**MyD88-dependent Toll-like receptor 4 signal pathway in intervertebral disc degeneration**

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Abstract. Lower back pain (LBP) is a common and remitting problem. One of the primary causes of LBP is thought to be degeneration of the intervertebral disc (IVD). The aim of the present study was to investigate the role of the myeloid differentiation primary-response protein 88 (MyD88)-dependent Toll-like receptor 4 (TLR4) signal pathway in the mechanism of IVD degeneration. IVD nucleus pulposus cells isolated and cultured from the lumbar vertebrae of Wistar rats were stimulated by various doses of lipopolysaccharide (LPS; 0.1, 1, 10 and 100 µg/ml) to simulate IVD degeneration. Cells were rinsed and cultured in serum-free Dulbecco's modified Eagle's medium/F12. Reverse transcription-quantitative polymerase chain reaction was used to determine the levels of TLR4, MyD88, tumor necrosis factor α (TNFα), and interleukin-1β (IL-1β) mRNA expression after 1, 3, 6, 9 and 12 h of incubation. Additionally, western blot and enzyme-linked immunosorbent assay analyses were used to determine the levels of TLR4, MyD88, TNFα, and IL-1β protein expression after 24, 48 and 72 h of incubation. The levels of TLR4, MyD88, TNFα and IL-1β mRNA all increased in the cells stimulated by 10 µg/ml LPS at 3, 6 and 9 h (all P<0.001). Furthermore, the levels of TLR4, MyD88, TNFα and IL-1β protein all increased at 24, 48 and 72 h (all P<0.001). Additionally, the mRNA and protein levels of TLR4, MyD88, TNFα and IL-1β increased significantly in the cells stimulated by 1, 10 and 100 µg/ml LPS compared with the control group, and reached a peak in the 10 µg/ml LPS group (all P<0.001). These results suggest that the MyD88-dependent TLR4 signal pathway is a target pathway in IVD degeneration. This pathway is time phase- and dose-dependent, and when activated can lead to the release of inflammatory factors that participate in IVD degeneration.

Introduction

Lower back pain (LBP) is a common and remitting problem that cannot be cured but is relieved by current treatments. Multiple studies have demonstrated that 80% of adults will have at least one episode of back pain during adulthood (1,2). One of the main causes of LBP is thought to be degeneration of the intervertebral disc (IVD) (3). However, current treatments for IVD degeneration and LBP are aimed at relieving symptoms; they are not curative and offer little hope of restoring the IVD to its original function (4). To date, there is no approved conservative therapy to prevent or inhibit IVD degeneration. Therefore, elucidating the mechanisms of IVD degeneration will be necessary for the development of agents to prevent and treat IVD degeneration.

The IVD is a composite tissue, composed of the nucleus pulposus (NP), annulus fibrosis, and cartilaginous end plate. Human IVD degeneration is characterized by changes in architecture and biochemical composition, which alter the disc's ability to bear weight (5). Studies characterizing the extracellular matrix (ECM) and the inflammatory environment of IVD tissue isolated from surgical patients found that degeneration was associated with a loss of proteoglycan (PG) content, an increase in degenerative fibrillation, decreased water content and upregulation of degradative enzymes (6-10). 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 the collagen fibrils surrounding and restraining large, hydrated aggregates of PG, principally aggrecan (11-14). Elevated levels of molecular mediators of inflammation have been described in pathological disc tissue, and have been shown to increase in correlation with the grade of degeneration (15,16). Similar results have been observed for interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), both of which have established roles in regulating nitric oxide (NO) and prostaglandin production, metalloproteinase expression, and apoptosis, all of which are changes that may contribute to the progressive pathology of the IVD (17). Therefore, it is crucial to identify and inhibit...
the key points of the signal pathway responsible for producing proinflammatory cytokines and matrix metalloproteases for the prevention and treatment of IVD degeneration.

The mammalian Toll-like receptors (TLRs), germline-encoded receptors expressed by cells of the innate immune system, are stimulated by structural motifs referred to as pathogen-associated molecular patterns (PAMPs), which are characteristically expressed by bacteria, viruses and fungi (18,19). Notably, TLR interactions trigger the expression of proinflammatory cytokines, as well as the functional maturation of antigen presenting cells of the innate immune system (19,20). TLR4 signals the presence of lipopolysaccharide (LPS) on the cell membrane of gram-negative bacteria and activates an inflammatory response (21). When the majority of TLRs are stimulated, they interact with an adapter protein referred to as myeloid differentiation primary response gene 88 (MyD88), which couples the TLR to downstream signaling kinases, eventually culminating in the activation (by translocation from the cytoplasm to the nucleus) of the transcription factor nuclear factor κB (NF-κB) (22). However, MyD88-independent signaling pathways for TLR3 and TLR4 are also known to exist (23). Therefore, TLR4 is able to affect signal transduction in two different ways, either through MyD88 or a TIR domain-containing adapter that induces interferon-beta (TRIF), while TLR3 can signal only through TRIF (24). Although the MyD88-dependent and -independent pathways utilize distinct adapter proteins, both signaling pathways involve the activation and nuclear translocation of NF-κB, leading to the expression of numerous proinflammatory cytokines. To date, it has been confirmed that the TLR4 gene is expressed in IVD NP cells, and that it plays an important role in the molecular mechanism of IVD degeneration (6). However, the signal transduction pathway that activates the TLR4 in NP cells in IVD degeneration remains unknown, as does the MyD88-dependent or -independent nature of this signal transduction pathway.

The aims of the current study were to determine whether the TLR4 signal pathway in IVD degeneration was MyD88-dependent or -independent, as well as to assess the consequences associated with activation of TLR4 in IVD NP cells. Achievement of these aims may provide direct evidence in support of the hypothesis that the MyD88-dependent TLR4 signal pathway is the target pathway underlying IVD degeneration, as well as providing a theoretical basis for researching the molecular mechanisms underlying IVD degeneration.

Materials and methods

Isolation and culture of IVD NP cells. Animal experiments were approved by the ethics review board of Guangzhou Medical University (Guangzhou, China) and were performed in accordance with the guidelines on animal use of Guangzhou Medical University. NP cells were isolated from 4-week-old female Wistar rat (weight, 200 g; Laboratory Animal Center, Sun Yat-sen University) lumbar discs using methods reported by Hiyama et al (25). Briefly, the rats were euthanized by injection with an overdose of pentobarbital sodium (100 mg/kg; Nembutal; Amresco LLC, Cleveland, OH, USA). The spinal column was then removed under aseptic conditions, and the lumbar IVDs were separated under microscopy. The obtained NP tissue was allowed to digest in a mixture of 0.01% trypsin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 15 min. The isolated cells (1x10^6 L^{-1}) were maintained in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12, 1:1) and 10% fetal bovine serum supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. When confluent, the NP cells were harvested and subcultured in 10-cm dishes.

Morphological observation. For the observation of morphology, 6-well culture plates with an additional coverglass in each well were used. The primary or P1 NP chondrocytes that adhered to the coverglasses were used for observation of morphological changes under an inverted phase contrast microscope (IX51; Olympus Corporation, Tokyo, Japan). For hematoxylin and eosin (HE) staining, the coverglasses were washed with phosphate-buffered saline (PBS) prior to fixation in 4% paraformaldehyde for 30 min, followed by consecutive staining in HE. For Oil Red O staining (Sigma-Aldrich), the coverglasses were washed with PBS and fixed for HE staining, and were immersed for 2 h in a 1% toluidine blue solution (KeyGen Biotech Co., Ltd., Nanjing, China) prior to rinsing in 95% ethanol. For immunohistochemistry staining of collagen II, the endogenous peroxidase was blocked by 3% H<sub>2</sub>O<sub>2</sub> in methanol, and then the coverglasses were incubated for 30 min with anti-human type II collagen antibody (ab34712; Abcam, Cambridge, MA, USA) at a 1:50 dilution. A secondary antibody linked with avidin-biotin-peroxidase (SV0002; Abcam) and 3,3'-diaminobenzidine substrate solutions was used to visualize the immunoreactivity, followed by counterstaining in hematoxylin. Negative control was processed without the anti-rat type II collagen antibody (26,27).

Co-culture of NP cells and LPS. A 6-well co-cultured system was used. LPS was suspended in sterile dH<sub>2</sub>O by sonication, diluted in serum-free media, and re-sonicated immediately prior to use. Cells were rinsed and cultured in serum-free DMEM/F12 (control) ± LPS (0.1, 1, 10 and 100 µg/ml) for 1, 3, 6, 9 or 12 h (n=3-6 wells per time phase point) prior to use of the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to determine mRNA (TLR4, MyD88, TNFα and IL-1β) expression levels, and ensure a best time phase point. In addition, cells were rinsed and cultured in serum-free DMEM/F12 (control) ± LPS (10 µg/ml) for 24, 48 and 72 h (n=3-6 wells per time phase point), prior to western blot and enzyme-linked immunosorbent assay (ELISA) analyses to determine protein (TLR4, MyD88, TNFα and IL-1β) expression levels, and ensure a best time phase point. In additional experiments, cells were rinsed and cultured in serum-free DMEM/F12 (control) ± LPS (0.1, 1, 10 and 100 µg/ml, n=3-6 wells per dose) for different lengths of time (as determined by the results of the qPCR experiment), prior to use of qPCR to determine the mRNA (TLR4, MyD88, TNFα and IL-1β) expression levels, and ensure a best dose of LPS. In other experiments, cells were rinsed and cultured in serum-free DMEM/F12 (control) ± LPS (0.1, 1, 10 and 100 µg/ml, n=3-6
wells per dose) for different lengths of time (determined by the results of the western blot and ELISA analyses), prior to the use of western blot and ELISA analyses to determine the protein (TLR4, MyD88, TNFα and IL-1β) expression levels, and ensure a best dose of LPS.

**RT-qPCR.** Total RNA was isolated from cell cultures at various time phase points using RNAiso Plus reagent (Takara Bio, Inc., Tokyo, Japan), prior to elution from the column. RNA was treated with RNase-free DNase I to remove genomic DNA. Absorbances at 260 and 280 nm were measured for RNA quantification and quality control. All RNA samples exhibited high quality RNA and were subsequently reverse transcribed to cDNA using the PrimeScript™ RT reagent kit (Perfect Real Time) according to the manufacturer’s instructions (Takara Bio, Inc.). Subsequently, qPCR was conducted to determine the levels of mRNA expression using an ABI Prism 7000 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA) in triplicate in 96-well plates in a final volume of 20 µl under standard conditions. qPCR was conducted on cDNA samples using the SYBR Green method with SYBR Green mastermix, 1 µl (6 µM) forward primer, 1 µl (6 µM) reverse primer, 6 µl water and 2 µl (5 ng/µl) cDNA. qPCR was performed as follows: Initial denaturation at 95°C for 30 sec for activation of AmpliTaq Cold DNA polymerase (Applied Biosystems; Thermo Fisher Scientific, Inc.), followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec, and extension at 95°C for 15 sec. Forward and reverse primer sequences are listed in Table I and were synthesized by Takara Bio, Inc. To normalize each sample, a control gene (GAPDH) was used, and the arbitrary intensity threshold of amplification was computed. The 2^ΔΔCq method was used to calculate the relative expression of each target gene, as described previously (28).

**Western blot analysis.** Following treatment, NP cells were immediately placed on ice and washed with cold PBS. Proteins were prepared using the CellLytic NuCLEAR extraction kit (Sigma-Aldrich). Protein quantification was performed using a microplate bicinchoninic acid protein assay kit (Pierce Biotechnology, Thermo Fisher Scientific, Inc., Rockford, IL, USA). All wash buffers and the final resuspension buffer included a 1X protease inhibitor cocktail (Pierce Biotechnology), NaF (5 mM) and Na3VO4 (200 mM). Nuclear or total cell proteins (50 µg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and were subsequently electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST: 50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) and were incubated overnight at 4°C in 5% BSA in TBST with an anti-β-catenin antibody (1:1,000; 9582; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h, followed by washing three times with TBS and incubation with a peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000; 111-035-003; Jackson Immunoresearch, Baltimore, MD, USA) for 2 h at room temperature. Immunolabeling was detected using enhanced chemiluminescence reagents (Amersham Biosciences; GE Healthcare, Little Chalfont, UK).

**ELISA.** The concentrations of TNFα and IL-1β in the NP cells were assayed using an ELISA kit (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) according to the manufacturer’s instructions.

**Statistical analysis.** Experiments were repeated three times in biological replicates to obtain mean values. Data are presented as the mean ± standard deviation. Differences among groups were assessed using a one-way analysis of variance. Least significant difference t-tests were used when a single control group was compared with all other groups. Statistical significance (P<0.001) as compared to control group is denoted with an asterisk (*). All statistical analyses were conducted using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA).

**Results**

**Characterization of IVD NP cells.** The third passage of NP cells appeared round or multi-angular with activity. HE staining appeared homogeneous with blue nuclei and pink cytoplasm; no cells size increases were observed. No inhomogeneous or poor light refraction in the cytoplasm was detected (Fig. 1A). Oil Red O staining (Fig. 1B), toluidine blue staining (Fig. 1C) and type II collagen immunohistochemistry staining (Fig. 1D) were all positive and maintained a good cell phenotype. These results indicated that the third passage of NP cells were active.

| Target | Forward primer (5'-3') | Reverse primer (5'-3') |
|--------|------------------------|------------------------|
| GAPDH  | ATGGGAAAGCTTGGTCAAC    | GTGGTTCACACCCCATCACAA  |
| TLR4   | GAGGACTGGGTGAGAAACGA   | AGATACACCAACGGCTCTGG   |
| MyD88  | GAGATCGCGAGTTTGAAC     | CTTGTCTGCTGTTGCCTGA    |
| TNFα   | CATCCTGCTGATACACAGTT   | TGAGCACAGAAGACGATGC    |
| IL-1β  | GGGTTTCCATGGAAGATCAAC  | CACCTCTCAAGCAGACAGCAG  |

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TLR4, Toll-like receptor 4; MyD88, myeloid differentiation primary response gene 88; TNFα, tumor necrosis factor α; IL-1β, interleukin-1β.
and homogeneous, thus were fit for researching the molecular mechanism of IVD degeneration.

**Time phase-dependent association between LPS and the TLR4 signal pathway (TLR4, MyD88, TNFα and IL-1β).** Using qPCR, mRNA levels for TLR4, MyD88, and the proinflammatory cytokines TNFα and IL-1β were detected in NP cells stimulated with 10 µg/ml LPS. LPS (10 µg/ml) significantly increased the mRNA levels of TLR4 at 1, 3, 6 and 9 h (P<0.001, P<0.001, P<0.001 and P=0.040, respectively), and MyD88 at 1, 3, 6, 9 and 12 h (all P<0.001). The mRNA levels of TNFα and IL-1β increased significantly at 1, 3, 6, 9 and 12 h (all P<0.001). The peak TLR4, MyD88, TNFα and IL-1β responses were observed at 3, 3, 6 and 6 h, respectively (Fig. 2).

Western blot analysis was used to detect the TLR4 and MyD88 protein levels. Compared with unstimulated control

![Figure 1. Morphological changes of intervertebral disc nucleus pulposus cells (magnification, x400). (A) Hematoxylin and eosin staining, (B) Oil Red O staining, (C) toluidine blue staining, and (D) type II collagen immunohistochemistry staining.](image1)

![Figure 2. Quantitative analysis of mRNA expression levels in lipopolysaccharide (10 µg/ml)-stimulated groups at different time points (1 , 3 , 6 , 9 and 12 h) by qPCR. (A) TLR4; (B) MyD88; (C) TNFα and (D) IL-1β. Glyceraldehyde 3-phosphate dehydrogenase was used as a control gene. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.001 vs. control. TLR4, Toll-like receptor 4; MyD88, myeloid differentiation primary response gene 88; TNFα, tumor necrosis factor α; IL-1β, interleukin-1β.](image2)
cells, the TLR4 and MyD88 protein levels increased significantly at 24, 48 and 72 h (all P<0.001). Peak protein expression levels were observed at the 48 h time point for TLR4 and MyD88. (Fig. 3A-C).

The levels of TNFα and IL-1β protein were confirmed via ELISA analysis of the cell supernatants. TNFα and IL-1β protein levels standardized against GAPDH increased significantly at 24, 48 and 72 h in the NP cells stimulated by 10 µg/ml LPS (all P<0.001). Levels of both proteins reached a peak at the 48 h time point (Fig. 3D and E).

**Dose-dependent association between LPS and the TLR4 signal pathway (TLR4, MyD88, TNFα and IL-1β).** The NP cells stimulated by 0.1, 1, 10 and 100 µg/ml LPS were assessed...
using qPCR. The mRNA levels of TLR4 and MyD88 were detected by qPCR after 3 h, and the mRNA levels of TNFα and IL-1β were detected after 6 h. The results indicated that the mRNA levels of TLR4, MyD88, TNFα and IL-1β increased significantly in the cells stimulated by 0.1, 1, 10 and 100 µg/ml LPS as compared to a control group, and reached a peak in the 10 µg/ml LPS group (all P<0.001; Fig. 4).

Using western blot and ELISA analyses, TLR4, MyD88, TNFα and IL-1β proteins were detected in NP cells stimulated with LPS (0.1, 1, 10 and 100 µg/ml) for 48 h. Western blot analysis was used to detect TLR4 and MyD88 protein levels (Fig. 5A), and ELISA analysis was used to confirm the presence of TNFα and IL-1β proteins. The results indicated that levels of all four target proteins were increased significantly in the LPS stimulated cells, most notably in the 10 µg/ml LPS group (TLR4, all P<0.001; MyD88, P=0.019, P<0.001, P<0.001 and P<0.001, respectively; TNFα and IL-1β, all P<0.001) (Fig. 5).

Discussion

The aims of the current study were to investigate whether TLR4 was expressed in IVD NP cells, and if so, whether its signaling was MyD88-dependent. Additionally, the response of NP cells to stimulation with a TLR4 ligand (LPS) was evaluated in vitro. To accomplish these goals, the presence of a functional TLR4 signal pathway in the IVD of rats was confirmed, and LPS was used to stimulate NP cells in order to assess the time phase-dependent association between LPS and the TLR4 signal pathway. The present results demonstrated that NP cells constitutively expressed TLR4 that could be activated by LPS at different time phase points. Subsequently, we used various concentrations of LPS to stimulate NP cells in order to ascertain the nature of the dose-dependent association between LPS and the TLR4 signal pathway. Indeed, TLR4 expression was modulated by stimulation with LPS in a dose-dependent manner via the MyD88-dependent signal pathway, resulting in upregulation of a coordinated set of proinflammatory mediators in vitro.

Currently, the treatment of symptomatic IVD degeneration consists of either conservative measures, such as the application of analgesics and physiotherapy or surgery in cases where conservative measures prove unhelpful (29). These approaches have not been shown to slow the degeneration process, and consequently, relapses or other adverse sequelae of discectomy, dynamic stabilization techniques, total disc replacement or fusion surgery may be expected (30-33).

As IVD degeneration has a high prevalence and is associated with major socioeconomic costs, the current study sought to elucidate causative mechanisms underlying IVD degeneration in order to identify targets for its prevention and therapy. Recent studies have partially elucidated the mechanisms of the LPS/TLR4 signaling pathway, and this understanding may be applied to model the regulation of additional TLR4 signaling pathways (34,35). As improper regulation of LPS/TLR4 signaling has the potential to induce massive inflammation and cause acute sepsis or chronic inflammatory disorders, it is crucial to investigate this pathway further and evaluate novel targets to counteract these conditions (36,37).
and the envelope protein from a mouse mammary tumor virus, TLR4 signaling may also be initiated by endogenous molecules that interact either directly or indirectly with TLR4, such as heat-shock proteins, hyaluronic acid and β-defensin 2 (34). LPS is among the most extensively studied immunostimulatory components of bacteria, and can induce systemic inflammation and sepsis if excessive signals occur (38). Furthermore, LPS is an important structural component of the outer membrane of Gram-negative bacteria, consisting of three parts; lipid A, core oligosaccharide and an O side chain. Lipid A is the main PAMP of LPS (34). Using the C3H/HeJ mouse strain, known to have a defective response to LPS, Beutler's group demonstrated that TLR4 was an important sensor for LPS (39). The active upregulation of TLR4 in IVD NP cells following LPS stimulation suggests that TLR4 may participate in these responses.

The ability of TLR4 activation by LPS to provoke the secretion of multiple cytokines may provide an opportunity to study broad aspects of the physiological inflammatory process in the IVD. Results from the current study suggest that in vivo inflammatory stimulation can induce degenerative changes in the IVD, without the use of physical destruction of disc integrity as an injury stimulus (6). Commonly used animal models of degeneration, where a stab or laceration lesion of the disc is performed, and reproduce morphological changes of IVD degeneration in general (40-43). These animal models have also been associated with transient increases in the expression or secretion of proinflammatory cytokines (44,45), results that differ from what is observed in human degenerative disc diseases. Clinical disc degeneration in humans is associated with chronically elevated levels of multiple proinflammatory cytokines, indicative of the crucial roles that inflammatory mediators play in degenerative etiology. The findings of the current study indicate that altering the stimulus of degeneration, from physical disruption to an inflammatory stimulant, can also initiate a degenerative process in the IVD in vivo. This suggests that inflammatory insults alone may be able to initiate degeneration of the IVD.

The current study had several limitations. For example, LPS was the only agent used to interfere with the studied signal pathway. Additionally, we did not study any aspect of other TLR4 signal pathways, including the MyD88-independent pathway. In a planned study, we will interfere with different location points (e.g., MyD88 and TRAF6) along the TLR4 signal pathway using siRNA, and will also study changes to the ECM following activation of the TLR4 signal pathway.

In conclusion, the present findings confirm the activity of the MyD88-dependent TLR4 signal pathway in IVD NP cells in IVD degeneration, and that this signal pathway was stimulated by LPS in vitro. Activation of this pathway was time phase- and dose-dependent. When activated, this signal pathway led to the release of inflammatory factors that participated in IVD degeneration. Although these findings cannot be used directly to prevent and treat IVD degeneration, they suggest a potential therapeutic benefit of inhibition of the MyD88-dependent TLR4 signal pathway in degenerative disc disease. Therefore, the results of the current study provide a foundation for further investigations into therapeutic agents designed to prevent or treat IVD degeneration.

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