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Toll-like receptor adaptor signaling molecule MyD88 on intervertebral disk homeostasis: In vitro, ex vivo studies

Michael B. Ellman^{a,b,1}, Jae-Sung Kim^{a,1}, Howard S. An^b, Di Chen^a, Ranjan KC^a, Jennifer An^{a,b}, Teju Dittakavi^a, Andre J. van Wijnen^e, Gabriella Cs-Szabo^{a,b}, Xin Li^a, Guozhi Xiao^b, Steven An^{a,b}, Su-Gwan Kim^f, and Hee-Jeong Im^{a,b,c,d,*}

^aDepartment of Biochemistry, Rush University Medical Center, Chicago, IL 606124, USA

^bDepartment of Orthopedic Surgery, Rush University Medical Center, Chicago, IL 606124, USA

^cDepartment of Internal Medicine (Section of Rheumatology), Rush University Medical Center, Chicago, IL 606124, USA

^dDepartment of Bioengineering, University of Illinois, Chicago, IL 60612, USA

^eDepartment of Cell Biology, University of Massachusetts Medical School, Worcester, MA 01655, USA

^fDepartment of Oral and Maxillofacial Surgery, School of Dentistry, Chosun University, GwangJu City, Republic of Korea

Abstract

MyD88 is an adapter protein that links toll-like receptors (TLRs) and Interleukin-1 receptors (IL-1Rs) with downstream signaling molecules. The MyD88 has been found to be an essential mediator in the development of osteoarthritis in articular cartilage. However, the role of the MyD88 pathway has yet to be elucidated in the intervertebral disk (IVD). Using in vitro techniques, we analyzed the effect of MyD88 pathway-specific inhibition on the potent inflammatory and catabolic mediator LPS and IL-1 in bovine and human nucleus pulposus (NP) cells by assessing matrix-degrading enzyme expression, including matrix metalloproteases (MMPs) and a disintegrin-like and metalloprotease with thrombospondin motifs (ADAMTS family). We also analyzed inhibition of MyD88 in the regulation of inducible nitric oxide synthase and TLR-2. Finally, we used an ex vivo organ culture model to assess the effects of MyD88 inhibitor (MyD88*i*) on catabolic factor-induced disk degeneration in mice lumbar disks. In bovine NP cells, MyD88*i* potently antagonizes LPS- or IL-1-mediated induction of cartilage-degrading enzyme production, including MMP-1, MMP-13, ADAMTS-4, and ADAMTS-5. MyD88*i* also attenuates the LPS- or IL-1-mediated induction of iNOS and TLR-2 gene expression. Our ex vivo findings reveal inhibition of MyD88 via counteraction of IL-1-mediated proteoglycan depletion. The findings from this study demonstrate the potent anti-inflammatory and anti-catabolic effects of inhibition of MyD88 pathway inhibition on IVD homeostasis, suggesting a potential therapeutic benefit of a MyD88*i* in degenerative disk disease in the future.

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*Corresponding author at: Department of Biochemistry, Rush University Medical Center, Cohn Research BD 516, 1735 W. Harrison, Chicago, IL 60612, USA. Tel.: +1 312 942 3091; fax: +1 312 942 3052. Hee-Jeong_Sampen@rush.edu (H.-J. Im)..

¹Equally contributed to this study.

Author contributions Study conception and design: Im, Kim, An H, Chen, Cs-Szabo, Wijnen

Acquisition of data: Kim, An J, Dittakavi, An S, KC

Analysis and interpretation of data: Im, Ellman, Chen, KC, An H, Kim, Cs-Szabo, Wijnen

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Keywords

MyD88 inhibitor; Intervertebral disk degeneration; Toll-like receptors; Low back pain; Signaling pathway

1. Introduction

The lifetime prevalence of back pain in the United States is 70–85% with roughly 10–20% of the population experiencing chronic symptoms (Andersson, 1999). While the etiology of back pain is multifactorial, it has been associated with degeneration of the intervertebral disk (IVD) (Freemont et al., 2001; Mooney, 1989). Disk degeneration results from an imbalance between degradation (catabolism) and synthesis (anabolism) of extracellular matrix (ECM) components by chondrocytes residing in the nucleus pulposus (NP) (Iatridis et al., 1997; Nomura et al., 2001). Via an upregulation of molecules such as proinflammatory cytokines and catabolic growth factors, homeostasis of the ECM shifts toward a degenerative, catabolic state with subsequent breakdown of ECM components, including collagen fibrils surrounding and restraining large, hydrated aggregates of proteoglycans (PGs), principally aggrecan (Goldring, 2000; Lee et al., 2009; Masuda et al., 2006). For example, a relative increase in destructive enzymes, such as matrix metalloproteases (MMPs) or a disintegrin-like and metalloprotease with thrombospondin motifs (ADAMTS family, aggrecanases), may alter the normal balance within the disk and result in degeneration (Crean et al., 1997; Im et al., 2007a, 2007b; Le Maitre et al., 2004, 2007; Martel-Pelletier et al., 2001; Muddasani et al., 2007). Recently, biological treatments capable of preventing disk degeneration by inhibiting catabolic processes and promoting ECM repair and regeneration have been considered (H. An et al., 2003; H.S. An et al., 2003; Masuda, 2008), and a greater understanding of the biochemical pathways involved in degeneration may help to identify potential biological treatments in the future.

Mammalian toll-like receptors (TLRs) comprise a family of at least 11 members that are involved in innate and adaptive immune responses, and specific members have been implicated in chronic inflammatory processes associated with cartilage degeneration (Kim et al., 2006; Liu-Bryan and Terkeltaub, 2010; Takeda and Akira, 2005; Takeda et al., 2003). All TLRs are type 1 transmembrane proteins that recognize distinct ligands extracellularly and mediate binding to adaptor proteins intracellularly. MyD88 is an adapter protein that links TLRs and Interleukin-1 receptors (IL-1Rs) with downstream signaling molecules. Following activation via binding of cytokine ligand to TLR, signaling pathways mediated by TLR-MyD88 culminate in NF κ B activation (Kim et al., 2006). Previous studies have demonstrated a capacity for TLRs to activate pro-inflammatory cytokines such as TNF α , IL-1, IL-6, and IL-8, thus mediating both inflammatory and pain pathways in cartilage (Kim et al., 2006; Liu-Bryan et al., 2005; Papathanasiou et al., 2011; Takeda and Akira, 2005; Takeda et al., 2003). TLRs have also been shown to upregulate production of collagenases (MMP-1 and MMP-13), and inducible nitric oxide synthase (Kokkinos et al., 1997), an enzyme that catalyzes the production of pro-inflammatory nitric oxide (NO) (Campo et al., 2011; Kim et al., 2006). The mechanism responsible for these downstream effects is not fully understood, but the MyD88 pathway has been shown to be an integral part of TLR activity (Abdollahi-Roodsaz et al., 2011).

In cartilage, TLR-2 and TLR-4 are highly upregulated in joints with advanced osteoarthritis (OA) (Kim et al., 2006). Similarly, marked upregulation of TLR-2 and TLR-4 gene expression are observed in degenerative human spine components, including disks, facet joints and facet joint capsules, compared to those in age-matched normal tissues (unpublished data). Both receptors are sensitive to binding by bacterial lipopolysaccharides

(LPS), which are microbial constituents found in the outer membrane of gram-negative bacteria. LPS has been implicated in both inflammatory and degenerative states in articular cartilage and bovine IVD tissue (Aota et al., 2006; Jasin, 1983), and the literature suggests that LPS induces catabolic effects via TLR-2 and 4, with destructive activity mediated mainly via LPS-induced activation of both receptor complexes in articular chondrocytes (Bobacz et al., 2007; Kim et al., 2006).

Joosten and colleagues previously identified both TLR-2 and MyD88 as essential components to the development of arthritis, and specifically the depletion of PG in the ECM, in a streptococcal wall (SCW)-induced arthritic mouse model utilizing different genetic ablations (Joosten et al., 2003). Activation of the TLR-2/MyD88 pathways has also been associated with the induction of pro-inflammatory cytokines such as IL-6 and destructive tissue MMPs in peripheral arthritis models (Kim et al., 2006; Papathanasiou et al., 2011). Most recently, PG synthesis was enhanced and matrix-degrading enzyme expression suppressed in MyD88 knockout mice, suggesting that MyD88 pathway inhibition may be a potential therapeutic treatment option for inflammatory cartilage disease (Abdollahi-Roodsaz et al., 2011).

Despite several studies identifying TLR activity in articular cartilage and arthritic joints, however, there have been no reports in the literature on the functional activity of TLRs in spine tissue. Based on ligand binding and known biological effects in articular cartilage, we hypothesize that TLRs and its key downstream cofactor, MyD88, may have particular significance in the pathogenesis of spine disk degeneration and low back pain. For example, inhibiting the activity of the TLR-2/MyD88 pathway via the use of antagonists may serve as a potential treatment strategy to slow disk degeneration and shift homeostasis away from catabolism. To date, however, studies addressing the responsiveness of disks to endogenous TLR ligands such as LPS, along with identification of the specific role of MyD88, are lacking in spine tissue. In this study, using in vitro and ex vivo organ disk culture models in human and mice spine disks, we are the first to elucidate the potential of a MyD88 inhibitor (MyD88i) to slow or reverse disk degeneration. The inhibitor functions as a decoy by binding to the MyD88 TLR domain, and subsequent binding of the endogenous MyD88 monomer to the inhibitor blocks homodimerization that is required for downstream signaling. The anti-inflammatory and anti-catabolic effects of MyD88 inhibition was assessed by downstream catabolic markers (aggrecanase, collagenase, iNOS) in bovine and human NP cells after stimulation with either LPS or IL-1, and these findings were generalized to a mouse organ culture model.

2. Materials and methods

2.1. IVD cell isolation and culture

Human spine segments (T11/12 to S1) were received within 24 h of death of donors from the Gift of Hope Organ and Tissue Donor Network (Elmhurst, Illinois), after obtaining consent from the families. Each of the lumbar segments underwent magnetic resonance imaging (MRI). Intact disk specimens (Thompsons grade 2 or 3) were removed and processed aseptically (Kim et al., 2009; Thompson et al., 1990). After dissection of the NP portion of disks either from human or bovine tails, NP cells were released by enzymatic digestion in Dulbecco's modified Eagle's medium [(DMEM)/Ham's F-12 (1:1)] culture medium with sequential treatments of 0.2% pronase and 0.025% collagenase P, as previously described (Kim et al., 2010; Loeser et al., 2003). Alginate beads and monolayers were prepared for long-term (21 days) and short-term studies (1–2 days) as previously described by our group (Im et al., 2003, 2007b, 2008; Kim et al., 2010; Li et al., 2008a).

For short-term monolayer cultures, isolated NP cells from either bovine tails or human spine disks were counted and plated onto 12-well plates at 8×10^5 cells/cm² as previously described (Im et al., 2003, 2007b). Cells were stimulated with either IL-1 (10 ng/mL, NCI) or LPS (10 µg/mL, Sigma-Aldrich, St. Louis, MO) in the presence or absence of MyD88 peptide inhibitor (MyD88i; IMGENEX, San Diego, CA) at the concentration of 100 µM, in 1 mL per well of serum-free medium for 24 h at 37°C under 5% CO₂. Supernatants were collected 24 h after the initiation of each treatment and subjected to Western blotting analyses for secreted matrix-degrading enzyme production. Cells were harvested and prepared for either cell lysates or total RNA extraction followed by real-time qPCR analyses, as previously described (Li et al., 2008b). 18s rRNA was used for normalization.

2.2. Real-time qPCR (quantitative polymerase chain reaction)

Total RNA was isolated from the cells using Trizol reagent (Invitrogen, Carlsbad, CA) following the instructions provided by the manufacturer. Reverse transcription was carried out with 1 µg total RNA using the ThermoScript™ RT-PCR system (Invitrogen, Carlsbad, CA) for first strand cDNA synthesis.

For real-time qPCR, cDNA was amplified using MyiQ Real-Time qPCR Detection System (Bio-Rad Hercules, CA). The synthesized cDNA was subjected to real-time qPCR utilizing the Bio-Rad iQ™ SYBR Green supermix (Bio-Rad Hercules, CA). A threshold cycle (Ct value) was obtained from each amplification curve using iQ5 Optical System Software provided by the manufacturer (Bio-Rad Hercules, CA). Relative mRNA expression was determined using the $\Delta\Delta C_T$ and calculated as follows: $\Delta\Delta C_T = \Delta C_T$ (gene of interest) - ΔC_T (endogenous control). Gene quantification was conducted for entire, individual treatment groups and expressed in terms of fold change from untreated control levels. Expression of 18s rRNA was used as the internal control. The deviations in samples represent 3 different donors in 3 separate experiments. The primer sequences and their conditions for use are summarized in Table 1.

2.3. Immunoblotting

Cell and tissue lysates were prepared using modified RIPA buffer, as previously described (Li et al., 2008a, 2008c). The total protein concentrations of cell lysates were determined by a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). An equal amount of protein was resolved by 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred to a nitrocellulose membrane for immunoblot analyses, as described previously (Im et al., 2007b). MMP-13 and MMP-1 antibodies were provided by courtesy of Dr. Gillian Murphy (Cambridge University, UK). Immunoreactivity was visualized using the ECL system (Amersham Biosciences, Piscataway, NJ, USA) and the Signal Visual Enhancer system (Pierce), which magnifies the signal.

2.4. Ex vivo analysis using an intradiskal injection organ culture model

Lumbar spine segments from deceased mice were dissected under sterile conditions within 12 h after sacrifice at the Rush University animal facility for ex vivo organ culture, as previously described (Chiba et al., 1998). The requirement for the pre-treatment of MyD88 has been previously described (Ahmad et al., 2007). Therefore, intradiskal pre-injection of MyD88i (150 µM per disk) en bloc was performed using a 30-gauge needle (30 G, 1.5 µL volume). Injected disks were then separated and incubated in DMEM/Ham's F-12 medium supplemented with 1% mini-ITS. After 24 h, the MyD88i-pre-injected disks were challenged with either IL-1 (100 ng/mL) or LPS (10 µg/mL) and further incubated for 6 days. Harvested disks were fixed in 4% paraformaldehyde and then decalcified in EDTA, which was changed every 5 days. The decalcified disks were paraffin embedded. Serial disk sections of exactly 5-µm thickness were obtained to prepare slides. Safranin O-fast green

staining was performed to assess general morphology and the loss of PG in disk ground substance.

On the last day of organ culture, the harvested mouse lumbar disk cells were assessed to evaluate cell viability with fluorescent microscopy using the LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) by modifying previously described methods (Del Carlo and Loeser, 2002; Junger et al., 2009). Briefly, sample disk tissues were dissected out and cells were isolated via enzymatic digestion (sequential treatments with pronase and collagenase). The cells were then incubated in serum free medium supplemented with 10 μ M calcein AM green and 1 μ M ethidium homodimer-1 for 30 min. Membrane-permeable calcein AM is cleaved by esterases in live cells to yield cytoplasmic green fluorescence, and membrane-impermeable ethidium homodimer-1 labels nucleic acids of membrane-compromised cells with red fluorescence. At least 100 cells were counted in triplicate for each data point.

2.5. Histologic analysis of injected disks

Harvested disks were fixed in 4% paraformaldehyde and decalcified in EDTA, which was changed every 5 days. The decalcified disks were paraffin embedded. Serial disk sections of exactly 5- μ m thickness were obtained to prepare slides. Safranin O-fast green staining was performed to assess general morphology and the loss of PG in disk ground substance, as previously described (Muddasani et al., 2007). All samples from disks that were stained were examined independently by two blinded observers.

2.6. Gelatin zymography

Gelatin zymography was then performed as previously described (Gupta et al., 2007). Briefly, an equal volume of cell culture supernatant was mixed with non-reducing sample buffer [4% SDS, 0.15 M Tris (pH 6.8), and 20% (volume/volume) glycerol containing 0.05% (weight/volume) bromophenol blue] and resolved on a 10% polyacrylamide gel containing copolymerized 0.2% (1 mg/mL) swine skin gelatin (Sigma). After electrophoresis of the conditioned medium supernatant samples, gels were washed twice, for 15 min each time, with 2.5% Triton X-100. Digestion was carried out by incubating the gel in the gelatinase buffer (50 mM Tris-HCl (pH 7.6), 10 mM CaCl_2 , 50mM NaCl, and 0.05% Brij-35) at 37 °C for 24 h. The gel was stained with 0.1% Coomassie brilliant blue R350 (GE Healthcare, Piscataway, NJ, USA), and the locations of gelatinolytic activity were revealed as clear bands on a background of uniform light blue staining. After development, gel images were captured and the clear bands were analyzed using “ImageJ” image analysis software (www.imagej.nih.gov), and were expressed in arbitrary optical density units. Data shown are cumulative of two experiments. p-Values presented as mean $\pm$ standard deviation; data without a common letter differ, $p < 0.05$.

2.7. Statistical analysis

Analysis of variance was performed using StatView 5.0 software (SAS Institute, Cary, NC). p-Values lower than 0.05 were considered significant.

3. Results

3.1. Inhibition of MyD88 pathway suppresses LPS- and IL-1-induced expression of matrix-degrading enzymes and TLR-2 in both bovine and human NP cells

LPS and inflammatory cytokine IL-1 both induce catabolic effects in cartilage via an upregulation of matrix-degrading enzymes such as MMP-1 and MMP-13, which are key matrix-degrading enzymes in articular cartilage as well as in the IVD (Le Maitre et al., 2004, 2007; Martel-Pelletier et al., 2001). Similar to collagenases, members of the ADAMTS

family (i.e. aggrecanases) induce cartilage degradation as well. Specifically, upregulation of ADAMTS-4 and -5 has been correlated with degradation of aggrecan (major component of PGs) in the IVD, ultimately resulting in disk degeneration (Le Maitre et al., 2004, 2007; Martel-Pelletier et al., 2001). Therefore, we assessed the capacity for MyD88*i* to inhibit catabolic factor-mediated upregulation of aggrecanase expression in NP cells.

Given that MyD88 is the key regulatory cofactor that transduces TLR signals to induce matrix-degrading enzyme expression and ECM degradation in articular chondrocytes (Abdollahi-Roodsaz et al., 2011; Liu-Bryan and Terkeltaub, 2010), we examined the potential for MyD88*i* to antagonize catabolic effects induced by LPS and IL-1 in bovine IVD cells. In initial experiments using monolayer cultures with different concentrations of peptide inhibitor of MyD88 (MyD88*i*), we found that 100 μ M of MyD88*i* is a minimal dose demonstrating maximal effects. Therefore, NP cells were treated with MyD88*i* (100 μ M), LPS or IL-1, individually, or combinations of LPS and MyD88*i* or IL-1 and MyD88*i* for 24 h. Resulting cellular responses were then analyzed for alterations in gene expression and/or secreted protein levels. Our data reveal the significant upregulation of collagenase (MMP-13) and aggrecanases (ADAMTS-4, ADAMTS-5) after stimulation with either LPS or IL-1 compared to control (untreated) in bovine NP cells (Figs. 1A–C). Treatment of cells with MyD88*i* significantly suppresses either LPS- or IL-1-induced upregulation of catabolic gene expression ($p < 0.01$ and $p < 0.05$, respectively).

The literature has suggested that TLR-2 is an essential component to the development of arthritis in peripheral joints, and is associated with upregulation of pro-inflammatory cytokines and destructive tissue enzymes in articular cartilage (Joosten et al., 2003; Kim et al., 2006). Treatment of NP cells with LPS or IL-1 leads to an upregulation of TLR-2 suggesting an auto- and paracrine-regulatory mechanism of TLR-2 pathway by inflammatory ligands (Fig. 1D). Pretreatment of these cells with MyD88*i*, however, appears to attenuate this auto- and paracrine-loop via a significant decrease in LPS- or IL-1-stimulated TLR-2 levels ($p < 0.01$ and $p < 0.05$, respectively). These data suggest MyD88*i* may antagonize the inflammatory response via downregulation of TLR-2.

Our data generated using bovine tail disk NP cells were further verified using human lumbar disk NP cells (Figs. 2–4). Similar to the cellular response in bovine NP cells, human NP cells reveal an increase in collagenases such as MMP-13 (Figs. 2A and B) and MMP-1 (Figs. 3A and B) at both mRNA and protein levels after stimulation with LPS or IL-1, with significant decreases in these catabolic gene expressions by co-treatment with MyD88*i* ($p < 0.01$ for LPS plus MyD88*i* and $p < 0.05$ for IL-1 plus MyD88*i*, respectively). Similarly, induction of ADAMTS-4 expression by LPS ($p < 0.05$) or IL-1 ($p < 0.01$) is significantly reduced by co-treatment with MyD88*i* in human NP cells (Fig. 4A; $p < 0.01$, $p < 0.05$, respectively). These data demonstrate potent anti-catabolic activity of MyD88*i* in both bovine and human NP cells in vitro, suggesting an important role of inhibition of MyD88 to limit collagen degradation in disks.

Nitric oxide (NO) is known to play an important role in cartilage metabolism and has been linked to inflammatory pathways and pain signaling (Castro et al., 2006; Del Carlo and Loeser, 2002; Kohyama et al., 2000). Increased expression of nitric oxide synthase (iNOS), a gene responsible for the production of NO, has been associated with cartilage destruction in knee joints (Pelletier et al., 1999) and IVDs (Kohyama et al., 2000), and a reduction of iNOS is closely linked to a reduction in tissue levels of multiple MMPs (Pelletier et al., 1999). We therefore examined the role of MyD88 inhibition in LPS- or IL-1-regulation of iNOS gene in human NP cells (Fig. 4B). After stimulation with LPS or IL-1, iNOS mRNA is significantly increased ($p < 0.05$; 2-fold) compared to control (untreated). Co-treatment with MyD88*i*, however, results in significant suppression of iNOS gene levels ($p < 0.01$ for

LPS; $p < 0.05$ for IL-1, respectively) compared to treatment with LPS or IL-1 alone, suggesting the role of MyD88 inhibition in an oxidative stress-related cellular response in disk cells.

3.2. MyD88 peptide inhibitor antagonizes LPS- or IL-1-induced catabolic protease activity in the IVD

Because MyD88*i* effectively suppresses expression and/or production of multiple cartilage degrading enzymes, TLR-2 and stress-associated genes after stimulation with either LPS or IL-1 (Figs. 1–4), we further analyzed the role of MyD88*i* in antagonizing catalytic enzyme activity by performing reverse zymography (Fig. 5). Human NP cells cultured in monolayer were stimulated with either LPS or IL-1 in the presence or absence of MyD88*i*, followed by zymographic analyses. Our results reveal that stimulation of NP cells with either LPS or IL-1 enhances catalytic activity up to 2-fold compared to control level (untreated) assessed by densitometry value (Fig. 5A; *lanes 2 and 3*), as represented by hydrolyzed clear bands of gelatin gel (Fig. 5B; *lanes 2 and 3*). Importantly, addition of MyD88*i* inhibits the catabolic effects mediated by either LPS or IL-1, as catalytic enzyme activity is markedly suppressed by the presence of MyD88*i* ($p < 0.01$; *lanes 5 and 6*). Taken together, these findings demonstrate the potent capacity of MyD88*i* to antagonize catabolic enzyme activity, in addition to its suppressive effects on mRNA and protein expression.

3.3. MyD88 inhibition antagonizes inflammatory cytokine-mediated proteoglycan depletion in mouse disk organ culture model

Given the in vitro results obtained above, we sought to elucidate potential physiologic effects of MyD88*i* in an organ culture model. Mouse disks were intradiskally injected en bloc with either MyD88*i* (150 μ M per disk) or control peptide (no cell permeability sequence, 150 μ M per disk) followed by subsequent stimulation with IL-1 (100 ng/mL) in culture media. After 6 days of lumbar disk organ culture, the presence of IL-1 with control peptide reveals severe PG depletion (Fig. 6B) compared to co-incubation with MyD88*i* (Fig. 6C) or control (Fig. 6A). Our ex vivo experiment results using a disk organ culture system suggest that injection of MyD88*i* significantly attenuates the PG loss that was induced by a potent inflammatory cytokine IL-1 and maintains the integrity of disk matrix homeostasis.

4. Discussion

The major new findings from this study reveal the potent anti-inflammatory and anti-catabolic effects of MyD88 pathway inhibition on IVD homeostasis, suggesting a potential therapeutic benefit of inhibition of the MyD88 pathway in degenerative disk disease. In the disk, MyD88*i* antagonizes LPS- and/or IL-1-mediated induction of cartilage-degrading enzymes, including MMP-1, MMP-13, ADAMTS-4, and ADAMTS-5, revealing a profound anti-catabolic role of MyD88*i* in NP cells from human as well as bovine tissues. Finally, MyD88*i* counteracts IL-1-mediated suppression of PG deposition in mouse disks, suggesting that inhibition of MyD88 pathway-induced anti-catabolic effects in disks may be generalizable across different species both in vitro and ex vivo.

Inhibition of the MyD88 pathway also suppresses the catabolic factor-mediated upregulation of iNOS and TLR-2, elucidating anti-oxidative, anti-inflammatory, and possibly anti-pain effects in disk homeostasis. Interestingly, our in vitro cell culture studies using human astroglial cells demonstrate that inhibition of MyD88 by a pharmacological peptide inhibitor effectively attenuates both LPS- and IL-1-induced iNOS and inflammatory cytokines (e.g., TNF α , MCP-1) (data not shown) that may further activate juxtaposed glial cell population (e.g., microglial), potentially influencing nociceptive pathways after peripheral tissue damage (disk degeneration). While the relevance of these findings is outside the scope of

this paper, further studies are warranted to elucidate the complex pain pathways involved in disk degeneration, and the effect in particular of MyD88*i* on glial cell processing of pain pathways in the brain and spinal cord.

Toll-like receptors have been found to play a beneficial immunological role in early host defense against invading pathogens (Takeda and Akira, 2005) as well as in inflammatory diseases involving bone and cartilage (O'Neill and Dinarello, 2000). In particular, TLR-2 signaling has been associated with synovial inflammation and cytokine production in septic arthritis and gouty arthritis, and is necessary for induction of matrix degradation and suppression of matrix synthesis in articular cartilage (Joosten et al., 2003; Liu-Bryan et al., 2005). Upon stimulation of human osteoarthritic cartilage with LPS, IL-1, TNF- α or peptidoglycan, TLR-2 level is significantly increased, suggesting that TLR-2 plays an important role in cartilage degeneration (Kim et al., 2006). Further, in a streptococcal cell wall (SCW)-induced joint inflammation model, Joosten and colleagues found that TLR-2-deficient mice were unable to develop either joint swelling or inhibition of cartilage matrix synthesis (Joosten et al., 2003). They also reported that mice deficient in MyD88 were unable to develop SCW-induced arthritis, as both joint swelling and disturbance of cartilage chondrocyte anabolic function were completely abolished. The authors concluded that MyD88 and TLR-2 are essential components in SCW-induced joint inflammation, and were the first to suggest targeting MyD88 as a novel therapy in inflammatory peripheral diseases (Joosten et al., 2003). The results of this study expand these findings to the IVD, suggesting that MyD88 may also be a target for biological therapy in the IVD.

The concentrations of LPS (10 μ g/mL) and IL-1 (10 ng/mL) appear to exert similar biological effects in terms of stimulation of cartilage degrading enzyme production. Our results consistently demonstrate a more potent capability of MyD88*i* to antagonize the effects of LPS ($p < 0.01$) compared to powerful inflammatory cytokine IL-1 ($p < 0.05$). The antagonistic potential of MyD88*i* on LPS-mediated activity may have significant potential with profound beneficial effects in the human body. First, LPS is a major component of the cell membrane of gram-negative bacteria, serving as a crucial target for the development of new antimicrobial agents. LPS induces a variety of cellular responses in vivo involving septic shock, and humans are known to be much more sensitive to LPS than other animals (e.g., mice, rat, rabbit, etc.). Thus, the development of an anti-LPS medication (i.e. MyD88*i*) may offer potential immunological benefits in fighting against unwanted bacteria. Second, as demonstrated in this study and others, inhibition of LPS-mediated effects via blockade of the MyD88 pathway may prevent or arrest joint degeneration in articular cartilage or disks. Several studies have demonstrated the potential for intra-articular injection of LPS to induce early inflammatory arthritis with synovitis and bone/cartilage destruction (Goldenberg et al., 1984; Idogawa et al., 1998; Matsukawa et al., 1993). Others have further shown that oral administration of LPS exacerbates collagen-induced inflammatory arthritis in mice (Yoshino et al., 1999). LPS not only stimulates the production of destructive enzymes in cartilage homeostasis, but also drives the expression of multiple pro-inflammatory cytokines and inhibits PG synthesis in human articular chondrocytes (Bobacz et al., 2007) and bovine disk NP cells (Aota et al., 2006). Liu et al. suggested that the compound icariin may exert protective effects on LPS-induced arthritis in neonatal mice chondrocytes via inhibition of nitric oxide and MMP synthesis (Liu et al., 2010). Here, we show similar results using MyD88*i*, revealing a potential therapeutic role of this inhibitor in disk degeneration.

Overexpression of MMPs and aggrecanases has been clearly associated with progressive degenerative conditions such as knee joint osteoarthritis and disk degeneration (Le Maitre et al., 2007). Research from our laboratory has demonstrated the potent catabolic and/or anti-anabolic effects of IL-1 and FGF-2 on human articular cartilage and spine tissue, particularly via upregulation of MMP-13 (Im et al., 2007b, 2008; Li et al., 2008b;

Muddasani et al., 2007). More recently, we elucidated the potential for bovine lactoferricin (LfcinB) to induce anabolic and anti-catabolic effects in bovine NP cells. LfcinB increases PG accumulation mainly via PG synthesis in a dose-dependent manner, decreases MMPs and ADAMTS expression, and simultaneously increases expression of multiple tissue inhibitors of metalloproteases (TIMPs) in a dose-dependent manner (Kim et al., 2011). Here, we demonstrate upregulation of MMPs and ADAMTS after treatment with LPS or IL-1 in NP tissue, and reveal a striking ability of MyD88*i* to inhibit the LPS- and IL-1-mediated upregulation of matrix-degrading enzyme gene expression, protein expression, and activity levels in bovine and human disks, revealing similar anti-inflammatory effects as LfcinB in spine tissue.

There are several recognized limitations of this study that must be taken into account. For example, the findings reported here relate only to in vitro and ex vivo culture models, which fail to adequately represent the complex variety of factors that may influence disk homeostasis in vivo. Indeed, results may not be generalizable to the complex interplay of factors in this setting. Also, the majority of our current studies is focused on NP cells and may not capture the effects of MyD88 inhibition on the entire disk, including the annulus fibrosis and neighboring endplates. Finally, we have reported the various effects on LPS- or IL-1-mediated activity by MyD88*i*, but the specific cell signaling pathways and detailed cellular mechanisms mediating these effects remain unknown. Further studies are warranted to elucidate these pathways.

In summary, we have shown that MyD88 pathway-specific inhibition exerts potent anti-inflammatory and anti-catabolic effects in vitro and ex vivo via the inhibition of LPS- or IL-1-mediated activity in bovine and human disk cells. Together with the results from ex vivo organ culture studies, our data elucidate a possible role of MyD88*i* in tissue engineering to help relieve degenerative disk disease in the future.

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Abbreviations

ADAMTS	a disintegrin-like and metalloprotease with thrombospondin motifs
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
FGF	fibroblast growth factor
IL	interleukin
iNOS	inducible nitric oxide synthase
IVD	intervertebral disk
LfcinB	bovine lactoferricin
LPS	lipopolysaccharide
MCP	monocyte chemotactic protein
MMP	matrix metalloprotease

MyD88i	MyD88 inhibitor
NO	nitric oxide
NP	nucleus pulposus
OA	osteoarthritis
PCR	polymerase chain reaction
PG	proteoglycan
SCW	streptococcal wall
TIMP	tissue inhibitor of metalloprotease
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha

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Fig. 1.

Inhibition of MyD88 pathway counteracts LPS- and IL-1-induced catabolic effects in bovine NP cells. The monolayer of bovine NP cells was treated with MyD88i (100 μ M), LPS (10 μ g/mL), IL-1 (10 ng/mL) and combinations of either LPS and MyD88i or IL-1 and MyD88i. After stimulation for 24 h, cells were harvested and total RNA was extracted to perform real time qPCR targeting key cartilage degrading enzymes including MMP-13 (A), ADAMTS-4 (B), ADAMTS-5 (C) and the MyD88 pathway receptor, TLR-2 (D). Values are the mean and SD and are representative of 3 different donors in 3 separate experiments. A value of $p < 0.05$ and $p < 0.01$ indicate a significant and a highly significant difference in ANOVA, respectively.

Fig. 2. Inhibition of MyD88 pathway suppresses LPS- and IL-1-induced expression of MMP-13 in human NP cells. The monolayer of human NP cells was treated with MyD88i (100 μM), LPS (10 μg/mL), IL-1 (10 ng/mL) and combinations of either LPS and MyD88i or IL-1 and MyD88i for 24 h. After stimulation, cells were harvested and subjected to either total RNA extraction for real time qPCR (A) or preparation of cell lysates for western blotting (B) to assess altered level of a potent collagen-degrading enzyme MMP-13 using human specific anti-MMP-13 antibody. Values are the mean and SD and are representative of 3 different donors in 3 separate experiments. A value of $p < 0.05$ and $p < 0.01$ indicate a significant and a highly significant difference in ANOVA, respectively.

Fig. 3.

The LPS- and IL-1-induced expression of matrix-degrading enzyme MMP-1 is significantly inhibited by MyD88 peptide inhibitor in human NP cells. The monolayer of human NP cells was treated with MyD88i (100 μ M), LPS (10 μ g/mL) and IL-1 (10 ng/mL) and combinations of either LPS and MyD88i or IL-1 and MyD88i for 24 h. A, After stimulation, cells were harvested and total RNA was extracted to perform real time qPCR targeting MMP-1 gene expression. B, Cell lysates were then prepared and analyzed by Western blotting with human specific anti-MMP-1 antibody. Values are the mean and SD and are representative of 3 different donors in 3 separate experiments. A value of $p < 0.05$ and $p < 0.01$ indicate a significant and a highly significant difference in ANOVA, respectively.

Fig. 4.

Inhibition of MyD88 pathway suppresses LPS- and IL-1-induced expression of aggrecanase (ADAMTS-4) and oxidative stress associated gene (iNOS) in human NP cells. Human NP cells in monolayer were treated with MyD88i (100 μ M), LPS (10 μ g/mL) or IL-1 (10 ng/mL) and combinations of either LPS and MyD88i or IL-1 and MyD88i for 24 h. After stimulation, cells were harvested and total RNA was extracted to perform real time qPCR to assess aggrecanase ADAMTS-4 (A) and oxidative stress associated gene iNOS (B). A value of $p < 0.05$ and $p < 0.01$ indicate a significant and a highly significant difference in ANOVA, respectively.

Fig. 5. MyD88 inhibitory peptide antagonizes LPS- and IL-1-induced protease activity in human NP cells. Human NP cells in monolayer were treated with MyD88*i* (100 μ M), LPS (10 μ g/mL) or IL-1 (10 ng/mL) and combinations of either LPS and MyD88*i* or IL-1 and MyD88*i* for 24 h. Gelatin zymography was performed by loading equal volumes of the conditioned media sample on polyacrylamide gel (A). Band images were digitally captured and the intensity of the bands (pixels/band) was obtained using the ImageJ densitometry analysis software in arbitrary optical density units (B). A value of $p < 0.01$ indicates a highly significant difference in ANOVA.

Fig. 6.

Histological assessments (original magnification: $\times 100$). Lumbar spine disks of mouse were dissected after en bloc intradiskal pre-microinjection with MyD88*i* at the concentration of 150 μ M per disk using 30 G needle (1.5 μ L in volume). The MyD88*i* pre-injected disks were challenged with either IL-1 (100 ng/mL) or LPS (10 μ g/mL) in DMEM/Ham's F-12 medium supplemented with 1% mini-ITS and further incubated for 6 days. Harvested disks were fixed, decalcified in EDTA and were paraffin embedded followed by serial disk sections of exactly 5- μ m thickness to prepare slides. Safranin O-fast green staining was performed to assess general morphology and the loss of PG in disk ground substance.

Table 1

The primer sequences for qRT-PCR.

Gene	Primer sequences	NCBI accession no.
Bovine	Forward: 5'-ACCCTTCCTTATCCCTTGATGCCA-3'	NM_174389.2
MMP-13	Reverse: 5'-AAACAGCTCTGCTTCAACCTGCTG-3'	
Bovine	Forward: 5'-AGTTCGACAAGTGCATGGTGTGTG-3'	NM_181667.1
ADAMTS-4	Reverse: 5'-ATGGTGACCACGTTGTTGTATCCGT-3'	
Bovine	Forward: 5'-TCACCAGCATTGACGCATCCAAAC-3'	NM_001166515.1
ADAMTS-5	Reverse: 5'-TTCGTGGTAGGTCCAGCAAACAGT-3'	
Bovine TLR-2	Forward: 5'-TAAGGATGCCTGGCCCTTCCTTCAAA-3'	AF368419
	Reverse: 5'-GGCCACTGACAAGTTTCAGGCATT-3'	
Human	Forward: 5'-ACCCTGGAGCACTCATGTTTCCTA-3'	NM_002427.2
MMP-13	Reverse: 5'-TGGCATCAAGGGATAAGGAAGGGT-3'	
Human	Forward: 5'-CATCCTGTTGTTGCTGTGGCTGAT-3'	NM_002421.2
MMP-1	Reverse: 5'-GCGTGGATGCCGCATATGAAGTTA-3'	
Human	Forward: 5'-AGTTTGAATGGGCCTTTGAGCACC-3'	NM_005099.4
ADAMTS-4	Reverse: 5'-TAGTCCTTGCCAGGGAAGTCACA-3'	
Human iNOS	Forward: 5'-5'-ATCACACGCCACAGAGATCCA-3'	NM_000625.4
	Reverse: 5'-GCTTCAGGCTGTTGAGCCATGT-3'	
Bovine 18s	Forward: 5'-GTAACCCGTTGAACCCATT-3'	NR_003286
rRNA	Reverse: 5'-CCATCCAATCGGTAGTAGCG-3'	
Human 18s	Forward: 5'-ATCCATTGGAGGGCAAGTCTGGT-3'	NR_003286
rRNA	Reverse: 5'-GCTCCCAAGATCCAACACTACGAGCTTT-3'	