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Neuraxial TNF and IFN-beta co-modulate persistent allodynia in arthritic mice

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Abstract

In rheumatoid arthritis, joint pain can persist despite resolution of swelling. Similarly, in the murine K/BxN serum transfer model, a persistent tactile allodynia is observed after the resolution of joint inflammation (post-inflammatory pain) in male mice. Here, we found female wild type (WT) mice show inflammatory, but reduced post-inflammatory tactile allodynia. The transition to the post-inflammatory phenotype is dependent on TLR4 signaling. At the spinal level, we found differences in TNF and IFNβ mRNA expression in WT and TLR4 deficient males. In wild type male and female mice, there is differential temporal spinal expression of TNF and IFNβ. In WT males, blockade of TNF or administration of IFNβ was insufficient to affect the persistent allodynia. However, co-administration of intrathecal (IT) IFNβ and anti-TNF antibodies in male WT mice permanently reversed tactile allodynia. IT IFNβ treatment induces expression of anti-inflammatory proteins, contributing to the beneficial effect. Together, these experiments illustrated differences in the transition to chronic tactile allodynia in male and female animals and the complexities of effective pharmacologic interventions.

Keywords

Toll-like receptors; allodynia; arthritis; interferon; sex differences

1 Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by joint inflammation and cartilage destruction. Pain is one of the primary reasons individuals with

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arthritis seek medical care, and pain often persists despite control or resolution of inflammation (American College of Rheumatology Pain Management Task, 2010; Heiberg and Kvien, 2002; Kojima et al., 2009; Lee et al., 2011; McWilliams et al., 2012). Furthermore, inadequately treated pain contributes to exacerbation of joint immobility, emotional distress, fatigue, and a decreased quality of life (Jia and Jackson, 2016; Margaretten et al., 2011; Minnock et al., 2015; Murphy et al., 2012). In arthritic disorders, women are more likely to report pain and experience more severe symptoms. In addition there is no single therapy in rheumatoid arthritis that induces sustained remission reflecting the complexity of this disease. To more effectively approach treatment of arthritis pain, it is paramount that we understand the underlying mechanisms and appreciate the inherent differences between males and females. Thus, the current set of experiments aimed to examine the role of innate immune signaling in the development of persistent, post-inflammatory pain in both male and female mice.

The passive K/BxN serum transfer model of arthritis produces transient paw inflammation (Korganow et al., 1999), which is accompanied, in male mice, by a robust mechanical allodynia (Christianson et al., 2010; Christianson et al., 2012; Park et al., 2016). This pain-like behavior persists after clinical and histological evidence of joint inflammation have resolved (Christianson et al., 2010). In addition, this post-inflammatory phase tactile allodynia co-varies with the appearance of activated spinal astrocytes, microglia, and increased expression of transcription factors in the dorsal root ganglia (DRG), consistent with the development of a neuropathic phenotype (Christianson et al., 2010). The inflammatory and post-inflammatory pain states display distinct analgesic pharmacologies. In the inflammatory phase (day 1–12), male arthritic mice respond to ketorolac, etanercept, and gabapentin with a transient reversal of tactile allodynia. However, in the post-inflammatory phase (day 18–28) only gabapentin is effective against elicited and spontaneous pain. This pharmacologic profile is recapitulated in models such as conditioned place preference, which define the aversive nature of the pain phenotype (Park et al. 2016), further supporting the hypothesis of a transition after the resolution of inflammation to a neuropathic pain state in male mice (Christianson et al., 2010).

Mounting evidence supports the role of Toll-like receptors (TLR) in the development of such persistent neuropathic pain states (Agalave et al., 2014; Cao et al., 2009; Christianson et al., 2011; Hutchinson et al., 2009; Hutchinson et al., 2008; Ji, 2015; Kato et al., 2016; Kato and Svensson, 2015; Lewis et al., 2012; Li et al., 2015a; Li et al., 2015b; Liu et al., 2012; Liu et al., 2016b; Miller et al., 2015; Park et al., 2014a; Park et al., 2014b; Sun et al., 2015; Tanga et al., 2005; Tanga et al., 2004; Wang et al., 2016; Watkins et al., 2009; Woller et al., 2015; Woller et al., 2016). When activated, the TLRs display a robust ability to initiate downstream signaling cascades, which facilitate neuraxial excitability (Hacker et al., 2006; Hutchinson et al., 2011; Kawai and Akira, 2006; O'Neill and Bowie, 2007; Takeuchi and Akira, 2010; Yamamoto et al., 2002). We previously identified a key role for TLR4 in mediating the transition from an acute to chronic, post-inflammatory allodynia in the passive serum transfer model in male mice (Christianson et al., 2011). Unlike WT animals, male *Tlr4*$^{-/-}$ mice did not develop persistent tactile allodynia and signs abated concurrently with the resolution of inflammation despite no significant clinical or histological differences with their WT counterparts. Amongst the TLR family members, TLR4 has been associated with

the development of neuropathic pain in multiple rodent models and has been the target for experimental interventions (Bettoni et al., 2008; Christianson et al., 2011; Hutchinson et al., 2008; Kuang et al., 2012; Lan et al., 2010; Lewis et al., 2012; Tanga et al., 2005; Woller et al., 2016; Wu et al., 2010). In addition, spinal TLR4 signaling cascades may be distinct in male and female mice (Sorge et al., 2011; Sorge et al., 2015; Woller et al., 2016), despite similar spinal expression levels (Sorge et al., 2011) allowing for different therapeutic responses between sexes.

Here, we have used the K/BxN passive serum transfer model to examine sex differences in hindpaw allodynia and potential downstream effector molecules using genetic and pharmacologic approaches. We find that acute inflammation and concurrent allodynia are indistinguishable between male and female WT mice. However, in the post-inflammatory phase, female WT mice display a significant reversal of the tactile allodynia. The ability to recover from the acute arthritic allodynia in both sexes utilizes redundant avenues that, *in vivo*, are related to the initial inflammation, reflecting an induction of TNF and then the ability to augment type I interferon signaling in the post-inflammatory phase. Specifically, co-modulation of spinal TNF and IFNβ are part of a complex cascade leading to the recovery from the acute phase or the subsequent transition to the chronic allodynia.

2 Materials and Methods

2.1 Mice

Male and female wild type (WT) C57BL/6 mice were purchased from Harlan (Indianapolis, IN), and were given at least 48 hours to acclimate to the vivarium before use. Other mice were obtained as breeder pairs and were bred/maintained under standard conditions in a University of California, San Diego Animal Facility that is accredited by the American Association for Accreditation of Laboratory Animal Care. KRN T cell receptor transgenic mice were a gift from Drs. D. Mathis and C. Benoist (Harvard Medical School, Boston, MA and Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). These mice were maintained on a C57BL/6 background (K/B). Arthritic mice were obtained by crossing K/B with NOD/Lt (N) animals (K/BxN). *Tnf*$^{-/-}$ mice were originally purchased from The Jackson Laboratory (Bar Harbor, Maine, USA).

All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. Mice were housed up to 4 per standard cage at room temperature and maintained on a 12:12 hour light:dark cycle. All behavioral testing was performed during the light cycle. Both food and water were available *ad libitum*.

2.2 Passive Serum Transfer of Arthritis

Groups of adult K/BxN transgenic mice were bled, the sera pooled, and transferred to recipient mice by intraperitoneal (IP) injection (100 μl on days 0 and 2). As an indicator of inflammation, ankle width was serially measured with a caliper on days 0–6, 9, 12, 15, 18, 21, 24, and 28. On the day of sacrifice the mice were bled and the hind paws were removed and fixed in 10% formaldehyde. The serum IL-6 levels were measured by capture (BD

Biosciences, La Jolla, CA. USA) (Chan et al., 2009). The hind paws were trimmed, decalcified, embedded, sectioned and stained with H&E and toluidine blue by Histotox Inc. (Boulder, CO). A blinded semi-quantitative scoring system was used to assess synovial inflammation, extra-articular inflammation, bone erosion and cartilage damage (0–5 scale), as previously described (Guma et al., 2009).

2.3 von Frey Behavioral Testing

Mechanical withdrawal thresholds were assessed on days 0–6, 9, 12, 15, 18, 21, 24, and 28 using the up-down method (Chaplan et al., 1994). Briefly, animals were placed in clear, plastic, wire mesh-bottomed cages for 45-min prior to the initiation of testing. Tactile thresholds were measured with a series of von Frey filaments (Seemes Weinstein von Frey anesthesiometer; Stoelting Co., Wood Dale, IL, USA) ranging from 2.44–4.31 (0.02–2.00 g). The 50% probability of withdrawal threshold was recorded. Mechanical values for the left and right hindpaws were measured and averaged to produce a single data point per day of measurement. In light of reports of the possible contribution of sex of the experimenter (Sorge et al., 2014), we note that both a male and female performed mouse behavioral testing, and all genotypes had similar baseline thresholds.

2.4 Druges and Drug Delivery

Needle placement for the intrathecal (IT) injections was performed as previously described (Hylden and Wilcox, 1980; Stokes et al., 2011). Briefly, anesthesia in mice was induced with 4% isoflurane (with 2% oxygen and 2% room air) in a chamber until a loss of the righting reflex was observed. Mice were then switched to 2.5% isoflurane maintenance and a 1”, 30-gauge needle attached to a 50μl Hamilton syringe was inserted between the L5 and L6 vertebrae evoking a tail flick reflex, and ensuring proper placement.

Type I Interferon (IFNβ) was purchased (Millipore, IF011) for IT administration (3600 units/5μl). IFNβ was diluted in 1% bovine serum albumin (BSA) and was administered on days 6, 9, and 12. Low endotoxin, azide free anti-TNF antibody was purchased from (BioLegend, San Diego, CA) for IT administration (5μg in 5μl). Rat IgG (1μg/μl, Vector Laboratories, Burlingame, CA) was used as a vehicle control.

2.5 RNA extraction and quantitative real-time PCR (qPCR)

RNA from flash frozen spinal cords (WT male (n=23), WT female (n=15), *Tlr4*$^{-/-}$ male (n=11), *Tlr4*$^{-/-}$ female (n=6), *Il10*$^{-/-}$ male (n=6), and *Il10*$^{-/-}$ female (n=6)) was extracted using QIAzol Lysis Reagent (QIAGEN), then the RNeasy Lipid Mini Kit (QIAGEN), according to the manufacturer’s protocol. Complementary DNA was prepared using qScript cDNA SuperMIx (Quanta Biosciences) and qPCR performed with TaqMan Universal PCR master mix and predesigned primer and probe sets (according to the manufacturer’s protocol; Applied Biosystems, Carlsbad, CA, USA), using a Bio-Rad iCycler with the MyiQ Optical Module (Bio-Rad, #576BR). Reactions were measured in duplicate and data normalized to the geometric mean of three housekeeping amplicons: 18S, Ywhaz (Mm03950126_s1), and β-actin (Mm02619589_g1; Applied Biosystems). Gene specific primer and probe sets included: Tnf (Mm00443258_m1), Ifnb (Mm00439552_s1), Il6

(Mm00446190_m1), Il10 (Mm00439614_m1), Il1b Mm00434228_m1), and Il1rn (Mm00446186_m1)(purchased from Applied Biosystems).

2.6 Immunohistochemistry

On the indicated days, mice were deeply anesthetized with Beuthanasia-D and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde. The spinal cords were removed, post fixed, and cryoprotected in sucrose. Lumbar sections (L4–L6) of the spinal cord were cut on a microtome (30μm) as free floating sections. Tissue sections were incubated with anti-GFAP antibody (1:1000 Sigma, St. Louis MO) or anti-Iba1 antibody (1:1000 Wako, Richmond, VA), washed, and then incubated with secondary antibodies conjugated with fluoro-Alexa-488 and Alexa-594 (1:500, Molecular Probes, Eugene, OR). Images were captured by Leica TCS SP5 confocal imaging system and quantified by a blinded investigator using Image-Pro Plus v.5.1 software. Microglia (Iba1) and astrocyte (GFAP) staining was quantified by measuring the total integrated intensity of pixels divided by the total number of pixels in a standardized area of the dorsal horn. Staining intensity was examined in lamina I-III of the superficial dorsal horn with 3 slices (separated by at least 180 μm) examined per animal and 5 – 6 animals per experimental condition. Only pixels above a preset background threshold were included. An increase in the integrated intensity / pixels for Iba1 and GFAP staining was interpreted to signify microglia and astrocyte reactivity, respectively.

2.7 Statistic

Results are represented as a mean ± SEM. Statistical analysis was performed using GraphPad Prism (version 6.0h; GraphPad Software, San Diego, CA, USA). For comparison of inflammation and tactile allodynia between sexes within a genotype, a 2-way (sex × time) repeated measures analysis of variance (ANOVA) was used. When appropriate, additional *post-hoc* comparisons were conducted to determine specific days of difference using Tukey correction. For qPCR comparisons, an unpaired *t*-test or one-way ANOVA were used. In all cases, $p < 0.05$ was considered significant.

3. Result

3.1 K/BxN inflammation and tactile allodynia resolve concurrently in WT female and Tlr4$^{-/-}$ mice, but not WT male mice.

In the passive K/BxN serum transfer model of arthritis, arthritogenic serum containing autoantibodies is injected into recipient mice, initiating a rapidly developing arthritis that resolves in approximately three weeks. Previously, we reported that male C57BL/6 (WT) mice develop persistent mechanical allodynia occurring with the onset, but persisting beyond the resolution of, inflammation (Christianson et al., 2010), which is replicated in the current study (Figure 1A and 1B). This chronic allodynic phenotype, which we will refer to as post-inflammatory phase tactile allodynia, is dependent on TLR4 signaling in male mice (Christianson et al., 2011). Recently, Sorge et al. highlighted a differential role for spinal TLR4 in the allodynic response of male and female mice (Sorge et al., 2011). Hence, we broaden our investigation to include female WT and *Tlr4*$^{-/-}$ mice. Our previous work showed that TLR4 deficiency had no effect on baseline thresholds or the magnitude and time

course of inflammation relative to WT males, but significantly attenuated the late phase allodynia (Christianson et al., 2011). Here, we demonstrate that there is no difference between strains or sexes in hindpaw swelling [F (3,546)=2.8481, p=0.062 two-way ANOVA] (Figure 1A). In addition all mice develop an initial tactile allodynia (Figure 1B); however, there are differences in the resolution of the allodynia in the post-inflammatory phase [F (3,572)=26.89, p<0.001 two-way ANOVA]. The female WT mice partially resolve their allodynia (significant differences on days 21 ($p = 0.0008$), 24 ($p = 0.0309$), and 28 ($p = 0.0024$) by two-way ANOVA and Tukey *post hoc* test compared to WT males), suggesting that they are relatively protected from the development of persistent tactile allodynia in this model. Of note, the female *Tlr4*$^{-/-}$ mice began to recover earlier than male *Tlr4*$^{-/-}$ mice on day with a significant difference on day 12 ($p<0.01$ by ANOVA and Tukey correction; Figure 1B).

We previously did not find any significant histological differences in the ankles on Day 28 between male *Tlr4*$^{-/-}$ mice and WT mice (Christianson et al., 2011). Here, we also do not see differences in the levels of ankle inflammation or joint damage in arthritic male and female mice on day 15 and day 28 (Supplementary Figure 1A–C). As an additional measure of inflammation, the sera levels of TNF and IL-6 were measured in mice sacrificed at different time points. The levels of IL-6 and TNF increase significantly on day 6 ($p < 0.001$) for both sexes of mice, but there are no difference between the sexes (Supplementary Figure 1D & E).

3.2 Changes in spinal cytokine transcripts.

In male mice, there is a pivotal point (~2 weeks) beyond which administration of a TLR4 antagonist (LPS-RS) can no longer abrogate the onset of a neuropathic pain state in this animal model (Christianson et al., 2011). Hence, we examined the spinal cords from arthritic mice on day 10 for differences in gene expression of cytokines associated with allodynia in male WT mice, as the WT male mice develop persistent allodynia, but WT female and *Tlr4*$^{-/-}$ mice do not. There is a significant decrease in the levels of *Tnf* mRNA transcripts [Figure 1C, F (3, 15) = 19.45, p=0.002; one-way ANOVA], and an increase in the levels of *Ifnb1* mRNA in the male *Tlr4*$^{-/-}$ mice [Figure 1D, F (3, 15) = 8.026, p=0.002 one-way ANOVA] compared to the other groups. WT female mice had fewer *Tnf* transcripts than their male counterparts (p<0.01 Tukey post hoc) at this time point. The female *Tlr4*$^{-/-}$ mice had a similar pattern of expression these two cytokines but at lower levels. IL-10 expression was increased in the *Tlr4*$^{-/-}$ male mice [F(3,12) = 6.549, p = 0.0072, one-way ANOVA] and statistically increased over that of WT male mice ($p < 0.05$, Tukey *post hoc*), and surprisingly the *Tlr4*$^{-/-}$ female mice ($p < 0.05$, Tukey *post hoc*). As this is only one time point there may have been an earlier increase in the *Tlr4*$^{-/-}$ female mice, since they began their resolution of allodynia earlier than the *Tlr4*$^{-/-}$ male mice (Figure 1B). The levels of mRNA expression for *Il1b* and *Il6* were equivalent in the two strains (Supplemental Figure 1 F and G). This survey indicates that the TLR pathways may be regulating more than one cytokine in the post-inflammatory phase.

3.3 Changes in dorsal horn glial activation in WT male and WT female mice.

The differences in the spinal cytokine mRNA expression in WT and *Tlr4*$^{-/-}$ mice suggested that the chronic allodynia may be centrally mediated. Accordingly, we next examined the spinal cords of naïve and arthritic WT male and WT female mice for glial activation by immuno-reactivity in laminae IIII for GFAP (astrocytes; Figure 2A) and Iba-1 (microglia; Figure 2B). Quantification of GFAP staining is elevated in the female mice on day 28 ($p < 0.05$; main effect one-way ANOVA F (2,25)=8.15; p=0.0019). However, Iba1 increased across days in both sexes [F (2, 27)=102.3; (p<0.0001) two-way ANOVA]. Iba1 is increased in female mice on both day 10 ($p < 0.05$) and on day 28 ($p < 0.05$), and on day 28 in male spinal cords ($p < 0.05$). In females, Iba1 staining increased on days 10 and 28 (p<0.0001) and on day 28 in male spinal cords (p<0.0001) compared to baseline. The immunostaining results suggest that there is persistent glial activity after the resolution of inflammation in WT male and female mice.

3.4 Changes in spinal pro-inflammatory cytokine transcripts over time in male and female WT mice.

Given the distinct temporal pattern of glial activation, and the differences in specific cytokine expression between *Tlr4*$^{-/-}$ and WT males, we next examine cytokine expression in WT male and female mice over time. The levels of *Tnf* and *Ifnb1* mRNA transcripts are measured in spinal cords collected on days 3, 10, 18, and 28 (Figure 2 E&F). WT male and female mice show an increase in *Tnf* amplicons peaking by day 18 (p<0.05) that then decline [F (7,48)=2.67 two-way ANOVA; p=0.02]. WT female animals have an increase in *Ifnb1* transcripts significantly peaking on day 28 [Figure 2F; F (7,47)=4.39; p=0.0008], and reaching higher levels than their male counterparts ($p < 0.05$).

3.5 Effects of spinal delivery of anti-TNF and IFNβ in male WT mice

These results indicate that spinal TNF and IFNβ are potential agents involved in the transition from acute to chronic tactile allodynia. Accordingly, we tested these targets in WT male mice as they transitioned to a chronic allodynic state; whereas female mice do not. We previously demonstrated that IP etanercept transiently alleviated allodynia during the period of acute inflammatory arthritis (day 4–10), but did not affect allodynia in male mice when given IP during the period of persistent allodynia (day 18–24) (Christianson et al., 2010). To test if the absence of a later response to anti-TNF agents is due to a lack of spinal penetrance of the drug, the direct intrathecal (IT) injection of anti-TNF antibody was tested in WT male mice. Direct IT injection of anti-TNF antibody did not produce an overall change in tactile reactivity in the early period [Figure 3A; F (1, 5) = 2.328, p =0.1876] or in the period of persistent allodynia [Figure 3B; F (1, 6) = 0.3604, p = 0.5702].

We noted that in the spinal cords of arthritic male *Tlr4*$^{-/-}$ and female WT mice there was an increase in *Ifnb1* transcripts (Figures 1C and 2C), which we postulated may have led to their spontaneous resolution of allodynia. To increase spinal levels of IFNβ in WT male mice, we administered IT IFNβ during the acute inflammatory and persistent allodynic phases. This treatment, however, did not produce any overall change in tactile reactivity (Figure 3C; F (1, 6) = 0.8920, p = 0.3814) two-way ANOVA). Together, these results suggested that manipulation of either cytokine alone (e.g. block of TNF or increased IFNβ) is not sufficient

to mediate reversal of the chronic allodynic phenotype in male WT mice. We then proceeded to examine concurrent modulation of TNF and IFNβ by injecting IT anti-TNF antibodies and IT IFNβ together into male WT mice. Arthritic male WT mice were injected intrathecally with both anti-TNF antibodies and IFNβ for two doses on days 6, and 12, which permanently reversed the allodynia with a significant effect from day 7 onward [$F(1,17)= 38.12$, $p < 0.0001$; Figure 3D)]. Confirming this outcome, the IFNβ injections in *Tnf*$^{-/-}$ male mice, had a marked (Supplementary Figure 2) and sustained effect from day 7 after IT delivery onward [$F(1,12)= 13.82$, $p < 0.003$)], indicating that abrogating TNF in the presence of sufficient IFNβ is key to circumventing the transition to the chronic allodynia state.

3.6 Spinal delivery of IFNβ can lead to expression of other beneficial effectors

At a minimum, dual modulation of TNF and IFNβ function was necessary to attenuate allodynia. This suggests that the reversal of the chronic allodynia phenotype may include other cytokines and regulatory pathways. Type I interferons have been attributed many pleotropic effects. In the K/BxN model we previously reported the peripheral effects of two IFN inducible genes *Il10* and *Il1rn* (Corr et al., 2011). Here, we tested the subsequent spinal expression of both transcripts (*Il10* and *Il1rn*) in male and female mice after the injection of IT IFNβ. As shown, there is a sharp rise in the expression of *Il10* mRNA in the spinal cords of female mice as early as 3h post injection (p=0.0052; Figure 4A). *Il1rn* transcripts show a progressive increase over time, with male mice showing a greater increase at 6 hours (p=0.046; Figure 4B). The role of spinal *IL1rn* expression on allodynia in the K/BxN mouse model had been previously reported (Lampa et al., 2012), but not *Il10*. Hence, we further examined K/BxN-induced arthritis and the corollary tactile allodynia in IL-10 deficient animals. Here, we found both males and females show similar levels of ankle inflammation, and both displayed a severe and persistent tactile allodynia that was expressed for the duration of the testing period without any difference between the sexes (Figure 4C and D). On day 28 the presence of transcripts for *Tnf* (Figure 4E) and *Ifnb* (Figure 4F) could be detected in the spinal cords of *Il10*$^{-/-}$ mice without differences between the sexes.

4. Discussion

Previous studies have shown male C57BL/6 mice transition from an acute inflammatory phenotype to a persistent allodynia with a neuropathic phenotype in response to passive transfer of K/BxN serum (Christianson et al., 2010; Christianson et al., 2012; Christianson et al., 2011; Park et al., 2014b). In the present work, we replicated this finding, and found, however, that WT female mice did not develop the post-inflammatory neuropathic allodynia, suggesting underlying differences in the systems processing nociceptive information in the two sexes. One caveat is that our findings in arthritic mice however, may not parallel the human situation, as females report greater sensation of pain in rheumatoid arthritis and other arthritic conditions (American College of Rheumatology Pain Management Task, 2010; Heiberg and Kvien, 2002; Kojima et al., 2009; Lee et al., 2011; McWilliams et al., 2012). In addition the influence of sex hormones was not addressed in our current studies, but may be an additional contributing factor in the sex differences that we observed.

TLR4 has long been implicated in neuropathic pain (Agalave et al., 2014; Cao et al., 2009; Christianson et al., 2011; Hutchinson et al., 2009; Hutchinson et al., 2008; Ji, 2015; Kato et al., 2016; Kato and Svensson, 2015; Lewis et al., 2012; Li et al., 2015a; Li et al., 2015b; Liu et al., 2016b; Miller et al., 2015; Park et al., 2014b; Sun et al., 2015; Tanga et al., 2005; Tanga et al., 2004; Wang et al., 2016; Watkins et al., 2009; Woller et al., 2015; Woller et al., 2016) and, as mentioned previously, mediates the transition to a neuropathic state in the K/BxN model (Christianson et al., 2011; Park et al., 2016). However, this appears to be limited to male mice, as we found that WT females behave largely like their *Tlr4*$^{-/-}$ counterparts e.g. no post-inflammatory allodynia. This is in concordance with studies showing spinal TLR4 signaling contributes to allodynia in the male, but not female mouse (Sorge et al., 2011; Sorge et al., 2015; Woller et al., 2016). To further understand the contributions of innate immune signaling to the development of persistent allodynic states in male and female mice, we examined the specific role of TLR4-associated pro-inflammatory cytokines. Our studies do not infer that these differences between male and female mice are limited to TLR4 signaling. The ability to recover from the acute arthritic allodynia in the WT female may apply to other TLRs (e.g. TLR3, TLR7, and TLR9) that, *in vivo*, share the degree of TNF induction and the ability to augment type I interferon signaling in the post-inflammatory phase (Kawai and Akira, 2008).

The anti-nociceptive effects of type I interferon activation and TNF blockade have been described in other acute models (Inglis et al., 2007; Stokes et al., 2013). Our studies, however, suggest that the transition to a chronic, post-inflammatory, allodynia represents the combined modulation of type I interferon and TNF, as neither alone was effective in WT male mice. The activated state of microglia and astrocytes by immunofluorescence suggests that resolution of the post-inflammatory allodynia and the avoidance of the development of a neuropathic phenotype is in fact an active regulatory process. Astrocytes within the spinal cord generate IFNα, and IFNα/β receptors are located on primary afferents in the spinal dorsal horn. Hence, the interferon effect may reflect a cascade from glial cells to afferent neurons (Liu et al., 2016a). Alternatively, IFNα has been reported to have an anti-nociceptive effect through the mu opioid receptors (Jiang et al., 2000). However, we believe that, as revealed in the present studies, that the post-inflammatory component of the K/BxN model may be robustly regulated by downstream effectors activated through interferon inducible factors, including, but not limited to IL-10 and IL-1Ra (Aman et al., 1996; Coclet-Ninin et al., 1997; Dayer et al., 2006; Palmer, 2004; Porrini et al., 1995). Importantly, these factors have been shown to regulate signaling underlying a neuropathic pain state (Gabay et al., 2011; Soderquist et al., 2010).

In male mice, the IT injection of IFNβ alone was insufficient to reverse tactile allodynia. The relative expression of downstream effectors may account for the lack of response in male mice. In our study, male mice demonstrated a greater increase in spinal *Il1rn* mRNA expression after intrathecal IFNβ injection, while the female animals had a greater IL-10 mRNA expression. In the K/BxN model IL-1Ra has been demonstrated to be beneficial in reducing peripheral arthritis (Corr et al., 2011) and expression is increased in the spinal cords of arthritic mice (Lampa et al., 2012); however, the endogenous production of IL-1Ra appears insufficient to overcome the persistent allodynia in male mice. Although we co-modulated type I IFN and TNF signaling, endogenous recovery is likely more complex

requiring multiple factors expressed at key regulatory checkpoints. Here, mice deficient in IL-10 rapidly developed mechanical allodynia that did not recover in either sex suggesting that this cytokine also plays a key role in the acute and chronic phases of pain-like behavior in this model. IL-10 is induced by IL-4 (Uceyler et al., 2011) as well as IFNβ; and is likely regulated by multiple other factors *in vivo*. As our mice were globally deficient in IL-10, our studies were limited by an inability to examine the temporal requirement for this cytokine or its locus of action There is likely a cascade of events some of which may contribute synergistically to regulating the response to a persistent inflammatory state.

5 Conclusions

Wild type male and female mice show differential development of persistent allodynia resulting from long-lasting inflammation. In females, allodynia subsides along with the resolution of inflammation, but male mice are not able to recover. Although the present work is limited to mice and may not completely recapitulate human disease, it contributes to a growing literature suggesting treatment strategies for clinical pain conditions may differ between male and female patients. Further, targeting TNF alone was not sufficient to alleviate the persistent pain state. The allodynia only subsided when anti-TNF therapy was combined with supplemental IFNβ, indicating the need to simultaneously modulate multiple pathways to gain control of the persistent pain. Finally, these studies show that therapeutically targeting peripheral inflammation will not necessarily affect persistent pain. Modulating multiple pathways at the *spinal* level, however, was effective in preventing the development of chronic pain, and suggests that development of treatments for spinal delivery may be necessary to adequately relieve pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Male K/BxN arthritic mice marked persistent allodynia compared to females
- TLR4 governs chronic tactile allodynia in arthritic mice
- Co-modulation of spinal TNF and IFNβ reverses allodynia but not independently

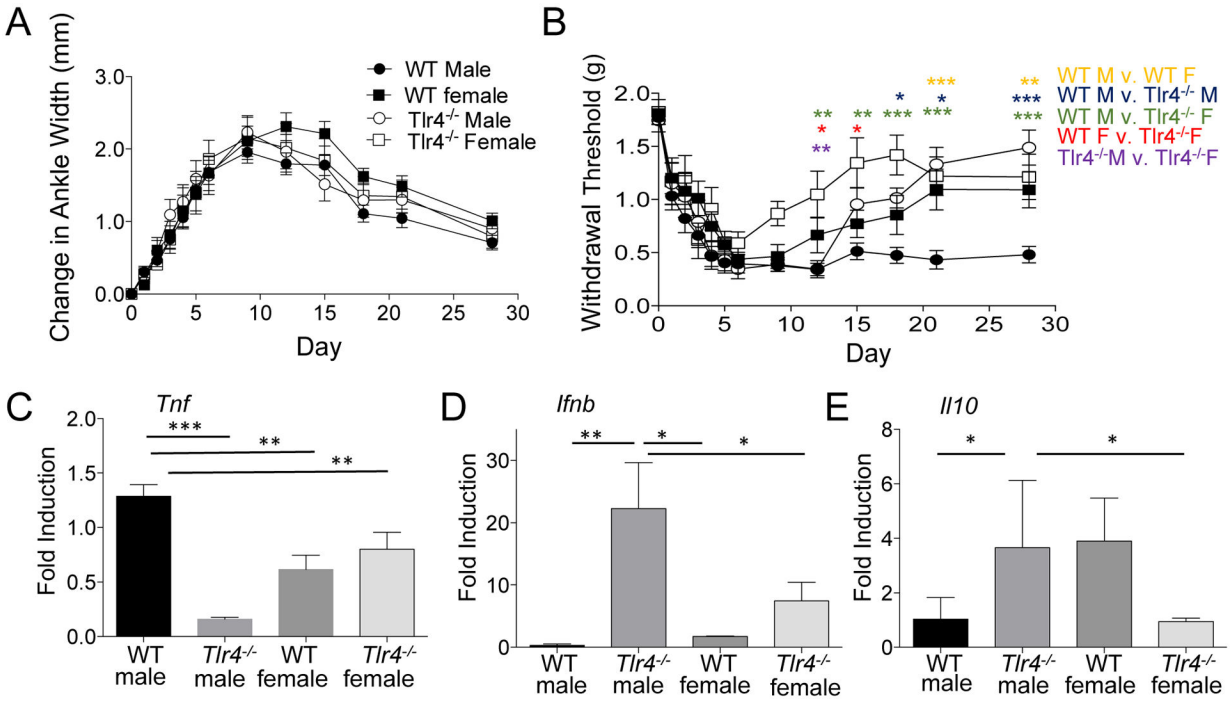

Figure 1. (Two column widths) WT male but not WT female mice develop persistent post-inflammatory allodynia.

(**A**) WT male (n=17), WT female (n=13), male *Tlr4*$^{-/-}$ (n=8) mice were injected on days 0 and 2 with K/BxN sera and developed equivalent levels of ankle swelling which begins to resolved around day 10. (**B**) Male and female mice show an initial tactile allodynia occurring with the onset of paw inflammation. Male WT mice continue to show tactile allodynia persisting after the resolution of inflammation, whereas female WT, and male *Tlr4*$^{-/-}$ and female *Tlr4*$^{-/-}$ mice recover as inflammation resolves at different rates. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with the color matching the corresponding legend for comparisons by way ANOVA for repeated measures and Tukey *post hoc* test; data are represented as mean ± SEM. (**C-E**) Spinal cords were harvested from WT and *Tlr4*$^{-/-}$ mice (4/group) on day 0 or day 10 after the injection of K/BxN serum. These tissues were analyzed for differebces in *Tnf (C), Ifnb1 (D), and Il10* (E) expression. WT male mice show significantly elevated *Tnf* mRNA on day 10 relative to *Tlr4*$^{-/-}$ mice, while *Tlr4*$^{-/-}$ mice show significantly elevated *Ifnb1* and *Il10*. * indicates $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; data are represented as mean ± SEM.

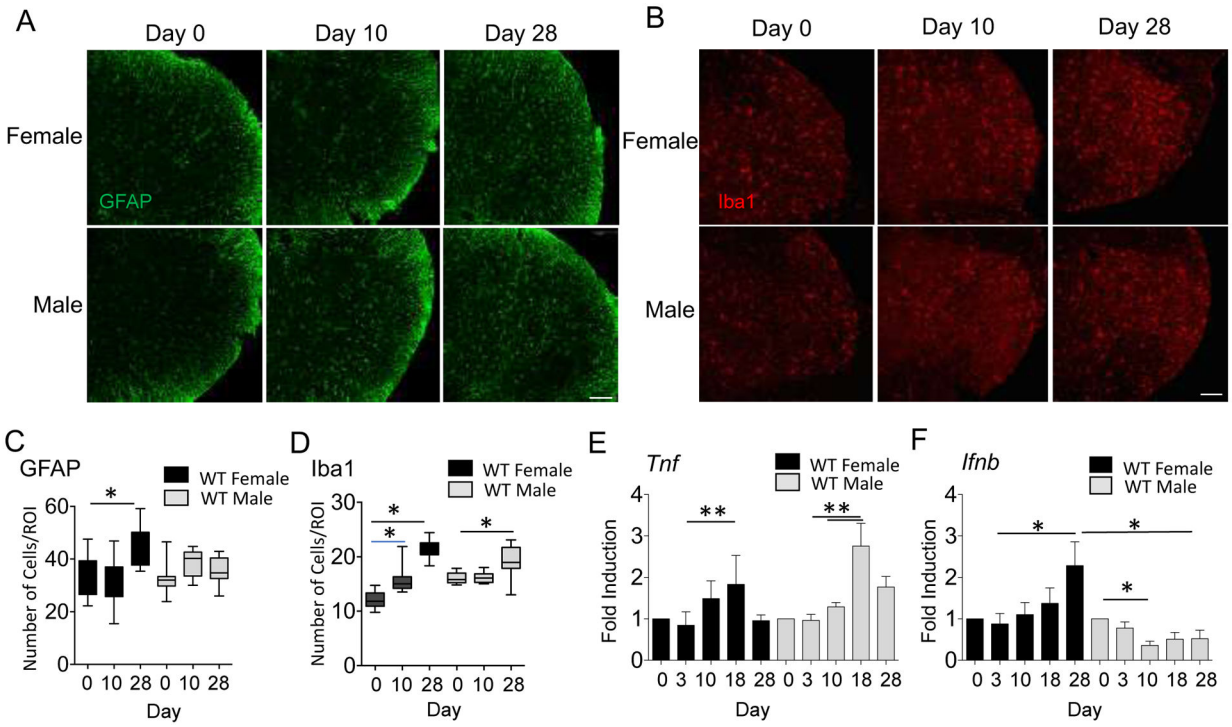

Figure 2. two columns Time course of WT male and WT female spinal cord glial activation and expression of TNF and IFNβ.

Spinal cords were harvested from WT male and WT female mice (5/group) on day 0, day 10, and day 28 and the lumber regions were incubated with antibodies against GFAP **(A)** and Iba1 **(B**). Representative staining images are shown (bar is 100 μM), which were used for the Iba-1 and GFAP quantification. **(C)** GFAP was elevated in the female mice on day 28 [F (2,25)=8.15 p=0.0019 by two way ANOVA *$p < 0.05$ with Tukey *post hoc* test]. **(D**) Iba1 staining increased in both groups [F (2,27)=102.3 (p<0.0001) two way ANOVA]. In females Iba1 staining increased on days 10 and 28 and on day 28 in male spinal cords compared to baseline (ANOVA *$p < 0.05$ with Tukey *post hoc* test). **(E&F)** Spinal cords were harvested from WT male and female mice (7/group) on days 3, 10, 18, and 28 and analyzed for changes in the levels of *Tnf* **(E)** and *Ifnb1* **(F)** mRNA transcripts. Both WT male and WT female mice increased the level of *Tnf* transcripts by day 18. WT female mice demonstrated a gradual increase in the levels of IFNβ mRNA transcripts attaining significance by Tukey test at day 28 compared to day 3 male and female. There was a decrease in the *Ifnb1* expression in male mice on day 10 when compared to day 3 male. * indicates $p < 0.05$, **$p < 0.01$; data are represented as mean ± SEM.

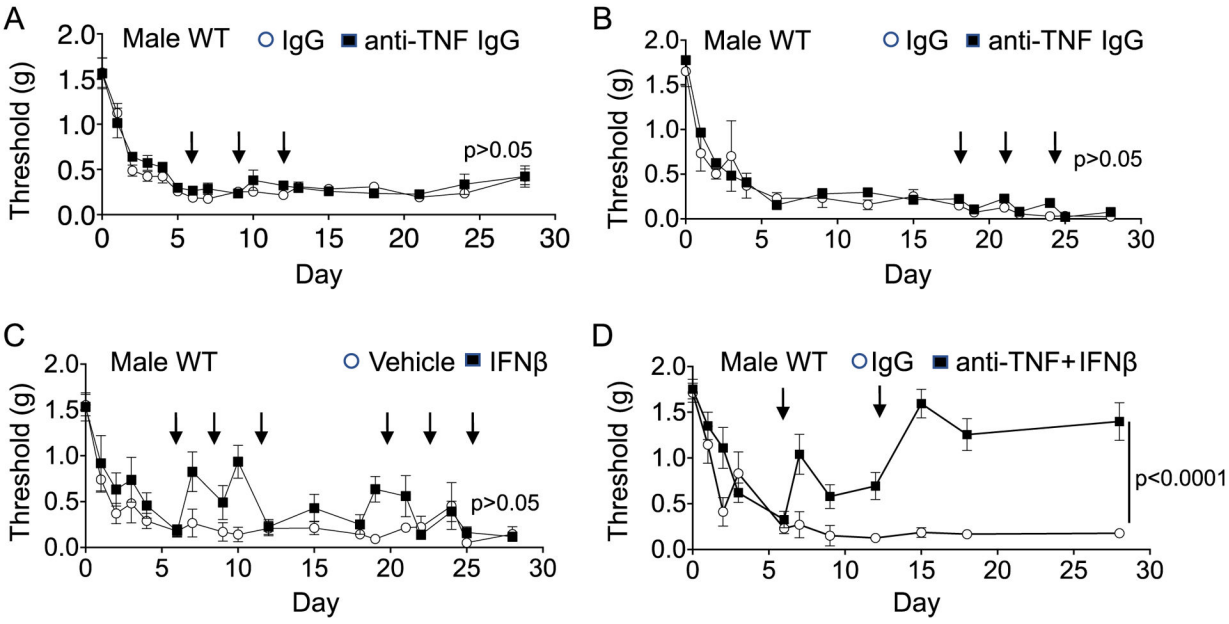

Figure 3: Co-modulation of TNF and IFNβ alters persistent, post-inflammatory allodynia. Two columns

(A and B) WT male mice (n =5–6/group) were administered K/BxN serum on days 0 and 2 and then treated with intrathecal (IT) injections of anti-TNF antibody (5μg in 5μl) or rat IgG (1μg/μl) on days 6, 9, and 12 **(A)** or on days 18, 21, and 24 **(B)** as indicated by the arrows. Neither treatment changed overall tactile reactivity. **(C)** IT administration of IFNβ (3600 units/5μl) or vehicle (1% BSA) on days 6, 9, 12, 18, 21, and 24 in arthritic WT male mice (n = 5–6/group) also did not produce an overall change in tactile reactivity. **(D)** IT injection of anti-TNF antibody (5μg in 5μl) and IFNβ (3600 units/5μl) or rat IgG (1μg/μl) and vehicle (1% BSA) on days 6, and 12 into male WT mice (n=6/group) had a significant effect from day 7 onward [$F(1,17) = 38.12$, $p < 0.0001$ by two-way ANOVA].

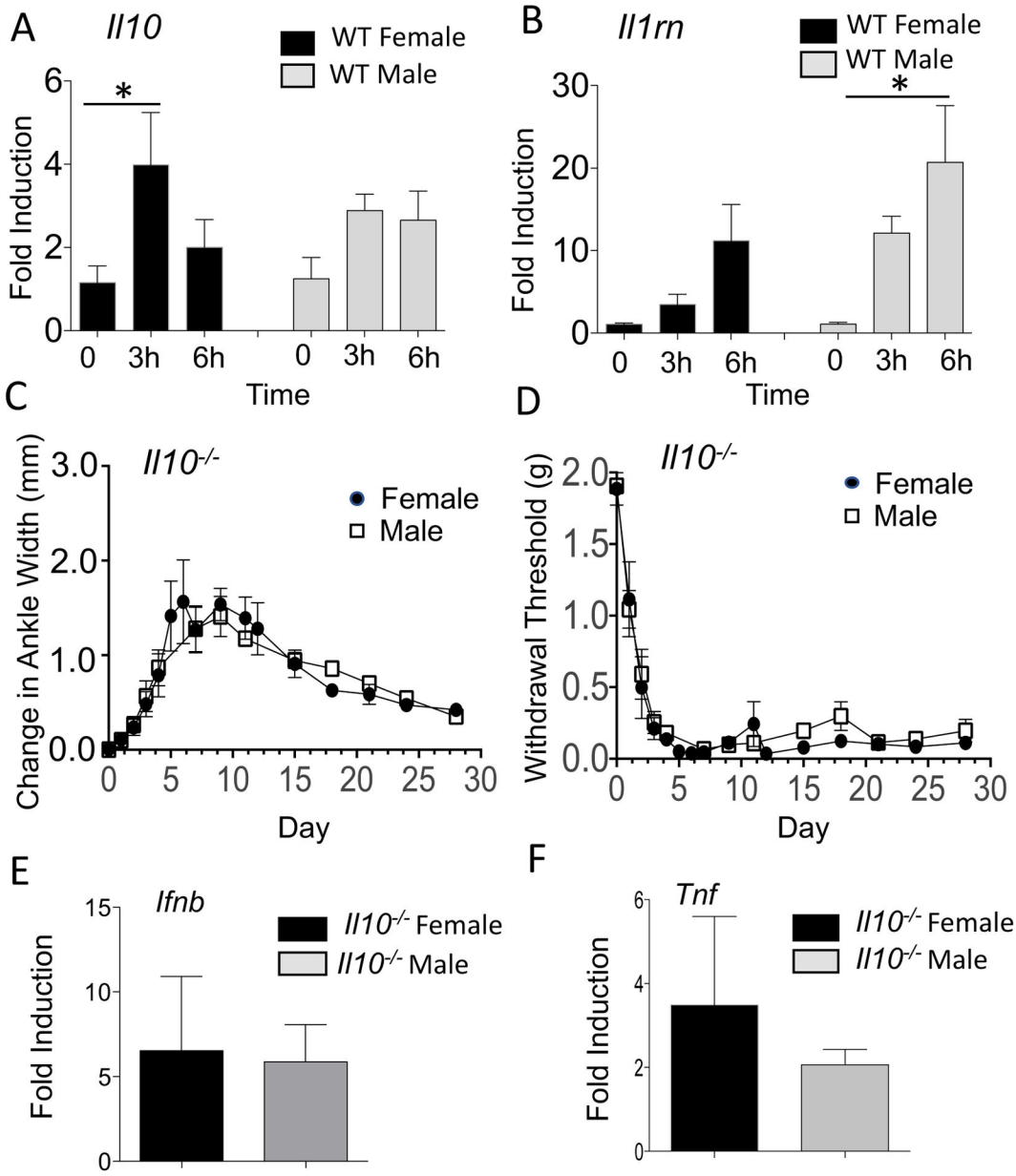

Figure 4. single column IL-10 contributes to recovery from inflammation induced allodynia.

Male and female WT mice (4/group) were injected intrathecally with IFNβ. The mice were sacrificed, and the lumbar cords analyzed for differences in *Il10* **(A)** and *Il1rn* **(B)** mRNA levels. Female mice had a sharp rise at 3h in *Il10* mRNA levels and males had a significant rise in *Il1rn* transcripts at 6h. * indicates $p < 0.05$. **(C)** Male and female *Il10*$^{-/-}$ mice (7/group) showed an equivalent degree of ankle inflammation following administration of K/BxN serum ($p > 0.05$). **(D)** Both sexes demonstrated profound allodynia without any recovery. On Day 28 mice were sacrificed, and the lumbar cords analyzed for differences in *Ifnb* **(E)** and *Tnf* **(F)** mRNA levels. Both cytokines were expressed and there was no difference between sexes ($p > 0.05$).