Role of Pattern Recognition Receptors in Chemotherapy-Induced Neuropathic Pain

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Supplementary material

Material & Methods

Oligodeoxynucleotides antisense to TLR4 and RAGE mRNA

A nucleotide BLAST search was performed to confirm that the mRNA sequence targeted by each AS-ODN, was not homologous to any other sequences in the rat database, and that MM-ODN control sequences were not homologous to any sequences in the rat database, as well. Before use, lyophilized ODNs were reconstituted in nuclease-free 0.9% NaCl and then administered intrathecally (i.t.) at a dose of 6 μg/μL, in a volume of 20 μL (120 μg/20 μL). As described previously [1], after rats were anesthetized with isoflurane (2.5% in O_2), ODN was injected, using a 0.3 mL syringe (300 units/μL; Walgreens, IL, USA) with a 29-gauge hypodermic needle, inserted between the L4 and L5 vertebrae into the subarachnoid space. The intrathecal site of injections were confirmed by elicitation of a sudden flick of the rat's tail, a reflex that is evoked by accessing the subarachnoid space and bolus injection [2]. A total of 120 μg of ODN, in a volume of 20 μL, was then injected. Rats regained consciousness approximately 2 minutes after anesthesia was discontinued. Use of the intrathecal route of administration of AS-ODN, to attenuate the expression of proteins essential for their role in nociceptor sensitization, is well supported by previous studies [3-11].

SDS-PAGE and Western blotting

DRGs were transferred into homogenization buffer (100mM NaCl, 1mM EDTA, 2% SDS, 50mM Tris-HCl, pH 7.4) supplemented with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IL), and manually homogenized with a hand-held plastic pistil. Proteins were solubilized by incubating the DRG homogenates for 2 h at 37^{o}C and 1400 rpm in an Eppendorf Thermomixer (Eppendorf AG, Hamburg, Germany). Solubilized proteins were extracted from insoluble cell and tissue components by a 15 min. centrifugation at 14000 rpm in a tabletop centrifuge. Protein concentration of all samples was determined using the micro–BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin (BSA) as the standard. Mixtures of 30 µg of protein per sample were denatured by boiling in sample buffer [3% SDS, 10% (v/v) Glycerol, 5% (v/v) β-Mercaptoethanol, 0.025 Bromphenol blue, 62.5 mM Tris-HCl pH 6.8] for 10 minutes and electrophoresed on 4-15% pre-cast polyacrylamide gels (Biorad, Hercules, CA) in 25mM Tris containing 192 mM glycine and 0.1% SDS. Proteins then were electrophoretically transferred to a nitrocellulose membrane using the semidry method [transfer time 2 h at 60mA/gel with 47.9mM Tris, 38.9 mM Glycine, 0.038% SDS and 20% (v/v) Methanol]. The nitrocellulose membranes were saturated by shaking in antibody dilution buffer [5% BSA in Tris-buffered saline containing 0.1% Tween 20, pH 7.4 (TBST)] for 1h at room temperature, cut in half at ~75kDa and probed with either a mouse monoclonal anti-TLR4 (NB100-56566, 1:500, Novus Biologicals, Littleton, CO) or a rabbit polyclonal anti-β actin (ab8227, 1:1000, Abcam, Cambridge, MA) antibody in antibody dilution buffer at 4 ^{o}C overnight. Blots probed with the anti-TLR4 antibody were rinsed with TBST (3 times at room temperature (RT), 15 minutes each) and probed with a biotinylated anti-mouse antibody (115-065-003, 1:2000, Jackson Immunoresearch Laboratories Inc.) in antibody dilution buffer for 2 h at RT. Blots were rinsed

with TBST (3 times at room temperature (RT), 15 minutes each) and probed with either horseradish peroxidase conjugated streptavidin (S2438, Sigma-Aldrich, 1:5000 in antibody dilution buffer) or a horseradish peroxidase conjugated anti-rabbit antibody (NA934V, GE Healthcare LifeSciences, Pittsburgh, PA, 1:2500 in antibody dilution buffer) for 2h at RT. The blotting membranes were rinsed with TBST (3 times at RT, 15 minutes each) and immunoreactivities visualized using the West femto chemiluminescence detection system (Pierce Biotechnology).

DRG neuron culture

In brief, under isoflurane anesthesia, rats were decapitated, and the dorsum of the vertebral column surgically removed; L_4 and L_5 DRGs were rapidly extracted, bilaterally, chilled and desheathed in Hanks' balanced salt solution (HBSS), on ice. Ganglia were then treated with 0.25% collagenase Type 4 (Worthington Biochemical Corporation, Lakewood, NJ, USA) in HBSS for 18 minutes at 37°C, and followed by treatment with 0.25% trypsin (Worthington Biochemical Corporation) in calcium- and magnesium-free phosphate buffered saline (PBS) (Invitrogen Life Technologies, Grand Island, NY USA) for 6 minutes, followed by three washes, and then trituration in Neurobasal-A medium (Invitrogen Life Technologies), to produce a single-cell suspension. This cell suspension was centrifuged at 1000 RPM for 3 minutes and re-suspended in Neurobasal-A medium supplemented with 50 ng/mL nerve growth factor, 100 U/mL penicillin/streptomycin, B-27, GlutaMAX and 10% FBS (Invitrogen Life Technologies). Cells were then plated on cover slips and incubated at 37°C in 3.5% CO_2 for at least 24 hours before use in experiments.

To parallel *in vivo* experiments, *in vitro* experiments were also performed using prevention and reversal protocols. For *in vitro* reversal of sensitization, LPS-RS Ultrapure (a selective inhibitor of TLR4; 10 μg/ml) was added to the culturing media 24 h before *in vitro* recordings. In *in vitro* experiments where oxaliplatin was administered *in vivo*, DRG culture was prepared 3 weeks after administration of oxaliplatin. For prevention experiments with TLR4, AS-ODN was administered *in vivo*, as in the behavioral studies. To prevent the effect of *in vitro* administered oxaliplatin using *in vivo* TLR4 AS-ODN, DRG neuron cultures were prepared the day after the last administration of TLR4 AS-ODN. For prevention of *in vitro* administered LPS-RS Ultrapure on the effect of oxaliplatin administered *in vitro* ("all *in vitro*" prevention), LPS-RS Ultrapure (10 μg/ml) was added to the culturing media 24 h before adding oxaliplatin (see next section for details).

In vitro patch-clamp electrophysiology

DRG from at least three rats (separate culture preparations) were used for each experimental series. Within the text, "n" refers to number of neurons. Cells were identified as neurons by their double birefringent plasma membranes [12,13]. After mounting a coverslip plated with cells in the recording chamber, the culture medium was replaced with external perfusion solution containing 140 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, and adjusted to pH 7.4 with NaOH; osmolarity is 310 mOsm/kg [14-16]. Drugs used *in vitro* were diluted to their final concentration in this solution, just prior to application. The volume of the recording chamber is 150 μL. The perfusion system is gravity-driven, with a flow rate of 0.5–1 mL/minute. Recording electrodes were fabricated from borosilicate glass capillaries (0.84/1.5 mm i.d./o.d., Warner Instruments, LLC, Hamden, CT, USA) using a Flaming/Brown P-87 puller (Sutter Instrument Co., Novato, CA, USA). Recording electrode resistance was ~3 MΩ after being filled

with a solution containing (in mM) 130 KCl, 10 HEPES, 10 EGTA, 1 $CaCl_2$, 5 MgATP, and 1 Na-GTP, pH 7.2 (adjusted with Tris-base), 300 mOsm (measured by Wescor Vapro 5520 osmometer, ELITech Group, Puteaux, France) [14,15]. Junction potential was not adjusted. Series resistance was below 10 MΩ at the end of recordings and was not compensated. Recordings were made with an Axon MultiClamp 700 B amplifier, filtered at 10 kHz, and sampled at 20 kHz using Axon Digidata 1550B controlled by pCLAMP 11 software (all from Molecular Devices LLC, Sunnyvale, CA, USA).

All parameters were measured at least 10 min after the establishment of whole cell configuration, at which time baseline current was stable. For experiments in which the effect of oxaliplatin administered *in vitro* was studied, oxaliplatin (50 μM) was added to the experimental chamber 3 h before recordings and remained in the external perfusion solution for the duration of the experiment.

Table, Figures & Figure Legends

Supplementary Table 1. *Electrophysiological parameters of small DRG neurons in control and in vivo oxaliplatin-treated (CIPN) groups, in vitro.*

	Control	CIPN	Difference [a]	T-test [f]	
				t	*p*
Input resistance at RMP, MOhm	156 ± 20	124 ± 27	-32 ± 34 *(-103 … 40)*	0.93	0.36
Input resistance at baseline, MOhm	155 ± 14	140 ± 27	-15 ± 28 *(-73 … 42)*	0.57	0.58
Series resistance, MOhm	4.3 ± 0.3	5.0 ± 0.4	0.6 ± 0.4 *(-0.3 … 1.6)*	1.44	0.16
AP overshoot, mV [b]	38 ± 1	35 ± 2	-3 ± 2 *(-8 … 2)*	1.22	0.24
AP amplitude from baseline, mV [c]	107 ± 1	106 ± 2	-1 ± 2 *(-6 … 4)*	0.44	0.67
AP amplitude from AP threshold, mV [d]	60 ± 4	73 ± 3	12 ± 6 *(-0.9 … 25)*	1.95	0.07
Baseline membrane potential, mV	-70 ± 1	-70 ± 1	-0.3 ± 1.5 *(-3.5 … 2.9)*	0.20	0.84
Holding current, pA [e]	-139 ± 16	-141 ± 29	-2 ± 30 *(-65 … 61)*	0.07	0.94

All parameters are presented as *mean ± S.E.M.* Number of values (cells) used for calculation: 14 in control and 8 in CIPN groups.

[a] Difference between the groups is value of the parameter in CIPN group minus value of the parameter in control. The 95% confidential interval *(in brackets)* indicates the range *("lower limit … upper limit"*) within of which the unknown "true" difference is expected with probability of 95%.

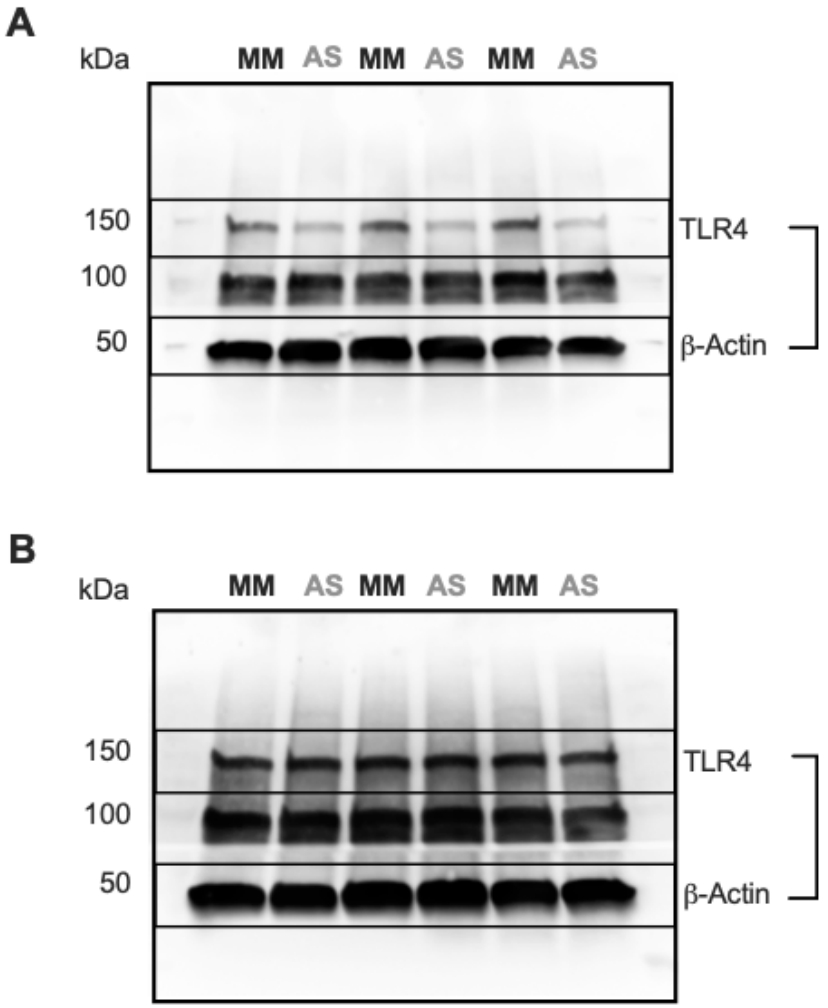
[b] AP overshoot is a peak AP potential above zero.

[c] AP amplitude from baseline is defined as "AP overshoot" – "baseline membrane potential".

[d] AP amplitude from AP threshold is defined as "AP overshoot" – "AP threshold potential".

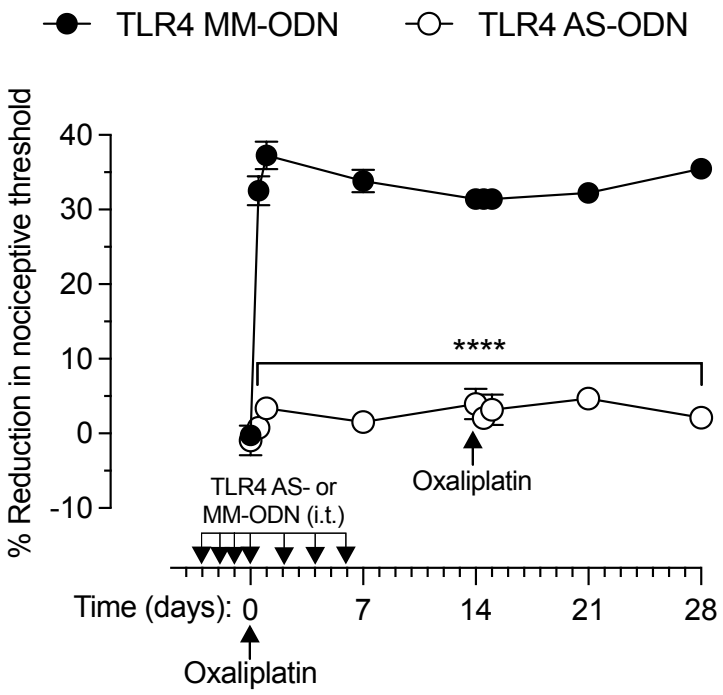
[e] Holding current (in current clamp mode) is the current required to achieve the baseline membrane potential from which all stimulation were performed.

[f] Values of each parameter were compared between two groups with unpaired two-tailed T-test reporting following parameters: number of the degrees of freedom, df = 20; t – value of t-statistics of T-test, *p* – the probability of getting that magnitude of discrepancy (Difference) by chance in case if actual means of two groups are the same. Importantly, statistically significant difference was not found in any of the analyzed parameters ($p > 0.05$) that does not prove absence of differences. In such case, 95% CI range of the difference could be used for further interpretation of the result. Of note, these comparisons are actually "stacked" multiple pairwise comparisons, however, we did not apply adjustments for multiple T-test comparisons here because it would make the comparisons even more strict and would not help to reveal any additional statistically significant differences compared to unadjusted ones.



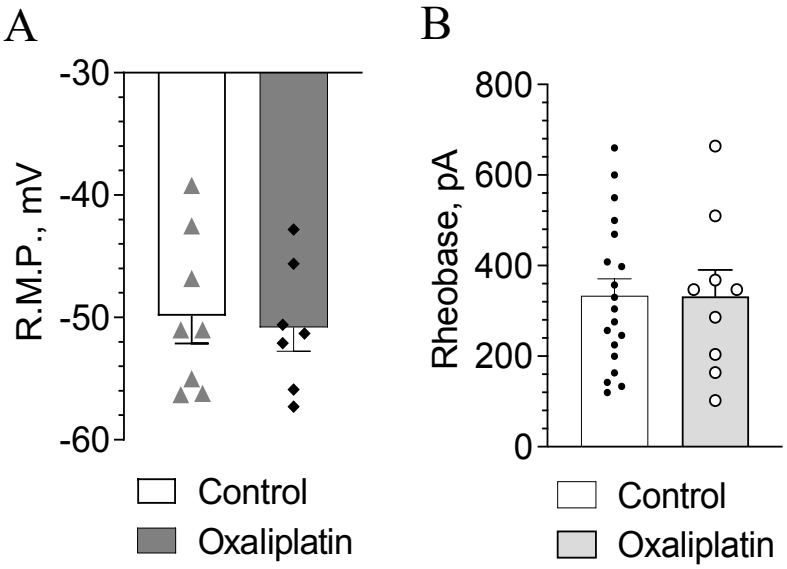
Supplementary Figure 3. ***TLR4 antisense attenuates TLR4 protein expression in L4 and L5 DRG and oxaliplatin induced CIPN.***

In Figures 3A and 3B of the publication, only the two rectangular boxes of the blots with the anti-TLR4 and anti-beta-actin immunoreactivities are shown. To determine the success of the blotting, a prestained protein marker from Bio-rad (cat. no. 161-0373; with prestained proteins of 250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa) was used. The mouse monoclonal anti-TLR4 antibody we used has been extensively characterized. At the time of this publication, there have been at least 126 publications in which the antibody has been used for various applications such as Western blotting, ELISA, immunohistochemistry and in situ hybridization on a range of human, mouse, rat and other tissues (see provider product page for more detailed information). The same also applies to the rabbit anti-beta-actin antibody from Abcam (further information can be found on the provider's product page).



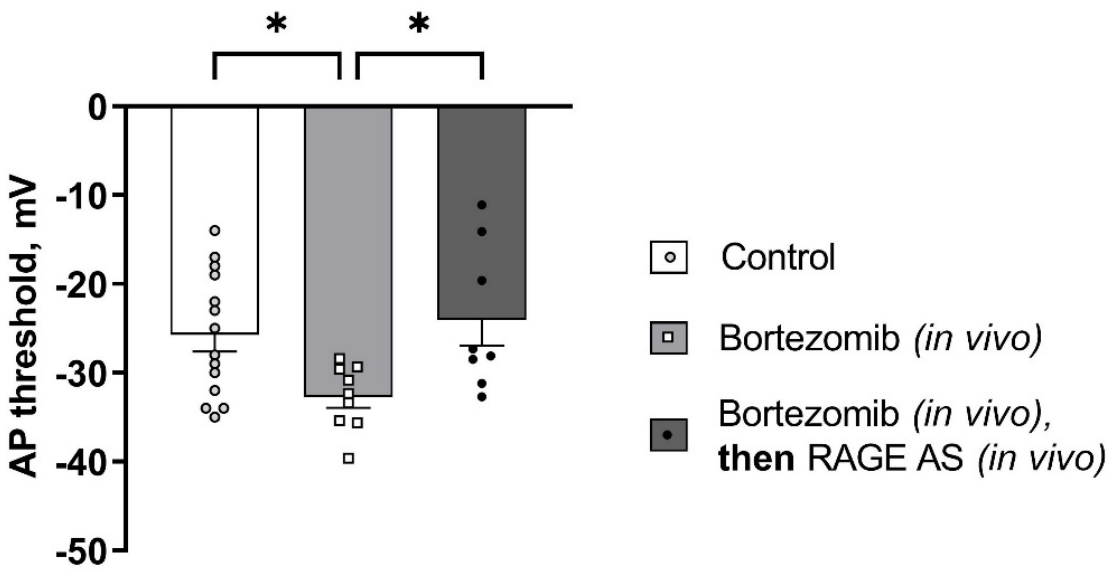
Supplementary Figure 4. ***TLR4 antisense two weeks prior to oxaliplatin blocks the hyperalgesia induced by its second administration.***

Male rats were treated intrathecally (i.t.) with AS-ODN or MM-ODN (both 120 µg in 20 µL/day) against TLR4 mRNA, once a day starting 3 days before the oxaliplatin, for 7 doses, over 10 days. On day 0, rats received oxaliplatin (2 mg/kg, i.v.) and mechanical nociceptive threshold was evaluated before the first dose of ODN (day -3) and then from day 0 to 28. In the TLR4 AS-ODN-treated group, oxaliplatin-induced hyperalgesia was inhibited when measured 30 min, 24 hours, 7 and 14 days after i.v. oxaliplatin. On day 14 after oxaliplatin (and 8 days after the last intrathecal TLR4 AS-ODN administration) only the TLR4 AS-ODN group received a second dose of intravenous oxaliplatin (2 mg/kg), and mechanical nociceptive threshold was evaluated 30 min and 24 hours later. In this group, the second dose of oxaliplatin did not induce hyperalgesia for the remainder of the 28-day testing period (data are mean ± SEM. Two-way repeated-measures ANOVA, time x TLR4 AS-ODN interaction, $F_{(8,80)} = 31.54$, $p < 0.0001$; TLR4 AS-ODN treatment, $F_{(1,10)} = 800.3$, $p < 0.0001$; Bonferroni's multiple *post hoc* comparisons test: ****$p < 0.0001$ [TLR4 MM-ODN vs AS-ODN]). N = 6 paws for each group.



Supplementary Figure 5. ***Resting membrane potential (RMP) and Rheobase of DRG neurons from naïve and in vivo oxaliplatin-treated animals, tested in vitro.***

Comparison of RMP (**A**) and Rheobase (**B**) between cultured DRG neurons from control (naïve) animals and animals treated with oxaliplatin *in vivo* (as described in **Fig. 2A**; neuronal culture was prepared 3 weeks after the treatment) did not reveal statistically significant differences, supporting the suggestion that oxaliplatin does not induce long lasting changes in these electrophysiological parameters. **A.** RMP in control and *in vivo* oxaliplatin-treated group was compared with unpaired two-tailed *t*-test, no statistical significance revealed: $t_{(13)} = 0.31$, $p = 0.76$ (8 cells in control and 7 cells in treated group). **B.** Rheobase in control and *in vivo* oxaliplatin-treated group was compared with unpaired two-tailed *t*-test, no statistical significance revealed: $t_{(26)} = 0.017$, $p = 0.99$ (19 cells in control and 9 cells in treated group).



Supplementary Figure 6. ***RAGE antisense reversal of bortezomib-induced nociceptor sensitization tested <u>in vitro</u>.***

Effect of bortezomib, administered *in vivo*, on action potential threshold and attenuation of this effect by RAGE AS-ODN administered *in vivo,* in the <u>reversal</u> protocol. Bortezomib induced reduction in action potential threshold, compared to a control group (untreated naïve animals) (one-way ANOVA: $F_{(2,28)} = 4.4$, $p = 0.02$; Holm-Šídák's *post hoc* test: $t_{(28)} = 2.5$, ** adjusted $p = 0.02$). RAGE AS-ODN administered intrathecally (120 µg in 20 µL/day; 7 doses, over 10 days,) once a day starting 3 days after the last dose of bortezomib (0.2 mg/kg, i.v., on days 0, 2, 4, and 6) produced a significant shift in action potential threshold back toward the control value ($t_{(28)} = 2.7$, $^{\#}$ adjusted $p = 0.02$ compared to *in vivo* bortezomib). Number of cells: 14 in control group (left bars), 9 in bortezomib group (middle bars), 8 in reversal group (RAGE AS-ODN administered after onset of bortezomib-induced hyperalgesia, as in **Fig. 7F**, *white diamond symbol*).

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