .05$; **$P < .01$).

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) (Fig. 6E), whereas the TLR3-L (Poly(I:C)) produced a robust effect.

3.6. 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 (Fig. 7A). This TA was transiently reversed by 100 mg/kg intraperitoneal gabapentin (Supplementary Fig. 3). The antiallodynic effects observed in these studies after systemic gabapentin are consistent with the presence of a facilitated state. In contrast to the *Trif*^{lps2} mice, the *Ifnar1*$^{-/-}$ mice developed prolonged allodynia after IT TLR3-L (Poly(I:C)) resembling the pattern of the IT TLR4-L (LPS)–treated mice (Fig. 7B). Together these results suggested that the TA after 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 after TLR3 signaling, an increase in spinal IFNβ was indeed detected by Western blot after TLR3-L (Poly(I:C)) (Supplementary Fig. 4).

3.7. Roles of spinal IFN in regulating spinal TLR–mediated TA

To test whether 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 (Fig. 5C), and have intact type I IFN receptor signaling. In *Trif*^{lps2} mice at 7 days after IT TLR4-L (LPS) or TLR2-L (HKLM), a posttreatment with IT IFNβ (100 ng/5 μL) reversed the TA (Fig. 8). In 2 separate TLR-induced TA pathways, IFNβ treatment temporarily alleviated the observed allodynia. Additionally, *Trif*^{lps2} mice were pretreated with IT IFNβ (100 ng/5 μL) 1 hour before IT LPS (Supplementary Fig. 5). Pretreatment 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 after TLR activation.

4. Discussion

In models of persistent inflammation, and mononeuropath/polyneuropathy, mice with defects in TLR expression show a prominent attenuation of behaviorally defined hyperpathy [4,8,11,28,36,49]. Although those studies used 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 antihyperpathic 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.

Previous reports of the contributions of 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 (Fig. 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 (Supplementary Table 2).

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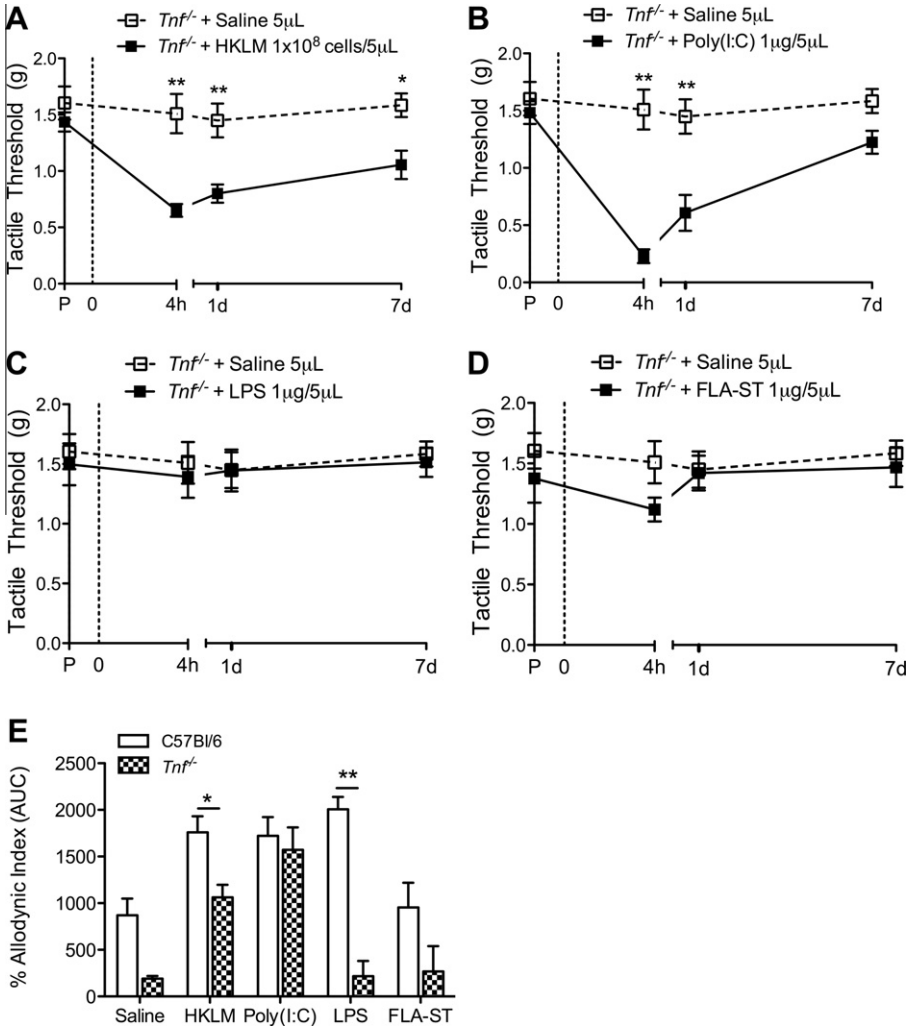

Fig. 6. Toll-like receptor (TLR) activation in $Tnf^{-/-}$ mice. TLR ligands were spinally delivered via intrathecal (IT) injection at the L5 level in $Tnf^{-/-}$ mice. The effect of IT administration of TLR ligands on tactile thresholds was measured by von Frey filament testing using the up-down method. (A to D) Data are expressed as mean ± SEM (n = 5 to 8 mice/group) and analyzed via 2-way analysis of variance, followed by a Bonferroni post hoc test to compare each time point to the IT saline group (*$P < .05$; **$P < .01$). The same IT saline group is represented in all 4 graphs. (E) Hyperalgesic indices were calculated for each mouse using their individual baseline threshold and calculating the area under the curve. Data are expressed as mean ± SEM. The hyperalgesic index was analyzed via 2-way analysis of variance, followed by a Bonferroni post hoc test to compare each C57Bl/6 treatment group to the same $Tnf^{-/-}$ treatment group (*$P < .05$; **$P < .01$).

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, although also a membrane TLR, produced only a short-lived (<4 hour) 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.

4.1. TLR coupling

Because 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 crosstalk 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 Fig. 9, IT TLR agonists in these mutant mice revealed several novel characteristics of these spinal TLR-effector cascades.

4.2. TIRAP signaling in spinal TLR-initiated allodynia

Consistent with coupling of TLR2 and TLR4, but not TLR3, through TIRAP, IT TLR2 and TLR4 agonist-initiated TA was absent in $Tirap^{-/-}$ mice, whereas 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 with 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

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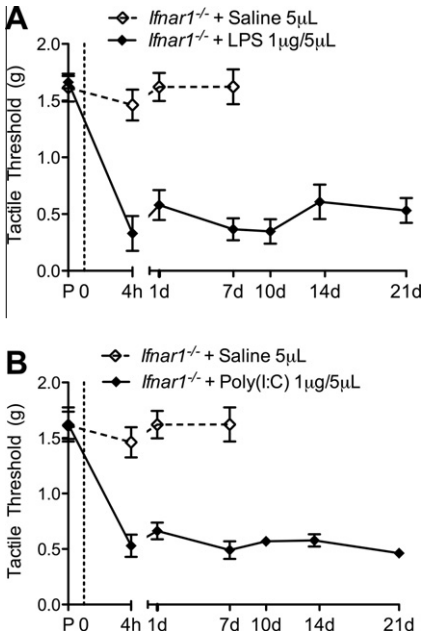

Fig. 7. Intrathecal (IT) Toll-like receptor (TLR) 3 and TLR4 agonist produce tactile allodynia (TA) in *Ifnar1*$^{-/-}$ mice. *Ifnar1*$^{-/-}$ mice were treated with IT TLR3-L (Poly(I:C)) and TLR4-L (LPS). (A) IT LPS produced a robust long-lasting TA, very similar to that observed in the *Trif*^{lps2} mice. (B) IT Poly(I:C) agonist also produced a robust TA, but was slower in onset when compared with IT LPS. The same IT saline group is represented in both graphs. Data are expressed as mean ± SEM (n = 6 to 7 mice/group). The same C57Bl/6 saline group is represented in this figure. Data analyzed via 1-way analysis of variance followed by a Bonferroni post hoc test to compare IT saline treatment group with IT LPS and IT Poly(I:C) treatment groups. For IT saline vs IT LPS and IT saline vs IT Poly(I:C), $P < .01$.

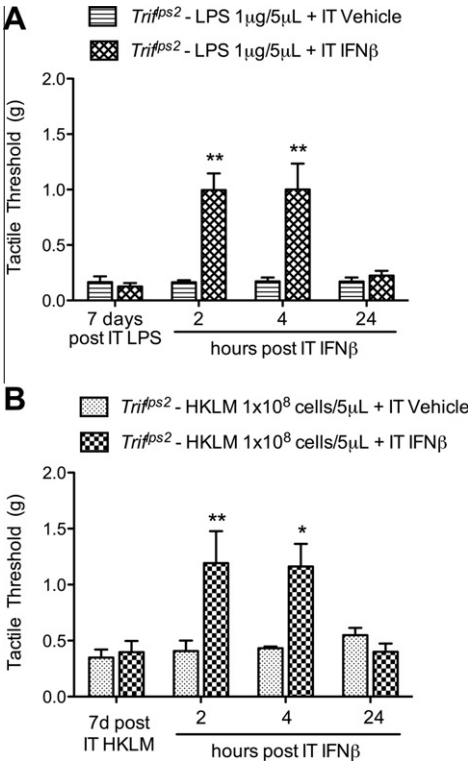

Fig. 8. Posttreatment with interferon (IFN) β blocks LPS- and HKLM-induced tactile allodynia (TA) in *Trif*^{lps2} mice. *Trif*^{lps2} mice were treated with either intrathecal (IT) LPS (1 μg/5 μL) or IT HKLM (1×10^8 cells/5 μL) 7 days before IT IFNβ (100 ng/5 μL). Posttreatment with IFNβ 7 days after IT LPS (1 μg/5 μL) transiently reversed the LPS-induced TA (A) and HKLM-induced TA (B). Data are expressed as mean ± SEM (n = 3 to 4 mice/group) and analyzed via 2-way analysis of variance, followed by a Bonferroni post hoc test to compare each time point after IFNβ posttreatment to the respective 7-day post–IT LPS or HKLM group ($^*P < .05$; $^{**}P < .01$).

[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 (Fig. 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 interleukin (IL)-1 and IL-6 also have 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 because 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 (eg, *Trif*^{lps2}), TLR4 stimulation continues to activate ERK and IκBα degradation and to produce TNF [19].

4.3. 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, downstream 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 that 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 on TRIF signaling. *Trif*^{lps2} mice do not produce IFNβ after TLR stimulation, whereas *Ifnar1*$^{-/-}$ mice produce IFNβ, but the IFN receptor is unable to signal. Because *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. Although *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 countermodulates the TIRAP-TNF cascade (Fig. 9).

With regard to spinal IFN, the effects of type I interferons are complex. Both IT IFNα and IFNβ inhibit CFA (complete Freund's adjuvant) 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].

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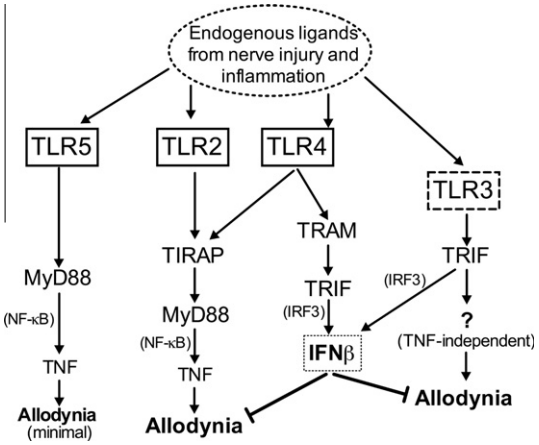

Fig. 9. Schematic of the Toll-like receptor (TLR) cascades emphasized in the present work. Endogenous ligands present in the injured and inflamed system activate resident TLRs, localized to glia and/or neurons. Based on the effects of intrathecal TLR ligands, it is hypothesized that TLRs signaling through Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP) and MyD88 lead to NF-κB–mediated cytokine tumor necrosis factor (TNF) release and to a TNF-dependent allodynia (eg, TLR2 and TLR4). However, TLR3 leads to a TNF-independent allodynia and interferon (IFN) β 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 intrathecal TLR3-L. As TLR4 activated both TIR-domain-containing adapter-inducing interferon-β (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.

4.4. TLRs and glial activation

Microglia show constitutive expression of virtually all TLRs, whereas 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. Although we specifically assessed TLR activation on primary glial cells, TLR3 expression by brain neurons [33,46] and expression of TLRs 3, 4, 7, and 9 in dorsal root ganglia [1,47] have been reported, suggesting that neurons can directly respond to TLR ligands. Cultured mouse dorsal root ganglia 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 TNF-independent facilitatory signaling, as well as an unexpected modulatory feedback through the TRIF pathway of TNF-dependent signaling. Although 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pain.2013.01.012.

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