Title

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Systemic TAK-242 prevents intrathecal LPS evoked hyperalgesia in male, but not female mice and prevents delayed allodynia following intraplantar formalin in both male and female mice: The role of TLR4 in the evolution of a persistent pain state

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Abstract

Objective—Pain resulting from local tissue injury or inflammation typically resolves with time. Frequently, however, this pain may unexpectedly persist, becoming a pathological chronic state. Increasingly, the innate and adaptive immune systems are being implicated in the initiation and maintenance of these persistent conditions. In particular, Toll-like receptor 4 (TLR4) signaling

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has been shown to mediate the transition to a persistent pain state in a sex-dependent manner. In the present work, we explored this contribution using the TLR4 antagonist, TAK-242.

Methods—Male and female C57Bl/6 mice were given intravenous (IV), intrathecal (IT), or intraperitoneal (IP) TAK-242 prior to IT delivery of lipopolysaccharide (LPS), and tactile reactivity was assessed at regular intervals over 72-hours. Additional groups of mice were treated with IP TAK-242 prior to intraplantar formalin, and flinching was monitored for 1-hour. Tactile reactivity was assessed at 7-days after formalin delivery.

Results—LPS evoked TNF release from male and female macrophages and RAW267.4 cells, which was blocked in a concentration dependent fashion by TAK-242. *In vivo*, IT LPS evoked tactile allodynia to a greater degree in male than female mice. TAK-242, given by all routes, prevented development of IT LPS-induced tactile allodynia in male animals, but did not reverse their established allodynia. TLR4 deficiency and TAK-242 treatment attenuated IT LPS-induced allodynia in male, but not female mice. In the formalin model, pre-treatment with TAK-242 did not affect Phase 1 or Phase 2 flinching, but prevented the delayed tactile allodynia in both male and unexpectedly in female mice (Phase 3).

Conclusions—Together, these results suggest that TAK-242 is a TLR4 antagonist that has efficacy after systemic and intrathecal delivery and confirms the role of endogenous TLR4 signaling in triggering the development of a delayed allodynia in both male and female mice.

Keywords

TLR4; LPS; TNF; TAK-242; tactile allodynia; formalin; mouse

1 Introduction

Tissue injury and inflammation induce ongoing traffic in nociceptive afferents leading to an ongoing pain state and an associated hyperpathia. Importantly, this pain phenotype resolves with resolution of the injury state. However, it is increasingly appreciated that there are important examples where an ongoing pain state may persist after the initial inflammation and injury has resolved. Pre-clinical behavioral models of these persistent pain states include the K/BxN serum transfer model of arthritis (Christianson et al., 2012; Christianson et al., 2011; Christianson et al., 2010), collagen antibody-induced arthritis (CAIA) (Bas et al., 2012; Agalave et al., 2014; Park et al., in press), and the intraplantar injection of formalin (Dubuisson & Dennis, 1977; Wheeler-Aceto & Cowan,1991; Wiertelak et al., 1994). In each model, animals display a pain state coincident with the inflammatory phenotype, but also display a hyperpathia, which persists long after the resolution of inflammation. The intraplantar injection of formalin leads to an initial period of high frequency afferent traffic with attendant flinching of the injected paw. After an interval of several days, a prominent tactile allodynia is observed (Ambriz-Tututi et al., 2013; Fu et al., 1999; Fu et al., 2000; Vierck et al., 2008; Wu et al., 2004; Zhang et al., 2007; Ambriz-Tututi et al., 2009). We believe that these preclinical observations parallel human conditions wherein acute tissue inflammation or injury may transition to a chronic pain state.

It is now appreciated that Toll-like receptors (TLRs) may be involved in the interaction of the immune response with neuraxial processing, and have emerged as potential mediators of

the transition between the acute and persistent pain phenotypes. Support for this hypothesized role of spinal TLRs, particularly TLR4, in pain processing arises from several convergent observations: i) intrathecal delivery of TLR4 ligands (e.g. ultra pure LPS, KDO2 lipid A) yields a persistent hindlimb hyperalgesia (Saito et al., 2010; Sorge et al., 2011; Stokes et al., 2013; Loram et al., 2011); ii) effects of spinal LPS are absent in TLR4 mutant or deficient mice (Sorge et al., 2011; Stokes et al., 2013; Wang et al., 2013); and iii) TLR4 KO or intrathecal delivery of a TLR4 antagonist results in an attenuated or absent hyperpathia in the post-inflammatory phase in rodent models of long lasting, but reversible inflammation (Christianson et al., 2011). The broad role of TLR4 is further evidenced by studies implicating it in pain models, such as K/BxN arthritis-induced inflammation (Christianson et al., 2010, 2011), nerve ligation mononeuropathy (Stokes et al., 2013; Tanga et al., 2005), and chemotherapeutic-induced polyneuropathy (Woller et al., 2015; Park et al., 2014; Li et al., 2015; Li et al., 2014).

The role of TLR4 is complex, however, as evidenced by two important caveats. First, the literature suggests that female mice do not respond to spinal LPS despite normal expression of spinal TLR4 (Sorge et al., 2011) and interpretation of results must thus be restricted to sex-specific mechanisms. Second, while it is evident that TLR4 deficiency can alter the hyperpathia presenting post-tissue and nerve injury, the precise role is more nuanced. For example, in the K/BxN model of inflammatory arthritis, TLR4 deficiency has minimal effects upon the early phase allodynia expressed during overt inflammation, and when the TLR4 antagonist LPS-RS is given IT after the established late phase, it has no effect. However, when IT LPS-RS is given during the inflammatory phase, it can prevent the transition to an allodynic state that otherwise persists beyond the resolution of inflammation (Christianson et al., 2011).

In the present study, we sought to extend these investigations on the role of TLR4, using TAK-242, a small molecule which selectively binds to Cys747 in the TIR domain of TLR4, changes the TIR domain conformation and inhibits the recruitment of signaling adaptors (Matsunaga et al., 2011; Kawamoto et al., 2008 Fekonja et al., 2012). Because of the TLR4 antagonistic action, TAK-242 was tested in a Phase III clinical trial in patients with septic shock (Wittebole et al., 2010; Rice et al., 2010; NCT00633477). Given the hypothesized role TLR4 plays in the development of persistent pain, TAK-242 may be useful in this regard. To date, little work has been done on TAK-242 to evaluate whether it affects the development of chronic pain, the effective routes of administration, duration of action, or dosing. Here, we examined TAK-242 for its effect upon LPS evoked release of TNF from male and female macrophage cultures. *In vivo*, we examined the TAK-242 effects after spinal (intrathecal) and systemic (intravenous and intraperitoneal) delivery against the spinally mediated tactile allodynia produced by intrathecal delivery of LPS in male and female mice. We also examined the effects of TLR4 blockade with TAK-242 on the flinching behavior produced by the intraplantar injection of formalin in male and female mice. The formalin model has minimal inflammatory covariates, but initiates a barrage of small afferent input which leads to an acute flinching behavior (Phase 1) followed shortly by a second phase of flinching (Phase 2), which is considered to reflect the initiation of a spinal state of facilitated processing (Puig & Sorkin, 1996; Abram et al., 1996). As noted, a third phase has been described wherein over a period of days, the animal displays the onset of a robust tactile

allodynia accompanied by glial activation (Tramullas et al., 2014; Fu et al., 2000; Wu et al., 2004; Fu et al., 2001). As will be noted, neither TAK-242 nor TLR4 deficiency had any effect upon either Phase 1 or, unexpectedly, on the facilitated state of spinal processing represented by Phase 2, but robustly prevented the tactile allodynia (Phase 3) in both male and female animals.

2 Materials & Methods

2.1 RAW264.7 Cell Culture

2.1.1 Cells and reagents—The male murine macrophage cell line RAW264.7 was purchased from American Tissue Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium (Life Technologies, Carlsbad, CA) with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotic, 1% antimycotic (Life Technologies) in a humidified incubator with 5% CO2. The TLR4 antagonist TAK-242 (ethyl(6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl]-cyclohex-1-ene-1-carboxylate)) (FW = 361.81 Da) (Yamada et al., 2005) was synthesized at Epigen Biosciences (San Diego, CA) and 10mM stock solution was prepared in N,N-dimethylformamide (DMF). LPS was purchased from Sigma Aldrich.

2.1.2 Measurement of TNF concentration in culture supernatants—RAW264.7 cells were cultured in 96 well plates at a concentration of 100,000 cells per well in RPMI 1640 with 10% FBS with 1% antibiotic and 1%antimycotic. The day of stimulation, cells were cultured in RPMI 1640 with 0.5% FBS with 1% antibiotic and 1% antimycotic in the presence or absence of LPS at 100ng/ml for 20 hours with or without TAK-242 (0.3–300 nM). Stock solutions of TAK-242 were diluted in RPMI 1640 to appropriate concentrations and then added to cultures. Control cultures were incubated with identical dilutions of DMF (vehicle control). Cell culture supernatants were used to determine tumor necrosis factor (TNF) concentrations (detection range 31.20–2000 pg/ml) using Enzyme Linked Immunosorbent Assay (ELISA) (R&D Systems) according to manufacturer's instructions. IC_{50}s were determined by plotting data using GraphPad Prism 3-parameter inhibition curve fit analysis. In preliminary studies, RAW264.7 cells stimulated with the TLR4 ligand LPS over a range of concentrations (10 to 1000 ng/ml). This treatment resulted in a concentration dependent increase in supernatant TNF concentration (data not shown). Based on these results the effects of antagonists on TLR4 activation were examined in the presence of LPS (100 ng/ml).

2.2 Primary Macrophage Culture

The femurs and tibia of three C57Bl/6 mice of each sex were sacrificed and the bone marrow cells were plated separately in DMEM+10%FBS, 1mM soduim pyruvate. +30% L929 cell conditioned media in 100mm petri dishes with 5×10^6 cells per plate. On day 7 the cells were trypsinized, washed and resuspended in RPMI+10%FCS and replated at 50,000 cells/well in a 96 well flat bottom plate for 3 days. On day 10, the cells were washed with PBS and stimulated. TAK-242 was titrated starting at 1μM in tripling dilutions and incubated for one hour. The cells were then stimulated with 100ng/ml LPS for 24-hours. Bone marrow enriched cells for each mouse were stimulated in triplicate for 24 hours with

titrated doses of LPS. The supernatants were harvested and assayed for TNF by ELISA (eBioscience).

2.3 Animals

All 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, with lights on at 07:00. All behavioral testing was performed during the light cycle. Both food and water were available *ad libitum*.

Wild type C57Bl/6 male (n = 98) and female (n = 33) mice were purchased from Harlan (Indianapolis, IN) at 20–25g. *Tlr4*$^{-/-}$ mice male (n = 4) and female (n=10) were a gift from Dr. S. Akira (Osaka University, Japan; see Hoshino et al., 1999 for a complete description) and were bred for 10 generations onto the C57Bl/6 background. All mice were held in the vivarium a minimum of 2 days before use.

2.4 Behavioral Tests

2.4.1 Mechanical Allodynia—For testing, 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 (0.02–2.00g). The 50% probability of withdrawal threshold was recorded. Mechanical values for the left and right paw 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 a female performed the mouse behavioral testing. In the present experiments, mechanical withdrawal thresholds were assessed prior to treatment and at 4-, 24-, and 72-hrs post treatment using the up-down method (Chaplan et al., 1994). In formalin experiments, thresholds were assessed on days 0 and 7 only.

2.4.2 Formalin Flinching—A metal band was placed around the left hindpaw of the mouse. After 1-hr acclimation with the metal band, the mouse received a single injection of intraplantar (IPLT) formalin (2.5%) to induce flinching. The movement of the metal band (mouse flinching) was detected by an automated device (Yaksh et al., 1985) for a period of 1-hr after delivery of formalin. Three treatments were examined: pre-treatment with intraperitoneal (IP) TAK-242 (3 mg/kg) or vehicle, and treatment with TAK-242 at 60-min post-formalin injection.

2.5 Drugs and Drug Delivery

2.5.1 TAK-242—For each route of delivery, TAK-242 (Epigen Biosciences Inc, San Diego, CA) was separately prepared in the desired concentration for delivery in 5% DMSO, 5% Tween80, and brought to a final volume using 0.9% saline. For intrathecal delivery, 1.5, 5, or 15μg/5μl was administered. An intraperitoneal dose of 0.3 or 3.0 mg/0.25ml/kg was administered. Similarly, for intravenous delivery, 0.3 or 3.0mg/kg was administered in 100μl. Stability of the formulated TAK-242 was confirmed by HPLC/MS (M+H 362.8). No adverse consequences associated with administration of TAK-242 (IV, IP, or IT) were observed.

2.5.2 LPS—Ultra pure lipopolysaccharide (LPS, *Escherichia coli* 0111:B4), shown to activate only the TLR4 pathway, was purchased from Invivogen, San Diego, SA, and was delivered via intrathecal injection at 1.0μg/5μl in 0.9% saline.

2.5.3 Mouse Intrathecal (IT) Injection—Mice were anesthetized using 4% isoflurane for induction and 2.5% for maintenance of anesthesia with mixture of 50% oxygen and 50% room air. The lower back was shaven and the animal was placed in a prone posture so that the pelvis could be held between the thumb and forefinger. The L5 and L6 vertebrae were identified by palpation and a 30G needle was inserted percutaneously on midline between the L5 and L6 vertebrae. Successful entry was assessed by the observation of a brisk tail flick. TAK-242, vehicle, or LPS were separately injected, each in a volume of 5μL, over an interval of ~30 seconds. Following recovery from anesthesia, mice were evaluated for normal motor coordination and muscle tone.

2.5.4 Mouse Intraperitoneal (IP) Injection—Mice were gently restrained and the mouse tilted so the head was facing downward and the abdomen exposed. A 30G needle was inserted though the abdominal skin and musculature on the right side of the animal and the fluid injected. Aspiration ensured the needle had not punctured a blood vessel, intestines, or bladder.

2.5.5 Mouse Intravenous (IV) Injection—Mice were lightly anesthetized (2.5% induction) and placed into a plastic restraining device. Upon waking, a 30G needle was inserted into the tail vein of the animal and 100μl TAK-242 or vehicle administered.

2.5.6 Mouse Intraplantar (IPLT) Injection—Mice were gently restrained and a 30G needle was inserted into the plantar surface of the paw subcutaneously and 20μl of 2.5% formalin was injected over 10 seconds.

2.6 Statistical Analysis

Data were compiled in and analyzed using GraphPad Prism V6.0g for Mac OSX. Behavioral data were analyzed using a two-way repeated measures analysis of variance (ANOVA) with a Bonferroni *post-hoc* analysis where appropriate. Critical values reaching a $p < 0.05$ level were considered to be statistically significant.

3 Results

3.1 TAK-242 inhibits LPS-mediated of TNF release by RAW264.7 cells

RAW264.7 cells incubated with vehicle media showed basal levels of TNF to be 77.4±12.9 pg/ml. Addition of 100 ng/mL LPS resulted in prominent increases in measured TNF to 2596.7±596.8 ng/ml. To confirm TAK-242 activity in blocking TLR4 activation, RAW264.7 cells were stimulated with the TLR4 ligand LPS (100ng/ml) in the presence or absence of increasing concentrations of TAK-242. In this experiment, TAK-242 inhibited LPS induced TNF production by RAW264.7 cells in a concentration-depended manner, with an IC_{50} 14.5nM (Figure 1A).

3.2 TAK-242 inhibits LPS-induced TNF release equally in male and female macrophages

RAW264.7 cells are a male murine cell line and it has been shown that male-derived macrophages produce higher levels of pro-inflammatory cytokines than females following LPS challenge (Marriot et al., 2006; Loram et al., 2012), so we also examined the response of male- and female-derived macrophages to LPS and TAK-242 application. Bone marrow from individual mice was harvested and enriched for macrophages for 10 days. The cells were treated with graded doses of TAK-242 and, after one hour, were stimulated for 24-hours with 100ng/ml purified LPS. The supernatants were tested for TNF by ELISA. We found male and female bone marrow-derived macrophages showed a similar degree of TNF release in response to LPS stimulation (female EC_{50}=2.2 pg/ml, male EC_{50}=1.1 pg/ml; Figure 1B). Macrophages from male and female *Tlr4*$^{-/-}$ were similarly tested, with no LPS-induced TNF response, but did respond to flagellin stimulation (data not shown). In addition, TAK-242 was equally effective in preventing TNF release in male and female-derived macrophages (female IC_{50} = 44nM, male IC_{50} = 93nM; Figure 1C).

3.2 Intrathecal TAK-242 prevents, but does not reverse intrathecal LPS-induced tactile allodynia in the male mouse

As previously reported, IT LPS (1.0μg in 5μl) results in a significant decrease in mechanical threshold observable 4-hrs though 72-hrs after recovery from light anesthesia in the male mouse (Stokes et al., 2011). In the current *in vivo* studies, we sought to determine whether the IT delivery of TAK-242 prior to IT LPS would reverse this effect, consistent with its role as a TLR4 antagonist. Following baseline threshold testing, animals were given an IT injection of TAK-242 (1.5, 5.0, or 15.0μg/5μl; n=4, n=9, n=4, respectively) or 5μl vehicle (n=4) immediately prior to the administration of IT LPS (1μg/5μl). As shown in Figure 2A, LPS induces a strong tactile allodynia persisting at least 72 hrs. The highest (15μg/5μl) dose of TAK-242 significantly reduced the development of tactile allodynia induced by IT LPS relative to vehicle-treated animals (p<.05). The allodynia was similarly prevented by the 5μg/5μl dose of TAK-242 ($p < 0.05$), while the low dose (1.5μg/5μl) had no effect (p>.05). Another group of male mice was given an IT injection of TAK-242 (5μg/5μl, n=4 or 15μg/5μl, n=4) 24-hrs *after* the IT delivery of LPS. Neither of these treatments had an effect on the established tactile allodynia (Figure 2B; $p > 0.05$).

3.3 Intravenous TAK-242 prevents the development of intrathecal LPS-induced tactile allodynia in the male mouse

After observing the effects of spinally delivered TAK-242 on IT LPS, we sought to determine whether it had similar efficacy after intravenous delivery against the centrally mediated, IT LPS-induced tactile allodynia in male mice. As shown in Figure 2C, after IV delivery, we found 3mg/kg TAK-242 delivered in 100μl through the tail vein immediately (< 5 min) prior to the IT administration of LPS significantly prevented the development of tactile allodynia seen in the vehicle control animals (p <.0001), while a lower 0.3mg/kg concentration had no effect (p > .05). These results suggest that after IV delivery, TAK-242 can block the effects of spinally delivered LPS, suggesting a potential central bioavailability.

3.4 Intraperitoneal TAK-242 prevents the development of intrathecal LPS-induced tactile allodynia

Following demonstration of the effects of IV TAK-242 against the spinally delivered LPS, we sought to determine whether a similar action could be obtained after IP delivery. A recent study indicated that mice receiving 3mg/kg TAK-242 delivered IP showed stable plasma concentrations from 3–8 hrs post administration (Hua et al., 2015). Based on these results, we began by examining IP administration of TAK-242 (3mg/kg) at various pre-treatment intervals to determine an approximate duration of antagonist action. After baseline threshold testing, male mice were treated with IP TAK-242 24-hrs (n=6), 3-hrs (n=6), or 5-min (n=6) prior to the administration of IT LPS. As shown in Figure 3A, the vehicle treated group (n=8) showed a decrease in tactile threshold, indicating the development of tactile allodynia. The animals receiving TAK-242 3-hrs prior to IT LPS showed the most robust effect. In this group, we found a complete prevention of allodynia; animals remained at their approximate baseline thresholds over the 72-hr testing period and were significantly different from vehicle controls ($p < 0.05$). Similarly, animals in the 5-min pre-treatment group showed a significant attenuation of allodynia relative to vehicle controls at all time points tested, but showed a decrease in thresholds over time. The 24-hr pre-treatment proved to be less effective as animals in this group were not significantly different from the vehicle controls until the 72-hr time point, indicating that they may be showing a quicker recovery from LPS than vehicle treated controls. In addition to different pre-treatment intervals, we tested a lower dose of TAK-242 (0.3 mg/kg; n=4). As shown in Figure 3B, this lower dose did not reduce the development of LPS-induced allodynia and treated animals were not different from vehicle-treated controls until the 72-hr time ($p > 0.05$), but were still significantly lower in threshold compared to animals treated with 3 mg/kg ($p < 0.05$).

3.5 Intrathecal LPS induces tactile allodynia in the female mouse, but this is not prevented by IP TAK-242

Previous reports have indicated that IT LPS does not induce tactile allodynia in female CD-1 mice (Sorge et al., 2011), suggesting that, at a spinal level, TLR4 mediates hyperpathic states in male, but not female mice. In the present studies, we examined whether IT LPS would induce tactile allodynia in female C57Bl/6 mice (n=8) and whether IP TAK-242 would have an effect (n=4). We found, unexpectedly, that IT LPS treatment in female animals does result in a decreased threshold relative to animals treated with vehicle injections, but does not induce the same level of tactile allodynia as their male counterparts (Figure 3C). Furthermore, the IP administration of TAK-242 3-hrs prior to the administration of IT LPS did not prevent this change in tactile reactivity, and appeared to decrease thresholds more than the IT LPS alone ($p < 0.0001$).

Given this result, we also examined $Tlr4^{-/-}$ female animals given IT vehicle or IT LPS. As shown in Figure 2D, we found that female animals deficient in TLR4 signaling continue to show LPS-induced tactile allodynia relative to the control injection, suggesting that TLR4 activation is not underlying the development of tactile allodynia following IT LPS administration in female mice. Given this paradoxical result, we note that, as found previously (Stokes et al., 2012) and unlike the female $Tlr4^{-/-}$ mice, $Tlr4^{-/-}$ male mice do not show a change in threshold resulting from IT LPS administration, starting at a baseline of

2.00±0.00g and falling to just 1.785±0.00g at 4-hours and 1.43±.57g at 24-hours (data not shown). In addition, we tested whether the administration of Kdo_2-Lipid A (KLA), the lipid component of LPS responsible for activation of TLR4, would produce similar results in male and female WT and *Tlr4*$^{-/-}$ mice. We confirmed that male WT mice show a decrease in threshold at 4 and 24-hours after IT KLA administration (1.81±0.14 g at baseline to 0.62±0.05 g at 4hrs), which, consistent with IT LPS, is present to a lesser degree in WT female mice (2.00±0.00 g at baseline to 1.10±0.15g at 4hrs). This effect was not seen in male *Tlr4*$^{-/-}$ receiving IT KLA, but was shown in female *Tlr4*$^{-/-}$ mice after IT KLA.

3.6 TLR4 deficiency and TAK-242 do not affect formalin flinching but do prevent the development of later tactile allodynia in both male and female mice

In each of the previous experiments, TAK-242 was tested for efficacy against the known TLR4 ligand, LPS. We next examined the efficacy of TAK-242 in a model of intraplantar formalin evoked spinal facilitation. In this experiment, baseline tactile thresholds were assessed then both male and female animals were given an IP injection of TAK-242 (n=16) or vehicle (n=16) 3-hrs prior to the IPLT injection of formalin. As shown in Figure 4A & 4B, none of the groups differed significantly from male vehicle controls in Phase 1 or Phase 2 flinching, indicating that the treatments did not affect flinching behavior. To confirm the absence of a role of TLR4 in formalin-induced flinching suggested by the TAK-242 studies, we also examined the Phase 1 and Phase 2 behavior in male *Tlr4*$^{-/-}$ mice (n=4). In this work, TLR4 deficiency had no effect as compared to WT males on either phase of formalin flinching (Figure 4A & 4B).

3.7 TLR4 deficiency and TAK-242 prevent the development of tactile allodynia observed 7 days after formalin in both male and female mice

Previous work has shown that animals will exhibit behavior indicative of tactile allodynia when tested seven-days after formalin administration (Ambriz-Tututi et al., 2013; Fu et al., 2000; Fu et al., 2001). To begin, we examined baselines across groups and found no differences in tactile reactivity on day 0 ($p > 0.05$). Next, because formalin was injected into one paw only, day 7 thresholds were initially examined individually by limb (e.g. ipsilateral versus contralateral limb). However, we found that, consistent with previous reports (Fu et al., 2000; Ambriz-Tututi et al., 2013), all mice showed bilateral effects (e.g. allodynia in both the ipsilateral and contralateral limbs in vehicle-treated mice) (data not shown), and we then averaged data from the two hindlimbs for analysis. In animals receiving IP vehicle prior to IPLT formalin, thresholds decreased on day 7, indicative of the development of tactile allodynia (Figure 4C), and were significantly lower than their baseline values ($p < 0.0001$). Unlike vehicle treated animals, both male and female mice pretreated with TAK-242 and TLR4 deficient mice showed a significant attenuation of tactile allodynia, showing thresholds on day 7 that are not different from baseline, and are significantly higher than vehicle treated controls (Figure 4C). The mice given TAK-242 after formalin showed a reduction in allodynia and had higher thresholds than vehicle-treated mice, but were also significantly lower than their baseline thresholds ($p = 0.0137$). After this testing was completed, mice were given vehicle or an IP injection of TAK-242 to determine whether the established allodynia could be reversed. As a 3-hr pre-treatment was most effective in the previous experiments, animals were tested a 3- and 24-hrs after IP injection. As shown in

Figure 4D, the TAK-242 had no effect on tactile reactivity ($p > 0.05$) at either time examined, indicating TAK-242 was not effective in reversing the established tactile allodynia.

4 Discussion

4.1 TLR4 and evolution of persistent pain states

In humans, acute injury or inflammation can lead to the development of an ongoing enhancement in pain sensitivity which persists following the resolution of the inflammatory condition. This persistent pain state has also been demonstrated in animal models of long-lasting inflammation, such as arthritis (K/BxN and CAIA; Christianson et al., 2011; Bas et al., 2012; Agalave et al., 2014; Park et al., in press). The distinct phases of inflammatory and post-inflammatory pain are characterized by different anti-hyperalgesic pharmacologies (Park et al., in press; Christianson et al., 2010) and by the appearance of epitopes consistent with nerve injury in the primary afferent and spinal cord (Wang et al., 2004; Obata et al., 2003; Ivanavicius et al., 2007). Furthermore, studies in the K/BxN inflammatory model of arthritis have demonstrated a pivotal role for TLR4 in the transition from the acute to persistent pain state. In this model, TLR4 deficiency has little effect on the development of the inflammatory tactile, but there is a reduction in the post-inflammatory phase allodynia normally seen in WT mice. Spinal TLR4 systems have been implicated in this transition as the IT delivery of a TLR4 antagonist (LPS-RS) prevented the transition to a chronic pain state in WT mice (Christianson et al., 2011). This role of spinal TLR4 in the evolution of a persistent pain state is consistent with the observation that delivery of TLR4 ligands will initiate a long-lasting change in tactile thresholds (Feldman et al., 2012; Tse et al., 2014; Liu et al., 2012; Kato & Svensson, 2015; Agalave & Svensson, 2015; Agalave et al., 2014), and that TLR4 ligands are reported to be associated with disease severity in humans (Ke et al., 2015). As will be noted below, the issue has several complicating factors, not the least of which is that the role of the spinal TLR4 receptor displays sex-dependent effects.

4.2 TLR4 signaling and the intraplantar formalin evoked changes in nociceptive processing

The use of persistent inflammation / injury models, such as the K/BxN, to define the acute to chronic pain transition has been useful. However, in the present studies, we sought to extend our appreciation of the role of the TLR4 in pain processing by considering its effects in the intraplantar formalin model. Here, the intraplantar delivery of formalin results in a three-phased response. In Phase 1, intraplantar formalin initiates an acute, high frequency afferent barrage lasting about 10 min, followed over the next hour by an ongoing, but diminished, level of afferent input (Puig & Sorkin, 1996). Dorsal horn wide dynamic range neurons display a strong response during both the first and second phase (Dickenson & Sullivan, 1987) and the behaving animals shows a corresponding robust flinching during Phase 1 and 2 (Yaksh et al., 1985). As the flinching response in Phase 2 is strong despite the diminished level of afferent traffic, and because Phase 2 is dependent upon the concurrent afferent traffic, it is argued that behavior in Phase 2 reflects a state of central facilitation (Tjølsen, et al. 1992; Pitcher & Henry, 2002) which is initiated by the robust conditioning input generated during Phase1 (see Abram & Yaksh, 1993; Buerkle, et al., 1998). Importantly, the

enhanced activity is reversed by spinal delivery of agents thought to target components mediating enhanced spinal excitability (e.g. NMDA antagonism (Yamamoto & Yaksh, 1992; Chaplan et al., 1997); p38 MAPK inhibitors (Svensson, et al., 2005); N-type calcium channel block (Ca^{2+}-activated chloride channels (García et al., 2014). Subsequent work has revealed that, over periods of days after the unilateral hind paw injection of formalin, a third phase could be observed which consists of the development of a bilateral hind paw tactile allodynia (Fu, et al., 1999; Fu, et al., 2000; Vierck, et al., 2008). Importantly, these delayed changes occurred in the absence of evident local inflammation, while the bilaterality emphasizes that the persistent secondary hyperaesthesia is likely mediated by central changes.

In the current studies, we found that, while Phase 1 and 2 were unaltered, the delayed allodynia (Phase 3) was absent in the male *Tlr4*$^{-/-}$ animals. These results were consistent with the effects of systemically delivered TAK-242 given at doses that blocked the effects of IT LPS. Several points should be emphasized. The fact that there was no change in Phase 1 and 2 flinching suggests that the delayed effects on hyperesthesia were not due to a block of either the acute input or processes leading to central facilitation. Further, the lack of effect of TAK-242 given at 7-days after the hyperesthesia had been established emphasizes that TLR4 signaling is involved in a transient activation that serves to promote the development of persistent pain states. In the formalin model, this cascade could be activated by the release of endogenous TLR4 ligands, leading to the initiation of a persistent allodynia (Yamasoba et al., 2016; Zhang et al, 2015).

4.3 TAK-242 as a TLR4 antagonist

TAK-242 has been shown to bind to the membrane bound TLR4 and prevent TLR4 ligand evoked signaling, including increases in TNF and IL-1β (Matsunaga et al., 2011; Kawamoto et al., 2008; Lin et al., 2015; Takashima et al., 2009). In the present work, we confirm the nM potency of TAK-242 in blocking the release of TNF from cell cultures exposed to LPS. System level studies have shown efficacy in rodent models of sepsis (Sha et al., 2011; Takishma et al., 2009; Sha et al., 2007). In addition, TAK-242 is reported to attenuate visceral pain when given IV and / or by intra-cranial delivery (Tramullas, et al., 2013).

In the present work we sought to determine the central activity of TAK-242 after intrathecal and peripheral routes of delivery and to estimate its functional duration of action. The present studies showed prominent activity after all routes of delivery with the time of onset in blocking the hyperalgesic actions of IT LPS occurring at intervals as short as 30-min and with a duration of pretreatment action being greater than 3-hrs. These studies showing the inhibitory effects upon the intrathecal LPS provide functional evidence that the drug, in fact, has access to sites within the blood brain barrier. This property would be consistent with its reported estimated CLogP of 3.1. However, we recognize that a caveat to this assertion is that LPS may perturb the blood brain barrier and thereby enhance TAK-242 penetration.

4.4 Sex and the effects of TAK-242

Previous reports suggested that, intrathecal LPS does not induce tactile allodynia in female mice, while administration to either the brain or hindpaw produce the same effect (allodynia)

in either sex (Sorge et al., 2011). In the present study, we found that LPS evoked TNF release from both male and female macrophages. These data thus suggest that any sex related differences to TLR signaling is not related to a lack of immune mediated signaling. Sorge and colleagues (2015) recently reported that unlike wild type female mice, T-cell deficient females appeared to develop a role for microglia in nociceptive signaling. In the present studies, the late phase allodynia was prevented in the female TLR4 deficient animal and after TAK-242. This suggests that, in this model of robust transient afferent activation, TLR4 plays a triggering role in the initiation of the late phase pain state in both male and female. An unexpected observation was that while activation of spinal TLR4 leads to robust allodynia, previous work has reported no effect of IT LPS in females. In the present study, however, we unexpectedly found a significant, reduction in the female thresholds. Importantly, we confirmed this effect using KDO2 lipid A, a highly selective TLR4 ligand. In neither case were these effects altered by the TLR4 KO or by TAK-242. While this phenomena has not, to our knowledge, been previously reported in the pain literature, LPS can signal though non-canonical activation of inflammasomes, leading to IL-1β production (Yang et al., 2015; Smith et al., 2015; Hagar et al., 2013; Kayagaki et al., 2013; Baker et al., 2015; Shi et al., 2014). Increased levels of IL-1β have been linked to the development of persistent pain (Watkins et al., 1994; Lu et al., 2014; Yan et al., 2014), and may be responsible for the tactile allodynia seen in the current experiments. However, the convergent outcomes of TLR4-deficient animals and the effects of TAK-242, suggest that the late “Phase 3” of the formalin model reflects an engagement of TLR4 signaling, presumably through a triggered release of endogenous TLR4 ligands known to induce pain-like behaviors in animals (Feldman et al., 2012; Tse et al., 2014; Liu et al., 2012; Kato & Svensson, 2015; Agalave & Svensson, 2015; Agalave et al., 2014).

5 Conclusions

This work demonstrates several important points: 1) TAK-242 delivered IT, IV, and IP can effectively prevent the development of LPS-induced tactile allodynia in male mice, 2) formalin flinching is not affected by TLR4 deficiency or antagonism, but TLR4 signaling is necessary for Phase III tactile allodynia in male and female animals, and 3) IT LPS is acting independently of the TLR4 receptor to induce tactile allodynia in female animals.

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Highlights

- TAK-242 prevents LPS evoked TNF release from RAW267.4 cells and male and female macrophages.
- IV, IT, and IP TAK-242 administration prevents LPS-induced allodynia in males.
- TLR4 deficiency and TAK-242 treatment prevents the delayed onset of formalin-induced tactile allodynia in both sexes.
- TAK-242 does not reverse established tactile allodynia in the current experiments

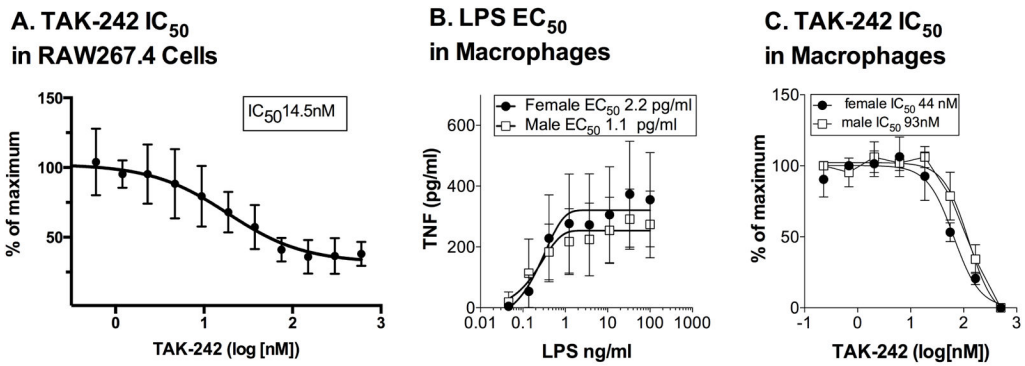

Figure 1.

In vitro inhibition of TNF production by LPS-activated RAW264.7 cells and macrophages: **A** RAW264.7 cells were stimulated with 100ng/ml LPS in the presence or absence of increasing concentrations of TAK-242. The IC_{50} TAK-242 was calculated using GraphPad Prism 3 parameter inhibition curve. Values are mean ± SD of three experiments. **B–C** Bone marrow from individual male or female mice was harvested and enriched for macrophages for 10 days. The cells were treated with graded doses of TAK-242 and, after one hour, stimulated for 24 hours with 100ng/ml purified LPS. The supernatants were tested for TNF by ELISA. The supernatant from bone marrow cells were tested in triplicate. Shown is the average for 3 mice / sex.

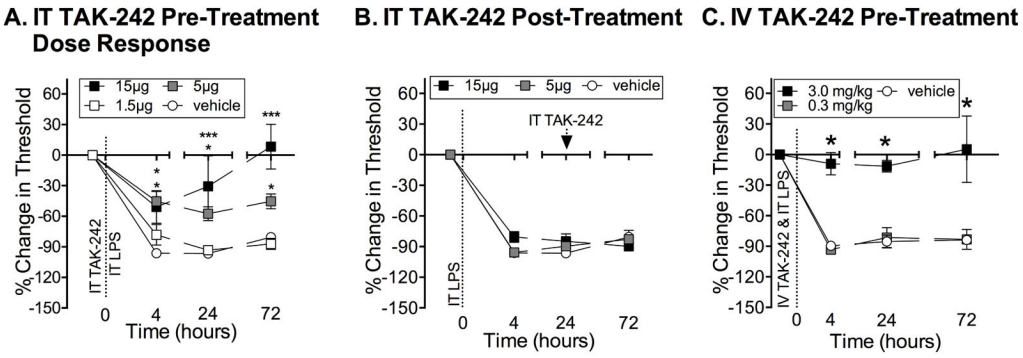

Figure 2.

A–B Intrathecal TAK-242 administration: **A** The IT administration of 5 and 15µg, but not 1.5µg, of TAK-242 immediately prior to IT LPS significantly prevented the development of tactile allodynia ($F(3, 17) = 12.33$, $p < 0.001$). **B** However, when given as a post-treatment, the allodynia was not revered by either 5µg or 15µg of IT TAK-242 ($p > 0.05$). **C** IV TAK-242 administration: The IV administration of 3.0mg/kg, but not 0.3 mg/kg, TAK-242 immediately prior to the administration of IT LPS (5µg) prevented the development of tactile allodynia, with TAK-242 animals receiving 3.0mg/kg remaining nearly at baseline threshold over a 72-hour period ($F(2, 7) = 40.10$, $p = 0.0001$). Values are mean ± SEM, * $p < 0.05$ *** $p < .0001$

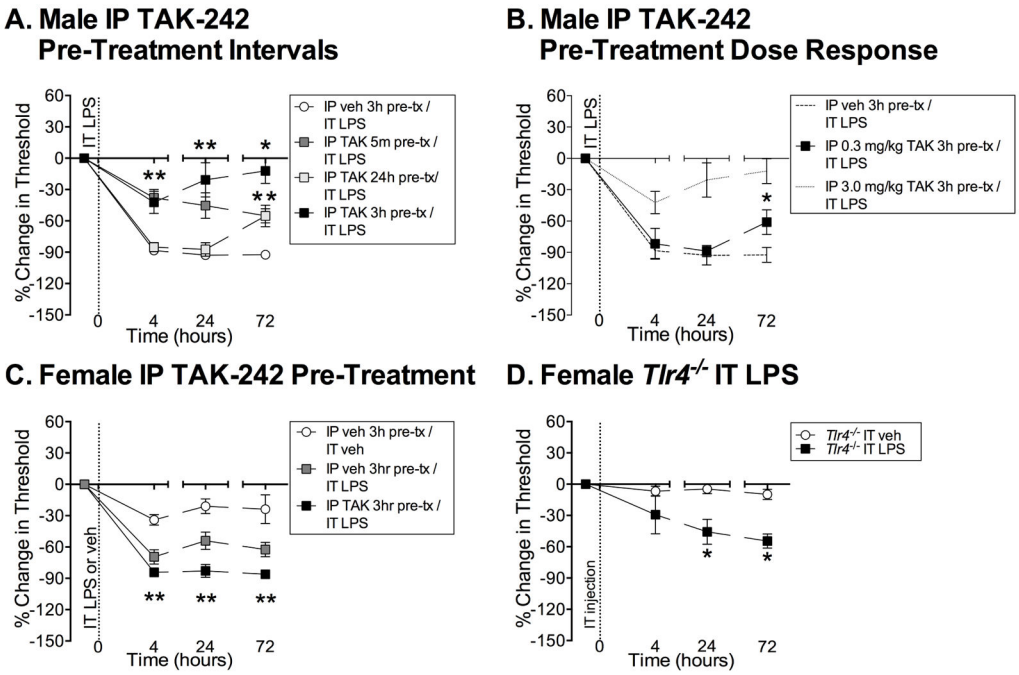

Figure 3.

A–B Male IP TAK-242 administration: **A** When testing the IP administration of TAK-242, we examined several pre-treatment intervals in male mice. Relative to the vehicle group, a 3-hour pre-treatment was most effective in preventing IT LPS-induced tactile allodynia. Both 5-min and 24-hour pre-treatments were less effective ($F(3, 22) = 26.92$, $p < 0.0001$). **B** In addition to different pre-treatment intervals, we examined whether a lower dose of TAK-242 would be as effective in male mice. Here 0.03 mg/kg was not effective in preventing the development of tactile allodynia, but did lead to a faster recovery ($p < 0.05$). **C–D** Female IP TAK-242 administration: **C** Female mice did not fully develop tactile allodynia following the administration of IT LPS, and TAK-242 pre-treatment (3 hrs prior) made this allodynia more severe ($F(1, 6) = 45.43$, $p < 0.001$). **D** TLR4 deficiency, in female animals, unlike their male counterparts, does not block the development of IT LPS-induced tactile allodynia. Values are mean ± SEM, * $p < .05$

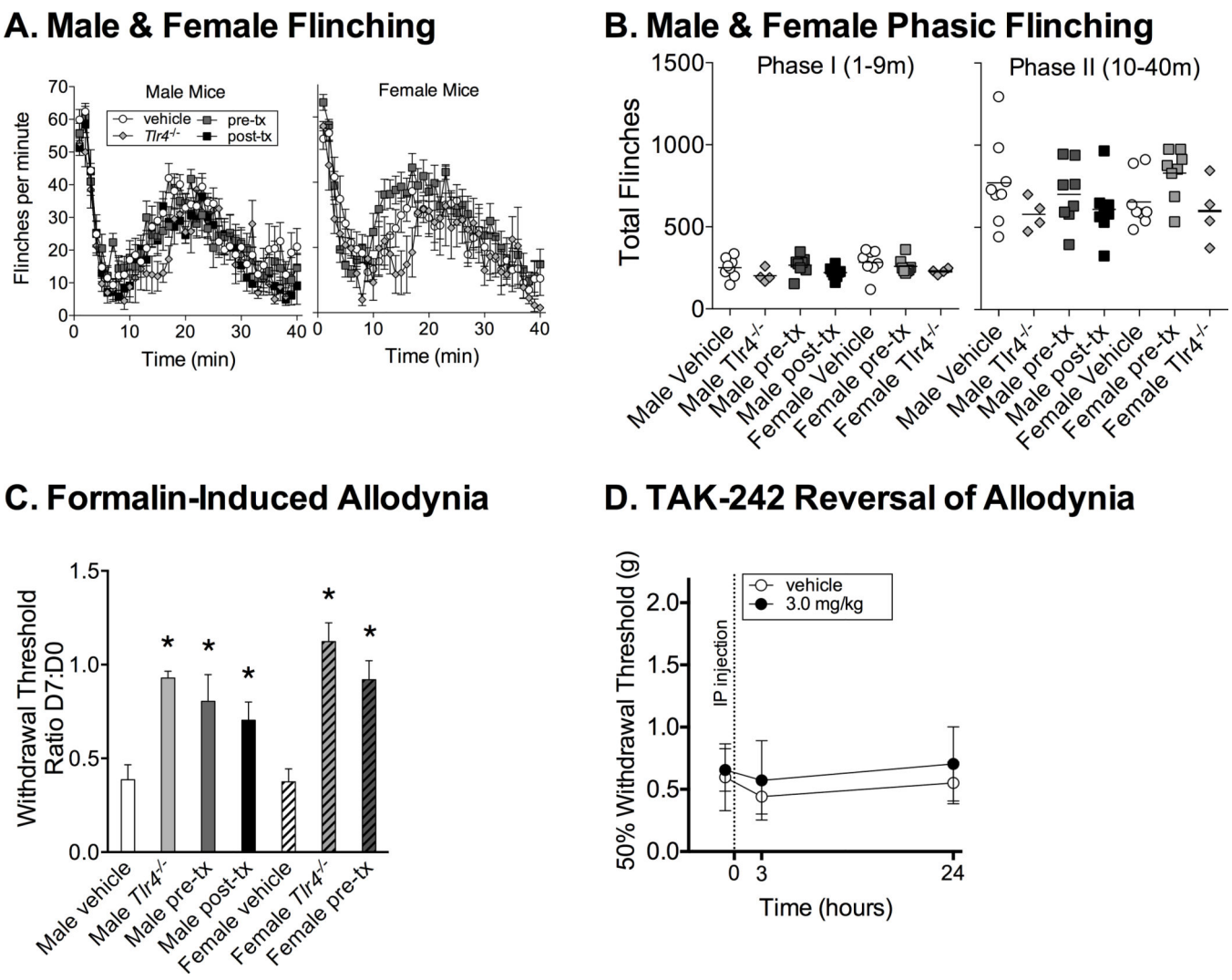

Figure 4.

A–D Phase I–III formalin flinching: **A** Intraplantar formalin induced both an immediate (Phase I) and delayed (Phase II) period of flinching. Flinching was similar in all animals regardless of treatment with TAK-242 or TLR4 deficiency. Flinching was also similar in male and female animals ($p < 0.05$). **B** The total number of flinches in both Phase I (1–9 min) and Phase II (10–40 min) are shown. There were no differences between the groups in either phase (Phase I ($F(5, 38) = 1.738$, $p > 0.05$); Phase II ($F(5, 38) = 1.807$, $p > 0.05$)). **C** Intraplantar formalin induced a bilateral tactile allodynia 7 days after injection in both male and female vehicle-treated animals. This allodynia was prevented in *Tlr4*$^{-/-}$ mice and animals receiving TAK-242 pre-treatment ($F(5, 38) = 5.93$, $p < .001$). **D** On day 7 post-formalin treatment, animals in the vehicle group were given IP TAK-242 or vehicle. This treatment did not affect tactile reactivity ($p > 0.05$).