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LIPOPOLYSACCHARIDE DIFFERENTIALLY MODULATES EXPRESSION OF CYTOKINES AND CYCLOOXYGENASES IN DORSAL ROOT GANGLION CELLS VIA TOLL-LIKE RECEPTOR-4 DEPENDENT PATHWAYS

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Abstract—We have examined the functional expression of Toll-like receptor 4 (TLR4) in adult male rat dorsal root ganglion (DRG) cells in culture by studying changes in pro-inflammatory cytokines and cyclooxygenase (COX)-dependent prostanoid production. In the mixed population of DRG neurons and glial cells, only DRG neurons expressed cell surface TLR4 along with MD-2 and CD14. This classical TLR4 signaling complex on DRG neurons responded to lipopolysaccharide (LPS) with a TLR4-dependent and time-dependent increase in interleukin-1β and tumor necrosis factor-α mRNA expression which was entirely dependent on NF-κB activity. In contrast, after 2-h incubation with DRG cells, LPS-stimulated COX-2 was regulated by both NF-κB and transactivation of epidermal growth factor receptor (EGFR) with potential downstream activation of ERK1/2 and p38 kinase. In contrast to this evidence for myeloid differentiation primary response gene-88 (MyD88)-dependent signaling, no evidence was obtained for TIR-domain-containing adaptor-inducing interferon-ß (TRIF)-dependent signaling from TLR4 in DRG neurons. LPS surprisingly produced a time-dependent decrease in COX-1 protein which likely facilitates the COX-2-dependent production of prostaglandin E_2 and prostacyclin. Our study is the first to demonstrate the activation of TLR4-dependent production of prostaglandin E_2 and prostacyclin in DRG cell cultures. Our findings support the concept that the activation of TLR4 on primary sensory neurons by endogenous ligands may underlie neuropathic and inflammatory pain states.

Key words: dorsal root ganglia, Toll-like receptor-4, neuroinflammation, lipopolysaccharide, cell signaling.

INTRODUCTION

Toll-like receptors (TLRs) are a family of pattern recognition receptors classically associated with cells of the innate immune system involved in detection of 'Danger signals' (Biswas and Tergaonkar, 2007). In addition, TLRs have now been identified in neuronal tissues such as the brain and spinal cord where they are expressed primarily by microglial cells (Lehnardt et al., 2003) but also by neurons (Tang et al., 2007) and play a role in neurodegenerative diseases (Okun et al., 2009). TLRs also have an emerging role in the control of pain (Liu et al., 2012; Nicotra et al., 2012), with inhibition of spinal cord microglial cell activity attenuating neuropathic pain in animal models of nerve injury (Tanga et al., 2005; Cao et al., 2009). Microglial cells express TLR4 which recognizes lipopolysaccharide (LPS) associated with Gram-negative bacteria; TLR4 is therefore a receptor for pathogen-associated molecular patterns (PAMPs). However, TLR4 can also respond to endogenous ligands with danger-associated molecular patterns (DAMPs) such as heat shock proteins, extracellular matrix molecules (hyaluronan), HMGB1, OxLDL and β-defensins (Miyake, 2007). And, some of these products of tissue damage are the possible ligands responsible for microglial cell activation during nerve injury.

Recently it has been recognized that primary sensory neurons in dorsal root ganglia (DRG) express TLR4 (Barajon et al., 2009; Nowicki et al., 2010; Ochoa-Cortes et al., 2010; Due et al., 2012) and both TLR4 and its co-receptor glycoprotein CD14 have been detected on human and rat trigeminal ganglion neurons (Wadachi and Hargreaves, 2006). In isolated DRG cells, TLR4 is also expressed by neurons rather than non-neuronal cells (Goethals et al., 2010; Nowicki et al., 2010; Due et al., 2012). As observed in intact DRG, TLR4 is co-localized with a subpopulation of small-diameter

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Abbreviations: BSA, bovine serum albumin; COX, cyclooxygenase; DAMPS, danger-associated molecular patterns; DRG, dorsal root ganglion; EDTA, ethylenediaminetetraacetic acid; EGFR, epidermal growth factor receptor; EIA, enzyme immunoassay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFNβ, interferon-β; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MD, myeloid differentiation protein; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PAMPs, pathogen-associated molecular patterns; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; PGI_2, prostacyclin; TLRs, Toll-like receptors; TNFα, tumor necrosis factor-α; TRPV1, transient receptor potential vanilloid receptor; TUJ-1, neuron-specific class III β-tubulin.

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transient receptor potential vanilloid receptor (TRPV1)-positive neurons, and some non-TRPV1 neurons, in adult rat trigeminal ganglia cells (Diogenes et al., 2011). Importantly, mouse DRG neurons express TLR4, myeloid differentiation protein (MD)-1 and CD14 mRNA and protein, but negligible MD-2 and no RP105 mRNA or protein (Acosta and Davies, 2008). The classical LPS receptor complex consists of TLR4, CD14 and MD-2, with CD14 concentrating LPS and presenting it to the cell surface TLR4/MD-2 complex where MD-2 is the primary ligand recognition molecule and TLR4 is the signal transducing molecule (Akashi-Takamura and Miyake, 2008).

Studies of LPS binding and TLR4-ir suggest that approximately 40% of DRG neurons express TLR4 (Acosta and Davies, 2008). LPS releases calcitonin gene-related peptide (CGRP) from DRG cell cultures (Hou and Wang, 2001), potentiates capsaicin-induced CGRP release from trigeminal ganglia cell cultures (Diogenes et al., 2011; Ferraz et al., 2011), increases the proportion of nociceptin-positive neurons in embryonic and adult mouse DRG cells (Acosta and Davies, 2008), and increases excitability of sensory neurons (Ochoa-Cortes et al., 2010; Due et al., 2012). A role for neuronal TLR4 in trigeminal ganglia in mediating pain induced by DAMPs, such as heat shock protein-70, has also been demonstrated (Ohara et al., 2013). This expression of functionally active TLR4 receptor complexes by primary sensory neurons therefore underlies an important neuroimmune involvement in pain (Hutchinson et al., 2013).

TLR4 is a transmembrane protein whose cytoplasmic domain contains a signal transducing Toll/IL-1 receptor homology domain (TIR) which can activate two signaling pathways: the myeloid differentiation primary response gene-88 (MyD88)/NF-κB/inflammatory mediator pathway and the TIR-domain-containing adaptor-inducing interferon-ß (TRIF)/IRF3/IFNβ pathway (Miyake, 2007). The purpose of the current investigation was to determine if rat DRG cells also express this unique LPS receptor complex of TLR4, CD14 and MD-1, and to determine if TLR4 activation in sensory neurons involves cell signaling via MyD88-dependent and/or TRIF-dependent pathways.

EXPERIMENTAL PROCEDURES

Materials

Ultrapure LPS (LPS) derived from *Escherichia coli* 0111:B4 strain and CLI-095 were purchased from InvivoGen (San Diego, CA, USA). AG1487 was purchased from Sigma Chemical Co. (St Louis, MO, USA) and LY294002 was from Cell Signaling Technology (Danvers, MA, USA). SB239063, SP600125, and U0126 were purchased from Tocris Bioscience (Bristol, UK). SC-560, NS-398, and Bay 11-7082 were purchased from Cayman Chemical (Ann Arbor, MI, USA). All other compounds, otherwise specified, were purchased from Invitrogen (Carlsbad, CA, USA) or Sigma Chemical Co. (St Louis, MO, USA).

DRG cell cultures

All experiments were conducted under license from the Government of Hong Kong SAR and approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong. The Chinese University of Hong Kong is in full compliance with standards for humane care and use of laboratory animals as reviewed and accepted by the US Office of Laboratory Animal Welfare. The ARRIVE guidelines have been followed for all aspects of this study. Briefly, a total of 100 male adult Sprague–Dawley rats were supplied by the Chinese University of Hong Kong Laboratory Animal Services Centre and were housed in a 12-h light/dark cycle under conditions controlled for temperature and humidity, with access to standard rodent chow and water *ad libitum*. Animals (150–200 g) were deeply anaesthetized with pentobarbitone (135 mg/kg, i.p.), and the DRG were excised from all levels of the spinal cord. Cells were prepared as described previously (Ng et al., 2010) and cultured in Ham's F14 media supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), and Ultroser G (4%). The number of neurons (phase-bright cells) and glial cells (phase-dark cells) in the cell pellet were counted using a haemocytometer; a typical culture of DRG cells contained approximately 52 ± 0.9% neurons and 48 ± 0.9% glial cells (n = 26) on the day of cell preparation. Unless otherwise specified, DRG cells were plated at a density of 5,000 neurons per well in 24-well tissue culture plates pre-coated with poly-DL-ornithine (500 μg/ml) and laminin (5 μg/ml). DRG cells were assayed after 2 days in culture in an atmosphere of 5% CO_2 at 37 °C. Please refer to Ng et al. (2010) for full characterization details of our DRG cell cultures.

Cell viability

The conversion of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) to a colored formazan product in metabolically active cells was determined, in duplicate, by incubation for 4 h in a CO_2 incubator at 37 °C. Absorbance was recorded at 560 nm with a 24-well microplate reader (Benchmark Plus Microplate Spectrophotometer System, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunocytochemistry

DRG cells were cultured on poly-DL-ornithine and laminin-coated 12-mm glass coverslips in 24-well tissue culture plates. DRG cells were washed with phosphate-buffered saline (PBS) and fixed in paraformaldehyde (4%) for 20 min. After washing with PBS–bovine serum albumin (BSA) (1%), cells were incubated for 30 min in PBS–BSA plus donkey serum (5%) containing 0.01% Triton X-100 (for extracellular antigens) or 0.3% Triton-X-100 (for intracellular antigens), respectively. Primary antibodies against neuron-specific class βIII-tubulin (TUJ-1, 1:3000; Sigma; Cat. No. T2200), CD14 (1:250; Santa Cruz, San Diego, CA, USA; Cat. No. sc-5749), cyclooxygenase (COX)-2 (1:100; Cayman; Cat. No.

CX229), glial acidic fibrillary protein (GFAP) (1:500, Sigma; Cat. No. G6171), MD-1 (1:250; Santa Cruz; Cat. No. sc-20615), MD-2 (1:250; Santa Cruz; Cat. No. sc-20668), RP105 (1:250; Santa Cruz; Cat. No. sc-27841), and TLR4 (1:250; Acris Antibodies Inc., San Diego, CA, USA; Cat. No. SM7124P) were incubated with the cells for 2 h at room temperature. Species-specific AF488 or AF647-conjugated secondary antibodies (1:1000; Invitrogen) were added for 1 h at room temperature. After washing with PBS, the coverslips were mounted on glass slides with Prolong Gold anti-fading reagent containing 4′,6′-diamidino-2-phenylindole (DAPI) (Invitrogen) to visualize cell nuclei. Cells were imaged using an Olympus FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan), and images were captured using excitation at 405 nm (for DAPI), 488 nm (for AF488) and 635 nm (for AF647). For z-stack imaging, optical slices of 1-μm thick were sequentially captured and reconstructed using Olympus FluoView software. The number of immuno-positive cells was determined by counting a minimum of 100 neurons in each of three to four independent experiments, by an observer blinded to the treatment protocol. Images were processed using ImageJ software (v1.45s, NIH, Baltimore, MD, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total messenger RNA from whole DRG, DRG cells and spleen was isolated using RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The amount and quality of the RNA were determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized from 300-ng RNA with SuperScript® III First-Strand Synthesis System (Applied Biosystems, Foster City, CA, USA) and used as the template for PCR with the FastStart High Fidelity PCR System (Roche Applied Science, Indianapolis, IN, USA). Primers for rat TLR4, MD-1, MD-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are as described in Acosta and Davies (2008) and CD14 as described in Zuo et al. (2001). Primers for rat RP105 were designed using Primer3Plus (forward: 5′-GGATTTAACCAGGTGC CAGA-3′; reverse: 5′-GGCTCAGATTAGTGGCTTGC-3′). The PCR products were resolved using a 1% agarose gel containing GelRed, and the relationship between the amount of PCR product and the number of amplification cycles was linear (data not shown).

Real-time PCR

Complementary DNA for real-time PCR was prepared with High-Capacity RNA-to-cDNA Kit (Applied Biosystems), and mRNA levels were quantified by real-time PCR using TaqMan Gene Expression Assays (Applied Biosystems) for COX-2 (Rn01483828_m1), interleukin-1β (IL-1β; Rn00580432_m1), interferon-β (IFNβ; Rn00569434_s1), TLR4 (Rn00569848_m1), and tumor necrosis factor-α (TNFα; Rn01525859_g1), with β-actin (Rn00667869_m1) as the endogenous control. COX-1 mRNA expression was determined using primers specific for COX-1 as described by Neeb et al. (2011) and GAPDH as described by Eberhardt et al. (2009) with Fast SYBR® SYBR Green Master Mix (Applied Biosystems). All real-time PCR experiments were performed using an Applied Biosystems® ViiA 7 Real-Time PCR System with Fast 96-Well Block. Analysis of real-time PCR data was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with target mRNA expression in each sample normalized against the endogenous control (β-actin or GAPDH).

Western blot analysis

Cell lysates were prepared in ice-cold lysis buffer (50 mM Trizma base, pH 7.4; 5 mM EDTA; 0.1 M NaCl; 67 mM $Na_4P_2O_7$; 1% Triton X-100) supplemented with protease inhibitors (Complete Tablets, EDTA-free; Roche) and shaken for 1 h on ice and then centrifuged at 14,000*g* for 10 min at 4 °C. Protein content of supernatants was determined using Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and 30–60 μg were resolved on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred to Hybond nitrocellulose membranes (GE Health Care, Piscataway NJ, USA). Membranes were blocked with 5% non-fat milk and incubated with primary antibodies specific for COX-1 (1:500; Cat. No. 160110) and COX-2 (1:500; Cat. No. 160126) from Cayman Chemical, and phospho-IkBα (Cat. No. 9246) and IkBα (Cat. No. 4812) from Cell Signaling Technologies. The blots were then probed with the corresponding horseradish peroxidase-linked goat anti-rabbit or anti-mouse secondary antisera (1:1000; Cell Signaling Technologies). β-actin was used as a protein loading control (1:5000, Cat. No. 4967; Cell Signaling Technologies). The immunoblots were visualized by chemiluminescence with an ECL kit (GE Health Care, Piscataway, NJ, USA) and the band intensities were quantified using Image J software.

Enzyme immunoassay (EIA)

DRG cells were plated at a density of 15,000 neurons per well in 24-well tissue culture plates, as described above. After 1 day in culture, DRG cells were incubated with inhibitors of COX-1 (10 nM SC-560) or COX-2 (3 μM NS-398) 30 min before the addition of LPS (1 μg/ml) for 24 h. The media was collected and assayed for 6-keto prostaglandin $F_{1\alpha}$ (stable metabolite of prostacyclin, PGI_2) and prostaglandin E_2 (PGE_2) by EIA kit, according to the manufacturer's instructions (Cayman Chemical).

Statistical analysis

Values reported are means ± SEM from assays repeated at least three times. Comparisons between groups were made using a one-way analysis of variance with Bonferroni's or Dunnett's *post hoc* tests, or Student's *t*-test, as appropriate, using GraphPad Prism software version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was taken as $p < 0.05$.

RESULTS

Rat DRG neurons express TLR4 signaling components

We first determined if rat DRG cells expressed the major TLR4 signaling components, and identified TLR4, CD14, MD-1, MD-2 and RP105 mRNA in isolated DRG cells, in DRG explants and in spleen (for reference) (Fig. 1a). Our DRG cell cultures are composed of GFAP-positive glial cells and TUJ-1-positive neurons (Fig. 1b). These cell types are readily identifiable by virtue of their shape and size (Ceruti et al., 2008), and it was clear that only DRG neurons expressed cell surface TLR4 (Fig. 1c). Because both TUJ-1 and GFAP are intracellular markers, we cannot co-stain with TLR4 antibodies to identify cell-type specific cell surface TLR4-ir. However, TLR4-ir is clearly detected in cells showing the same phenotype as the TUJ-1-positive cells shown in Fig. 1b, and is absent in the smaller GFAP-positive glial cells. Based on our results that DRG cells may express all the major TLR4 signaling components at mRNA level but only DRG neurons express TLR4 on the cell surface, we proceeded to identify whether TLR4-positive DRG neurons also express these components at the protein level.

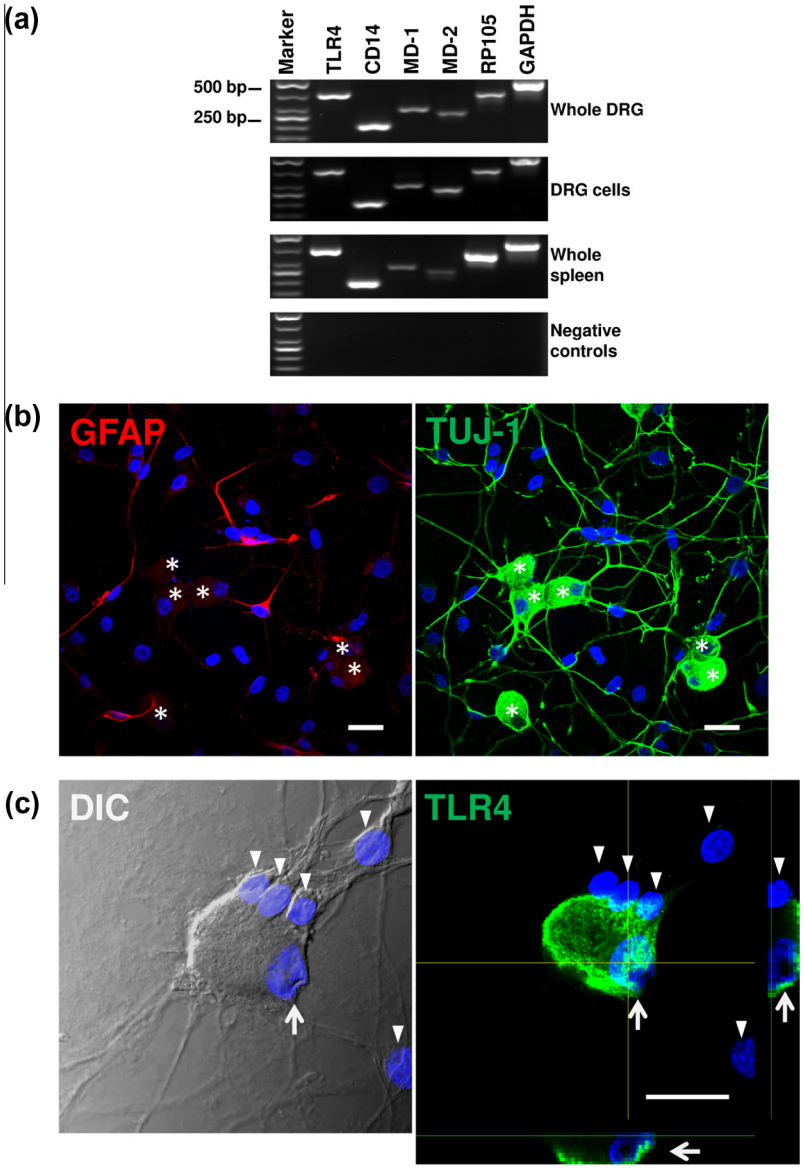

Fig. 1. Rat DRG neurons express TLR4 signaling components. (a) mRNA expression of TLR4 (384 bp), CD14 (189 bp), MD-1 (282 bp), MD-2 (250 bp), RP105 (365 bp) and GAPDH (452 bp) in whole DRG, DRG cells and spleen. Negative controls were performed in the absence of template cDNA. Gels are representative of three independent experiments. (b) A representative image of a typical DRG cell culture, with neurons marked with an asterisk and cell nuclei identified by blue DAPI staining. *Left* (red) shows GFAP-positive glial cells and *Right* (green) shows TUJ-1-positive neurons. Scale bar = 20 μm. (c) A representative higher magnification image: *Left* differential interference contrast image (DIC) shows a neuronal cell body (arrow) surrounded by glial cells (arrowheads); *Right* a *z*-stack confocal micrograph shows the concentration of TLR4-ir on the plasma membrane of the neuron and the absence of TLR4-ir on the surface of glial cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We observed that the expression of TLR4, as well as CD14, MD-2, RP105 and MD-1, were mostly localized on the cell bodies of neurons of various sizes and on parts of the extended neurites (Fig. 2a–d). Quantification of the

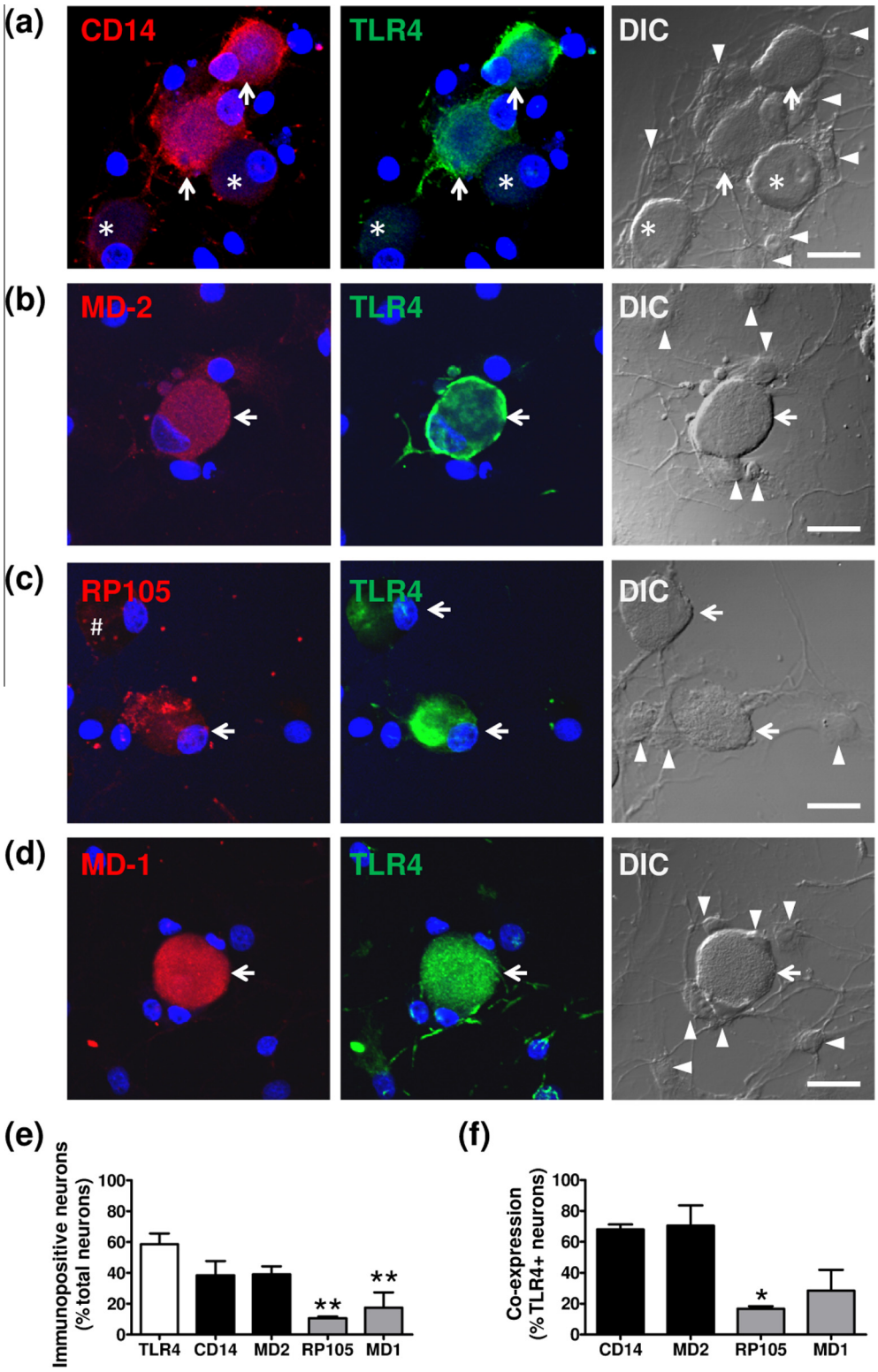

Fig. 2. DRG neurons principally express TLR4 in conjunction with CD14 and MD-2. TLR4-positive neurons co-expressing (a) CD14, (b) MD-2, (c) RP105 and (d) MD-1, respectively (red). In (a)–(d), images are marked for TLR4-positive neurons (arrows), TLR4-negative neurons (*) and glial cells (arrowheads). In (c) a TLR4-positive but RP105-negative neuron is marked (#). Scale bar, 20 μm. (e) Quantification of proportion of neurons expressing TLR4 signaling molecules (**$p < 0.01$ compared to TLR4 group), and (f) evidence that the majority of TLR4-positive neurons co-express CD14 and MD-2, not RP105 or MD-1 (*$p < 0.05$ compared to CD14 and MD-2 groups). Data are means ± SEM from three independent cell preparations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

double immunofluorescence study determined that approximately 60% of all neurons were TLR4-positive but significantly fewer neurons expressed either MD-1 or RP105 (Fig. 2e). The majority of TLR4-positive neurons co-expressed CD14 or MD-2 rather than RP105 or MD-1 (Fig. 2f), with a significantly greater co-expression of TLR4 with CD14 or MD-2.

LPS activates TLR4-dependent MyD88-dependent signaling pathways in DRG neurons

Using ultrapure LPS to avoid potential off-target effects of standard grade LPS (Ochoa-Cortes et al., 2010; Saito et al., 2010), we found that DRG cells showed a concentration-dependent increase in COX-2 mRNA after 4 h incubation with LPS (0.01–1 μg/ml), with a significant increase of 3.9 ± 0.4-fold basal ($p < 0.01$) and 6.7 ± 0.9-fold basal ($p < 0.001$) at 0.1 and 1 μg/ml, respectively ($n = 3$). DRG cell viability was assessed using the MTT assay, and 24-h incubation with LPS (0.1 and 1 μg/ml) produced a minor 6% and 9% loss of viability ($p < 0.05$; $n = 3$). No obvious signs of cell death were visible at this time, therefore, 1 μg/ml LPS was selected for all subsequent experiments.

Activation of the MyD88-dependent pathway classically involves NF-κB activation and subsequent expression of inflammatory mediators. DRG neurons responded to LPS with a time-dependent increase in IκBα phosphorylation which was statistically significant at 30–120 min, and was mirrored by a fall in IκBα protein (Fig. 3a, b). LPS produced a rapid increase in expression of both IL-1β and TNFα mRNA which fell sharply after 2 h (Fig. 3c, d). IL-1β and TNFα mRNA increased sharply at 2 h ($p < 0.05$) which preceded the

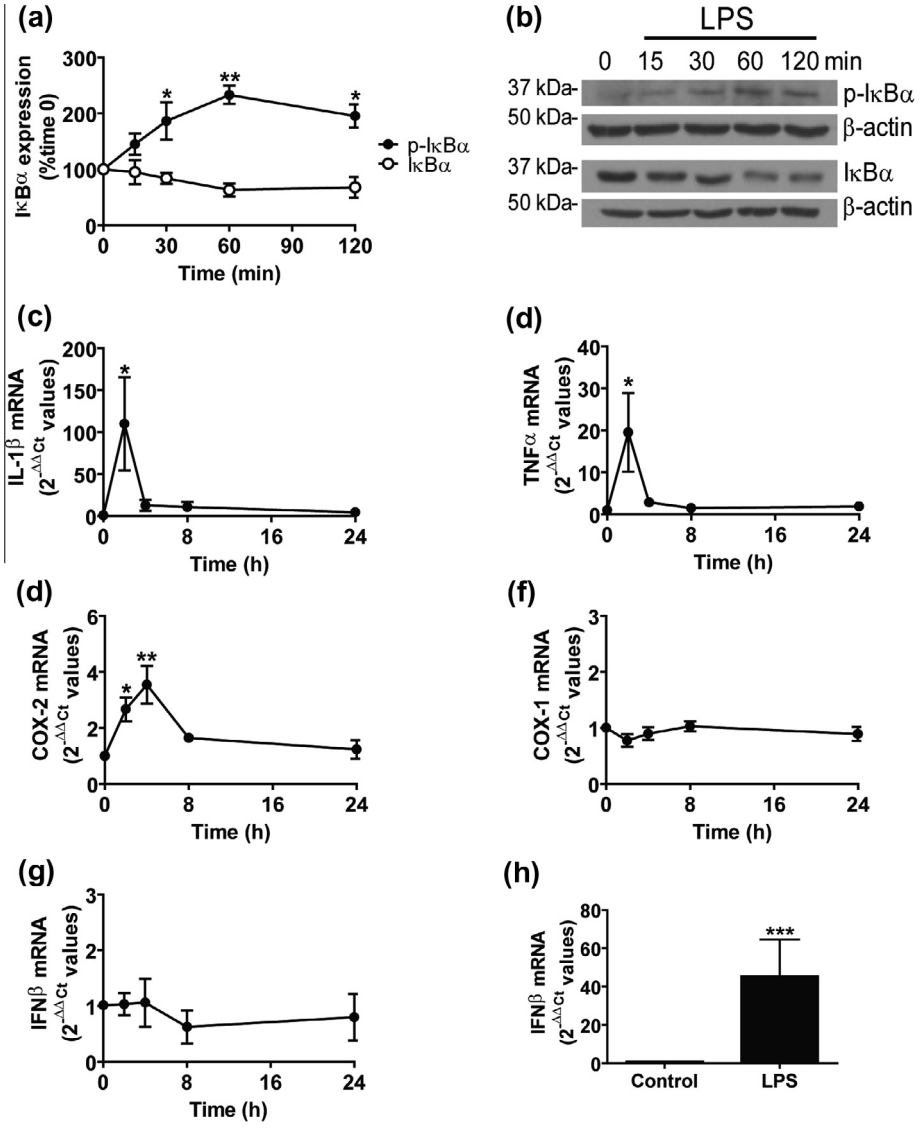

Fig. 3. LPS activates MyD88-dependent signaling pathways in rat DRG cells. Cells were incubated with LPS (1 μg/ml) for distinct time periods, then processed for Western blotting or real-time PCR as described in Experimental procedures. (a, b) Western blot analysis shows time-dependent increase in IκBα phosphorylation and accompanying loss of IκBα protein in response to LPS. Time-dependent effect of LPS on expression of (c) IL-1β, (d) TNFα, (e) COX-2, (f) COX-1 mRNA and (g) IFNβ mRNA. (*$p < 0.05$, **$p < 0.01$ compared to 0 time point). (h) Stimulation of rat leukocytes with LPS (1 μg/ml) for 4 h significantly increased expression of IFNβ mRNA (***$p < 0.001$). Data are means ± SEM, $n = 3$.

peak increase in COX-2 mRNA ($p < 0.01$) at 4 h. LPS had no significant effect on COX-1 mRNA (Fig. 3f). Activation of the TRIF-dependent pathway should result in activation of the Type I interferons. However, LPS failed to increase IFNβ mRNA expression over 24 h (Fig. 3g). To confirm the validity of this negative result, we incubated rat leucocytes (prepared according to Davenpeck et al. (1998)) with 1 μg/ml LPS for 4 h and found a 45 ± 19-fold increase in IFNβ mRNA ($p < 0.001$; mean ± SEM, $n = 3$).

To demonstrate the TLR4- and NF-κB-dependence of responses to LPS, we incubated DRG cells with the TLR4 inhibitor CLI-095 (also known as TAK-242; Kawamoto et al., 2008)) or the NF-κB inhibitor Bay 11-7082 in the presence or absence of LPS. CLI-095 significantly inhibited LPS-stimulated COX-2, IL-1β and TNFα mRNA expression (Fig. 4a–c). Bay 11-7082 also inhibited LPS-stimulated COX-2, IL-1β and TNFα mRNA expression (Fig. 4d–f), but its effects on cytokine production did not reach statistical significance due to the more variable effects of LPS to stimulate cytokine production at this 2-h time point. Notably, CLI-095 also inhibited basal IL-1β mRNA ($2^{-\Delta\Delta Ct}$ value: 0.32 ± 0.12) and TNFα mRNA ($2^{-\Delta\Delta Ct}$ value: 0.67 ± 0.12), suggesting the presence of an endogenous TLR4 agonist in the dissociated DRG cell cultures. Taken together, these results indicate that LPS stimulates TLR4 to initiate classical MyD88-dependent signaling pathways in DRG cells.

LPS differentially increases COX-2 and cytokine mRNA expression

It was notable that Bay 11–7082 had far greater effect on LPS-stimulated IL-1β and TNFα mRNA expression (83% and 87% inhibition, respectively) compared with its effect on COX-2 mRNA (42% inhibition). We therefore investigated possible alternative pathways for LPS-stimulated COX-2 mRNA using inhibitors specific for mitogen-activated protein kinase kinase 1/2 (MEK1/2; U0126), p38 kinase (SB239063), c-Jun N-terminal kinase (JNK; SP600125) and phosphatidylinositide 3-kinase (PI3K; LY294002). Only U0126 and SB239063 significantly inhibited LPS-stimulated COX-2 mRNA expression (46% and 67% inhibition, respectively), implicating a role for extracellular signal-regulated kinases-1/2 (ERK1/2) and p38 kinase in addition to NF-κB (Fig. 5). TLR4-dependent activation of COX-2 is partially dependent on the transactivation of epidermal growth factor receptor (EGFR) in renal medullary collecting duct cells (Küper et al., 2012) and in enterocytes (McElroy et al., 2012). The EGFR inhibitor AG1478 significantly inhibited LPS-stimulated COX-2

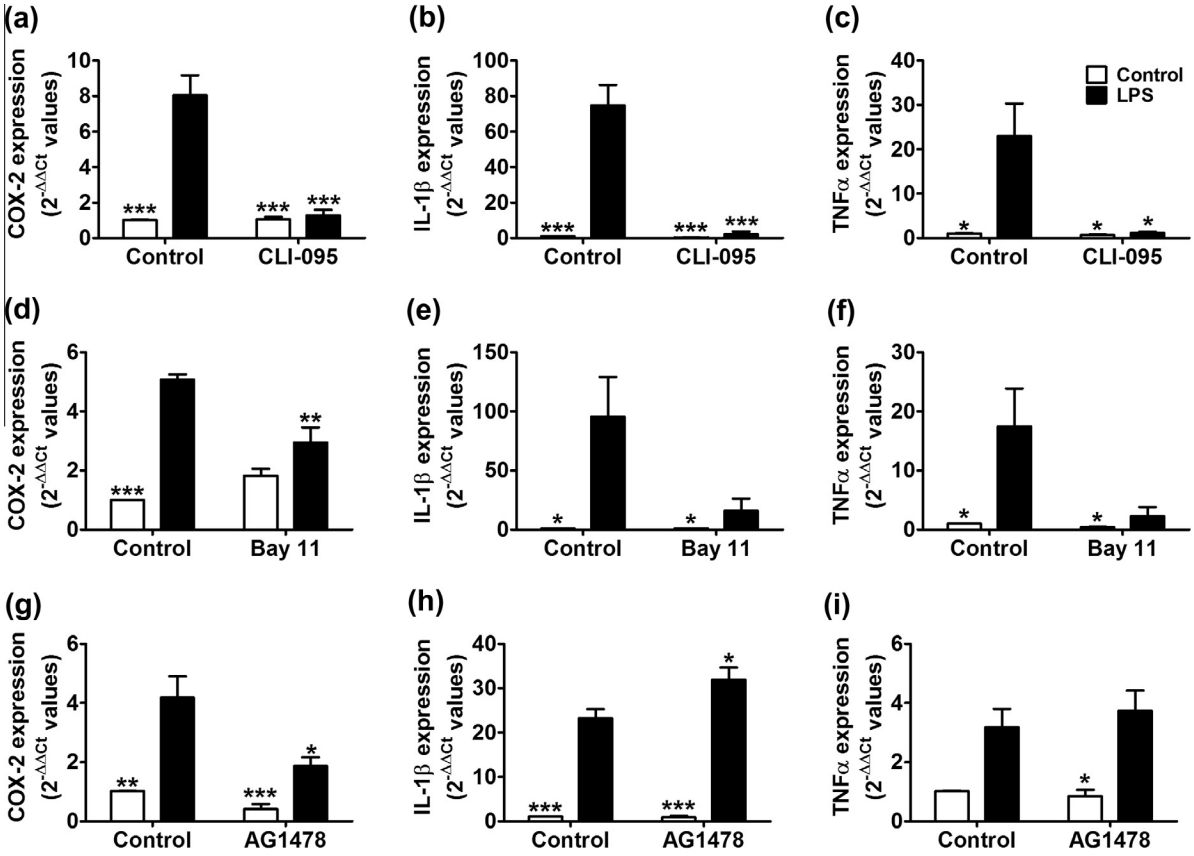

Fig. 4. LPS activates different TLR4-dependent signaling pathways to increase COX-2, IL-1β and TNFα mRNA expression. DRG cells were incubated ± CLI-095 (TLR4 inhibitor; 1 μM), Bay 11–0782 (NF-κB inhibitor; 10 μM) or AG1478 (EGFR inhibitor; 10 μM) for 30 min prior to the addition of control medium or LPS (1 μg/ml) for 2 h. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to LPS group). Data are means ± SEM, $n = 3$, of assays performed in duplicate.

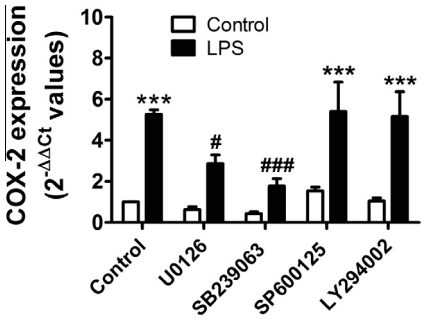

Fig. 5. LPS-stimulated COX-2 mRNA is ERK1/2 and p38 kinase-dependent. DRG cells were incubated with U0126 (ERK1/2 inhibitor; 25 μM), SB239063 (p38 kinase inhibitor; 10 μM), SP600125 (JNK inhibitor; 10 μM) or LY294002 (PI3K inhibitor; 10 μM) for 30 min prior to the addition of control medium or LPS (1 μg/ml) for 2 h. (***$p < 0.001$ compared to control group; #$p < 0.05$, ###$p < 0.001$ compared to LPS group). Data are means ± SEM, $n = 3$, of assays performed in duplicate.

mRNA by 54% (Fig. 4g), increased IL-1β mRNA by 38% (Fig. 4h) but had no effect on TNFα mRNA (Fig. 4i).

LPS differentially regulates COX-1 and COX-2 in DRG cells

LPS produced a significant time-dependent increase in COX-2 protein, plateauing at 8 h (Fig. 6b). In contrast, LPS significantly inhibited COX-1 protein, again plateauing at 8 h (Fig. 6a).

After 24-h incubation of DRG cells with LPS, PGE_2 and 6-keto $PGF_{1\alpha}$ production increased 5.2 and 4.2-fold, respectively ($p < 0.001$; Fig. 6c, d). Although this increase in prostanoid production was completely inhibited by the COX-2 inhibitor NS-398 ($p < 0.001$), the COX-1 inhibitor SC-560 also inhibited PGE_2 ($p < 0.01$) and 6-keto $PGF_{1\alpha}$ ($p < 0.05$) production by 59%.

DISCUSSION

Recent studies show that low-dose endotoxin potentiates capsaicin-induced hyperalgesia and allodynia in man (Hutchinson et al., 2013), clearly demonstrating that the neuronal expression of receptors more commonly associated with immune cells are involved in the neuroimmune modulation of nociceptive pathways (Nicotra et al., 2012). In contrast to recent findings in murine DRG neurons (Acosta and Davies, 2008), we found that rat DRG neurons expressed the classical LPS receptor complex of TLR4, MD-2 and CD14 and estimate that approximately 40% of DRG neurons are TLR4-signaling competent by virtue of cell surface co-expression of TLR4 with MD-2 or CD14-ir. The expression of TLR4-ir was not restricted to any subpopulation of neurons, confirming the observations of Due et al. (2012) showing that LPS stimulated Ca^{2+} signaling in both capsaicin-sensitive and capsaicin-insensitive DRG neurons. We observed a time-dependent elevation in COX-2, IL-1β and TNFα mRNA in response to LPS which was completely inhibited by

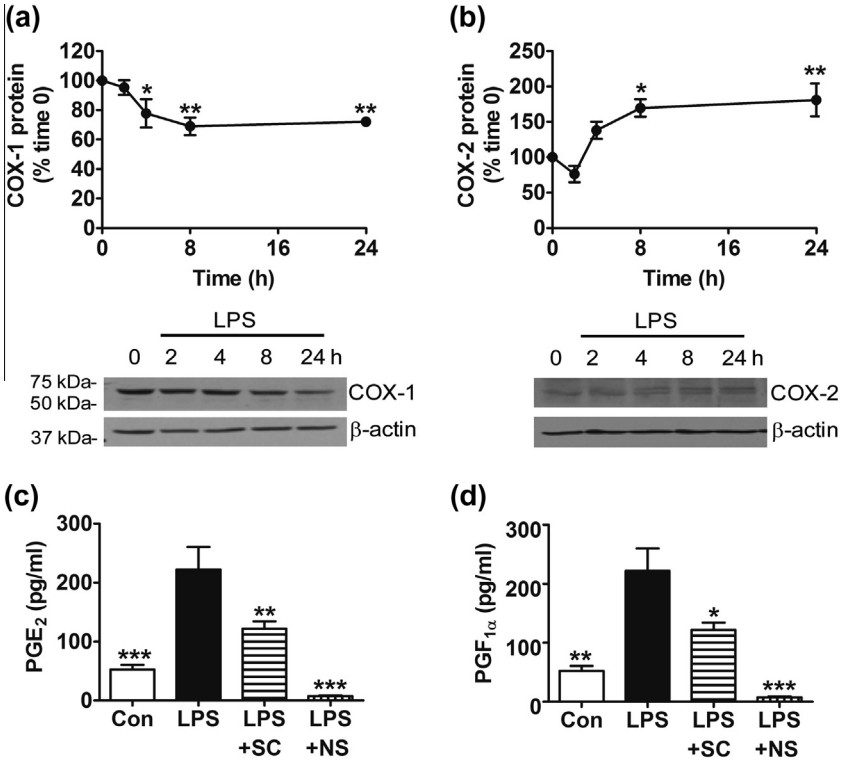

Fig. 6. LPS differentially regulates COX-1 and COX-2 protein expression to enhance COX-2-dependent production of PGE_2 and PGI_2. DRG cells were incubated ± LPS (1 μg/ml) for periods up to 24 h and (a) COX-1 or (b) COX-2 protein detected by Western blotting. Data are means ± SEM, $n = 3$, with representative gels shown below. (c, d) DRG cells were incubated with the COX-1 inhibitor SC-560 (10 nM) or the COX-2 inhibitor NS-398 (3 μM) for 30 min prior to addition of LPS (1 μg/ml). After 24 h, supernatants were harvested and assayed by EIA for (c) PGE_2 and (d) 6-keto $PGF_{1\alpha}$. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to LPS group). Data are means ± SEM, $n = 3$, of assays performed in duplicate.

the TLR4 inhibitor CLI-095, indicating that the responses to LPS were TLR4-dependent. Furthermore, LPS increased phosphorylation of IκBα indicating that DRG neuronal TLR4 activates the MyD88-dependent signaling pathway. In contrast, LPS failed to increase in IFNβ mRNA and therefore does not activate a TRIF-dependent signaling pathway in DRG cells. In contrast, LPS dramatically increased IFNβ mRNA expression in rat leucocytes, suggesting the downstream signaling pathways from TLR4 are cell-type specific. These different signaling outcomes for TLR4 activation of DRG neurons may be required for these cells to distinguish PAMPs and DAMPs during infection and tissue damage, in order to achieve a balance between immune intervention and tissue damage repair (Okun et al., 2011).

DRG neurons express multiple TLRs (Barajon et al., 2009; Qi et al., 2011) which would be expected to allow for the detection of PAMPs as well as endogenous DAMPs (Miyake, 2007). Coordinated interaction between different TLRs could contribute to overall responses to painful stimuli. For example, while intrathecal TLR4 ligands evoked persistent TNFα-dependent tactile allodynia, this response was considerably prolonged in TRIF-deficient mice, implicating a role for IFNβ in response resolution, most likely generated via TLR3 activation (Stokes et al., 2013b). Curiously, spinal processing of inflammatory and neuropathic pain was TLR4-dependent in male mice, but TLR4-independent in female mice (Sorge et al., 2011; Stokes et al., 2013a), but any role for hormonal regulation of TLR4 responses in the DRG is currently unknown and warrants further investigation.

Following nerve damage, the up-regulation of IL-1β and TNFα represents the earliest events by sensory neurons in response to injury (Miller et al., 2009). Similarly, intrathecal LPS is known to rapidly enhance pain responses within 1–4 h, and this is principally through the generation of cytokines although the cell source is uncertain (Saito et al., 2010; Loram et al., 2012). Rat DRG neurons release TNFα (Fernyhough et al., 2005) and isolated mouse colonic DRG neurons increase TNFα mRNA within 5 min of LPS stimulation (Ochoa-Cortes et al., 2010). But pain models *in vivo* suggest that TNFα is predominantly produced by DRG satellite glial cells, not neurons (Li et al., 2004; Ohtori et al., 2004; Dubový et al., 2006). The lack of cell surface TLR4-ir on DRG glial cells in our study is consistent with previous reports (Acosta and Davies, 2008; Ochoa-Cortes et al., 2010), and suggests that sensory neurons are the primary responder to LPS and other TLR4 ligands. However, although we estimate that approximately 40% of DRG neurons should be TLR4-responsive, we were unable to confirm that DRG neurons were the sole source of LPS-stimulated prostanoids at 24 h due to the poor sensitivity of COX-2 antibodies for immunocytochemistry. Both COX-1 and COX-2-ir have been identified in whole DRG by immunohistochemistry (Araldi et al., 2013) but we could not detect LPS-stimulated COX-2-ir in isolated DRG cells (data not shown) despite our data showing LPS-stimulated COX-2 mRNA and COX-2 protein, and COX-2-dependent prostanoid production. While only DRG neurons express cell surface TLR4-ir and must be the first responders to LPS, the intimate relationship between DRG neurons and satellite glial cells *in vivo* (Hanani, 2005) suggests that paracrine communication may serve to regulate/amplify this initial response and contribute to increased neuronal sensitivity. Further studies are therefore underway to investigate the role of DRG neuron-glial cell interactions contributing to the overall production of cytokines and COX-2 products following the activation of neuronal TLR4.

In DRG cells, TLR4-induced COX-2 mRNA expression was dependent on EGFR transactivation in addition to the canonical NF-κB, ERK1/2 and p38 kinase pathways. Such TLR4-mediated EGFR-dependent COX-2 expression was similarly found in renal medullary collecting duct cells (Küper et al., 2012). Transactivation of EGFR via tumour necrosis factor alpha-converting enzyme (TACE)-induced ectodomain shedding of pro-transforming growth factor-α and subsequent downstream activation of ERK1/2 and p38 kinase, were required for this TLR4-dependent COX-2 expression. And, in optic nerve astrocytes, EGFR stimulation rapidly induced COX-2 expression which was also dependent on downstream activation of ERK1/2 and p38 kinase (Zhang and Neufeld, 2007). In DRG cells, TLR4-dependent COX-2 expression was also EGFR-dependent and was similarly inhibited by blockers of ERK1/2 and p38 kinase. Therefore, we hypothesize that EGFR is upstream of ERK1/2 and p38 kinase in DRG cells. In contrast, TLR4-dependent IL-1β and TNFα mRNA expression were not attenuated by inhibiting EGFR; highlighting the differences in signaling pathways used to elevate cytokines and other inflammatory mediators. In murine astrocytes, inhibiting NF-κB also failed to completely inhibit LPS-stimulated COX-2 expression although this response was completely lost in MyD88$^{-/-}$ mice (Font-Nieves et al., 2012), further suggesting that mechanism(s) in addition to the activation of NF-κB, such as transactivation of EGFR, contribute to TLR4-dependent COX-2 expression in astrocytes and DRG cells.

The relative roles of COX-1 and COX-2 in the spinal cord *versus* DRG regarding pain control has been debated for many years (Svensson and Yaksh, 2002; Ma and Quirion, 2008; Araldi et al., 2013). A possible complicating factor comes from our observations that LPS caused a significant decrease in COX-1 protein expression in a manner similar to that reported for murine astrocytes (Font-Nieves et al., 2012). Using astrocytes derived from MyD88$^{-/-}$ mice, Font-Nieves et al. (2012) demonstrated that LPS-stimulated loss of COX-1 was MyD88-dependent but MAPK-independent. This loss of COX-1 further enhanced COX-2-dependent PGE_2 production because COX-1 exerts an inhibitory tone over COX-2 activity (Font-Nieves et al., 2012). In most reports, LPS has no obvious effect on COX-1, but a decrease of COX-1 mRNA detected by *in situ* hybridization in kidney inner medulla cells has been noted despite no change in other kidney cell types (Ichitani et al., 2001). And, in COX-1 siRNA-transfected

human follicular dendritic cells, LPS also significantly increased COX-2 mRNA and PGE_2 production compared to wild-type cells (Cho et al., 2011). It is likely that these complex cell type-specific responses to LPS have confounded previous attempts to understand the relative roles of prostanoid-generating COX-1 and COX-2 in acute and chronic pain conditions. Here we showed that LPS-stimulated PGE_2 and PGI_2 production in DRG cells was completely inhibited by the specific COX-2 inhibitor NS-398. Although the COX-1 inhibitor SC-560 also partially inhibited LPS-stimulated prostanoid production, SC-560 inhibits COX-2 in a cell type-specific manner (Brenneis et al., 2006) which likely accounts for the inhibitory effect observed herein.

CONCLUSION

Rat DRG neurons express the classical TLR4 signaling complex of TLR4, MD-2 and CD14, and in response to LPS stimulation generate TLR4-dependent and time-dependent increases in COX-2, IL-1β and TNFα and a time-dependent decrease in COX-1 expression in DRG cells. In the mixed population of DRG cells, the localization of TLR4 in DRG neurons rather than glial cells further emphasizes the role of primary sensory neurons as detectors of danger signals. In our putative model of acute TLR4 signaling in DRG cells, we propose that TLR4 activates the transcriptional regulator NF-κB which induces IL-1β and TNFα expression. TLR4 also increases COX-2 via a NF-κB-dependent pathway but additionally transactivates EGFR with downstream activation of ERK1/2 and p38 kinase. To further potentiate COX-2-dependent production of prostanoids, TLR4 also decreases COX-1 expression to relieve its negative regulation of COX-2. Although these conclusions are based on our studies using isolated adult rat DRG cells, we can see that such studies can point to novel approaches for pain control in man; indeed the role of NF-κB in inflammatory and neuropathic pain is already well established (Tegeder et al., 2004) and targeting EGFR inhibition showed dramatic relief of neuropathic pain in patients (Kersten et al., 2013). Dissociation of DRG explants has been known for some time to produce increased excitability of neurons (Zheng et al., 2007), and our studies suggest this could result from tonic TLR4-dependent production of IL-1β and/or TNFα. Further studies are underway to evaluate the presence of endogenous factors capable of activating TLR4 signaling the DRG and to investigate the neuron-glial cell interactions contributing to the overall production of cytokines and COX-2 products following the activation of neuronal TLR4.

CONFLICT OF INTEREST

There is no conflict of interest.

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