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Arthritis Rheumatol. Author manuscript; available in PMC 2016 November 01.

Published in final edited form as:
Arthritis Rheumatol. 2015 November ; 67(11): 2933–2943. doi:10.1002/art.39291.

Damage-Associated Molecular Patterns Generated in Osteoarthritis Directly Excite Murine Nociceptive Neurons through Toll-like Receptor 4

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Abstract

Objective—The aim of this study was to determine whether selected damage-associated molecular patterns (DAMPs) present in osteoarthritic (OA) joints excite nociceptors through toll-like receptor (TLR)-4.

Methods—The ability of S100A8 and α_2-macroglobulin to excite nociceptors was determined by measuring: (1) Release of monocyte chemoattractant protein (MCP)-1 by cultured dorsal root ganglion (DRG) cells; (2) Intracellular calcium $(Ca)_i$ levels in cultured DRG neurons from naïve mice or mice 8 weeks after destabilization of the medial meniscus (DMM). The role of TLR4 was assessed using *Tlr4*$^{-/-}$ cells or a TLR4 inhibitor. $(Ca)_i$ levels in neurons within *ex vivo* intact DRG were measured using Pirt-GCaMP3 mice. Neuronal *Tlr4* expression was determined by *in situ* hybridization. DMM surgery was performed in wild-type and *Tlr4*$^{-/-}$ mice; mechanical allodynia was monitored, and joint damage was assessed histologically after 16 weeks.

Results—Both naïve and DMM DRG neurons expressed *Tlr4*. Both S100A8 and α_2-macroglobulin stimulated release of the pro-algesic chemokine, MCP-1, by DRG cultures and neurons rapidly responded to S100A8 and α_2-macroglobulin with increased $(Ca)_i$. Blocking TLR4 inhibited these effects. Neurons within intact DRG responded to the TLR4 agonist, lipopolysaccharide. In both calcium-imaging assays, it was primarily the nociceptor population of neurons that responded to TLR4 ligands. *Tlr4*$^{-/-}$ mice were not protected from mechanical allodynia or from joint damage associated with DMM.

Conclusion—Our experiments suggest a role for TLR4 signaling in the excitation of nociceptors by selected DAMPs. Further research is needed to delineate the importance of this pathway in relation to OA pain.

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Keywords

pain; osteoarthritis; innate immunity; mouse models; other

INTRODUCTION

Pain represents a protective response to potentially damaging stimuli and is therefore essential to health. Under normal circumstances, both the stimuli that cause pain and the ensuing response are relatively transient in nature. However, pain may also be experienced in the context of chronic pathology. In these chronic pain syndromes, nerves that normally transmit painful information to the brain undergo numerous changes in their properties, resulting in hypersensitivity to painful stimulation or in allodynia - defined as pain in response to a normally innocuous stimulus - or even in pain in the absence of a stimulus (1).

Chronic joint disease is frequently accompanied by pain. Notably, osteoarthritis (OA), the most common form of arthritis, is a major source of chronic pain (2). The lifetime risk of developing symptomatic knee OA is approximately 45% based on Johnston County Osteoarthritis Project data (3) and worldwide, OA is a leading cause of chronic pain (4). Treatment options for OA pain include acetaminophen, nonsteroidal anti-inflammatory drugs, opioids, and intra-articular therapy with glucocorticoid or hyaluronan preparations, but their efficacy is limited and chronic use can be associated with adverse effects (5).

In order to develop improved analgesics, a better understanding of mechanisms that generate and maintain OA pain is necessary. Current evidence suggests that ongoing nociceptive input from the joint contributes to the maintenance of OA knee pain (6). The pathology of knee OA affects all joint tissues, including cartilage, subchondral bone, menisci, ligaments, and synovium (7). With the exception of articular cartilage, joint tissues are innervated by sensory neurons. The cell bodies of these afferents are contained in the dorsal root ganglia (DRG). These neurons are pseudounipolar, extending one axon toward the periphery and the other to the dorsal horn of the spinal cord, where synapses are formed with second-order neurons. Nociceptive neurons, either small unmyelinated C-fibers or medium-sized lightly myelinated Aδ-fibers, are a subtype of sensory afferents that detect mechanical, chemical or thermal noxious stimuli in the innervated tissues (1). Therefore, it is of great interest to define mediators in the OA joint that can sensitize nociceptors and lead to changes that contribute to pain. Numerous inflammatory mediators, including a number of cytokines and chemokines present in the arthritic joint, are capable of activating nociceptors, and may thus contribute to joint pain (8–10).

In addition to traditional inflammatory cytokines like IL-1β, IL-6, and TNF-α, damage-associated molecular patterns (DAMPs) (11, 12) have been implicated in driving the chronic, low-level inflammation associated with OA (11, 13). DAMPs can activate pattern recognition receptors (PRR) on chondrocytes and synovial macrophages, thus promoting cartilage degradation and synovitis in OA (14). These PRR include toll-like receptors (TLR) and the receptor for advanced glycation end products (RAGE). In particular, TLR4 has been highlighted as a potential target for disease-modifying OA drugs (15).

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Since it has recently been reported that DRG neurons also express PRR (16–19), including TLR4 (16, 20, 21), we hypothesized that DAMPs generated in the OA joint can directly excite nociceptors and thus provide a putative link between tissue damage and pain development. To test this hypothesis, we chose to investigate two endogenous activators of TLR4 present in OA joints, namely, S100A8 (22) and α_2-macroglobulin (23). Synovial biopsies taken from patients with early, symptomatic knee OA had significantly elevated expression of the alarmin, S100A8, which correlated with the presence of synovitis (24). Patients undergoing total joint replacement also had abundant S100A8 synovial expression (24). The plasma protein, α_2-macroglobulin, is detectable in synovial fluid in both early- and late-stage knee OA (23, 25–27). S100A8 and α_2-macroglobulin were shown to promote catabolic pathways in macrophages through TLR4 (22, 23). Therefore, we investigated the ability of these DAMPs to excite sensory neurons through TLR4 and promote a pro-algesic state. We have previously shown that wild-type mice develop sustained mechanical allodynia for 16 weeks after destabilization of the medial meniscus (DMM), but not after sham surgery, indicating that maintenance of mechanical allodynia is associated with joint damage in this model (28, 29). Therefore, we tested the role of TLR4 in the development of mechanical allodynia, a behavioral measure of sensitization, in the DMM model.

MATERIALS AND METHODS

Animals and surgery

A total of 125 mice were used. All animal experiments were approved by the Institutional Animal Care and Use Committees at Rush University Medical Center and Northwestern University. Animals were housed with food and water *ad libitum* and kept on 12-hour light cycles. Wild-type C57BL/6, *Tlr4*$^{-/-}$ mice (Jackson and courtesy of Dr. David Hackam, University of Pittsburgh, C57BL/6 background (30)), and Pirt-GCaMP3 mice (courtesy of Dr. Xinzhong Dong, Johns Hopkins University, C57BL/6 background (31)) were used. DMM surgery was performed as previously described (29, 32) in the right knee of 10-week old male mice. Briefly, after medial parapatellar arthrotomy, the anterior fat pad was dissected to expose the anterior medial meniscotibial ligament, which was severed. The knee was flushed with saline and the incision closed.

DRG cell culture and stimulations

Cells were isolated from knee-innervating dorsal root ganglia (L3-L5) of 3–4 mice (male or female naïve C57BL/6 or *Tlr4*$^{-/-}$ mice at least 10 weeks of age or male C57BL/6 mice 8 weeks after DMM surgery), plated onto glass coverslips, and cultured in adult neurogenic medium, as previously described (29). Overnight stimulations from days 3–4 were carried out with S100A8 (0–1 μg/mL, Prospec, East Brunswick, NJ) or α_2-macroglobulin (0–100 μg/mL, EMD Millipore, Billerica, MA) ± the selective small molecule TLR4 inhibitor, Tak242 (1 μM, EMD Millipore). Stimulation with 10 ng/mL IL-1α (Peprotech, Rocky Hill, NJ) was used as a positive control. Similar results were obtained ± polymyxin B (inhibitor of lipopolysaccharide (LPS) and used to control for endotoxin contamination) (10 μg/mL, Sigma-Aldrich, St. Louis, MO). In addition, endotoxin levels in S100A8 and α_2-macroglobulin were confirmed to be <0.01 EU/μg by LAL assay (Fisher Scientific, Pittsburgh, PA). As a positive control for TLR4 activation, stimulations were performed

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using the TLR4 ligand LPS (1 μg/mL) (Ultra-pure LPS from E. coli O111:B4 purified so that only TLR4 is activated (33); InvivoGen, San Diego, CA, cat#tlrl-3pelps). Supernatants were collected for protein analysis. Three independent experiments were performed per stimulus.

Protein analysis of supernatant

Total protein levels were determined by BCA assay (Thermo Fisher Scientific, Inc., Rockford, IL), and MCP-1 protein levels were determined via ELISA (R&D Systems Inc, Minneapolis, MN).

In situ hybridization

Ipsilateral L3-L5 DRG were harvested, embedded, and sectioned as previously described (29). For the generation of *Tlr4* probes, a 405-bp *Tlr4* cDNA fragment (GenBank no. NM_021297) was cloned by PCR by using mouse brain cDNA. The resulting PCR product was subcloned into a pGEM-T Easy Vector (Promega Madison, WI) and verified by restriction analysis and automated DNA sequencing (Perkin Elmer, Boston, MA). The *Tlr4* template was linearized with Xba I to generate an antisense probe by using SP6 polymerase. The sense probe was linearized with Hind III by using T7 polymerase. *In situ* hybridization histochemistry for *Tlr4* was performed by using digoxigenin-labeled riboprobes (Roche Applied Science, Indianapolis, IN) as previously described (34).

In vitro calcium imaging

The response of cultured DRG neurons to selected DAMPs was recorded though intracellular Ca^{2+}-imaging, following standard protocols using Fura-2AM (2 μM; Life Technologies, Grand Island, NY) (29, 35). S100A8 (1 μg/mL) or α_2-macroglobulin (100 μg/mL) was applied for 3 min by adding 0.5–1 mL of solution to the bath chamber. Cells were washed with balanced salt solution before applying controls (potassium (50 mM) and capsaicin (10 μM)). LPS (1 μg/mL) was used as a positive control for TLR4 activation. Three independent experiments, each using DRG pooled from 4 naïve wild-type C57BL/6, three independent experiments each using DRG pooled from 4 wild-type C57BL/6 mice 8 weeks after DMM surgery, and two independent experiments using DRG pooled from 4 $Tlr4^{-/-}$ naïve mice, were performed.

Ex vivo calcium imaging of intact DRG

Intact DRG (L4 or L5) were isolated from naïve male or female Pirt-GCaMP3 mice and equilibrated in artificial cerebrospinal fluid (ACSF) (31) bubbled with 95% O_2/5% CO_2 on ice. After 30 minutes, explants were placed in a perfusion chamber within ACSF and imaged using a CSU-X1 spinning disk confocal microscope (Intelligent Imaging Innovations, Inc., Denver, CO) at 20x magnification at the 488 nm wavelength. Explants were stimulated by injecting 10 μL of LPS solution into a continuously running perfusion chamber with a volume of 1 mL (LPS = 50 μg/mL). Positive controls (potassium and capsaicin) were applied as for *in vitro* calcium imaging. Image analysis was performed using an ImageJ (36) macro to determine change in fluorescence intensity with time. Neurons with spontaneous responses to perfusion buffer were excluded.

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von Frey testing

Wild-type or *Tlr4*$^{-/-}$ mice were tested for mechanical allodynia using von Frey fibers as previously described (29). Baseline thresholds were assessed prior to surgery, and weeks 2, 4, 8, 12, and 16 after DMM. Results are representative of two independent experiments.

Histopathology of the knee

Sixteen weeks after DMM, histopathology of the knee was evaluated based on OARSI recommendations (39) (Alison Bendele, Bolder BioPATH, Inc., Boulder CO). Joints were fixed in 10% formalin, decalcified, embedded in the frontal plane, sectioned, and stained with Toluidine blue, as described (29). Medial and lateral femoral condyles and tibial plateaux were scored for severity of cartilage degeneration on a scale of 0–5, with 5 representing the most damage (maximum summed score=60). Scoring of the osteophytes on the medial and lateral sides (largest on tibial or femoral surface under evaluation) and categorization into small, medium and large was done with an ocular micrometer and scored from 0–3 (3 = large).

Statistical analysis

For MCP-1 stimulation experiments, one-way ANOVA with Bonferroni post-tests or unpaired t-tests assuming equal variances were used to compare the groups of interest. For calcium imaging experiments, Fisher's exact tests were used to compare the number of responses in capsaicin-sensitive neurons *versus* non-capsaicin-sensitive neurons. For von Frey testing, one-way ANOVA with Bonferroni post-tests was used to compare each time point to time 0. For joint histopathology, an unpaired t-test assuming equal variance was used to compare *Tlr4*$^{-/-}$ mice to wild-type mice. A p-value < 0.05 was considered significant for all tests. All analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Results are presented as mean ± standard error of the mean.

RESULTS

MCP-1 production by cultured DRG cells

In order to determine whether selected DAMPs can promote a pro-algesic state in DRG cells, we stimulated primary cultures of DRG cells and measured release of the chemokine MCP-1 (CCL2) into culture medium, since we previously found that MCP-1 is upregulated by DRG neurons in the DMM model and acts as a key mediator of pain (29). Overnight stimulation of DRG cells with S100A8 or α_2-macroglobulin induced release of MCP-1, in a concentration-dependent manner (Figs 1A,B). IL-1α was included as a positive control for MCP-1 stimulation (Fig 1A). A subset of experiments was performed in the presence of polymyxin B to test for endotoxin contamination. Similar results were found with or without polymyxin B (Supp. Fig 1). In three independent experiments, both S100A8 and α_2-macroglobulin induced significantly greater amounts of MCP-1 compared to unstimulated cells (Fig 1C).

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Role of Toll-like receptor 4 in MCP-1 production

Since the TLR4 pathway has been reported to mediate cytokine production in response to these DAMPs in other cell types, including macrophages and phagocytes (22, 23, 40), we investigated whether the observed MCP-1 upregulation was mediated through TLR4. A small molecule TLR4 inhibitor, Tak242 (1 μM), significantly inhibited MCP-1 production induced by S100A8 (83±2% inhibition, in 3 independent experiments) and by α_2-macroglobulin (88±2% inhibition, in 3 independent experiments) (Fig 1A,B). The pattern-associated molecular pattern (PAMP) lipopolysaccharide (TLR4-selective LPS from E. coli O111:B4 (33)) was tested as a positive control for the ability of the TLR4 pathway to induce MCP-1 release. LPS stimulated the production of MCP-1 19.0±6.6-fold over unstimulated cells, and Tak242 completely blocked LPS-stimulated MCP-1 release (Fig 1D).

In *Tlr4*$^{-/-}$ cells, stimulation of MCP-1 production by S100A8 and by α_2-macroglobulin was inhibited to a similar extent as seen with Tak242, while IL-1α still provoked robust stimulation of MCP-1 (Fig 1E). Specifically, S100A8 stimulated only 3.0±0.3-fold production of MCP-1 compared to unstimulated *Tlr4*$^{-/-}$ cells, whereas α_2-macroglobulin stimulation was completely inhibited (1.4±0.03-fold compared to unstimulated *Tlr4*$^{-/-}$ cells).

Together, these results suggest that S100A8 and α_2-macroglobulin primarily signal through TLR4 on DRG neurons.

Blocking TLR4 reduces MCP-1 produced by DRG cells harvested 8 weeks after DMM surgery

We have previously shown that, 8 weeks after DMM surgery, cultured DRG cells produce increased amounts of MCP-1 compared to sham and to age-matched naïve neurons (29). Here, we confirmed our previous results, finding that DRG cells taken from DMM mice 8 weeks after surgery produce more MCP-1 than naïve cells (Fig 1A,F). Incubating DMM-DRG cultures with Tak242 significantly reduced MCP-1 production, suggesting that part of the upregulated expression of MCP-1 by these cells in OA is driven by activation of TLR4 (Fig 1F).

DRG neurons express Tlr4

In order to determine which cells in the DRG express *Tlr4*, *in situ* hybridization was performed. Staining DRG sections from naïve mice with the anti-sense probe revealed that *Tlr4* was widely expressed by small-to-medium-diameter neurons (Fig 2A), which is consistent with previous reports utilizing an immunohistochemical approach (20, 21). A sense probe was used as a negative control (Fig 2B). Similar numbers and sizes of neurons expressed *Tlr4* 8 weeks after DMM compared to naïve expression (Fig 2C).

Calcium imaging in cultured naïve DRG neurons

We next sought to determine whether S100A8 and α_2-macroglobulin can directly excite DRG neurons by examining their ability to elicit calcium signals. Cultured DRG neurons rapidly responded to S100A8 (1 μg/mL) and to α_2-macroglobulin (100 μg/mL), as indicated by increased intracellular calcium ($(Ca)_i$) levels, suggesting that DRG neurons express

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receptors for these proteins (Fig 3A,B). Response to potassium (K) depolarization to activate voltage-dependent Ca-channels was used to confirm that each traced cell was a viable sensory neuron. Both S100A8 and α_2-macroglobulin induced responses in small-to-medium-diameter neurons, which is consistent with the size of nociceptors (Fig 3C,D).

Further, we divided responses into two categories, responses by capsaicin-sensitive neurons (cap; marker for transient receptor potential vanilloid-1 (TRPV1)-expressing nociceptive neurons) or by non-capsaicin-sensitive neurons. Both S100A8 and α_2-macroglobulin induced more responses in capsaicin-sensitive neurons compared to non-capsaicin-sensitive neurons (Table 1), although only α_2-macroglobulin induced significantly greater responses in capsaicin-sensitive neurons. LPS-induced $(Ca)_i$ increases were also primarily observed in capsaicin-sensitive neurons (Table 1).

In DRG neurons taken from *Tlr4*$^{-/-}$ mice, responses to S100A8 and α_2-macroglobulin were seen in a reduced number of neurons compared to wild-type neurons (Data combining two independent experiments: S100A8: 3/113 neurons (2.7%); α_2-macroglobulin 1/99 neurons (1.0%); compare to wild-type responses in column 5 of Table 1), again confirming that S100A8 and α_2-macroglobulin primarily signal through TLR4 on DRG neurons.

Calcium imaging in cultured DMM DRG neurons

We also tested whether DRG neurons taken from DMM mice 8 weeks after surgery could be directly excited by S100A8 or by α_2-macroglobulin. Increased numbers of neurons taken from DMM mice responded to S100A8 compared to naïve neurons (p=0.0004, Table 1). In contrast, similar numbers of neurons from DMM mice responded to α_2-macroglobulin compared to naïve neurons, perhaps reflecting differences in the way these two stimuli engage and signal through the TLR4 receptor. In both cases, responses were primarily in small-to-medium-diameter, capsaicin-sensitive neurons, similar to the naïve findings (Fig 3E,F; Table 1).

Ex vivo calcium imaging in intact DRG explants

In order to produce a more complete picture of the effects of TLR4 activation on sensory ganglia, *ex vivo* calcium imaging was performed using intact DRG explants from naïve Pirt-GCaMP3 mice. Pirt-GCaMP3 mice express a genetically-encoded fluorescent calcium indicator (GCaMP3) in almost all primary sensory neurons via the Pirt promoter, which is selective for DRG neurons and absent from other peripheral tissues, glia, and the central nervous system (31). In contrast to *in vitro* calcium imaging of cultured cells, this technique visualizes $(Ca)_i$ changes by DRG neurons *in situ*, providing additional evidence that TLR4 expression and activation by DRG neurons is not a cell-culture artifact. Using this technique, LPS induced rapid $(Ca)_i$ increases in DRG neurons (representative screen shot and trace shown in Fig 4A,B, Videos 1–4). Responses to LPS were seen in small-to-medium-diameter neurons (Fig 4C). All neurons responding to LPS also responded to capsaicin (Table 1, Fig 4D). Together, this is consistent with LPS inducing responses specifically in TRPV1-expressing nociceptors.

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Role of TLR4 in mechanical allodynia associated with experimental osteoarthritis

In order to assess whether TLR4 is necessary for the development of mechanical allodynia, DMM surgery was performed in *Tlr4*$^{-/-}$ mice and mechanical allodynia was monitored for 16 weeks. Mechanical allodynia developed by 2 weeks after surgery and was maintained through 16 weeks, similar to what was seen in wild-type mice (29) (Fig 5A,B, representative of two independent experiments). In addition, 16 weeks after DMM, *Tlr4*$^{-/-}$ mice (cartilage degeneration score = 19.1±3.6; osteophyte score = 1.9±0.3; n=9) showed similar joint damage as wild-type mice (cartilage degeneration score = 19.1±2.6; osteophyte score = 1.9±0.3; n=11).

DISCUSSION

In these experiments, we have demonstrated for the first time that DRG neurons can respond to representative molecules from two classes of DAMPs present in osteoarthritic joints: alarmins, represented by S100A8, and plasma proteins, represented by α_2-macroglobulin. TLR4 is the primary receptor through which S100A8 and α_2-macroglobulin excite DRG neurons, as shown by blockade using a small-molecule TLR4 inhibitor and *Tlr4*$^{-/-}$ mice. Our previous results demonstrated that MCP-1 and its receptor, CCR2, are upregulated by DRG neurons 8 weeks after DMM surgery (29). In addition, *Ccr2*$^{-/-}$ mice are protected from persistent pain in the DMM model. The ability of DAMPs such as S100A8 and α_2-macroglobulin to upregulate MCP-1 production in DRG neurons, and the ability of a TLR4 inhibitor to reduce MCP-1 released by DRG cells 8 weeks after DMM surgery, suggests that the TLR4 pathway may be an important upstream pathway for the promotion of pain. However, *Tlr4*$^{-/-}$ mice were not protected from developing mechanical allodynia associated with joint damage after DMM surgery, indicating that pathways other than TLR4 contribute to the development of OA-associated mechanical allodynia in this model.

Chondrocytes demonstrate increased *S100a8* mRNA expression during the first two weeks after DMM, while chondrocyte immunostaining in load-bearing areas of cartilage was lost during this time (41). This led the authors of that study to speculate that S100A8 is secreted into the extracellular space where it may act as a cytokine-like molecule during early OA (41). Extracellular homodimeric S100A8 and S100A9, but not the heterodimer S100A8/A9, have been shown to induce catabolic responses in human (42) and ovine (41) chondrocytes. S100A8 and S100A9 stimulation of chondrocyte monolayers from OA patients upregulated MCP-1 expression, and S100A9 stimulation was mediated through TLR4 signaling (S100A8 stimulation of MCP-1 was not tested for TLR4 dependency in that study) (42). S100A8 has also been associated with RAGE signaling under certain circumstances (43). Here, we demonstrate that S100A8-stimulated MCP-1 release by DRG neurons is primarily mediated by TLR4. Our findings suggest that, in addition to acting on chondrocytes, extracellular S100A8 may be able to act directly on joint nociceptors in early OA.

Synovial fluid levels of the endoproteinase inhibitor, α_2-macroglobulin, are elevated in both early- and late-stage OA (23, 25–27). α_2-Macroglobulin inhibits a wide range of proteases present in synovial fluid, including MMPs and ADAMTSs (27, 44, 45) and intra-articular administration of α_2-macroglobulin has been found to inhibit cartilage degradation in the rat anterior cruciate ligament transection model, consistent with its protease-inhibiting ability

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(46). Conversely, this protein may also promote inflammation by stimulating macrophages to produce cytokines (47) through TLR4 signaling (23). Here, α_2-macroglobulin stimulated DRG cells to produce MCP-1 through TLR4.

Using calcium imaging assays, we demonstrated that in both naïve and DMM cells $(Ca)_i$ increases in response to S100A8 and to α_2-macroglobulin occur in small-to-medium-diameter neurons, and that the majority of responses occur in neurons that express TRPV1 (capsaicin-sensitive neurons). Concordantly, *in situ* hybridization showed that mainly small-to-medium-diameter neurons express *Tlr4*, both in naive mice and in mice 8 weeks after DMM. Together, these findings suggest that it is the nociceptor population that is able to directly respond to these DAMPs. This is in line with recent studies showing that the majority of calcium responses by cultured neurons to another TLR4 agonist, HMGB1, were in capsaicin-sensitive neurons (16, 48). In addition, 6% of neurons responded to HMGB1, which again, is consistent with the percentage of naïve neurons shown here to respond to S100A8 (5%) and to α_2-macroglobulin (15%). Although the numbers of neurons responding to S100A8 and to α_2-macroglobulin were decreased in *Tlr4*$^{-/-}$ mice, a few neurons still responded, indicating that other pathways, including RAGE, may also facilitate responses to these DAMPs.

Through the use of genetically modified Pirt-GCaMP3 mice, it was possible to visualize $(Ca)_i$ increases in DRG neurons within seconds of application of LPS, a TLR4 ligand, while neurons were still contained in intact DRGs. Intact DRG are more relevant to the *in vivo* situation, providing evidence that these functional responses to TLR4 ligands are not an artifact of cell isolation and culture and that neurons are able to directly respond to these stimuli. These results support previous work in cell culture showing TLR4-mediated LPS-induced $(Ca)_i$ increases in dissociated DRG neurons (16, 20, 49, 50). Again, the fact that responses were seen primarily in capsaicin-sensitive neurons suggests that LPS-responsive neurons are a subpopulation of TRPV1-expressing nociceptors, which is consistent with recent reports studying calcium fluxes induced by LPS in cell culture (16, 20). Although we have not identified the source of the $(Ca)_i$ signal in cell culture or whole ganglion experiments, published observations that activation of TLR4 can depolarize populations of DRG neurons indicate that a rise in $(Ca)_i$ represents direct excitation of neurons by this mechanism (20).

Collectively, our findings suggest a role for TLR4 in mediating nociceptor sensitization by tissue damage products such as S100A8 and α_2-macroglobulin. Although our experiments and other reports clearly establish that the cell bodies of DRG neurons express TLR4 (16, 20, 21), their presence and location on joint termini needs to be investigated.

Based on our *in vitro* findings, we tested whether *Tlr4*$^{-/-}$ mice developed mechanical allodynia, an evoked measure of sensitization, after DMM. We found that *Tlr4*$^{-/-}$ mice developed secondary mechanical allodynia in the presence of experimental OA after DMM and this was maintained for 16 weeks, just like in wild-type mice (29). This may reflect the great complexity of the OA joint milieu, where many tissue damage products are generated in the course of progressive disease. In addition to the TLR4 pathway, OA DAMPs can signal through TLR2 and RAGE (11), and other pro-algesic cytokines and chemokines

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signal through additional pathways, which often involve the transcription factor NFκB (9, 51, 52). Our results are consistent with the idea that it is unlikely that OA-associated pain would arise from damage products signaling through a single pathway. Further, secondary mechanical allodynia in the hindpaw also reflects central sensitization (53). Although we focused on the role of TLR4 in the peripheral nervous system, there is evidence that this receptor can play a role in regulation of neuro-immune processes in the central nervous system. In a nerve transection model (54) and an inflammatory arthritis model (55), mechanical allodynia and spinal microglial activation were reduced in *Tlr4*$^{-/-}$ mice. Likewise, intrathecal injection of a TLR4 antagonist reduced mechanical allodynia associated with several acute inflammatory models (56). Here, we did not observe a reduction in mechanical allodynia in *Tlr4*$^{-/-}$ mice in the DMM model, which may be due to differences in both peripheral and central levels of inflammation in the DMM model compared to the models above.

It is interesting to note that *Tlr4*$^{-/-}$ mice developed similar joint damage as wild-type mice by 16 weeks after surgery. This is consistent with previous reports that state that *S100a9*$^{-/-}$ mice (which are also functional *S100a8*$^{-/-}$ mice (57)) were not protected from joint damage after DMM (24, 58). In another murine surgical OA model, the partial meniscectomy model, *Tlr4*$^{-/-}$ mice also developed similar joint destruction compared to wild-type mice (59). In contrast, in collagenase-induced arthritis, which has more synovitis than the DMM model, synovial expression of S100A8 and S100A9 has been shown to be upregulated for prolonged periods (24, 58), and *S100a9*$^{-/-}$ mice were protected from synovitis, cartilage degradation, and osteophyte formation in this model (24, 58). Together, this suggests that in a joint environment with low-level inflammation, other pathways besides the TLR4 pathway are likely driving joint damage. *Tlr4*$^{-/-}$ mice have yet to be tested in the collagenase-induced model.

In summary, our experiments suggest a role for TLR4 signaling in mediating the excitation of DRG neurons by the DAMPs, S100A8 and α_2-macroglobulin, which may contribute to nociceptor sensitization in OA. Further research is needed to understand the relative importance of this pathway in comparison to other PRR pathways in the development of mechanical allodynia and other OA-associated pain behaviors. In addition, the complex and diverse actions of DAMPs on a variety of cell types indicate that more research is needed to understand how intervening in these PRR pathways at different stages of disease and in varying inflammatory environments augments both pain and joint damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Rachel Miller was supported by an Arthritis Foundation Postdoctoral Fellowship and by the US National Institutes of Health/National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) (F32AR062927). Anne-Marie Malfait (R01AR064251 and R01AR060364) and Richard Miller (R01AR064251) were supported by NIAMS.

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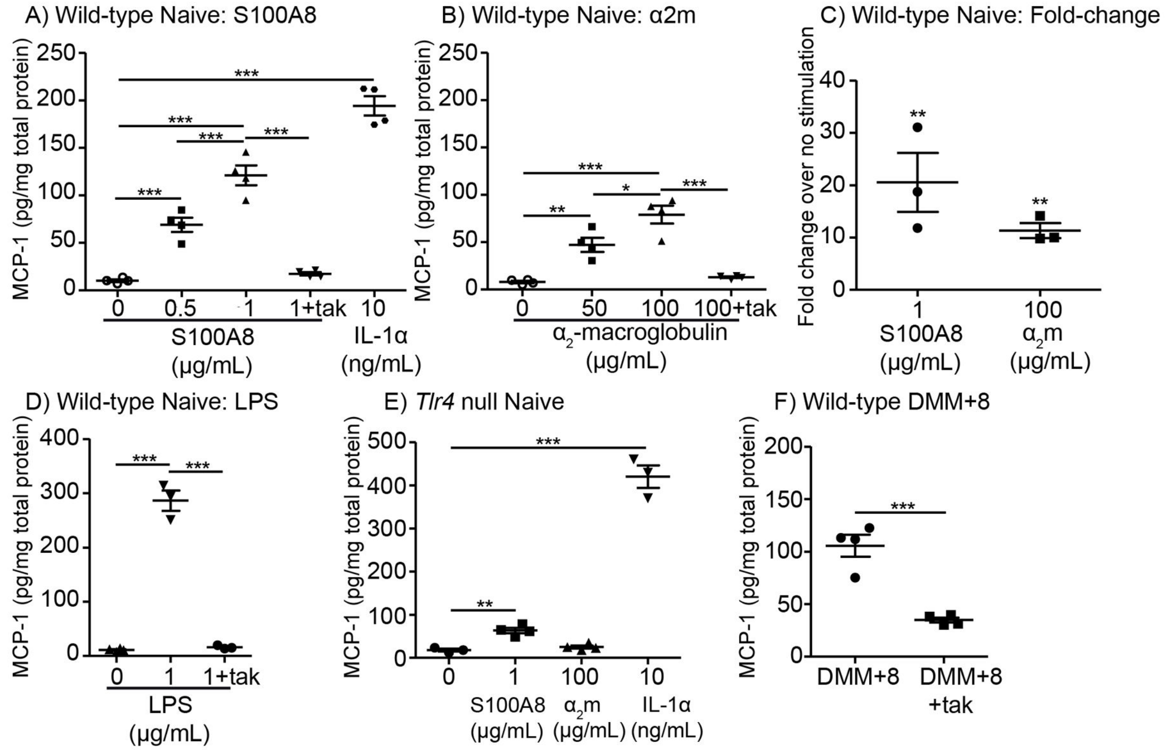

Figure 1.

Candidate osteoarthritis DAMPs induce MCP-1 production by DRG cells primarily through TLR4. **A)** Wild-type DRG cells were stimulated with 0, 0.5, or 1 µg/mL S100A8 ± the small molecule TLR4 inhibitor Tak242 (Tak, 1 µM) or with 10 ng/mL IL-1α. Representative of three independent experiments. **B)** Wild-type DRG cells were stimulated with 0, 50, or 100 µg/mL α_2-macroglobulin, ± Tak242 (Tak, 1 µM). Representative of three independent experiments. **C)** Fold-change plot combines data from the three independent experiments for S100A8 and α_2-macroglobulin (α_2m); **p<0.01 vs no stimulation. **D)** Wild-type DRG cells were stimulated with 1 µg/mL LPS ± 1 µM Tak242. Representative of two independent experiments. **E)** *Tlr4*$^{-/-}$ DRG cells were stimulated with 1 µg/mL S100A8, 100 µg/mL α_2-macroglobulin, or 10 ng/mL IL-1α. Representative of three independent experiments for S100A8 and two experiments for α_2-macroglobulin. **F)** DRG cells taken from mice 8 weeks after surgery were incubated ± 1 µM Tak242. Representative of three independent experiments. *p<0.05, **p<0.01, ***p<0.001; mean±SEM.

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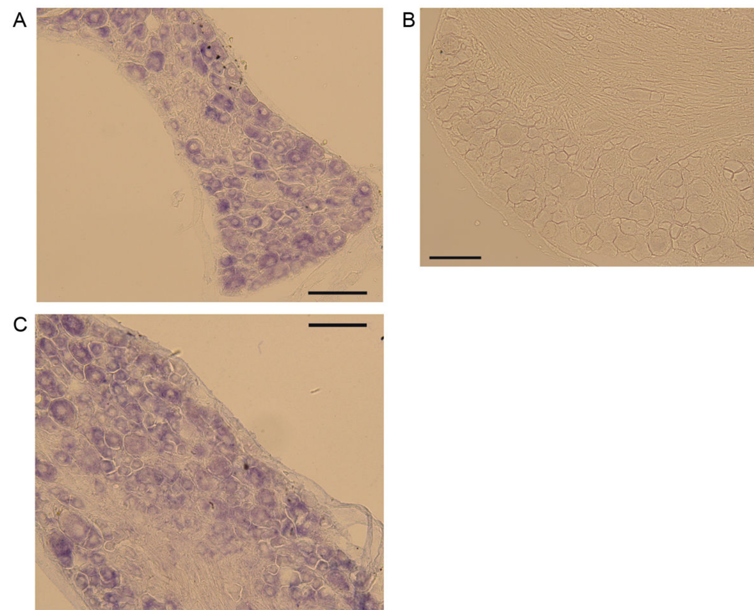

Figure 2.

A) Representative image of *in situ* hybridization using an anti-sense probe for *Tlr4* in DRG sections taken from wild-type naïve mice, n=2. B) Sense probe control. **C)** Representative image of *in situ* hybridization using an anti-sense probe for *Tlr4* in DRG sections taken from wild-type DMM mice 8 weeks after surgery, n=2. Magnification 20x. Scale bars, 100 μm.

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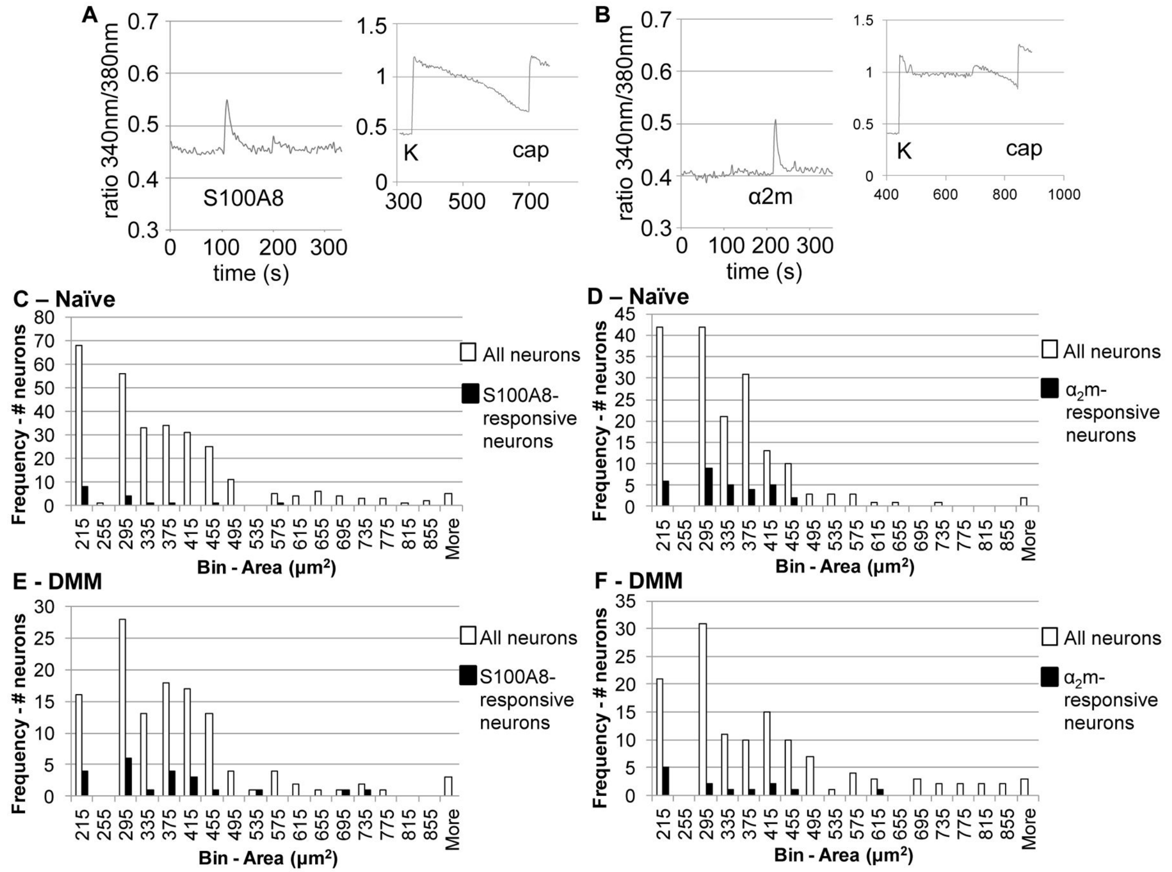

Figure 3.

Candidate osteoarthritis DAMPs induce intracellular calcium $(Ca)_i$ increases in cultured primary DRG neurons. Representative traces showing $(Ca)_i$ increases in a wild-type DRG neuron in response to **A)** S100A8 and to **B)** α_2-macroglobulin (α2m). Insets show response to 50 mM potassium (K), which was used to verify neuronal identity and viability, and response to 10 μM capsaicin (cap), which was used to identify TRPV1-expressing nociceptors. Histograms showing areas of naïve neurons (in μm^2) responding to **C)** S100A8 and to **D)** α_2-macroglobulin relative to histograms showing the areas of all imaged neurons, both responsive and non-responsive. Histograms showing areas of neurons from DMM mice 8 weeks after surgery (in μm^2) responding to **E)** S100A8 and to **F)** α_2-macroglobulin relative to histograms showing the areas of all imaged neurons, both responsive and non-responsive.

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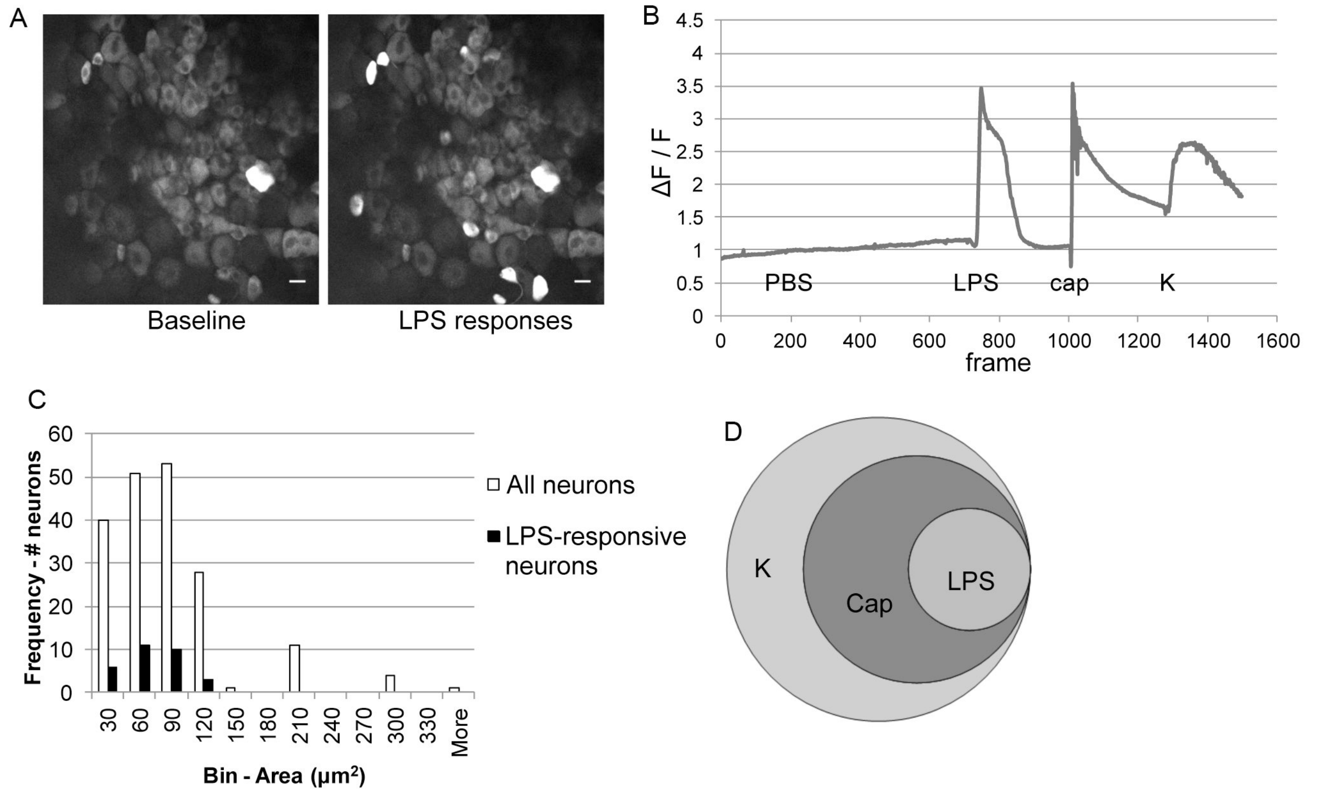

Figure 4.

The TLR4 agonist LPS induces intracellular calcium $(Ca)_i$ increases in neurons contained within intact DRG. Representative **A)** images (scale bar = 10 μm) and **B)** traces of Pirt-GCaMP3 DRG neuron responses to LPS (50 μg/mL). The trace plots the relative change in fluorescence (F/F), indicative of $(Ca)_i$ increases. Response to 10 μM capsaicin (cap) was used to identify TRPV1-expressing nociceptors, and response to 50 mM potassium (K) was used to verify neuronal identity and viability. Representative of two independent experiments. **C)** Histogram showing areas of neurons (μm^2) responding to LPS compared to histogram showing size of all imaged neurons. **D)** Venn diagram showing the relationship among LPS-, capsaicin- (cap), and potassium- (K) responding neurons.

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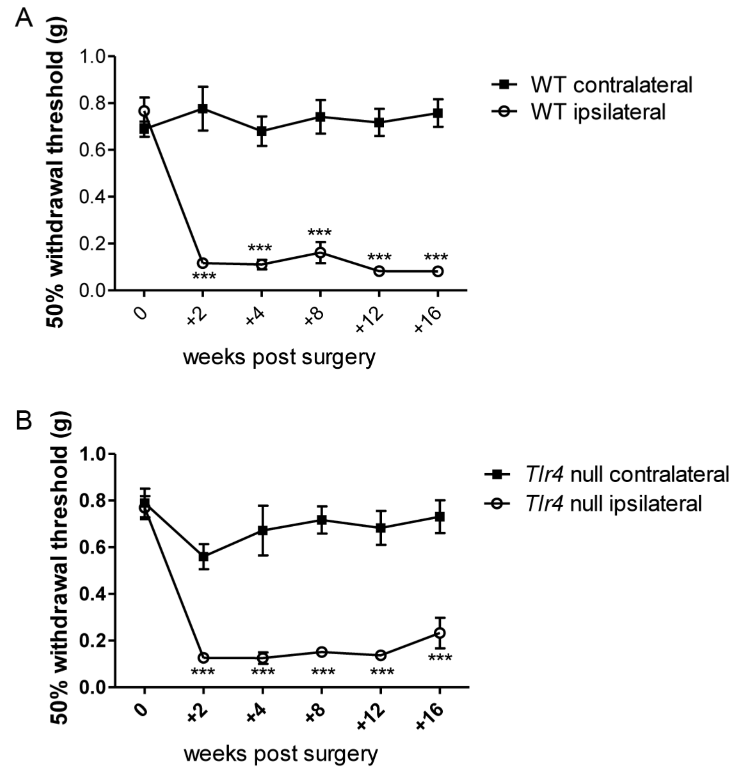

Figure 5.
Wild-type (WT) (**A**) and *Tlr4*$^{-/-}$ mice (**B**) develop ipsilateral mechanical allodynia (n=6–12 mice/time point) through 16 weeks post DMM surgery. ***p<0.001 vs time 0. mean±SEM. Representative of two independent experiments.

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Table 1

Calcium imaging of DRG neurons

Type of Imaging	Protein tested	(# neuronal responses)/ (total # capsaicin-sensitive neurons), (%)	(# neuronal responses)/ (total # non-capsaicin-sensitive neurons), (%)	(# neuronal responses)/ (total # neurons), (%)	p-value (capsaicin-sensitive vs non-capsaicin-sensitive)	number of independent experiments
Naïve *in vitro*	S100A8 (1 μg/mL)	10/127 (8%)	4/134 (3%)	14/261 (5%)	0.1011	3
DMM+8 *in vitro*	S100A8 (1 μg/mL)	18/82 (22%)	5/52 (10%)	23/134 (17%)	0.0984	3
Naïve *in vitro*	α_2-macroglobulin (100 μg/mL)	23/98 (23%)	10/120 (8%)	33/218 (15%)	**0.0023**	3
DMM+8 *in vitro*	α_2-macroglobulin (100 μg/mL)	15/76 (20%)	7/81 (9%)	22/157 (14%)	0.0646	3
Naïve *in vitro*	LPS (1 μg/mL)	5/52 (10%)	1/54 (2%)	6/106 (6%)	0.11	1
Naïve *ex vivo*	LPS (50 μg/mL)	30/104 (29%)	0/82 (0%)	30/186 (16%)	**<0.0001**	2