Expression of Toll-like receptors 2 and 4 in stifle joint synovial tissues of dogs with or without osteoarthritis

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**Objective**—To investigate the presence or absence of Toll-like receptor (TLR)-2 and TLR-4 in synovial tissues collected from stifle joints (SJs) of dogs with or without osteoarthritis.

**Animals**—21 purpose-bred research dogs, 3 client-owned dogs with SJ osteoarthritis, and 3 dogs without SJ osteoarthritis.

**Procedures**—Research dogs underwent arthroscopic surgery in 1 SJ to induce osteoarthritis via cranial cruciate ligament transection (CrCLt; n = 5), femoral condylar articular cartilage groove creation (6), or release of the caudal horn of the medial meniscus (5); 5 dogs underwent sham surgery. Synovial tissue specimens were obtained from both stifle joints of each dog 12 weeks after surgery, and TLR-2 and TLR-4 gene expression were determined via real-time reverse transcription PCR assays. Expression of TLR-4 protein was determined via an immunofluorescence technique in additional specimens obtained from osteoarthritic SJs of dogs with cranial cruciate ligament insufficiency and from dogs with nonosteoarthritic SJs.

**Results**—Synovial tissues from CrCLt-treated joints had significantly higher TLR-4 gene expression, compared with the contralateral control SJs or any other joint group. TLR-2 gene expression did not differ significantly among groups. Toll-like receptor-4 protein was detected in synovial tissues of osteoarthritic SJs but was rarely evident in nonosteoarthritic SJs.

**Conclusions and Clinical Relevance**—Increased TLR-4 gene expression in the synovial tissue of SJs with osteoarthritis secondary to CrCLt suggests that activation of innate immunity may play a role in the pathophysiology of SJ osteoarthritis in at least a subset of dogs. (Am J Vet Res 2010;71:750–754)
Materials and Methods

Animals—All procedures were approved by an institutional animal care and use committee. Twenty-one purpose-bred female hound dogs (mean body weight, 23.3 kg; age range, 2 to 4 years) were used for surgical induction of osteoarthritis. Dogs were kept in individual kennels and allowed 15 minutes of on-leash exercise 5 d/wk for the entire study period. Three client-owned adult dogs that had SJ osteoarthritis secondary to CrCL insufficiency were also included in the study; prior to enrollment in the study, informed client consent was obtained and documented in the medical records. Additionally, 3 adult dog cadavers were obtained immediately after euthanasia for use in the study. The dogs were euthanatized for reasons unrelated to the study and had grossly normal SJs.

Procedures in research dogs—Each of the 21 hound dogs was premedicated, anesthetized, and prepared for aseptic surgery of 1 pelvic limb. Dogs were assigned to undergo 1 of 4 arthroscopic procedures on 1 SJ: CrCLt (n = 5), GR (ie, creation of two 6- to 8-mm-long full-thickness grooves in the medial femoral condyle cartilage with a 3-mm-diameter ring curette [6]), MR (ie, meniscal transection at the caudal horn junction with the caudal meniscotibial ligament [5]), or SS (ie, probe manipulation of all joint landmarks including cruciate ligaments, femoral condyles, and medial meniscus without insult [5]). The efficacy of the 3 osteoarthritis induction procedures has been reported.8–10 The contralateral SJ was not surgically manipulated and was used as an internal control for each dog. Twelve weeks after surgery, dogs were euthanatized by IV administration of an overdose of phenytoin and pentobarbital. Synovial tissue samples were collected from both SJs of each dog and stored in an RNA-preserving solutiona until used for RNA extraction.

Procedures in dogs with naturally occurring SJ osteoarthritis and dogs with nonosteoarthritic SJs—Stifle joint synovial tissues were collected from 3 adult dogs with SJ osteoarthritis secondary to CrCL insufficiency. These biopsy specimens were obtained during surgical procedures performed at our veterinary medical teaching hospital to address the osteoarthritis and joint instability of an SJ in each dog. Synovial tissue samples were collected from 1 grossly stiff SJ of each of 3 apparently healthy adult dogs immediately after euthanasia performed at a local humane society. The dogs were euthanatized by use of IV administration of an overdose of phenytoin and pentobarbital for reasons unrelated to the study.

TLR-2 and TLR-4 gene expression analysis—The RNA was extracted from each synovial tissue sample by use of a standard method.11 Reverse transcription was performed on total RNA samples (500 ng) by use of a reverse transcriptaseb according to the manufacturer’s protocol. After the RT reaction, the samples were diluted to 200 µL with RNase-free water and analyzed by use of a real-time RT-PCR procedure involving SYBR green and a thermal cycler.8 The reaction mixture consisted of 4 µL of diluted cDNA, forward and reverse primers (0.5 µL each), and 5 µL of SYBR green (total volume, 10 µL). The PCR profile consisted of 5 minutes at 95°C; 2 minutes at 94°C; 30 cycles of 3 seconds at 94°C (melting), 3 seconds at 60°C (annealing), and 10 seconds at 72°C (extension); and a melt curve analysis from 70° to 95°C. Fluorescence was detected during the extension step of each cycle and during the melt curve analysis at 470 nm (excitation) and 510 nm (emission) for SYBR green. Melt curve analysis was performed to ensure specific amplification. Take-off point was determined by use of the software provided with the thermal cycler. Melt curve analysis was performed with the melt curve analysis function provided with the thermal cycler software. Dog-specific TLR-2 primers (forward, 5′-AACCCCGGACCGAGTTGTG-3′; reverse, 5′-ATGTTTTGGCGCTCTTTC-3′) and dog-specific TLR-4 primers (forward, 5′-GTCTGTGCTGCATCATTCTC-3′; reverse, 5′-CATTCAGGATACTTTC-3′) were used. The sequences of the TLR-4 primers were constructed on the basis of a reference canine TLR-4 sequence (NM_001002950.1) deposited in GenBank.12 Gene expression levels were determined by comparison to the housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase (forward, 5′-CCACGCCATTCGAGCGAC-AG-3′; reverse, 5′-GGGTCCTCCGGATGCCCTGCTTC-3′). Specimens of whole blood and spleen obtained from an adult dog cadaver donated to the institution for research use and necropsy examination were used as positive controls for TLR-2 and TLR-4 PCR assays.13,14 The no-RT controls were negative for the primer set used, indicating that genomic DNA contamination was undetectable. All RT-PCR assays were performed in duplicate. To confirm specific amplification, a representative sample for each PCR product was purified by use of an RNA purification kit and sequenced.

![Figure 1](image-url)

Figure 1.—Mean ± SEM relative TLR-4 gene expression in synovial tissue specimens from SJs of 18 dogs in each of which 1 SJ (black bars) underwent CrCLt (n = 4), GR (6), or MR (3) to induce osteoarthritis or SS (5) and the contralateral SJ (gray bars) did not undergo surgical manipulation. *Value for the SJs that underwent CrCLt was significantly (P < 0.01) greater than values for the contralateral control SJs in that group and values for the surgically manipulated and respective contralateral control SJs in any other procedure group.
Immunofluorescence detection of TLR-4 protein—To determine whether TLR-4 gene expression was associated with the presence of TLR-4 protein in synovial tissues of osteoarthritic SJs, immunofluorescence testing was performed on biopsy samples of synovial tissue collected from 3 dogs with SJ osteoarthritis secondary to CrCL insufficiency (3 SJs) and synovial tissue from 3 dog cadavers (3 SJs). Cryosections (10 µm in thickness) of synovial tissues were placed on slides and incubated with anti–TLR-4 primary antibody. The primary antibody has been reported to react with canine TLR-4 protein. After incubation overnight (approx 15 hours) at 4°C, sections were washed in 50mM Tris-HCl (pH, 7.6) with 0.1% Tween 20. Sections were then incubated with goat anti-rabbit fluorescein isothiocyanate antibody for 1 hour followed by a rinse in Tris-HCl with Tween 20. The sections were mounted by use of mounting medium with 4', 6-diamidino-2-phenylindole. Sections of spleen tissue obtained from the same adult dog cadaver used for PCR assay control were stained and used as a positive control. Staining of samples in the absence of primary antibody provided negative controls.

Statistical analysis—All statistic analyses were performed by use of a computer software program. Among the research dogs in each procedure group (ie, dogs that underwent CrCLt, GR, MR, or SS), data obtained from the manipulated SJs were combined, and mean ± SEM values of both relative TLR-2 and TLR-4 gene expressions were determined. Similarly, data obtained from the contralateral control SJs in each procedure group were combined, and mean values of each relative gene expression were determined. A 1-way ANOVA was performed to determine differences in TLR-2 or TLR-4 expression levels in manipulated and control SJs among the procedure groups. When significant differences among groups were detected, a Tukey pairwise multiple comparison test was performed to determine which groups differed from each other. Significance was set at a value of P < 0.05.

Results

TLR-2 and TLR-4 gene expressions—Among the research dogs, sufficient amounts of RNA were extracted from both SJs of 18 dogs (4, 6, 3, and 5 dogs that underwent unilateral CrCLt, GR, MR, and SS, respectively) for relative gene expression analysis. The TLR-4 gene expression levels in synovial tissues obtained from SJs following CrCLt were significantly (P < 0.01) higher than levels in the contralateral control SJs and higher than levels in SJs that underwent any other procedure or their respective contralateral control SJs (Figure 1). In contrast, there was no significant difference in TLR-2 gene expression levels among manipulated SJs and their contralateral control groups in the 4 procedure groups (Figure 2).

Figure 2—Mean ± SEM relative TLR-2 gene expression in synovial tissue specimens from SJs of 18 dogs in each of which 1 SJ (black bars) underwent CrCLt (n = 4), GR (6), or MR (3) to induce osteoarthritis or SS (5) and the contralateral SJ (gray bars) did not undergo surgical manipulation. There was no significant difference among the groups; TLR-2 was constitutively expressed in synovial tissues, regardless of the presence or absence of osteoarthritis.

Figure 3—Representative photomicrographs of immunofluorescence detection of TLR-4 protein in canine synovial tissue specimens from an SJ with osteoarthritis secondary to CrCL insufficiency (A) and a grossly normal SJ (B). Green fluorescence (positive result; A) was frequently observed in synoviocytes lining the synovial tissues in joints with osteoarthritis; the synovium in affected joints formed multiple papillary projections covered by hyperplastic synoviocytes. In contrast, no immunofluorescence (negative result; B) was evident in synovial tissues obtained from healthy stifle joints; the synovium in unaffected joints was covered by a thin layer of synoviocytes. Nuclei are counterstained with 4', 6-diamidino-2-phenylindole (blue fluorescence). Bar (applies to both panels) = 200 µm.
Discussion

Results of the present study clearly indicated that gene expression levels of TLR-4 are significantly increased in synovial tissues in SJs of dogs after CrCLt. The dogs used for the gene expression analyses were purpose-bred research dogs, and the possibility of microbial joint infection that could activate TLRs in response to microbe-associated molecular pattern molecules was unlikely. We did not detect elevated expression of TLR-4 in SJs after they received the other types of surgical insult, and gene expression levels among their contralateral joints were similar and not different from findings for the contralateral SJs in the dogs in the CrCLt group. Moreover, there was no difference in the expression level of TLR-2 gene among the groups of manipulated SJs and the respective groups of control SJs. This suggests that CrCL insufficiency has a unique characteristic—whether it be exposed ligament, marked joint instability, or severity of the induced osteoarthritis—that incites the TLR-4 pathway as part of the mechanism of disease. This was further verified by evidence of TLR-4 protein expression (detected via immunofluorescence testing) in synovial tissues obtained from client-owned dogs with osteoarthritis secondary to CrCL insufficiency.

A recent finding regarding the initiation of the inflammatory response against endogenous DAMPs (which act as physiologic danger signals) has shed light on a potential role for TLRs in various inflammatory diseases and tissue injuries. However, the importance of TLRs in the pathogenesis of osteoarthritis is unknown. Among the known potential DAMPs, hyaluronan molecules are particularly abundant in synovial fluid, and the presence of hyaluronan fragments in synovial fluid is a hallmark change associated with development of osteoarthritis. A recent study in dogs performed in our laboratory revealed that a unique, low–molecular-weight hyaluronan fragment was present in synovial fluid of osteoarthritic joints. It is known that TLR-2 and TLR-4 are responsible for alveolar macrophage inflammatory gene expression in response to hyaluronan fragments. In another study, TLR-2 gene expression levels in synovial fluid from the SJs of dogs affected with osteoarthritis were not significantly different from findings in the SJs of healthy dogs. Similarly, TLR-2 gene expression levels in synovial tissues from osteoarthritic and nonosteoarthritic SJs did not differ in the present study. We found that the TLR-2 gene was constitutively expressed in the synovial tissue, regardless of the presence or absence of osteoarthritis. It can be speculated that TLR-2–mediated immune responses are readily provoked by exogenous or endogenous ligands in the synovial joint and that TLR-2 might be a potential target for the treatment of joint inflammation. Further investigation of the amounts of TLR-2 protein in osteoarthritic and nonosteoarthritic joints and the functional roles of TLR-2 in development of osteoarthritis is necessary to elucidate these possibilities. The TLR-2 protein assays were not used in the present study because, in contrast to immunohistochemical analysis of TLR-4, immunohistochemical testing with anti–TLR-2 antibodies in canine tissues has not been validated, to our knowledge. Interestingly, among the SJs that underwent CrCLt, GR, or MR, TLR-4 gene expression was increased significantly (compared with the contralateral joints that did not undergo surgical manipulation) only in the CrCLt group. It has been reported that TLR-4 protein expression by chondrocytes in osteoarthritic joints typically increases in parallel with increases in severity of microscopic cartilage damage as determined via immunohistochemical analyses. Joint destruction and synovial inflammation progress more effectively in joints that undergo CrCLt, compared with changes in joints that undergo GR or MR. Although we do not know whether the increase in TLR-4 gene expression in synovial tissues is directly related to the severity of the tissue injury or initial insult in joints that undergo CrCLt, we speculate that the initial mechanical injury is followed by exuberant inflammatory responses after a threshold level of local immune system activation is exceeded. For example, transection of the CrCL may release endogenous DAMP molecules including hyaluronan fragments, wear particles (eg, fibronectin), and plasma mediator products (eg, fibrinogen) in greater abundance, compared with effects of the other 2 surgical manipulations. Ligand binding to TLRs at the cell surface leads to recruitment of MyD88, the adaptor molecule, which interacts with interleukin-1 receptor–associated kinase 4 and then with tumor necrosis factor receptor–associated factor 6, resulting in activation of transcription factors and expression of various genes pertaining to inflammatory responses. Chemokines, such as monocyte chemoattractant protein-1/CCL2 and interleukin-8/CXCL8, are important local inflammatory mediators in various disease conditions including osteoarthritis, and their expression in response to hyaluronan fragments via TLR-4 in endothelial cells and melanoma cells has been reported. A recent unpublished study in dogs performed in our laboratory revealed significantly increased concentrations of monocyte chemoattractant protein-1/CCL2 and interleukin-8/CXCL8 in synovial fluid obtained from SJs with osteoarthritis induced via CrCLt, compared with synovial fluid concentrations in SJs that underwent SS. These find-
ings suggest a possible link between TLR-4 signaling and chemokine expression in joints with osteoarthritis. Synovial tissue infiltration by helper T cells in patients with osteoarthritis has been reported and is implicated in the disease mechanism. Microscopic changes in synovial tissues in SJs with osteoarthritis can vary from little discernible change to synovial tissue hyperplasia with lymphoplasmacytic inflammation. What determines the apparent involvement of the adaptive immune responses in at least a subset of patients with osteoarthritis is an important question to be answered. Activation of TLR pathways may be a key innate immune response that initiates and perpetuates the adaptive immune responses with persistent inflammation in some patients with osteoarthritis. This might explain why surgical interventions to correct joint malalignment and instability or medical interventions to reduce inflammation often fail to fully address the problems of patients with osteoarthritis. Elucidation of the specific roles of TLR pathways in the disease mechanisms of osteoarthritis is warranted.

References