TLR2 Signaling in Chondrocytes Drives Calcium Pyrophosphate Dihydrate and Monosodium Urate Crystal-Induced Nitric Oxide Generation

Ru Liu-Bryan, Kenneth Pritzker, Gary S. Firestein and Robert Terkeltaub

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The deposition of microcrystalline monosodium urate (MSU) \(^3\) is associated with variable forms of articular inflammation, including characteristically self-limited episodes of neutrophilic inflammatory synovitis (acute gout) \((1, 2)\). A remarkably similar form of acute inflammatory arthritis (psuedogout) \((3, 4)\) is associated with deposition of microcrystals of calcium pyrophosphate dihydrate (CPPD) in chondrocalcinosis and osteoarthritis (OA) articular cartilages \((3, 4)\). Both CPPD and MSU crystals stimulate acute inflammation in large part via their capacity to directly activate resident articular connective tissue cells as well as leukocytes \((1–5)\). Furthermore, CPPD crystals originating from deposits formed by chondrocytes in their pericellular matrix and MSU crystals deposited in synovial and cartilaginous tophi can directly activate chondrocytes and synovial lining cells, thereby promoting cartilage degradation via induction of IL-1, TNF-\(\alpha\), matrix metalloproteinases (MMPs), and certain other mediators \((1–5)\).

MSU crystals stimulate articular chondrocytes in part through activation of proline-rich tyrosine kinase \((\text{Pyk}2)\) and Src tyrosine kinase, and subsequent activation of p38 MAPK, thereby promoting the expression of MMP-3 and the NF-\(\kappa\)B-regulated gene-inducible NO synthase as well as robust generation of NO \((6)\). In chondrocytes, increased NO production stimulates redox stress, promotes chondrocyte apoptosis and expression and activation of MMPs, depresses matrix synthesis, and inhibits responsiveness to growth and anabolic factor insulin-like growth factor I \((7)\). Up-regulated NO production also induces transglutaminase activity, which modulates chondrocyte differentiation \((8)\) and matrix calcification \((9)\) in chondrocytes.

The objective of this study was to examine how MSU and CPPD crystals use chondrocyte plasma membrane proteins for signal transduction that culminates in NO release. In this context, macrophage NO release in response to proinflammatory components of several pathogenic microorganisms is mediated by signaling of the TLR family of type I transmembrane receptors \((10)\). TLRs bear extracellular leucine-rich repeat motifs that recognize pathogen-associated molecular patterns \((11)\). In TLRs, the cytoplasmic Toll/IL-1R domain transduces ligand-induced activation of signaling pathways leading to activation of the transcription factor NF-\(\kappa\)B \((11)\), which promotes the expression of inducible NO synthase and a variety of inflammatory cytokine and MMP genes \((12–14)\). This downstream signaling pathway involves myeloid differentiation factor 88 \((\text{MyD}88)\), IL-1R-associated kinase \((\text{IRAK})\), and TNF receptor-associated factor 6 \((\text{TRAF}6)\), which synergize to activate I\(\kappa\)B kinases \((\text{IkKs})\). Activated IkKs phosphorylate the NF-\(\kappa\)B inhibitor I\(\kappa\)B, resulting in I\(\kappa\)B degradation and the release and translocation of active NF-\(\kappa\)B to the nucleus \((11)\). Another pathway involving the Rho family GTPase Rac1, the ubiquitous lipid kinase

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*Abbreviations used in this paper: MSU, monosodium urate; CPPD, calcium pyrophosphate dihydrate; IKK, I\(\kappa\)B kinase; IRAK, IL-1R-associated kinase; MMP, matrix metalloproteinase; OA, osteoarthritis; PBD, protein binding domain; PDTC, pyridil-dithiocarbamate; Pyk2, proline-rich tyrosine kinase; Tollip, Toll-interacting protein; TRAF6, TNF receptor-associated factor 6; HEMA, 2-hydroxyethylmethacrylate.
P3K, and the downstream protein kinase Akt also transduces TLR2 signaling to NF-κB activation (15). Interestingly, both P3K and Akt are involved in MSU and CPPD crystal-induced stimulation of neutrophils (16, 17).

Although many TLR ligands are microbial products (11), nonbacterial ligands for certain TLRs have been identified, such as heat shock protein 70, fatty acids, etc. (18, 19). In addition, MSU crystals appear to directly engage certain integrins and the FcR CD16 in cells of hemopoietic origin (20, 21). Recently, the expression of certain TLRs was discovered in normal and rheumatoid arthritis synovial fibroblasts (22, 23), indicative of potential innate immune responses driven by mesenchymally derived cells in arthritis. Hence, we hypothesized that normal articular chondrocytes also normally express TLRs and that MSU and CPPD crystals use TLRs to stimulate chondrocytes. Below, we demonstrate constitutive expression by articular chondrocytes of TLR2, and we observe that TLR2 signaling centrally mediates CPPD and MSU crystal-induced NO release in chondrocytes. Our findings identify an innate immune response pathway of the articular cartilage chondrocyte that may contribute to inflammatory and degenerative tissue reactions in the joint in both gout and pseudogout.

Materials and Methods

Reagents

All chemical reagents were obtained from Sigma-Aldrich, unless otherwise indicated. Monoclonal CPPD crystals were prepared as previously described (24) and treated for 2 h at 200°C after crystallization, followed by suspension at 25 mg/ml in sterile, endotoxin-free 10 mM sodium PBS, pH 7.4. Triclinic MSU crystals were prepared under pyrogen-free conditions using uric acid treated for 2 h at 200°C before crystallization (25) and were suspended at 25 mg/ml in PBS. The suspended MSU and CPPD crystals were verified to be free of detectable LPS contamination (<0.025 endotoxin unit/ml) by the Limulus amoebocyte lysate assay (BioWhittaker).

The pharmacological inhibitor to PI3K LY294002, and pyrrolidine dithiocarbamate (PDTC) were purchased from Calbiochem. Phosphospecific Akt (Ser73), p65 NF-κB (Ser536), and IκBα (Ser32) polyclonal Abs and total Akt and p65 NF-κB polyclonal Abs were purchased from Cell Signaling Technology. mAb to IKK1, IKK2, Rac1, and MyD88; mAb to total Akt and p65 NF-κB are from Upstate Biotechnology. Polyclonal Abs to IKK1, IKK2, Rac1, and MyD88; mAb to TLR2; and HRP-conjugated goat anti-mouse IgG, and anti-rabbit IgG were obtained from Santa Cruz Biotechnology. Functional blocking mAb to TLR2; and HRP-conjugated goat anti-rabbit IgG and anti-mouse IgG were obtained from eBioscience.

Cell culture and transfection and assay of NO production

Normal human knee articular chondrocytes and adult bovine knee articular chondrocytes (Animal Technologies) were isolated as described previously (6). For immunoprecipitation assays, 250 μg of total RNA were reverse transcribed as previously described (6). PCRs were performed using for RT-PCR are shown in Table I. Aliquots of 600 ng of total RNA were reverse transcribed as previously described (6), where first passage chondrocytes in DMEM supplemented with 1% FCS, streptomycin, and penicillin, as described above, were stimulated with MSU and CPPD crystals. For transfection of bovine chondrocytes, aliquots of 4 × 10⁵ primary cells were plated in 60-mm dishes and allowed to adhere for 18 h, after which cells were transfected using FuGene 6 and hyaluronidase, as previously described (26). Transfection efficiency, evaluated in control samples via β-galactosidase transfection and staining (26), was >25%. Twenty-four hours after transfection, medium was replaced with fresh complete DMEM high glucose medium containing 10% FCS, and the cells were allowed to recover for another 24 h. NO production was measured as the concentration of nitrates in conditioned medium by the Griess reaction (27) using NaNO₂ as standard.

Where a higher transfection efficiency was needed for plasmids, chondrocytes were transfected using the Nucleofection system (Amaxa), following the manufacturer’s protocol optimized for chondrocytes. The Amaxa Nucleofection system yielded a transfection efficiency of >70%. Adenoviral gene transfer for expression of wild-type and dominant negative IKK1 and IKK2 and control adenoaviral GFP (provided by Dr. B. Benett, Celgene, Signal Research Division) was performed in chondrocytes, with transfection efficiency >80%.

We cultured the human embryonic kidney cell line HEK293 (American Type Culture Collection) in DMEM with 10% FCS. For transfection, aliquots of 3 × 10⁶ HEK293 cells were plated in each well of six-well plates overnight. Cells were then cotransfected with plasmids including the NF-κB binding site-containing reporter construct ELAM-1-luciferase, pRLTK (used as an internal control), and TLR2 using the transfection reagent SuperFect (Invitrogen Life Technologies) according to the manufacturer’s instructions, which yielded a transfection efficiency of >80%.

Immunohistochemistry

Frozen sections from human knee cartilage sections (5 μm) were obtained from normal joints at autopsy or from osteoarthritic joints at the time of total knee arthroplasty, prepared as previously described (28). Sections were fixed with ice-cold acetone, washed with PBS, and treated with 0.1% H₂O₂ in PBS for 10 min at room temperature. After serial washes in PBS, the sections were blocked with PBS containing 10% goat serum and incubated with TLR2 Ab at 4°C overnight. Washed sections were then incubated for 1 h at room temperature with biotinylated goat anti-mouse IgG, followed by a 1-h incubation with peroxidase-conjugated avidin. Peroxidase activity was detected using the Sigma Fast 3,3'-diaminobenzidine staining kit, according to the manufacturer’s instructions.

Assays for TLR family member mRNA expression

RT-PCR analyses for TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR9, CD14, and the housekeeping gene L30 were performed on RNA isolated from normal and rheumatoid arthritis synovial fibroblasts. Using uric acid treated for 2 h at 200°C before crystallization (25) and were suspended at 25 mg/ml in PBS. The suspended MSU and CPPD crystals were verified to be free of detectable LPS contamination (<0.025 endotoxin unit/ml) by the Limulus amoebocyte lysate assay (BioWhittaker).

The pharmacological inhibitor to PI3K LY294002, and pyrrolidine dithiocarbamate (PDTC) were purchased from Calbiochem. Phosphospecific Akt (Ser73), p65 NF-κB (Ser536), and IκBα (Ser32) polyclonal Abs and total Akt and p65 NF-κB polyclonal Abs were purchased from Cell Signaling Technology. mAb to IKK1, IKK2, Rac1, and MyD88; mAb to TLR2; and HRP-conjugated goat anti-rabbit IgG and anti-mouse IgG were obtained from Santa Cruz Biotechnology. Functional blocking mAb to TLR2 were transfected using the Nucleofection system (Amaxa), following the manufacturer’s protocol optimized for chondrocytes. The Amaxa Nucleofection system yielded a transfection efficiency of >70%. Adenoviral gene transfer for expression of wild-type and dominant negative IKK1 and IKK2 and control adenoaviral GFP (provided by Dr. B. Benett, Celgene, Signal Research Division) was performed in chondrocytes, with transfection efficiency >80%.

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Assays for TLR family member mRNA expression

RT-PCR analyses for TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR9, CD14, and the housekeeping gene L30 were performed on RNA isolated from chondrocytes using TRizol (Invitrogen Life Technologies). Primers used for RT-PCR are shown in Table I. Aliquots of 600 ng of total RNA were reverse transcribed as previously described (6). PCRs were performed for 30 cycles; 95°C for 5 min, 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 72°C for 5 min.

Preparation of cell lysates, immunoprecipitation, SDS-PAGE/ Western blot analyses, and in vitro kinase assays

Preparation of cell lysates and SDS-PAGE/Western blot analyses were performed as previously described in detail, using HRP-conjugated secondary Ab (Santa Cruz Biotechnology) and detection by the ECL system (Pierce) (6). For immunoprecipitation assays, 250 μg of protein of cell lysates was used as previously described (6). For IKK1 or IKK2 in vitro kinase assays, the cell lysates (250 μg) were first precipitated with IKK1

Table I. Primers for RT-PCR analyses

| TLR1  | TCAAGGTGTTTCGCAAATTTACCACG | GTCCTCTTTTTCGCCATAGG | 283 |
| TLR2  | GAGCCTCATCCTGCTGGCAAGTGG | CGAGTGGATCTTGGTTCCTC | 254 |
| TLR3  | CCTCGACTTCTCCTCAACACACG | AAGGAGGATGAACTGGCAAGGT | 392 |
| TLR4  | CACCTGATGCTTCTTGCTG | TGCTCGAAGACCTGCAAGG | 314 |
| TLR6  | AAGAGAATAATGCAACACTT | TAAAGGTGGACCTTGGT | 336 |
| TLR9  | CACTGGACTCATTCAAACCGCC | AAAGGGCTGCTGCTTGTAGCTT | 476 |
| L30   | GAAAGTACGCTGCTGGATTTCAACAACACT | ATTCGAATACCCTGGTCATTTGATAGCCAG | 235 |
or IKK2 Ab. IKK1 or IKK2 activity was then determined using GST-IκBα as a substrate as described previously (29). For PI3K assay, aliquots of 250 μg of cell lysate protein were precipitated with p85α Ab using protein A/G agarose (Santa Cruz Biotechnology). PI3K activity was examined using the method described previously (30).

Rac1 activation assay

Cell lysates (250 μg) were subjected to affinity precipitation with GST-protein binding domain (GST-PBD; 10 μg) in binding buffer containing 25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 30 mM MgCl2, 40 mM NaCl, and 1% Nonidet P-40, proteins bound to GST-PBD beads were separated by SDS-PAGE and immunoblotted for bound Rac1.

Statistical analysis

Numerical data were uniformly expressed as the mean ± SD. Statistical analyses were performed using two-tailed Student’s t test.

Results

Constitutive expression of TLR2 in human articular chondrocytes and knee cartilage in situ

We consistently detected TLR2 mRNA expression in different donors of normal human primary articular chondrocytes by RT-PCR (Fig. 1A). However, TLR1, TLR3, TLR4, TLR6, and TLR9 mRNA expression were consistently below limits of detection in human primary chondrocytes by RT-PCR analysis under these conditions (Fig. 1A). Immunohistochemical analysis demonstrated constitutive TLR2 expression in normal human knee articular cartilages (Fig. 1B).

TLR2 mediates induction of NO production by MSU and CPPD crystals in chondrocytes

We confirmed that pyrogen-free MSU crystals induced NO release by normal bovine knee articular chondrocytes (6) and observed a similar response to pyrogen-free CPPD crystals (Fig. 2A). Next, we transfected bovine articular chondrocytes with Tollip, an adaptor protein known to inhibit both TLR2- and TLR4-mediated signaling (31, 32). Chondrocytes transfected with Tollip and then stimulated with either MSU or CPPD crystals for 24 h demonstrated marked inhibition of NO production in response to both MSU and CPPD crystals (Fig. 2A). TLR2 functional blocking Ab also suppressed MSU and CPPD crystal-induced NO production (Fig. 2B). Next, using a gain-of-function approach, we observed that overexpression of TLR2 (but not TLR1, TLR3, or TLR9) via transient transfection in bovine articular chondrocytes was associated with a significant increase in NO production in response to both MSU and CPPD crystals (Fig. 2C).

Chondrocyte MyD88, IRAK-1, and TRAF6 mediate NF-κB activation and induction of NO by MSU and CPPD crystals

Chemiluminescent assays of bovine chondrocytes stimulated with MSU crystals demonstrated rapidly up-regulated NF-κB p65 subunit activity, which reached a peak by 60 min (Fig. 3A). Furthermore, pretreatment of bovine chondrocytes with the NF-κB inhibitor PDTC markedly inhibited both MSU and CPPD crystal-induced NO production (Fig. 3B). MyD88, IRAK, and TRAF6 have been observed to mediate TLR2 signaling that promotes NF-κB activation (11). We observed that dominant negative mutants of MyD88, IRAK1, and TRAF6 inhibited NF-κB transcriptional activation and NO generation induced by both crystal types (Fig. 3, C and D).

IKK2 mediates induction of NO production by MSU and CPPD crystals in chondrocytes

IKKs function downstream of TRAF6 in mediating TLR2 signaling to activate NF-κB-dependent genes (11). Via in vitro kinase assays, we demonstrated that both IKK1 and IKK2 were activated by MSU crystals (Fig. 4A), with similar results seen for IKK activation by CPPD crystals (not shown). To assess whether IKK1 or IKK2 mediates NF-κB activation by MSU crystals, bovine chondrocytes were infected with recombinant adenovirus of kinase-dead mutants of IKK1 and IKK2 before stimulation with the...
NF-κB crystals (0.5 mg/ml) at the times indicated, and cell lysates were prepared as described in Materials and Methods. Nonradioactive chemiluminescent NF-κB p65 transcription factor assays determined the activity of p65 subunit of NF-κB, as described in Materials and Methods. B, Bovine primary chondrocytes cultured under nonadherent conditions on poly-HEMA-coated plates in medium containing 1% FCS were treated with MSU crystals (0.5 mg/ml) for 24 h with and without the pretreatment with the NF-κB inhibitor PDTC (100 μM). NO release was determined in the conditioned medium as described above. C, HEK293 cells were cotransfected with TLR2, pELAM-Luc, pRLTK (internal control), and dominant negative mutants of MyD88, IRAK1, TRAF6, and vector control (Vec) using FuGene 6 and hyaluronidase as described above. Two days after transfection, the cells were treated with MSU or CPPD crystals (0.5 mg/ml) for 6 h, and cell lysate luciferase activity was determined, as described in Materials and Methods. The cells were then stimulated with MSU or CPPD crystals (0.5 mg/ml) for 6 h, and cell lysate luciferase activity was determined, as described in Materials and Methods. D, Bovine chondrocytes were transiently transfected with dominant negative mutants of MyD88, IRAK1, TRAF6, and vector control (Vec) using FuGene 6 and hyaluronidase as described above. Two days after transfection, the cells were treated with MSU or CPPD crystals using the same conditions as described in Fig. 3B. NO release was determined in the conditioned medium as described above. All data are representative of three independent experiments with three different donors. *, p < 0.05.

FIGURE 3. MyD88, IRAK1, and TRAF6 signaling mediates NF-κB activation and NO production by MSU and CPPD crystals in chondrocytes. A, Bovine knee articular chondrocytes cultured under nonadherent conditions on poly-HEMA-coated plates in medium containing 1% FCS were treated with MSU crystals (0.5 mg/ml) for 24 h with and without the pretreatment with the NF-κB inhibitor PDTC (100 μM). NO release was determined in the conditioned medium as described above. C, HEK293 cells were cotransfected with TLR2, pELAM-Luc, pRLTK (internal control), and dominant negative mutants of MyD88, IRAK1, TRAF6, and vector control (Vec) using FuGene 6 and hyaluronidase as described above. Two days after transfection, the cells were treated with MSU or CPPD crystals (0.5 mg/ml) for 6 h, and cell lysate luciferase activity was determined, as described in Materials and Methods. The cells were then stimulated with MSU or CPPD crystals (0.5 mg/ml) for 6 h, and cell lysate luciferase activity was determined, as described in Materials and Methods. D, Bovine chondrocytes were transiently transfected with dominant negative mutants of MyD88, IRAK1, TRAF6, and vector control (Vec) using FuGene 6 and hyaluronidase as described above. Two days after transfection, the cells were treated with MSU or CPPD crystals using the same conditions as described in Fig. 3B. NO release was determined in the conditioned medium as described above. All data are representative of three independent experiments with three different donors. *, p < 0.05.

Rapid signaling complex assembly of TLR2, MyD88, Rac1, and PI3K in chondrocytes stimulated with MSU crystals

Upon stimulation with TLR2 ligands, MyD88 is recruited to the cytosolic domain of TLR2 (11), and PI3K subunit p85α, Rac1, and the cytosolic domain of TLR2 form a stimulus-dependent signaling complex (15). Immunoprecipitation studies (Fig. 5) demonstrated rapid signaling complex assembly that involved TLR2, MyD88, IRAK1, and TRAF6 in chondrocytes stimulated with MSU crystals (Fig. 5). Transient association of p85α with MyD88 was detected and reached a maximum at 15–30 min; a similar pattern of association of p85α with TLR2 was also observed, whereas the association of p85α and Rac1 was rapid and transient within 5 min (Fig. 5).

PI3K and Akt mediate induction of NO production by MSU and CPPD crystals in chondrocytes

MSU and CPPD crystals both triggered rapid PI3K activation in chondrocytes (Fig. 6A). Inhibition of PI3K using the selective pharmacological inhibitor LY294002 or by transient transfection of chondrocytes with dominant negative mutant of PI3K subunit p85α (Δp85α) prevented NO production in response to MSU and CPPD crystals (Fig. 6B). MSU and CPPD crystals also induced rapid phosphorylation of Akt in chondrocytes, which was demonstrated to be PI3K-dependent via inhibition using LY294002 (Fig. 7A). Inhibition of Akt activity by transfection with a kinase-dead mutant of Akt (Akt-KM) also attenuated the induction of NO production by MSU and CPPD crystals in chondrocytes (Fig. 7B).

Rac1 critically mediates induction of NO production by MSU and CPPD crystals in chondrocytes

Rac1 was activated in chondrocytes stimulated with MSU or CPPD crystals (Fig. 8A). Rac1 not only can regulate the activation of PI3K (35), but also can be activated by PI3K (36). We observed that activation of Rac1 induced by MSU crystals was not inhibited by LY29004 (Fig. 8B), consistent with PI3K being a downstream target of Rac1 in MSU crystal-stimulated chondrocytes. Transfection of the constitutively activated Rac1V12 mutant slightly enhanced both MSU and CPPD crystal-induced NO production, whereas transfection with the dominant negative Rac1N17 significantly attenuated induction of NO production expression by both crystals (Fig. 8C). Furthermore, Rac1N17 and PI3K Δp85α, inhibited MSU and CPPD crystal-induced phosphorylation of NF-κB p65 (Fig. 8D), consistent with a joint role of Rac1 and PI3K in crystal-induced NF-κB activation.

Collectively, the results obtained in this study were consistent with a model of crystal-induced chondrocyte activation via TLR2-mediated parallel signaling chains in which NF-κB activation was a critical downstream event in NO production (Fig. 9).

Discussion

In this study we demonstrated that human knee articular cartilage chondrocytes constitutively express TLR2. Moreover, we observed that TLR2 signaling is centrally used to stimulate chondrocytes by microcrystals of CPPD and MSU. This conclusion were...
phagocytes were lessened by blocking Abs specific for the leukocyte MSU crystal-induced secretory and respiratory burst responses in diates crystal-induced secretion of platelet granule contents (20).

FIGURE 4. Activation of NF-κB via IKK2 plays a major role in the induction of NO release in chondrocytes in response to MSU and CPPD crystals. A, Bovine primary chondrocytes cultured on poly-HEMA-coated plates were treated with MSU crystals (0.5 mg/ml) at the times indicated, and cell lysates were prepared. An in vitro kinase assay was conducted to examine the activities of IKK1 and IKK2 using GST-IκBα as substrate, as described in Materials and Methods. B, Bovine chondrocytes in monolayer culture were infected with recombinant adenovirus of mutants of IKK1, IKK2, and their control virus (GFP) for 16 h before being transferred to nonadherent culture conditions and stimulated with MSU crystals (0.5 mg/ml) for 1 h, as described above. Phosphorylation of NF-κB was determined by SDS-PAGE/Western blot analysis from cell lysates using Abs to phospho-specific p65 (Ser536) and total p65 NF-κB. C, Bovine chondrocytes were adenovirally infected under the same conditions as described in B, then stimulated with the crystals for 24 h, at which time NO release was determined as described above. The data are representative of three independent experiments with three different donors. *p < 0.05.

FIGURE 5. MSU crystals stimulate rapid signaling complex assembly involving TLR2, MyD88, Rac1, and PI3K in chondrocytes. Bovine chondrocytes cultured under nonadherent conditions in medium containing 1% FCS, as described above, were treated with MSU crystals (0.5 mg/ml) at the times indicated, and cell lysates were analyzed by coimmunoprecipitation assays using Abs specific for TLR2, Rac1, and MyD88. The immunoprecipitates were then separated by SDS-PAGE, and Western blot analyses using Ab to p85α of PI3K followed, as described in Materials and Methods. The data are representative of two independent experiments with two different donors.

supported by complementary TLR2 signaling loss-of-function approaches via Tollip transfection and treatment with TLR2-blocking Ab, and TLR2 gain-of-function via comparison of several transfected TLRS.

TLR2 can form heterodimers with TLR1 and TLR6 (11), thereby conferring discrimination among different types of agonists (11, 37–39). Hence, the lack of up-regulated crystal-induced NO production in TLR2-expressing chondrocytes transfected with TLR1 reinforced the specificity of the TLR2-mediated effects in this study. Whether TLR6 could cooperate with TLR2 in mediating crystal-induced NO production has not been determined.

The negatively charged surfaces of MSU and CPPD crystals form hydrogen and electrostatic binds with a multitude of proteins (40). In this context, direct engagement by MSU crystals of the platelet integrin αIbβ3 has been demonstrated, an event that mediates crystal-induced secretion of platelet granule contents (20). MSU crystal-induced secretory and respiratory burst responses in phagocytes were lessened by blocking Abs specific for the leukocyte β2 integrin CD11b/CD18 (21) and FcR CD16 (21), which provided additional direct effects on cells mediated via engagement of plasma membrane proteins by MSU crystals. Given our results and the rapidity of crystal-induced chondrocyte signaling through TLR2, we speculate that direct physical interaction of MSU and CPPD crystals with the extracellular domain of TLR2 mediates activation of chondrocytes, but we have not excluded a role for rapid crystal-induced release of one or more endogenous TLR2 ligands, such as heat shock protein 70 or specific saturated fatty acids (18, 19).

IKK2, but not IKK1, has been shown to be critical for cytokine-induced NF-κB activation and expression of a variety of NF-κB-regulated genes in fibroblast-like synovial lining cells (41). In this study, crystal-induced TLR2-mediated signaling cascades culminating in NF-κB activation included activation of both IKK1 and IKK2 in chondrocytes, but only IKK2 activation was required to mediate crystal-induced NO production. It should be noted that the IL-1R and TLRs share a common signaling pathway, leading to NF-κB activation involving MyD88, IRAK, TRAF6, and IKKs (11). Furthermore, incubation of chondrocytes with MSU crystals induces IL-1β expression (6). Significantly, the triggering of MSU crystal-induced NO production in chondrocytes is not IL-1 dependent in chondrocytes, as we demonstrated previously using soluble IL-1R antagonist (6). However, it remains possible that autocrine signaling by cytokines, including effects of IL-1 through MyD88, could amplify NO production in chondrocytes after the early responses to MSU and CPPD crystals.

We demonstrated transient complex formation among TLR2, MyD88, Rac1, and PI3K (through its subunit p85α) in chondrocytes in response to MSU crystals. We also demonstrated that Rac1 and PI3K mediated chondrocyte activation in response to MSU and CPPD crystals, consistent with a direct crystal-induced Rac1/PI3K/Akt signaling cascade that transduced TLR2 signaling...
FIGURE 6. PI3K mediates the induction of NO in response to MSU and CPPD crystals in chondrocytes. 

A, Bovine primary chondrocytes cultured under nonadherent conditions in medium containing 1% FCS, as described above, were treated with MSU crystals (0.5 mg/ml) at the times indicated. The PI3K assay was conducted on cell lysates, as described in Materials and Methods. B, Bovine chondrocytes were stimulated with MSU crystals (0.5 mg/ml) for 24 h with or without pretreatment with the PI3K inhibitor LY294002 (25 μM) for 1 h. Where indicated, the cells were transiently transfected with wild-type and mutant PI3K subunit p85α (specifically p85α-WT and p85α, which lacks a region that is necessary for its association with PI3K activity) or vector control (Vec). Two days after transfection, the cells were treated with MSU or CPPD crystals (0.5 mg/ml), and NO release was determined as described above. The data are representative of three independent experiments with three different donors.

\[ p < 0.05. \]

FIGURE 7. Akt mediates induction of NO in response to MSU and CPPD crystals in chondrocytes. 

A, Bovine chondrocytes cultured under nonadherent conditions in medium containing 1% FCS were stimulated with MSU crystals (0.5 mg/ml) for the times indicated with or without pretreatment with the PI3K inhibitor LY294002 (25 μM) for 1 h. Cell lysates were analyzed by SDS-PAGE/Western blotting using Abs to phospho-specific Akt (Ser473) and total Akt. B, Bovine chondrocytes were transiently transfected with wild-type and kinase-dead mutant-type Akt (Akt-WT and Akt-KM) or vector control (Vec) using FuGene 6 and hyaluronidase, as described above. Two days after transfection, the cells were treated with MSU or CPPD crystals (0.5 mg/ml), and NO release was determined as described above. The data are representative of three independent experiments with three different donors.

\[ p < 0.05. \]

FIGURE 8. Rac1 acts upstream of PI3K to mediate induction of NO in response to MSU and CPPD crystals in chondrocytes. 

A and B, Bovine chondrocytes cultured under nonadherent conditions in medium containing 1% FCS were stimulated with MSU or CPPD crystals (0.5 mg/ml) for the times indicated. Where indicated, pretreatment with LY294002 (25 μM) was performed. Rac1 activation (pull-down) assay was performed on cell lysates (250 μg) prepared from these cells using GST-PBD, as described in Materials and Methods. SDS-PAGE/Western blotting analyses were then performed on the GST-PBD pull-down samples as well as the control cell lysates (aliquots of 30 μg of protein) from each sample using Rac1 Ab. C, Bovine chondrocytes were transiently transfected with constitutively activated or dominant negative Rac1 (Rac1V12 and Rac1N17, respectively) or vector control (Vec) using FuGene 6 and hyaluronidase as described above. Two days after transfection, the cells were treated with MSU or CPPD crystals (0.5 mg/ml), and NO release was determined as described above. D, Bovine chondrocytes were transiently transfected with dominant negative mutants of Rac1 (Rac1N17) and p85α (Δp85α) or vector control (Vec) using the Amaxa Nucleofection system, as described in Materials and Methods. Two days after transfection, the cells were transferred to nonadherent culture conditions in medium containing 1% FCS, then stimulated with MSU or CPPD crystals (0.5 mg/ml) for 1 h. Phosphorylation of NF-κB was determined by SDS-PAGE/Western blot analysis from cell lysates using Abs to phosphor-specific p65 (Ser326) and total p65. Data are representative of two independent experiments with two different donors.

\[ p < 0.05. \]
to induce NO production in chondrocytes. In previous studies of chondrocytes, we observed that MSU crystals induce tyrosine phosphorylation of paxillin (6), an adaptor protein for focal adhesion kinases, including Pyk2 (42). MSU crystal-induced activation of Pyk2 is pivotal for downstream p38 MAPK activation involved in the induction of NO production and MMP-3 expression in chondrocytes (6). Significantly, MyD88-dependent phosphorylation of paxillin by TLR2 ligands has been observed in macrophages (43), and we have observed that the dominant negative mutant of MyD88 inhibits phosphorylation of paxillin induced by both MSU and CPPD crystals in chondrocytes (R. Liu-Bryan, unpublished observations). Because paxillin can associate with Pyk2, it will be of interest to determine whether Pyk2 activation by the crystals is also mediated through TLR2 signaling.

We speculate that the strikingly similar modes by which pyrogen-free MSU and CPPD crystals were shown in this study to induce NF-κB activation and NO production in chondrocytes may be mirrored in the remarkable phenotypic similarity of synovitis in acute gout and pseudogout. Both of these forms of joint inflammation are mediated by transcriptionally NF-κB-regulated cytokines, such as TNF-α and IL-8 (25, 44–47). However, this study was based on observations with cultured cells. We stimulated chondrocytes with crystals under nonadherent culture conditions in this study. Nevertheless, crystal-induced cell-signaling events observed in this study in cultured chondrocytes could differ from those in chondrocytes in their cartilage matrix in situ. Another limitation of this study was the primary reliance on transfection of cultured cells. Due to the limitations imposed by the low yields of primary articular chondrocytes (e.g., <1000 cells/knee) (8) from mouse joints, studies of crystal-induced activation of TLR2 signaling in cultured chondrocytes has not yet been performed.

Innate immunity, modulated directly by MSU crystal-induced activation of the membrane attack complex of complement, was recently identified as a major mediator of neutrophil ingress in experimental acute gouty knee synovitis in the rabbit model (48). Taken together with findings in this study, we conclude that innate immunity, including TLR2-mediated activation of chondrocytes with articular cartilage triggered by free MSU and CPPD crystals, has the potential to contribute to degradation of cartilage matrix and to joint inflammation in gout and pseudogout.

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Disclosures
The authors have no financial conflict of interest.

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