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Painful Pathways Induced by TLR Stimulation of Dorsal Root Ganglion Neurons

Jia Qi,* Kristzina Buzas,*† Huiting Fan,*,† Jeffrey I. Cohen,‡ Kening Wang,‡ Erik Mont,‡ Dennis Klinman,§ Joost J. Oppenheim,* and O. M. Zack Howard*

We hypothesize that innate immune signals from infectious organisms and/or injured tissues may activate peripheral neuronal pain signals. In this study, we demonstrated that TLRs 3, 7, and 9 are expressed by human dorsal root ganglion neurons (DRGNs) and in cultures of primary mouse DRGNs. Stimulation of murine DRGNs with TLR ligands induced expression and production of proinflammatory chemokines and cytokines CCL5 (RANTES), CXCL10 (IP-10), IL-1α, IL-1β, and PGE₂, which have previously been shown to augment pain. Further, TLR ligands upregulated the expression of a nociceptive receptor, transient receptor potential vanilloid type 1 (TRPV1), and enhanced calcium flux by TRPV1-expressing DRGNs. Using a tumor-induced temperature sensitivity model, we showed that in vivo activation of a TLR9 antagonist, known as a suppressive oligodeoxynucleotide, blocked tumor-induced temperature sensitivity. Taken together, these data indicate that stimulation of peripheral neurons by TLR ligands can induce nerve pain. The Journal of Immunology, 2011, 186: 6417–6426.

Toll-like receptors play a fundamental and essential role in host defense during pathogen infection by regulating and linking innate and adaptive immune responses (1, 2). The 12 mammalian TLRs belong to a family of receptors that recognize pathogen-associated molecular patterns and can be divided into those that are expressed in the cell membrane and those located in endosomes. The ones located in endosomes, TLR3, TLR7/8, and TLR9, are activated by double-stranded and single-stranded nucleotides of viral or cellular origin. Innate immune cells sense viral infection by detecting viral proteins and/or nucleic acids. TLR3 is known to be a major mediator of the cellular response to viral infection because it responds to dsRNA, a common by-product of viral replication (3), whereas TLR7 and TLR9 are activated by ssRNA and cytosine-guanosine (CpG) DNA, respectively.

Pain is generated by a combination of sensory and affective components and classified as physiological, normal, or chronic pain. Chronic pain, including tissue injury-associated inflammatory pain and nerve injury-associated neuropathic pain, is often more intense than the underlying tissue damage would predict. The vanilloid receptor 1 (VR1), which is also known as transient receptor potential vanilloid type 1 (TRPV1), is an ion channel receptor that has been validated as a pain target by chemical stimulation, using capsaicin (CAP) or by endogenous anandamide (Ana), and by genetic deletion (4). Our earlier studies have shown that signals initiated by chemokine receptors (5, 6), which are expressed by both immune and nervous tissue, enhance expression and function of TRPV1 (7). This led us to question if pain sensation in peripheral nervous system neurons could also be enhanced by cross-talk between classic innate immune receptors like TLRs and TRPV1.

There is considerable evidence showing that TLRs participate in nerve injury in the peripheral nervous system and CNS (8–10) but little evidence showing that neurons respond to innate immune stimuli. TLR3 has a role in the activation of spinal glial cells and the development of tactile allodynia, which is pain in response to inoffensive stimulation after nerve injury (11). Intrathecal administration of TLR3 agonist polyinosinic-polycytidylic acid (poly-I:C) induced behavioral, morphological, and biochemical changes similar to those observed after nerve injury (11). Conversely, downregulation of TLR3 inhibited spinal nerve injury induced by proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α (11). Furthermore, TLR3 antisense oligodeoxynucleotide (ODN) suppressed nerve injury-induced tactile allodynia and decreased the phosphorylation of p38 MAPK in spinal glial cells (11). Lafon et al. reported that human neurons, in the absence of glia, expressed TLR3 and sensed viral dsRNA, thus neurons have the intrinsic machinery to trigger robust inflammatory, chemotactic, and antiviral responses (12). However, whether TLR3 contributes to pain signals remains unknown. By examining the role of spinal cord glial cells in neuropathic pain and opioid actions, Hutchinson et al. demonstrated that TLR4-dependent glial activation is pivotal to the maintenance of neuropathic pain, and TLR4-dependent opioid-induced glial activation is fundamental to reducing morphine analgesia and producing dependence (13).

*Laboratory of Molecular Immunoregulation, Cancer and Inflammatory Program, Center for Cancer Research, National Cancer Institute-Frederick, Frederick, MD 21702; †Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; ‡Nova Scotia Medical Examiner Service, Halifax, Nova Scotia B3J1H6, Canada; and §Laboratory of Experimental Immunology, Cancer and Inflammatory Program, Center for Cancer Research, National Cancer Institute-Frederick, Frederick, MD 21702

1Current address: Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary.

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Address correspondence and reprint requests to Dr. O.M. Zack Howard, Laboratory of Molecular Immunoregulation, Cancer and Inflammatory Program, Center for Cancer Research, National Cancer Institute-Frederick, P.O. Box B, 1050 Boyles Street, Frederick, MD 21702. E-mail address: howardr@mail.nih.gov

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Abbreviations used in this article: Ana, anandamide; CAP, capsaicin; DRG, dorsal root ganglion; DRGN, dorsal root ganglion neuron; NGS, normal goat serum; ODN, oligodeoxynucleotide; poly:IC, polynosinic-polycytidylic acid; Sup ODN, suppressive oligodeoxynucleotide; TRPV1, transient receptor potential vanilloid type 1.
Thus, some TLRs provide a key link between the innate immune system and the nervous system (14–16). This led us to hypothesize that TLR ligands generated by viral infections or cell death may induce painful signals in the peripheral nervous system by stimulating peripheral sensory neurons exemplified by dorsal root ganglion neurons (DRGNs). We therefore investigated whether DRGNs express TLRs and whether the TLRs participate in the pain signals when stimulated by TLR 3, 7, or 9 ligands.

In the current study, we demonstrate that both human and mouse DRGNs express TLR3/7/9 and that stimulating mouse DRGNs with TLR3/7/9 ligands increased TLR3/7/9 expression. Murine DRGNs stimulated with TLR ligands increase mRNA expression and protein production of many inflammatory cytokines and chemokines, which have previously been identified as mediators of pain hypersensitivity. Further, TLR ligands upregulated the expression of TRPV1, a nociceptive receptor, and also enhanced calcium (Ca$^{2+}$) flux mediated by TRPV1. These results provide new insights into the role of TLRs in pain signaling by peripheral neurons.

Materials and Methods

Primary DRG culture

These studies were performed in compliance with the principles and procedures outlined in the National Research Council’s Guide for the Care and Use of Laboratory Animals and were approved by the National Cancer Institute-Frederick Animal Care and Use Committee under Animal Study Protocols 08-005 and 10-218. DRG cultures were prepared as described previously (17, 18) with modifications (19). A detailed description of the procedure for the removal of rat dorsal root ganglia (DRGs), used in this article for both mice and rats, can be found elsewhere (20) as can concerns about plating surface coatings (21). Briefly, DRGs were dissected from 13- to 15-day-old mouse embryos of NIH Swiss mice (NIH Swiss is N:NIIH(S), which was derived from the N:GP(S) colony in 1936; C57BL/6N mice (NCI Cancer Research Institute-Frederick Animal Care and Use Committee under Animal Study Protocols 08-005 and 10-218) or MyD88 knockout mice (from Dr. S. Akira, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; further back-crossed to C57BL/6N by Dr. D. Klinman, National Cancer Institute, Frederick, MD). DRGs were then rinsed in DMEM (Mediatech, Herndon, VA) for 10 min. After centrifugation at 350 × g for 5 min, the cells were resuspended in DMEM (Mediatech, Herndon, VA) containing 5% heat-inactivated 10% FBS (nucleus containing; HyClone, Thermo Scientific, Logan, UT), 4 mM t-glutamine (Quality Biological, Gaithersburg, MD), and penicillin–streptomycin (100 U/ml and 100 μg/ml, respectively; Quality Biological). The cells (7.5 × 10^5) were then plated onto 4-well or 8-well chambered coverglass (Nunc, Rochester, NY) precoated with poly-l-lysine (Sigma-Aldrich, St. Louis, MO) and collagen type I (Innaemed Biomaterials, Fremont, CA) for 1 h. Subsequently, cells were grown in neurobasal medium (Life Technologies) supplemented with 10% heat-inactivated horse serum (nucleus containing; Sigma-Aldrich), 2% B27 supplement (Life Technologies), 3 μg/ml glucose, 0.5 μg/ml mitotic inhibitor (Sigma-Aldrich) to inhibit cell division, and 100 ng/ml nerve growth factor (R&D Systems, Minneapolis, MN) to promote neuronal survival and differentiation. DRG cultures were maintained in the medium in a 5% CO2 incubator at 37°C for 4 d, at which point well-differentiated dendrites were observed. Primary DRG neuron cultures were used on the fifth day of culture.

TLR ligand treatment

TLR ligands, poly-IC (25 μg/ml, TLR3), gardiquimod (3 μg/ml, TLR7 and TLR8), ODN 1826 (32 μg/ml, TLR9 agonist), and ODN 2088 (50 μg/ml, TLR9 antagonist) (InvivoGen, San Diego, CA) were dissolved in media and added to the cultures on day 5 for 16 h. Suppressiv oligodeoxy-nucleotides (Sup ODNs) for in vivo studies were provided by Dr. Klinman (22, 23). Sup ODN A151 had a phosphorothiate backbone of sequence 5'-TTAGGGTTAGGGTTAGGGTTAGGG-3' and was synthesized by the Center for Biologics Evaluation and Research Core Facility.

Immunohistochemistry

Immunohistochemical analysis was performed on primary cultured embryonic murine DRGNS and adult human DRG sections. Human ganglia were collected at autopsy less than 24 h after death, frozen on dry ice, and stored at −80°C before cryostat sections were obtained. The Office of Human Subjects Research at the National Institutes of Health deemed this research exempt. Cultured DRGNS and human DRG cryostat sections were fixed with 4% paraformaldehyde for 10 min, washed with PBS three times each for 5 min, permeabilized with 0.1% Triton X-100 in PBS containing 1% normal goat serum (NGS) (Sigma-Aldrich) for 10 min, and washed with PBS three times each for 5 min. Samples were then blocked in 5% NGS at room temperature for 1 h. After blocking, the samples were then incubated with primary Abs, which included rabbit polyclonal Ab against TLR3 (cat. no. 3643), TLR7 (cat. no. 3269), TLR9 (cat. no. 3739) (1:200, respectively; Proscei, Poway, CA), these polyclonal Abs are mouse and human reactive), TRPV1 (cat. no. NB100-98866, 1:1000, human and mouse reactive; Novus Biologicals, Littleton, CO), and mouse monoclonal anti-neuron–specific β-III tubulin (1:200; R&D Systems) in 2% NGS overnight at 4°C. Rabbit IgG (1:200; R&D Systems) was used as the secondary Ab for all studies. The samples were then washed with PBS three times each for 5 min and incubated with the appropriate secondary Abs including Alexa Fluor 546 goat anti-mouse (Invitrogen, Eugene, OR) or Alexa Fluor 488 goat anti-rabbit (Invitrogen) at a dilution of 1:1000 in 2% NGS in PBS for 1 h at room temperature. Samples were then washed with PBS three times each for 5 min and mounted using prolong gold antifade reagent with DAPI (P36935; Invitrogen) and examined with a Zeiss LSM 510 laser-scanning microscope (Carl Zeiss MicroImaging, Thornwood, NY) equipped with argon (excitation 488 nm), DPSS (excitation 561 nm), and diode (excitation 405 nm) lasers. We used the following procedure to calculate the average intensity of TRPV1 in the neuron surface and dendrites before and after stimulation with TLR ligands: Otsu’s global thresholding algorithm (24) was used to identify the β-III tubulin in the red channel. The resulting binary mask of the red channel was then used to calculate the average intensity of TRPV1 in the neuron surface and dendrites (green channel). All image-processing steps were performed using a custom ImageJ (National Institutes of Health; http://rsb.info.nih.gov/ij/) script.

Western blot analysis

DRG cultures were lysed in ice-cold lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT), briefly sonicated, and centrifuged at 17,000 × g at 4°C for 10 min. Total protein in the supernatant was determined using the BCA Protein assay kit (Pierce, Thermo Scientific, Logan, UT). As a positive control, spleen extract was treated as described above. Equal amounts of protein were subjected to electrophoresis on NuPAGE Novex Bis-Tris (Invitrogen, Carlsbad, CA) in 4–12% gradient gels in MOPS buffer and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). The membranes were blocked in blocking buffer (TBST, 0.25 M Tris base, 1.37 M NaCl, 0.03 M KCl, 0.01% Tween 20, pH 7.4, with 5% nonfat dry milk) at room temperature for 1 h. The membranes were then incubated with rabbit polyclonal Ab against TLR3, TLR7, and TLR9 (1:1000; Proscei, rabbit anti-TRPV1 (1:1000; Novus Biologicals), and rabbit anti-GAPDH (1:1000; cat. no. 2118; Cell Signaling Technology, Danvers, MA) overnight at 4°C. The blots were washed three times in TBST and incubated with HRP-conjugated anti-rabbit antibody (1:10,000, Cell Signaling Technology) for 1 h at room temperature. Blots were then visualized with ECL reagent (GE Healthcare, Pittsburgh, PA).

To quantify Western blot data, developed films were scanned, the immunoreactive bands were digitized, and the densitometry was performed using a custom ImageJ (National Institutes of Health; http://rsb.info.nih.gov/ij/) script. The signal for each lane was calculated by summing the area × intensity of immunoreactivity (gray level of immunoreactive band background level) of TLR3/7/9 and TRPV1 and normalized with internal control bands (GAPDH). The results, which were presented as mean ± SEM, were expressed as percentages of levels in control group (100%) and statistically analyzed with Student t test for two-group comparisons. The level of significance was taken as p < 0.05. All experiments were performed at least three times.

PGF2α detection

Sera from rats pretreated with TLR ligands, poly-IC, gardiquimod, ODN 1826, or ODN 2088 (InvivoGen, San Diego, CA) were collected, respectively, and PGF2α was detected with PGF2α enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) as indicated by the manufacturer.

RNA isolation, cDNA synthesis, and quantitative real-time PCR array

DRG cultures were treated with TLR ligands for 16 h. Total RNA was isolated from samples by RT-qPCR-Grade RNA isolation kit
(SABiosciences, Frederick, MD). After checking the purification and quality by nanodrop, RNA was reverse-transcribed into cDNA (PTC-200; MJ Research, Watertown, MA) using the RT² first strand kit (SABiosciences). Each PCR array for quantitative PCR reaction (mouse Inflammatory Response and Autoimmunity PCR Array, cat. no. PAMM3803; SABiosciences) was carried out in a 10-μl final volume per well containing cDNA (50 ng) and RT Real-Time SYBR Green/Rox PCR Master Mix (SABiosciences). Thermal cycling was performed in 384-well format plates using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Beverly, MA) according to the manufacturer’s instructions. All data were captured using the ABI Prism 7900HT Sequence Detector Software version 2.1 (Applied Biosystems) (25) and analyzed by RT² Profiler PCR Array Data Analysis software (SABiosciences).

**Cytokine quantification**

The expression of selected cytokines, chemokines, and their receptors in the supernatant of DRGN cultures was determined by multiplex array (Aushon Biosystems, Woburn, MA).

**Ca²⁺ flux**

DRGNs were maintained in 8-well chambered coverglass for 5 d. Time-lapse images (phase contrast) were captured with an Olympus Confocal Laser Scanning Microscope, Fluoview FV1000 (Olympus, Center Valley, PA; equipped with a heated stage maintained at 25°C and with a constant CO₂ source [5%]). For Ca²⁺ imaging, the culture medium was removed, and DRGNs were washed with Krebs–Ringer solution (124 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 1.3 mM NaH₂PO₄, 26 mM NaHCO₃, 10 mM dextrose, pH 7.4) (26). DRGNs in the coverglass chambers were loaded with fluo-3-AM (final concentration 5 μM) in Krebs–Ringer solution) dissolved in 0.1% DMSO (Sigma-Aldrich) at 37°C for 30 min. DRGNs were then gently washed using Krebs–Ringer solution to remove free dye. Cultures were illuminated with 488-nm light from a multi Ar+HeNe (argon and HeNe) laser through an epifluorescence Olympus FV1000 IX81 inverted microscope with a 10×, 0.4 numerical aperture Olympus UPLAPO objective. Light passing through the aperture was filtered by a 505–600 nm band-pass filter (BA505-600). The basal fluorescence level was recorded for 90 s just before the application of each reagent. Subsequently, 50 μL CAP (Sigma-Aldrich, Steinheim, Switzerland; final concentration 0.03, 0.1, 1, and 10 μM dissolved in 0.1% DMSO) or Ana (Sigma-Aldrich; final concentration 1.86, 5.57, 16.7, and 50 μM dissolved in 0.1% DMSO) were added for 10 s. To confirm the Ca²⁺ flux was mediated by TRPV1, we investigated the effects of SB-366791 (Biomol International, Plymouth Meeting, PA), the selective TRPV1 antagonist (27). After being washed following dye fluo-3-AM loading, the neurons were incubated for 30 min at 25°C with SB-366791 (1 μM) or without the compound (control). The chambers were then placed onto the microscope plate before the addition of various activators of TRPV1. Images were analyzed for changes in intensity of Ca²⁺-mediated fluorescence using Fluoview version 1.7b software (Olympus, Center Valley, PA) and converted into Rainbow color (26).

**Tumor-induced temperature sensitivity**

A tumor-induced temperature sensitivity model was adapted to our laboratory from earlier studies (28–30) to investigate whether TLR9 antagonist (Sup ODN) could decrease temperature sensitivity, which is mediated by TRPV1. This model is considered a neuropathic pain model because the tumor-bearing mice experience temperature hypersensitivity. The model was developed in conjunction with and approved by the National Cancer Institute-Frederick Animal Care and Use Committee (Animal Study Protocol 10-218). Two million S-180 cancer cells were inoculated into the muscular tissue of female Swiss Webster mice in the immediate vicinity of the sciatic nerve near the trochanter, immediately distal to where the posterior biceps semitendinosus branches off the common sciatic nerve. A negative control group was injected with PBS instead of tumor cells. Paw withdrawal latencies to radiant heat stimulation at 55˚C were measured before any procedure and on days 2, 5, 7, 9, 11, 13, and 15 after tumor inoculation. Treatment groups consisted of 8 animals. Synthetic Sup ODNs were delivered i.p. in a dose range from 5 μg/mouse to 320 μg/mouse (22); the tumor control groups received PBS i.p.

**Statistics**

Statistical determination of PGE₂, and protein levels in supernatants of DRGN cultures were performed using Student t test comparison with control (GraphPad Prism, version 4.0c; GraphPad, San Diego, CA). The p values <0.05 were considered statistically significant. Data for RNA array were analyzed by RT² Profiler PCR Array Data Analysis software (SABiosciences) and standard deviations calculated as recommended (31). Ca²⁺ responses in activated cells were individually identified and their correspondence with 488-nm emission measured using the Fluoview version 1.7b software. Data from activated cells on 8-well coverglass

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**FIGURE 1.** DRGNs express TLR3, TLR7, and TLR9. Confocal images of primary cultured mouse DRGNs isolated from E13 embryos are shown. DRGNs were stained with TLR3 (A), TLR7 (B), and TLR9 (C) (green), neuron-specific β-III tubulin (red), and DAPI (blue). Shown are separate monochrome images of the green, red, and blue fluorescence channel and merged color images from all channels. Scale bars, 50 μm.
chambers in three dependent experiments (a total of 30 cells) were analyzed in each condition (CAP, Ana only, or pretreated with TLR ligands), and Ca\textsuperscript{2+} responses (mean of the peak values) were plotted by using SigmaPlot 8.0 software (Fig. 8). The comparison of ligands-induced activation of TRPV1 between nontreated and TLR ligands-pretreated cells was carried out by two-way ANOVA with SPSS version 13.0. Linear regression and least squares comparison (GraphPad Prism, version 4.0c; GraphPad) were used to determine the slope and correlation within

**FIGURE 2.** Negative control staining for TLRs and TRPV1 in DRGNs (A), TRPV1 in human DRGN sections (B), and magnified images from human sections (C). Scale bars, 50 µm (A), 100 µm (B), and 10 µm (C), respectively.

**FIGURE 3.** Human DRG sections express TLR3, TLR7, and TLR9. Confocal images of DRG cryostat sections. Samples were stained with TLR3 (A), TLR7 (B), and TLR9 (C) (green), neuron-specific β-III tubulin (red), and DAPI (blue). Shown are separate monochrome images of the green, red, and blue fluorescence channel and merged color images from all channels. Scale bars, 50 µm.
treatment groups and one-way ANOVA with post tests used to determine the probability and dosing trend.

**Results**

**TLR3/7/9 expressed by DRGNs**

To show that DRGNs express TLR3/7/9, we established primary cultures from day 13 mouse embryos. In these cultures, >95% of the cells showed phenotypic properties of neurons (32, 33). Immunofluorescence staining on day 5 showed that TLR3, TLR7, or TLR9, which are pseudo-colored green in the micrographs, are expressed on neurons isolated from DRGs (Fig. 1A, 1B, 1C, respectively). Immunofluorescent controls are shown in Fig. 2A. We also found that TLR 3, 7, and 9 were expressed by neurons in adult human DRG tissue sections (Fig. 3A, 3B, 3C, respectively; negative controls shown in Fig. 2B, 2C). β-III tubulin, which is a neuron-specific marker, is pseudo-colored red in the micrographs, verifying that the cells expressing TLRs were neurons.

**TLR ligands enhanced TLR3/7/9 expression by DRGNs**

Based on our pilot studies (K. Buzas, J. Cohen, O.M.Z. Howard, and J.J. Oppeneheim, reported at the 2007 National Institutes of Health-Immunology Interest Group, Warrenton, VA), we deter-

**FIGURE 4. TLR ligands induce corresponding receptor expression by DRGNs.** Western blot showing TLR3, TLR7, and TLR9 expression by DRGNs after ligand stimulation are shown. A, Poly-IC (25 μg/ml) induced TLR3 protein expression in DRGNs. B, Gardiquimod (3 μg/ml) induced TLR7 protein expression in DRGNs. C, ODN 1826 (32 μg/ml) induced TLR9 expression in DRGNs. D, Densitometry from the blots. Cells were stimulated for 16 h then harvested, lysed, and the protein extracts were probed with anti-TLR3, anti-TLR7, and anti-TLR9 Ab. Control (Con) samples were cultured for 16 h without additional stimulation. Protein loading is reported using an anti-GPDH Ab. Spleen lysate was used as a positive control. One representative of more than three independent experiments is shown. Image densitometry was performed using ImageJ software. The densitometry results were determined for five sites within each band, presented as mean ± SEM, and are expressed as percentages of levels in control group (100%). GAPDH was used as internal control. Statistics analysis was performed using Student t test for two-group comparisons. **p < 0.01, ***p < 0.001 (compared with control).

**FIGURE 5. TLR ligands induce PGE2 production by DRGNs.** Supernatants of DRGN cultures were collected and PGE2 was detected using enzyme immunoassay after stimulation by poly-IC (25 μg/ml), gardiquimod (3 μg/ml), or ODN 1826 (32 μg/ml) for 16 h. Each bar represents the mean ± SEM (n = 10). A, PGE2 production by DRGNs from Swiss embryonic mice. B, PGE2 production by DRGNs from C57 and MyD88 knockout embryonic mice. Statistical analysis was performed using Student t test for two-group comparisons. **p < 0.01, ***p < 0.001 (compared with corresponding control), ##p < 0.01 (compared with corresponding C57 mice treated with gardiquimod and ODN 1826).
mimined the optimal nontoxic dose of stimulants to be 25 μg/ml poly:IC, 3 μg/ml gardiquimod, or 32 μg/ml ODN 1826 over a 16-h incubation period. We applied these individual treatments to poly:IC, 3 μg/ml gardiquimod, or 32 μg/ml ODN 1826 (32 μg/ml), which is an antagonist of TLR9, for 16 h with poly:IC, gardiquimod, or ODN 1826, respectively. We also determined the PGE2 concentration in the supernatants of DRG cultures after stimulation by poly-IC (25 μg/ml), gardiquimod (3 μg/ml), or ODN 1826 (32 μg/ml) by 31.8% compared with ODN 1826 alone of maximum stimulation (p < 0.005 compared with control (n = 3)).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>CCL5</th>
<th>CXCL10</th>
<th>IL-1α</th>
<th>IL-1β</th>
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<tr>
<td>mRNA Fold Increase Compared with Control</td>
<td></td>
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<tr>
<td>Poly:IC (25 μg/ml)</td>
<td>289.9 ± 7.4*</td>
<td>73.8 ± 5.4*</td>
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<td>Gardiquimod (3 μg/ml)</td>
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<tr>
<td>ODN 1826 (32 μg/ml)</td>
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<td>7.6 ± 0.17*</td>
<td>6.4 ± 1.7*</td>
<td>6.6 ± 1.4*</td>
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### Protein Concentration (pg/ml)

<table>
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<th>Control</th>
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<th>1.2 ± 0.34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly:IC (25 μg/ml)</td>
<td>1,899.6 ± 762.6*</td>
<td>26,455.8 ± 9,714.8*</td>
<td>2.3 ± 1.2*</td>
<td>7.8 ± 2.9*</td>
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<tr>
<td>Gardiquimod (3 μg/ml)</td>
<td>76.8 ± 25.8</td>
<td>505.7 ± 225.1</td>
<td>3.2 ± 0.17*</td>
<td>2.8 ± 0.52*</td>
</tr>
<tr>
<td>ODN 1826 (32 μg/ml)</td>
<td>25.0 ± 5.2</td>
<td>410.0 ± 206.0</td>
<td>1.6 ± 0.34*</td>
<td>3.3 ± 0.69*</td>
</tr>
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</table>

DRG cultures were treated with TLRs ligands for 16 h. Total RNA was isolated from neurons by RNA isolation kit and reverse-transcribed into cDNA. Each PCR array for quantitative PCR reaction was carried out. The protein levels in supernatants of DRG cultures were determined by multiplex array. The results represent the mean ± SD. Data for RNA array were analyzed by RT² Profiler PCR Array Data Analysis software (SABiosciences). Statistical analysis for the protein levels was performed by Student t test.

**TLR3/7/9 ligands stimulated PGE₂ release by mouse DRGN cultures**

PGE₂ is a central mediator of febrile response triggered by the inflammatory process, and intradermal PGE₂ is hyperalgesic in the peripheral nervous system (34, 35). We determined the PGE₂ levels in the supernatants of 5-d DRGN cultures after stimulation for 16 h. As shown in Fig. 5A, PGE₂ concentration increased after stimulation by each of the TLR3/7/9 ligands compared with that of the control group (p < 0.005), which suggests that ligands for endosomal TLRs could induce DRGNs to produce this mediator of hyperalgesia and inflammation, namely PGE₂. Moreover, we found that ODN 2088 (50 μg/ml), which is an antagonist of TLR9, reduced PGE₂ levels induced by ODN 1826 (32 μg/ml) by 31.8% compared with ODN 1826 alone of maximum stimulation (p = 0.009). We also determined the PGE₂ concentration in the supernatants of DRGN cultures of MyD88 knockout mice. As shown in Fig. 5B, gardiquimod and ODN 1826 failed to induce PGE₂ production significantly in MyD88 knockout mice (p < 0.01), but as anticipated poly:IC did induce PGE₂ in the knockout DRGN.

**TLR ligands upregulated mRNA levels and protein expression of proinflammatory cytokines and chemokines by DRGNs**

To confirm that ligands of endosomal TLRs induce a proinflammatory response by DRGNs, we performed real-time quantitative PCR (mouse Inflammatory Response and Autoimmunity PCR Array) to analyze mRNA levels in DRG neurons after stimulation for 16 h with poly:IC, gardiquimod, or ODN 1826, respectively. TLR ligands dramatically increased CCL5, CXCL10, IL-1α, and IL-1β mRNA levels (Table I) (GSE27579, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE27579). These factors were previously identified as important mediators of pain hypersensitivity (5–7, 36, 37). Furthermore, as shown in Table I, TLR3/7/9 ligands also increased IL-1α and IL-1β protein production. Moreover, poly:IC increased CXCL10 and CCL5 protein production, markedly. These data indicate that DRGNs respond to ligands of endosomal TLRs by producing high levels of proinflammatory mediators.

**TLR ligands increased TRPV1 expression and translocation by DRGNs**

The effect of TLR stimulation on TRPV1, the pain detector and integrator, was evaluated. As shown in Fig. 6, TLR ligands markedly increased TRPV1 (~100 kDa) expression by DRGNs. Moreover, TLR ligands enhanced translocation of TRPV1 proteins from cell bodies in DRGNs to sensory nerve endings (Fig. 7). The elevated TRPV1 expression and translocation suggested that the nociceptive neurons stimulated by TLR ligands are better able to
respond to pain stimuli. We next evaluated an in vitro correlate for pain sensation, calcium flux.

**TLR ligands enhanced Ca\textsuperscript{2+} influx induced by CAP and Ana**

For studies of Ca\textsuperscript{2+} influx, neurons were maintained in culture for 5 d and only small to medium-sized neurons were studied. Murine DRGNs responded with a persistent and concentration-dependent increase in Ca\textsuperscript{2+} influx after stimulation with CAP as indicated by an increase in the fluorescence intensity of fluo-3 emission at 488-nm excitation (Fig. 8). This demonstrated that TRPV1 in primary cultured DRGNs was functional. As shown in Fig. 8A, DRGNs responded to 0.1 \textmu M CAP with a much greater change in fluo-3 fluorescence intensity when pretreated with poly:IC, gardiquimod, or ODN 1826 (Fig. 8B) (**p = 0.008, p = 0.039, and p = 0.041, respectively**). We further confirmed the enhanced sensitization of TRPV1 induced by TLR ligands by using an endogenous ligand of TRPV1, Ana. DRGNs consistently responded to Ana with an enhanced Ca\textsuperscript{2+} influx when pretreated with poly:IC, gardiquimod, or ODN 1826 (Fig. 8C). The Ca\textsuperscript{2+} influx in neurons pretreated with TLR ligands was higher in the presence of 1.86, 5.57, or 16.7 \textmu M Ana (Fig. 8C) (**poly:IC p = 0.009, gardiquimod p = 0.029, or ODN 1826 p = 0.048**). The Ca\textsuperscript{2+} influx induced by CAP in DRGNs was totally blocked by SB-366791 (Supplemental Fig. 1), a selective TRPV1 antagonist. Conversely, no Ca\textsuperscript{2+} influx in DRGNs was observed when TLR ligands alone were applied (Supplemental Fig. 2). These studies show that in addition to inducing the production of inflammatory pain-inducing mediators, TLR ligands also upregulated expression of functional TRPV1 pain signaling receptors.

**Sup ODN treatment blocks tumor-induced pain sensitivity**

Sup ODNs, which mimic the suppressive effect of self-DNA by decreasing the immune activating signals of TLR9, were tested in a mouse model of tumor-induced neuropathic pain for their ability to decrease temperature sensitivity. As can be seen in Fig. 9, fibrosarcoma tumor cells implanted near the sciatic nerve result in decreased time of paw withdrawal, indicating hypersensitivity to heat in the tumor-bearing mice. When compared with negative control or naive mice, the tumor-bearing mice show a significantly faster withdrawal from the hot plate that increases over time (**p < 0.001**). We were unable to evaluate longer time points because tumor-bearing mice begin to show stress by day 15 and must be euthanized. Treating the tumor-bearing mice with Sup ODNs, which block TLR9-mediated immune stimulation, resulted in an

**FIGURE 7.** TRPV1 is expressed by DRGNs and relocates after TLR ligands stimulation. Confocal images of TRPV1 expressed by primary cultured mouse DRGNs are shown. DRGNs were stained with TRPV1 (green), neuron-specific \(\beta\)-III tubulin (red), and DAPI (blue). Shown are monochrome images of the green fluorescence channel and merged color images from all channels. A–D, Control (A), poly:IC (25 \mu g/ml) (B), gardiquimod (3 \mu g/ml) (C), and ODN 1826 (32 \mu g/ml) (D) stimulation for 16 h induced TRPV1 expression in DRGNs. Scale bars, 50 \mu m. E, The average intensity of TRPV1 in the neuron surface and dendrites. Image intensity analysis was performed using ImageJ software. Statistical analysis was performed using Student t test for two-group comparisons. **p < 0.01 (compared with control).
increase in time of paw withdrawal from the heat challenge, indicating a reduction in heat sensitivity. Fig. 9 shows a dose response from no treatment to treatment with 320 mg/mouse Sup ODN. Beginning at the 20 mg/mouse dose, the difference between the negative control and the tumor-bearing and treated groups becomes suppressed, indicating that treatment with Sup ODN reduced tumor-induced pain. The reduction in heat sensitivity becomes significant at the 80 mg/mouse dose. This was not due to inhibition of tumor growth because a reduction in tumor growth was not observed until the highest tolerated dose (320 mg/mouse) of Sup ODN (data not shown). The paw withdrawal latencies for naive mice receiving 320 mg/mouse Sup ODN were not significantly different from those of the control group (data not shown). Thus, our data indicate that suppression of TLR9 leads to reduced sensitivity to heat in a neuropathic pain model despite the presence of a growing tumor when the Sup ODNs are delivered outside of the CNS. Our observations suggest a connection between suppression of a heat-activated pain receptor in the peripheral nervous system, when a mouse is treated with a suppressive TLR9 oligonucleotide.

**Discussion**

One of the cardinal signals of inflammation is pain. Pathogens, injury, and stressors stimulate TLRs on innate immune cells thereby initiating the proinflammatory signal-transduction pathways that ultimately trigger cytokine production. In the current study, we found that TLRs, which are also expressed by peripheral neuronal cells in response to synthetic ligands, produce cytokine and chemokine protein products, such as IL-1α, IL-1β, RANTES, mean ± SEM (n = 30). The data were analyzed by two-way ANOVA. *p < 0.05, **p < 0.01 (compared with control).
and IP-10, which have previously been shown to act as pain mediators (5, 6, 36–38).

The current results provide the first evidence, to our knowledge, that primary cultured mouse DRGNs constitutively express TLR3/7/9 and respond to their ligands. We focused on embryonic DRGNs because the resulting cultures generate highly pure (>95%) single neurons, thereby allowing us to investigate selectively neuronal TLR expression and responsiveness to TLR ligands. Our results indicate that DRGNs have the potential to recognize viral and cellular products and initiate an inflammatory response in the peripheral nervous system without prior activation of immune cells to produce proinflammatory cytokines. Indeed, in our study, stimulation by the TLR ligands (poly-IC, guradquimod, and ODN 1826) induced cytokine (IL-1α and IL-1β) and chemokine (CCLS and CXCL10) gene expression and protein production by DRGNs. Moreover, the activation of TLRs produced PGE2, which acts as a pain inducer. TLR ligand stimulation also enhanced expression and translocation of TRPV1. Furthermore, TRPV1 relocated from the DRGN cell bodies into dendrites after TLR ligand stimulation. TRPV1 relocalization has been reported to be involved in the development of hyperalgesia in vivo (39, 40). Notably, pretreatment with TLR ligands also enhanced the TRPV1-mediated Ca2+ flux induced by CAP in DRGNs. Thus, peripheral neurons function to bridge the innate immune and sensory systems.

Previous studies demonstrated that TLRs are widely expressed in the CNS, particularly by microglia (41). Activation of TLRs on microglia and astrocytes leads to production of cytokines, cellular adhesion molecules, chemokines, and the expression of surface Ags that results in a nervous system immune cascade. Therefore, TLRs also invoke inflammation in the nervous system (42, 43). TLRs expressed on microglia appear to trigger microglial activation, which might be a driving force of chronic pain. Acosta et al. have shown that LPS, by stimulating TRL4, regulates the expression of the peptide nociceptin/orphanin FQ, which contributes to feeding behavior. These observations show that TLR4 potentially acts as a key initiator of behavioral responses mediated by DRGNs (44). In addition, immunohistochemical analysis of human and rat trigeminal neurons demonstrated that CAP-sensitive nociceptors express TLR4, thus enabling sensory neurons to respond to tissue levels of bacterial substances such as LPS (45).

Other cells in the peripheral nervous system, including Schwann cells, respond to TLR ligands by producing proinflammatory factors. Poly-IC is responsible for stimulating inducible NO synthase gene expression and NO production in Schwann cells, which exerts neurotoxic effects on DRGNs (46). Our results confirm and extend earlier observations that TLR3/7/9 are expressed by DRGNs (47). Our studies demonstrate that DRGNs respond to the TLR ligand stimulation, indicating that they are functional, and suggest that DRGNs through their response to TLR ligands mediate neuronal function.

Ca2+ influx induced by TRPV1 activation is one of the causes of pain perception in the nervous system (48). We observed Ca2+ influx in DRGNs after stimulation with CAP or Ana, which indicates that the primary cultured DRGNs respond to TRPV1 ligands. SB-366791, a selective TRPV1 antagonist, totally blocked Ca2+ influx, suggesting that Ca2+ influx was mediated by TRPV1. A dramatic increase in Ca2+ was observed in DRGNs after pretreatment with TLR ligands. Therefore, TLR ligands not only upregulated TRPV1 expression but also enhanced its activity. At the same time, Ca2+ influx through TRPV1 in the nociceptive neuron endings is known to cause the release of mediators of inflammation such as substance P and calcitonin gene-related peptide synergize to induce a phenomenon called neurogenic inflammation that results in increased blood–brain barrier permeability (49). Investigating the effect of delivering TLR agonists to the peripheral nervous system on blood–brain barrier permeability will require future in vivo studies.

Inflammation induces neuropathic pain, which is experienced as hypersensitivity to thermal, mechanical, or chemical stimuli. The model of neuropathic pain used in this study, implantation of fibrosarcoma S-180 cells near the sciatic nerve, is thought to induce pain through two pathways, mechanical restriction of the nerve (28) as the tumor grows and tumor-produced soluble factors like PGE2 (50) and chemokines (51). In this study, we have shown that suppressive oligonucleotides that block TLR9-mediated immune activation (22) also block sensitivity to heat, which is thought to be mediated by TRPV1 (52). Taken with our in vitro data, these in vivo data indicate that there is functional cross-talk between innate immune receptors (TLR) and pain sensory receptors (TRPV) in peripheral neurons.

In summary, our findings provide an intriguing example of a set of receptors shared between cells of the immune and the nervous systems that are likely to enhance the responses of both systems. These studies show that TLRs have the potential to have both direct and indirect effects on pain initiation and regulation. Therefore, DRGNs and TLRs are likely to be important contributors to chronic pain and a rewarding target in the development of novel therapeutic strategies in the prevention of development and in reduction of chronic pain.

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Disclosures

The authors have no financial conflicts of interest.

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**Fig. S1.** The inhibitory effects of SB-366791, the selective TRPV1 antagonist. The Ca$^{2+}$ responses of primary DRGNs upon stimulation by 0.1 μM CAP at 90 second were recorded by confocal laser scanning microscope. After pre-incubated with SB-366791 for 30 min, DRGNs were treated with CAP, shown in the red line. The blue line represents the DRGNs without the influence of SB-366791.

**Fig. S2.** Effects of TLR ligands administration alone on Ca$^{2+}$ flux. (A) TLR3 ligand (poly I:C 12.5, 25 and 50 μg/ml), (B) TLR7 ligand (gardiquimod 1.5, 3 and 6 μg/ml), and (C) TLR9 ligand (ODN 1826 16, 32 and 64 μg/ml) administered at 90 second, and the Ca$^{2+}$ responses were recorded for 500 sec. CAP (0.1 μM) stimulated the Ca$^{2+}$ responses were found after administered at 590 second.
The graph shows the relative fluorescence intensity over time for two treatments: CAP and SB-366791/CAP. A significant increase in fluorescence intensity is observed for CAP (0.1 μM) at a specific time point, indicated by an arrow.