Introduction: Intervertebral disc (IVD) degeneration is accompanied by chronic inflammation [1, 2]. Studies have shown that tissue harvested from degenerated discs express elevated levels of multiple pro-inflammatory cytokines (e.g. TNFα, IL-1β, IL-6). While the response of disc cells to stimulation by a single cytokine has been studied in vitro [3, 4], the response of disc cells to multiple cytokines mimicking the inflammatory environment of degeneration has not been explored. In this study, we utilize lipopolysaccharide (LPS), an inflammatory stimulus to provoke secretion of multiple cytokines (e.g. IL-1β, IL-6, TNF-α) by disc cells. LPS binds to Toll-Like Receptor-4 (TLR-4), a pattern recognition receptor that is activated in innate immunity. Recent studies have shown that TLRs are expressed by human articular chondrocytes [5], and are upregulated in osteoarthritic canine joints [6]. To date, no study has examined the expression of TLR-4 in the intervertebral disc. The goal of this study is to (a) measure the baseline expression of TLR-4 in IVD cells from the annulus fibrosis (AF), nucleus pulposus (NP) and end plate (EP), and (b) to measure the dose-dependent response of disc cells to LPS, thus more closely mimicking the microenvironment of disc degeneration. This approach provides an opportunity to study broad aspects of pathophysiology of degeneration.

Methods: IVD Cell Isolation: Intervertebral disc tissue was isolated from bovine lumbar spines. AF, NP and EP tissues were separated, minced and digested using standard enzymatic digestion. Cells were cultured in 6-well plates (1x10^5 cells per well) in DMEM+10% FBS. LPS challenge: After two days in culture, FBS containing media was replaced with serum-free DMEM containing LPS (0, 0.01, 0.1, 1, or 10 μg/mL). NP cells were also stimulated with individual recombinant cytokines (TNFα or IL-1β, 10 ng/ml) for comparison. Gene Expression: The gene expression of cells ± LPS was examined with quantitative RT-PCR. Total RNA was isolated using RNEasy kit (Qiagen) from cultured cells 24 hours post exposure to inflammatory stimuli. Bovine primers for extracellular matrix (ECM: AggreCan, COL1A, COL2A) and pro-inflammatory cytokine (IL-1β, IL-6, TNF-α), TLR-4, and GAPDH were designed for assay with the Universal Probe Library (Roche). Quantitative PCR was performed with ABI Prism 7900 Detector, Eurogentec Master Mix and FAM probe/TAMRA quencher reagents (Applied Biosystems), following recommended protocols. Quantitative PCR results were normalized to GAPDH transcript level (ΔCt), and expression changes in LPS groups were normalized to control groups using the ΔΔCt method. Statistical analysis was performed, with p<0.05.

Results: We found that TLR-4 was expressed in all IVD cell types (NP, AF, EP, Figure 1). The baseline expression of TLR-4 in NP cells was significantly greater than in AF or EP cells (Figure 1). Exposure to LPS for 24 hours resulted in significant up-regulation of inflammatory genes and down-regulation of ECM genes. Cells cultured in highest dose of LPS (10μg/mL) had lower up-regulation vs. other dosages, due to partial loss of cell viability. TNF-α and IL-1β expression were up-regulated in all cell types by LPS in a dose-dependent manner (0.01, 0.1, and 1μg/mL, Figure 2, 3). IL-1β expression was greatest in NP and EP cells, with AF cells demonstrating a lower level of stimulation in LPS (Figure 3). IL-6 was upregulated in all cell types due to LPS challenge, with NP cells having a significantly higher level of stimulation vs. AF and EP (Figure 4). ECM genes were downregulated in a dose-dependent manner. AggreCan expression was diminished in NP and AF cells exposed to LPS (0.1, 1, and 10μg/mL), with EP cells demonstrating little change in Ag expression (Figure 5). Col-2 was downregulated in all disc cells exposed to LPS (Figure 6). Col-1 expression was inhibited in AF cells by LPS (not shown). Stimulation of IVD cells with LPS resulted in differential up-regulation of TLR-4 expression, with NP cells overexpressing TLR-4 compared to EP or AF cells (Figure 7). Expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in NP cells after stimulation with individual recombinant cytokines was significantly lower than that seen for stimulation with LPS (Figure 8). The results of this study indicate that LPS can trigger cells to initiate catabolic pathways seen in IVD degeneration [1,2]. LPS was also found to be a more potent inflammatory stimulant than single recombinant cytokine in NP cells (Figure 8).

Discussion: The goal of this study was to measure the baseline expression of TLR-4 in IVD cells, and to examine the dose-dependent response of disc cells to multiple inflammatory stimuli. Our findings indicate that IVD cells express TLR-4 differentially, with NP cells possessing a higher baseline expression than AF or EP cells. This differential expression may be responsible for the greater pro-inflammatory stimulation response seen in NP cells when cultured with LPS (Figure 2-4). Alternatively, TLR-4 activation may trigger different pathways in each cell type (MyD88 dependent vs. independent pathways). Overall, NP cells were most sensitive to LPS stimulation, as indicated by the greatest upregulation of cytokine and downregulation of ECM expression. Additionally, at 1μg/mL LPS induced the largest number of cytokines in all cell types, with the loss of cell viability (not shown). Interestingly TLR-4 expression was significantly upregulated by inflammatory stimulation, suggesting that NP cells may actively change their response to inflammation. Our findings also indicate the ECM genes aggrecan, collagen-1, and collagen-2 are most significantly downregulated at 1μg/mL LPS exposure. This change in expression suggests that LPS can trigger cells to initiate catabolic pathways seen in IVD degeneration [1,2]. LPS was also found to be a more potent inflammatory stimulant than single recombinant cytokine in NP cells (Figure 8).