Cancer Chemother Pharmacol (2014) 73:25–34
DOI 10.1007/s00280-013-2304-9

ORIGINAL ARTICLE

Toll-like receptor signaling regulates cisplatin-induced mechanical allodynia in mice

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Received: 4 April 2013 / Accepted: 25 September 2013 / Published online: 27 October 2013

Abstract
Purpose Cisplatin-treated mice develop a persistent pain state and a condition wherein otherwise innocuous tactile stimuli evoke pain behavior, e.g., tactile allodynia. The allodynia is associated with an up-regulation of activation transcription factor 3 (ATF3) in the dorsal root ganglia (DRG), a factor, which is activated by Toll-like receptors (TLRs). Accordingly, we sought to examine the role of the TLR signaling cascade on allodynia, weight, and changes in DRG ATF3 in cisplatin-treated mice.
Methods Cisplatin (2.3 mg/kg/day × 6 injections every other day) or vehicle was administered to male wild-type (WT) C57BL/6, *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, *Trif*^{lps2} and *Myd88/Trif*^{lps2} mice. We examined allodynia and body weight at intervals over 30 days, when we measured DRG ATF3 by immunostaining.
Results (1) WT cisplatin-treated mice showed tactile allodynia from day 3 through day 30. (2) The *Myd88/Trif*^{lps2} mice did not show allodynia. (3) In *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, and *Myd88*$^{-/-}$ mice, withdrawal thresholds were elevated toward normal versus WT cisplatin-treated mice, but remained decreased as compared to vehicle mice. (4) In *Trif*^{lps2} mice, cisplatin allodynia showed a delayed onset, but persisted. (5) In *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, and *Myd88/Trif*^{lps2} mice, the increase in DRG ATF3 was abolished. (6) Weight loss occurred during cisplatin administration, which was exacerbated in mutant as compared to WT mice.
Conclusions Cisplatin evoked a persistent allodynia and DRG ATF3 expression in WT mice, but these effects were reduced in mice with TLR signaling deficiency. TLR signaling may thus be involved in the mechanisms leading to the cisplatin polyneuropathy.

Keywords Allodynia · Cisplatin · Toll-like receptor · Mouse · Dorsal root ganglion

Introduction

Cisplatin and its homologs are important components of cancer therapy. Its use can result in a polyneuropathy and weight loss [1, 2]. The polyneuropathy is a severe dose-limiting adverse effect for this cancer therapeutic. The major manifestations of cisplatin-induced peripheral neuropathy are disturbances in both sensory and motor functions [3]. Sensory disturbances range from mild tingling to spontaneous painful burning paresthesia and hypersensitivity to non-painful stimuli, also known as tactile allodynia [4]. Changes in sensory function after nerve injury are often accompanied by important changes in the biology of the spinal cord and dorsal root ganglion (DRG), typically manifested in spinal glial activation and increased expression of activation of products such as activation transcription factor 3 (ATF3). In previous work with cisplatin in the mouse, we validated the presence of a persistent tactile allodynia in cisplatin-treated mice and noted no changes in spinal glial (astrocytes and microglia), but observed a significant increase in ATF3 protein expression in the dorsal root

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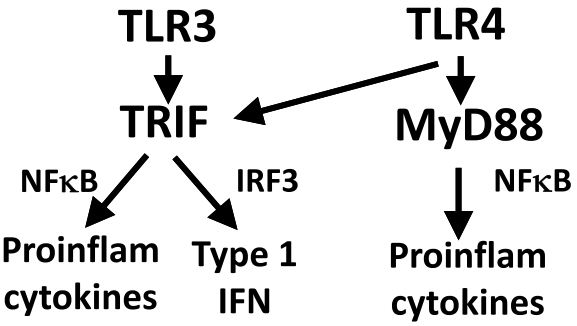
ganglia (DRG) [5]. Similar increases have been reported by others [6]. ATF3 has been implicated not only as a marker of nerve injury, but as playing a potential role in regulating intracellular cascades that are initiated by Toll-like receptor (TLR) activation [7]. This increased expression of ATF3 after cisplatin led us to consider the potential role of the TLRs in this polyneuropathy pain state.

There are at least 13 known mammalian TLRs, which play a key role in the innate immune system. TLRs 1/2, 2/6, 4, and 5 are found on the cell membrane, while TLRs 3, 7/8, and 9 are found in the cell endosome [8]. The TLR signaling pathways branch at two key adapter proteins. The myeloid differentiation primary response gene (88) (MyD88)-dependent pathway leads to the activation of NF-kB and subsequently the production of proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-1. The MyD88-independent pathway, which uses the TIR-domain-containing adapter-inducing interferon-β (TRIF) adapter protein, results in the production of type 1 interferon (IFN) [9]. All TLR signaling uses one or both of these pathways. Besides inflammatory processing, it has been shown that TLRs might play a role in the signaling cascades initiated by nerve injury [10–13].

To study the role of the TLR signaling cascade, we examined the enhanced mechanical sensitivity and changes in DRG ATF3 otherwise produced by cisplatin treatment in WT and in *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, *Trif*^{lps2}, and *Myd88/Trif*^{lps2} mice (Table 1). The use of *Myd88/Trif*^{lps2} afforded us the ability to examine the role of any TLR signaling in establishing cisplatin-induced neuropathy. To further define which TLR pathway was necessary, the *Myd88*$^{-/-}$ and *Trif*^{lps2} mice were examined, and finally TLR3- and TLR4-deficient mice were utilized as these are the only TLRs that utilize the TRIF pathway, and TLR4 also signals through MyD88. While the primary aim of the present work related to the role of TLR signaling in polyneuropathy, we also were interested in determining whether the change in TLR function would alter weight loss, a side effect that often accompanies chemotherapy. Loss of normal weight gain and failure to feed normally has been taken as a direct marker of nausea in non-vomiting rodents [14].

The present work indicates that both the MyD88 and the TRIF pathways play pivotal roles in the aberrations in nociceptive signaling observed in cisplatin polyneuropathy and support a potential role of DRG ATF3 in this cascade.

Table 1 Toll-like receptor (TLR) signaling and cascades targeted in mice used in these studies



Target protein	Genotype nomenclature	Type of protein deficiency
TLR3	*Tlr3*$^{-/-}$	TLR3
TLR4	*Tlr4*$^{-/-}$	TLR4
MyD88	*Myd88*$^{-/-}$	MyD88 adaptor protein
TRIF	*Trif*^{lps2}	Point mutation in the *Ticam 1* gene leading to defective TRIF adaptor protein function
MyD88/TRIF	*Myd88/Trif*^{lps2}	Combined deficiency in MyD88 and TRIF signaling

TLR4 is found on the cell surface, whereas TLR3 is in the cell endosomes. MyD88 is a key adaptor protein for all TLRs, except TLR3, signaling to release proinflammatory cytokines. TRIF mediates all TLR3 and a portion of TLR4 signaling, resulting in production of type I interferon and some proinflammatory cytokines

Methods

Animals

The protocol was approved by the Institutional Animal Care and Use Committee at the University of California, San Diego. Mice were housed up to four per standard cage with soft bedding, and maintained on a 12:12-h light/dark cycle. All the procedure and testing were conducted during the light cycle. Food and water were available freely. Wild-type C57BL/6 mice (male, 25–30 g) were purchased from Harlan (Indianapolis, IN). The mutant mice are summarized in Table 1. The *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, and *Myd88*$^{-/-}$ mice were a gift from Dr. S. Akira (Osaka University, Japan) and were backcrossed for 10 generations onto the C57BL/6 background. *Trif*^{lps2} mice have a point mutation in *Ticam1* disabling the function of the TRIF protein and were a gift from Dr. B. Beutler (University of Texas Southwestern, TX) and were directly generated on the C57BL/6 background. *Myd88*$^{-/-}$ mice and *Trif*^{lps2} mice were intercrossed to generate *Myd88/Trif*^{lps2} mice.

Cisplatin treatment for peripheral neuropathy

Cisplatin (Spectrum Chemical MFG. Gardena, CA, USA) or saline was administered to wild-type (WT) male C57BL/6 and the *Tlr3*$^{-/-}$ *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, *Trif*^{lps2}, and *Myd88/Trif*^{lps2} mice. The protocol for drug treatment was as follows. The cisplatin (2.3 mg/kg) was given intraperitoneally (IP) six times, once every other day (e.o.d.). Vehicle

animals received saline vehicle in place of the cisplatin. In between cisplatin injection days, lactated ringer solution (0.25 ml) was injected subcutaneously to maintain hydration and to prevent kidney and liver damage. This follows a protocol that we have previously validated [5]. Cisplatin-treated mice showed no impairment in mobility or motor function during the course of these observations as evidence by lack of change in placing and stepping reflexes, pinnae or blink reflexes.

Behavioral tests

To assess the mechanical threshold in grams required to produce paw withdrawal by the mouse, mice were placed in a clear plastic containers with wire mesh bottoms. After initial acclimation, the 50 % mechanical threshold for paw withdrawal was assessed using von Frey filaments, which deform at calibrated pressures (0.02–2.0 g). Each filament was pressed perpendicularly against the mid-hind paw plantar surface with sufficient force for 6–8 s to cause slight bending. Flinching or withdrawal of the paw in response to the hair was designated a positive response. Stimuli were applied using the Dixon up–down paradigm. Thus, the absence of a response after filament application was cause to present the next consecutive *stronger* stimulus; a withdrawal or flinch was cause to present the next *weaker* stimulus. This up–down sequence was repeated 5 additional times. The resulting pattern of responses was tabulated and the 50 % response threshold computed using the previously published formula [15, 16]. Thresholds were assessed in both hind paws, and the results were reported as the mean of the two hind paws. Threshold measurements were accomplished before and at intervals over the ensuing 30 days after the initiation of the cisplatin injection. When tactile thresholds fell to approximately 0.5 g or less, the mouse was said to display tactile allodynia.

Immunohistochemistry

On day 30, mice were anesthetized with Euthasol© and perfused intracardially with 0.9 % saline followed by 4 % paraformaldehyde. The L4–L6 DRGs were removed, post-fixed in 4 % paraformaldehyde and cryoprotected in 30 % sucrose. DRGs were frozen and cut in transverse sections (10 μm). DRGs were mounted on glass slides. Non-specific binding was blocked by incubation in 2 % normal goat serum in phosphate-buffered saline with 0.3 % Triton X-100 followed by incubation with primary ATF3 antibody (generated in rabbit, 1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C under gentle agitation. Binding sites were visualized with anti-rabbit IgG antibodies conjugated with Alexa-488 (1:500, Invitrogen). Nuclei were counterstained using Topro3 (1:500; Invitrogen). All images were captured by Leica TCS SP5 confocal imaging system and quantified using Image-Pro Plus v.5.1 software. ATF3 staining was quantified by measuring the total integrated intensity of pixels divided by the total number of pixels in a standardized area. The investigator was blinded to experimental conditions during the quantification. ATF3 data were presented as percentage change from the corresponding control group. Statistics were performed on raw data values.

Statistical analysis

Results are expressed as the mean ± SEM. For comparisons of the time courses of mechanical allodynia, and weight loss, two-way analysis of variance (ANOVA) was used for multiple group comparisons with Bonferoni post hoc comparisons for multiple pairwise comparisons. For comparisons of multiple groups, a one-way ANOVA was used, followed by post hoc Bonferroni multiple comparisons test. A P value of <0.05 was considered to be statistically significant. Statistical analysis was performed using GraphPad Prism (version 5.0, GraphPad Software, San Diego, CA).

Results

Morbidity

All cisplatin-treated mice survived the intended time frame of the study. These mice showed no impairment in mobility or motor function during the course of these observations as evidenced by lack of change in placing and stepping reflexes, pinnae or blink reflexes. As noted below in the following section, cisplatin-treated mice showed a change in normal weight gain.

Weight loss in the cisplatin-treated mice

After study initiation (first IP administration), WT mice receiving saline (vehicle) showed a gradual increase in body weight over the 30-day period of observation (Fig. 1a, g). However, the rate of weight gain in the mutant mice treated with saline, especially TLR3, was slower than that for WT mice. Cisplatin-injected WT mice typically showed a reduction in the normal increase in body weight otherwise expected over the twelve day period of drug delivery. However, the weight loss was greater in the TLR signaling-deficient mice ($Myd88^{-/-}$, $Trif^{lps2}$, and $Myd88/Trif^{lps2}$) with a more modest effect in the single TLR-deficient ($Tlr3^{-/-}$ and $Tlr4^{-/-}$) strains (Fig. 1b–g). Thus, comparing day 15 to day 0 revealed no

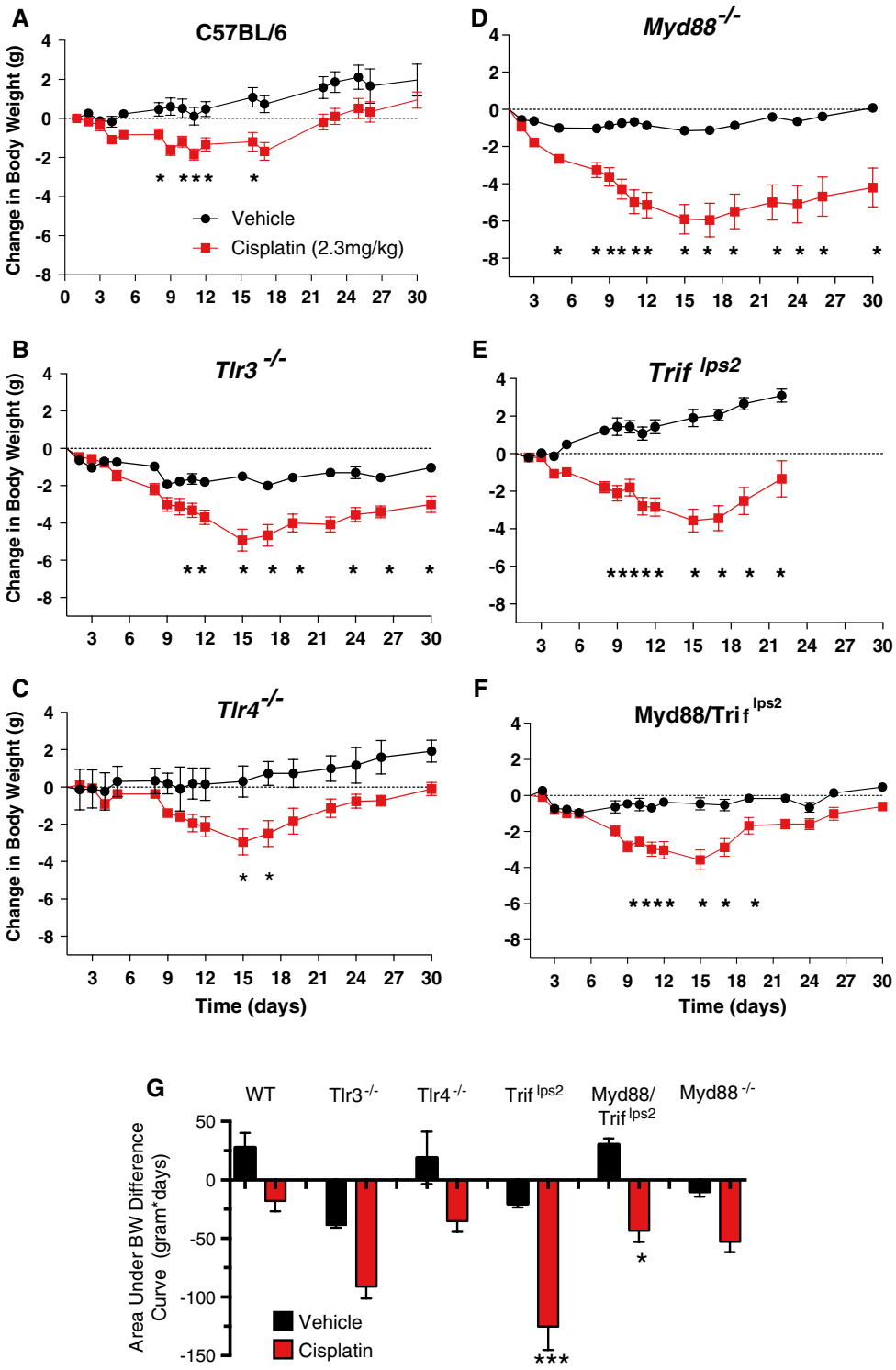


Fig. 1 The cisplatin-injected mice showed a modestly retarded weight gain. Following cisplatin treatment C57BL/6 wild-type (WT) animals showed either no change in body weight or a slight fall (**a**). Cisplatin-injected mice typically showed a loss of body weight during the initial treatment phase in all animals and this fall appeared to be greatest in the *Myd88*$^{-/-}$ mice. After termination of cisplatin treatment, all animals began to display an increase in body weight over the remaining portion of the 30-day interval. Each graph shows the body weights of WT (**a**), *Tlr3*$^{-/-}$ (**b**), *Tlr4*$^{-/-}$ (**c**), *Myd88*$^{-/-}$ (**d**), *Trif*^{lps2} (**e**), and *Myd88/Trif*^{lps2} (**f**) mice. Data are expressed as mean ± SEM ($n = 3$–8 mice/group) and analyzed via 2-way ANOVA, followed by Bonferroni post hoc test to compare each time point to the respective control group (* $P < 0.05$). **g** The area under the weight–time curve for each mouse was calculated and the averages ± SEM are shown. Comparison is made by ANOVA with Bonferroni post hoc comparison to the respective saline-injected control group (*$P < 0.05$, **$P < 0.01$, or ***$P < 0.001$)

change in WT ($P > 0.05$) and a significant reduction in *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, *Trif*^{lps2}, and *Myd88/Trif*^{lps2} mice (Fig. 1a–f, $P < 0.05$). After termination of cisplatin treatment, all animals began to display an increase in body weight over the remaining portion of the 30-day interval.

Myd88/Trif^{lps2} mice are protected from developing neuropathy after cisplatin

The weight loss seen after cisplatin injection validated that a biologic response occurred in all of the drug-treated mice albeit to different degrees. Parenteral

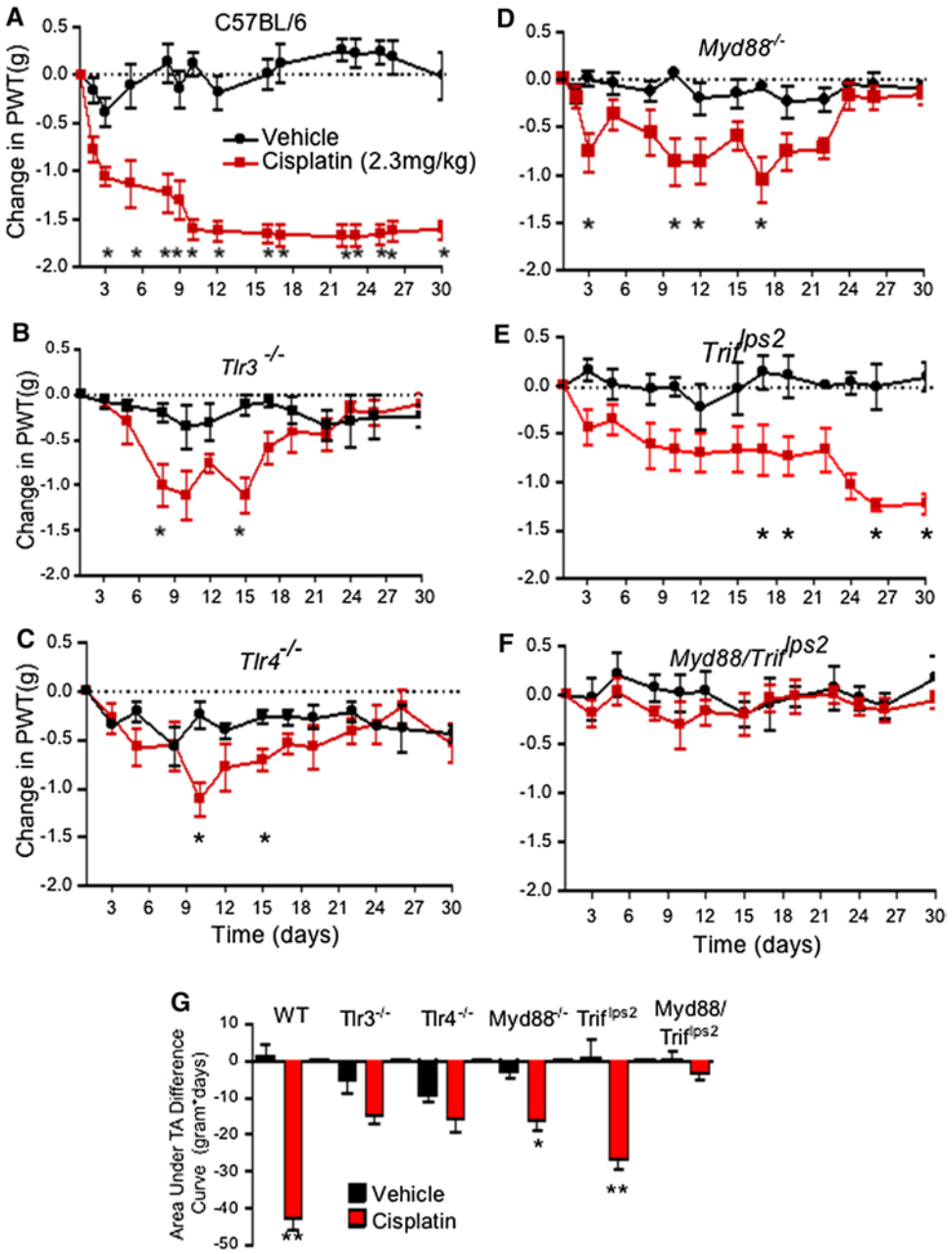


Fig. 2 Mechanical allodynia observed following cisplatin treatment in C57BL/6 wild-type mice was reduced or abolished in TLR signaling-deficient mice. Cisplatin treatment was performed on **a** WT, **b** *Myd88/Trif*^{lps2}, **c** *Tlr3*$^{-/-}$, **d** *Tlr4*$^{-/-}$, **e** *Myd88*$^{-/-}$, and **f** *Trif*^{lps2} mice. Mice were tested from day 1 to day 30 with the von Frey up–down method. The *solid black circle line* and *red rectangular line* represent the vehicle- and cisplatin-injected mechanical thresholds, respectively, on (**a–f**). **a** WT mice show a robust mechanical allodynia beginning 3 days after cisplatin injection ($P < 0.05$). **b** *Myd88/Trif*^{lps2} mice did not show mechanical allodynia through the 30 day follow-up period of cisplatin treatment as compared to baseline and controls ($P > 0.05$). **c** *Tlr3*$^{-/-}$, **d** *Tlr4*$^{-/-}$, and **e** *Myd88*$^{-/-}$ mice showed a modest reduction in mechanical thresholds during cisplatin treatment and recovery of thresholds around at day 24. **f** *Trif*^{lps2} produced a delayed initial onset of the allodynia beginning day 26 after cisplatin injection. Data are expressed as mean ± SEM ($n = 3$–8 mice/group) and analyzed via 2-way ANOVA, followed by Bonferroni post hoc test to compare each time point to the respective control group (*$P < 0.05$). **g** The area under the threshold–time curve for each mouse was calculated, and the averages ± SEM are shown. Comparison is made by ANOVA with Bonferroni post hoc comparison to the respective saline-injected control group (*$P < 0.05$ and **$P < 0.01$)

cisplatin, but not saline, produced a robust and persistent mechanical allodynia in the WT mice. Tactile thresholds significantly declined from day 3 to day 9 and then persisted without improvement through day 30 after cisplatin injection, as compared to the baseline ($P < 0.0001$, Fig. 2a, g).

In contrast to cisplatin-treated WT mice, cisplatin-injected *Myd88/Trif*^{dps2} mice did not show mechanical hyperalgesia or allodynia at any time through the 30-day follow-up period of cisplatin treatment as compared to baseline and controls ($P > 0.05$, Fig. 2f, g).

MyD88 and *TRIF* play distinct roles in allodynia development and recovery

In cisplatin-treated *Myd88*$^{-/-}$ mice, withdrawal thresholds were significantly increased at day 3, 10, and 17 ($P < 0.05$, Fig. 2d). The overall trend was an increase in threshold up to day 17 and then a full recovery by day 30. In T*rif*^{dps2} mice, cisplatin treatment prominently delayed the onset of the allodynia as compared to the cisplatin-treated WT mice, such that withdrawal thresholds did not become significantly reduced until days 24, 26, and 30 ($P < 0.05$, Fig. 3e). Similar to the WT mice, these mice did not demonstrate any recovery from the cisplatin-induced allodynia despite the very slow and delayed onset.

Treatment of individual TLR-deficient mice with cisplatin resulted in a trend similar to MyD88 null mice. In *Tlr4*$^{-/-}$ mice, withdrawal thresholds were significantly increased (e.g., normalized) at day 10 ($P < 0.05$, Fig. 2c). In *Tlr3*$^{-/-}$ mice, mechanical withdrawal thresholds were decreased compared to saline-treated KO mice and statistically different at day 8, 10, and 15 ($P < 0.05$, Fig. 2b). In both of these strains, there was a full recovery to baseline by day 30. These results suggest that the induction of allodynia likely requires the contribution of more than one TLR signaling pathway, and TLR3 and TLR4 can compensate for each other to allow recovery through TRIF signaling (Fig. 2g).

ATF3 induction in the DRG after cisplatin in wild type, but not TLR-deficient strains

In WT, cisplatin-treated mice showed significant activation of neuronal ATF3 as compared to the control group ($P < 0.05$, Fig. 3a, g). However, in the *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$-, *Myd88*$^{-/-}$, and *Myd88/Trif*^{dps2} mice, the number of ATF3 (+) cells was significantly reduced ($P > 0.05$, Fig. 3b–e, g). The *Trif*^{dps2} mice showed a significant increase in DRG ATF3 with cisplatin treatment ($P < 0.05$, Fig. 3f, g), which was still suppressed as compared to cisplatin-treated WT animals ($P < 0.05$, Fig. 3g).

Discussion

This study shows, as previously reported, that cisplatin treatment resulted in a prominent and persistent mechanical allodynia in WT mice, lasting >30 days, without detectable effects upon motor function [5]. There are four key observations reflecting the role of TLR receptors in this robust cisplatin-induced polyneuropathy.

1. Disruption of all downstream TLR signaling by disruption of the *Myd88* and *Trif* adaptor genes completely prevented the onset of cisplatin-initiated allodynia at any time.
2. In TLR3-, TLR4-, or MyD88-deficient mice, mechanical withdrawal thresholds were significantly elevated during the first 15–18 days, but the magnitude of this allodynia was reduced as compared to WT and unlike the WT, displayed complete recovery over the ensuing 15 days (see Fig. 2) when compared to WT cisplatin-treated mice.
3. In TRIF-defective mice, there was a substantial delay in the onset of cisplatin-induced allodynia, but there was no reversal.
4. Cisplatin-treated animals displayed a significant increase in the expression of ATF3 in the dorsal root ganglion of WT mice. This increase was also seen in the Trif mutant mice, but not in any of the other strains tested.

TLR pathways involved in allodynia

Toll-like receptors (TLRs) have been identified in spinal glia and DRG cells [17, 18]. Activation of TLR4 in primary microglia and astrocyte cultures leads to a prominent release of cytokines though the MyD88-NFkB cascade. In contrast, TLR3 signaling through interferon regulatory factors (IRF) will increase IFNß secretion [9]. In previous work, we have shown that the activation of spinal TLR3 and 4 by the intrathecal delivery of their respective ligands initiated a long lasting (7 days) tactile allodynia in mice [13]. The TLR4-ligand effect was mediated by entirely by tumor necrosis factor (TNF); however, the TLR3-ligand-induced allodynia was TNF-independent [13]. Our work in TLR signaling-deficient animals with cisplatin now shows parallel results. The anti-hyperpathic agent, gabapentin, was able to completely reverse the cisplatin-initiated tactile allodynia [5]. However, blockade of TNF function by systemically delivered etanercept had significant, but incomplete effects upon the cisplatin-induced allodynia emphasizing that mediators other than TNF were involved [5]. The involvement of other TLR signaling systems (such as TLR3) is involved is supported by the observation that the cisplatin-induced allodynia was absent in mice that completely lacked TLR signaling (*Myd88/Trif*^{dps2}), but not in either individual pathway or single TLR-deficient strain (*Tlr3*$^{-/-}$ and *Tlr4*$^{-/-}$). It should be noted that these effects are consistent with recent reports that TLR4 inhibitors can diminish the behavioral hyperpathia in mononeuropathies [19].

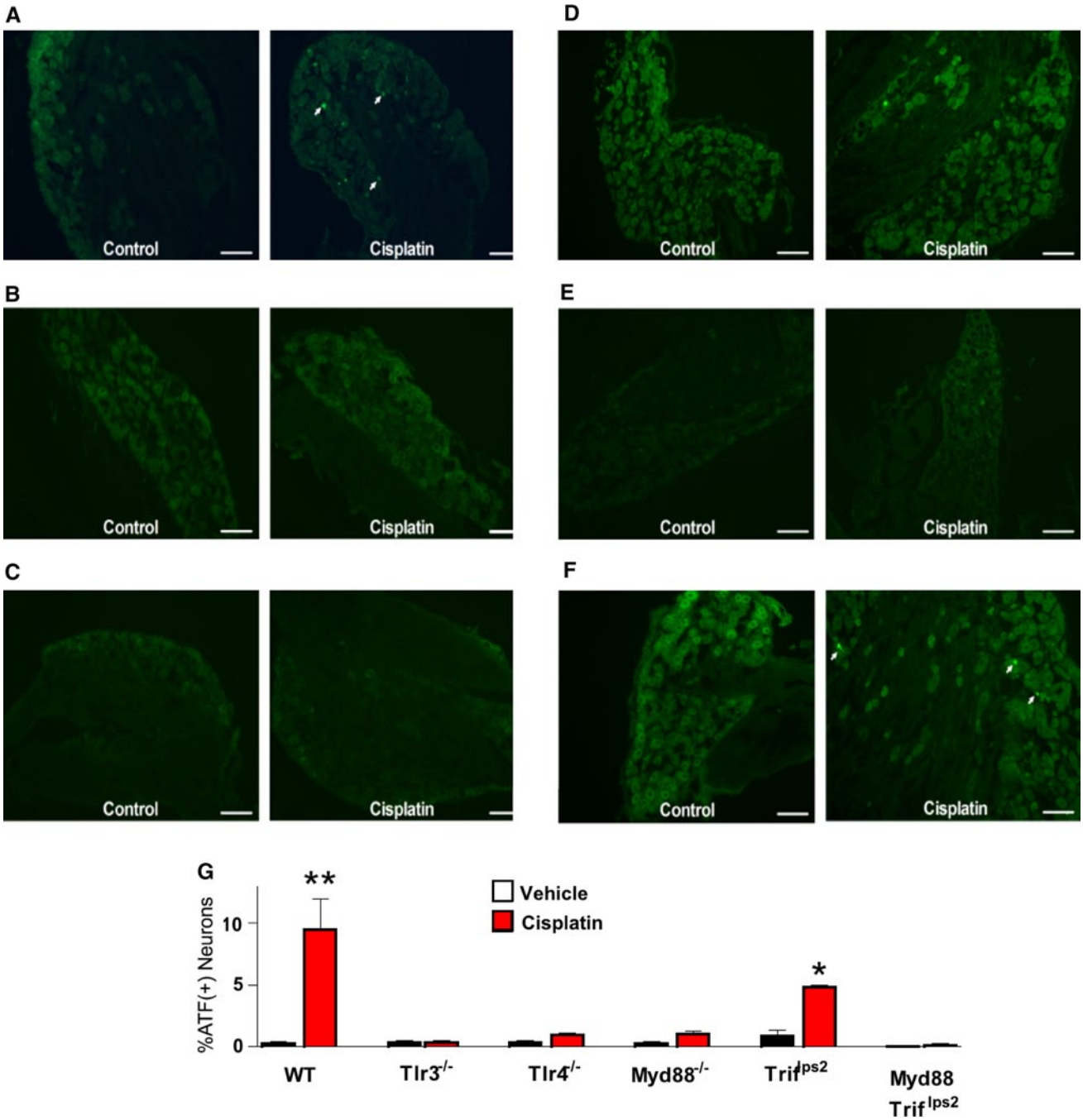


Fig. 3 Toll-like receptor signaling-deficient mice have less ATF3 (+) nuclei following cisplatin-treated neuropathy. At day 30, following cisplatin-treated neuropathy lumbar DRGs were harvested and incubated with an antibody against ATF3. ATF3 immuno-reactivity is visualized with Alexa-488 (*green*) and recognized as an intense fluorescent mark in the nuclei region. The *white arrows* point to examples of ATF3 staining. **a** WT cisplatin-treated DRGs showed significant activation of neuronal ATF3 as compared to control group. *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, and *Myd88/Trif*^{lps2} DRGs display less ATF3 immunoreactivity in their cisplatin-treated DRGs when compared to WT (n = 3–6 mice/group). Meanwhile, the *Trif*^{lps2} mice showed modest increase in DRG ATF3 with cisplatin treatment, which was still suppressed as compared to cisplatin-treated WT animals. Representative stained images from the DRGs of WT (**a**), *Myd88/Trif*^{lps2} (**b**), *Tlr3*$^{-/-}$ (**c**), *Tlr4*$^{-/-}$ (**d**), *Myd88*$^{-/-}$ (**e**), and *Trif*^{lps2} (**f**) mice are presented. Examples of ATF3 (+) nuclei are marked with a *white arrow* in (**a**) and (**f**). **g** WT cisplatin-treated DRGs displayed significantly increased incidence of neuronal ATF3 immuno-reactivity as compared to control group. In contrast, cisplatin-treated *Tlr3*$^{-/-}$, *Tlr4*$^{-/-}$, *Myd88*$^{-/-}$, and *Myd88/Trif*^{lps2} mice all displayed highly significant reductions in ATF3 counts as compared to the WT animals. Data expressed as mean ± SEM (n = 3–6 mice/group) and analyzed via 1-way ANOVA followed by Newman–Keuls multiple comparison test (*$P < 0.05$ and ***$P < 0.001$)

ATF3 is augmented in DRGs of mice with residual cisplatin-induced neuropathy

As reviewed, ATF3 is considered to be a DRG marker for events leading to impairment of the integrity of the sensory afferent. As a transcription factor, this agent is also considered to play an important role in regulating signaling, particularly though NFkB [20]. ATF3 null animals have been shown to display a significant enhancement of the response of the cell systems to TLR4 activation. Such animals for example will display cytokine storms and enhance the lethality associated with TLR4 activation (e.g., in septic shock) [7]. In vivo, increased ATF3 expression occurs in the DRG during chronic inflammation, in osteoarthritis [21] and after nerve injury in models of mononeuropathy (nerve ligation) and as with chemotherapy-induced polyneuropathy [6, 22, 23]. Further, afferent traffic as initiated by injury and inflammation has the ability to initiate this activation in subpopulations of sensory neurons [24]. Thus, the increase in ATF3 expression in nerve injury pain state raises the intriguing likelihood that the ATF3 response serves to regulate the magnitude of the response of the organism initiated by TLR4 activation after nerve injury. After 30 days, only the WT and the *Trif*^{lps2} mice had persistent cisplatin-induced allodynia and elevated ATF3 in the DRGs. The TLR3-, TLR4-, and MyD88-deficient strains had recovered from the chemotherapy-induced hyperpathia and had no elevation in the ATF3 levels. This would suggest that the neuronal ATF3 itself is regulated by the activation of the TLR-MyD88/TRIF cascades in the DRG. It will be interesting to assess the effects of elimination of the DRG-ATF3 response on the behavioral sequelae arising after nerve injury.

Role for endogenous TLR ligands in nerve injury-induced allodynia

The present studies raise the issues of what endogenous agents are mediating the effects of nerve injury that are blocked in the TLR signaling-deficient mice. Neurons and glia are activated by peripheral nerve injury and chronic inflammation [25, 26]. Several products have been shown to be released from such cell systems, which can interact with the distinct TLRs. Such agents include the high mobility group box 1 (HMGB1), tenascin C, peroxiredoxin (Prx) family proteins, β-amyloid (Aβ), hyaluronan, DNA or RNA, heat shock proteins, and heparan sulfate [27–35]. Indeed, mRNA and protein expression of HMGB1 spinal dorsal horn and DRG were increased in nerve-injured rats [36].

In our studies, both the *Myd88*$^{-/-}$ and the *Trif*^{lps2} mice developed cisplatin-induced allodynia, albeit to varying extents. However, the *Myd88/Trif*^{lps2} mice were completely protected. These results suggested that both MyD88 and TRIF are required for the full manifestations of cisplatin-induced pain. The *Myd88*$^{-/-}$ mice recovered with full resolution of hyperpathia, whereas the *Trif*^{lps2} and WT mice remained fully sensitized at day 30. These results suggest that TRIF signaling is necessary but not sufficient for recovery or reversal of cisplatin-induced pain. In addition, there are likely several TLRs involved in the full induction of the hypersensitivity as disruptions in TLR3 or TLR4 alone had modest effects. The other TLRs did not fully compensate for the loss of either of these TLRs. In injured tissues, it is likely that there is release of multiple endogenous TLR ligands that contribute to the full manifestation of this deleterious side effect.

TLRs and cisplatin-induced weight loss

The present work demonstrated that cisplatin resulted in a loss of the normal weight gain over the period of drug delivery. The loss of weight has been ascribed to gastrointestinal distress and the consequence of the extreme emetogenic activity of this chemotherapeutic [16]. The mechanism of this action is not well understood. Cisplatin has been shown to stimulate intestinal enterochromaffin cells to release serotonin, which through an action on vagal 5-HT3 receptors initiates brainstem reflexes to alter gastrointestinal motility [37]. The exacerbation of this weight loss in the mutant animals was unexpected. Activation of TLR4 has, for example, been associated with emetic activity in humans [38]. Other TLRs have been shown to play important roles in mediating bowel pathology and permeability [39]. We would note that while many downstream effects mediated through MyD88 are commonly associated with pro-inflammatory events, coupling through both TRIF and MyD88 can also lead to activation of interferon pathways, which may play an immunomodulatory role that is lost in the *Myd88/Trif*^{lps2} mice [40].

In conclusion, our work reveals that KO of TLR signaling leads to a prominent effect on both the response to the injury as measured by changes in the DRG transcription factor ATF3 and by the ability to persistently alter the polyneuropathic hyperpathia otherwise induced by the chemotherapeutic cisplatin.

Acknowledgments This work was supported by Grants from National Institutes of Health: NS16541 and DA02110.

Conflict of interest None.

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