Nociceptive and Inflammatory Factors and Toll-like Receptors: Their Role in Intervertebral Disc Degeneration and Chronic Low Back Pain

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Abstract

Intervertebral disc degeneration is one of the most common causes of chronic low back pain, which is a leading cause of pain and morbidity worldwide. Current therapeutic strategies are limited by poor etiological understanding and by the lack of disease-modifying drugs. During disc degeneration the extracellular matrix is degraded and fragmented, and the disc undergoes biochemical and structural changes that ultimately lead to biomechanical failure. Non-degenerate discs are minimally innervated, but as they degenerate innervation increases. Increased innervation is a possible pain-generating mechanism. Proteases, proinflammatory cytokines and neurotrophins all increase within the tissue during degeneration. Proteases degrade the extracellular matrix of the disc. Cytokines and neurotrophins function as nociceptive factors, potentially leading to the development of neuronal sensitization and chronic pain. However, the regulation of these components in disc degeneration, especially during early disease stages, is poorly understood. Increased understanding of these mechanisms will identify possible disease-modifying therapeutic targets.

This thesis hypothesized degenerating discs produce and secrete proinflammatory and pronociceptive factors that further degeneration and lead to chronic low back pain. This hypothesis was investigated by carrying out four specific aims.

1) Characterized proinflammatory cytokine and neurotrophin release from non-degenerating and degenerating human discs and investigated the effects of the disc secretome on neurons.

2) Determined if NGF is regulated by toll-like receptors (TLR).

3) Determined if TLR2 activation induces degenerative changes in non-degenerating human discs.

4) Investigated if TLR inhibition acts as a disease-modifying strategy in a mouse model.

We found degenerating discs from chronic low back pain patients secrete increased levels of inflammatory and nociceptive factors that promote pain-related neurochemical changes in sensory neurons and increased neurite growth. Nerve growth factor (NGF) was identified as a key protein in regulating these changes (Manuscript I). However, little was known about the regulation of NGF in pathogenic contexts. We then investigated whether TLRs regulate NGF expression. TLRs are activated by endogenous danger signals called 'alarmins' that are generated in response to cellular stress and/or injury. Examples found in the disc include fragmented aggrecan, fibronectin, and extracellular HMGB1. Discs constitutively express TLRs and TLR1, 2, 4, and 6 expression increases with degeneration grade. Using *in vitro* cell culture assays, we found TLR2 activation regulates NGF mRNA and protein secretion through NF-κB (Mauscript II). This study, along with a number of previous *in vitro* studies suggest a role for TLRs in disc degeneration and chronic low back pain.

Due to their constitutive expression, TLRs could potentially play a role during the early stages of disc degeneration, which is an ideal target for disease modifying interventions. However, two questions remained. First, would TLR activation be sufficient to induce degenerative changes in intervertebral discs? Second, would TLR inhibition slow the progression of disc degeneration and reduce chronic low back pain? Injecting TLR2 ligands into isolated, intact non-degenerate human discs induces degenerative changes in *ex vivo* organ culture that are characterized by extracellular matrix destruction and protease and cytokine increases (Manuscript III). Furthermore, inhibiting TLR4 in the SPARC-null mouse model of degeneration reduces behavioral signs of chronic back pain. TLR4 inhibition also decreases cytokine secretion by degenerating mouse discs, suggesting TLR4 inhibition slows the progression of degeneration (Manuscript IV).

The work in this thesis found degenerating intervertebral discs secrete proinflammatory and nociceptive factors that are regulated by disc cells expressing TLRs *in vitro,* e*x vivo* and *in vivo*. These results increase the understanding of how disc derived nociceptive factors are regulated and how degenerating discs act on neurons to cause pain. This increased understanding can help identify new therapeutic strategies. Specifically, we found TLRs regulate many hallmarks of disc degeneration and their inhibition decrease pain in a mouse model. Therefore, TLRs are potential therapeutic targets to slow the progression of intervertebral disc degeneration and manage the associated chronic low back pain.

Résumé

La dégénérescence des disques intervertébraux est une des causes les plus communes de la douleur chronique lombaire. Cette maladie contribue fortement à la morbidité et à la douleur chronique dans le monde. Les stratégies thérapeutiques courantes sont limitées par le manque de connaissance étiologique et de médicaments modifiant la maladie. Lorsque le disque se dégénère, la matrice extracellulaire se dégrade et se fragmente ce qui apporte des changements structuraux et biochimiques résultant en échec biomécanique. Alors que les disques sains contiennent peu de nerfs sensoriels, les disques en dégénérescence deviennent innervés. Cette accumulation de nerfs est un mécanisme potentiel de douleur. Il y a également une augmentation de protéases, de cytokines pro-inflammatoires et de neurotrophines dans le tissu en voie de dégénérescence. Les cytokines et neurotrophines agissent comme facteurs nociceptifs qui peuvent potentiellement apporter le développement de sensibilisation neuronale et de douleur chronique. Toutefois, la régulation de ces composantes lors de la dégénérescence du disque, spécialement durant les premiers stages, reste mal comprise. Une meilleure compréhension de ces mécanismes va aider à identifier des cibles thérapeutiques pouvant modifier la maladie.

Cette thèse hypothétise que les disques souffrant de dégénérescence produisent et sécrètent des facteurs pro-inflammatoires et pro-nociceptifs qui contribuent à la dégénérescence et mènent à la douleur chronique du dos. Cette hypothèse fut investiguée par l'entremise de quatre buts spécifiques.

1) Caractérisation de la libération de cytokines pro-inflammatoires et de neurotrophines par des disques humains dégénérés et non-dégénérés et investigation des effets du sécrétome du disque sur des neurones.
2) Déterminer si NGF (nerve growth factor) se fait réguler par les TLRs(Toll-like receptors).
3) Déterminer si l'activation de TLR2 induit des changements dégénératifs dans des disques humains non-dégénérés.
4) Déterminer si l'inhibition des TLR pourrait agir comme stratégie de modification de la maladie dans un modèle murin.

Nous avons découvert que les disques venant de patients souffrant de douleur lombaire chronique sécrètent plus de facteurs pro-inflammatoires et nociceptifs, ce qui promouvoit des changements neurochimiques reliés à la douleur chez les neurones ainsi qu'une croissance des neurites. NGF (Nerve Growth Factor) fut identifié comme étant une protéine clé dans la régulation de ces changements (Manuscrit 1). Pourtant, très peu de connaissances existent sur la régulation de NGF dans un contexte pathogénique. Nous avons ensuite investigué si les TLRs (toll-like receptors) régulent NGF. Les TLRs sont activés par des « alarmins » endogènes trouvées dans le disque comme des fragments d'aggrecan, fibronectin et HMGB1 extracellulaire. Le disque exprime constitutivement les TLRs, alors que l'expression de TLR1, 2, 4 et 6 augmente avec l'indice de dégénérescence. En utilisant des tests *in vitro* en culture cellulaire, nous avons trouvé que l'activation de TLR2 régule NGF au travers de NF- κB. (Manuscrit 2)

Cette étude, en complémentarité avec plusieurs autres études in vitro suggèrent la présence du rôle des TLRs durant la dégénérescence des disques intervertébraux et de la douleur chronique lombaire.

Vu que les TLRs sont exprimés de manière constitutive dans les disques, ils pourraient potentiellement jouer un rôle dans les premiers stages de dégénérescence, ce qui est une cible idéale pour des interventions pouvant modifier la maladie. Toutefois, deux questions restent à répondre. Premièrement, est-ce que l'activation des TLRs seraient suffisante pour induire des changements dégénératifs chez les disques intervertébraux? Deuxièmement, est-ce que l'inhibition de TLRs ralentirait la progression de le la dégénérescence et réduirait la douleur lombaire chronique? En injectant des agonistes de TLR2 dans des disques humains non-dégénérés, intacts et isolés, nous avons découvert l'induction de changements dégénératifs caractérisés par la destruction de matrice extracellulaire et de l'augmentation de protéases et de cytokines. (Manuscrit III) En plus, l'inhibition de TLR4 chez le modèle murin de dégénérescence SPARC-null réduit les signes comportementaux reliés à la douleur. L'inhibition de TLR4 décroit également la sécrétion de cytokines par les disques dégénérés de souris, suggérant le ralentissement de la dégénérescence. (Manuscrit IV)

Le travail complété dans cette thèse a trouvé que les disques intervertébraux dégénérés sécrètent des facteurs pro-inflammatoires et nociceptifs qui sont régulés par les cellules du disque qui expriment les TLRs *in vitro,* e*x vivo* et *in vivo.* Ces résultats aident à mieux comprendre comment les facteurs nociceptifs dérivés du disque sont régulés et comment les disques dégénérés agissent sur les neurones résultant en douleur. Cette meilleure compréhension peut aider à identifier des nouvelles cibles thérapeutiques. Plus précisément, nous avons découvert que les TLRs régulent plusieurs caractéristiques de la dégénérescence du disque et leur

inhibition diminue la douleur dans un modèle murin. Finalement, les TLRs sont des cibles potentielles pour ralentir la progression de la dégénérescence des disques intervertébraux et contrôler la douleur chronique lombaire qui y est associée.

Acknowledgements

Science is a team effort and the work in this thesis would not have been possible without the fantastic people I have been surrounded by. Many people have had lasting influences on me and only some will be listed here. Dr. Lisbet Haglund has been the best Ph.D. supervisor and mentor I could have imagined, and she is largely responsible for why I have enjoyed graduate school so much. She has given me a lot of scientific, career and life advice. Lisbet can also be incredibly caring; I once missed the last direct bus from the airport to the hotel in Xi'an, China, took another bus, got lost and ended up taking a motorized rickshaw across the city. I arrived in the middle of the night to a very concerned and upset Lisbet wondering where I was. Dr. Jean Ouellet, my co-supervisor, lent his expertise to project design and manuscript writing and managed the Transplant Quebec program. My thesis committee consisted of Drs. Laura Stone, Michael Weber, and Jake Barrelet (Chair). Laura has been an excellent mentor. She encouraged my interest in pain research from the beginning and has spent a lot of time helping me to pursue a path in pain research. Dr. Weber asked questions that would force me to think about the bigger picture and came on a number of spine harvests. Dr. Barrelet always made sure I was on track to finish. More importantly, he gave me a lot of anecdotal advice ranging from "Do fewer western blots and write your thesis" (Dr. Jake Barrelet, a paraphrased quote, 2016) to "Always make sure to have some data stashed away that your supervisor does not know about for after vacations or bad weeks" (Dr. Jake Barrelet, a paraphrased quote, ~Dec. 22, year unknown).

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Preface and Contribution of Authors

This thesis-based manuscript consists of four multi-authored manuscripts. Each manuscript consists of several original and distinct contributions of knowledge that will be highlighted and explained throughout this thesis. For each manuscript I (E. Krock) conducted the majority of experiments and data analysis, and wrote the initial draft of each manuscript. However, none of the work would have been possible without the contributions my coauthors made in designing and conducting experiments, analyzing data and providing funding. D.H. Rosenzweig, A-J. Chabot-Doré, and M. Millecamps provided training for many of the experimental techniques I used in addition to performing experiments. Below is a categorical list of each authors' contribution for every manuscript.

Manuscript I: Painful, degenerating intervertebral discs up-regulate neurite sprouting and CGRP through nociceptive factors (Published in the Journal of Cellular and Molecular Medicine, 2014)

Study design: E. Krock, D.H. Rosenzweig, J.A. Ouellet, L.S. Stone, L. Haglund

Conducted experiments and analyzed data: E. Krock, D.H. Rosenzweig, A-J. Chabot-Doré

Human Tissue Collection: J.A. Ouellet, M.H. Weber, P. Jarzem

Writing the manuscript: E. Krock, D.H. Rosenzweig, L.S. Stone, L. Haglund

Funding: J.A. Ouellet, L.S. Stone, L. Haglund

Manuscript II: Nerve Growth Factor Is Regulated by Toll-Like Receptor 2 in Human Intervertebral Discs (Published in the Journal of Biological Chemistry, 2016)

Study design: E. Krock, D.H. Rosenzweig, L.S. Stone, L. Haglund

Conducted experiments and analyzed data: E. Krock, D.H. Rosenzweig, J.B. Currie

Human Tissue Collection: J.A. Ouellet, M.H. Weber

Writing the manuscript: E. Krock, D.H. Rosenzweig, L.S. Stone, L. Haglund

Funding: E. Krock, J.A. Ouellet, L.S. Stone, L. Haglund

Manuscript III: Toll-like Receptor 2 Activation Induces Human Intervertebral Disc Degeneration (In preparation)

Study design: E. Krock, D.H. Rosenzweig, L. Haglund

Conducted experiments and analyzed data: E. Krock, D.H. Rosenzweig, J.B. Currie

Human Tissue Collection: J.A. Ouellet

Writing the manuscript: E. Krock, D.H. Rosenzweig, L. Haglund

Funding: E. Krock, J.B. Currie, J.A. Ouellet, L. Haglund

Manuscript IV: Toll-like Receptor 4 Inhibition Decreases Disc Degeneration and Pain in a Mouse Model (In preparation)

Study design: E. Krock, M. Millecamps, L.S. Stone, L. Haglund

Conducted experiments and analyzed data: E. Krock, M. Millecamps, J. B. Currie

Writing the manuscript: E. Krock, L.S. Stone, L. Haglund

Funding: E. Krock, M. Millecamps, J. B. Currie, L.S. Stone, L. Haglund

List of Abbreviations

ADAMTS - A Disintergrin and Metalloproteinase with Thrombospondin Motifs

AEB – acetone evoked behavior

AF- Annulus Fibrosus

BDNF – Brain Derived Neurotrophic Factor

CGRP – Calcitonin Gene-Related Peptide

DAMP – Danger Associated Molecular Pattern

DMEM – Dulbecco's Modified Eagle Medium

DRG – Dorsal Root Ganglia

ECM – Extra Cellular Matrix

GAG- Glycosaminoglycan

GFAP – glial fibrillary acidic protein

HMGB1 – High Mobility Growth Box 1

HSP – Heat Shock Protein

HTRA1 – High-Temperature Requirement A serine protease 1

IFN – Interferon

IL- Interleukin

iNOS – inducible Nitric Oxide Synthase

IR – Immunoreactivity

ITS – Insulin-Transferrin-Selenium

MAPK – Mitogen-Activated Protein Kinase

MMP – Matrix Metalloproteinase

MRI – Magnetic Resonance Imaging

MRI – Magnetic resonance imaging

NF-κB – Nuclear Factor-light-chain-enhancer of activated B cells

NGF – Nerve Growth Factor

NP – Nucleus Pulposus

NT – Neurotrophin

PAMP – Pathogen Associated Molecular Pattern

PBS = Phosphate Buffered Saline Solution

SLRP – Small Leucine-Rich Repeat Protein

SPARC – Secreted Protein Acidic and Rich in Cysteine

TIR – Toll/Interleukin Receptor

TLR – Toll-like Receptor

TNF – Tumor Necrosis Factor

Trk – Tropomyosin receptor kinase

TRPV1 – Transient receptor cation channel subfamily V member 1

Chapter 1: Introduction

1.1 The Problem of Chronic Low Back Pain and Disc Degeneration

Chronic low back pain is the leading cause of disability worldwide according to the Global Burden of Disease study [2,3]. Up to 80% of people will experience an episode of low back pain in their lifetime and 15-30% of people develop chronic low back pain. The high prevalence results in massive direct healthcare costs and indirect costs, such as days of work lost. Examples of annual costs associated with chronic low back pain include $85.9 billion in the United States, £12.3 billion in the United Kingdom, and €16.5-50 billion in Germany [4,5]. The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." Pain is considered chronic when it "persists beyond the normal healing time" and is defined by the International Classification of Disease as "recurring or persistent pain that lasts longer than three months." The North American Spine Society Clinical Care Guidelines defines chronic low back pain as "pain of musculoskeletal origin that can extend from the bottom rib through the lumbar region, and at time extends as somatic referred pain, also known as radiating pain, in the thigh." Despite chronic low back pain being a widespread and costly health problem, current treatments are limited.

Many tissues can contribute to chronic low back pain including facet joints, ligaments, muscle and intervertebral discs. Of these structures, intervertebral discs, through disc degeneration and herniation, are one of the most common pathological causes of chronic low back pain [6]. As such, several therapeutic strategies have been developed to manage disc degeneration and chronic low back pain. Strategies range from conservative approaches such as physical therapy and analgesic drug regimens, to more invasive surgical approaches including

discectomies, lumbar fusions and disc replacement. Conservative strategies often fail to sufficiently manage patients' pain while surgical interventions are highly invasive, do not always lead to pain resolution, and have high reoperation rates [7,8]. Lumbar fusions may alleviate patients' pain but they are also a risk factor for the adjacent discs to degenerate, which results in more pain and surgery. Currently, no disease modifying drugs that slow the progression of degeneration and/or alleviate pain are available. Thus, existing therapeutic strategies do not satisfactorily manage disc degeneration and chronic pain. Poor understanding of the pathological mechanisms underlying disc degeneration and chronic low back pain limits the potential of therapeutic strategies. Increased understanding of these mechanisms will lead to the identification of new therapeutic targets and approaches.

1.2 Comprehensive Literature Review and Background

1.2.1 Spine and Intervertebral Disc Structure

The human spine is composed of 33 vertebra divided into five sections: 7 cervical, 12 thoracic, 5 lumbar and 5 fused sacral vertebra and 4 fused vertebra in the coccyx. Adjacent vertebrae are separated by 23 intervertebral discs, with the exception of the cervical 1 and 2 vertebrae, sacrum and coccyx [1]. Intervertebral discs are pads of fibrocartilage composed of the central nucleus pulposus (NP), the outer annulus fibrosus (AF), which surrounds and contains the NP. Two cartilaginous endplates are found anteriorly and posteriorly of the NP and AF, separating the disc from the vertebral bodies (Fig. 1). Discs function as motion segments, similar to a joint, and allow the spine to compress, twist and bend. Adult discs are almost aneural and avascular, with only a few fibers and vessels found in the out edge of the AF [9]. Due to the lack of vasculature, the disc is an immune privileged tissue, meaning there are no resident immune cells and no direct source of immune cells [10]. Each tissue is composed of distinct cell types (NP cells,

AF cells and chondrocytes) with distinct lineages. All three cell types are responsible for both anabolic and catabolic processes in their respective tissues [6]. The unique structure of each tissue type allows the disc to perform its biomechanical functions.

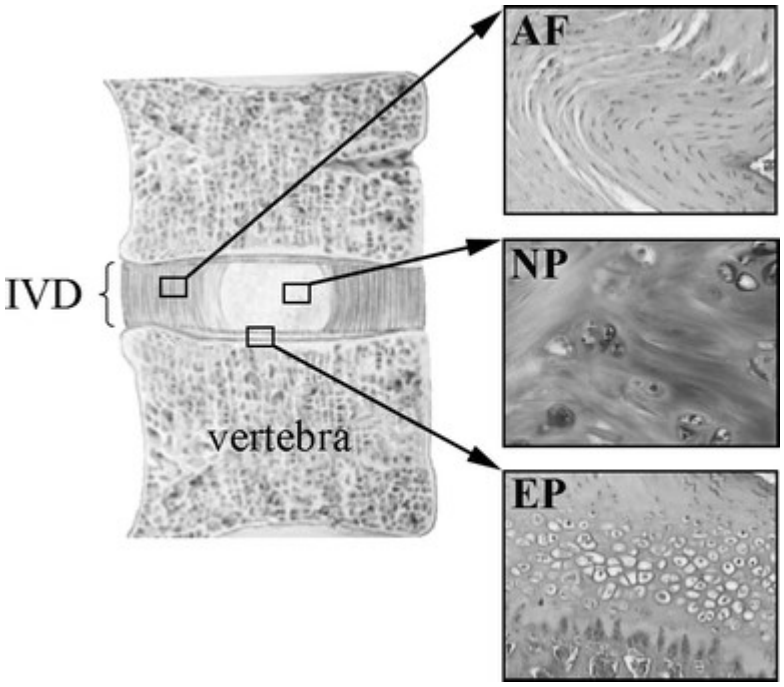

Figure 1. Schematic of Intervertebral Disc Structure. The intervertebral disc (IVD) is sandwiched between two adjacent vertebra. The disc is made up of the central nucleus pulposus (NP), the annulus fibrosus (AF), which surrounds the NP, and a thin layer of hyaline cartilage called the cartilaginous endplate. The image on the left illustrates the laminar structure of the AF. The micrographs on the right show the laminar structure of the AF, the random structure of the NP and the transition between bone and in the disc that in the endplate. This image was used with permission from the publisher.

1.2.1.1 Nucleus Pulposus

The fetal NP is fluid and becomes more gel-like in juveniles. As collagen content increases with age, the NP increasingly become less fluid-like and more gel-like until it shows fibrotic characteristics in adulthood. The biochemical composition of the NP confers its

biomechanical function to act as a shock absorbing tissue [1]. The extracellular matrix (ECM) of the NP (Fig.1 and 2) consists of randomly distributed type II collagen fibers, elastin and has high proteoglycan content. The high proteoglycan content in the NP is illustrated by a proteoglycan to collagen ratio of 27:1, compared to 2:1 in hyaline cartilage [11]. The main proteoglycan found in the NP is aggrecan [6,12]. Aggrecan is loaded with anionic keratin sulfate and chondroitin sulfite glycosaminoglycan (GAG) chains. The anionic charge of the GAG chains makes the NP a hydrophilic tissue, resulting in high water content. Small leucine-rich repeat proteins (SLRP) also contribute to the development and structure of NP ECM. The ECM composition leads to the NP's gel-like structure that allows for compression when the spine is loaded and reswelling when load is removed. During development, notochordal cells populate the NP. In humans notochordal cells begin to disappear and are replaced by small, round chondrocyte-like cells within the first ten years of life[6]. Recent studies have suggested that notochordal cells found in the NP serve as NP progenitor cells and give rise to the small chondrocyte-like NP cells that are found in mature individuals [13]. In an adult, the NP cell density is low (~3000 cells/mm^3) and cells are randomly distributed [14]. NP cells synthesize, repair and regulate the NP ECM. The unique cell type and ECM structure of the NP confer its function. However, these same characteristics contribute to disc degeneration and hamper tissue repair strategies.

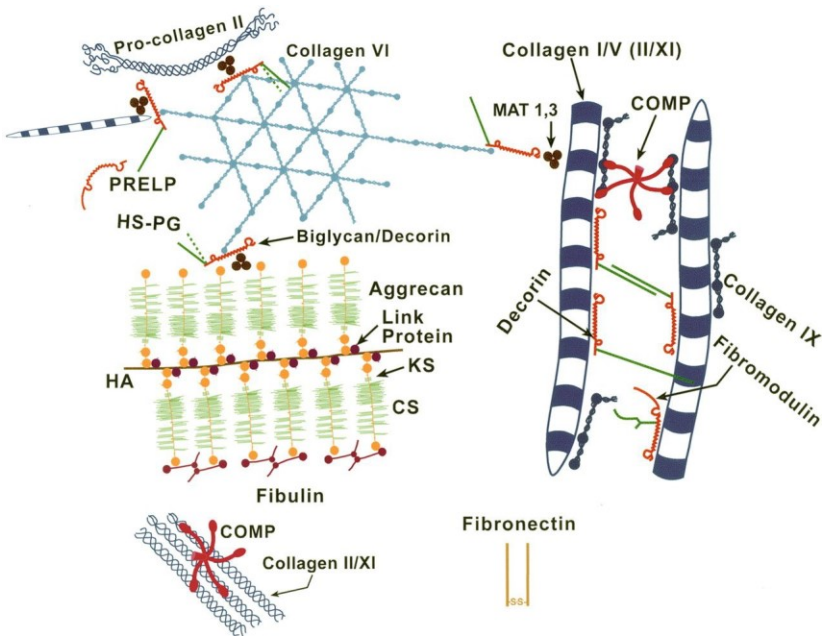

Figure 2. Schematic of key extracellular matrix components in the disc. NP ECM is primarily composed of hyaluronic acid backbones connected with link protein to aggrecan containing keratin sulfate (KS) and chondroitin sulfate (CS) GAG chains and a type II collagen network. AF ECM has proportionately less aggrecan and type II collagen and more type I collagen. Several other proteins including cartilage oligomeric matrix protein (COMP), fibulin, fibronectin, decorin, fibromodulin, matrilin (MAT), biglycan, proline arginine-rich end leucine-rich repeat protein (PRELEP), and chrondroadherin (not picture) contribute to ECM assembly and stability. Many of these proteins are proteolyticly cleaved and degraded during degeneration, leading to ECM destabilization and GAG loss, which results in water loss and ultimately biomechanical failure. This image was used with permission from the publisher [15].

1.2.1.2 Annulus Fibrosus

The AF surrounds the NP and is subdivided into the inner AF and the outer AF. As a whole, the AF has less type II collagen and less aggrecan and more type I collagen than the NP [16]. The proteoglycan to collagen ratio is approximetly 1.6:1 [11]. The inner AF serves as a

transition zone between the NP and outer AF. Compared to the outer AF, the inner AF has a less organized ECM and a higher proteoglycan and type II collagen content [1,6]. The resident inner AF cells are round chondrocyte-like cells similar to the NP, but are of mesenchymal in origin. The outer AF has elongated fibroblastic-like cells and AF cell density is ~5000 cells/mm^3 in young adults [14]. The outer AF has more collagen than proteoglycan by dry weight, and unlike the NP and inner AF, type I collagen is the predominant collagen species [6,17]. The collagen fibrils are organized into fibers that form 15-25 concentric lamellar rings alternating 30-45^0 to the axis of the spine. This organization give the AF a high tensile strain that allows for bending and twisting of the spine, and for the AF to resist NP bulging when the NP is compressed.

1.2.1.3 Cartilaginous Endplate

The cartilaginous endplate is a thin (~0.6 mm) layer of hyaline cartilage capping intervertebral discs at either end. It covers the NP and inner AF but not the outer most part of the outer AF. The endplate contains chondrocytes found at a density of ~10 000 to 18 000 cells/mm^3 [14]. Its ECM is composed of type II collagen, aggrecan and other proteins and proteoglycans. The proteoglycan to type II collagen ratio of approximately 2:1[11]. The endplate has several important functions. First, it protects the disc from the adjacent vertebrae, which could damage the NP and inner AF. Secondly, the endplates contain the NP and prevent it from herniating into the vertebral body. Thirdly, the endplate serves as the primary location for nutrient diffusion into the disc and waste diffusion out of the disc [18]. Blood vessels found in the vertebrae adjacent bud into the outer layer of the endplate and deliver nutrients and absorb waste.

1.2.2 Intervertebral Disc Degeneration and Degenerative Disc Disease

Intervertebral disc degeneration is defined by Michael Adams and Peter Roughley as “an aberrant, cell-mediated response to progressive structural failure. A degenerate disc is one with

structural failure combined with accelerated or advanced signs of aging. Degenerative disc disease should also be applied to a degenerate disc that is also painful" [6] This definition is a useful framework to discuss disc degeneration and degenerative disc disease because it draws attention to the key characteristics and mechanisms of degeneration. Degenerating disc height decreases, discs bulge, or herniate (Fig. 3) and they lose their biomechanical function. NP hydrostatic pressure is lost and compressive loads are redistributed from the NP to the AF [19]. Structural failure is also characterized by annular or endplate tears and fissures, which can lead to NP herniation through the AF or endplate [6]. Large-scale ECM remodeling mediates the degenerative changes that lead to structural failure [6,16]. The inclusion of a cell-mediated process in the definition implies several things. Cells respond to mechanical and chemical signals, and adverse signals promote degenerative processes [20-23]. The definition also indicates cells actively contribute to disc degeneration. Importantly, the definition says degenerative disc disease should be applied to discs that are painful. Pain is what brings patients to a clinic and results in a disc degeneration diagnosis. Since Adams and Roughley published this definition in 2006 several other factors have been suggested as critical hallmarks of disc degeneration. These factors still fit within the framework but are more specific. Increases in matrix proteases and proinflammatory cytokines and most recently immune cell infiltration are suggested to be defining characteristics of disc degeneration [10]. These changes will be discussed in greater detail below.

Several macroscopic and microscopic changes occur during disc degeneration. Macroscopic changes include end-plate thinning, loss of demarcation between the inner AF and NP, disc thinning, disc bulging, and loss of NP water content. Both the NP and AF become discolored from non-enzymatic glycosylation and are increasingly calcified. The NP also loses its gelatinous characteristics and becomes more fibrotic. Compressive loads that are redistributed

to the AF further exacerbate AF degeneration. Additionally, disc pH and nutrition decrease, making the disc an adverse environment for self-repair [6,10,18,24,25]. Degeneration can be visualized and classified using imaging techniques and grading schemes, such as the commonly used Pfirrmann Grade. The Pfirrmann Grade is determined by using T2-weighted magnetic resonance imaging (MRI), which gives insight into morphological changes and water loss [26]. Beyond the structural changes described above, several adverse biochemical changes occur. These include degradation of the ECM, and increased levels of matrix proteases, proinflammatory cytokines and chemokines, and neurotrophins.

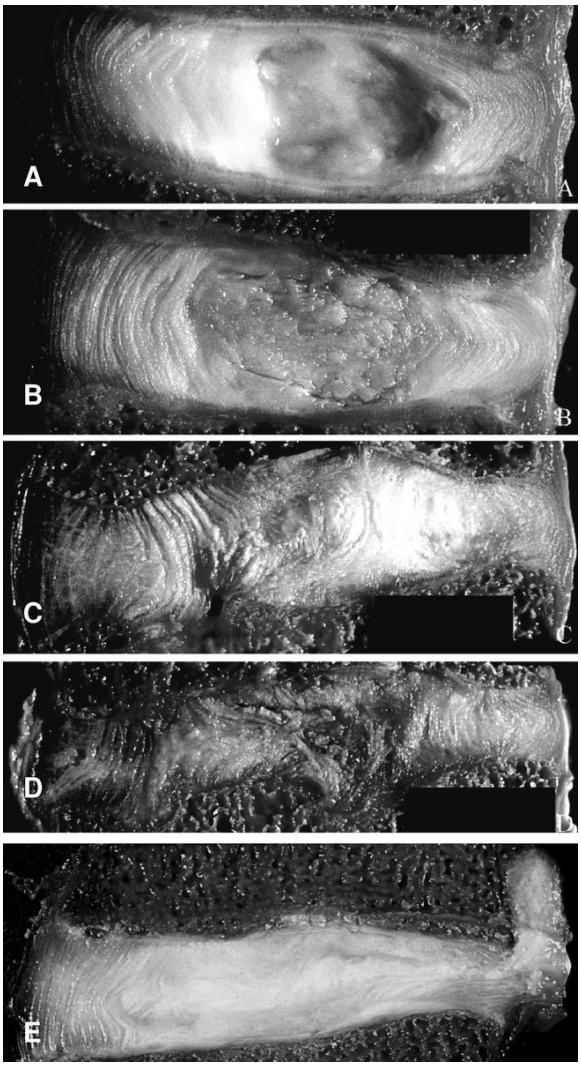

Figure 3. Images of cadaveric lumbar discs illustrating different stages of degeneration. A is a disc from a young 35 year old male. B is a 47 year old male. Note how the NP has lost it gel like structure that is evident in A. C and D are examples of degenerate and severly degenerate discs. Demarcation between the NP and the AF is lost, and endplate disruption is evident. E is an image of a herniated disc. Note how the AF and NP division is clear on the left, and the NP still looks gel like. This image was used with permission from its original source [1].

1.2.2.1 Matrix Catabolism

During degeneration the AF, NP and cartilage end-plate ECM undergo massive remodeling. In both the NP and the AF proteoglycan synthesis and content decline. In the NP and inner AF Type II collagen is gradually replaced with type I collagen. Collagen fibrils thicken, and become increasingly cross-linked. Furthermore, the NP, inner and outer AF become progressively calcified. All of these changes cause the NP to become fibrotic, contributing to loss of mechanical function [6,24]. ECM components are also degraded and fragmented. For example, fragmented collagen, aggrecan, fibronectin, biglycan and versican products are all increased in degenerating discs compared to non-degenerating discs [6,12,27]. These fragments themselves have biological activity (discussed below in the toll-like receptor section) and result in ECM destabilization. GAG chains are also cleaved and lost as aggrecan is degraded. Additionally, as degeneration progresses ECM synthesis declines. Degradation of aggrecan, other proteoglycans and GAGs reduces the anionic charge of this disc, thus reducing the disc's hydrophilic properties. Decrease hydrophilic properties result in water and hydrostatic pressure loss in the NP, altering NP biomechanical characteristics. These matrix changes result in load redistribution and contribute to the mechanical failure of the disc.

Matrix proteases including matrix metalloproteinases (MMP)-1, -3, -7, -9, and -13, cathepsins [28,29], high-temperature requirement serine protease A1 (HTRA1) [30-32] and a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS) 4 and 5, also termed aggracanases, [33,34] and ADAMTS 1, 9 and 15, all increase during disc degeneration. The activity of many of these proteases is required for normal matrix turnover. However, when protease levels increase or protease inhibition mechanisms are reduced (i.e. tissue inhibitor of metalloproteinase TIMP)), they degrade the disc ECM, as described above. Thus, proteases

increases are a hallmarks of disc degeneration. Combined, proteolytic degradation and decreased matrix synthesis are responsible for ECM changes in degenerative discs.

Endplate changes during degeneration are less characterized than NP and AF changes. Similar to the NP and AF endplate ECM undergoes remodeling. Due to size, the endplate itself is difficult to image but endplate defects are thought to be a source of Modic changes. Modic changes are vertebral bone marrow lesions visualized by MRI that are not associated with marrow malignancies, pyogenesis, or seropositive rheumatic disorders, are characterized by inflammation and adverse bone remodeling, and are strongly associated with chronic low back pain. Endplate defects also allow for the nucleus to herniate through the endplate into the vertebral body. Herniations are then calcified, resulting in the formation of Schmorl's nodes, which can also be imaged and are also linked to chronic low back pain [35,36]. Effects of endplate ECM degradation and calcification on diffusion of nutrients into the disc and waste products out of the disc are unclear. Diffusion has been suggested to decrease, and more recently endplates have been suggested to become more permeable as they degenerate [37,38]. Either situation has negative implications for degeneration. Decreased diffusion decreases nutrient delivery, making it more difficult for cells to survive, whereas increased permeability could allow for cytokines and neurotrophins to diffuse into the vertebral bodies and immune cells to migrate into the disc. Regardless, it seems likely the endplate has an important but poorly characterized role in disc degeneration.

1.2.2.2. Sterile Inflammation

A hallmark of painful disc degeneration is sterile inflammation, which is the increase of proinflamamotry cytokines and chemokines in the absence of pathogens. In disc degeneration Interleukin-1β (IL-1β) and Tumor Necrosis Factor α (TNFα) are the most investigated cytokines

[10]. Both IL-1β and TNFα are produced by NP and AF cells and their expression increases during degeneration [39-41]. Their initial induction in the early stages of degeneration is poorly understood, but it is likely several mechanisms contribute to their increases. Mechanisms behind cytokine increases include genetic alleles, such as IL-10, IL-1 and IL-6 [42-48], adverse mechanical strain [20,21,23] and smoking [49]. Their expression is increased by other cytokines, such as IL-6, and by activation of their own receptors. IL-1β and TNFα increase protease levels, decrease matrix synthesis [50-52], and increase production of other proinflammatory cytokines, chemokines and neurotrophins [10]. Both have been investigated extensively as therapeutic targets. Clinical trials have tested TNFα inhibitors and are discussed below. IL-1β inhibitors, such as Anakinra, have not been tested in humans with disc degeneration.

A large number of other proinflammatory cytokines, including CCL2, 5, and 7 and, Interferon γ (IFNγ), IL-2, -4, -6, -7, -8, -12 and -17, and CXCL1 and 9, increase in degenerating discs [10,22]. The roles of only some of these factors have characterized roles in degeneration. For example, IL-6 and IL-8 regulate other cytokines and catabolic factors, and can act as chemoattractant factors for immune cells [22]. Furthermore, they are both associated with pain in animal models of disc degeneration or herniation [53,54]. It is likely IL-1β, TNFα and other cytokines and chemokines work together to contribute to degeneration and pain. Therefore, it may by insufficient to target only one cytokine or chemokine to slow or stop degeneration.

Non-degenerate discs are considered an immune privileged tissue, meaning they lack resident immune cells and vasculature to supply immune cells. However, an increasing amount of evidence suggests that following disc herniation neovascularization occurs and immune cells infiltrate the disc [55]. Whether immune cells infiltrate degenerating, non-herniated discs is less clear. Cells infiltrating herniated disc may include T cells, neutrophils, macrophages and mast

cells [10]. Evidence of immune cell infiltration is not straight forward. For example, many early studies investigating immune cell infiltration used CD68 as a marker for marcrophages. CD68 is expressed on membranes of lysozymes and Nerlich *et al* found CD68+ cells in NP tissue of degenerating, non-herniated discs. Cell morphology characteristics of the cell lead them to propose that the CD68+ cells are not immune cells but resident NP cells that have undergone a phenotypic change [56]. More recently Chen *et al* found NP cells can display macrophage like behaviors characterized by a phagocytic phenotype and morphological characteristics [57]. More recent studies have found immune cells infiltrate herniated disc tissues using CD11b, which is a marker for leukocytes [55,58]. Immune cells are likely attracted by the increase of proinflammatory cytokines and chemokines produced by resident NP and AF cells. They may recognize disc tissue as 'non-self' and will further increase proinflammatory factors that are found in degenerating discs [10], further contributing sterile inflammation, matrix catabolism and the development of pain.

1.2.2.3. Possible Mechanisms Leading to Pain

Non-degenerating discs are almost anural, with only the outer few lamina of the AF being innervated [9]. However, a number of studies found degenerating discs from low back pain patients have increased innervation and innervation of normally aneural areas (Fig. 4). Some of the nerve fibers innervating the disc also contain pain-related neuropeptides like substance P [59-61]. Provocative discography demonstrates non-degenerating discs are not painful, but when degenerating discs are provoked, low back pain is elicited [6] . Discs are primarily innervated by small-diameter, nociceptive dorsal root ganglia (DRG) neurons [9]. A recent study also found neurofilament-200 immunoreactive fibers, suggesting that discs may also be innervated by large diameter, myelinated neurons [62]. DRG neurons project onto the spinal cord of the dorsal horn

where nociceptive signals can be transmitted and result in pain. The development of chronic low back pain can be attributed to two distinct but over lapping causes: mechanical/neuropathic injury or inflammatory sensitization of nerve fibers.

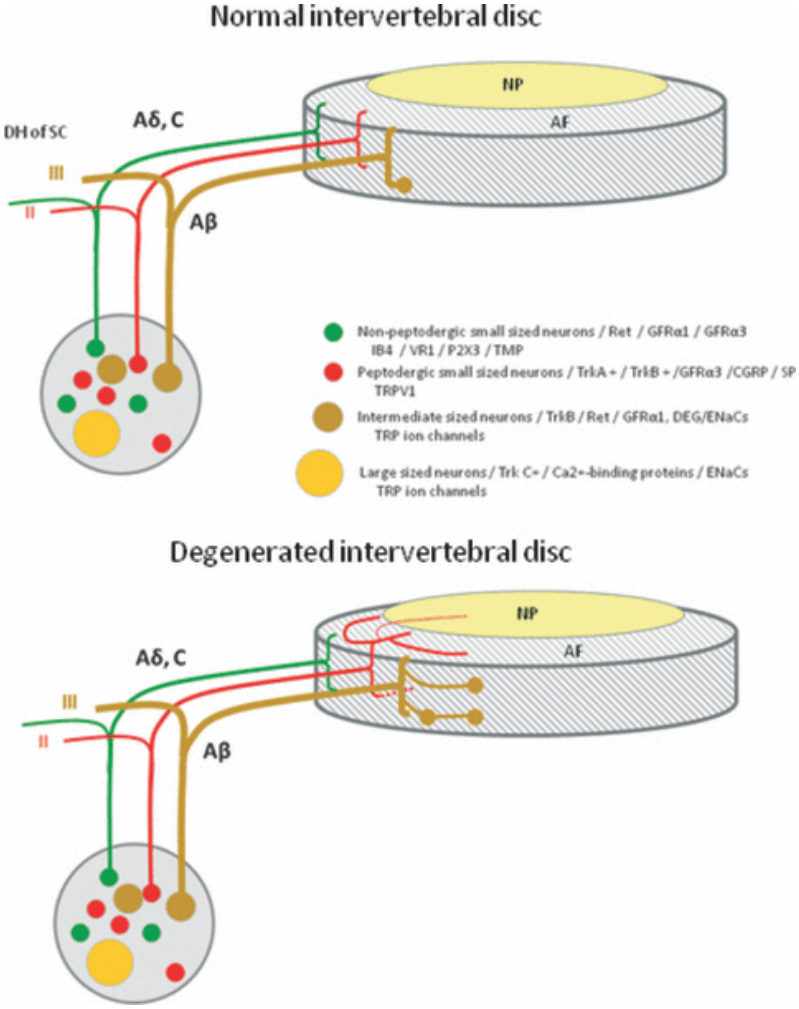

Figure 4. Sensory Innervation of Normal and Degenerating Intervertebral Discs.

Only the outer layers of the annulus fibrosus are innervated by DRG sensory neurons (top). As discs degenerate they become increasingly innervated in the outer layers of the AF and in areas of the disc that are normally aneural. Different neuron-types are represented in different colors. This image was used and adapted with permission of the publisher [9].

1.2.2.3.1 Mechanical Sources of Pain

Mechanical injury to the nervous system can cause neuropathic pain. In the context of disc degeneration, this can occur when disc herniation, collapse, loss of height or bulge compresses the spinal cord, DRG or dorsal root [6]. These mechanical injuries can result in both radiating pain in the leg and low back pain. The initial neuropathic injury causes pain, and the subsequent sterile inflammation will further contribute to the development of neuronal sensitization and chronic pain [22]. The work in this thesis does not address mechanical injury and pain but instead focuses on the role of cytokines and neurotrophins in pain.

1.2.2.3.2 Neurotrophic and Inflammatory Pain

As addressed above, a hallmark of disc degeneration is sterile inflammation. In addition to increases in cytokines and chemokines, several neurotrophins increase in the inflammatory milieu of degenerating discs. The neurotrophin family is comprised of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5. Neurotrophins bind their specific high affinity receptors TrkA (NGF), TrkB (BDNF/NT-3) and TrkC (NT-4/5) and they all bind the low-affinity receptor $p75^{NTR}$ [63]. During development neurotrophins promote neuronal survival, growth, maturation and innervation of target tissues. However, neurotrophins are also peripheral mediators of pain, and NGF is the best characterized. NGF is often found in the inflammatory milieu following nerve injury or other sources of inflammation related to pain. Through TrkA binding NGF promotes a rapid sensitization of peripheral nociceptive fibers, although the precise mechanisms are not fully understood. One proposed mechanism functions through TRPV1(Transient Receptor Potential cation channel subfamily V member 1) an ion channel involved in heat and inflammatory pain. TrkA activation is thought to increase TRPV1 sensitization and insertion into the membrane leading to neuronal sensitization. Also, retrograde transport of NGF to the DRG soma increases expression of several

pain-related proteins such as TRPV1, substance P and calcitonin gene-related peptide (CGRP) [64,65]. Preclinical studies indicated NGF likely contributes to pain in degenerative disc disease. NGF gene and protein expression is increased in degenerating human discs [55,66-69] and NGF controls expression of neurochemical pain markers, like calcitonin gene-related peptide (CGRP) in animal studies [70,71].

Cytokines and chemokine have multiple roles in the development of pain. Cytokines increase pronociceptive factors such as prostaglandins, bradykinin and NGF [72]. Additionally, cytokine receptors, such as those for IL-1β, IL-6 and TNFα, are expressed by subsets of DRG neurons. Cytokines directly act on neurons to increase their activity and persistent sensitization [65,73]. DRG neurons also express chemokine receptors. For example the receptors for CCL2 (CCR2), and CXCL8/IL-8 and CXCL1 (CXCR1 and 2) are expressed by DRG neurons and activation leads to increased activity, sensitization and TRPV1 activity modulation [74]. Preclinical and clinical evidence suggest that cytokines and chemokines directly contribute to chronic muscoskeletal pain including low back pain or osteoarthritis [10,75]. For example, animal models of disc herniation and injury have suggested IL-6 and TNFα have direct roles in pain [54,76,77].

1.2.2.4 Disease Modifying Drugs

No disease modifying drugs for intervertebral disc degeneration and chronic low back are approved. Disease modifying drugs can be defined as a drug that alters the course of an underlying pathology in a beneficial manner [78]. With regards to disc degeneration and chronic low back pain, a disease-modifying drug should slow or inhibit the progress of disc degeneration. While a drug would ideally lead to disc regeneration, this would be unlikely due to the low cell population, poor nutrition and low pH [25,79,80]. Outcome measures for success of a drug would need to relate to slowing or inhibiting the progression of degeneration. For example, this could

be defined as decreased proinflammatory cytokines, proteases and neurotrophins, or less progress of degeneration by MRI compared to a control group of patients. Slowing or inhibiting degeneration could then make the disc more permissible for tissue engineering strategies to rebuild the disc, such as stem cell therapy via intradiscal injection. A disease modifying drug that also reduces chronic low back pain, the clinical symptom, would be ideal and possible. For example, proinflammatory cytokines such as TNFα or IL-1β can act as nociceptive factors and they also regulate NGF. Therefore, a drug that targets the proinflammatory environment of the disc may reduce pain. However, as discussed above, there can also be a mechanical component to chronic low back pain, which a drug may not resolve. A disease-modifying drug could exist as an inhibitor of a specific target or biological process or it could exist as a biologic factor that may affect disease progress. An example of the latter could perhaps be Link-N, which is an N-terminal fragment of Link protein that disc cells to produce *de novo* ECM and decrease the adverse effects of IL-1β [81]. However, only the former will be reviewed here. Several targets have been identified as candidates for disease modifying drug targets and a range of evidence exists in support for or against each.

Cytokines have been targeted in clinical trials with patients suffering from disc degeneration, low back pain and sciatica. Cytokines may be disease modifying drug targets due to their regulation of proteases, neurotrophins and matrix synthesis. Furthermore, cytokines may directly cause neuronal sensitization, and this effect could be blocked. TNFα is the most investigated cytokine target. Several TNFα inhibitors have been tested in low back pain patients, including Etanercept, a receptor decoy, and Adalimumab and Infliximab, which are monoclonal antibodies targeting TNFα. Of these Etanercept is the most studied. For example, epidural injections of Etanercept improve low back pain, leg pain and numbness in lumbar spinal stenosis

patients [82]. A subsequent study found intradiscal injection of Entanercept decreases low back pain scores and disability scores 1 day, and 1, 2, 4, and 8 weeks after injection [83]. A recent meta-analysis strongly recommends further investigation of Etanercept to treat low back pain and sciatica [84]. Il-6 has also been targeted using Tocilizumab, a monoclonal antibody blocking the activation of the IL-6 receptor (IL-6R). Epidural administration decreases leg and back pain due to sciatica and decreased leg numbness 3 days, and 1, 2, 4 and 8 weeks after injection [85]. A single intradiscal injection of Tocilizumab also improves low back pain and disability scores 2 and 4 weeks after injection [86]. Initial studies suggest IL-6 may be a feasible target to manage low back, sciatica and disability associated with disc degeneration, although larger studies are required. Both the TNFα and IL-6 inhibition studies show that these may be promising analgesic targets. However, it is not possible to determine whether these drugs have disease-modifying effects or only analgesic effects since disc degeneration was not evaluated in these initial trials.

NGF has been a heavily investigated therapeutic target for a variety of muscoskeletal pain conditions for the past decade including rheumatoid arthritis, osteoarthritis and chronic low back pain. As mentioned above, as well as in manuscripts within this thesis, NGF has a well-established role in pain, and including disc degeneration and pain. However, NGF is associated with chronic pain rather than mechanisms of degeneration [10]. Nevertheless, since NGF is strongly linked to the development of pain, anti-NGF therapeutics that have gone through clinical trials that are worth discussing.

Several humanized monoclonal antibodies against NGF have been developed including Tanezumab, Fulrunumab, and Fasinumab. All have been investigated in osteoarthritic pain but less so for low back pain [87]. Of these, Tanezumab has been the most studied, with clinical trials beginning 10 years ago. Anti-NGF therapeutics are effective at reducing pain and disability

associated with osteoarthritis. However, in 2010 the Food and Drug Administration placed all anti-NGF clinical trials on partial hold due to the occurrence of adverse side effects, with another hold in 2013. Adverse side effects included osteonecrosis, rapidly progressing osteoarthritis, and parasthesias, but the overall incidence was low [87].While anti-NGF therapeutics have been successful for OA pain, their success in low back pain is less clear. Tanezumab has been shown to reduce back pain [88], but only a few clinical trials specific to low back pain have been conducted. A 2014 meta-analysis found low to moderate evidence supporting the use of Tanezumab but no evidence to support the use of fasinumab or fulranumab [89]. The small number of studies and mixed results have been suggested to be a reflection of the complicated multifactorial nature of low back pain [87]. Following the lift on the clinical hold on anti-NGF trials, several new trials have begun or are recruiting patients to evaluate anti-NGF therapeutics to manage low back pain. While preclinical data suggests NGF is an important pain-management target in low back pain, we will have to wait and see if they will be as successful in low back pain as they were for OA.

1.2.2.5 Toll-like Receptors

Toll-like receptors (TLR) are pattern recognition receptors originally characterized as having microbial components as their ligands, which are termed pathogen associated molecular patterns (PAMPs). PAMPs include peptidoglycan, lipopolysaccharide and viral ribonucleic acid (RNA) [90]. More recently TLRs were found to be activated by endogenous danger associated molecular patterns (DAMPS), also termed alarmins. Examples of alarmins include degradation products of ECM components, such as fragmented hyaluronic acid, fibronectin, and aggrecan; and normally cytosolic or nuclear proteins that are secreted or released into the extracellular space, such as high mobility group box 1 (HMGB1) or heat shock proteins (HSP) [91,92]. Humans express 10

TLRs (TLR1-10) and TLRs1, 2, 4 and 6 are most commonly associated with alarmin recognition. A number of immune cell types express TLRs and a number of other cell types, including NP and AF cells, chondrocytes and neurons.

1.2.2.5.1. Toll-like Receptor Signaling

TLRs recognize PAMPs and alarmins either as homodimers or heterodimers [90]. TLR2 functions as a homodimer or a TLR1/TLR2 or TLR2/TLR6 heterodimers while TLR4 is thought to signal mainly as a homodimer but recent evidence suggests that it can also signal as a TLR4/TLR6 or TLR2/4 heterodimer (Fig. 5)[93]. TLRs consist of a single transmembrane domain and an extracellular domain composed of 19-25 tandem leucine rich repeats that contribute to pattern recognition [94]. In the cytoplasm, TLRs share many similarities with the IL-1receptor (IL-1R) family. Both TLRs and IL-1R contain a cytoplasmic TIR domain and in a MyD88 dependent manner, although at least TLR4 can signal in a MyD88 independent manner. MyD88 recruitment to the TIR signaling complex results in activation of the (nucleur factor-light-chain-enhancer of activated B cells (NF-κB) and mitogen activated protein kinase (MAPK) signaling pathways. Upon activation by PAMPs or DAMPs, TLR signaling increases expression of proinflammatory cytokines such as IL-1β and TNF and interferon family genes (Fig. 5). Recent work suggests that TLR signaling could contribute to regulation of inflammation in arthritis and most recently disc degeneration.

1.2.2.5.2 Toll-like Receptors in Cartilage and Disc Degeneration

TLRs have only recently begun to be investigated in the context of disc degeneration. However, their role in osteoarthritis and rheumatoid arthritis has been investigated in greater detail. While many differences exist between cartilage and discs, many similarities also exist that may help in understanding the role of TLRs in disc degeneration. Importantly, this previous work suggests

that TLRs have an important role in the degradation of connective tissues [91]. In osteoarthritis, which shares a number of features with disc degeneration, TLR1-9 expression increases with cartilage degeneration [95]. Several studies have shown that chondrocyte TLR activation by bacterial ligands like lipopolysaccharide or peptidoglycan and endogenous ligands such as fragmented aggrecan or fibronectin, increases cytokine and protease expression and decreases matrix synthesis [96-99]. Cartilage literature also demonstrated non-immune cell expressed TLRs and that chondrocytes respond to many alarmins including HMGB1, S100 proteins, fibronectin fragments and low molecular weight hyaluronan [91,100]. These studies indicate TLR activation by endogenous ligands leads to the hallmarks of cartilage degradation. They also importantly have characterized a role for TLRs and alarmins in a non-immune cell context, which creates a more relevant background to investigate TLRs and alarmins in intervertebral discs.

Compared to arthritis, less is known about the role of TLRs in disc degeneration. A recent study detected TLR1, 2, 3, 4, 5, 6, 9 and 10 gene expression in disc cells (NP and AF cells were not separated). TLRs1, 2, 4 and 6 are constitutively expressed and their expression correlates with the grade of degeneration in samples obtained from surgical low back pain patients [101]. Adverse mechanical strain, treatment of cells with IL-1β or TNFα, or TLR2 activation increases TLR2 and 4 gene expression [21,101,102], but it remains unclear how and why TLR expression increases *in vivo* during degeneration. Many alarmins that could potentially activate TLRs are found in the disc, including 30 kDa fibronectin fragments, aggrecan fragments, low molecular weight hyaluronic acid fragments, and HMGB1 [12,102-104]. Treatment of human disc cells (mixed population) with low molecular weight hyaluronic acid increases IL-1β, -6 and -8 gene expression through TLR2. *In vivo* injection of 30 kDa fibronectin fragments into rabbit discs induces degenerative changes, but whether this effect was through TLR activation was not

investigated [105]. Effects of other alarmins have not been extensively characterized in the disc. More commonly TLR agonists, such as LPS, PGN or Pam2CSK4, are used to activate TLR2 or TLR4. TLR2 or TLR4 in bovine or human disc cells (either mixed or NP human cells and bovine NP cells) increases MMP-1, -3, -13, ADAMTS-4 and -5, IL-6, IL-8 and iNOS expression (Fig. 5) [97,101,102,106]. These previous studies indicate TLRs may have a role in regulating disc degeneration. However, it remains unknown whether TLRs are involved in degenerative mechanisms leading to pain, whether TLR activation leads to degenerative changes in human discs cultured, and if TLRs are potential disease modifying targets.

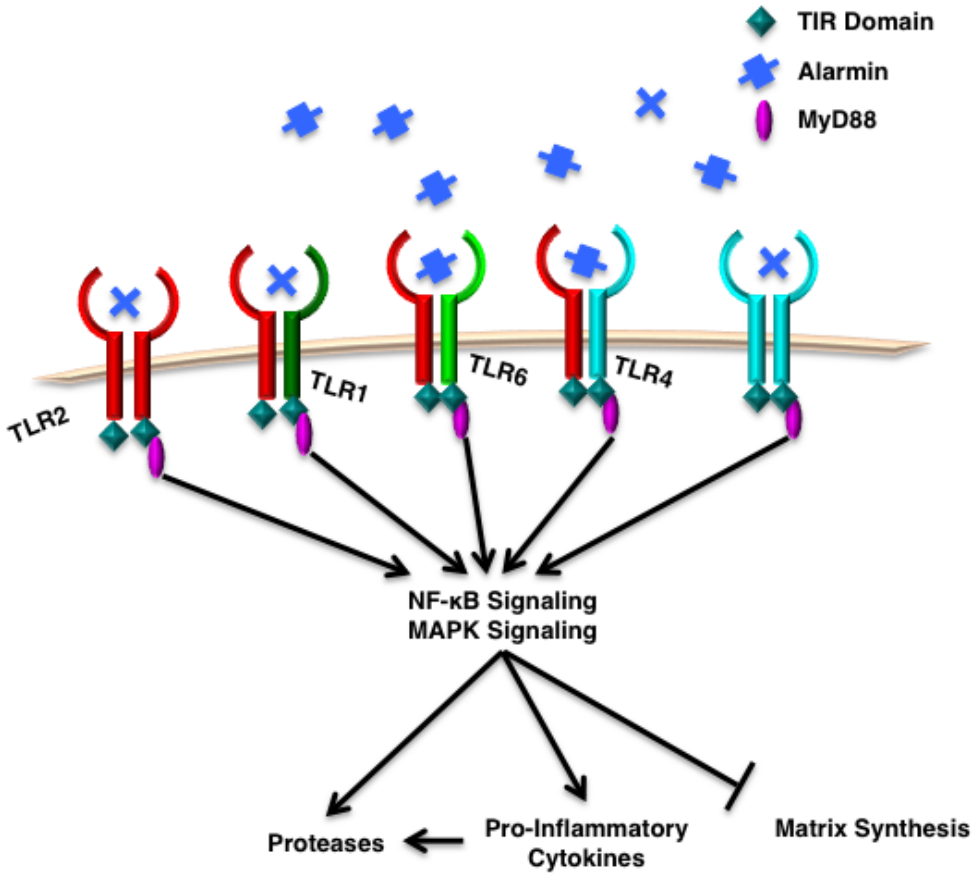

Figure 5. Schematic of TLR Activation. TLRs function as heterodimes as shown. Red is TLR2, dark green TLR1, bright green TLR6, and teal is TLR4. Following activation by alarmins or pathogens the toll-interleukin receptor domain (TIR) and MyD88 adapater protein activate

NF-κB and MAPK signaling. Pro-inflammatory cytokines, such as IL-1β, and proteases, such as MMP3, increase. At the same time extracellular matrix synthesis is decreased. This figure has been adapted from presentations that Dr. Lisbet Haglund and I have given between 2015 and 2017.

1.2.2.6. Models of Disc Degeneration

Models of disc degeneration range in species size (bovine to mouse), from *in vitro* cell culture to *in vivo* disc injury and genetic models. Most large animal models use *ex vivo* intervertebral disc organ cultures, including bovine caudal discs, ovine discs and porcine discs. Labs with access to human tissue have also developed a number of human disc organ culture systems [107]. Following disc excision, degeneration can be induced enzymatically, mechanically, or with proinflammatory factors. Human disc organ and cell culture, and small animal models were used for the work in this thesis. Several rodent models of disc degeneration exist. Rabbits, rats and mice have all been used, however only mice and rats have been used extensively for investigating pain and disc degeneration. Rat and mouse models can be divided into two groups based on the induction of degeneration; mechanical/surgical or genetic.

There are a number of mechanical and surgical rodent models of degeneration. A common way to induce disc degeneration is with a stab or puncture injury delivered a needle to the disc. There are several variations and techniques to this model, but they typically produce a strong degeneration of the disc and mimic a herniation or annular rupture. Some investigators have found a stab injury produces a robust pain phenotype as well [108,109]. A caveat to a stab or puncture model is that it is an acute injury and therefore does not mimic the slow progression of disc degeneration, but more likely models a rapid traumatic injury. Another strategy is to mechanically induce disc degeneration. For example, groups have developed a rat tail

compression model using an Illizarov-type device [110]. Looping the tail of a mouse back onto itself for 4-12 weeks also induces disc degeneration through asymmetric loading [111]. Compared to puncture models these mechanical induction models lead to a more progressive onset of degeneration, similar to the human disc degeneration. A final surgical model that is used commonly to model disc herniation and pain is a nucloectomy model in rats. The NP of tail discs is removed and placed or compressed onto the sciatic nerve, resulting in a profound pain phenotype[112,113]. This model is meant to mimic disc herniation and is used to study pain rather than degeneration.

All of these models have several advantages and disadvantages. For example, disc puncture models allow for investigation of patterns of cytokine expression, innervation and vascularization, and animal pain behavior. However, they may not be appropriate to study tissue repair strategies, progressive degenerative processes or disease-modifying drugs. Similarly, tail compression models may allow for study of progressive degeneration and tissue repair strategies, but are ill-suited to study pain behavior related to lumbar disc degeneration. All of these models have different positive and negative attributes; therefore models should be chosen to suit the research question that is being asked.

A number of genetic mouse models of disc degeneration have been developed, including the SPARC-null mouse used in this thesis. SPARC-null mice lack the gene encoding Secreted Protein Acidic and Cysteine Rich (SPARC), also known as Osteonectin. The SPARC-null mice were originally developed to study the cataractogensis [114] and were later discovered to develop progressive disc degeneration. Compared to wild-type mice, SPARC-null mice loose disc height, have histological changes indicating proteoglycan loss, disc herniations (Fig. 6), increased pain-related spinal cord and DRG neuroplasticity and develop robust axial and radiating pain

phenotype (pain behavior assays reviewed below) [115-119]. Due to the progressive degeneration of SPARC-null disc degeneration, these mice serve as an interesting and representative model of human disc degeneration. Col9a1-null mice also develop disc degeneration and display altered gait and increased mechanical sensitivity compared to wild-type mice. However, they also develop osteoarthritis, making it difficult to interpret functional and behavioral data [120]. A tissue-specific strategy to create genetic models of disc degeneration is to use notochordal-cell specific knock out mice [121]. Mouse NP cells are derived from notochordal cells, allowing for NP specific knock out of genes of interest. Notochordal specific knockouts have been used to investigate the roles of CCN2 [122] and HIF-1α [123] in disc degeneration and development. Notochordal knock out specific mice allow investigation of pain-behavior and drug treatments while being able to attribute effects to the disc. Genetic models offer advantages over surgical and mechanically induced degeneration models since they do no require any invasive procedures. Furthermore, genetic models are more useful for longitudinal studies since disc degeneration can progressively develop, as is the case with SPARC-null and CCN2 notochordal knockout mice. This may allow researchers to better mimic the long term, progressive nature of human disc degeneration. Small rodent models have several limitations (highlighted in the discussion), but serve as useful tool to further understand disc degeneration and low back pain mechanisms.

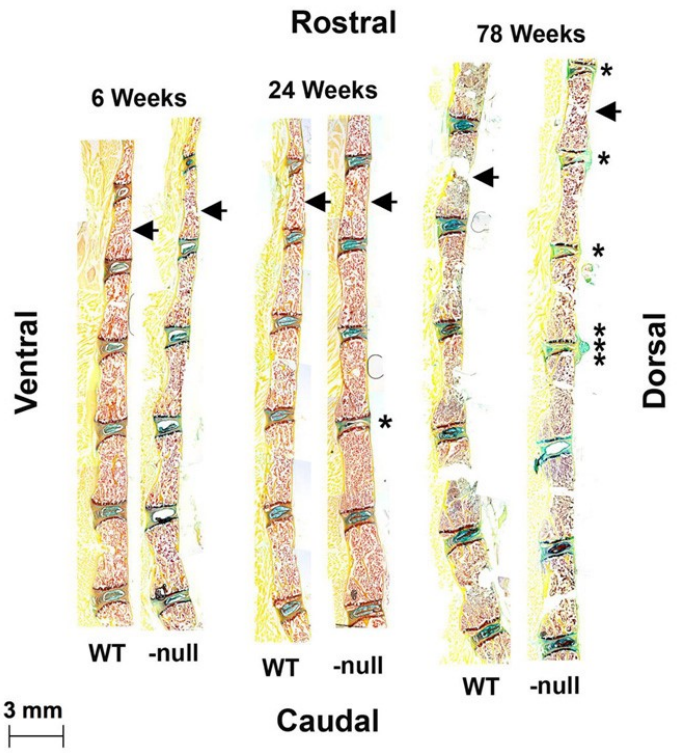

Figure 6. Histological Staining of Whole Lumbar Spinal Columns. SPARC-null and age matched wild-type mice lumbar spinal column sections were stained with FAST stain. Staining shows age and strain dependent proteoglycan loss in the discs. Arrow heads indicate L1 vertebrae, * indicates degenerating discs and *** indicates herniated discs. Image modified from original publication [117] and used with permission.

1.3 Rational

Chronic discogenic low back pain is a prevalent and costly condition that has limited therapeutic options. As this thesis work began it was evident degenerating discs express cytokines, chemokines and neurotrophins and that their expression increased with degeneration. However, most of this work was performed using histological sections, cell culture, gene expression data or non-human models [22]. Understanding of whether discs secrete factors that act as neuroattractants, chemoattractants, and nociceptive factors was limited. Furthermore, how many of these factors are regulated was not clear. IL-1β and TNFα undoubtedly have important roles in disc

degeneration but they are not constitutively expressed, leaving the possibility that other factors are involved in the beginning stages of disc degeneration and regulation of the disc's inflammatory milieu.

1.4 Hypothesis

Degenerating intervertebral discs produce and secrete proinflammatory and pronociceptive factors that further disc degeneration and induce neuronal sensitization and chronic pain.

1.5 Specific Aims

1) Characterize proinflammatory cytokine and neurotrophin release from non-degenerating and degenerating human discs and investigate the effect of the degenerating disc secretome on neurons.
2) Determine if NGF is regulated by TLRs.
3) Determine if TLR2 activation is sufficient to induce degenerative changes in non-degenerating human discs.
4) Investigate if TLR inhibitors act as a disease-modifying drug in a mouse model.

Chapter 2: Nociceptive Factors in Discogenic Low Back Pain Patients

2.1 Preface

The poor understanding of chronic low back pain mechanisms limits therapeutic strategies. As described above inflammatory factors, and increased disc innervation are linked to the development of chronic low back pain. Vertebral endplates are also disrupted during degeneration, changing diffusion between discs and the adjacent, richly innervated vertebral bodies. A variety of cytokines, chemokines and neurotrophins increase during disc degeneration. Many of these proteins may act as nociceptive or neuroattractant factors during degeneration if degenerating discs secrete them. However, previous studies have had several limitations. Cytokines and neurotrophins were commonly evaluated by gene expression or histological techniques; thus secretion of these factors by degenerating human discs was poorly characterized. Furthermore, the effects of secreted nociceptive factors on neurons were not well understood. The use of neuron-like cell lines further limits conclusions due to differences between DRG nociceptive neurons and cell lines. Previous studies also often lacked non-degenerate, non-painful control human discs. Furthering the understanding of degenerating discs' secretion profile and how discs affect neurons could provide the basis for new therapeutic targets.

In this study we hypothesized that as discs degenerate, they secrete factors that actively promote neurite growth and neuronal sensitization. We characterized factors secreted by non-degenerating discs from organ donors and degenerating discs from chronic low back pain patients. We then looked at the role of conditioned culture medium on cultured DRG neurons on the PC12 neuron-like cell line.

2.2 Manuscript I: Painful Degenerating Intervertebral Discs Upregulate Neurite Sprouting and CGRP Through Nociceptive Factors

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Abstract:

Intervertebral disc (IVD) degeneration can result in chronic low back pain, a common cause of morbidity and disability. Inflammation has been associated with IVD degeneration, however the relationship between inflammatory factors and chronic low back pain remains unclear. Furthermore, increased levels nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) are both associated with inflammation and chronic low back pain, but whether degenerating discs release sufficient concentrations of factors that induce nociceptor plasticity is unclear. Degenerating IVDs from low back pain patients and healthy, painless IVDs from human organ donors were cultured *ex vivo*. Inflammatory and nociceptive factors released by IVDs into culture media were quantified by enzyme-linked immunosorbent assays and protein arrays. The ability of factors released to induce neurite growth and nociceptive neuropeptide production was investigated. Degenerating discs release increased levels of tumor necrosis factor-α, interleukin-1β, NGF and BDNF. Factors released by degenerating IVDs increased neurite growth and calcitonin gene-related peptide expression, both of which were blocked by anti-NGF treatment. Furthermore protein arrays found increased levels of 20 inflammatory factors, many of which have nociceptive effects. Our results demonstrate that degenerating and painful human IVDs release increased levels of NGF, inflammatory and nociceptive factors *ex vivo* that induce neuronal plasticity and may actively diffuse to induce neo-innervation and pain *in vivo*.

Keywords: Intervertebral disc degeneration, discogenic pain, human, nerve growth factor, CGRP, inflammatory cytokines

Introduction

Low back pain has a lifetime prevalence of 60-80% and is associated with profound socioeconomic costs [124]. Intervertebral disc (IVD) degeneration is a major cause of low back pain [125]. The IVD is composed of two distinct regions; the outer annulus fibrosus (AF) and the central nucleus pulposus (NP). Healthy, pain-free IVDs are mostly avascular and aneural with neurites penetrating only the outer layers of the AF. However, evidence suggests that degenerating, painful IVDs are innervated [126], supporting a relationship between discogenic pain and increased IVD innervation. The extracellular matrix of healthy IVDs contains high concentrations of negatively charged proteoglycans, providing an unfavourable environment for neurite growth [127]. However, with degeneration proteoglycans are fragmented and released from the tissue [125], potentially creating an environment more permissive to neurite ingrowth.

Several animal models for disc degeneration show increased intervertebral disc innervation by the identification of calcitonin gene related peptide (CGRP) and Substance P-expressing fibers [128-130], the majority of which are thought to be nociceptors. Nociceptive fibers have also been reported in degenerate human disc tissue [126,131,132], suggesting a mechanistic role in pain associated with degenerative disc disease. However, the direct mechanism of discogenic pain *in vivo* has yet to be established.

Nerve damage and neuronal sensitization are hypothesized to play a role in chronic pain associated with degenerative disc disease [133,134]. As degenerating IVDs lose height or herniate, the dorsal root ganglion (DRG), nerve root or spinal cord can be compressed, leading to neuropathic pain [135,136]. Furthermore, the inflammatory processes involved in IVD degeneration have been hypothesized to contribute to chronic back pain [135]. These factors can induce neuronal sensitization, leading to the development of inflammatory pain, a process separate from

neuropathic pain. *In vitro* studies on either treated disc cells (human NP and AF cells or mixed populations, NP and AF cells of non-human species) or cells isolated from degenerating IVDs (usually mixed populations) have shown increases in pro-inflammatory and pro-nociceptive factors such as interleukins IL-1β, IL-6, tumor necrosis factor-α (TNF-α), nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) [135,137,138]. NGF and BDNF are neurotrophins that promote neuronal development, survival and growth. NGF and BDNF produced *in vitro* by cultured NP and AF cells stimulate neurite growth in neuronal cell lines [139]. They can also modulate pain and increase the expression of the nociceptive neuropeptides Substance P and CGRP in neurons [133]. Therefore inflammatory and neurotrophic factors may play a significant role in discogenic pain. However, it is unclear whether degenerating human IVDs release sufficient concentrations of NGF and BDNF to stimulate neurite growth and nociceptive peptide production.

The present study investigates the factors actively released by degenerating and painful IVDs, and determines whether they can directly induce innervation and activation of pain-sensing fibers. We show in real-time that *ex vivo,* degenerating IVDs release factors that induce neurite growth and nociceptor plasticity, as compared to healthy disc cultures. From this study, we propose that inflammation associated with IVD degeneration may directly contribute to the development of chronic low back pain.

Methods

Tissue Sources

This study was approved by McGill University Institutional Review Board (IRB# A04-M53-08B) project titled "Human Intervertebral Discs used for Culture and Extracellular Matrix". 8 degenerating IVDs from 6 females and 2 males, ages 33-58 years (mean 40.4 years) were resected en-block from consenting patients undergoing discectomy, interbody arthoplasty, or fusion for chronic discogenic axial low back pain. Throughout this study these surgically removed samples are called degenerating, painful IVDs. Eleven healthy, pain-free IVDs from 6 female and 2 male organ donors, ages 20-50 years, (mean 35.6 years) were obtained through the Transplant Quebec Organ Donation Program from individuals who had undergone sustained brain death. IVDs from organ donors were inspected for visual signs of degeneration. X-rays of the lumbar spinal segments were evaluated for signs of disc degeneration, loss of disc height, endplate spurs and intra-discal calcification. In addition, a family member completed a back pain questionnaire about pain history and treatment for back pain of the donor. IVDs that showed signs of degeneration or came from donors with a history of back pain were excluded from the study

IVD Isolation and Culture

Healthy control IVDs were isolated from organ donors as previously described [140]. Briefly, both degenerating and healthy IVDs were cultured using a method that has more than 95% cell viability after 7 days in culture [140,141]. Briefly, intact discs were washed in phosphate buffer saline (PBS) supplemented with 5 µg/mL Gentamicin (Gibco, Burlington, ON, Canada) and 0.125 µg/mL fungizone (Gibco) for five minutes, then twice in Hanks Balanced Salt Solution (HBSS, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5 µg/mL Gentamicin

(Gibco) and 125 ng/mL fungizone (Gibco) for five minutes. Discs were cultured in 3.5 mL per gram of tissue in serum-free IVD media (Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich), 50 µg/mL ascorbic acid, 5 µg/mL Gentamicin (Gibco), 0.125 µg/mL fungizone (Gibco), and 1× glutamax (Gibco) as previously described [140,141]. 3.5 mL media per gram of tissue is a sufficient volume to completely submerge discs and to maintain long term cell viability [140,141]. Cultures were maintained at 37°C and 5% CO_2 for 48 hours. Degenerating, painful IVD conditioned media and healthy, pain-free IVD conditioned media was collected and frozen as individual samples at -80°C for later analysis. The conditioned medium was filter sterilized and supplemented with 5 µg/mL Gentamicin (Gibco), penstrep (25 U/mL Penicillin, 25 µg/mL Streptomycin, Gibco), 0.125 µg/mL fungizone (Gibco) prior to use in neuronal cultures.

Conditioned Media Analysis

The concentration of TNF-α (RayBiotech, ELH-TNFALPHA-001), NGF (RayBiotech, ELH-BNGF-001), BDNF (RayBiotech, ELH-BDNF-001) and IL-1β (RayBiotech, ELH-IL1BETA-001) in the culture medium was quantified using enzyme-linked immunosorbent assays (ELISA) according to manufacturers' instructions. Duplicate 100 µl samples of each conditioned medium were incubated in ELISA plates overnight at 4°C. Colormetric absorbance was measured with a Tecan Infinite M200 PRO (Tecan, Männedorf, Switzerland) and analyzed with i-control 1.9 software (Tecan). Duplicates were averaged and the mean concentrations for healthy and degenerating IVDs were calculated. To calculate the amount of a given protein per gram of disc tissue weight the amount of protein per mL was multiplied by the amount of culture media used per gram of disc tissue (3.5 g/mL, see above).

RayBio Human Cytokine Array 1 Maps (RayBiotech, Inc., Norcross, GA, USA, product code: AAH-CYT-1) were used to determine the relative quantities of 23 cytokines according to

manufacturer's instructions. Arrays were imaged with the provided enhanced chemiluminescence kit using an ImageQuant LAS4000 (GE Healthcare, Baie d'Urfe, Qc, Canada). ImageQuant TL array analysis software (GE Healthcare) was used to analyze the blots. The relative quantity of each factor present in each media sample was calculated using the controls included on the protein arrays. Mean relative quantities of each factor for the degenerating and healthy groups were then calculated.

Mouse DRG Neuron Isolation

Studies were approved by the Animal Care Committee at McGill University, and conformed to the ethical guidelines of the Canadian Council on Animal Care and the Committee for Research and Ethical Issues of IASP. Mouse DRG cell cultures were derived as previously described by Malin *et al.* [142]. DRGs were dissected from the spine, digested in papain (Worthington Biochemical Corporation, Lakewood, NJ), followed by collagenase type II (Worthington). Cells were mechanically dissociated by pipetting the solution up and down, the suspension was then passed through a cell strainer to separate the neurons from remaining debris. The neurons were collected by centrifugation and resuspended in F12 medium (Gibco) supplemented with 10% FBS, 10 U/mL Penicillin and 10 µg/mL Streptomycin (Gibco). The isolated neurons were cultured in eight well chamber slides (BD Biosciences, Bedford, MA) coated with 5 µg/mL Laminin (BD Biosciences) and 5 µg/mL Poly-d-Lysine (BD Biosciences) at 37^{o}C and 5% CO_2.

Cell Culture

Rat adrenal pheochromocytoma (PC12) cell line expresses the receptor for and responds to NGF. When exposed to NGF they take on a neuronal-like phenotype. They are commonly used to study neuronal differentiation and neurite sprouting [143-145]. PC12 cells (ATCC, Manassas,

VA) in passages 2-7 were cultured on six well plates (Nunc) or 8-well chamber slides (Nunc) coated with 50 µg/mL rat tail collagen type I (Gibco) and 10 µg/mL Poly-L-Lysine (Sigma). The cells were maintained for 24 hours in RPMI (Gibco) media containing 10% horse serum (Gibco), 5% FBS (Gibco) and 1 × antibiotic/antimycotic (anti-anti) solution (Gibco).

PC12 and neuronal culture media was replaced after a 24 hour acclimatization period, with IVD medium supplemented with 0.1% FBS (Gibco) containing either no NGF, 2.5 ng/mL (Bioshop, Burlington, ON) (PC12 cells), 100 pg/mL (PC12 cells and neurons) or 10 ng/mL NGF (neurons), degenerating IVD conditioned medium or healthy IVD conditioned medium. 2.5 ng/ml NGF for PC12 cells was selected as the positive control based on a serial dilution that showed an effect similar to higher doses of NGF (data not shown). 10 ng/ml NGF was selected for neuronal cultures based on the literature [146]. PC12 cells were exposed for 24 or 48 hours to the different media (n=3 in each IVD media group, n=2 for each control for 24 hour cultures and n=6 in each IVD media group, n=3 for each control for 48 hour cultures). DRG neurons were exposed for 48 hours to the different media (n=3 in each IVD media group, n=2 for each control group) For conditioned media cultures, samples from the low, middle and high range of NGF concentrations were used.

NGF neutralization experiments were performed over 48 hours using a mouse monoclonal NGF antibody (Exalpha Biologicals Inc., Shirley, MA, USA) raised against human NGF. A 200-fold molar excess compared to 10 ng/mL was used. The antibody was pre-incubated with media containing 10 ng/ml and 100 pg/ml NGF (n=2 for each) and IVD conditioned media (n=3 for PC12 cells, n=2 for neurons) for 1 hour at room temperature prior to applying to neuronal cultures. Anti-NGF was added to media with NGF to ensure antibody efficacy. Pre-

immune mouse IgG (Sigma) used at the same concentration as NGF antibody was incubated with NGF containing media prior to application to cultures.

PC12 Culture Image Acquisition and Neurite Analysis

Each medium was applied in duplicate wells and two random images per well were taken using a Zeiss Axiovert 40 C inverted light microscope with a Canon PowerShot A640 camera and 52mm Soligor adaptor tube. The percentage of cells with neurites was determined and then averaged for each experimental condition.

Reverse Transcription and Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed after 24 and 48 hours on the same cultures used to quantify neurite growth (n=3 in each IVD media group, n=2 for each control for 24 hour cultures and n=6 in each IVD media group, n=3 for each control for 48 hour cultures). RNA was extracted from PC12 cultures using TRIzol Reagent (Invitrogen, Burlington, On, Canada). Approximately 500 ng of RNA was reverse transcribed to cDNA (qSqript cDNA, Quanta Biosciences, Gaithersburg, MD, USA) using an Applied Biosystem Veriti thermal cycler (Applied Biosystems, Carlsbad, CA, USA). qRT-PCR was performed as previously described [147]. Briefly, qRT-PCR was performed with PerfeCTa SYBR Green FastMix (Quanta Biosciences) on an Applied Biosystems StepOnePlus using specific primers [148] to Neurofilament Light Chain (NF-L), plasminogen activator, urokinase receptor (Plaur), polo-like kinase 2 (Plk2) poliovirus receptor (PVR), vaccinia growth factor (VGF), which are associated with neurite growth [148]. β-actin was used as an endogenous control and average fold change of each gene was normalized to the no NGF control according to the $2^{-\Delta\Delta Ct}$ method [149].

Immunohistochemistry and Image Acquisition of DRG Cultures

DRG cultures were fixed for 10 minutes in 4% paraformaldehyde at room temperature, washed three times in PBS and incubated at room temperature for 1 hour in blocking buffer containing 0.3% Triton X-100, 1 % bovine albumin serum, 1% normal donkey serum, 0.1% sodium azide in PBS. The slides were then incubated with a Protein Gene Product (PGP 9.5) rabbit monoclonal antibody (1:2000, Ultraclone Limited, Isle of Wight, England, catalogue number RAB95101) and a CGRP sheep polyclonal antibody (1:1000, Enzo Life Sciences, Inc, Farmingdale, NY, USA, catalogue number CA1137, lot 12031227) in blocking buffer overnight at 4^{o}C. Slides were washed three times in PBS and incubated with the secondary antibodies (Alexa Flour® 488-conjugated Donkey anti-rabbit, catalogue number 711-545-152, and Cy™3-conjugated Donkey Anti-Sheep, product number 713-165-147, Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA) for 1.5 hours at room temperature followed by exposure to DAPI (1:5000, Sigma-Aldrich) in PBS for ten minutes and then two washes with PBS. Slides were mounted using Aqua Polymount (Polysciences, Inc., Warrington, PA, USA) and images were acquired using an Olympus BX51 (Tokyo, Japan) microscope equipped with a colour digital camera (Olympus DP71. Five random images (20x magnification) were taken of each well, making a total of 10 images per condition tested. A total of 20 images of each control were taken and 30 images of degenerating and 30 images of healthy media-treated cells were taken. For anti-NGF neutralization experiments, 10 images per control condition and 20 images per degenerating media-treated cells were taken.

DRG Culture Image Analysis

The acquired images of each location were combined in Photoshop CS2 and ImageJ was used to establish thresholds for positive staining of each marker. PGP 9.5 (green) and CGRP (red) immunoreactive cells were counted in separate channels. Images were assessed in a blinded

and randomized manner. CGRP immunoreactive neurons are reported as a percentage of all neurons (PGP 9.5 immunoreactive cell bodies). All CGRP immunoreactive cell bodies were also PGP 9.5 immunoreactive.

Statistical Analysis

Graphpad Prism 6 (La Jolla, CA) was used for all statistical analyses. Unpaired t-tests were used to test for differences between degenerative and healthy groups for each factor present on antibody arrays and TNF-α and NGF ELISAs. Unpaired Mann-Whitney test was used for BDNF ELISA analysis. Differences between neurite sprouting and CGRP expression were tested using one-way ANOVAs with post-hoc Tukey tests. Gene expression from 2.5 ng/ml NGF, healthy IVD media and degenerating IVD media cultures was compared to the –NGF control using a two-tailed T-test. For all tests significance was established at $p<0.05$. All data is presented as the mean value ± standard error of the mean in the text. Data is graphed as mean values with the 95% confidence intervals.

Results

Degenerating IVDs release Increased Quantities of TNF-α, IL-1β, NGF and BDNF

Since isolated IVD cells can release inflammatory and neurotrophic factors [150,151] and histological analyses of degenerate tissue have confirmed this [131,152], we quantified the ability of degenerating IVDs to actively release these factors *ex vivo* into culture media. IVDs were cultured on a volume per weight. ELISA analysis revealed that degenerating, painful IVDs released a significantly greater amount of TNF-α (138.3±24.7 pg/mL, p=0.01, Fig. 1A) compared to healthy, pain-free IVDs (69.2±22.6 pg/mL). NGF was released at a significantly greater amount by degenerating, painful IVDs (44.2±6.5 pg/mL, p<0.001, Fig. 1B) compared to healthy, pain-free IVDs (12.5±4.5 pg/mL). BDNF was significantly higher in the media from degenerating, painful IVDs (1.54±0.026 ng/mL, p=0.004, Fig. 1C), compared to control IVDs (0.67±0.039 ng/mL). Degenerating, painful IVDs released detectable amounts of IL-1β, but not all healthy, pain-free IVDs released IL-1β above the detection limit (0.48 pg/mL) of the assay (data not shown). Therefore the difference between the two groups was not determined.

Figure 1

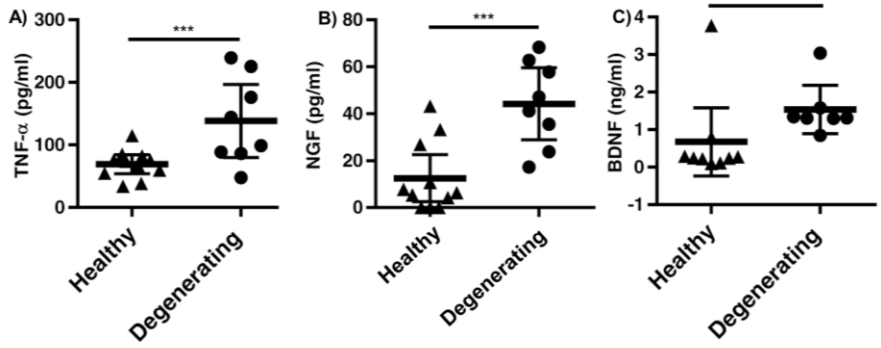

Figure 1. TNF-α (A), NGF (C) and BDNF (E) mean concentrations in media from healthy pain-free or degenerating painful IVDs. Mean concentrations of TNF-α (B), NGF (D) and BDNF (F) released into culture media per gram of IVD tissue from healthy pain-free or degenerating painful IVDs. n=8 in degenerate, painful group and n=11 in healthy, pain-free group for TNF-α

and NGF ±95% CI, unpaired t-test. n=7 in degenerating, painful group and n=9 in healthy, pain-free group for BDNF, ±95% CI, Mann-Whitney test. ** indicates $p<0.01$, *** indicates $p<0.001$.

Degenerating, painful IVD conditioned media increases neurite sprouting in PC12 cells

Since degenerating, painful IVDs release increased amounts of NGF and BDNF *ex vivo*, the conditioned media was tested for the ability to stimulate neurite growth in PC12 cells. After 48 hours of culture 20±3% of untreated cells, 78±4% of NGF treated cells, 29±2% of cells cultured in healthy, pain-free IVD media and 64±2% of cells cultured in degenerating, painful IVDs had neurites (Fig. 2A, B, n=6 IVDs for each conditioned media group). A significantly greater proportion of cells treated with NGF extended neurites when compared to untreated cells ($p<0.0001$). There was no significant difference in the proportion of cells with neurites between untreated and healthy IVD media groups ($p=0.25$). NGF-treated cultures had a greater proportion of cells compared to degenerating media groups ($p<0.01$). However, degenerating, painful IVD media induced neurite sprouting in a significantly greater percentage of cells compared to cells cultured in healthy, pain-free IVD media ($p=<0.0001$) (Fig. 2) indicating that painful degenerating IVDs produce factors that promote neurite growth.

The ability of NGF released by degenerating IVDs to induce neurite sprouting was determined through NGF sequestration. In these experiments 100 pg/mL NGF was used as an additional control because this is similar to the highest concentration measured in degenerating IVD conditioned media (Fig 1B). However, 100 pg/mL NGF alone was not sufficient to drive a significant increase in neurite sprouting in 48 hours compared to untreated cultures (28±3% vs 19±3%, $p=0.667$). Anti-NGF treatment significantly reduced the percentage of cells with neurites (9±1%) in cultures treated with 2.5 ng/mL NGF ($p<0.001$), whereas normal IgG had no effect on

neurite sprouting (70±2%, p=0.8517). Anti-NGF treatment of degenerating, painful IVD media significantly reduced the percentage of cells with neurites compared to degenerating IVD media without antibody (25±5% vs 64±2%, $p<0.001$, n=3 IVD samples for each group). There was no significant difference in the percentage of cells with neurites between healthy IVD media and anti-NGF treated degenerating media cultures (p=0.929, Fig. 2). Interestingly, this data indicates that NGF released by degenerating IVDs is required for increased neurite growth but that additional factors are required to allow such low concentrations to stimulate neurite growth.

Figure 2

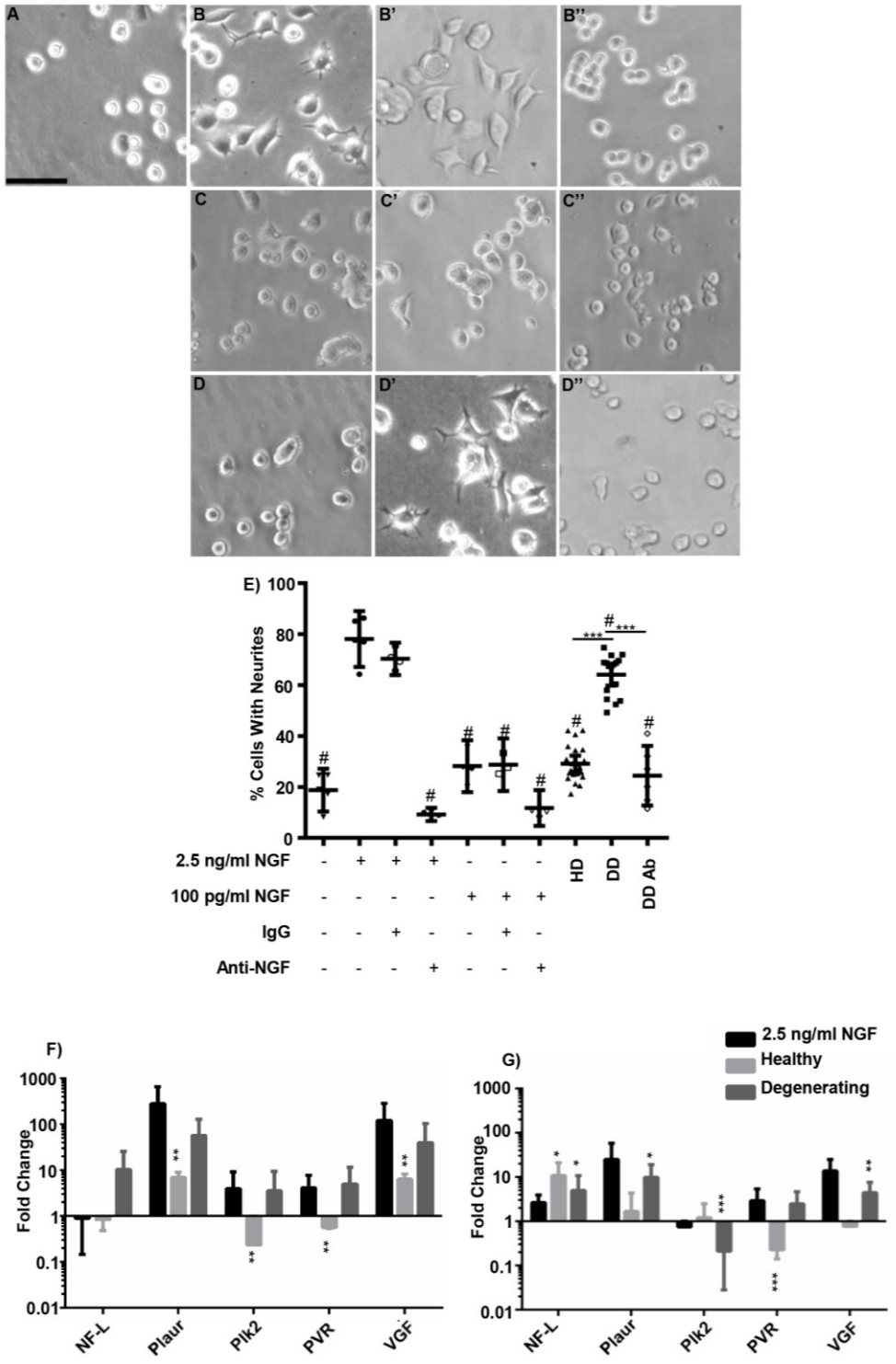

Figure 2. PC12 neurite growth after 48 hours of culture. Representative phase contrast image of untreated cultures (A), cultures treated with 2.5 ng/ml NGF (B), 2.5 ng/ml NGF and normal IgG

(B'), 2.5 ng/ml NGF and anti-NGF antibody (B"), 100/ml NGF (C), 100 pg/ml NGF and normal IgG (C'), 100 pg/ml NGF and anti-NGF antibody (C"), healthy pain-free IVD media conditioned media (D), degenerating painful IVD conditioned media (D') and degenerating painful IVD condition media treated with anti-NGF antibody (D"). Scale bar: 62.5 μm. E) Quantification of the proportion of cells with neurites after 48 hours of culture. Untreated cultures, cultures with 2.5 ng/ml NGF, 100 pg/ml NGF, IgG, and anti-NGF antibodies in different combinations were quantified as indicated. Control cultures healthy pain-free IVD media (HD) cultures, degenerating painful IVD media cultures DD and degenerating painful IVD media cultures treated with anti-NGF (DD Ab) were quantified as indicated. Fold changes of marker genes compared to –NGF control for PC12 neuronal differentiation and growth measured by qRT-PCR after 24 hours (F) and 48 hours (G). N=3 in each IVD media group, n=2 for each control in 24 hour cultures. N=6 in each IVD media group, n=3 for each control for 48 hour cultures. N=3 for DD Ab and n=2 for 2.5 ng/ml and 100 pg/ml NGF and NGF IgG. Error bars; ±95% C.I, one-way ANOVA. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. # indicates $p<0.001$ when compared to 2.5 ng/ml NGF control.

Gene expression associated with neurite growth is increased in degenerating painful IVD media cultures

To confirm that degenerating, painful IVD media induces neuronal differentiation, gene expression commonly used neuronal markers of PC12 differentiation was assessed. After 24 hours of culture, degenerating, painful IVD media caused strong trends for increased expression of of NF-L, Plaur, Plk2, PVR and VGF (Fig. 2F). Healthy media caused both up and down regulation of gene expression. At 48 hours, degenerating IVD media caused a significant

upregulation of NF-L, Plaur and VGF, whereas healthy media caused a significant upregulation of only NF-L.

Degenerating, painful IVD media increases CGRP expression in mouse DRG neurons

CGRP is a neurotransmitter that acts as a pain modulator and increased production can cause hyperexcitability and sensitization. To determine the effect of degenerating painful IVD media on CGRP expression, DRG neurons were exposed to this media and compared healthy pain-free IVD media. After 48 hours, neuronal cultures were analyzed for the expression of the general neuronal marker PGP 9.5 (green) and CGRP (red) (Fig 3A). The proportion of neurons that showed CGRP expression was analyzed for each treatment. 16±1% of untreated neurons, and 30±2% of 10 ng/mL NGF-treated neurons expressed CGRP. Similar to untreated controls, 18±2% of neurons cultured in healthy, pain-free IVD media expressed CGRP. Similar to NGF-treated controls, 29±2% of neurons cultured in degenerating, painful IVD media were CGRP immunoreactive 10 ng/mL NGF-treated neurons had a significantly higher percentage of CGRP-immunoreactive cells compared to untreated controls ($p=0.0045$). There was no difference in CGRP-immunoreactivity between non-treated and healthy, pain-free media groups ($p=0.9999$) or between NGF-treated and degenerating, painful media groups ($p>0.9999$). A significantly greater proportion of cells cultured in degenerating, painful IVD were CGRP-immunoreactive compared to neurons cultured in healthy, pain-free IVD media ($p=0.007$, Fig 3B).

Figure 3

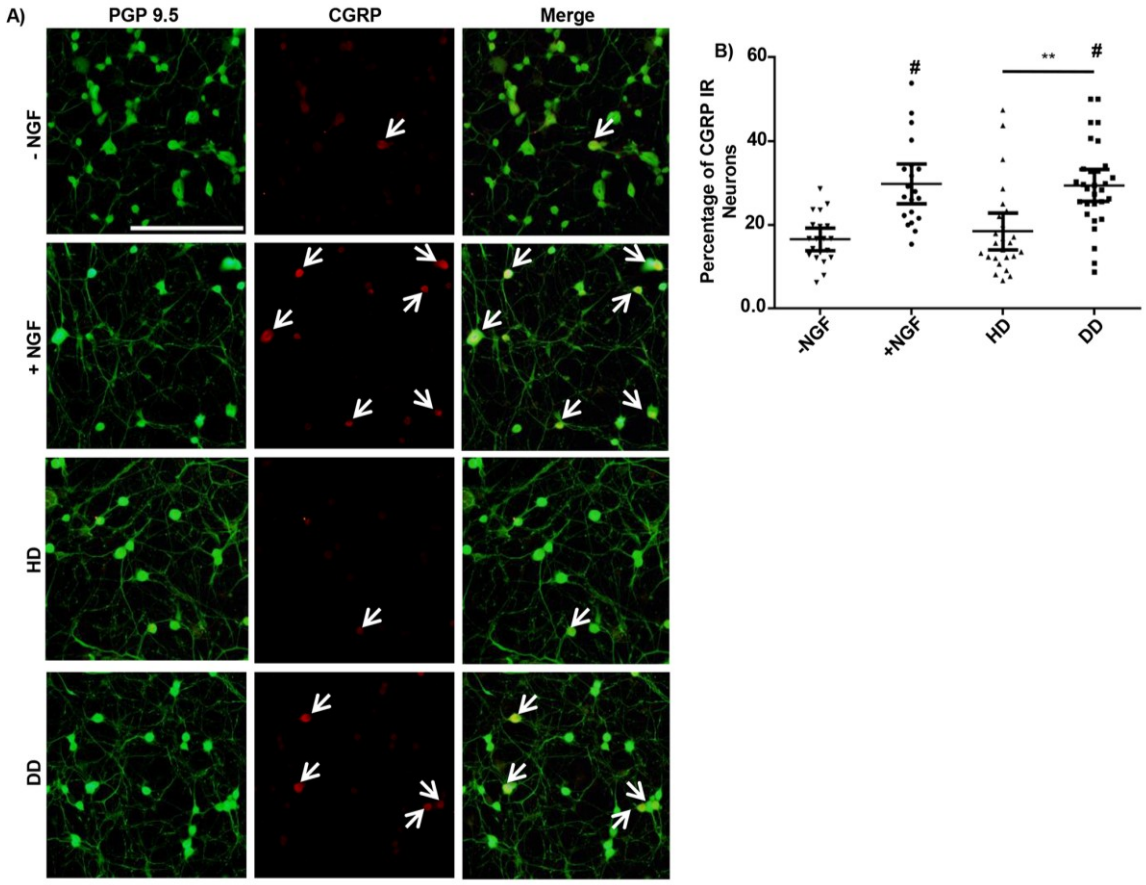

Figure 3. CGRP immunoreactivity in mouse DRG neurons after 48 hours of culture. A) Representative fluorescent images of neuronal cultures that were untreated (row 1), treated with 10 ng/mL NGF (row 2), maintained in healthy, pain-free IVD conditioned media (row 3) or in degenerating, painful IVD media (row 4). PGP 9.5 (green) is a general neuronal marker and CGRP (red) is nociceptive neuropeptide. PGP 9.5 and CGRP are overlaid in merged images. White arrows indicate CGRP immunoreactive neurons. Scale bar: 200 μm. B) Quantification of CGRP immunoreactivity for each group. –NGF; untreated media, +NGF; media supplemented with NGF, HD; healthy disc conditioned media, DD, degenerating, painful IVD conditioned media. n=3 samples per group, tested in duplicate, with 5 fields counted per duplicate totaling 10

fields counted per condition, error bars; ±95% C.I, one-way ANOVA. ** indicates $p<0.01$. # indicates $p<0.001$ when compared to –NGF control.

In order to determine if NGF released from degenerating IVDs is contributing to the increase in CGRP-immunoreactivity, experiments using an anti-NGF antibody were conducted. Like in PC12 cultures, 100 pg/ml of NGF was used as an additional control in this set of experiments. Following treatment with 100 pg/mL of NGF, 26±1% of neurons were CGRP immunoreactive, which was not significantly different from 10 ng/mL NGF-treated neurons ($p=0.97$). Incubating 100 pg/mL NGF media with normal IgG did not significantly alter CGRP-immunoreactivity ($p=0.99$). In contrast, incubating media containing 100 pg/mL of NGF with an anti-NGF antibody, CGRP expression was significantly reduced to 13±3% ($p=0.038$). Similarly, addition of anti-NGF antibody to degenerating, painful IVD media significantly reduced CGRP expression to 20±2% ($p=0.048$) when compared to degenerating media without the antibody (Fig 4, n=2 IVD samples in each group).

Figure 4

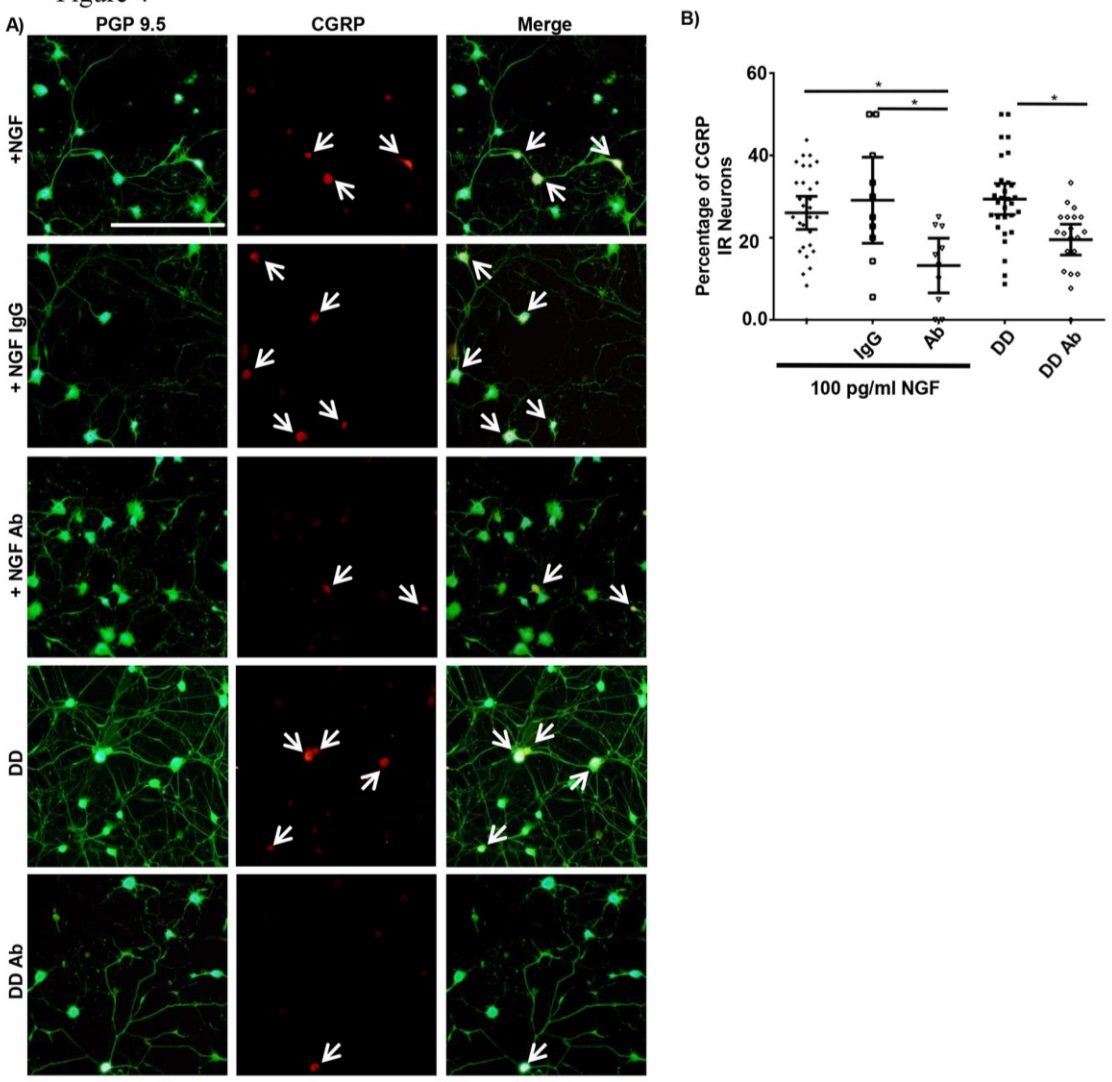

Figure 4. CGRP expression in mouse DRG neurons after 48 hours of culture. A) Representative fluorescent images of neuronal cultures treated with 100 pg/mL NGF (+NGF, Row 1)), 100 pg/mlLNGF and normal IgG (+NGF IgG, Row 2)), 100 pg/mL NGF and anti-NGF antibody (+NGF Ab, Row 3), media conditioned by degenerating, painful IVDs (DD, Row 4), or degenerating IVD media with anti-NGF antibody (DD Ab, Row 5). PGP 9.5 (green) is a general neuronal marker and CGRP (red) is pain neurotransmitter. PGP 9.5 and CGRP are overlaid in merged images. White arrows indicate CGRP immunoreactive neurons. Scale bar: 200 μm. B) Quantification of CGRP immunoreactivity for each group. n=2 samples per group, tested in

duplicate, with 5 fields counted per duplicate totaling 10 fields counted per condition, error bars; ±95% C.I, one-way ANOVA. * indicates $p<0.05$.

Degenerating IVDs release a multitude of pro-inflammatory and pro-nociceptive factors

Although the degenerating IVD media induced neurite growth, the same concentration range of NGF alone was insufficient. Therefore, protein arrays were used to identify potential cooperative factors [153]. Degenerating and painful IVDs released significantly higher levels of 20 of these factors (Fig 5A, Table 1). 15 factors had a p-value below 0.01 (GCSF, GM-CSF, IFN-γ, IL-2 , IL-3 , IL-5, IL-6 , IL-7 , IL-15, CCL2, CCL7, CCL8, MIG, RANTES and TNF-β), and 5 factors had a p-value between 0.01 and 0.05 (IL-1α, IL-13, TNF-α , GRO and CXCL1). There was no difference in the relative quantities of IL-8, IL-10 and TGF-β1 between the two groups (Fig. 5D, Table 1). The relative mean quantities and a summary of previous studies implicating specific factors with either degenerating IVDs and/or pain are listed in Table 1. Of particular interest are IFN-γ, IL-6, CCL2 and CXCL1 due to their suggested role in IVD degeneration, neuronal sensitization and pain [133,154-160]. Figure 1B shows the individual donor variation of these factors. IFN-γ and CXCL1 showed a fairly large donor variation especially in the degenerate samples whereas the levels of IL-6 and CCL2 were much more homogeneous in their expression levels within each of the two groups.

Factor	P Value	Sig	Change	Relative Mean Densitometry Units of Healthy IVD Media	Relative Mean Densitometry Units of Degenerating IVD Media	IVD	Pain
GCSF	0.0025	**	Up	0.012±0.003	0.1±0.03		[161]
GM-CSF	0.0013	**	Up	0.012±0.003	0.09±0.03		[44]
IFN-γ	0.0022	**	Up	0.014±0.004	0.21±0.07	[135]	[156,157]
IL-1α	0.0303	*	Up	0.0089±0.003	0.034±0.02	[138]	
IL-2	0.0069	**	Up	0.007±0.003	0.085±0.04	[135]	[162]
IL-3	0.0012	**	Up	0.023±0.005	0.18± 0.05		
IL-5	0.0015	**	Up	0.023±0.007	0.24±0.08		

IL-6	0.0019	**	Up	0.87±0.2	2.3±0.4	[135,163]	[159,164]
IL-7	0.0002	***	Up	0.06±0.01	0.34±0.08	[165]	
IL-8	0.2434	N		1.2±0.2	1.4± 0.1	[135]	[166]
IL-10	0.1169	N		0.047±0.0046	0.081±0.03	[163]	[134]
IL-13	0.0218	*	Up	0.0082±0.002	0.047±0.021		[167]
IL-15	0.0054	**	Up	0.015±0.005	0.11±0.04		[39]
CCL2	0.0005	***	Up	0.20±0.05	0.59±0.1	[135]	[134, 153,160]
CCL8	0.0029	**	Up	0.024±0.009	0.29±0.1		
CCL7	0.0049	**	Up	0.007±0.003	0.24±0.09	[168]	[169]
CXCL9	0.0028	**	Up	0.014±0.005	0.17±0.06	[48]	
CCL5	0.0071	**	Up	0.039±0.01	0.13±0.04	[170]	[134, 170]
TGF-ß1	0.2251	N		0.018±0.006	0.03±0.02	[157,165]	[171]
TNF-α	0.0473	*	Up	0.016±0.0057	0.05±0.02	[151,163]	[13]
TNF-ß	0.0085	**	Up	0.017±0.005	0.11±0.04		
GRO	0.0286	*	Up	0.242±0.07	0.55±0.2		[153-155]
CXCL1	0.0304	*	Up	0.018±0.002	0.22±0.1		[153-155]

Table 1. Comparison of 23 factors released by degenerating painful or healthy pain-free IVDs

Relative mean densitometry unit quantities of 23 factors secreted by either healthy IVDs or degenerating, painful IVDs. Relative quantity of factors was analyzed by RayBio Human Cytokine Array 1 Maps. P values were calculated using unpaired t-tests.. The columns named, IVD and Pain, provide references describing previous studies implicating that factor with either degenerating IVDs and/or pain. Reviews were used when possible. n=8 in degenerating, painful group and n=11 in healthy, pain-free group, ±SEM, unpaired t-test. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Figure 5

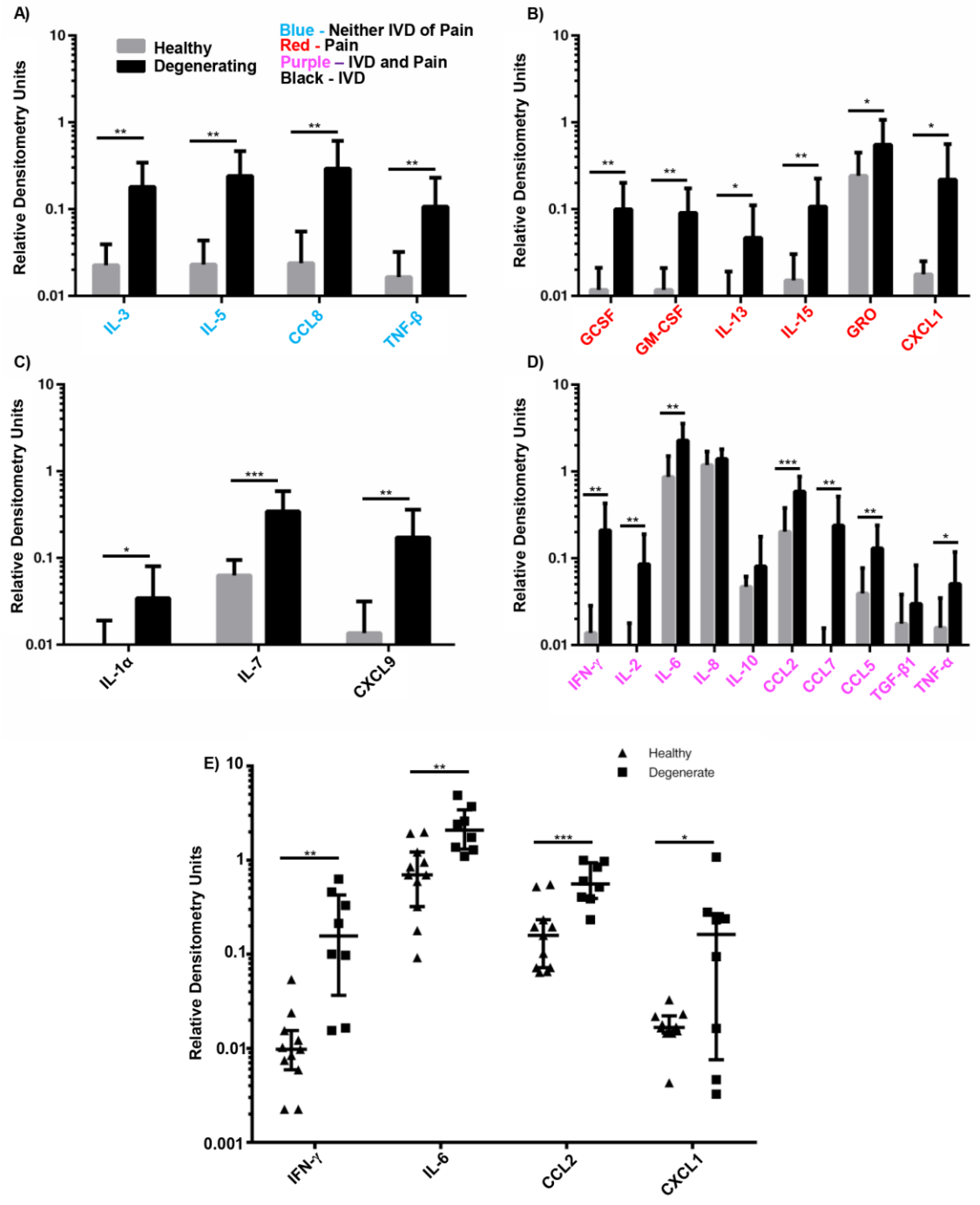

Figure 5. Comparison of factors released by healthy, pain-free and degenerating, painful IVDs measured by protein arrays. The mean relative quantity of each factor released by healthy pain free IVDs (grey bars) and degenerating painful IVDs (black bars) are presented (A-D). Factors in

blue have not been previously associated with disc degeneration or pain (A), factors in red have been associated with pain, but not disc degeneration (B), factors in black have been associated with disc degeneration (C) and factors in purple have been associated with both disc degeneration and pain (D). E) Mean relative quantities of select factors involved in nociception are plotted to show individual variation between donors. n=8 in degenerating, painful group and n=11 in healthy, pain-free group, S.E.M. for A-D, ±95% CI for E, unpaired t-test. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Discussion

Low back pain associated with IVD degeneration is a leading cause of chronic pain and morbidity, but how disc degeneration causes pain is not fully understood. Pain arises through a complex interplay between IVD matrix remodeling, the production of inflammatory, nociceptive and neurotrophic factors, nerve root and sensory neuron compression and disc innervation. Here we show that degenerating IVDs surgically removed from axial low back pain patients release increased levels of several pro-inflammatory and pro-nociceptive factors that are able to drive neurite sprouting of PC-12 cells and increase CGRP expression in primary neurons compared to healthy IVDs from donors who did not suffer from back pain. Moreover, we show that NGF is required to drive these observed effects. We demonstrate a direct link between disc degeneration and nociception by comparing degenerating and healthy human discs *ex vivo*.

Multiple studies have suggested that inflammatory and neurotrophic factors are present in degenerating disc tissue [131,152], and isolated IVD cells can actively secrete these factors in culture [150,151]. Moreover, treating cells isolated from either degenerating or healthy IVDs with cytokines, such as IL-1β or TNF-α, induces production of the neurotropic factors NGF and BDNF [68,172]. To establish a more direct link to *in vivo* disc degeneration the present study uses a whole organ, *ex vivo* culture approach to compare the profile of factors released from degenerating and painful

discs to healthy discs obtained from pain-free transplant donors. This approach leaves the IVD cells in their native environment and minimizes the effects of cell isolation and culture. Additionally, the whole organ culture approach maintains *in vivo* cell densities whereas cell culture studies maintain much higher relative cell densities. These variables potentially affect IVD inter- and intracellular interactions, possibly influencing the profile of secreted factors.

While previous studies have implicated some of the factors in Figure 1 and 5 and Table 1, NGF and BDNF in disc degeneration through *in vitro* cell culture studies or histological analysis, direct release of all of these factors has yet to be determined *in vivo.* Our *ex vivo* organ culture model demonstrates that degenerating IVDs from patients with chronic axial low back pain are releasing significantly higher levels of inflammatory and nociceptive factors compared to healthy discs. At this point it is not clear if all degenerating discs produce inflammatory and nociceptive factors or they are only produced by discs found in low back pain patients. We were unable to include degenerating IVDs from pain-free individuals in the present study because degenerating discs are not removed from individuals not suffering from chronic back pain and it is not possible to obtain a reliable long term back pain history of an organ donor post-mortem. Many of these factors can potentially modulate neurite growth, nociceptive related neuroplasticity and chronic pain. These results suggest that inflammatory and nociceptive factors may be secreted *in vivo* from degenerating discs in low back pain patients, where they are likely playing a direct role in discogenic low back pain.

NGF and BDNF are neuronal survival and growth factors. Previous studies demonstrated AF and NP cells isolated from degenerating human discs can increase neurite growth in co-cultures with the neuron-like SH-SY5Y cell line [139,173]. Among the factors identified in IVD conditioned media, degenerating painful IVDs had elevated levels of NGF and BDNF.

Surprisingly, the relatively low concentrations of these factors in the conditioned media significantly induced sprouting in PC12 cells. Anti-NGF treatment was sufficient to reduce neurite sprouting, demonstrating and important role for NGF in degenerating IVDs, even at low concentrations. However, when NGF is added to PC12 cultures at similar concentration to the one measured in degenerating IVD conditioned media, neurite sprouting was not induced. This data suggests that other factors released by degenerating IVDs are required in addition to NGF to induce neurite sprouting at concentrations below 100 pg/mL. Further studies are required to fully elucidate the mechanisms of painful degenerating IVD media on neurite sprouting.

In addition to finding increased neurite growth, this study demonstrated that degenerating, painful IVDs secrete a combination of factors that increase CGRP expression in primary mouse DRG neurons. CGRP functions as a neurotransmitter and is strongly associated with pain. CGRP expression can be increased by pro-nociceptive factors like NGF, TNF-α and CCL2 [133,160,174]. Understanding of nociceptor plasticity and CGRP changes associated with disc degeneration is mostly limited to animal models. Increased numbers of CGRP immunoreactive neurons have been shown to innervate rat IVDs treated with complete Freund's adjuvant [175], a model for IVD inflammation. CGRP expression is also increased in a rat model of injury-induced IVD degeneration [176]. The present study demonstrates that degenerating and painful human IVDs also release factors that increase CGRP levels in neurons, thus further supporting the central hypothesis that degenerating and painful IVDs secrete factors known to contribute to nociception.

NGF is a potent inducer of CGRP [133], and we therefore hypothesized that it mediates increased CGRP expression in cultures treated with degenerating, painful media. In contrast to previously published cell culture studies that use NGF concentrations higher than that found in

disc media, we used a more similar concentration (Fig. 1B) of 100 pg/mL. 100 pg/mL NGF was sufficient to increase CGRP expression to similar levels observed in cultures with 10 ng/mL NGF (Fig. 4B, Fig 5B). This suggests that degenerating, painful IVDs secrete sufficient amounts of NGF to alter CGRP-expression. Inhibiting NGF in degenerating disc media by incubation with an anti-NGF antibody caused a significant decrease in the percentage of CGRP-immunoreactive neurons compared to media without the antibody. This demonstrates that NGF found in media conditioned by degenerating, painful IVDs is sufficient to increase CGRP expression. This data suggests that NGF may play an important role *in vivo* in the development of chronic pain associated with intervertebral disc degeneration.

Since 100 pg/mL of NGF is insufficient to drive neurite growth and other factors can sensitize neurons, we used protein arrays to identify additional factors that may play a role. Conditioned media from degenerating, painful IVDs and healthy, pain-free IVDs were analyzed using cytokine and chemokine protein arrays. 20 factors were found to be upregulated in degenerating, painful samples, of which 10 (IFN-γ, IL-6 and -15, CCL2 and -7, CCL5, TNF-α, GRO and CXCL1, GCSF and GM-CSF) have been associated with increased nociception [133,134,154-162,164,166,167,169,171]. Some of the 20 factors have been previously associated with IVD degeneration [135,138,163,165,168,170], however 10 of the 20 factors (GCSF, GM-CSF, IL-3, IL-5, IL-13, IL-15, CCL8, TNF-β, GRO and CXCL1) have not previously been described in the IVD and 2 (CCL7 and CXCL9) [168] have not been described in degenerative disc disease. Of the 12 factors not described in pain associated with disc degeneration, 7 (IL-13, IL-15, CCL7, GRO, CXCL1, GCSF and GM-CSF) have been associated with a variety of pain conditions (Figure 1, Table 1). This data demonstrates that degenerating, painful IVDs secrete elevated levels of several pro-inflammatory and pro-nociceptive factors that are only secreted in very low basal levels by

healthy, pain-free IVDs. Since many of these factors have not been previously described in IVD degeneration; further investigation is warranted to understand their role in discogenic pain.

Multiple factors (including IFN-γ, IL-6, CCL2 and CXCL1, TNF-α, IL-1β, NGF and BDNF) known to be involved in nociception, development of chronic neuronal sensitization and hyperexcitability and chronic pain were increased in degenerating IVD conditioned media. For example, Robertson *et al.* have demonstrated that intrathecal injections of IFN-γ increase pain-related behavior in mice [156] and Vikam *et al.* demonstrated that IFN-γ can induce increased excitability in dorsal horn neurons [157], suggesting that IFN-γ has a modulatory role in nociception. Similarly, in a rodent model of arthritis, IL-6 contributes to inflammatory pain, neuronal hyperexcitibility and increased neuronal CGRP levels [158,159]. CCL2 can also increase CGRP production [153], and potentially acts as a pain related neurotransmitter in DRG neuronal cultures [160]. CXCL1 has also been shown to induce increase nociceptor excitability and play an important role in inflammatory pain and neuronal sensitization [154,155]. While this study found inhibiting NGF was sufficient to inhibit degenerating disc induced CGRP increases, these other factors could contribute to the development of chronic low back pain through other mechanisms. However, further investigation of such mechanisms is required.

The present study shows that degenerating painful IVDs secrete increased levels of multiple cytokines, chemokines and neurotrophins and that these factors increase neurite sprouting and CGRP expression. Furthermore, NGF secretion by degenerating, painful IVDs is sufficient to increase neurite sprouting and CGRP expression, which both can be blocked by anti-NGF antibody treatment. Taken together, this data suggests that factors actively released by degenerating and painful IVDs may induce innervation and pain *in vivo.* Furthermore, NGF may play an important role in nociception associated with IVD degeneration *in vivo.* Our data

supports further development of anti-NGF therapeutics to manage pain in degenerative disc disease [177-179]. A greater understanding of the molecular mechanisms driving pain associated with IVD degeneration may lead to improved therapies and quality of life for individuals with discogenic pain.

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Chapter 3: Regulation of NGF

3.1 Preface

Nerve growth factor (NGF) is a neurotrophin that has a well described role in nociception and neuronal sensitization. In our previous study we identified an important role for NGF secreted by degenerating discs in regulating neuronal sensitization and neurite growth. Several other previous preclinical studies have also suggested an important role for NGF in disc degeneration and low back pain. Clinical trials also targeted NGF in an attempt to decrease chronic low back pain, but have had mixed results. Despite its importance, little is known about the regulation and induction of NGF in a pathogenic context, like that of a degenerating intervertebral disc. NGF is induced by IL-1β and TNFα receptor activation on NP and AF cells, but IL-1β and TNFα are not constitutively expressed in discs. Therefore other mechanisms likely regulate NGF in degenerating discs. The signaling mechanisms regulating NGF expression during disc degeneration have also not been investigated. Increased understanding of mechanisms regulating NGF could identify novel NGF inhibition strategies.

TLRs are activated by endogenous components of degenerating discs such as fragmented aggrecan, fibronectin and HMGB1. Upon activation, TLRs increase many proinflammatory factors found in degenerating discs. TLRs are also constitutively expressed and the expression of TLRs 1, 2, 4 and 6 increase with degeneration. We therefore hypothesized that TLRs regulate NGF in disc cells. We also examined cell-signaling pathways that regulate NGF.

3.2 Manuscript II: Nerve Growth Factor Is Regulated by Toll-Like Receptor 2 in Human Intervertebral Discs

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Running title: TLR2 Regulates NGF Via NF-κB

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ABSTRACT

Nerve growth factor (NGF) contributes to the development of chronic pain associated with degenerative connective tissue pathologies, such as intervertebral disc degeneration and

osteoarthritis. However, surprisingly little is known about the regulation of NGF in these conditions. Toll-like receptors (TLR) are pattern recognition receptors classically associated with innate immunity, but more recently were found to be activated by endogenous alarmins such as fragmented extra-cellular matrix proteins found in degenerating discs or cartilage. In this study we investigated if TLR activation regulates NGF and which signaling mechanisms control this response in intervertebral discs. TLR2 agonists, TLR4 agonists, or IL-1β (control) treatment increased NGF, BDNF and IL-1β gene expression in human disc cells isolated from healthy, pain-free organ donors. However, only TLR2 activation or IL-1β treatment increased NGF protein secretion. TLR2 activation increased p38, ERK1/2 and p65 activity and increased p65 translocation to the cell nucleus. JNK activity was not affected by TLR2 activation. Inhibition of NF-κB, and to a lesser extent p38, but not ERK1/2 activity blocked TLR2-driven NGF upregulation at both the transcript and protein levels. These results provide a novel mechanism of NGF regulation in the intervertebral disc and potentially other pathogenic connective tissues. TLR2 and NF-κB signaling are known to increase cytokines and proteases, which accelerate matrix degradation. Therefore, TLR2 or NF-κB inhibition may both attenuate chronic pain and slow the degenerative progress in vivo.

Introduction

Intervertebral disc degeneration is a major cause of chronic low back pain, for which current therapeutics are largely ineffective. Intervertebral discs have two distinct areas; the central gelatinous nucleus pulposus (NP), rich in proteoglycans and collagen type II, and the surrounding fibro-cartilaginous annulus fibrosus (AF), rich in collagen type I. The causes of disc degeneration are multifaceted and not well understood, yet genetics, mechanical load or traumatic injury to the disc, along with several life style choices are known to contribute to the

etiologies of disc degeneration [6]. During degeneration there is a large increase in catabolic proteases including matrix metalloproteinases (MMPs) [33,180], cathepsins [28,29], high-temperature requirement serine protease A1 (HTRA1) [30,31], and disintergrin and metalloproteinase with thrombospondin motifs (ADAMTSs) [34,181]. Protease activity degrades the extracellular matrix (ECM), and the generated ECM fragments can potentially function as endogenous danger associated patterns (DAMPs) also known as 'alarmins' [94]. Alarmins have been shown to activate toll-like receptors (TLRs) in other tissues, resulting in a robust increase of inflammatory cytokines including IL-1β and TNFα [27,101]. TLRs have recently been proposed to contribute to disc degeneration, and their activation may play an important role in the induction of inflammatory cytokines and pain mediators implicated in painful disc degeneration.

Low back pain can develop through multiple mechanisms including compression of the dorsal nerve root, dorsal root ganglia or spinal cord and through neuronal sensitization via cytokines, chemokines and neurotrophins. Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are neurotrophic factors best characterized for promoting neuronal survival, maturation and growth. However, they also induce chronic neuronal sensitization in mature peripheral afferent fibers, which results in the development of chronic pain [63]. Accordingly, NGF and BDNF have both been implicated in chronic low back pain associated with degeneration. Immunohistochemical staining of NGF and BDNF is stronger in degenerating or herniated discs [66,67,182] and degenerating discs secrete greater amounts of NGF and BDNF compared to healthy discs [183]. Healthy discs are primarily aneural, but several studies have reported that they become increasingly innervated as discs degenerate [22,59,116]. In vitro human studies and in vivo animal studies have suggested that NGF and BDNF contribute to both innervation of degenerating discs and neuronal sensitization [183-185]. These preclinical studies have

made NGF and BDNF attractive therapeutic targets to treat low back pain. In fact, clinical trials using monoclonal antibodies against NGF have shown some efficacy to treat low back pain [88,89]. These prior studies demonstrate an important role for neurotrophins in the development of chronic low back pain, however little is known about their regulation in pathologies such as painful disc degeneration.

Inflammatory mediators, such as IL-1β and TNFα are known to increase NGF and BDNF gene expression in isolated disc cells [66,77,186,187]. However, it is also possible that other mechanisms increase neurotrophin expression during degeneration. For example, NGF secretion is elevated following mechanical injury to human discs or high mechanical strain to isolated disc cells [20,21]. The signaling mechanisms regulating neurotrophin transcription in pathogenic target tissues like degenerating discs are also unknown. A possible mechanism that has not been explored is that TLR signaling directly increases neurotrophin expression.

TLRs are pattern recognition receptors that were originally characterized in the innate immune system and are activated by pathogen associated molecular patterns (PAMPs), such as bacterial cell wall components, in addition to alarmins. In humans, 10 TLRs have been described. Gene expression of TLRs 1, 2, 3, 4, 5, 6, 9, and 10 have been detected in human disc cells where expression of TLRs 1, 2, 4 and 6 are correlated with increasing degree of degeneration [101]. Alarmins include proteolysis-generated ECM fragments, such as fragmented hyaluronic acid, fibronectin, biglycan, tenacin C, versican, heparan sulphate and aggrecan, and high-mobility group B1 (HMGB1), and the heat shock proteins (HSP) 60 and 70 [90,92,94]. Many alarmins, including fibronectin and biglycan fragments and HMGB1, have been found in degenerating discs [103,104,188,189]. TLR2 and TLR4 are thought to be the primary TLR subtypes that recognize ECM alarmins, where TLR4 functions as a homodimer and TLR2 functions as a

homodimer or heterodimer with TLR1 or 6 [94]. TLR activation increases the catabolic proteases MMP-1 and -13 as well as IL-1β, IL-6 and IL-8 in disc cells [101,102]. Interestingly, in vivo injection of fibronectin fragments into rabbit discs induces degenerative changes [105] and exposure of NP cells to fibronectin fragments decreases proteoglycan synthesis and increases proteoglycan degradation [190]. However, these studies did not investigate the mechanism responsible for these changes.

The potential role of TLR signaling in early stages of disc degeneration led us to hypothesize that TLR activation induces neurotrophin expression, either directly or as a secondary effect via cytokines. In this study we investigated TLR agonist- and cytokine-induced neurotrophin induction in human disc cells and determined that TLRs directly regulate neurotrophin expression. The signaling mechanisms regulating NGF were then explored. This study has identified novel mechanisms contributing to NGF regulation, offering alternative strategies to target NGF in disc degeneration to treat chronic pain.

Experimental Procedures

Tissue Collection and Cell isolation

All procedures were approved by the institutional review board of McGill University (IRB# A04-M53-08B) project titled 'Human Intervertebral Discs used for Culture and Extracellular Matrix.' Human lumbar spines were harvested from organ donors following donor and familial consent via a collaboration with Transplant Quebec. Donor information is presented in Table 1. NP and AF cells were isolated separately as previously described [21]. Briefly, discs were excised from the lumbar spine and NP and AF tissues were separated. Cells were cultured using 'disc cell media' composed of Dulbeco's Modified Eagle Medium (DMEM, Sigma, St.

Louis, MO, USA) supplemented with 10% fetal bovine serum, 1X glutamax and 25 μg/mL gentamicin (Life Technologies, Carlsbad, CA, USA).

Table 1: Information on Donors Used in this Study

Donor	Age	Sex	Cause of death	Time Course	TLR2 Inhibition	Cell Signaling	IL-1β Inhibition	Signaling Pathway Inhibition
1	19	M	suicide	•		•		•
2	17	M	suicide	•	NP only	•	•	•
3	50	F	Cerebral hemorrhage	•		•		NP only
4	24	M	head trauma	•			•	•
6	29	M	suicide		•	•	•	•
7	27	M	suicide	•	AF only		•	•
8	25	F	MVA, head trauma		•			AF only

In vitro Cell Experiments

Experiments were performed with NP and AF cells separately within passages 2-4. Approximately 250 000 cells per well were seeded into six well plates and allowed 12-24 hours to adhere. Cells were than serum starved in serum-free disc cell media supplemented with 1X Insulin-Transferrin Selenium (ITS, Life Technologies) for 12 hours. For initial studies examining the effects of different treatments cells were left untreated or treated with IL-1β (10ng/ml), TNFα (10ng/ml, both from Peprotech, Rocky Hill, NJ, USA), PGN (5μg/ml, Sigma) or LPS (10μg/ml,

Sigma). RNA was extracted following 6, 12 and 24 hours of treatment with TRIzol Reagent (Life Technologies) and media was collected following 12, 24 and 48 hours of treatment. For cell signaling experiments, cells were treated with IL-1β or PGN. Cell lysates were collected 30, 60, 120 and 360 minutes following treatment using NuPage LDS Loading Buffer (Life Technologies).

Antibody Neutralization

Serum-free disc cell media supplemented with ITS containing polyclonal antibodies against TLR2 (5µg/ml, Invivogen, San Diego, CA, USA, catalogue # pab-hstlr2) or normal IgG were added to cultures 2 hours prior to the addition of PGN. For IL-1β inhibition experiments, serum-free disc cell media supplemented with ITS containing no treatment, IL-1β or PGN was incubated with monoclonal neutralizing antibodies against IL-1β (5µg/ml, Invivogen, mabg-hil1b-3) or normal IgG for one hour and then applied to cells. RNA was extracted following 6 hours of treatment.

Cell Signaling Inhibition

Cells were serum starved with disc cell media supplemented with ITS for 12 hours and then pretreated with SB203580 (10 µM, p38 inhibitor, Life Technologies), PD98059 (10 µM, MEK1/2 inhibitor, Life Technologies) or BMS-345541 (5 µM, NF-κB inhibitor, Sigma-Aldrich) for two hours. Cultures were then challenged with IL-1β or PGN alone or in combination with inhibitors. RNA was extracted following 6 hours of treatment and conditioned culture media was collected after 48 hours for protein analysis.

Real-Time Quantitative Polymerase Chain Reaction

qRT-PCR was performed as previously described [183]. Briefly, RNA was extracted using TRIzol Reagent (Life Technologies) and isolated using a chloroform extraction method as

previously described [183]. Approximately 500 ng of RNA was reverse transcribed to cDNA using qSqript cDNA synthesis kits (Quanta Biosciences, Gaithersburg, MA, USA) with an Applied Biosystems Verti thermal cycler (Life Technologies). qRT-PCR was performed using an Applied Biosystems StepOnePlus (Life Technologies) with PerfeCTa SYBR Green FastMix (Quanta Biosciences) and previously published primers specific against 18s, IL-1β, NGF and BDNF [191-193](Life Technologies) or with TaqMan Gene Expression Master Mix and commercially available primers specific against 18s, TLR1, 2 4 and 6 (Life Technologies). Data was normalized to 18s expression or analyzed using the $2^{-\Delta\Delta Ct}$ method [194].

Protein Analysis

NGF enzyme-linked immunosorbent assays (ELISAs, RayBiotech, Norcross, GA, USA catalogue # ELH-BNGF) and BDNF ELISAs (Millipore, Billerica, MA, USA, catalogue #CYT306) were used to quantify protein concentrations in conditioned cell culture media according to manufacturer's instructions. For NGF western blots, protein in the conditioned culture media was precipitated using 100% ethanol overnight at 4^{o}C. Samples were then spun down and the pellet was resuspended in NuPage LDS Loading Buffer (Life Technologies). Samples were boiled and resolved on a 10-20% gradient gel (Life Technologies). For cell signaling, samples were boiled and resolved on a 10% poly-acrylamide gel. Protein was then transferred to a nitrocellulose membrane (General Electric, Mississauga, ON, Canada). Membranes were blocked using 3% bovine serum albium (BSA). A rabbit antibody against NGF (1:500, Santa Cruz, Dallas, TX, USA, Catalogue # sc-548, lot # l1113) was incubated overnight to detect NGF. For signaling proteins phosphorylation specific antibodies were used to detect phosphorylated p38 (Cell Signaling, Beverly, MA, USA), ERK1/2 (Cell Signaling), p65 (recognizes phosphorylation at Ser536, Santa Cruz), and JNK (Cell Signaling). Blots were than

stripped using Restore PLUS Western Blot Stripping Buffer (Sigma-Aldrich) and reprobed with antibodies detecting total p38, ERK1/2, p65 and JNK (all from Cell Signaling). α –tublin was probed as a loading control. All membranes were incubated with appropriate horseradish peroxidase conjugated secondary antibodies, exposed to Western Lightning Plus-ECL (PerkinElmer, Inc., Waltham, MA, USA) and imaged using a LAS 4000 Image Quant system (General Electric). Densitometric analysis was carried out using the ImageQuant TL program (General Electric). Samples were normalized to the background of the blot.

Immunofluorescence

NP and AF cells in 8-well chamber slides (Nunc) were serum starved and then treated with IL-1β, PGN or left untreated. After 1 hour cultures were fixed with ice cold methanol for 15 minutes. Cultures were blocked in PBS with 1%BSA and 0.1% Triton-X100 (Sigma-Aldrich) for 1 hour at room temperature and then incubated with an antibody specific against p65 (Santa Cruz) for 1 hour at room temperature. Following washing with PBS, slides were incubated with an appropriate Alexa Fluor® 488 conjugated secondary antibody for 1 hour at room temperature and then counterstained with DAPI. Cover slips were mounted using Aqua Polymount (Polysciences, Inc., Warrington, PA, USA) and images were captured with an Olympus BX51 (Olympus, Tokyo, Japan) microscope equipped with a colour digital camera (Olympus DP71). Two random images of each well were taken and p65 and DAPI images were overlaid in Photoshop.

Statistical Analysis

Data was analyzed using Graph Prism 6 (Graph Pad, La Jolla, CA, USA). Paired t-tests were used to analyze two groups and the Kruskal-Wallis test was used to analyze data from two or more groups.

Results

Induction of Neurotrophins

Proteolysis of ECM proteins has been suggested to be an early event in disc degeneration where the resulting ECM fragments could induce TLR signaling. To determine if TLR activation induces neurotrophin expression, NP and AF cells from healthy human discs were treated with the TLR agonists PGN (TLR2 agonist) and LPS (TLR4 agonist) for 6, 12, 24 and 48 hours. Cells were treated with IL-1β and TNFα to provide positive controls, and untreated cells served as the baseline. Both TLR and cytokine receptor activation are known to increase IL-1β gene expression. Therefore, IL-1β expression was measured to ensure that the cells express functional receptors for both cytokines and TLR agonists. As expected, cytokine treatment and TLR activation increased IL-1β gene expression. In some experiments, baseline IL-1β expression was un-detectable in untreated cells, and therefore IL-1β expression could not be normalized to baseline levels, rendering 2-ΔΔCt values impossible to calculate for those experiments. This reduces the sample size for IL-1β gene expression to n=3. Nevertheless, IL-1β expression was always detected in treated cells, suggesting that both NP and AF cells from non-degenerate discs express functional TLR receptors in addition to cytokine receptors (Fig 1A and B).

To determine if TLR2 or TLR4 activation increased neurotrophin expression we treated cells with peptidoglycan (PGN, TLR2 agonist) or lipopolysaccharide (LPS, TLR4 agonist). TLR2 activation significantly increased NGF expression in NP cells compared untreated cells following 6, 12 and 24 hours of treatment. TLR4 activation also increased NGF expression, but the increase was not statistically significant (Fig. 1C). In comparison, IL-1β increased NGF expression at all time points, while TNFα promoted a strong early trend for increased NGF

expression that was significantly increased by 24 hours compared to untreated cells. IL-1β and TLR2 activation significantly increased BDNF expression following 12 and 24 hours of treatment, while TLR4 activation and TNFα promoted less pronounced BDNF increases compared to controls (Fig. 1E). In AF cells, IL-1β significantly increased NGF after 6 hours, while TNFα and TLR2 activation promoted an increase in NGF expression. Following 12 hours, IL-1β and TNFα increased NGF expression, and after 24 hours IL-1β, TNFα and TLR2 activation significantly increased NGF expression (Fig. 1D). TLR2 activation never significantly increased BDNF expression in AF cells (Fig. 1F) and similarly to NP cells, TLR4 activation had a variable effect on neurotrophin induction (Fig 1D and F).

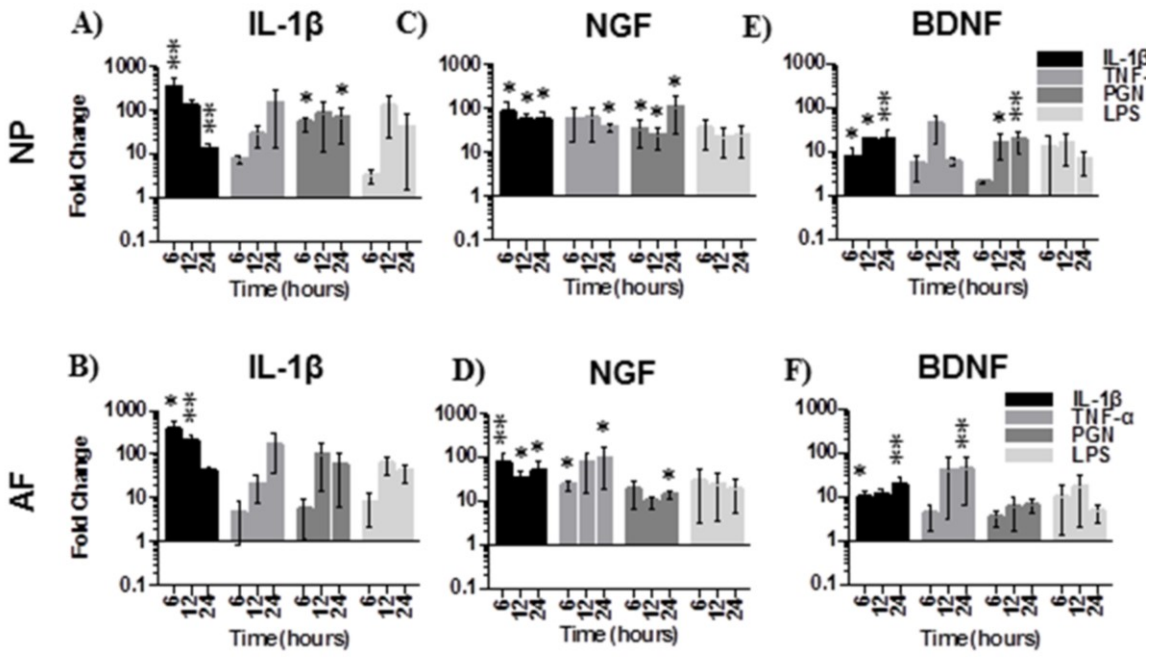

Figure 1

Gene Expression in NP and AF Following Cytokine and TLR Agonist Treatment IL-1β (A, B), NGF (C, D) and BDNF (E, F) gene expression in NP (A, C, E) or AF cells (B, D, F) following 6, 12 and 24 hours of treatment with IL-1β, TNF, PGN or LPS. Data is presented as

fold change normalized to 18s gene expression and untreated cells using the $2^{-\Delta\Delta Ct}$ method. Data was analyzed used the Kruskal-Wallis test for multiple comparisons of non-parametric data. Data is presented at mean ± SEM, n=4, except for IL-1β gene expression where n=3. * indicates $p<0.05$, ** indicates $p<0.01$.

To further examine TLR regulation of neurotrophins, NGF and BDNF concentrations in conditioned culture media of NP and AF cells was quantified by ELISA. In NP cells, NGF was undetectable or barely detectable in untreated cells, whereas NGF was detected as early as 12 hours following treatment and was significantly increased after 48 hours of TLR2 activation and treatment with IL-1β. Similarly, in AF cells NGF had a low baseline expression while IL-1β significantly increased NGF after 48 hours, while TLR2 induction of NGF showed a strong trend compared to untreated cells after 48 hours ($p=0.06$). TNFα also increased NGF secretion although not to statistically significant levels. TLR4 activation increased NGF protein secretion slightly, but variably, compared to untreated cells (Fig. 2). In all but a few samples BDNF was below the detection threshold (0.015 ng/ml) of the ELISA (data not shown). Therefore, we proceeded to focus on TLR2 regulation of NGF.

NGF is post-transnationally cleaved from proNGF to mature NGF (mNGF), and these two isoforms exert different biological effects [195]. Therefore, we used western blot to determine the relative predominance of proNGF or mNGF in disc cell-conditioned medium following 48 hours of treatment. Only mNGF was detected in the conditioned medium (Fig. 2C, D). Furthermore, densitometry analysis supported our findings that IL-1β and TLR2 activation more strongly increase NGF compared to other treatments (Fig. 2E, F). Taken together, these results demonstrate that NGF transcription and translation is induced by TLR2 activation in intervertebral disc cells.

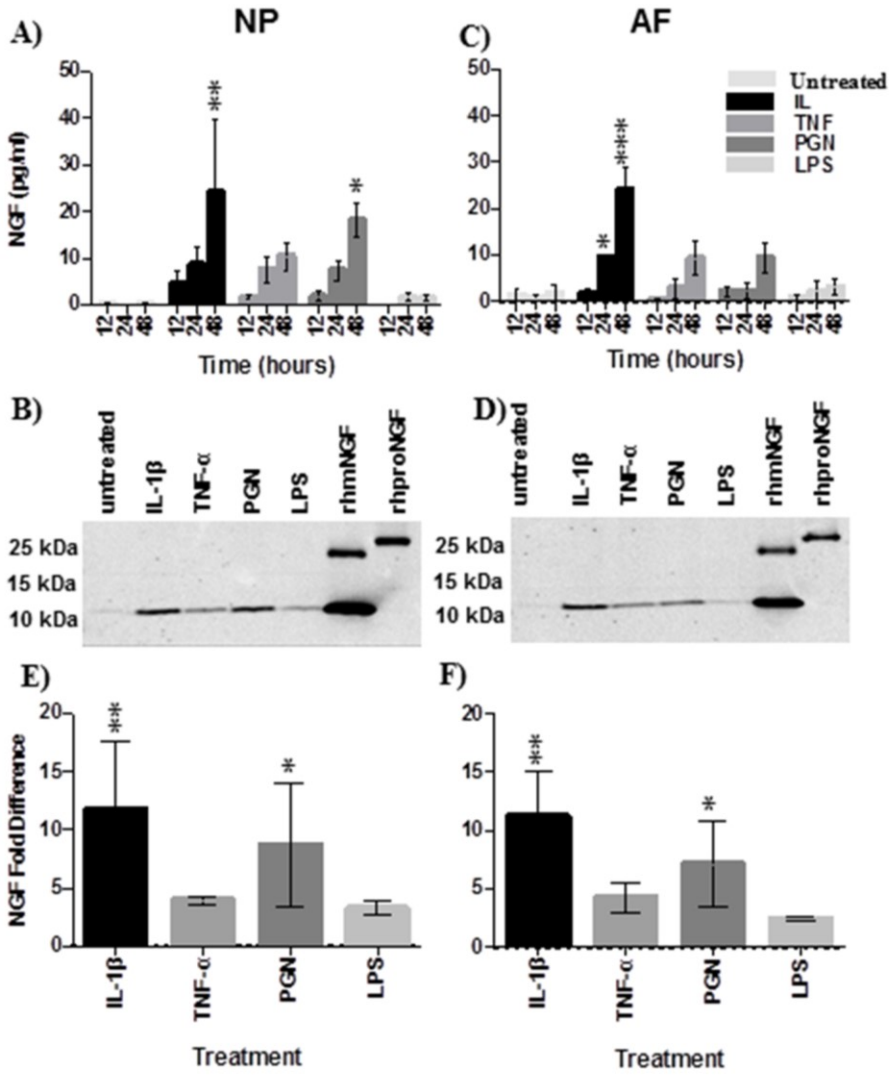

Figure 2

NGF Protein Secretion by NP and AF Following Cytokine and TLR Agonist Treatment

Conditioned cell culture media was collected 12, 24 and 48 hours following treatment with IL-1β, TNF, PGN and LPS. NGF protein levels in the media was analyzed by ELISA (A, B) and western blot (C-F). NP (A, C, E) and AF (B, D, F) media were both analyzed. Conditioned media following 48 hours of treatment was used for western blots. Recombinant human mature NGF (rhmNGF) and proNGF (rhproNGF) were used as controls for western blots. Western blots were analyzed by densitometry and normalized to untreated cells in E (NP cells) and F (AF

cells). Data was analyzed used the Kruskal-Wallis test for multiple comparisons of non-parametric data. Data is presented at mean ± SEM, n=4 for ELISA, n=3 for western blot. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Toll-like Receptor Expression

Increased TLR1, 2, 4, and 6 gene expression has been correlated with an increasing degree of degeneration [101]. TLR2 can signal as a homodimer or heterodimer with TLR1 or TLR6 whereas TLR4 signals primarily as a homodimer [94]. To determine the presence of individual TLR subtypes, and the level of induced gene expression, TLR1, 2, 4 and 6 expression was analyzed in NP and AF cells following treatment with IL-1β and PGN. TLR1, 2, 4 and 6 were all expressed in NP and AF cells, treated or untreated. The expression was variable between donors but TLR2 or IL-1β receptor activation strongly increased TLR2 expression compared to untreated cells (Fig 3A and B).

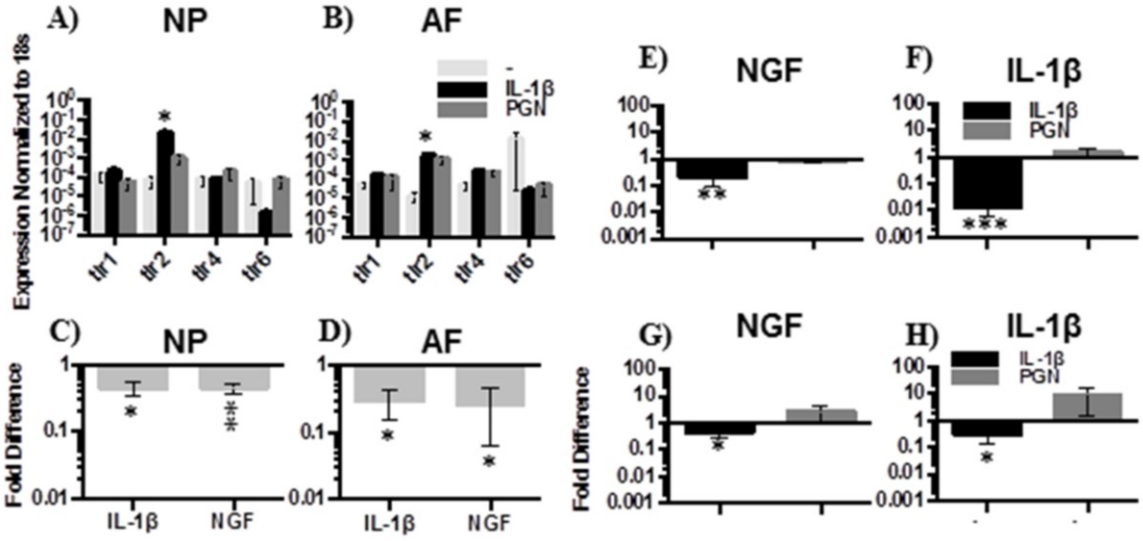

Figure 3

TLR Gene Expression and TLR2 Inhibition Gene expression of TLR1, 2, 4 and 6 in NP (A) and AF (B) cells following 6 hours of treatment with IL-1β, PGN or left untreated. Data was

normalized to 18s gene expression as endogenous housekeeping gene and presented as $2^{-\Delta Ct}$, n=4. For TLR2 inhibition (C and D), cells were pretreated for two hours with polyclonal neutralizing antibodies against TLR2 or normal IgG and then treated with PGN. IL-1β and NGF gene expression in NP (C) and AF (D) cells was analyzed using the $2^{-\Delta\Delta Ct}$ method and presented as fold difference compared to cells that were treated with PGN and normal IgG, n=3. To examine if IL-1β is required for TLR2 activation to induce IL-1β and NGF gene expression, NP (E and F) and AF (G and H) cells were treated with IL-1β or PGN in combination with an IL-1β neutralizing antibody or normal IgG. NGF (E and G) and IL-1β (F and H) gene expression was evaluated using the $2^{-\Delta\Delta Ct}$ method and presented as fold difference compared to cells that were treated with IL-1β or PGN and normal IgG. Differences in gene expression were assessed ANOVA (A and B) or paired t-tests (C-H), n=4. * indicates $p<0.05$, ** indicates $p<0.01$, *** $p<0.001$.

TLR2 is Required for PGN-Induced NGF Expression

PGN can potentially activate other pattern recognition receptors besides TLR2, such as NOD receptors [196]. Neutralizing polyclonal antibodies against TLR2 were used to confirm that PGN is acting through TLR2 in disc cells. NP and AF cells treated with PGN together with TLR2 neutralizing antibodies express decreased levels of IL-1β and NGF compared to cells treated with PGN alone (Fig. 3C and D). These results demonstrate that TLR2 activation can induce NGF expression.

IL-1β is Not Required for TLR2-Induced NGF Expression

TLR2 induction of IL-1β is well characterized in other cell types and occurs in disc cells (Fig. 1 A, B). Since IL-1β induces NGF, as does TLR2 activation, it is possible that TLR2 induces NGF through a feedback loop via IL-1β. To evaluate a potential feedback loop

neutralizing antibodies against IL-1β were used in cultured NP and AF cells treated with IL-1β (control) or PGN to determine if TLR2 requires IL-1β for NGF induction. As expected, in both NP and AF cells treated with IL-1β, NGF gene expression was decreased following treatment with neutralizing IL-1β antibodies. However, neutralizing antibodies had no effect on TLR2 induced NGF (Fig. 3 E-H), indicating IL-1β is not required for TLR2 induced NGF.

PGN Induces NF-κB, p38 and ERK1/2 Signaling

Signaling mechanisms downstream of TLRs in intervertebral discs have only recently begun to be investigated. In other tissues TLR or IL-1Receptor (IL-1R) activation can induce NF-κB, p38, ERK1/2 and JNK signaling [90]. To evaluate the pathway activated in IVD cells, NP and AF cells were treated with IL-1β or the TLR2 agonist PGN, and cell lysates were analyzed following 30, 60, 120, and 360 minutes of treatment. Western blot analysis revealed that IL-1β and PGN strongly increase NF-κB (p65), p38 and ERK1/2 phosphorylation compared to untreated cells after 30 minutes. p65 and p38 phosphorylation remained elevated after 360 minutes where as ERK1/2 phosphorylation decreased after 60 minutes. JNK phosphorylation was undetectable or minimal following TLR2 activation whereas IL-1β induced JNK phosphorylation after 30 minutes, which then declined to undetectable levels (Fig. 4A, B, C). These results suggest that TLR2 signaling in disc cells is predominantly through NF-κB, p38 and ERK1/2 signaling.

To further investigate NF-κB induction, p65 translocation to the nucleus was examined. Following 1 hour of treatment p65 translocation was examined by immunofluorescence. TLR2 activation and IL-1β both induced p65 translocation to the nucleus in NP and AF cells, whereas p65 remained dispersed throughout the cytoplasm in untreated cells (Fig 5). These results further support that TLR2 activates NF-κB signaling in disc cells.

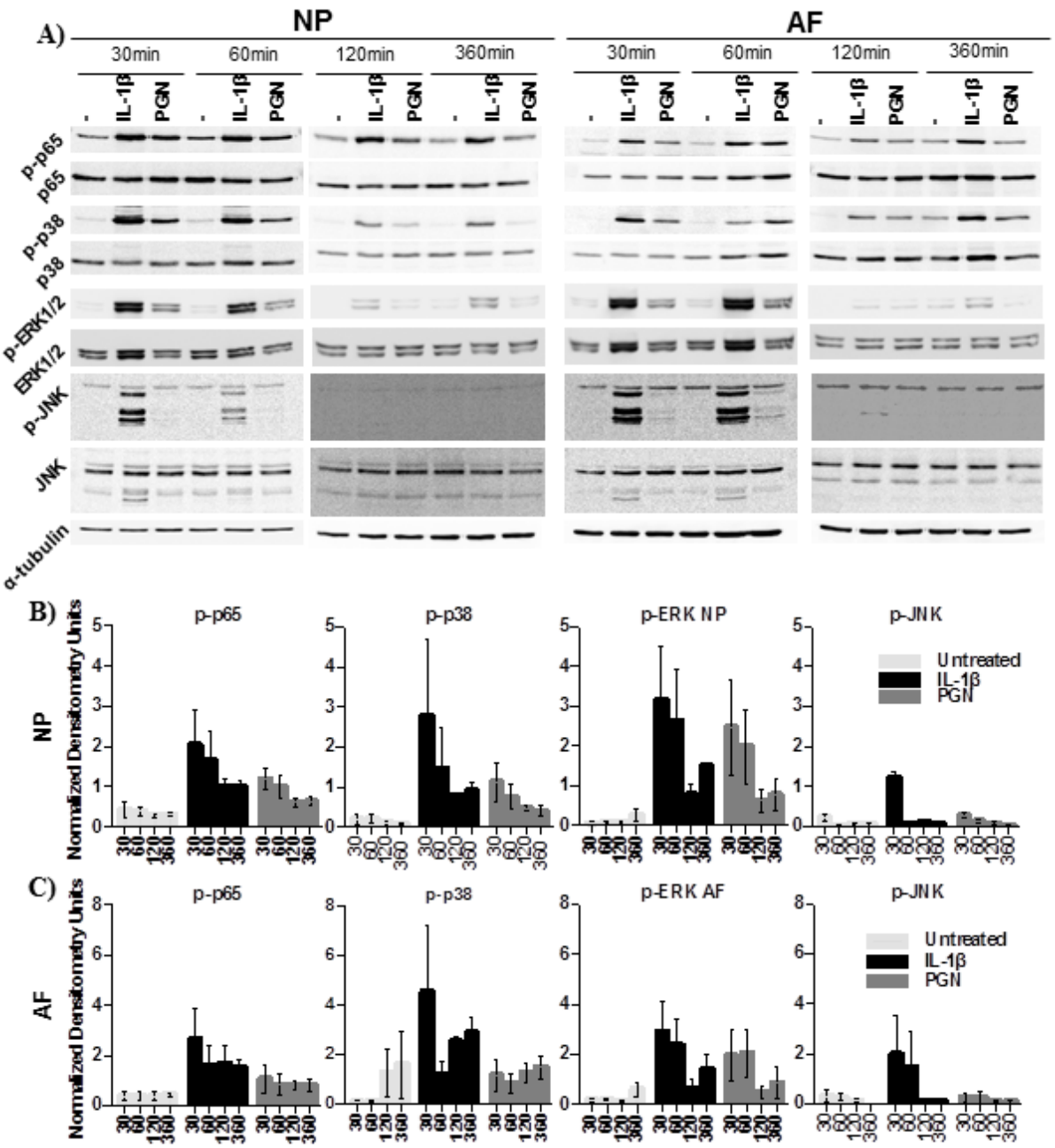

Figure 4

Signaling Pathway Activity p65, p38, ERK1/2 and JNK activity was evaluated using phosphorylated-specific antibodies and antibodies recognizing total p65, p38, ERK1/2 and JNK protein.. Cell lysates of NP and AF cells were collected following 30, 60, 120 and 360 minutes of treatment with IL-1β, PGN or left untreated (A). Cell lysates were probed using α-tubulin was

used as a loading control. Densitometry data of phosphorylated protein is normalized to total p65, p38, ERK1/2 or JNK in NP (B) and AF (C) cells. n=4.

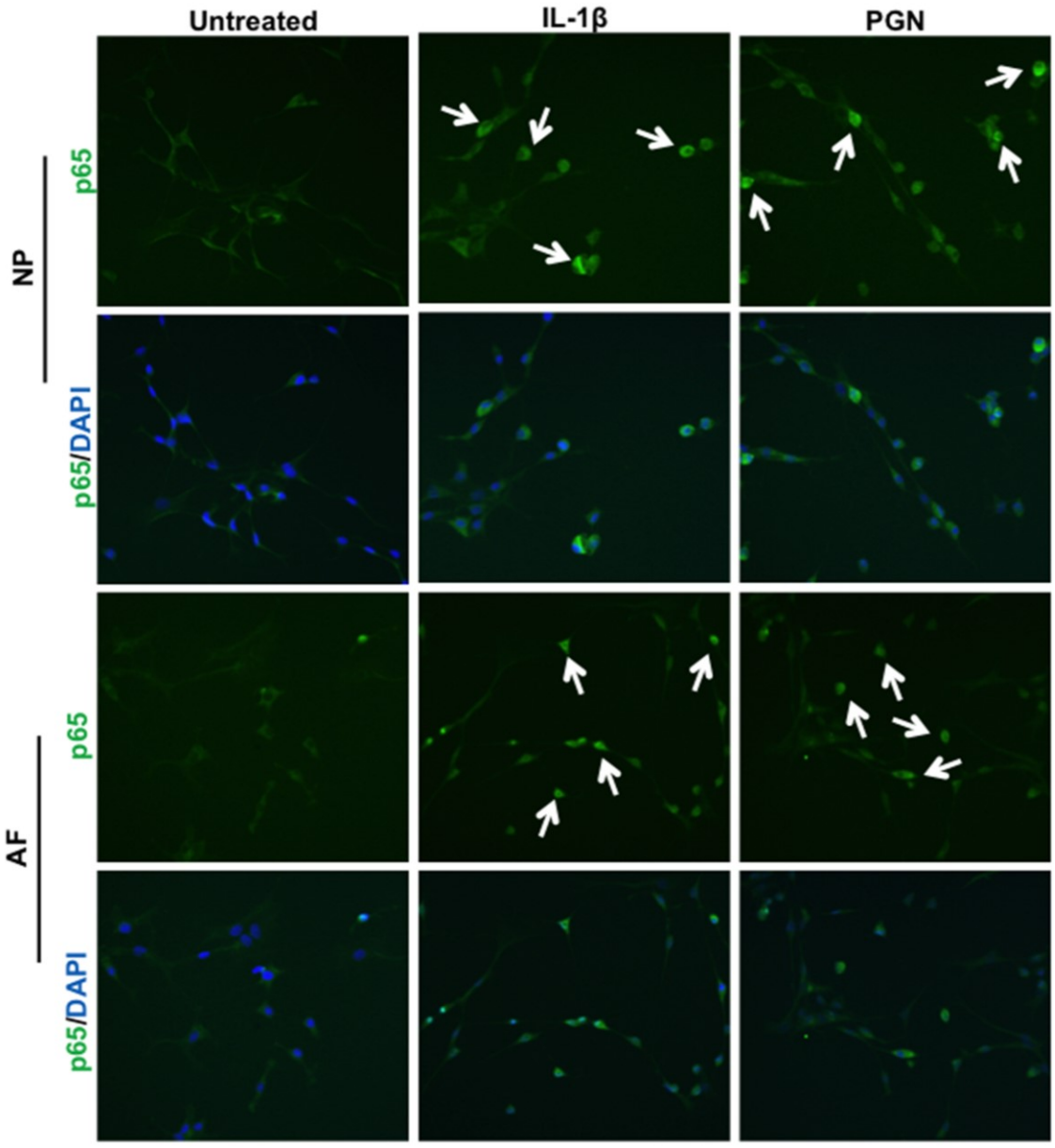

Figure 5

p65 Translocation to the Cell Nucleus Cells were treated with IL-1β, PGN or left untreated or left untreated for 1 hour and then stained for p65 localization (green). Cultures were counter

stained with the nuclear DNA stain DAPI (blue). White arrows indicate examples of p65 translocation to the nucleus. n=3.

Signaling mechanisms regulating NGF expression

While regulatory mechanisms controlling NGF expression in the central nervous system have been investigated, little is known about its regulation in degenerating tissues such as the intervertebral disc. To evaluate if TLR2 and IL-1β regulate NGF through p38, ERK1/2, NF-κB or another mechanism, NP and AF cells were pre-treated with small molecule inhibitors that specifically block either p38, ERK1/2 (MEK) or NF-κB activity. They were then exposed to IL-1β and PGN. In NP cells treated with IL-1β, p38 inhibition significantly decreased NGF expression compared to IL-1β alone and decreased NGF expression in AF cells. Interestingly, p38 inhibition did not alter TLR2 induced NGF expression in either NP or AF cells (Fig 6, A, B). Similarly, ERK1/2 inhibition did not effect NGF expression in NP cells (Fig. 6A), but surprisingly caused an increase in NGF expression in AF cells (Fig. 6B). To further investigate the role of MAPK signaling in NGF regulation and possible p38-ERK1/2 interactions, both pathways were inhibited together. Co-inhibition of p38 and ERK1/2 resulted in a decrease of NGF expression in IL-1β treated, but not PGN treated NP cells (Fig. 6A). In the AF cells, co-inhibition resulted in a small increase of NGF following IL-1β treatment and a small decrease following PGN treatment. Unlike inhibition of MAPK signaling pathways, NF-κB inhibition greatly reduced NGF gene expression compared to IL-1β or PGN treated cells without inhibitors in NP and AF cells (Fig. 6A and B), suggesting an important role for NF-κB regulation of NGF in disc cells.

To examine NGF protein secretion conditioned culture medium was probed by western blot after 48 hours. ERK1/2 inhibition had little effect or slightly increased NGF secretion in IL-

1β or PGN treated cells. Similarly, co-inhibition of p38 and ERK1/2 resulted in a small decrease in NP cells treated with IL-1β but did not affect NGF secretion induced by TLR2 activation or IL-1β in AF cells (Fig. 7). p38 inhibition resulted in small decreases of NGF secretion in NP cells (Fig. 7A, C). Interestingly, p38 inhibition resulted in significant decreases in both IL-1β and PGN treated AF cells. However, similar to NGF gene expression, NF-κB inhibition reduced NGF secretion by IL-1β or PGN treated cells to levels similar to untreated cultures (Fig. 7A, B). Important to note, NP and AF cells treated with cell signaling inhibitors only secreted mNGF like cells treated with IL-1β or PGN alone. These results suggest that IL-1β and TLR2 regulate NGF through NF-κB signaling.

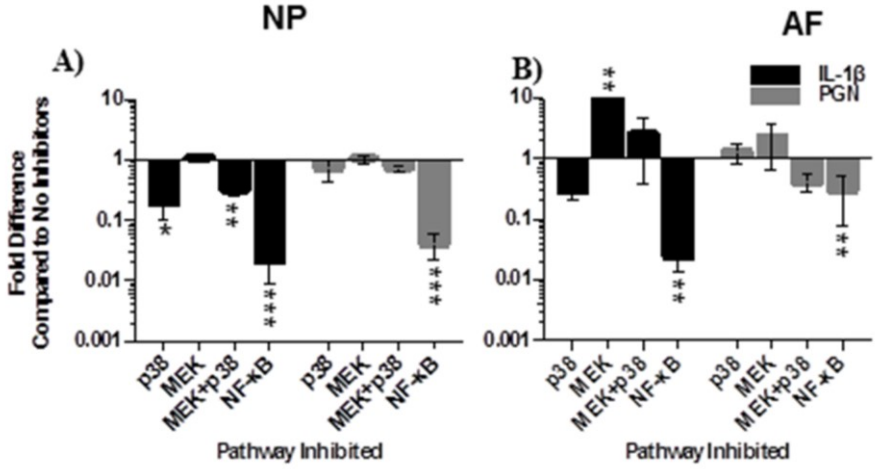

Figure 6

NGF Gene Expression Following p38, ERK1/2 and NF-B Inhibition Small molecule signaling inhibitors SB203580 (10 μM, p38), PD98059 (10 μM, MEK), SB203580 and PD98059,or BMS-345541(5 μM, NF-κB) were added to cultures 2 hours prior to treatment with IL-1β or PGN. NGF gene expression data following 6 hours of treatment with IL-1β or PGN in NP (A) and AF (B) cells was normalized to GAPDH expression and analyzed using the $2^{-\Delta\Delta Ct}$. Data is presented as fold difference compared to IL-1β or PGN treatments without signaling

inhibitors. Data was analyzed used the Kruskal-Wallis test for multiple comparisons of non-parametric data. Data is presented at mean ± SEM, n=4. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

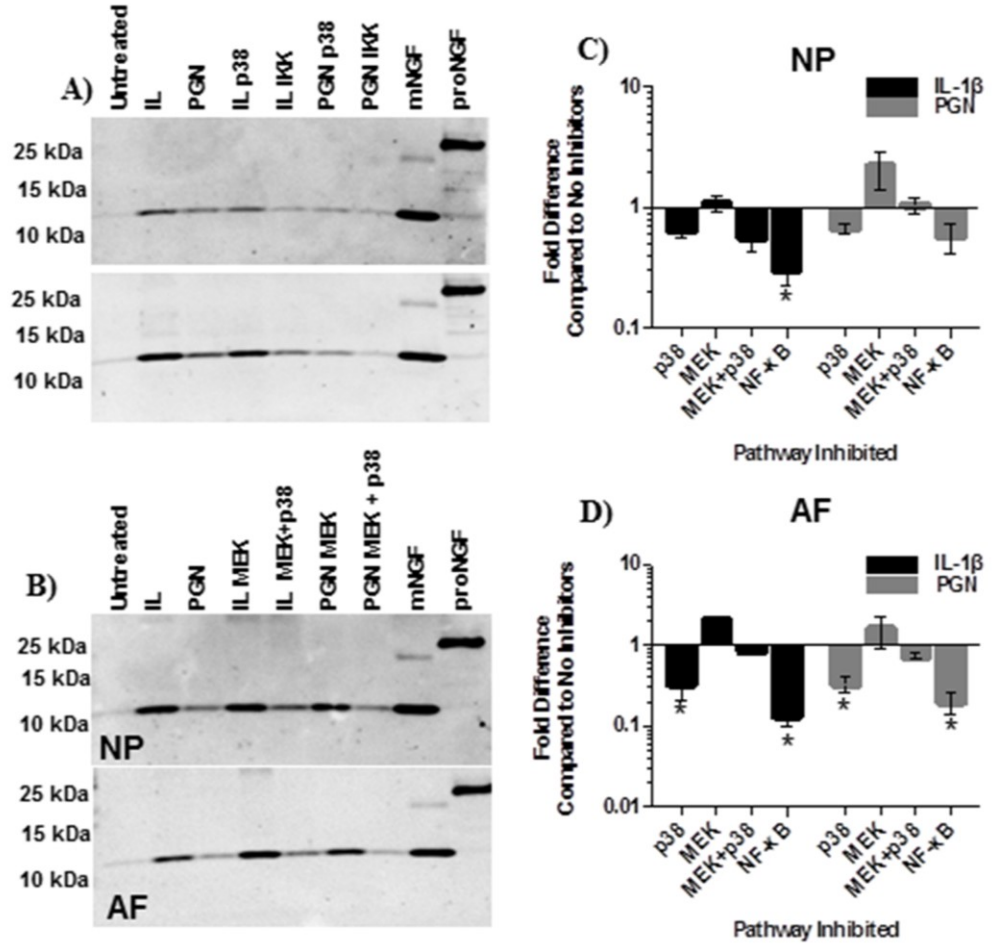

Figure 7

NGF Secretion following p38, ERK1/2 and NF-κa Inhibition Small molecule signaling inhibitors SB203580 (10 μM, p38), PD98059 (10 μM, MEK), SB203580 and PD98059,or BMS-345541(5 μM, NF-κB) were added to cultures 2 hours prior to treatment with IL-1β or PGN. Protein was precipitated from conditioned culture media after 48 hours of treatment. NGF secretion following p38 or NF-κB inhibition in NP and AF cells (A) and following ERK1/2 or ERK1/2 and p38 in NP cells (B) was assessed by western blot. Western blots were analyzed

using densitometry for both NP (C) and AF (D) conditioned media. Data was normalized to NGF secretion from cells treated with IL-1β or PGN but no signaling inhibitors.

Discussion

The results from this study demonstrate that TLR2 activation induces NGF and BDNF gene expression and NGF protein secretion in human intervertebral disc cells. To determine how NGF is regulated, p38, ERK1/2, JNK and NF-κB signaling activity was assessed. NF-κB activity is required for NGF expression induced by TLR2 as well as IL-1β. Taken together, these results identify a new regulatory mechanism mediating pathological NGF expression in NP and AF cells (Fig. 8) that could be used to target NGF to treat low back pain associated with disc degeneration.

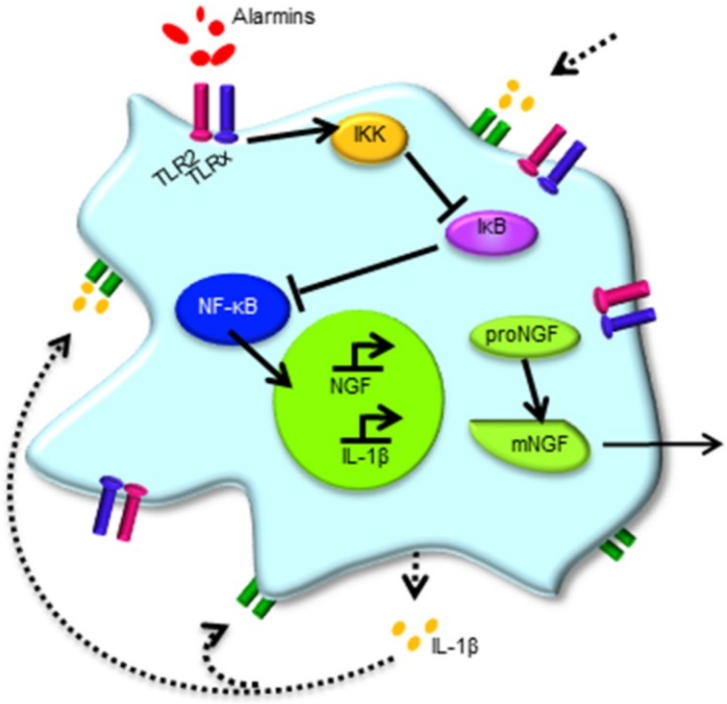

Figure 8

Schematic of TLR2 Regulation of NGF Alarmins activate TLR2 (blue) –TLRx (pink), where TLRx is TLR1, 2 or 6. TLR activation induces the NF-κB signaling cascade, promoting NGF synthesis. NGF is translated as proNGF and is cleaved intracellularly to mNGF and then secreted from the cell. Additionally, TLR2 activation results in increased IL-1β gene expression, which may result in increased IL-1β protein that could create a feedback loop, indicated by the dotted line, to further increase NGF.

NGF increases during disc degeneration and has been implicated in animal models of painful disc degeneration, where it is thought to induce neuronal sensitization [67,183,185]. NGF has also been suggested to increase innervation of degenerating discs [183,184], and has been the target of clinical trials to treat low back pain associated with degeneration [88,89]. However, little is known about the regulation of NGF in tissues that become pathologically innervated with painful diseases like disc degeneration or osteoarthritis. Exposure of disc cells to the pro-inflammatory cytokine IL-1β increases NGF and BDNF expression. Some studies report that also TNFα increases neurotrophin expression while other studies found that it does not. This discrepancy could be explained by differences in tissue source, experimental TNFα concentrations, and the time at which NGF expression was evaluated [66,187]. Our results show that TNFα induces NGF and to a lesser extent BDNF expression. However, the signaling mechanisms regulating NGF expression in pathological connective tissues like the disc were unknown until now. It was also not known that TLR2 activation increases NGF and BDNF expression in disc cells.

A current hypothesis is that ECM produced alarmins, potentially generated by mechanical trauma, activate TLR signaling in early stages of disc degeneration [27,101,103]. TLR activation can result in a robust increase of inflammatory cytokines and catabolic proteases,

which in turn can drive further degeneration. Potential roles for TLR signaling have long been suggested in rheumatoid arthritis and osteoarthritis and most recently in disc degeneration. For example, a recent study found HMGB1, a TLR2 ligand, increases with grade of degeneration in surgical specimens [103]. Furthermore, TLR2 activation increases IL-1β, IL-6, IL-8, MMP-1 and -13, and COX-2 gene expression and IL-6 protein in a mixed population of NP and AF cells [101,102]. These prior studies are indicative that TLR2 could play an important role in the increase of inflammatory mediators and catabolic enzymes that contribute to disc degeneration.

Here, we examined the role of TLR2 and 4 in regulating the pain mediators NGF and BDNF. TLR2 activation increases NGF as early as 6 hours in the NP and follows the same temporal pattern as IL-1β, demonstrating that TLR2 induces NGF directly, rather than increasing NGF secondarily through cytokine activation. TLR2 also increased BDNF gene expression. To confirm PGN functions through TLR2, we inhibited TLR2 activation and found TLR2 is required for PGN induced NGF expression. Interestingly, LPS, a TLR4 ligand, had a highly variable effect on NGF and BDNF gene expression while having little effect on NGF and BDNF protein expression. This suggests either TLR4 does not induce neurotrophins as readily as TLR2, or that there are variable levels of TLR4 on disc cell surfaces. When cells were challenged with PGN and IL-1β, only TLR2 expression increased, which agrees with other studies that found TLR2, but not TLR4, gene expression is readily inducible in disc cells [101]. Taken together, these results suggest TLR2 has a more prominent role in human disc degeneration compared to TLR4. TLR2 can signal as a homo- or heterodimer, but which combination of receptors function in intervertebral discs is an area requiring further investigation. TLR2 activation leads to increased protease and cytokine levels that will contribute to ECM breakdown and degeneration. Therefore, TLR2 activation can potentially contribute to the development of both chronic low

back pain via NGF synthesis and to accelerated degeneration via protease and cytokine induction.

TLR2 and IL-1R (IL-1β receptor) both contain an intracellular Toll-interleukin receptor (TIR) intracellular domain. As expected, the downstream signal mechanisms are similar and include the NF-κB pathway and p38, ERK1/2 and JNK MAP kinase signaling, which can cause a variety of transcription factors to translocate to the nucleus [90,94]. Activation of these pathways in discs results in an increase in inflammatory mediators and proteases [197]. While signaling mechanisms of TLR2 have been extensively investigated in other cell types, they are only now being elucidated in intervertebral disc. Quero *et al.* found that fragmented hyaluronic acid activation of TLR2 increases JNK, p38 and ERK1/2 phosphorylation, but not p65 phosphorylation or translocation [102] and Pam3CSK4 (TLR2/TLR1 agonist) also did not stimulate p65 [101]. These studies suggest TLR2 activation functions through MAP Kinase signaling and not NF-κB signaling in disc cells. However, the current study found TLR2 activation in NP and AF cells increases p38, ERK1/2 and NF-κB activity and induces p65 translocation, while JNK phosphorylation did not increase compared to controls. Therefore, while the current study confirms previous findings that TLR2 activates p38 and ERK1/2 signaling, it conflicts with previous data suggesting that NF- κB is not activated in disc cells. This contradiction may be due to several factors: 1) the current study uses NP and AF cells from non-degenerating discs whereas the previous studies used surgical samples from patients with symptomatic disc degeneration, disc herniation or trauma; 2) previous studies did not separate NP and AF cells; 3) different ligands were used. Due to the tissue sources, there was likely different TLR expression between tissues at baseline. This, along with different ligands, could influence what signaling pathways are activated by TLR2 signaling. Importantly, TLR2 activation provides another

mechanism for activating p38, ERK1/2 and NF-κB signaling during disc degeneration. Furthermore, phosphorylation of NF-κB is sustained (greater than 30 minutes) which agrees with other studies that have found prolonged NF-κB activation in NP and AF cells [198,199]. For other pathologies, both p38 (rheumatoid arthritis) and NF-κB (atopic dermatitis) inhibitors have gone to clinical trials [197]. If successful, these inhibitors may also positively affect disc degeneration.

Until now, signaling mechanisms regulating NGF in pathogenic connective tissues had not been investigated. However, NGF synthesis has been investigated in other tissues including the central nervous system. In astrocytes, PKC activation was associated with increased NGF, whereas increased cAMP levels did not affect NGF expression [200]. In astrocytoma cells, c-fos activation increases NGF and inhibiting c-fos reduced β-adrenergic receptor activation of NGF [201]. In glioma cells PKA, PKC or increased [Ca2+] mediate AP-1 (c-fos/c-jun) binding a consensus sequence in the NGF gene [202]. Similarly in fibroblasts, AP-1 binds a consensus sequence in the first intron of NGF and mutating this binding site reduces NGF promoter activity [203]. NGF mRNA synthesis has also been shown to be mediated by cAMP in Schwan cells [204]. Aside from AP-1 mediated NGF transcription, β-adrenergic receptor stimulation also activates PKA and induces NGF via C/EBPδ and CREB in glioma cells and in the cerebral cortex. Importantly, a C/EBPδ binding motif is also present in the NGF promoter region [205]. Taken together, these studies demonstrate central nervous system or fibroblast NGF synthesis is likely mediated by a number of different pathways that converge on AP-1, C/EBPδ, or CREB.

Here we investigated NGF synthesis in peripheral target tissues when induced by inflammatory mediators. We found that both TLR2 and IL-1β increase p38 and ERK1/2 activity, which can cause AP-1 translocation to the nucleus, and others have found evidence of CREB and C/EBPδ activity in intervertebral disc cells [206,207]. However, despite these *trans*-activating

factors, we found that NF-κB signaling is required for TLR2 and IL-1β to increase NGF expression. Furthermore, NF-κB inhibition had the largest effect on NGF synthesis of all pathways inhibited. TLR2 induced NF-κB-dependent NGF synthesis in both NP and AF cells differs from other cell types suggesting that NGF is regulated in a context-dependent or cell type-dependent manner. This has important implications when considering NGF as a therapeutic target. Diseases of the central nervous system such as Alzheimer's disease are associated NGF dysregulation [208], whereas increased NGF can cause chronic pain in peripheral tissues. Thus, being able to target NGF in a context dependent manner is advantageous.

NGF is translated into the ~26kDa proNGF, which is then cleaved to the ~13kDa mature form of NGF. ProNGF and mNGF exert different biological effects, where ProNGF has apoptotic functions and mNGF promotes neurite growth and survival. mNGF is classically thought to sensitize neurons via the TrkA receptor and proNGF may also affect inflammatory pain via the p75NTR-sortillin receptor complex in addition to having apoptotic effects [209]. In the central nervous system evidence suggests that proNGF is secreted and processed extracellularly by plasmin to mNGF [208]. In contrast, others have found proNGF is cleaved to mNGF by Furin prior to secretion in the central nervous system [210]. The mechanisms and location of NGF maturation have not yet been investigated in pathological connective tissues such as degenerating discs. In this study, only mNGF was detected in conditioned culture media and preliminary data also has detected mNGF in cell lysates of disc cells, suggesting that proNGF can be cleaved intracellularly. However, additional investigation is required to develop a complete understanding of NGF maturation in discs.

The results from this study show for the first time that TLR2 activation directly increases NGF gene expression and protein levels in human cells. Furthermore, NF-κB signaling is a novel

regulatory mechanism of NGF. This novel mechanism of NGF regulation is outlined in Figure 7. NGF undoubtedly plays an important role in the development of low back pain and many other painful degenerative connective tissue disorders. As clinical trials with monoclonal antibodies against NGF have shown, targeting NGF could be an effective therapeutic strategy. By better understanding how NGF is regulated, new therapeutics could be developed to target NGF production and to treat low back pain. An additional benefit to targeting TLR2 is that its activation also increases cytokines and catabolic proteases, which further contribute to disc degeneration. By using TLR2 as a therapeutic target, not only could NGF levels be potentially reduced, levels of inflammatory cytokines and catabolic proteases could also be similarly reduced, thus slowing the progression of disc degeneration in addition stopping the associated low back pain.

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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions: EK, JBC, and DHR performed experiments and collected data. EK, LSS, DHR and LH designed the study and wrote the manuscript. MHW and JAO assisted in sample collection and results interpretation. All authors reviewed, edited and approved the final manuscript.

Chapter 4: Toll-like Receptors in Early Stage Disc Degeneration

4.1 Preface

There are many risk factors that contribute to the development of disc degeneration and chronic low back pain, creating difficulties in characterizing disease onset. In turn, this leads to poor understanding of the early stages of degenerative disc disease. Better understanding of the early mechanisms will identify potential disease-modify drug targets. The previous manuscript and previous studies from our group and others identified a role for TLR2 in disc degeneration. When TLR2 is activated *in vitro,* disc cells secrete NGF and BDNF, proinflammatory cytokines and proteases, and decrease matrix synthesis. Furthermore, disc cells constitutively express TLR2 and TLR4, creating the possibility they are activated by alarmins during the early stages of disc degeneration. However, whether TLR activation is sufficient to induce degenerative changes in human discs remained unknown. TLRs also function as heterodimers and which dimers are more readily activated human discs had not been determined. Understanding activation of TLR heterodimers and the role of TLR activation in the early stages of degeneration will indicate whether they are potential disease modifying targets.

In this study we hypothesized TLR activation will induce degenerative changes in human intervertebral discs. We use ligands to activate specific TLR heterodimers. We then inject TLR agonists into human discs that lacked signs of degeneration and cultured them *ex vivo*. To determine if TLR activation is sufficient to cause degenerative changes we characterized protease and cytokine production as well as extracellular matrix degradation.

4.2 Manuscript III: Toll-like Receptor 2 Activation Induces Human Intervertebral Disc Degeneration

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Key Words: toll-like receptor, intervertebral disc degeneration, inflammation, cytokines, proteases, extracellular matrix

The etiology of intervertebral disc degeneration and chronic low back are poorly understood, thus no disease modifying drugs exist. Toll-like receptors (TLR) are activated by endogenous alarmins such as fragmented extracellular matrix compounds found in the degenerating disc. TLRs regulate cytokine, neurotrophin, and protease expression in disc cells *in vitro*, and thus can control key factors in disc degeneration. However, whether TLR activation leads to degenerative changes in intact human discs is unclear. The current study investigated whether TLR activation induces degenerative changes in intact human discs *ex vivo*. Non-degenerate human discs from organ donors were injected with PBS, Pam2CSK4 (TLR2/6 and TLR2 agonist) and 30 kDa fibronectin-fragments (FN-f, a TLR2 and TLR4 alarmin), and cultured for 28 days. TLR activation increases proteoglycan release into the culture media and decreases proteoglycan content in the extra cellular matrix. Proteases that degrade disc extracellular matrix, including MMP3, 13 and HTRA1, are expressed at higher levels following TLR activation. In addition, proinflammatory cytokine levels, including IL-1, TNFα and IFNγ, increase following TLR activation. Taken together, these results indicate that TLR activation induces degenerative changes in human discs, possibly representing an early event in disc degeneration. Therefore, TLRs represent potential disease-modifying therapeutic targets to slow disc degeneration.

Introduction

Chronic low back is a leading cause of disability and morbidity world-wide [2,3], of which intervertebral disc degeneration is one of the main etiologies [6]. Despite the high prevalence and the associated health care and social costs, limited knowledge of disc pathology and pain restrict therapeutic approaches. Conservative approaches, such as NSAID regimes or physical therapy often do not effectively relieve patients' suffering, and surgical interventions are costly, invasive, and have a high reoperation rate. Currently no disease modifying drugs exist. The early stages of disc degeneration are the ideal disease stage for non-surgical, disease modifying interventions that stop or slow the progression of degeneration. However, early stages of degeneration are particularly poorly understood.

The disc is a fibrocartilaginous tissue consisting of the central, gelatinous nucleus pulposus (NP) and the outer annulus fibrosus (AF), which surrounds the NP. The NP is composed primarily of proteoglycans, especially aggrecan, and randomly organized type II collagen fibers. As a result the NP is a highly hydrophilic tissue, with high water content and gel-like characteristics, allowing for compression of the spine. The AF is composed primarily of type I collagen fibers organized in a laminar structure. This composition confers a high tensile strength that contains the NP. The NP and AF are separated from the vertebral bodies by a thin layer of hyaline cartilage called the cartilaginous endplate [1,6]. These distinct structures give intervertebral discs their biomechanical function. When the structures begin to degenerate and fail the disc loses its biomechanical function.

In non-degenerating discs there is a balance between ECM synthesis and degradation, resulting in a slow, physiological ECM turnover. During disc degeneration ECM turnover is no longer balanced and catabolism outpaces ECM synthesis. The proteoglycans, glycosaminoglycans (GAG), and other ECM proteins, such as fibronectin, collagen species and a number of small leucine-rich repeat proteins (SLRP), in the NP and the AF are degraded and fragmented [6,16]. Upregulated proteases, including cathepsins, MMP-3 and -13, ADAMTS-4 and -5 [12,33] and HTRA1 [20,30], degrade the ECM.

Sterile inflammation, defined as an increase of proinflammatory factors in the absence of infection, is another hallmark of disc degeneration. Proinflammatory cytokines, such as IL-1β, TNFα, IL-6 and IL-8, angiogenic factors and neurotrophins all increase [39-41,50,66,67,183]. Cytokines increase expression of proteases, neurotrophins, cytokines and cytokine receptors. Angiogenic factors promote vascularization and neurotrophins promote innervation of the normally avascular and anueral disc. Furthermore, cytokines, neurotrophins and innervation are directly linked to pain [9,10,22,65]. The sterile inflammation of the disc creates a feed-forward loop that increases ECM degeneration and could lead to pain. However, cytokines like IL-1β and TNFα are expressed at low to undetectable levels in non-degenerating discs [58,183,211]. Therefore cytokines alone do not explain the sterile inflammation that

occurs during early stages disc degeneration. Increased understanding of sterile inflammation and early stages of disc degeneration could identify new therapeutic targets.

ECM degradation is a key event even the earliest stages of disc degeneration. ECM fragments can act as danger associated molecular patterns (DAMPs), also termed alarmins. Due to poor waste exchange, matrix fragments also may slowly accumulate during physiological matrix turnover. Alarmins can activate pattern recognition receptors including toll-like receptors (TLRs). TLRs were originally characterized for their role in innate immunity, but more recently alarmins were also found to activate TLRs. Extracellular matrix fragments that act as alarmins include fragmented aggrecan [92], fibronectin, biglycan [212] and low molecular weight hyaluronic Acid [94]. Extracellular HMGB1 also activates TLRs and likely functions as an alarmin in disc degeneration [103]. Human disc cells express TLRs 1-6, -9 and -10 and TLRs 1, 2, 4 and 6 increase with the grade of degeneration [101]. Interestingly, cells from non-degenerating discs express TLR1, 2, 4 and 6 and adverse mechanical strain increases the expression of TLR2 and 4 [21]. TLR2 and 4 are the TLRs primarily associated with alarmin activation. TLR2 activation in human NP or AF cells activates NF-κB, p38 and ERK signaling, which are associated with disc degeneration [197,211]. Furthermore TLR2 activation increases NGF, BDNF, IL-1β, TNFα, IL-6, MMP-1-3, and -13 and COX-2 in human disc cells [30,101,211]. TLR4 activation in human NP and AF cells also increases neurotrophin and cytokine gene expression, although inconsistently [211]. These previous studies demonstrate that TLR activation increases key components of disc degeneration (proteases, cytokines and neurotrophins) *in vitro*, which could create a proinflammatory feed-forward loop *in vivo*. TLRs therefore likely play a role in disc degeneration and chronic low back pain. Due to ECM fragment and alarmin turnover during physiological and pathological matrix degradation, TLRs are potentially activated during the early stages of degeneration. However, it remains unclear if TLR activation is sufficient to induce degenerative changes in human discs.

In the current study we hypothesized activation of TLR2 will induce degenerative changes in human intervertebral discs. This study investigated the effects injecting TLR2 agonists and 30 kDa

fibronectin fragments (a TLR2 and TLR4 agonist) into non-degenerate human discs in an *ex vivo* organ culture system. We found TLR activation causes degenerative changes in the NP tissue, release of specific ECM components, and increases in proteases and sterile inflammation.

Methods

Tissue Collection

All procedures were approved by the institutional review board of McGill University (IRB#s A04-M53-08B andA10-M113-13B) for the projects titled "Human Intervertebral Discs used for Culture and Extracellular Matrix. " and "Spinal Tissue Repository and Databank Protocol." Lumbar spinal segments (T12-S1) were removed from human organ donors following familial consent. Spines were harvested within 4 hours of aortic cross clamping. Only discs lacking radiographic and visual signs of degeneration were used.

In vitro Cell Culture

NP and AF tissues were separated, mechanically diced and digested overnight with 1.5 mg/ml Collagenase Type II. Cells were then cultured and passaged in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) containing 4.5 g/L glucose, 1x Glutamax (Thermo Fisher Scientific), and 50 µg/ml gentamicin (Thermo Fisher Scientific) and 10% fetal bovine serum (FBS, Thermo Fisher Scientific). All experiments were performed in passage 1 or passage 2. Prior to treatment cells were serum starved in DMEM containing 4.5g/L gluclose, 5 µg/ml Glutamax, 50 µg/ml Gentamicin and 1X insulin transferrin selenium (Thermo Fisher Scientific) for 2 hours. Cells were then treated in the same serum free media with 5µg/ml PGN (general TLR2 agonist, Sigma-Aldrich), 100 ng/ml Pam3CSK4 (TLR1/2 specific agonist, Invivogen) or 100 ng/ml Pam2CSK4 (TLR2/6 specific agonist, Invivogen) for 6 hours. RNA was then collected in TRIzol reagent (Thermo Fisher Scientific).

Quantitative PCR

RNA was extracted using the TRIzol chloroform extraction method according to manufactures instructions (Thermo Fisher Scientific). 500 ng of RNA was reverse transcribed using a qScript CDNA

Synthesis Kit (Quanta Biosciences) with an Applied Biosystems Verti Thermocycler (Thermo Fisher Scientific). Real-time quantative PCR was performed using an Applied Biosystems StepOnePlus machine (Thermo Fisher Scientific) with PerfecCTa SYBR Green Fast Mix (Quanta Biosciences). Primers (Thermo Fisher Scientific) are described in table 2. Data was analyzed using the $2^{-\Delta\Delta CT}$ method. Gene expression was normalized to GAPDH and then to vehicle treated cells.

Table 2

Gene	Primer Sequence 5' to 3'	Primer Reference
GAPDH	F: TCCCTGAGCTGAACGGGAAG R: GGAGGAGTGGGTGTCGCTGT	[213]
IL-1β	F: ACAGATGAAGTGCTCCTTCCA R: GTCGGAGATTCGTAGCTGGAT	[214]
NGF	F: AAGTGCCGGGACCCAAAT R: TGAGTTCCAGTGCTTTGAGTCAA	[191]

Ex Vivo Organ Culture and Disc Injection

Three human intervertebral discs from the same spine (n=7 spines, 21 discs) were isolated and cultured as previously described [20]. Briefly, discs were excised from the spine, boney endplates were removed and discs were washed. Discs were then culture in DMEM containing 4.5 g/L of glucose supplemented with 5% FBS, 1X Glutamax, 50 µg/ml gentamicin and 50 µg/ml ascorbic acid. After 4-6 days and at least one media change discs from the same spine were injected with PBS, Pam2CSK4 (100ng/g disc tissue) or 30 kDa FN-f (1nM/g disc tissue) in a total volume of 200µl PBS. Discs were then cultured in DMEM supplemented with 1x glutamax, 50 µg/ml gentamicin and 1% FBS for 28 days. Media was changed and collected every 3-4 days. On day 28 tissue punches were taken from the NP and the inner AF regions for analysis (Fig. 2).

Protein Extraction

Protein from tissue punches was extracted with 4M GuHCl extracted on a wet-weight per volume basis using 9 volumes extraction buffer (4 M guanidinium chloride, 50 mM sodium acetate, pH

5.8, 10 mM EDTA (all chemicals from Sigma-Alrdich), and COMPLETE® protease inhibitors [Roche, Laval, QC]). Samples were incubated for at least 72 hours at 4°C with gentle rocking.

DMMB

Dimethyl methylene blue (DMMB) assays were used according to Mort and Roughley [215] to quantify sulfated glycosaminoglycans. Chondroitin sulfate (Sigma-Aldrich) was used to make the standard curve and 4M GuHCl was added to standard curves when quantify tissue GAG content. All samples were diluted to fall in the middle of the linear portion of the standard curve. Media samples were pooled from days 4 to 28 prior to GAG measurement. GAG release in media was normalized to the GAG in media at Day 0 and then normalized to PBS injected discs from the same spine. NP GAG content was normalized to PBS injected discs from the same spine.

Histological Staining

4mm tissue punches were fixed and stored in 80% methanol at 4°C. Tissues were cyroprotected in 10% (1h), then 20% (6h), and then 30% (overnight) sucrose. Samples were embedded in optimal cutting temperature medium (OCT, Thermo Fischer) and sectioned at 14µm and thaw mounted on glass slides. Samples were then stained with Safranin-O (Ssigma-Aldrich). Sections were imaged using a Zeiss Axioskop 40 and images were taken with an AxioCam MR (Zeiss), and processed using AxioVision LE64 software (Zeiss).

Mass Spectrometry

Proteins in media samples were reduced with DTT, alkylated with iodoacetic acid and then digested with trypsin with re-solubilization in 0.1% aqueous formic acid/2% acetonitrile. The peptides were loaded onto a Thermo Acclaim Pepmap (Thermo, 75uM ID X 2cm C18 3uM beads) precolumn and then onto an Acclaim Pepmap Easyspray (Thermo, 75uM X 15cm with 2uM C18 beads) analytical column separation using a Dionex Ultimate 3000 uHPLC at 220 nl/min with a gradient of 2-35% organic (0.1% formic acid in acetonitrile) over 3 hours. Peptides were analyzed using a Thermo Orbitrap Fusion mass spectrometer operating at 120,000 resolution (FWHM in MS1, 15,000 for

MS/MS) with HCD sequencing all peptides with a charge of 2+ or greater. The raw data were converted into *.mgf format (Mascot generic format) and searched using Mascot 2.3 against human sequences (Swissprot). The database search results were loaded onto Scaffold Q+ Scaffold_4.4.8 (Proteome Sciences) for spectral counting and data visualization.

ELISA

MMP3 and MMP13 enzyme linked immunosorbent assays (ELISA) were performed according to manufactures instructions (Abcam PLC). Conditioned media from day 4 through day 28 was pooled together. Colorimetric absorbance was measured with a Tecan Infinite M200 PRO (Tecan) and analyzed with i-control 1.9 software (Tecan). Protein levels were then normalized to PBS injected discs from the same spine.

Protein Arrays

Media collected from day 4 to day 28 was pooled together and analyzed use the RayBio Human Cytokine Antibody Array C6 (RayBiotech, Inc.) according to manufactures instructions. Arrays were imaged using an ImageQuant LAS4000 Image Analyzer (GE) and analyzed with ImageQuant TL array analysis software (GE). Data was normalized to cytokine secretion from PBS injected discs from the same spine.

Statistical Analysis

Data was analyzed with Graph Pad Prism Version 7. Data was analyzed by repeated-measures one-way ANOVA with Fisher's least significant difference post-hoc text unless otherwise indicated, normalized to untreated cells of PBS injected discs, and presented at mean ± standard error of the mean (SEM).

Results

Pam2CSK4 increases proinflammatory and pronociceptive gene expression

Previously we used the non-soluble TLR2 agonist, peptidoglycan (PGN). Agonists likely have to be soluble to have an effect following injection into a disc. We therefore assessed the ability of the

soluble TLR2 agonists Pam3CSK4, a TLR2/1 agonist, and Pam2CSK4, a TLR2/6 or TLR2 agonist, to induce inflammatory and nociceptive gene expression. As previously reported [211], PGN increases NGF and IL-1β expression in NP cells compared to untreated cells (Fig. 1). Pam3CSK4 had no effect on NGF or IL-1β expression whereas Pam2CSK4 increased NGF (NP: 7.2±0.9-fold, AF: 5.8±0.9-fold, Fig. 1a and b) and IL-1β (NP: 9.1±3.4, AF: 4.9±1.1, Fig. 1c and d) expression.

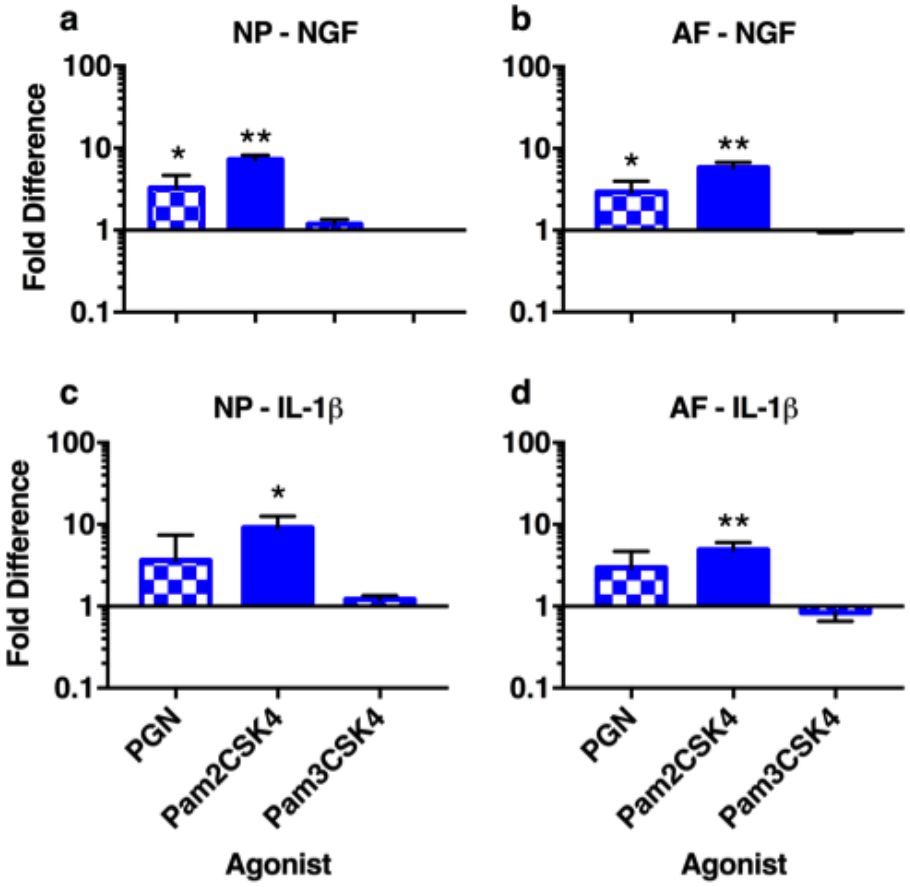

Figure 1. NGF and IL-1β expression following TLR stimulation. NP (A, C) and AF (B, D) cells were isolated from non-degenerate human discs and treated with PGN (TLR2 agonist), Pam2CSK4 (TLR2/6 and TLR2 agonist) and Pam3CSK4 (TLR1/2 agonist). NGF (a, b) and IL-1β (c, d) gene expression were evaluated with RT-qPCR. Data is presented as mean ± SEM and was analyzed by repeated measures one-way ANOVA. * indicates $p<0.05$, ** indicates $p<0.01$, n=4.

TLR agonists induce Glycosaminoglycan and Proteoglycan loss

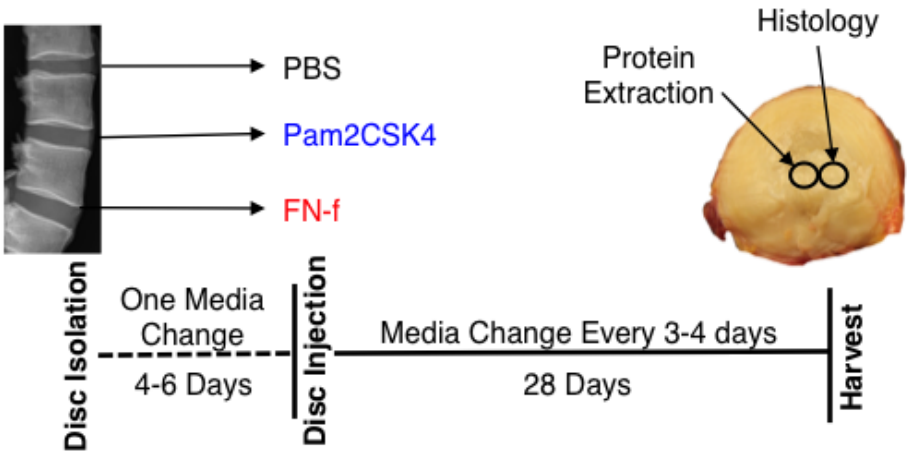

Figure 2. Schematic of the *ex vivo* organ culture experiment. Lumbar spines from organ donors were assessed radiographicly for signs of degeneration. 3 discs per experiment lacking signs of degeneration were isolated from a spine, cultured for 4-6 days and injected with PBS, Pam2CSK4 of 30 kDa fibronectin fragments. Discs were then cultured for 28 days, with media changes every 3-4 days. NP tissues punches were taken at the end of the experiment.

To determine if TLR activation induces degenerative changes in human discs, lumbar discs lacking signs of degeneration were excised from human spinal segments and cultured. Following 4-6 days of *ex vivo* whole disc organ culture, discs were injected with PBS, Pam2CSK4 (TLR2/6 and TLR2 agonist) or 30 kDa FN-f (TLR2 and 4 agonist). The experimental setup is outlined in Figure 2. After 28 days GAG content was assessed by DMMB assay. Pam2CSK4 and FN-f injection reduce GAG content in the NP tissue compared to discs injected with PBS from the same spine (0.63±0.09 fold-decrease and 0.60±0.09 fold-decrease Fig. 3a). Conditioned media from day 4 through day 28 was pooled and also assessed by DMMB. Compared to PBS injected discs Pam2CSK4 increases (4.99±2.2 fold-increase) and FN-f creates a trend of increased GAG release into media (Fig. 3b).

We further assessed NP ECM degeneration following TLR activation with Safranin-O staining. Safranin-O binds negatively charged molecules, which are primarily represented by proteoglycan in the NP. 28 days after Pam2CSK4 and FN-f injection tissue punches from the NP were taken (Fig. 2) and

histological sections were stained. Both Pam2CSK4 and FN-f decrease Safranin-O stain compared to PBS injected discs, indicating that TLR activation leads to a loss of proteoglycan (Fig. 3c). Taken together, these results indicate that Pam2CSK4 and FN-f injection decrease proteoglycan content in the NP, suggesting the TLR activation results in NP matrix degeneration.

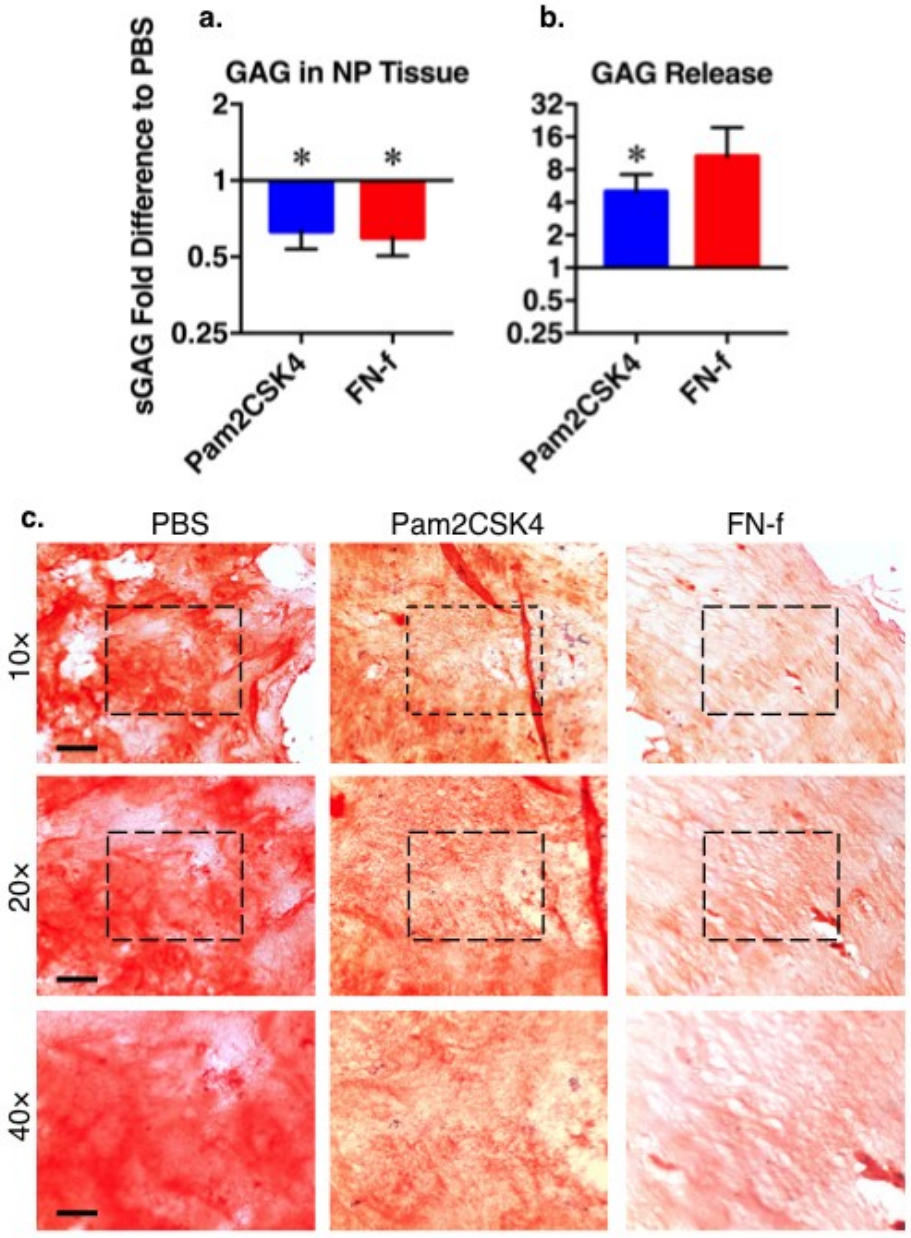

Figure 3. Effect of TLR activation on NP matrix. Glycosaminoglycan (GAG) levels in the NP were assessed by DMMB and normalized to discs injected with PBS from the same spine (a). Conditioned culture media was pooled from days 4 to 28 and GAG release was assessed by DMMB and normalized

to day 0 GAG concentration and then to the GAG concentration of PBS injected disc media from the same spine (b). Histological sections were stained with Safranin-O and imaged with 10x, 20x, and 40x objectives. Representative images are shown. Scale bars represent 200, 100, 50 μm under 10×, 20× and 40× magnification. Data is presented as mean fold difference ± SEM and was analyzed by repeated measures one-way ANOVA. * indicates $p<0.05$, ** indicates $p<0.01$, n=6.

TLR activation promotes release of ECM proteins

Pooled conditioned media was assessed by mass spectrometry to better understand the effect of TLR activation on disc ECM components. Compared to PBS injected discs, Pam2CSK4 and FN-f injections increase the release of fibronectin, matrix scaffolding proteins, small leucine-rich repeat proteins, and a number of collagen species as indicated by increased red on the heat map (Fig. 4a). Release of the proteoglycans aggrecan and versican did not increase although lubrican increase did. Decorin, CILP and COMP were also found in higher amounts (Fig. 4b). While many proteins had increasing trends, only type II collagen release (3.4±1.4) following Pam2CSK4 injection was significantly different. Taken together, as illustrated by Figure 2a and b, Pam2CSK4 and FN-f injection increase ECM component release indicating that TLR activation leads to overall changes in the ECM composition.

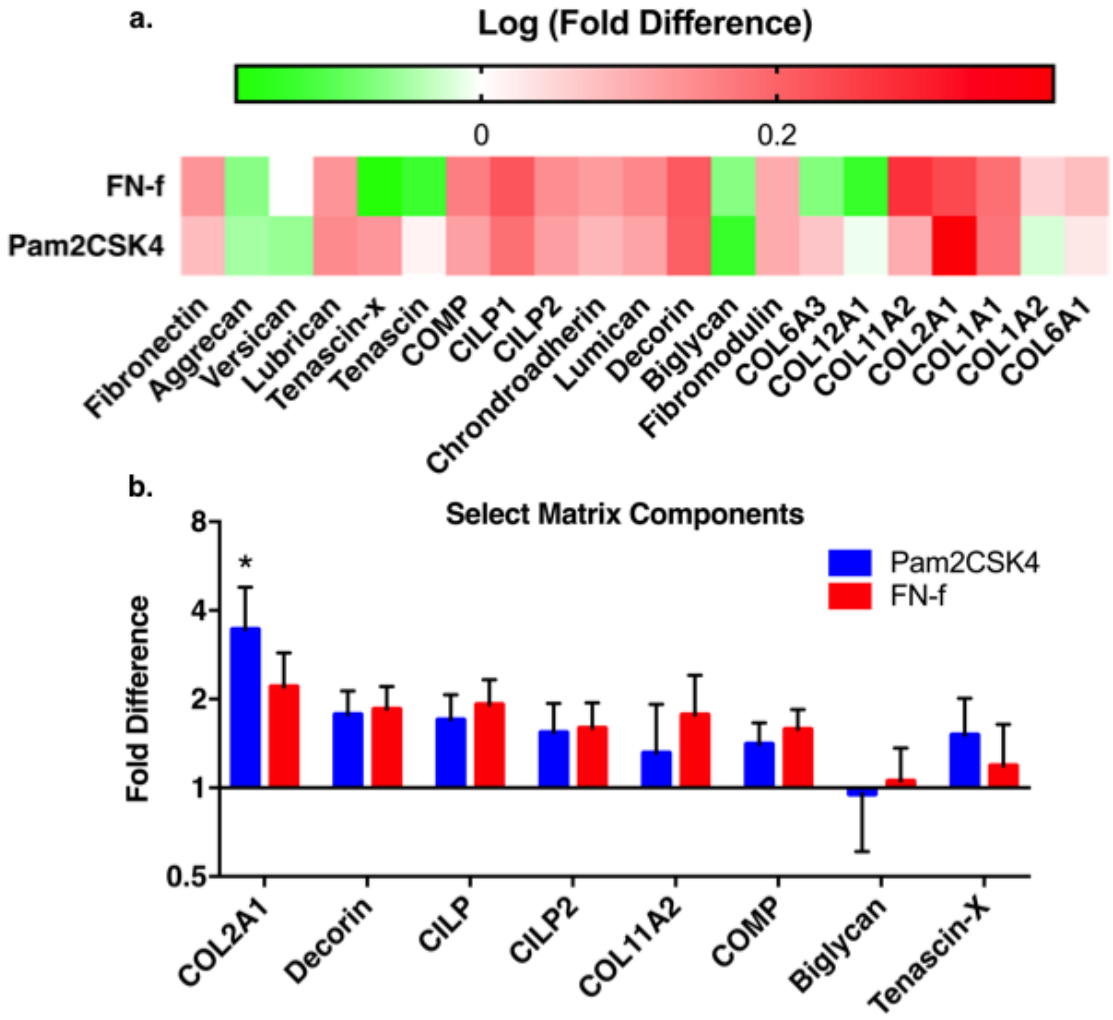

Figure 4. Release of matrix components following TLR activation. Conditioned culture media was pooled from days 4 to 28 and analyzed by mass spectrometry. Spectral counts were normalized to PBS injected discs from the same spine. Data is presented as the log of the fold difference in a. Select matrix components are shown as mean fold difference ± SEM (b). Data was analyzed by repeated measures one-way ANOVA. * indicates $p<0.05$, n=6.

TLR agonists increase protease levels

Since *ex vivo* TLR activation results in the production of matrix degrading enzymes, we hypothesized Pam2CSK and FN-f increase protease levels. We further examined our mass spectrometry data to evaluate protease changes. A number of proteases were detected in conditioned

culture media, including several with described roles in disc degeneration. MMP2 slightly increases following agonist injection of both agonists (Fig. 5a). Pam2CSK4 increases the expression of MMP 3 (1.82±0.1), HTRA1 (2.75±0.9) and Cathepsin D (1.78±0.3). FN-f also increases HTRA1 (2.65±0.7) secretion and non-significantly increases MMP 3 and Cathepsin D.

Along with MMP 3, MMP 13 is one of the main proteases described in disc degeneration [10,22], but it was not detected by mass spectrometry. To evaluate the effect of TLR activation on MMP13 secretion and to further quantify MMP3, ELISAs were used. Confirming the mass spectrometry data, MMP3 secretion is increased by Pam2CSK4 injection (1.64±0.4 fold increase, 45.2±9.6 ng/ml) and FN-f (1.49±0.31 fold-increase, 49.66±11.6 ng/ml) compared to PBS (31.4±7.2 ng/ml). Pam2CSK4 injection also increases MMP13 secretion (1.82±0.5 fold-increase, 3.7±0.69 ng/ml) compared to PBS injected discs (2.3±0.55 ng/ml). The MMP13 concentrations measured by ELISA are an order of magnitude lower than MMP3, possibly explain why MMP13 was not detected by mass spectrometry. Together, mass spectrometry and ELISA data show that TLR activation in intact IVDs result in increased levels of proteases that could degrade the disc ECM.

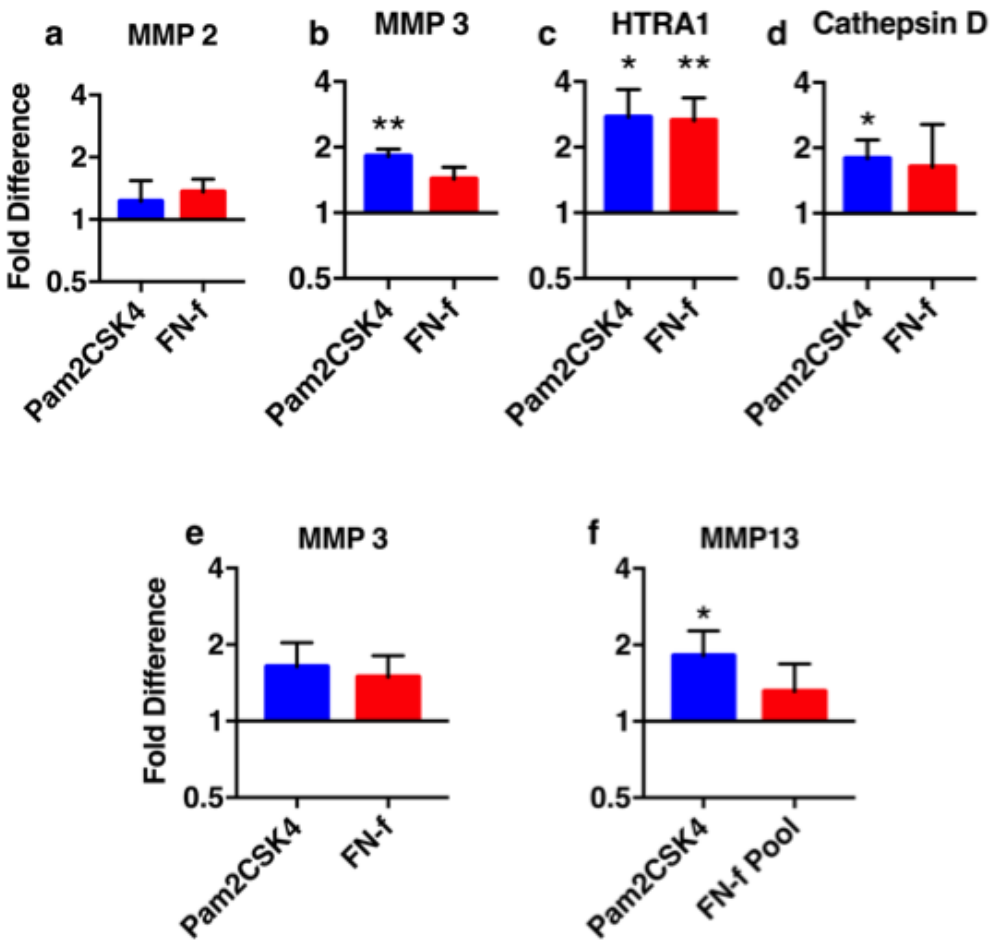

Figure 5. The effect of TLR activation on protease secretion. Conditioned media was pooled from days 4 through 28 and analyzed with mass spectrometry (a-d) and ELISA (e, f). Mass spectrometry data was analyzed using spectral counting. All data was normalized to PBS injected discs from the same spine and is presented as the mean fold difference ± SEM. * indicates $p<0.05$, ** indicates $p<0.01$, n=6.

TLR activation induces a sterile inflammatory response

In addition to increased matrix degradation and protease production, sterile inflammation is another hallmark of disc degeneration [22]. In fact, *ex vivo* degenerating discs from chronic low back pain patients secrete increased levels of many proinflammatory cytokines [183]. To evaluate whether TLR activation by Pam2CSK4 or FN-f increases cytokine secretion, conditioned media from day 4 through day 28 was pooled and assessed by antibody protein arrays. Compared to PBS injected discs

Pam2CSK4 robustly increases secretion of many cytokines (Fig. 6a) and chemokines (Fig. 6b) including IFN-γ (78.4±46.3 fold-increase), GM-CSF (11.1±4.3) and CXCL1 (21.6±14.6) and to a lesser extent increases others including IL-1α, TNFα and CCL8. FN-f injection at this concentration showed a less pronounced increase in cytokine release, although it robustly increased CXCL9 (73.2±70.4) secretion and to a lesser extent increased IL-1α, IFNγ, and TNFα. Sterile inflammation often results in activation of the complement system. Mass spectrometry analysis of pooled conditioned media showed that Pam2CSK4 and FN-f injection increase secretion of a number of complement factors including complement C3, C4 and C5 (Fig. 6c). These results further support that activation of TLRs in human discs induces sterile inflammation leading to IVD degeneration.

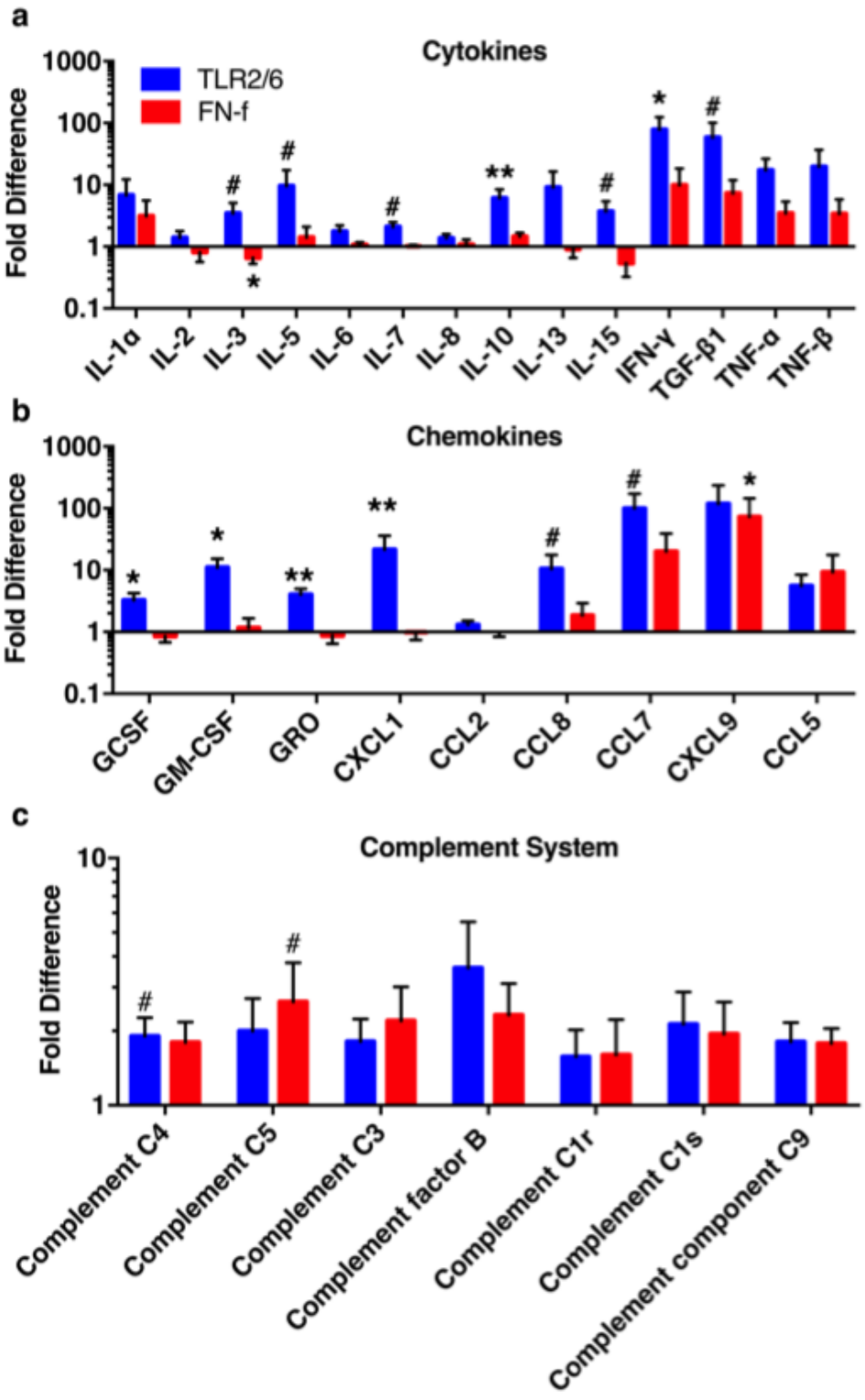

Figure 6. Inflammatory factor secretion following TLR activation. Conditioned media was pooled from days 4 through 28 and analyzed for inflammatory factors. Cytokines (a), chemokines (b) and components of the complement system (c) were analyzed by protein arrays and complement system

factors were analyzed with mass spectrometry with spectral counting. Data was normalized to PBS injected discs from the same spine and are show as the mean fold difference ± SEM. Data was analyzed by repeated measures one-way ANOVA. # indicates $p<0.1$, * indicates $p<0.05$, ** indicates $p<0.01$, n=6.

Discussion

Despite disc degeneration and chronic low back pain being a leading cause of morbidity no disease modifying drugs exist. This is partly due to the poor understanding of the early stages of disc degeneration. Decreased matrix synthesis, ECM degradation, increases in proteases and sterile inflammation are hallmarks of disc degeneration [22], but how physiological matrix turnover and disc aging transitions to pathological characteristics is poorly understood. Here, we investigated if TLR activation leads to degenerative changes in non-degenerate human discs. We found that injection of Pam2CSK4 and 30 kDa Fibronectin fragments, an endogenous alarmin found in the degenerating disc, decreases proteoglycan and content in the NP, increases general matrix degradation, increases protease secretion and results in sterile inflammation. Thus TLR activation can induce the pathological hallmarks and drive the feed-forward loop that results in disc degeneration as depicted in figure 7.

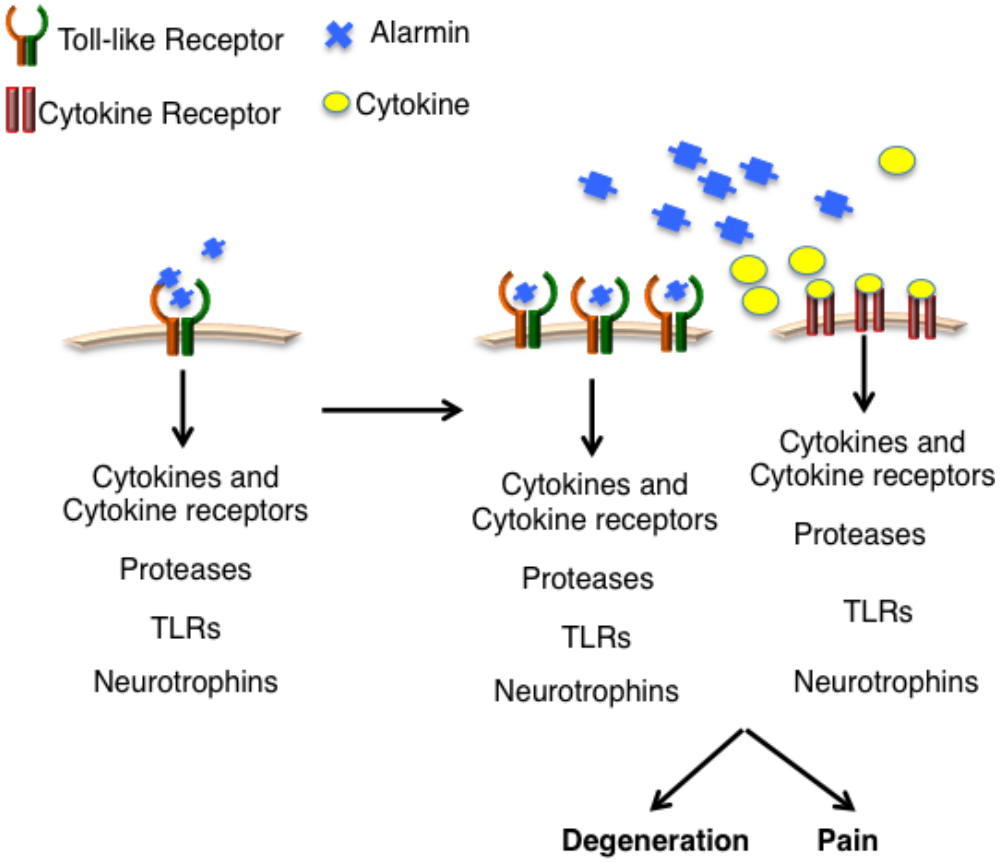

Figure 7. Schematic of a TLR regulated feed-forward degeneration cycle. Early activation of TLRs leads to initial increases in cytokines, cytokine receptors, TLRs, neurotrophins and proteases. In turn, this creates more alarms, leading to enhanced TLR activation. Cytokine receptor activation increases cytokines, cytokine receptors, TLRs, neurotrophins and proteases as well, creating a viscous feed-forward degeneration cycle that results in disc degeneration and pain.

Proteoglycans, especially aggrecan make up the bulk of the NP, as illustrated by a proteoglycan to collagen ratio of 27:1in healthy NP tissue [216]. This ECM composition confers a high anionic charge to the NP, resulting in high water content and the NP's gel like characteristics. Loss of proteoglycan is an early event in IVD degeneration leading to loss of biomechanical function. Mass spectrometry did not find increased proteoglycan loss likely because aggrecan and versican are highly glycosylated making detection by mass spec difficult. It is the glycosylated parts that are first lost from the tissue

while the non-glycosylated fragments are retained in the ECM as is evident by the accumulation of aggrecan G1 region in degenerating IVDs. However, DMMB and Safrainin-O staining show TLR activation we decreases proteoglycan content in the NP and increases GAG release into condition media. These results show that anionic GAG-chains responsible for water retention are lost following TLR activation.

Release of several specific ECM components further supports the conclusion TLR activation leads to degenerative changes. Type II collagen is the primary collagen in the NP and its decrease is a hallmark of disc degeneration [6], which is modeled by TLR activation. A number of ECM proteins involved in matrix stability and organization also are released following TLR activation. Cartilage oligomeric matrix protein (COMP) is involved in collagen fibrillogenesis and links a number of other matrix proteins. Furthermore, it correlates with osteoarthritis severity and may have a similar function in disc degeneration [15]. Similarly, cartilage intermediate layer protein (CILP) is another ECM scaffolding protein and increased cleavage is associated with osteoarthritis and disc degeneration [15]. SLRPs are also involved in matrix stability, collagen fibril formation and cell-ECM interactions and SLRP degradation is another specific characteristic of disc degeneration [15,217]. For example, chondroadherin fragmentation at a specific site is a marker of disc degeneration [31]. Taken together, our data indicate that TLR activation leads to cleavage and release of specific matrix components from the disc. This conclusion is further supported by the global decreases in proteoglycan content.

Proteases increase following TLR activation, providing a possible mechanism to explain the increase in matrix components found in the culture media. MMP3 cleaves proteoglycans, such as aggrecan, and collagens and MMP13 cleaves primarily fibrillar collagen [218]. Cathepsin D also cleaves aggrecan and a number of other matrix proteins. HTRA1 cleaves a number of important disc ECM components including fibronection, chondroadherin, type II collagen, decorin, aggrecan and fibromudulin [219-221]. For example, HTRA1 produces specific chrondroadherin fragments in degenerating discs [31], it cleaves fibronectin in disc tissue and increases cytokine gene expression and

MMP gene expression in intervertebral disc cells [30]. These proteases degrade key components of the disc ECM, including many of those detected in the media by mass spectrometry. Increased protease activity also explains decreased proteoglycan content in the NP following TLR activation. Furthermore, these proteases cleave the ECM to produce fragments that may act as alarmins, including fibronectin, decorin, aggrecan and fibromodulin. Therefore, proteases upregulated by TLRs likely contribute to a feed-forward mechanism resulting in disc degeneration by degrading the ECM and creating new alarmins.

Sterile inflammation is a key characteristic of disc degeneration defined by increases in proinflammatory cytokines and chemokines [27]. *In vitro* human disc cell studies have found TLR2 activation increases IL-1β, TNFα, IL-6, and COX-2, but whether this occurs in intact human disc was unknown. Here, we show a robust increase in proinflammatory cytokines and chemokines following TLR activation. Furthermore, increased components of the complement system were detected in media from discs, suggesting that also the complement system is activated following TLR stimulation in human discs. The complement system is activated downstream of TLR activation and has roles in regulating inflammatory cytokines in osteoarthritis and rheumatoid arthritis [96,222], and could have a role in disc degeneration. Cytokines, such as IL-1β, increase cell-surface cytokine receptors, TLRs, and other cytokines and chemokines, further driving the feed-forward cycle of sterile inflammation and disc degeneration [101,197,211]. Furthermore, cytokines also directly increase ECM proteases, such as MMPs and decrease ECM synthesis [22,50,51,223]. TLR regulated sterile inflammation therefore likely contributes to the pathogenesis of disc degeneration.

The results from the current study clearly demonstrate that TLR activation induce degenerative changes in intact human discs resembling early degenerative changes. The changes in matrix composition and increases in proteases and sterile inflammation suggest a complex feed-forward loop downstream of TLR activation. Therefore, TLR activation may represent an early event in disc degeneration. Breaking this loop may slow disc degeneration and reduce inflammation. Currently, a

number of tissue engineering strategies are being investigated to repair discs. However, it is likely that progression of degeneration needs to be slowed and sterile inflammation reduced for these strategies to succeed [27]. Furthermore, TLR2 regulates nerve growth factor and brain-derived neurotrophic factor, two potent nociceptive factors found in disc degeneration [211]. Taken together, TLRs are potential disease-modifying therapeutic targets that could slow disc degeneration and reduce chronic low back pain.

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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions: EK, DHR and JBC performed experiments and collected data. EK and LH designed the study and wrote the manuscript. JAO assisted in sample collection and results interpretation.

Chapter 5: Targeting TLRs to Slow Degenerative Disc Disease and Reduce Pain

5.1 Preface

Despite being a leading cause of morbidity world wide, therapeutic options for degenerative disc disease are limited by the lack of disease modifying drugs. *In vitro* disc cell activation of TLR2 increases proteases and cytokines, while decreasing matrix synthesis. We recently found TLR2 regulates NGF and other nociceptive factors, linking TLR activation in discs to pain. We also found TLR activation induces degenerative changes in non-degenerate discs. Combined, these previous studies demonstrate TLRs regulate key components of disc degeneration and may be involved in the early stages of disc degeneration. However, whether TLR inhibition slows or stops the progression of disc degeneration and/or reduces chronic back pain remained unclear. Based on the existing knowledge we hypothesized inhibiting TLR activation will slow the progression of disc degeneration and reduce pain in an animal model.

What TLRs are most readily activated in mice had not yet been explored. Previous work suggests species differences exist in TLR activation in the disc and in cartilage (see manuscript IV and thesis discussion). Upon finding mouse discs are more responsive to TLR4 ligands than TLR2 ligands, we inhibited TLR4 in the SPARC-null mouse model of degeneration and pain. We conducted acute (1 injection) and chronic (3 injections/week for 8 weeks) experiments and evaluated pain behavior throughout. We also analyzed changes in the profile of cytokines that discs secreted profile to evaluate whether TLR4 inhibition affected the progression of disc degeneration.

5.2 Manuscript IV: Toll-like Receptor 4 Inhibition Decreases Signs of Disc Degeneration and Pain in a Mouse Model

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Objectives: Chronic low back pain due to intervertebral disc degeneration is a leading cause of disability worldwide, but no disease modifying drugs exist. Toll-like receptors (TLR) on disc cells are activated by endogenous alarmins and regulate many components of degeneration *in vitro*, and therefore represent a potential drug target. This study investigates whether TLR inhibition slows disc degeneration and reduces pain.

Methods: 7-month old wild-type and SPARC-null (a mouse model of disc degeneration and pain) mice were treated with TAK-242 (TLR4 inhibitor) for 8 weeks. Behavioral signs of back pain were assessed weekly. At the end of treatment discs were excised and secretion of proinflammatory cytokines was analyzed by protein arrays. Spinal neuroplastic changes associated with chronic pain were evaluated with immunofluorescence.

Results: TLR4 inhibition decreases signs of radiating and axial back pain in SPARC-null mice when compared to vehicle. TAK-242 treatment decreases inflammatory and nociceptive factor secretion, including IL-1β, TNFα, KC and LIX. CGRP and GFAP immunoreactivity decrease in the dorsal horn following 8 weeks of TLR4 inhibition.

Conclusions: Chronic TLR4 inhibition decreases behavioral and neuroplastic signs of back pain, and reduces characteristics of disc degeneration in SPARC-null mice. TAK-242 likely inhibits TLR4 activation in discs, as evidenced by decreases in cytokine release from the disc tissue. Reversal of pain related changes in the dorsal horn suggest decreased spinal pain signaling. Furthermore, decreased CGRP suggests nociceptive input from the periphery decreases. Taken together, these results suggest TLRs are a potential disease modifying drug targets to slow disc degeneration and reduce pain.

Introduction

Intervertebral disc degeneration is one of the most common causes of chronic low back pain [6], which is the leading cause of disability worldwide [2,3]. However, current therapeutic strategies lack desirable efficacy and no disease modifying drugs exist. Painful disc degeneration etiology is multifactorial; genetics, adverse mechanical load, traumatic injury and certain lifestyle choices are all risk factors [27]. A hallmark of painful disc degeneration is sterile inflammation of the disc tissue, which is defined by an increase in proinflammatory factors in the absence of infection and sometimes immune cells. How a degenerating disc reaches this state is poorly understood though. During degeneration, the extracellular matrix (ECM) of the disc is degraded and fragmented resulting in a loss disc height and biomechanical function. Proinflammatory cytokines, including IL-1β and TNFα, and neurotrophins increase and stimulate innervation of the normally aneural disc [9,39,183]. These nociceptive factors also promote neuronal sensitization and can lead to the development of chronic pain [63,72]. Matrix proteases also increase, including MMPs [33,180], ADAMTSs [34,181] and HRTA1 [20], and are regulated in part by cytokines. Increased protease levels result in further ECM degradation. The concurrent increase of cytokines, neurotrophins and proteases is believed to lead to a feed-forward loop promoting ECM degradation, inflammation and pain [27]. However, in non-degenerating discs cytokines, such as IL-1β and TNFα, are expressed at very low or undetectable levels, and thus do not alone explain the changes. Interestingly, ECM fragments found in degenerating discs can act as danger associated molecular patterns (DAMPs), also termed alarmins, and activate toll-like receptors (TLRs) [94], which in turn could lead to disc degeneration.

TLRs are pattern recognition receptors originally characterized for their ability to recognize pathogen components and more recently were found to be activated by alarmins. Examples of alarmins found in discs include fragmented ECM proteins and glycosaminoglycan, such as aggrecan, fibronectin and low molecular weight hyaluronic acid, and normally nuclear or cytosolic proteins that are secreted like HMGB1 and heat shock proteins [91,92,94]. TLR1-6, -9 and -10 are expressed by disc cells and TLR1,

-2, -4 and -6 all increase as discs degenerate [101]. Multiple studies have confirmed TLR1, 2, 4 and 6 are expressed by disc cells from non-degenerating discs, unlike IL-1β and TNFα [211]. TLR2 and 4 are the TLRs most often found to be activated by alarmins. Following TLR2 activation, cultured human disc cells produce increased amounts of IL-1β, IL-6, TNFα, nerve growth factor, brain derived neurotrophic factor, MMP1-3, -9 and -13 and ADAMTS4 and 5 [101,102,224]. TLR4 when activated by ECM alarmins in connective tissues such as cartilage, result in increased inflammatory and catabolic mediators [91,96], and as TLR4 is also expressed by IVD cells it may play a role in disc degeneration. This evidence indicates TLRs are likely involved in disc degeneration and are possible therapeutic targets to break the sterile inflammatory loop. However, little is known about the *in vivo* role of TLRs in disc degeneration and pain.

SPARC (Secreted Protein Acidic and Rich in Cysteine) is a matricellular protein that decreases in human discs as a function of age and disc degeneration [225]. SPARC-null mice develop progressive disc degeneration that mimics the human condition. As the mice age their discs loose proteoglycan content, distinct disc structure, and disc height compared to wild-type mice. SPARC-null mice also display increased axial pain assessed by tail suspension and grip force assays and radiating pain assessed by acetone evoked behavior [115,117,119]. The aim of the present study was to investigate if disease modifying drugs targeting TLRs can decrease disc degeneration and pain in SPARC-null mice.

Methods

Animals

All experiments performed received approval from the Animal Care Committee of McGill University and followed the ethical guidelines of the Canadian Council of Animal Care. For initial disc culture studies 12-week-old male mice were used. For TLR4 inhibition 7-9 month old male mice were used for this study. SPARC-null mice were bred as previously described [114,119] and bred in-house. Age-matched wild-type C57Bl/6 mice were used as controls. Mice were housed in a temperature-controlled room with a 12-hour light/dark cycle in a temperature-controlled room, 2-5 per ventilated

polycarbonate cage (Allentown), and with corncob bedding (Envigo), cotton nesting squares. Mice were give *ab libitum* access to food (Global Soy Protein-Free Extruded Rodent Diet, Irradiated) and water.

Mouse Disc Culture and TLR Agonist Treatment

Briefly, 12-week-old mice were euthanized with isoflurane and decapitated. The spine was isolated and overlying muscle was removed to reveal the intervertebral discs. The four upper lumbar discs (L1-2, L2-3, L3-4, L4-5) were excised, washed once with Phosphate Buffered Solution and twice with Hanks Balanced Salt Solution, both supplemented with 20 U/ml penicillin and 20 µg/ml streptomycin. Each was 5 minutes long. Discs were placed in 24 well plates and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1X glutamax and 10 U/ml penicillin and 10 µg/ml streptomycin. Discs were challenged with 100 ng/ml Pam3CSK4 (TLR1/2 specific agonist), 100 ng/ml Pam2CSK4 (TLR2/6 specific agonist), 10 µg/ml LPS (lipopolysaccharide, TLR4 specific agonist) or vehicle for 72 hours and conditioned media was collected. Media was analyzed by cytokine array as described below.

Drug Treatment and Pain Behavior Schedule

7-9-month-old SPARC-null and wild-type mice were given *i.p.* injections of TAK-242 (10mg/kg), a TLR4 specific inhibitor [226], dissolved at 1mg/ml in saline with 5% DMSO and 5% Tween 80 or vehicle (n=5-6/wild-type treatment group, 9-10/SPARC-null treatment group). Dosage and solution were based on the literature [227,228]. No adverse effects were observed. Mice were randomized into the TAK-242 group or vehicle group, and given a single injection. Grip strength, acetone evoked behavior and mechanical sensitivity were assessed 1, 3, 6 and 24 hours after injection. Following a 10-day washout period mice were re-randomized into either group and treated 3 times per week for 8 weeks. Grip strength, acetone evoked behavior and mechanical sensitivity were assessed during weeks 1, 2, 4, 6, and 8 on non-treatment days and 1 hour after injection during week 1. Grip strength, acetone evoked behavior and mechanical sensitivity were also assessed 1 hour after injection during week 1.

Distance travelled in open field was measured in weeks 3 and 7 on non-treatment days. SPARC-null mice underwent the rotorod test 1 hour after the last injection in week 1. The experimental paradigm is depicted in Fig. 1A.

Behavior

Grip Force Axial pain was measured by allowing the mice to grip a bar with their forepaws and gently stretching them by pulling on their tail until they release the bar. The force at which they released was recorded in grams [117].

Acetone Evoked Behavior Cold sensitivity was used as a measure for referred or radiating pain in SPARC-null mice. Acetone was sprayed on the right hind paw and seconds of evoked behavior, including paw lifting, shaking and scratching were recorded [117].

Von Frey Mechanical sensitivity was assessed on the plantar surface of the left hind paw using the up-down method for Von Frey filaments [229].

Rotorod Locomotive capacity was measured using an accelerating rotorod. The time that the mouse fell from the cylinder was recorded as previously described [117].

Open Field Mice were placed in a plexiglass box with 9 8x8 cm squares on the bottom for five minutes. The total distance travelled was analyzed using AnyMaze software as previously described [117].

Disc height analysis

Following 8 weeks of treatment lateral x-ray radiographs of the lumbar region were taken using a Faxitron MX-20 (Faxitron X-Ray, LLC). Disc height index was calculated using the formula DHI=((D2a+D2b+D2c)/3/(D1a+D1b+D1c+D3a+D3b+D3C)/6)*100 where D represents a disc and lower case letter represent the three measurements of the same disc. D1 and D3 are the discs adjacent to D2 [117,230]

Ex vivo *Disc Culture and Cytokine Analysis*

Following the completion of the chronic drug treatment and behavioral testing, the L1-2, 2-3 and 3-4 discs were cultured as described above. The media was collected and analyzed using the 62 protein RayBiotech Mouse Cytokine Antibody Array C3 according to manufactures instructions (RayBiotech). Arrays were imaged using an ImageQuant LAS4000 Image Analyzer (GE) and analyzed with ImageQuant TL array analysis software (GE) according to manufactures instructions. Data was then normalized to either wild-type vehicle treated mice or SPARC-null vehicle treated mice to calculate the fold difference of each protein.

Immunofluorescence

After euthanasia, spinal cords were fixed in 4% paraformaldehyde for 24 hours, sucrose protected in 30% sucrose at 4°C, embedded in optimum cutting temperature medium (OCT) and sectioned at 14µm and thaw mounted on gel coated slides. 3 randomly selected sections per animal spread across ~1mm of the lumbar spinal cord were selected for each antibody. Sections were incubated for 1 hour at room temperature in blocking buffer containing 0.3% Triton X-100, 1% bovine albumin, 1% normal donkey serum and 0.1% sodium azide in PBS. Slides were then incubated with anti-calcitonin gene-related peptide (CGRP) sheep polyclonal antibody (1:1000), anti-glial fibrillary acidic protein (GFAP) goat polyclonal antibody (1:1000), or anti-CD11b rat monoclonal antibody (1:1000) in blocking buffer overnight at 4^0C, with only one antibody per slide. Slides were incubated for 1.5 hours at room temperature with appropriate secondary antibodies in blocking buffer. Antibody details are outlined in table 1. DAPI (1:50000, Sigma-Aldrich) was briefly applied. Coverslips were mounted using Aqua Polymount (Polysciences Inc.). Images were taken at 10x magnification using an Olympus BX51microscope equipped with an Olympus DP71 camera (Olympus).

Table 1: Details of Antibodies used for Immunofluorescence

Primary Antibody	Manufacturer	Catalogue #	Lot #	Secondary Antibody	Secondary Manufacturer	Secondary catalogue #
Sheep anti-CGRP	Enzo Life Sciences	BML-CA1137010 0	0807 B74	Donkey anti-sheep Cy3	Jackson Immunoresearch	713-165-147
Goat anti-GFAP	Sigma-Aldrich	SAB250046 2	7478 52C2 G2	Donkey anti-goat AlexaFlour 594		705-585-144
Rat anti-CD11b	BioRad	MCA711G	0614	Donkey anti-Ray Alexafluor 488		712-225-153

Using ImageJ, a region of interest was drawn around the dorsal horn and a threshold was established to differentiate between positive immunoreactivity (ir) and background. The % area of the region of interest at, or above, the threshold was quantified to measure CGRP, GFAP or CD11b-immunoreactivity. The average % area immunoreactivity across the three sections from each animal was calculated.

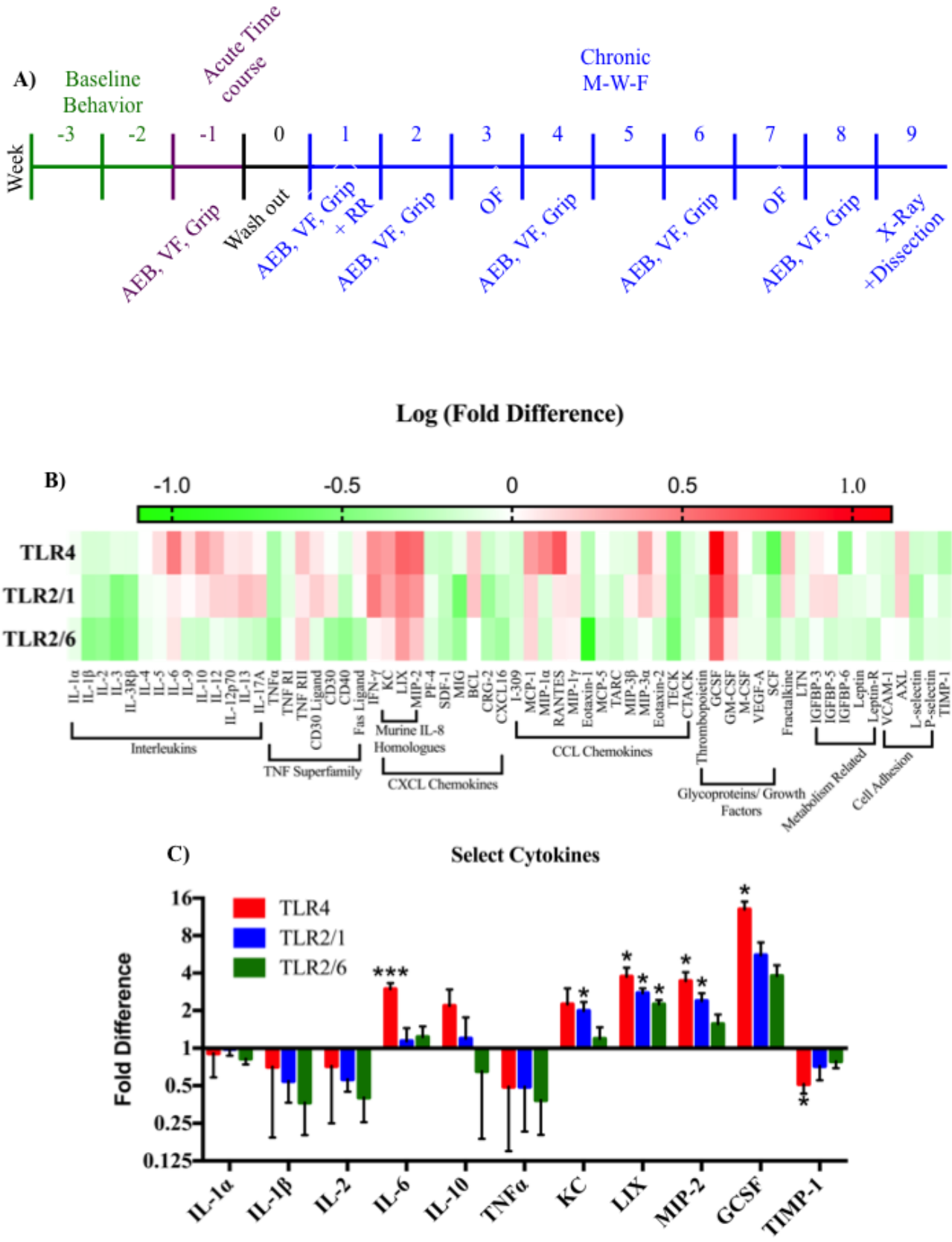
Statistical Analysis

All data was analyzed using GraphPad Prism version 7 with $p \leq 0.05$ being considered statistically different. Data is presented as mean ± SEM. Data was analyzed using statistical hypothesis testing as indicated in figure legends.

Results

TLR 4 Activation Increases Proinflammatory Cytokine Release by Mouse Discs

TLR2 and TLR4 both recognize alarmins but it is unclear if TLR 2 and/or 4 can be activated in rodent disc tissue. Mouse discs were treated with the specific TLR agonists LPS (TLR4 agonist), Pam3CSK4 (TLR1/2 agonist), and Pam2CSK4 (TLR2/6) for 72 hours. Conditioned media was assessed using antibody protein arrays. Compared to control discs, TLR4 activation increases proinflammatory cytokine secretion from discs. TLR 2/6 activation also causes increases, although not as robustly as LPS. TLR1/2 activation has little effect on the overall secretion of proinflammatory cytokines (Fig. 1B). Specific examples of increased cytokines include IL-6 (LPS, 2.96±0.346 fold increase, p=0.009), the murine IL-8 homologues KC (Pam2CSK4, 1.99±0.349, p=0.044), LIX (LPS, 3.74±0.7, p=0.058, Pam2CSK4, 2.758±0.25, p=0.0046, Pam3CSK4, 2.24±0.185, p=0.016,) and MIP-2 (LPS, 3.452±0.626, p=0.043, Pam2CSK4, 2.385±0.367, p=0.017), MCP-1 (LPS, 2.24±0.238, p=0.01) and GCSF (LPS, 13.02±1.982, p=0.01). Interestingly LPS treatment also decreased TIMP-1 (0.0514±0.082, p=0.033, Fig. 1C) an inhibitor of MMPs. Overall, the TLR4 agonist, LPS, resulted in the greatest overall increase in proinflammatory cytokine release from mouse discs. We therefore set out to evaluate if TLR4 disease modifying drugs can decrease progression of disc degeneration and behavioral signs of pain in SPARC-null mice.


A)
Baseline Behavior
Acute Time course
Chronic M-W-F
Week
-3
-2
-1
0
1
2
3
4
5
6
7
8
9
AEB, VF, Grip
Wash out
AEB, VF, Grip + RR
AEB, VF, Grip
OF
AEB, VF, Grip
AEB, VF, Grip
OF
AEB, VF, Grip
X-Ray +Dissection
Log (Fold Difference)
B)
-1.0
-0.5
0
0.5
1.0
TLR4
TLR2/1
TLR2/6
IL-1α
IL-1β
IL-2
IL-3
IL-3Rβ
IL-4
IL-5
IL-6
IL-9
IL-10
IL-12
IL-12p70
IL-13
IL-17A
TNFα
TNF RI
TNF RII
CD30 Ligand
CD30
CD40
Fas Ligand
IFN-γ
KC
LIX
MIP-2
PF-4
SDF-1
MIG
BCL
CRG-2
CXCL16
I-309
MCP-1
MIP-1α
RANTES
MIP-1γ
Eotaxin-1
MCP-5
TARC
MIP-3β
MIP-3α
Eotaxin-2
TECK
CTACK
Thrombopoietin
GCSF
GM-CSF
M-CSF
VEGF-A
SCF
Fractalkine
LTN
IGFBP-3
IGFBP-5
IGFBP-6
Leptin
Leptin-R
VCAM-1
AXL
L-selectin
P-selectin
TIMP-1
Interleukins
TNF Superfamily
Murine IL-8 Homologues
CXCL Chemokines
CCL Chemokines
Glycoproteins/ Growth Factors
Metabolism Related
Cell Adhesion
C)
Select Cytokines
Fold Difference
16
8
4
2
1
0.5
0.25
0.125
TLR4
TLR2/1
TLR2/6
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*
IL-1α
IL-1β
IL-2
IL-6
IL-10
TNFα
KC
LIX
MIP-2
GCSF
TIMP-1

Figure 1) Schematic of mouse treatment and behavior schedule, and the effect of TLR agonists on mouse discs. The *in vivo* mouse study consisted of baseline behavior (green), an acute time course where behavior was assessed 1, 3, 6 and 24 hours after TAK-242 treatment (purple), and a chronic treatment (blue) with injections Monday (M), Wednesday (W), and Friday (F) and behavior as indicated (A). AEB indicates acetone evoked behavior, VF indicate Von Frey, RR indicates rotorod test and OF indicates open field. Mouse discs from young wild-type mice were treated with LPS (TLR4 agonist), Pam2CSK4 (TLR2/6 and TLR2 agonist) and Pam3CSK4 (TLR1/2 agonist) for 72 hours and conditioned media was analyzed by protein array. The heat map illustrates the log fold difference compared to vehicle treated discs (B). C illustrates changes in select cytokines and shows data as the mean fold difference ± SEM compared to vehicle treated discs. * indicates $p<0.05$, *** indicates $p<0.001$, n=5.Data was analyzed by repeated measures one-way ANOVA.

Single Injection of TAK-242 does not decrease behavioral signs of back pain

TAK-242 is TLR4 antagonist and as such a potential disease modifying drug. TLR4 antagonists have been suggested to have an acute analgesic effect in the serum transfer model of arthritis [231], neuropathic pain models [232,233] and in migraine models [234]. To determine if TAK-242 has an acute analgesic effect on pain in SPARC-null mice a single injection was given and grip strength (axial discomfort), acetone evoked behavior (radiating pain), and mechanical sensitivity were evaluated 1, 3, 6 and 24 hours following treatment. As previously demonstrated, SPARC-null mice have increased radiating pain and axial discomfort, but unchanged mechanical sensitivity at baseline compared to wild-type mice. A single injection of TAK-242 treatment did not decrease radiating pain or axial discomfort in SPARC-null mice compared to vehicle (Fig. S1)

Chronic TAK-242 treatment does not affect disc height, locomotion or weight

Similar to previous studies, L2/3 and L3/4 disc height index decreases from 8.206±0.324 to 5.6±0.565 and 7.859±0.323 to 5.623±0.565 in SPARC-null mice compared to wild-type ($p=0.003$ and $p=0.014$). TLR4 inhibition did not reverse the loss following 8 weeks of treatment. (Fig. 2A).

TAK-242 treated SPARC-null mice and wild-type mice travelled similar distances to vehicle treated mice in an open field (Fig. 2B) and performed similarly on a rotorod test (Fig. S2) indicating TAK-242 did not have a sedative effect. Drug treated mice also maintained weights comparable to vehicle treated mice (Fig. 2C).

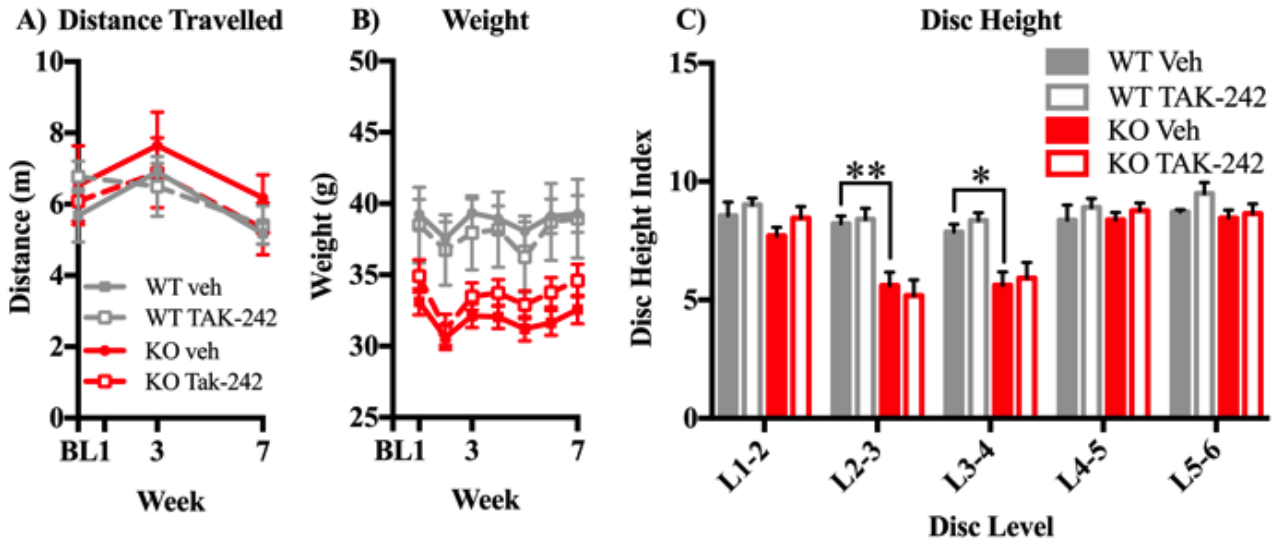

Figure 2) Effect of TAK-242 on locomotion, weight and disc height. To examine the effect of TAK-242 on locomotion the distance travelled was assessed in a five-minute open field test during weeks 3 and 7 of treatment (A). Weight was assessed weekly (B). Following 8 weeks of chronic TLR4 inhibition the lumbar spines of mice were imaged with x-ray and disc height index was determined (C). Data is presented as mean ± SEM. * indicates $p<0.05$, ** indicates $p<0.01$. Data was assessed by repeated measures two-way ANOVA (A and B) or one-way ANOVA (C).

Chronic TLR4 inhibition decreases behavioral signs of back pain

Since a single TAk-242 injection has no acute analgesic effect we continued to evaluate if TLR4 inhibition could decrease behavioral signs of back pain by acting on the disc following chronic treatment. After a ten-day washout period mice were treated 3 times/week for 8 weeks. At baseline both treated and untreated SPARC-null mice displayed increased axial (76.79±4.22 g vs. 90.17±2.95 g, $p=0.025$, Fig. 3Di) and radiating pain (5.26±0.53 s vs. 1.964±0.33, $p=0.027$, Fig. 3Ai), but not mechanical sensitivity compared to wild-type. Similar to a single acute injection, there was no effect on

pain behavior during the first week 1 hour (Fig. S3A and B) or 24 hours (Fig. 3A and D) after injections or on non-injection days. All behavior in the remainder of the chronic treatment was evaluated on non-injection days, approximately 24 hours after TAK-242 delivery. Chronic TLR4 inhibition in SPARC-null mice gradually increases grip strength, which was significantly different from SPARC-null vehicle after 6 (57.76±6.25 g vs. 45.244±4.09, p=0.014) and 8 (53.43±3.31 g vs 37.00±2.52 g, p=0.004) weeks of treatment (Fig. 3A). Similarly, chronic TLR4 inhibition gradually decreases acetone evoked behavior in SPARC-null mice compared to vehicle with significant decreases after 6 (2.65±1.57 s vs. 6.08±3.28 s, p=0.006) and 8 (2.748±1.287 s vs. 6.216±2.79 s, p=0.006) weeks of treatment (Fig. 3D).

Area under the curve calculations further indicate that SPARC-null mice have increased axial (401.3±16.7 g×week vs 552.3±35.38 g×week, p<0.001, Fig. S4A) and radiating pain (44.7±3.87 s×week vs 28.3±6.39 s × week, p=0.011, Fig. S4B) compared to wild-type. TLR4 inhibition decreases axial and radiating pain area under the curve compared to SPARC-null vehicle (460.4±22.81 g×week vs. 401.3±16.7 g×week, p=0.041 and 26.51±2.56 s×week vs. 44.7±3.87 s×week, p=0.0012, Fig. S4). Separating the area under the curve measurements between baseline to week 4 and week 4 to week 8 further shows that TLR4 inhibition reduces pain behavior with increasing effectiveness over time (Fig. 3B,C,E,F). Mechanical sensitivity of SPARC-null mice did not differ from wild-type mice at baseline. However, mechanical sensitivity did vary at different time points, resulting in a difference in the area under the curve between SPARC-null and wild-type treat mice. TLR4 inhibition did not affect mechanical sensitivity (Fig. S4C). Taken together, these results indicate chronic TLR4 inhibition reduces signs of back pain.

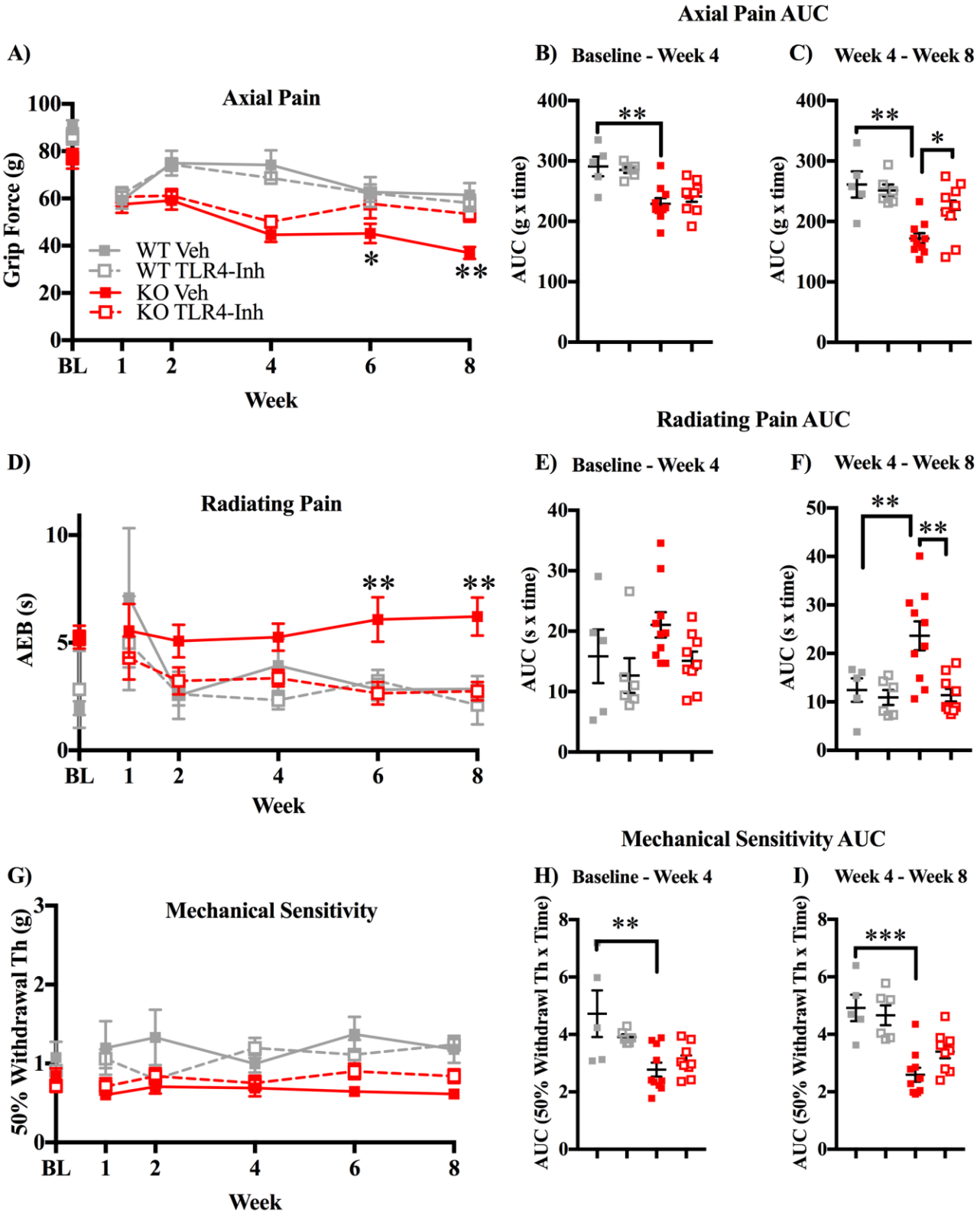

Figure 3. Behavioral Signs of Back Pain During Chronic TLR4 Inhibition. Tak-242 was injected 3 times/week for 8 weeks and axial pain (grip strength, A), radiating pain (acetone evoked behavior, D)

and mechanical sensitivity (Von Frey, G) were assed on non-injection days during weeks 1, 2, 4, 6 and 8. Baseline to week 4 and week 4 to week 8 area under the curve was calculated using the trapezoid method for axial pain (B, C), radiating pain (E, F) and mechanical sensitivity (H, I). Data is presented as mean ± SEM and analyzed by repeated measures two-way ANOVA (A, D, G) or one-way ANOVA (B, C, E, F, H, I). * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, n=5-10.

Chronic TLR4 inhibition decreases proinflammatory cytokine secretion from discs

The observation that chronic but not acute TLR4 inhibition decreases behavioral signs of back pain suggests that TAK-242 treatment may act directly on intervertebral disc cells to decrease release of pro-nociceptive and pro-inflammatory mediators. To evaluate this L1/2, L2/3 and L3/4 discs were excised and placed in culture media in the ninth week of treatment. Conditioned media was analyzed with protein arrays. Discs from SPARC-null mice secreted higher levels of multiple proinflammatory cytokines when compared to wild type animals as indicated in the heat map by increased red color (Fig. 4A) Specific examples of mediators with characterized roles in disc degeneration and pain include IL-1α which increased 2.38±0.48-fold ($p=0.026$), IL-1β (5.717±1.07-fold, $p=0.003$), IL-2 (5.75±1.49-fold, $p=0.009$), and the murine IL-8 homologues KC showed a non-significant trend (non-significant) and LIX increased 3.6±1.42-fold ($p=0.037$) (Fig 4C). This increase was normalized following chronic TLR4 inhibition in SPARC-null mice as indicated in the heat map (Fig. 4B) by increased green color. Specific examples of cytokines with documented importance for disc degeneration and pain that decrease following TLR4 inhibition compared to SPARC-null vehicle mice include IL-1α which decreased 0.601±0.16-fold ($p=0.046$), IL-1β (0.345±0.15-fold, $p=0.003$), TNFα (0.257±0.108-fold, $p=0.06$) and the murine IL-8 homologues KC (0.486±0.093-fold, $p=0.047$) and LIX (0.0447±0.075-fold, $p=0.06$) (Fig. 4D). TLR4 inhibition also resulted in increased expression of the anti-inflammatory cytokine IL-10, although this failed to reach significance. Interestingly, TLR4 inhibition in SPARC-null mice also led to decreased expression of cytokines that were not significantly increased in

comparison to wild-type vehicle treated mice. Taken together, these results suggest chronic TLR 4 inhibition decreases sterile inflammation in degenerating SPARC-null discs.

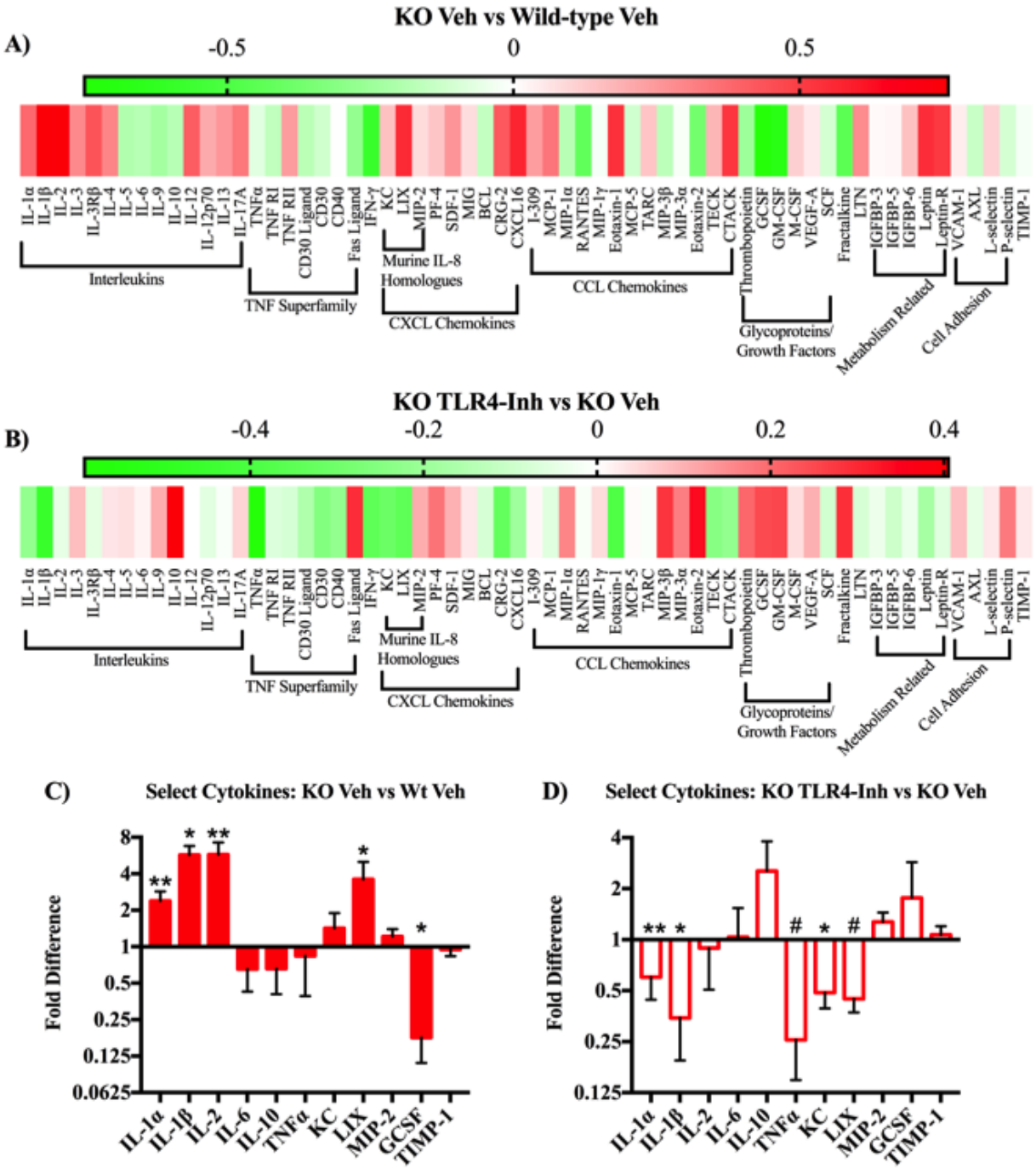

Figure 4. Disc Secretion of Inflammatory Mediators After Chronic TLR4 Inhibition. Discs were extracted following chronic TLR4 treatment and cultured for 48 hours. Conditioned culture media was analyzed with protein arrays. Heat map data is presented as the log (fold difference) of SPARC-null

vehicle compared to wild-type vehicle treated mice (A) or of SPARC-null TAK-242 treated mice compared to SPARC-null vehicle (B). Select cytokines are show as mean fold difference ± SEM for SPARC-null vehicle compared to wild-type vehicle treated mice (C) or SPARC-null TAK-242 treated mice compared to SPARC-null vehicle (D). # indicated $p<0.1$, * indicates $p<0.05$, ** indicates $p<0.001$. Data was analyzed by one-tail T-tests.

Chronic TLR4 inhibition reverses pain-related spinal neuroplasticity

Previous work has shown that SPARC-null mice undergo spinal cord changes associated with pain compared to wild-type mice, including increased microglia and astrocyte numbers and increased calcitonin gene-related peptide (CGRP) in the dorsal horn [116]. Knowing that TLR4 inhibition decreases back pain and sterile disc inflammation, we investigated whether pain related dorsal horn changes improved following treatment. CGRP is a neuropeptide produced by primary nociceptive DRG neurons that functions as a pain-related neurotransmitter in the spinal cord [235]. CGRP-ir increases in the dorsal horn of SPARC-null vehicle treated mice (21.91±2.42%) compared to wild-type mice (13.23±0.90%, $p=0.043$, Fig. 5A, B). Chronic TLR4 inhibition of SPARC-null mice significantly reduces CGRP-ir (14.5±2.41%) to levels detected in wild type animals ($p=0.027$, Fig. 4A, B), suggesting there is decreased nociceptive input to the dorsal horn.

Spinal glia, namely astrocytes and microglia, are also involved in spinal pain signaling and synaptic modulation in the dorsal horn [236]. The % area of immunoreactivity for GFAP (astrocyte marker) or CD11b (microglia marker) accounts for both cell proliferation and morphological changes where an increased area indicates increased reactivity. Immunoreactivity area increased for GFAP (16.98±2.39% vs. 8.702±1.48%, $p=0.034$, Fig. 5A, C) and CD11b (3.61±0.95% vs. 0.786±0.19%, $p=0.048$, Fig. 5A, D) in SPARC-null mice compared to wild-type mice, indicating increased astrocyte and microglia activity in SPARC-null dorsal horn. Chronic TAK-242 treatment significantly decreases GFAP-ir area compared to SPARC-null vehicle (7.92±1.68%, $p=0.0045$, Fig. 5A, C), whereas CD11b-ir area was unaffected (Fig. 5A, D). These results suggest that chronic TLR4 inhibition decreases

astrocyte, but not microglia, activity. Taken together these results indicate that chronic TLR4 inhibition decreases neuroplastic changes in the dorsal horn that are associated with pain.

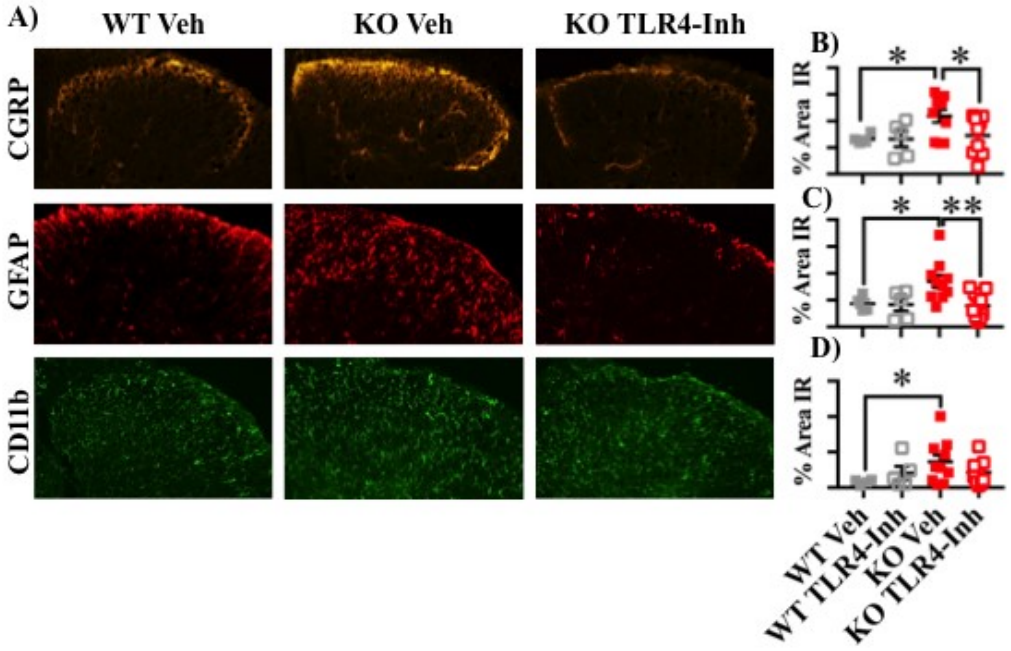

Figure 5. Neuroplastic Changes in the Spinal Cord Dorsal Horn. Calcitonin gene-related peptide (CGRP, a neurotransmitter involved in pain), glia fibrillary acidic protein (GFAP, an astrocyte marker) and CD11b (a microglial marker) immunoreactivity were assessed by immunofluorescence in the dorsal horn (A). % area of immunoreactivty was calculated for CGRP (B), GFAP (C) and CD11b (D) by comparing the area stained above a threshold to the total area of the dorsal horn (B-D). n=4-9 animals, the dorsal horn of each animal was analyzed on three separate slides and the mean value was used to calculate the mean of the treatment group. Data is presented as mean ± SEM and was analyzed by one-way ANOVA, * indicate $p<0.05$, ** indicates $p<0.01$.

Discussion

There are presently no disease modifying drugs to treat patients with intervertebral disc degeneration and chronic low back pain. Here, we show that chronic TLR4 inhibition decreases behavioral signs of back pain in SPARC-null mice, decreases proinflammatory cytokine production in degenerating discs and decreases pain-related neuroplasticity in the spinal cord. We propose these effects are due to decreased sterile inflammation in degenerating discs, leading to decreased nociceptive input from sensory fibers innervating and surrounding degenerating discs (Fig. 6) The current and previous studies support TLRs as potential therapeutic targets to manage symptomatic disc degeneration.

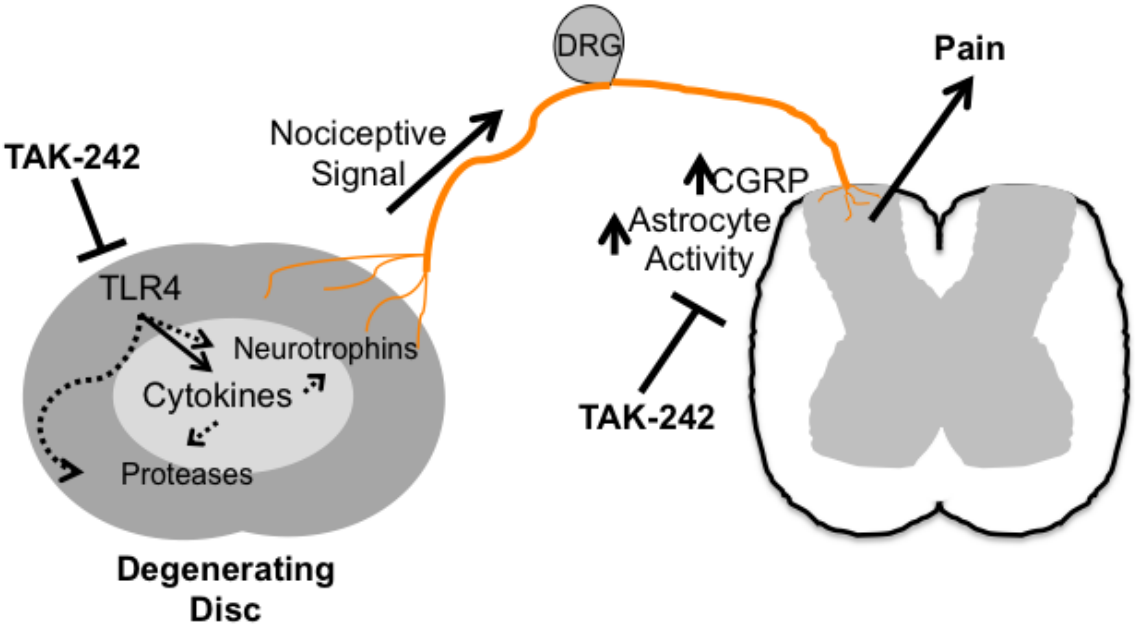

Figure 6. Proposed mechanism of how chronic TLR4 inhibition reduces pain and disc degeneration. TAK-242 inhibits TLR4 activation by alarmins in intervertebral discs, thus decreasing cytokine, neurotrophin and protease levels. Decreased proteases will decrease matrix degradation. Fewer cytokines will decrease the catabolic and inflammatory effects within the disc. Furthermore, decreased cytokine and neurotrophin levels will decrease nociceptive signaling through DRG fibers innervating and surrounding the disc, resulting in decreased pain-related spinal signaling and pain. It

remains possible TAK-242 inhibits astrocyte activity as well. In the disc, dotted arrows represent established regulatory pathways from the literature and solid arrows represent pathways investigated in the current study.

TLRs are pattern recognition receptors for endogenous alarmins that are found in degenerating discs. TLR2 activation of human disc cells *in vitro* increases cytokine, neurotrophin and protease production [30,101,224]. In contrast to human, rodent and bovine disc cells are more responsive to TLR4 activation and *in vivo* injection of the TLR4 agonist LPS into rat discs increases IL-1β and TNFα [97,237,238]. Here we tested TLR2 and 4 agonists to determine which TLR is more responsive in mouse discs and whether TLR increases expression of degeneration related factors. LPS elicits a stronger response than TLR2 agonists when applied *in vitro* to young, wild-type mouse discs. We only tested one single but high dose of agonists, leaving the possibility that different dosages of TLR2 ligands could give a larger effect. Regardless, these results indicate TLR activation causes changes protein expression in mouse discs similar to those seen in human disc cells and degenerating, painful disc [101,102,183,224].

SPARC-null mouse discs secrete increased levels of proinflammatory cytokines compared to wild-type mice, indicating degenerating SPARC-null discs have sterile inflammation. This reflects human disc pathogenesis where degenerating discs from low back pain patients secrete increased levels of proinflammatory cytokines compared to non-degenerating discs *ex vivo* [183]. Similar to LPS treated discs, SPARC-null discs secrete more proinflammatory cytokines; however several differences in the secretion profiles exist. For example, short term LPS exposure does not induce a measurable increase IL-1α and IL-1β, whereas SPARC-null discs secrete more IL-1α and IL-1β, when compared to age matched wild-type mice. On the other hand TIMP-1 secretion increases following LPS exposure but was not different between aged wild type and SPARC-null discs. These differences may reflect age or degenerative changes in both wild type and SPAC null mice. The SPARC-null and age matched wild

type discs were analyzed after TAK-242 treatment were from 9-11 months old animals whereas the LPS treated discs were from 12-week-old wild-type animals. Therefore, the effect of 72 hours of LPS treatment could mimic TLR4 activation in the initiating stage of disc degeneration whereas aged SPARC-null discs would represent a later stage when pain and degeneration is well established. *Ex vivo* exposure to LPS also likely does not model all important factors involved in disc degeneration.

TLR4 is expressed many cell types throughout the body including immune cells, astrocytes, microglia, chondrocytes and synoviocytes. Thus, TAK-242 could act in multiple locations and potentially reduce pain behavior either acutely or chronically. For example, inhibiting TLR4 signaling in spinal cord microglia decreases the development of pain in some models [228,239]. TAK-242 acutely reduces pain immediately after induction of neuropathic pain [232], but was unable to reverse tactile allodynia 7 days after formalin injection (model of prolonged pain) [228]. Acute TLR4 inhibition does not reduce pain behavior when arthritis has been established for more than 2 weeks [231]. We did not see an acute analgesic in SPARC-null mice. Instead a pronged treatment is required to obtain pain relief. As TLR4 is an upstream regulator of inflammatory and nociceptive factors a prolonged treatment is likely required to reduce the production of pain mediators secreted by the degenerating discs. SPARC-null mice display behavioral signs of back pain from 2 months of age [117], so it is not surprising that acute TLR4 inhibition failed to reverse the well-established pain in 7 month-old animals.

Chronic TLR4 inhibition decreases proinflammatory cytokine secretion by degenerating discs; suggesting that TAK-242 inhibits TLR4 regulated cytokine production. Furthermore, many cytokines that are reduced by TAK-242, such as IL-1β and TNFα, act as nociceptive factors [10,65,74]. NGF, another potent nociceptive factor, is also increased by TLR activation, and TAK-242 may also reduce NGF expression. Reduced cytokine secretion suggests that TAK-242 is acting directly on degenerating discs by reducing sterile inflammation, which would decrease nociceptor sensitization. Chronic TLR4 inhibition also decreases CGRP expression in the dorsal horn of SPARC-null mice. This suggests the peripheral nociceptive input is diminished [235,240], perhaps due to decreased disc inflammation.

However, the possibility of TAK-242 acting spinally on astrocytes for example or elsewhere remains. Regardless, the evidence from the current study indicates TAK-242 affects the disc, which likely contributes to reduced pain behavior in SPARC-null mice.

Disease modifying drugs need to manage underlying pathologies (disc degeneration) and symptoms (pain). We began TAK242 treatment at 7-9 moths of age when signs of back pain and disc height loss is already present in SPARC-null mice [117]. It is therefore not surprising that TLR4 inhibition failed to recover disc height. Future studies will investigate if earlier or longer TLR4 inhibition would prevent or reverse the loss of disc height. However, despite being a sign of painful disc degeneration, loss of disc height is not linked to pain in humans [241]. Therefore, recovery of disc height may not be an appropriate preclinical measurement of potential therapeutic efficacy. Several components of disc degeneration likely contribute to the development of pain. For example, as discs become innervated, nerve fibers are exposed to many nociceptive factors. Changes in the vertebral end plate may also allow for nociceptive factors to diffuse from the disc into adjacent vertebral bodies, which are innervated by nociceptors. By reducing disc inflammation, TLR4 inhibition potentially acts on both of these mechanisms.

We found TLR4 inhibition decreases a number of proinflammatory cytokines that are linked to disc degeneration in humans, including IL-1β, TNFα, IFN-γ and mouse IL-8 homologues LIX and KC [22,183]. Cytokines are known to increase protease production, decrease matrix synthesis and act as chemoattractants for immune cells to infiltrate degenerating discs. By reducing cytokine expression chronic, TLR4 inhibition would slow the progression of disc degeneration, consistent a disease modifying effect.

In conclusion, we demonstrate chronic TLR4 inhibition decreases signs of disc degeneration and pain in SPARC-null mice. These results indicate that alarmins likely contribute to disc degeneration *in vivo* and that targeting alarmin receptors decreases sterile inflammation in the disc. Decreasing inflammation and slowing degenerating is likely required for regenerative medicine

strategies to repair degenerating discs. TLRs represent a potential disease-modifying target to slow disc degeneration and improve pain and functioning in patients with degenerative disc disease.

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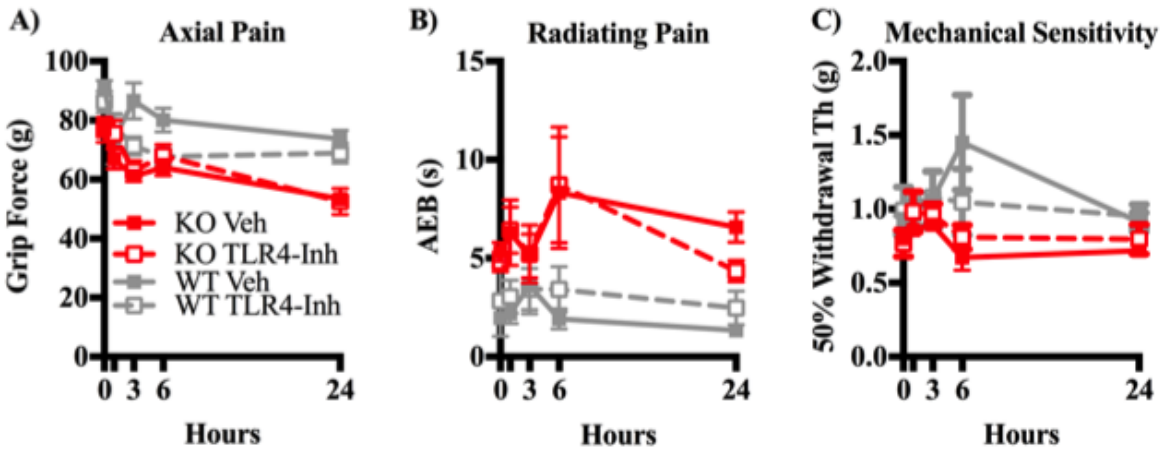
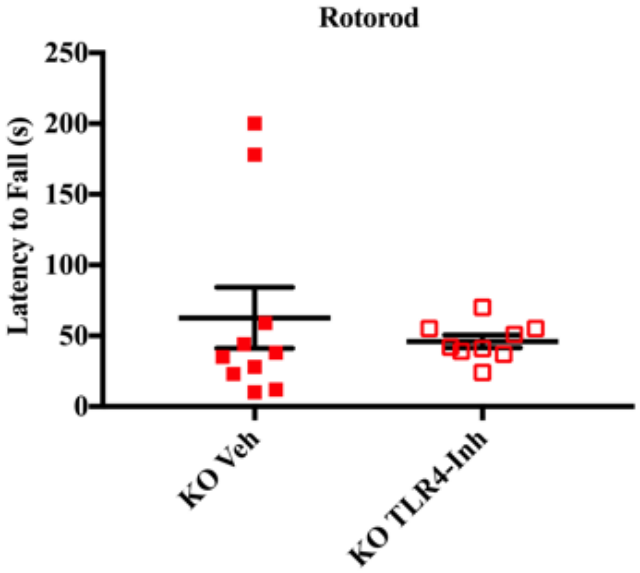
Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

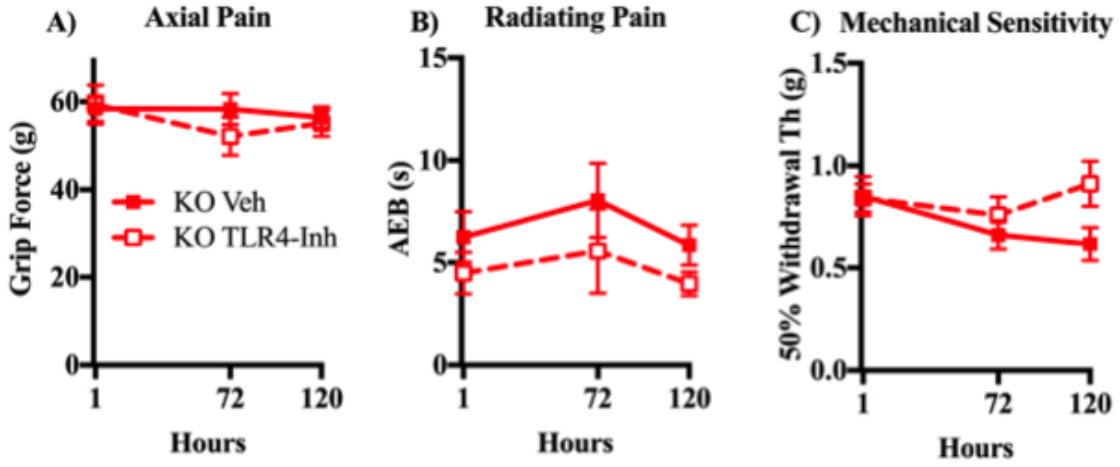
Author Contributions: EK, MM and JBC performed experiments and collected data. EK, MM, LSS and LH designed the study. EK, LSS and LH wrote the manuscript.



Supplemental Figure 1. Affect of acute TAK-242 on pain behavior. A single injection of TAK-242 was given and axial pain (A), radiating pain (B) and mechanical sensitivity (C) were evaluated 1, 3, 6 and 24 hours following injection. Data is presented as mean ± SEM and was analyzed by repeated measures two-way ANOVA, n=6-10.

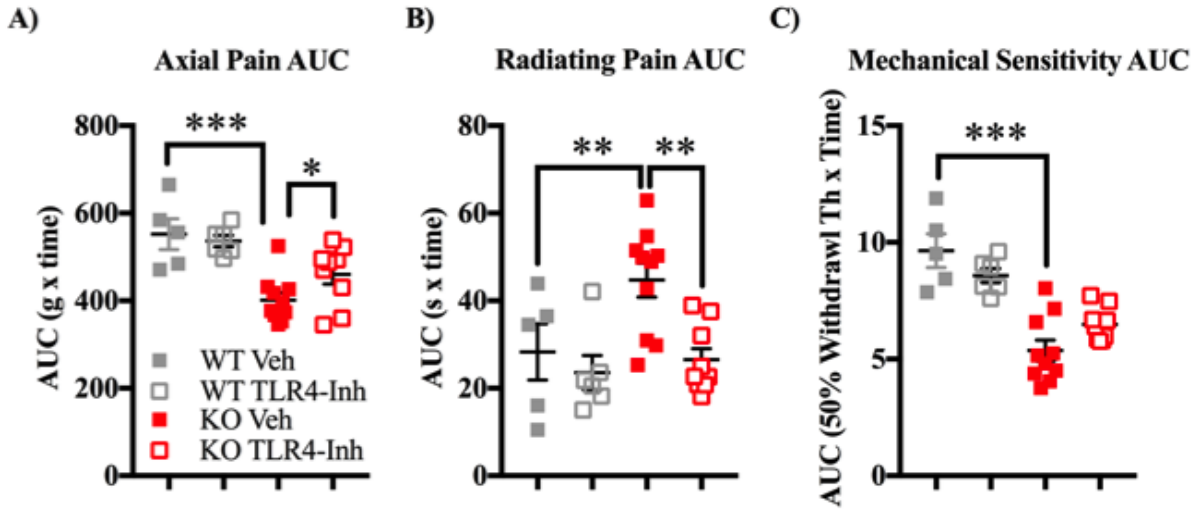


Supplemental Figure 2. Rotorod test following TAK-242 treatment. 1 hour following the third injection of TAK-242 locomotive capacity was assed with the rotorod test in SPARC-null vehicle and TAK-242 treated mice. Data is presented as mean±SEM and was analyzed by one-way ANOVA, n=9-10.



Supplemental Figure 3. Affect of TAK-242 on pain behavior following multiple injections. Axial pain (A), radiating pain (B), and mechanical sensitivity were assessed 1 hour after TAK-242 injection

during the first week of chronic TLR4 inhibition in SAPRC-null vehicle and TAK-242 treated mice. Data is presented as mean ± SEM and was analyzed by repeated measures two-way ANOVA, n=9-10.



Supplemental Figure 4. Area under the curve analysis from baseline to week 8. Area under the curve was calculated using the trapezoid method for axial pain (A), radiating pain (B), and mechanical sensitivity (C) for baseline to week 8. Data is presented as mean ± SEM and was analyzed by one-way ANOVA, n=5-10.

Chapter 6: Conclusions and Discussion

6.1 Summary

Despite being a leading cause of disability [2,3,242,243], the mechanisms of disc degeneration and chronic low back pain are poorly understood. The hypothesis for this thesis is that degenerating discs produce and secrete proinflammatory and pronociceptive factors that lead to the development of chronic low back pain. Our studies aimed to better characterize factors that degenerating disc secrete, how these factors are regulated, and how discs affect neurons,.

We first characterized differences in proinflammatory and pronociceptive factor released by non-degenerating and degenerating discs from low back pain patients. We found degenerating discs secrete increased levels of many proinflammatory and pronociceptive factors, including NGF, BDNF, IL-1β, TNFα, CCL2 and CXCL1. Furthermore, we found media conditioned by degenerating discs increases neurite growth in PC12 cells and CGRP expression in mouse DRG neurons compared to media from non-degenerating discs, which had little effect on PC12 cells and neurons. NGF is required for these effects[183].

Our first study highlighted the importance of NGF, but little was known about the regulation of NGF in pathogenic contexts. A possible role for TLRs in disc degeneration was being suggested at the time [21,101,102], so we investigated TLR regulation of NGF. We found TLR2 directly regulates NGF via NF-κB signaling. This work discovered a novel regulatory mechanism of NGF and added to the growing body of evidence suggesting TLRs are involved in the mechanisms of painful disc degeneration [224]. Furthermore, our results identified a new mechanism that could be used to therapeutically target NGF.

A number of *in vitro* studies, including our own, have identified a role for TLRs in painful disc degeneration, but whether TLR activation leads to degenerative changes in human discs was unknown [21,27,101,102]. We found injecting a TLR2/6 specific agonist or 30 kDa fibronectin fragments leads to degenerative changes in human discs *ex vivo.* Degeneration was characterized by GAG loss and

increases in cytokines and proteases (Krock *et al.,* in preparation). These results indicate TLR activation is sufficient to begin a degenerative cascade of events.

We also determined TLRs have an *in vivo* role in disc degeneration and pain. We found chronic TLR4 inhibition in SPARC-null mice reduces behavioral signs of chronic radiating and axial pain. TLR4 inhibition also decreases the proinflammatory cytokine release by degenerating SPARC-null discs and changes pain-related spinal neuroplasticity in SPARC-null mice to more resemble wild-type mice (Krock *et al.,* in preparation). The results from this study indicate TLRs are involved in the pathogenesis of disc degeneration and chronic low back pain and are potential therapeutic targets.

6.2 Discussion

6.2.1 Sterile Inflammation

Sterile inflammation is a common focus of this thesis and is defined as an increase in proinflammatory factors and other inflammatory processes in the absence of pathogenic infection. In the case of intervertebral discs, the resident cells create a sterile inflammation during disc degeneration [22]. Additionally, it is suggested that following disc herniation a variety of immune cells, including macrophages, neurtrophils and T-cells, infiltrate degenerating discs [10,244]. Autoantibodies against ECM proteins, like type II collagen and aggrecan, have also been found in NP tissue of degenerating discs [245] further supporting the role of immune cells and sterile inflammation. The combination of resident NP and AF cells alone as well as in combination with immune cells increase many proinflammatory cytokines, proteases and neurotrophins that promote disc degeneration, innervation and vascularization of the disc.

Here, we demonstrated that *ex vivo* degenerating discs from low back pain patients secrete increased levels of cytokines and neurotrophins compared to non-degenerating discs [183]. The uniqueness of this study stems from the comparison to non-degenerating discs and that we examined cytokine and neurotorphin release rather that look at gene expression or protein *in situ.* Comparing to non-degenerate human tissue increased understanding in the differences between pathogenic and non-

pathogenic tissues. Analyzing factor release increased understanding of what factors can promote vascularization, innervation and immune cell infiltration. Assuming these processes are linked to degeneration progression and development of pain, knowing whether specific factors are released from the disc or not is an important consideration for developing potential therapeutic targets. Secreted factors could act directly on nerve fibers and have other adverse effects once secreted from the disc.

Secreted nociceptive and inflammatory factors could have negative effects in endplates and vertebral bodies. For example, abnormal endplates are innervated [246] and disc derived nociceptive factors could act on these fibers. Modic changes in the end plates and the vertebral bodies are linked to disc derived inflammation and associated with chronic low back pain [247,248]. Modic changes, pathological changes in vertebral bodies and secreted factors from degenerating discs could all contribute to the development of chronic pain. Adult bone, such as vertebra, is innervated by pain-sensing TrkA (NGF receptor) and CGRP expressing fibers (Aδ and C-fibers)[249]. Sensitization of these fibers by NGF and cytokines, could lead to the development of chronic pain. Our results placed in the context of existing literature support the hypothesis that secreted cytokines have adverse effects outside of the disc.

In vitro or *ex vivo* TLR2 activation increases production and secretion of NGF, BDNF, IL-1β, TNFα, and many other cytokines and proteases [101,102,211](Krock *et al*, in preparation), and TLR4 inhibition in mice decreases the signs of sterile inflammation (Krock *et al*, in preparation). *In* vitro studies demonstrate NP and AF cell both express functional TLR receptors. Immune cells that infiltrate herniated discs also likely express TLRs and could be activated by endogenous alarmins. Taken together, the results from our studies indicate TLRs are involved in the regulation of sterile disc inflammation.

Our work as well as that of many others has led to the theory that the initial onset of sterile inflammation can be mediated by alarmins and can lead to the development of a vicious cycle resulting in degeneration and pain. The initial increase in alarmins, cytokines and proteases can further increase

alarmins, cytokines, proteases, neurotrophins, as well as alarmin and cytokine receptors. Increased receptor expression would accelerate the cycle. As these factors increase, the ECM of the disc would be further degraded, ultimately resulting in the structural failure. While ECM from non-degenerate discs repel nerve fibers, the ECM of degenerate discs has lost this ability, likely due to the decreased anionic charge [250]. Furthermore, semaphorins are guidance and repulsive cues for axon growth and semaphorin expression changes during disc degeneration and is partially regulated by cytokines like IL-1β [251,252]. Sterile inflammation increases neurotrophic factors that promote innervation, like NGF and BDNF [66,67,183,211], and vascularization factors like VEGF [108,253,254]. A variety of chemotactic factors that attract and activate immune cells such as CXCL1, CCL2, and CCL8 also increase [183]. Vascularization allows for immune cell infiltration, resulting in more cytokines [10,255]. Innervating DRG nerve fibers could also have proinflammatory neurogenic effects in the disc, although this has not been investigated. Therefore, innervation and vascularization can further the proinflammatory and prodegenerative cycle. To slow or stop the progression of disc degeneration the proinflammatory cycle would have to be broken.

Beyond slowing or stopping the progress of disc degeneration, sterile inflammation likely needs to be reduced for regenerative medicine strategies to succeed [27]. Current anti-inflammatory strategies, such as NSAIDs, do not appear to be sufficient. Currently several disc repair and regeneration strategies focusing on NP cell or mesenchymal stem cell (MSC) implantation are being pursued [4,27]. As discussed and investigated throughout this thesis, cytokines and alarmins act directly on NP and AF cells to increase production of proteases, while cytokines and alarmins decrease matrix synthesis in NP and AF cells [22,27,224]. Therefore implanting non-degenerate NP or AF cells into an environment full of cytokines and alarmins may not have the desired regenerative effect.

The effect of implanting MSCs into the environment of a degenerating disc is less clear. MSCs may have anti-inflammatory effects and have been shown to beneficially modulate disc cytokine production *in vitro* [256]. However, *in vitro* studies do not fully recapitulate the poor nutrition, low pH, oxygen

levels, and osmolality of a degenerating disc [10,22], which negatively effects the expression of NP matrix molecules in MSCs [257,258]. Furthermore, IL-1β and TNFα promote osteogenic differentiation of MSCs *in vitro*. Hypoxic conditions like those found in the disc and cytokines also increase MSC expression of TLRs. TLR activation drives MSCs towards an osteogenic or proinflammatory phenotype [259,260]. Taken together, the feasibility and effectiveness of implanting naïve MSCs into degenerating disc is unclear and may be detrimental. Modulating the sterile inflammation of degenerating discs is likely required for cell transplantation regenerative strategies to be effective.

6.2.2 Nerve Growth Factor

Nerve growth factor has been targeted in a number of clinical studies to manage chronic pain conditions including chronic low back pain, osteoarthritis, cancer pain and others [261]. Initial clinical studies yielded promising results for osteoarthritis and cancer pain; however the results of anti-NGF therapeutics to treat chronic low back pain are mixed [87,89]. Despite these clinical results a number of preclinical studies suggest NGF is a promising therapeutic target [10,55,66,67,69-71,187]. We found degenerating discs from low back pain patients secrete increased levels of NGF compared to non-degenerating discs in an *ex vivo* organ culture system. NGF released by the degenerating discs from low back pain patients increases CGRP in cultured DRG neurons, suggesting that NGF could sensitize neurons *in vivo* [183]. Our evidence and previous studies support the continued development of anti-NGF therapeutics to treat chronic low back pain. Lack of efficacy in anti-NGF clinical trials for low back pain may be due to the many etiologies of low back pain and the lack of selection for specific causes of low back pain in previous clinical trials [87]. The studies discussed above investigated NGF in degenerating discs and do not suggest a role for NGF in other causes of chronic low back pain. Therefore specific patient selection, for example patients suffering from disc degeneration and low back pain, may increase the efficacy of anti-NGF therapeutics in clinical trials.

Despite the large amount of evidence implicating NGF as a main contributor to chronic low back pain associated with disc degeneration it may not be a disease-modifying drug. Evidence

supporting a catabolic role for NGF in disc degeneration is limited. A single study found NGF increases MMP3 and Chi3L1 gene and protein expression in rat AF cells and human disc explant cultures. From this the authors conclude NGF has a catabolic effect. However, they only looked at Col1a1 gene expression and no other matrix proteins in the AF and did not examine the effect on NP at all [262]. Furthermore, the role of Chi3L1 in connective tissue degeneration is poorly understood and may have a protective role [263,264]. Evidence also suggests NGF may have an anti-inflammatory role by down regulating NF-κB signaling [265]. Taken together, there is insufficient evidence to indicate NGF has a protective or catabolic role in degenerating discs. Further work is required to determine whether NGF contributes to the pathogenesis of disc degeneration. Even if it does not, evidence strongly supports it as a pain management target.

6.2.3 Toll-like Receptors and Alarmins

The etiology disc degeneration and chronic low back pain is multifactorial and risk factors include specific genetic alleles, adverse mechanical loading, traumatic injuries and several lifestyle choices. A combination of risk factors likely leads to disc degeneration, just as a combination of risk factors likely lead to chronic low back pain [6,42,266]. Therefore the onset and early stages of disc degeneration are poorly understood, limiting the ability to develop disease-modifying drugs. An underlying characteristic of disc degeneration, regardless of etiology, is ECM degradation. Traumatic injury also results in rapid ECM degradation [20]. In addition to pathological ECM degradation, physiological ECM degradation occurs during normal ECM turnover. ECM turnover potentially increases alarmins slowly due to the poor waste exchange from the disc [6]. As alarmins increase from normal and pathogenic matrix turnover they can then activate TLRs, thus potentially contributing to early stages of disc degeneration.

They key outcome of TLR activation is increased production of proinflamamtory cytokines. Additionally TLR activation in discs increases protease production, decreases ECM production and as we recently showed, increases NGF and BDNF [101,102,224] (Krock *et al,* in preparation). Proteases, such

as MMP3 and MMP13, cleave disc ECM components, including type II collagen, aggrecan and fibronectin, potentially resulting in more alarmins. In a pathogenic state, it is possible that an initial activation of TLRs increases cytokines, proteases, TLRs and cytokine receptors. Cytokines in turn will lead to increased TLR and cytokine receptor expression, cytokines and proteases. At the same time proteases lead to the production of more alarmins, which can then activate TLRs. This series of events would result in a sterile proinflammatory feed-forward loop discussed above and illustrated in figure 7 of the manuscript *Toll-like Receptor 2 Activation Induces Human Intervertebral Disc Degeneration***.** We show TLR2 activation in non-degenerate human discs induces degenerative changes, indicating TLRs could have a role during the early stages of disc degeneration. Therefore, TLR2 represents a potential disease-modifying therapeutic target.

The work in this thesis primarily focused on TLR2, but a number of other TLRs and pattern recognition receptors function as alarmin receptors. TLR4 is activated by many ECM alarmins. Arthritis and cartilage research has found TLR4 is activated by S100 proteins, HMGB1, annexins II, V, and VII, uric acid, fibronectin fragments, low-molecular weight hyaluronan, biglycan, and Tenascin [91]. Many of these alarmins also activate TLR2. We briefly investigated TLR4 in human disc cells by treating cells with LPS and found LPS did increase cytokine and neurotrophin gene expression but not protein. Quero *et al.* has found TLR2 is also preferentially activated on human disc cells by alarmins, such as low molecular weight HA, despite this being previously described as a TLR4 ligand [102]. Differences in TLR2 and TLR4 between human disc studies and other studies have a few possible explanations. Studies, including our own, have identified a role for TLR4 in rodent disc degeneration and TLR2 in human discs, so a species difference may exist. Another explanation is that discs may express TLR co-receptors that lead to TLR2 activation over TLR4 activation. A third explanation is the effects of only a few alarmins on disc cells have been investigated and perhaps others will activate TLR4. Finally, preliminary work from our group (performed by my colleagues, not myself) suggests isolation, culturing and passaging NP and AF cells affects TLR expression. However, context and

tissue specificity is likely important. For example, alarmins have different effects on synoviocytes compared to chondrocytes [91]. While the role of TLR4 needs to be further investigated, evidence thus far has suggested an important role for TLR2 in human disc degeneration.

In addition to TLR2 and TLR4 there are other alarmin receptors. TLR5 was recently found to be a receptor for HMGB1 [267]. The receptor for advanced glycation end products (RAGE) acts as an alarmin receptor for AGEs, S100 proteins and HMGB1 in joint cells. Purinoceptors, such as P2X receptors for ATP, can also be considered alarmins receptors depending on the context [91]. The common characteristic between ECM and non-ECM alarmins is that they are required for normal physiological function but are modified (ie ECM cleavage) and/or found in non-physiological locations (ie extracellular HMGB1, soluble biglycan). Thus far, alarmin and alarmin receptors have been little characterized in intervertebral discs. The role alarmins play in disc degeneration likely goes far beyond only TLR2 and should be investigated. Since alarmins are implicitly pathogenic, there is much knowledge to gain from characterizing their profiles in degenerating discs. Regardless, the work within this thesis demonstrated that TLR2 activation a) directly increases NGF and other nociceptive factors, b) leads to degenerative changes in intervertebral discs and that c) blocking TLR4 in a mouse model reduces signs of disc degeneration and chronic back pain. Taken together, these results strongly suggest a role for TLRs in disc degeneration and indicate TLRs are potential disease-modifying therapeutic targets.

6.2.4 Animal Models for Disc Degeneration and Pain

Several models were mentioned in the introduction and the manuscript *Toll-like Receptor 4 Inhibition Decreases Disc Degeneration and Pain in a Mouse Model.* Each has strengths and weaknesses. The progression of disc degeneration measured by x-ray, histology and MRI, pain phenotype, pain-related spinal cord neuroplasticity and pharmacological profiles have been characterized in SPARC-null mice [115-119]. The progressive, age-related degeneration in SPARC-null mice models the human progressive, age-related degeneration of human discs [6]. This information

allowed us to plan our experiments in order to investigate TLR4 at an age when SPARC-null mice have had disc degeneration and pain for several months, but the discs have not reached end-stage degeneration. We chose this timing for our intervention in an attempt to mimic human chronic low back pain and disc degeneration, where patients have been suffering for greater than 3 months when seeking treatment but surgery is not yet warranted.

Several other rodent models exist. Most commonly disc degeneration is induced with an acute injury, such as a needle puncture or a prolonged, adverse strain. A few major issues exist with injury models. First, young rodents are most commonly used to model pathologies that afflict middle age and elderly adults [109,110,268]. Changes in the nervous system and pain occur as a function of age. Nociceptor sensitization undergoes age-dependent changes and age also affects chronic pain behaviors in rodents [269]. Humans experience gray matter and white matter changes in the brain [270,271], which may affect chronic pain perception. Furthermore, age affects pain perception, pain ratings and pain tolerance in humans [272,273]. Therefore the use of young rodents introduces a potential confounding factor when attempting to make clinically relevant interpretations with young rodent models. Another caveat of injury models is that they are acutely induced, and therefore do not reflect progressive or age-related changes to intervertebral discs that humans experience. However, injury induced models of disc degeneration do offer several advantages: a) which disc affected by degeneration is known and can be limited to one disc or many, b) the onset of degeneration is controlled, c) puncture models can be used to model traumatic disc injuries which are another common source of low back pain. The temporal and anatomical control of injury models offer advantages when elucidating specific mechanisms or looking at treatment strategies.

Our study using the SPARC-null mouse to investigate TLR4 has several limitations. First, SPARC is expressed throughout the body; therefore other structures are affected by the deletion, potentially confounding our interpretations of our data. Deletion of SPARC affects mouse vision, bone density, skin, periodontal ligaments and adipose tissue [274,275]. However, SPARC-null mice do not

appear to develop osteoarthritis [115]. SPARC also decreases during human disc degeneration, suggesting it is related to disease pathogenesis [276]. Several cell types also express TLR4 and TAK-242, the TLR4 inhibitor we used, was delivered systemically. Therefore, TAK-242 could act in other tissues to alter their pain phenotype. However, we found chronic TLR4 inhibition decreases disc inflammation so we are confident that TAK-242 did affect the disc in SPARC-null mice. Human disc herniation generally occurs before end stage degeneration because sufficient hydrostatic pressure is required for the NP to rupture through the AF or the endplate [6,277]. Conversely, SPARC-null mice experience herniation later in their degenerative process. This difference likely did not affect our study since we used SPARC-null mice at an age where their discs had not herniated in previous studies [115,117]. A strategy around the potential effects of using a global gene deletion would be to delete a gene in notochordal cells to have an effect only in the NP. This strategy has been employed to delete CCN2 specifically in the NP and it results in disc degeneration [122]. Tissue specific deletions of SPARC or TLR4 would alleviate concerns of effects from other tissues that are affected in SPARC-null mice.

A caveat of all rodent models is the cellular composition of their disc is different from humans. The NP cell density of mouse and rat discs is much higher than humans and notochordal cells are present in the NP throughout the life span of rodents whereas this may not be the case in humans [13,278]. Differences in cell density and cell-type likely result in differences in the self-repair capacity of rodent discs compared to human discs. This may be why some groups inject TNFα into discs when inducing degeneration in a puncture model, ensuring a robust degeneration [108]. SPARC-null mice experience well-characterized disc degeneration, but increased cell density could still create differences in cytokine, protease, neurotrophin and ECM production compared to humans. Increased cell density could change how discs respond to therapeutic strategies. As described here, no single animal model of disc degeneration is perfect, but having the ability to use several different animals to ask specific questions will increases the understanding of disc degeneration and pain mechanisms.

Pain and loss of function are what lead patients to seek medical attention for disc degeneration and should therefore be considered and evaluated when using animal models. Here we used a number of behavioral assays including Von Frey, acetone evoked behavior and grip strength. Our group has used these behavioral assays extensively to validate them in SPARC-null mice [115-119] and similar to previous studies we found SPARC-null mice have increased radiating and axial pain but do not consistently have increased mechanical sensitivity compared to wild-type mice. Acetone evoked behavior is thought to be a measure of radiating pain due to disc degeneration because it is localized, is modality specific (ie no mechanical sensitivity), and is not the result of general nervous system changes that would be reflected by motor impairment or increased sensitivity in other modalities [117,119]. Interestingly, inflammatory mediators can evoke cold sensitivity in absence of mechanical sensitivity [279] and we showed here degenerating discs from SPARC-null mice secrete many inflammatory cytokines. The global deletion of SPARC could result in other structures contributing to the pain phenotype. However, it is unlikely that decreased bone density contributes to pain behavior since models of osteoporosis have non-localized thermal and mechanical sensitivity [115]. Of course, the possibility that other structures such as ligaments, could contribute to the pain phenotype in SPARC-null mice. Interestingly, injury induced rodent models of disc degeneration that have investigated pain behavior have observed a mechanical sensitivity phenotype [268]. These differences may reflect different mechanisms leading to pain between models. Regardless of these differences and limitations, using a variety of animal models will increase understanding of disc degeneration and disc injury relate to pain.

The behavioral assays we have and others have used in models of disc degeneration are largely evoked measures of pain. Evoked measure of pain, such as acetone evoked behavior and Von Frey, are convenient, reproducible tools to study pain in rodents and provide insights into neuronal sensitization and nervous system reorganization. However, spontaneous pain and changes in function are what lead patients to seek medical attention and evoked pain measures in rodents may not reflect these experiences. A number of assays attempt to measure spontaneous pain and functional changes in

rodents. For example, open field can be used to track changes in locomotion, spontaneous rearing, scratching, and other behaviors. Burrowing and nest building are natural behaviors of rodents that are altered in some chronic pain models [280]. Horizontal, vertical and rearing activity can also be evaluated using automated activity boxes [281,282]. The benefit of burrowing and automated activity boxes is that they measure function. Inferences about the pain state of an animal are made from changes in function. However, different rodent strains display these behaviors differently and not all chronic pain conditions may have a phenotype. For example, strain, sex and likely pain severity differences affect nesting behaviors [283-285]. By considering the benefits and disadvantages of spontaneous and evoked measure of pain it seems likely the best approach to understanding animal pain behavior is a combination of evoked and spontaneous measures.

6.2.5 The Use of Human Tissue and Animal Models for Translational Science

Chronic low back pain and disc degeneration are leading causes of disability worldwide [2,3]. Therefore, investigations of disc degeneration should have translational research aims. The goal of translational, preclinical research is to discover and use basic science concepts to further understanding of a clinical problem in order to better treat patients. We used this notion when designing the projects for this thesis. Examples include the use of primary neurons, rather than only relying on cell lines which are quite different from DRG neurons [286], investigating the induction of degenerating changes by using non-degenerate human discs rather than using cells derived from discs that were already degenerate, and the use of an animal model where disc degeneration and pain had developed months prior to beginning an intervention. One of the major strengths of this thesis was the use of human and rodent *in vitro* cell culture, human *ex vivo* organ culture and *in vivo* animal models. Using a combination of models allowed us to investigate several components of disc degeneration that no one model would and ultimately increased our understanding of disc degeneration and chronic low back pain. It is important to note that our group is incredibly fortunate to have collaboration with Transplant Quebec that gives us access to non-degenerate human intervertebral discs. This allows us to study the

onset and regulatory mechanisms of disc degeneration and make comparisons between pathogenic and non-pathogenic tissues that would not otherwise be possible. Continuing to use a variety of tissue sources, cell and organ culture, and animal models will be important in the future to provide translational knowledge.

6.3 Future Directions

There are a number of questions that could be addressed to continue where the work in this thesis ended.

1. *Does NGF contribute to the sterile inflammatory feedback loop as a pro- or anti-inflammatory factor or is it only a nociceptive product of disc degeneration?*

Evidence supports the role of NGF as an analgesic target. Understanding whether NGF contributes to the sterile inflammatory feedback loop, and whether blocking NGF is sufficient to break the loop, will provide insight on whether NGF could also function as a disease-modifying drug.

2. *Will blocking TLR activation prevent the onset of disc degeneration?*

Blocking TLR4 activation in SPARC-null mice from a young age (i.e. 8 weeks) would further investigate our hypothesis that TLR activation plays an important role in the early stages of disc degeneration.

3. *Comprehensively characterize the alarmin profile of degenerating discs.*

Alarmins are a diverse group of molecules that share the characteristic of being present where they are not found in a physiological state, thus acting as a danger signal. Profiling alarmins in disc degeneration would provide insight into other pattern recognition receptors involved in degeneration. Also, characterizing alarmins found in degenerating discs increase understanding of degenerative processes since alarmins are found in pathogenic states.

4. *Investigate the role of other Pattern Recognition Receptors.*

Investigating the role of other pattern recognition receptors in degenerating discs goes hand-in-hand with characterizing alarmins and will increase understanding of cytokine, protease and neurotrophin regulation in disc degeneration.

5. Development of TLR2 inhibitors.

TLR2 is likely involved in the pathogenesis of disc degeneration, but few specific inhibitors of TLR2 exist. Development of specific TLR inhibitors would allow for preclinical research to further elucidate TLR2's role in disc degeneration. To target TLR2 clinically a specific inhibitor or specific inhibition strategy would also be required.

6. What is the role of spinal glia to chronic low back pain?

Little is known about the role spinal glia in disc degeneration and chronic low back pain beyond that they are in an altered state in animal models of degeneration. Defining the roles of microglia and astrocytes to the development and maintenance of chronic low back pain with disc degeneration could lead to new pain management strategies.

7. Better characterize systemic effects of disc inflammation.

Sterile inflammation is a hallmark of disc degeneration. However, the systemic effects of local disc inflammation are poorly understood. Characterizing systemic markers of disc inflammation in serum of cerebral spinal fluid would further characterize the clinical phenotype of disc degeneration and low back pain.

6.4 Contribution to the Scientific Literature

The work in this thesis consists of several novel contributions to the field of disc degeneration and chronic low back pain. The contributions and their significance have been explained and discussed in the context of existing knowledge in the manuscripts and discussion above. Here, they are concisely summarized.

1. Nerve growth factor is secreted by degenerating discs from low back pain patients and is required to increase CGRP in DRG neurons, suggesting disc derived NGF likely sensitizes nociceptive fibers.

E. Krock, D.H Rosenzweig, A-J. Chabot-Doré, P Jarzem, M.H. Webber, J.A. Ouellet, L.S. Stone, Lisbet Haglund; Painful Degenerating Intervertebral Discs Upregulate Neurite Sprouting and CGRP Through Nociceptive Factors; *Journal of Cellular and Molecular Medicine,* 2014; Vol 18, No 6, 2014 pp. 1213-1225

2. Degenerating discs from low back pain patients release increased amounts of several proinflammatory cytokines. We found several cytokines increase that had not previously been associated with disc degeneration or pain.

E. Krock, D.H Rosenzweig, A-J. Chabot-Doré, P Jarzem, M.H. Webber, J.A. Ouellet, L.S. Stone, Lisbet Haglund; Painful Degenerating Intervertebral Discs Upregulate Neurite Sprouting and CGRP Through Nociceptive Factors; *Journal of Cellular and Molecular Medicine,* 2014; Vol 18, No 6, 2014 pp. 1213-1225

3. TLR2 regulates NGF through NF-κB. We were the first to discover NGF is regulated by TLR2 and by NF-κB signaling in any tissue.

E. Krock, J.B. Currie, M.H. Weber, J.A. Ouellet, L.S. Stone, D.H. Rosenzweig, L. Haglund; Nerve Growth Factor is Regulated by Toll-Like Receptor 2 in Human Intervertebral Discs; *Journal of Biological Chemistry,* 2016; VOL. 291, NO. 7, pp. 3541–3551

4. TLR2 activation is sufficient to induce degenerative changes of intervertebral discs. This suggests TLRs could contribute to the early stages of disc degeneration.

E. Krock, D.H Rosenzweig, J.B. Currie, J.A. Ouellet, Lisbet Haglund; Toll-like Receptor 2 Activation Induces Human Intervertebral Disc Degeneration; *In preparation*

5. TLR4 inhibition reduces signs of disc degeneration and pain in SPARC-null mice. This was the first study to show TLR4 inhibition reduces signs of degeneration and pain in any model. Furthermore, it supports inhibiting TLRs as a disease modifying therapeutic strategy.

E. Krock, M. Millecamps, J.B. Currie, L.S. Stone, L. Haglund; Toll-like Receptor 4 Inhibition Decreases Disc Degeneration and Pain in a Mouse Model; *in preparation*

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Chapter 8: Appendices

8.1 Related Works

During my Ph.D. I contributed as a coauthor to two manuscripts that complemented my thesis:

B. Alkhatib, D.H. Rosenzweig, **E. Krock**, P.J. Roughley, L. Beckman, T. Steffen, M.H. Weber, J.A. Ouellet, and L. Haglund; Acute mechanical injury of the human IVD: Link to Degeneration and Pain; *European Cells and Materials,* 2014; (28) 98-11 https://www.ncbi.nlm.nih.gov/pubmed/25214017

R. Gawri, D.H. Rosenzweig, **E. Krock**, J.A. Ouellett, L.S. Stone, T.M. Quinn, L. Haglund; Excessive Mechanical Strain of Primary Intervertebral Disc Cells Promotes Secretion of Inflammatory Factors Associated with Disc Degeneration and Pain; *Arthritis, Research and Therapy,* 2014; 16(1):R21http://www.ncbi.nlm.nih.gov/pubmed/24457003

I also authored a review article with Derek Rosenzweig and Lisbet Haglund that is related to my thesis work:

E. Krock, D.H. Rosenzweig, L. Haglund; Stem cell-based therapeutics for intervertebral disc repair: is it feasible within the inflammatory milieu of the degenerate disc?; *Current Stem Cell Research and Therapy*, 2015; 10(4):317-28 http://www.ncbi.nlm.nih.gov/pubmed/25670061

I have also completed a side project that examines the role of IL-8 and the mouse homologues of IL-8. The manuscript is currently in preparation.

E. Krock, M. Millecamps, L. Haglund, L.S. Stone; CXCR1/2 Inhibition Decreases Signs of Disc Degeneration and Chronic Low Back Pain Behavior in a Mouse Model; *in preparation*

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