Evaluation of Senolytic Drugs o-Vanillin and RG-7112 in Painful Intervertebral Disc Degeneration

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Abstract:

Low back pain is a global health problem related to intervertebral disc (IVD) degeneration. It is experienced by approximately 80% of individuals at some time in their lifespan. Despite its prevalence, little is known about the mechanisms leading to painful IVD degeneration, leaving surgical removal and vertebral fusion in end-stage disease as the most common treatment. There is growing recognition that senescent cells accumulate during tissue degeneration, contributing directly to disorders like heart disease, cancer, and osteoarthritis. In ageing and degenerating IVDs, tissue homeostasis is disrupted by the accumulation of senescent cells producing inflammatory and nociceptive factors that cause pain and inflammation, along with proteases degrading the tissue. Previously, our lab has shown that senolytic drugs RG-7112 and o-Vanillin target and remove senescent cells from the IVDs, improving tissue homeostasis and relieving inflammatory processes.

The **overall objective** of this thesis is to evaluate the potential of senolytics as a treatment option in painful degenerate IVDs *in vitro* and *in vivo*. The specific chapter objectives are:

1) To investigate a potential link between Toll-like receptor (TLR) expression and cellular senescence from patients undergoing surgery for low back pain.

2)

2.1 To determine if treatment with o-Vanillin and/or RG-7112 has the potential to alter factors secreted from cells from degenerating human IVDs that cause innervation.

2.2 To determine if combination treatment with o-Vanillin and RG-7112 is more potent than a single senolytic at reducing senescent phenotypes and SASP factors in cells from degenerating human IVDs.

3) To evaluate if combining o-Vanillin and RG-7112 can selectively remove senescent cells, reduce inflammatory mediators, and relieve pain in middle-aged SPARC-null mice with back pain *in vivo* better than a single senolytic.

Chapter 1 shows higher levels of SASP factors, TLR-2 gene expression, and protein expression following 48h induction with TLR-2/6 agonist. Treatment with o-Vanillin reduces the number of senescent cells and increases matrix synthesis in IVD cells from back pain patients. Treatment with o-Vanillin after induction with TLR-2/6 agonist reduces gene and protein expression of SASP factors and TLR-2. Co-localization staining of $p16^{INK4a}$ and TLR-2 demonstrates that senescent cells have a high TLR-2 expression.

In **Chapter 2**, compared to the single treatments, the combination of o-Vanillin and RG-7112 significantly reduces the amount of senescent IVD cells, pro-inflammatory cytokines, and neurotrophic factors in cell pellets from degenerating human IVDs. Moreover, single and combination treatments significantly reduces neuronal sprouting in PC-12 cells from degenerate human IVD cell pellets.

In **Chapter 3**, we show that treatment of middle-aged SPARC-null mice with either o-Vanillin or RG-7112 can reduce pain behaviour, IVD degeneration and senescence, spinal cord senescence and spinal cord pain marker expression (CGRP, CD11-b, GFAP) when compared to non-treated controls. Combination treatment was found to produce a more potent effect than the single senolytic treatment groups.

The data presented in this thesis suggests that single and combination treatment with o-Vanillin and RG-7112 demonstrates promising results in vitro to in vivo models for IVD degeneration which is commonly associated with low back pain. If proven successful, senolytic

therapy could not only be a major advance in back pain treatment for millions of patients but also provide general improvement of tissue homeostasis and possible longevity.

Résumé

La lombalgie est un problème de santé mondial lié à la dégénérescence des disques intervertébraux (DIV). Environ 80% des personnes en souffrent à un moment ou à un autre de leur vie. Malgré sa prévalence, on connaît peu les mécanismes conduisant à la dégénérescence douloureuse de la DIV, ce qui fait que l'ablation chirurgicale et la fusion vertébrale au stade terminal de la maladie constituent le traitement le plus courant. Il est de plus en plus reconnu que les cellules sénescentes s'accumulent au cours de la dégénérescence des tissus, contribuant directement à des troubles tels que les maladies cardiaques, le cancer et l'arthrose. Dans les DIV vieillissantes et dégénératives, l'homéostasie tissulaire est perturbée par l'accumulation de cellules sénescentes produisant des facteurs inflammatoires et nociceptifs à l'origine de la douleur et de l'inflammation, ainsi que des protéases dégradant le tissu. Auparavant, notre laboratoire a montré que les médicaments sénolytiques RG-7112 et o-Vanilline ciblent et éliminent les cellules sénescentes des DIV, améliorant l'homéostasie des tissus et soulageant les processus inflammatoires.

L'objectif global de cette thèse est d'évaluer le potentiel des sénolytiques en tant qu'option de traitement, seuls ou en combinaison avec des IVD douloureuses et dégénérées, in vitro et in vivo. Les objectifs spécifiques du chapitre sont les suivants:

1) Étudier le lien potentiel entre l'expression des récepteurs Toll-like (TLR) et la sénescence cellulaire chez des patients opérés pour une lombalgie.

2)

2.1 Déterminer si le traitement à l'o-Vanilline et/ou au RG-7112 peut modifier les facteurs sécrétés par les cellules des DIV humaines en dégénérescence qui provoquent l'innervation.

2.2 Déterminer si un traitement combiné par o-Vanilline et RG-7112 est plus puissant qu'un sénolytique unique pour réduire les phénotypes sénescents et les facteurs SASP dans les cellules des DIV humaines en dégénérescence.

3) Évaluer si la combinaison de l'o-Vanilline et du RG-7112 peut éliminer sélectivement les cellules sénescentes, réduire les médiateurs inflammatoires et soulager la douleur chez les souris SPARC-null d'âge moyen souffrant de maux de dos in vivo, mieux qu'un sénolytique unique.

Le **chapitre 1** montre des niveaux plus élevés de facteurs SASP, d'expression des gènes TLR-2 et d'expression des protéines après 48 heures d'induction avec l'agoniste TLR-2/6. Le traitement à l'o-vanilline réduit le nombre de cellules sénescentes et augmente la synthèse matricielle dans les cellules des DIV de patients souffrant de lombalgie. Le traitement à l'o-vanilline après induction par l'agoniste TLR-2/6 réduit l'expression des gènes et des protéines des facteurs SASP et TLR-2. La colocalisation de $p16^{INK4a}$ et de TLR-2 démontre que les cellules sénescentes ont une forte expression de TLR-2.

Dans le **chapitre 2**, par rapport aux traitements uniques, la combinaison de l'o-vanilline et du RG-7112 réduit de manière significative la quantité de cellules sénescentes des DIV, de cytokines pro-inflammatoires et de facteurs neurotrophiques dans les pelotes de cellules provenant de DIV humaines en dégénérescence. En outre, les traitements simples et combinés réduisent de manière significative la croissance neuronale dans les cellules PC-12 à partir de pelotes de cellules de DIV humaines en dégénérescence.

Dans le **chapitre 3**, nous montrons que le traitement de souris SPARC-null d'âge moyen par o-Vanilline ou RG-7112 peut réduire le comportement douloureux, la dégénérescence et la sénescence des DIV, la sénescence de la moelle épinière et l'expression des marqueurs de la

douleur dans la moelle épinière (CGRP, CD11-b, GFAP) par rapport à des contrôles non traités. Le traitement combiné s'est avéré produire un effet plus puissant que les groupes ayant reçu un seul traitement sénolytique.

Les données présentées dans cette thèse suggèrent que le traitement unique et combiné avec l'o-Vanilline et le RG-7112 donne des résultats prometteurs dans les modèles in vitro et in vivo pour la dégénérescence de la DIV qui est communément associée à la lombalgie. En cas de succès, la thérapie sénolytique pourrait non seulement constituer une avancée majeure dans le traitement de la lombalgie pour des millions de patients, mais aussi apporter une amélioration générale de l'homéostasie tissulaire et une éventuelle longévité.

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understanding, and patience, which helped me to stay focused and motivated throughout this degree.

Contribution to Original Knowledge

The manuscript presented here is a compilation of three multi-authored papers. To our knowledge, each paper contains unique and original contributions that will be thoroughly discussed in this thesis. In conducting the experiments and analyzing the data for each manuscript, I (M. Mannarino) played a major role and drafted the initial manuscript. However, my coauthors' collaborative efforts were crucial to this work's success. Their contributions included designing and executing experiments, analyzing data, and providing funding. For clarity, each author's contributions to each manuscript are listed below.

Contribution of Authors

The data included in this thesis is exclusive to this thesis and will not be reproduced in any other thesis. All co-authors have agreed to these conditions.

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All authors read and approved the final manuscript. Matthew Mannarino received a studentship from MITACS. Matthew Mannarino and Hosni Cherif designed the study, conducted experiments, analyzed data, and wrote the manuscript. Li Li and Kai Sheng conducted experiments. Oded Rabau, Jean Ouellet, Michael Weber and Peter Jarzem helped to design the

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List of Abbreviations

ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motifs AEB – Acetone Evoked Behaviour
AF	Annulus Fibrosus
BDNF	Brain Derived Neurotrophic Factor
CIHR	Canadian Institute of Health Research
CGRP	Calcitonin Gene-Related Peptide
CXCL	Chemokine Ligand
DAMP	Danger Associated Molecular Pattern
DDR	DNA Damage Response
DMEM	Dulbecco's Modified Eagle Medium
DMMB	Dimethyl-Methylene Blue Assay
DRG	Dorsal Root Ganglia
ECM	Extracellular 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
ICC	Immunocytochemistry
IDD	Intervertebral Disc Degeneration
IFN	Interferon
IL	Interleukin

iNOS	Inducible Nitric Oxide Synthase
IR	Immunoreactivity
IVD	Intervertebral Disc
MAPK	Mitogen-Activated Protein Kinase
MMP	Matrix Metalloproteinase
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
RAGE	Receptor for Advanced Glycation End Products
ROS	Reactive Oxygen Species
SASP	Senescence-associated Secretory Phenotype
sGAG	Sulfated Glycosaminoglycans
SIPS	Stress-induced Premature Senescence
SLRP	Small Leucine-Rich Repeat Protein
SPARC	Secreted Protein Acidic and Rich in Cysteine
TIMP	Tissue Inhibitor of Metalloproteinase
TIR	Toll-Interleukin Receptor
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor

TRK	Tropomyosin Receptor Kinase
TRPV1	Transient Receptor Potential Cation Channel Subfamily V Member 1

Introduction

1. The Burden of Disease: Disc Degeneration and Chronic Low Back Pain

The Global Burden of Disease study identifies chronic low back pain as the most significant cause of disability worldwide[1,2]. Up to 80% of people will have low back pain at some point, and between 15% and 30% will develop chronic low back pain[3]. The high prevalence of lower back pain results in enormous healthcare expenses. Chronic low back pain is associated with annual costs ranging from £12.3 billion in the United Kingdom and €16.5 to €50 billion in Germany, and $85.9 billion in the United States[4,5].

Pain is defined as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage," by the International Association for the Study of Pain (IASP). Whereas chronic pain is defined as persistent pain or recurring pain lasting longer than three months. Furthermore, chronic low back pain is defined by the North American Spine Society Clinical Care Guidelines as "musculoskeletal pain that can extend from the bottom rib through the lumbar area, and at times as somatic referred pain, also known as radiating pain, in the thigh." Despite the high prevalence of back pain, it remains a widespread and critically important health issue for which therapeutic options remain limited.

Facet joints, ligaments, muscles, and intervertebral discs are all tissues that might contribute to chronic low back discomfort. Intervertebral disc degeneration and herniation are among the most common pathological causes of chronic low back pain among these tissues[6]. Consequently, therapeutic approaches have been developed to treat disc degeneration and chronic low back pain. Strategies range from conservative methods, such as physical therapy and analgesic drug regimens, to more invasive surgical procedures, such as arthroscopic surgery, discectomies, lumbar fusions, and disc replacement. Conservative methods frequently

fail to adequately manage patients' pain, whereas surgical interventions are highly invasive and do not always relieve pain, leading to high reoperation rates[7,8]. For example, while lumbar fusions may relieve patients' discomfort, they can also increase the likelihood that the neighboring discs will deteriorate, resulting in more pain and surgery. Of note, no currently approved disease-modifying medications decrease the progression of deterioration and/or alleviate discomfort. Thus, conventional therapeutic techniques cannot control disc degeneration and persistent pain appropriately. Insufficient knowledge of the pathogenic mechanisms behind disc degeneration and chronic low back pain restricts the efficacy of treatment interventions. A more profound comprehension of these mechanisms will lead to discovering novel treatment targets and methods.

2. Comprehensive Literature Review and Background

2a. Structure of the Spine and Intervertebral Disc

The human spine comprises thirty-three vertebrae organized into five sections: seven cervical, twelve thoracic, five lumbar, five fused sacral, and four fused coccygeal vertebrae. Twenty-three intervertebral discs are located between adjacent vertebrae, except between the cervical first and second vertebrae, sacrum, and coccyx[9]. Intervertebral discs comprise a core nucleus pulposus (NP) and the annulus fibrosus (AF), which surrounds and contains the NP. The NP and AF's anterior and posterior cartilaginous endplates separate the disc from the vertebral bodies. Discs are analogous to joints, given that they are motion segments which allow the spine to compress and twist flexibly. Adult discs are mostly aneural and avascular, with only a few nerve fibres and blood vessels at the AF[10]. Each section of disc tissue has unique cell types (NP, AF, and chondrocytes) with separate lineages. Each cell type is accountable for anabolic and catabolic activities in its specific tissue. The disc can fulfill its

biomechanical tasks due to the particular structure of each tissue type.

2a.i. Nucleus Pulposus

The fetus NP is fluid, while the juvenile NP is more gel-like. As collagen content increases with age, the NP becomes less fluid-like and more gel-like, eventually exhibiting fibrotic properties. The NP's biological makeup provides its biomechanical function as a shock-absorbing tissue[9]. NP's extracellular matrix (ECM) comprises randomly distributed type II collagen fibres, elastin, and high proteoglycan content. A ratio of 27:1 between proteoglycan and collagen demonstrates the high proteoglycan content of NPs, as contrasted to a ratio of 2:1 in hyaline cartilage[11]. The predominant proteoglycan in the NP is aggrecan[6,12]. Aggrecan has an abundance of anionic keratin sulphate and chondroitin sulphate glycosaminoglycan (GAG) chains. Given the anionic charge of the GAG chains, NP is a hydrophilic tissue with a high-water content. Small leucine-rich repeat proteins (SLRP) also contribute to the formation and structure of the NP extracellular matrix (ECM). The ECM composition results in the gel-like structure of the NP, which permits compression and reswelling when the spine is loaded and unloaded, respectively.

NP is populated by notochordal cells during embryogenesis. Within the first decade of human life, notochordal cells begin to vanish and are replaced by small, round chondrocyte-like cells[6]. The literature indicates that notochordal cells in the NP function as NP progenitor cells**,** giving rise to the tiny chondrocyte-like NP cells present in mature humans[13]. In adults, the NP cell density is modest (3000 cells/mm^3), and the distribution of cells is random[14]. The NP ECM is synthesized, repaired, and regulated by NP cells. NP's function is determined by its distinct cell type and ECM structure.

2a.ii Annulus Fibrosus

The AF encompasses the NP and is separated into an inner and an outer portion. Overall, the AF has less type II collagen and aggrecan, and more type I collagen, than NP[15]. The ratio of proteoglycan to collagen is around 1.6:1. The inner AF functions as a transition zone between the NP and outer AF. The inner AF has a less structured ECM and a larger proteoglycan and type II collagen concentration than the outer AF[6,9]. The inner resident AF cells are round chondrocyte-like cells comparable to the NP but of mesenchymal origin. The outer AF has elongated fibroblast-like cells, and the AF cell density in young adults is 5,000 cells/mm^3. The outer AF contains more collagen than proteoglycan by dry weight, and, unlike the NP and inner AF, type I collagen is the main form of collagen[6,16]. The collagen fibrils are arranged into fibres, creating fifteen to twenty-five concentric lamellar rings that alternate thirty to forty-five degrees to the spine axis. This structure provides the AF with a high tensile strain that enables bending and twisting of the spine and resistance to NP bulging when the NP is compressed.

2a.iii Cartilaginous Endplate

The cartilaginous endplate is a thin (0.6 mm) layer of hyaline cartilage that caps each end of the intervertebral disc. Not included is the outermost portion of the outer AF. The density of chondrocytes on the endplate is between 10,000 and 18,000 cells/mm^3. Its extracellular matrix comprises type II collagen, aggrecan, and other proteins and proteoglycans. The ratio of type II collagen to proteoglycan is around 2:1[11]. The endplate serves multiple vital tasks. First, it safeguards the disc from the surrounding vertebrae, which could cause harm to the NP and inner AF. Second, the endplates prevent the NP from herniating into the vertebral body by containing it. Thirdly, the endplate is the main site for nutrition diffusion into the disc and waste

diffusion out of the disc[17]. Blood vessels near the vertebrae feed nutrients and absorb waste into the endplate's outermost layer.

2b. Intervertebral Disc Degeneration and Degenerative Disc Disease

Michael Adams and Peter Roughley define intervertebral disc degeneration as "an abnormal, cell-mediated response to gradual structural breakdown." A degenerated disc has a structural breakdown that can be accelerated by age[6]. This concept provides a helpful foundation for discussing disc degeneration and degenerative disc disease, as it highlights the essential traits and mechanisms of degeneration. The height of degenerating discs decreases, they bulge or herniate, and they lose their biomechanical function. Loss of NP hydrostatic pressure causes the redistribution of compressive pressures from the NP to the AF[18]. In addition to the annulus or endplate tears and fissures, structural failure is also marked by NP herniation through the AF or endplate[6]. Large-scale ECM remodelling facilitates the degenerative alterations that result in structural collapse[6,15]. In response to mechanical and chemical cues, cells undergo degenerative processes[19-22]. In addition, the definition specifies that cells actively contribute to disc degeneration. Pain draws patients to a clinic and thus leads to a diagnosis of disc degeneration. Since Adams and Roughley presented this classification in 2006, several additional defining characteristics of disc degeneration have been proposed. These elements remain compatible with the framework but are more specific. Disc degeneration may be characterized by increases in matrix proteases, proinflammatory cytokines, immune cell infiltration and, more recently, cellular senescence[23]. These modifications will be addressed in depth in the following section.

Multiple macroscopic and microscopic alterations accompany disc degeneration. Macroscopic alterations include endplate thinning, lack of demarcation between the inner AF

and NP, disc thinning, disc bulging, and NP water content decrease. The NP and AF become discoloured and increasingly calcified due to non-enzymatic glycosylation. Additionally, the NP loses its gelatinous properties and becomes more fibrotic. The redistribution of compressive pressures to the AF exacerbates AF deterioration. In addition, disc pH and nutrients drop, creating an unfavourable environment for disc self-repair[6,17,23-25]. Imaging techniques and grading scales, such as the widely used Pfirrmann Grade, can be utilized to visualize and classify degeneration. Using T2-weighted magnetic resonance imaging (MRI), which provides insight into morphological alterations and water loss, the Pfirrmann Grade is determined[26]. In addition to the structural changes discussed previously, various detrimental metabolic changes occur. Among these are the breakdown of the ECM and elevated levels of matrix proteases, pro-inflammatory cytokines and chemokines, and neurotrophins.

2b.i Matrix Catabolism

AF, NP, and cartilage end-plate ECM undergo extensive change during degeneration. Both the NP and the AF exhibit a reduction in proteoglycan production and content. Type II collagen is gradually replaced by type I collagen in the NP and inner AF. Collagen fibrils grow progressively dense and cross-link. In addition, the NP and the inner and outer AF gradually become calcified. These alterations result in fibrosis of the NP, which contributes to the loss of mechanical function[6,24]. Additionally, ECM components are damaged and fragmented. Degenerated discs contain more fragmented chondroadherin, collagen, aggrecan, fibronectin, biglycan, and versican products than non-degenerated discs[6,12,27]. Some of these fragments have biological activity *(described more in section 2b.iii Toll-like Receptors)* and lead to ECM degradation. GAG chains are also broken and lost during aggrecan degradation. In addition, when degeneration advances, ECM synthesis decreases. The degradation of aggrecan, other

559 proteoglycans, and GAGs decreases the disc's anionic charge and hydrophilic characteristics.
560 Reduced hydrophilic capabilities lead to water and hydrostatic pressure loss in the NP, affecting
561 the biomechanical properties of the NP. These matrix modifications cause load redistribution
562 and contribute to the disc's mechanical failure.

563 During disc degeneration, there are a large number of matrix proteases found in the
564 IVD, such as matrix metalloproteinases (MMP)-1, -3, -7, -9, and -13, cathepsins[28,29], high-
565 temperature requirement serine protease A1 (HTRA1)[30-32] and a disintegrin and
566 metalloproteinases with thrombospondin motifs (ADAMTS)-4 and -5, also known as
567 aggrecanases[33,34], and ADAMTS-1,-9 and -15. The activity of a number of these proteases is
568 necessary for the regular turnover of the matrix. However, disc ECM is degraded when protease
569 levels increase or protease inhibitory mechanisms (i.e., tissue inhibitor of metalloproteinase
570 TIMP) decrease. Thus, an increase in proteases is a characteristic of disc degeneration. ECM
571 alterations in degenerative discs result from a combination of proteolytic degradation and
572 reduced matrix synthesis. Alterations to the endplate during degeneration are less well
573 understood than NP and AF changes.

574 Like the NP and AF, the endplates ECM undergoes remodeling. The endplate itself is
575 difficult to image because of its size; however, endplate defects are believed to be the source
576 of Modic alterations. Modic alterations are vertebral bone marrow lesions seen by MRI that are
577 unrelated to marrow malignancies, pyogenesis, or seropositive rheumatic illnesses, are
578 characterized by inflammation and unfavorable bone remodeling, and are strongly correlated
579 with chronic low back pain. Endplate abnormalities also permit herniation of the nucleus
580 through the endplate into the vertebral body. The calcification of herniations leads to the
581 creation of Schmorl's nodes, which can also be scanned via MRI and are similarly associated

582 with chronic low back pain[35,36]. The effects of endplate ECM degradation and calcification on
583 the diffusion of nutrients into the disc and waste out of the disc are uncertain. As endplates
584 degenerate, it has been hypothesized that diffusion will decrease and that they will become
585 more porous[37,38]. Reduced diffusion reduces nutrient delivery, making it more difficult for cells
586 to survive. However, increased permeability could allow cytokines, neurotrophins, and immune
587 cell diffusion into the vertebral bodies and migration into the disc. Regardless, it appears
588 probable that the endplate plays a significant but poorly understood role in disc degeneration.
589
590 **2b.ii Sterile Inflammation**
591 Sterile inflammation, which constitutes the rise of pro-inflammatory cytokines and
592 chemokines in the absence of pathogens, is a hallmark of painful disc degeneration. Interleukin-
593 1 (IL-1) and Tumor Necrosis Factor (TNF) are the most studied cytokines in disc
594 degeneration[23]. NP and AF cells generate IL-1 and TNF, and their expression rises during
595 degeneration[39-41]. Their induction in the early stages of degeneration is poorly understood,
596 although it is probable that multiple pathways contribute to the increases in these cytokines.
597 Gene expression of IL-1, and IL-6[42-48], unfavorable mechanical strain[19,20,22], and smoking[49]
598 contribute to cytokine increases. IL-1 and TNF raise protease levels, reduce matrix
599 synthesis[40,50,51], and enhance the production of pro-inflammatory cytokines, chemokines, and
600 neurotrophins[23]. Both IL-1 and TNF have been widely studied as potential therapeutic targets.
601 TNF inhibitors have also been evaluated in clinical trials, as described below. Anakinra and
602 other IL-1 inhibitors have not been evaluated in humans with disc degeneration.
603 Other pro-inflammatory cytokines, including CCL-2, -5, and -7, Interferon (IFN), IL-
604 2, -4, -6, -7, -8, -12, and -17, chemokine (C-X-C motif) ligand (CXCL)-1, -9 and -10 rise in

degenerating discs[21,23]. Only a few of these factors have been assigned roles in degeneration. IL-6 and IL-8, for example, can influence other cytokines and catabolic factors and act as chemoattractant factors for immune cells[21]. In addition, both are linked to pain in animal models of disc degeneration or herniation[52,53]. IL-1, TNF, other cytokines, and chemokines probably collaborate to cause degeneration and pain. Therefore, targeting a single cytokine or chemokine may not be sufficient to delay or stop degeneration.

Discs that have not degenerated are considered immune-privileged tissues, as they lack resident immune cells and the vasculature that supplies immune cells. However, research suggests neovascularization and immune cells permeate the disc following disc herniation[54]. T cells, neutrophils, macrophages, and mast cells can be among the cells that infiltrate a herniated disc[54]. Numerous early investigations on immune cell infiltration, for instance, utilized CD68 as a marker for macrophages. CD68 is expressed on the membranes of lysozymes, and Nerlich et al. discovered CD68+ cells in the NP tissue of non-herniated, degenerating discs[55]. They hypothesize that CD68+ cells are not immune cells but rather resident NP cells that have undergone a phenotypic shift due to the cell's shape[56]. Chen et al. discovered that NP cells could exhibit macrophage-like actions defined by a phagocytic phenotype and physical features[57]. As per more recent studies, immune cells infiltrate herniated disc tissues utilizing CD11b, a leucocyte marker[54,58]. The increase in pro-inflammatory cytokines and chemokines released by resident NP and AF cells is likely to attract immune cells. Immune cells may identify disc tissue as 'non-self' and elevate the pro-inflammatory factors seen in degenerating discs, thereby contributing to sterile inflammation, matrix catabolism, and the development of discomfort[23].

2b.iii Toll-like Receptors

Toll-like receptors (TLR) are pattern recognition receptors that were initially identified as having microbial components as their ligands, also known as pathogen-associated molecular patterns (PAMPs). PAMPs include peptidoglycan, lipopolysaccharide, and viral ribonucleic acid (RNA)[59]. It has been shown that endogenous danger-associated molecular patterns (DAMPS), also known as alarmins, activate TLRs. Examples of alarmins include fragmented hyaluronic acid, fibronectin, and aggrecan, as well as 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)[60,61]. Humans express ten TLRs (TLR1-10), and TLRs-1, -2, -4 and -6 are most frequently involved with detecting alarmins. Many immune cells and other cell types express TLRs, including NP cells, AF cells, chondrocytes, and neurons.

2b.iii.i Toll-like Receptor Signaling

TLRs can identify homodimers or heterodimers of PAMPs and alarmins[59]. TLR-2 works as a homodimer, a TLR-1/2 heterodimer, or a TLR-2/6 heterodimer, whereas TLR-4 is believed to signal mainly as a homodimer. However, recent studies suggest TLR-4 can also signal as a TLR-4/6 or TLR-2/4 heterodimer (**Figure 1**)[62]. TLRs consist of a single transmembrane domain and an extracellular domain with 19-25 tandem leucine-rich repeats that contribute to pattern recognition[63]. TLRs share numerous characteristics with the IL-1 receptor (IL-1R) family. Both TLRs and IL-1R have a cytoplasmic TIR domain and signal in a MyD88-dependent manner, but TLR-4 can signal independently of MyD88. MyD88 recruitment to the TIR signaling complex results in the activation of the NFκ-B and mitogen-activated protein kinase (MAPK) signaling pathways. TLR signaling enhances the production of proinflammatory cytokines such as IL-1 and TNF and interferon family genes upon activation

by PAMPs or DAMPs (**Figure 1**). Recent studies suggest that TLR signaling may contribute to the regulation of inflammation in rheumatoid arthritis and disc degeneration.

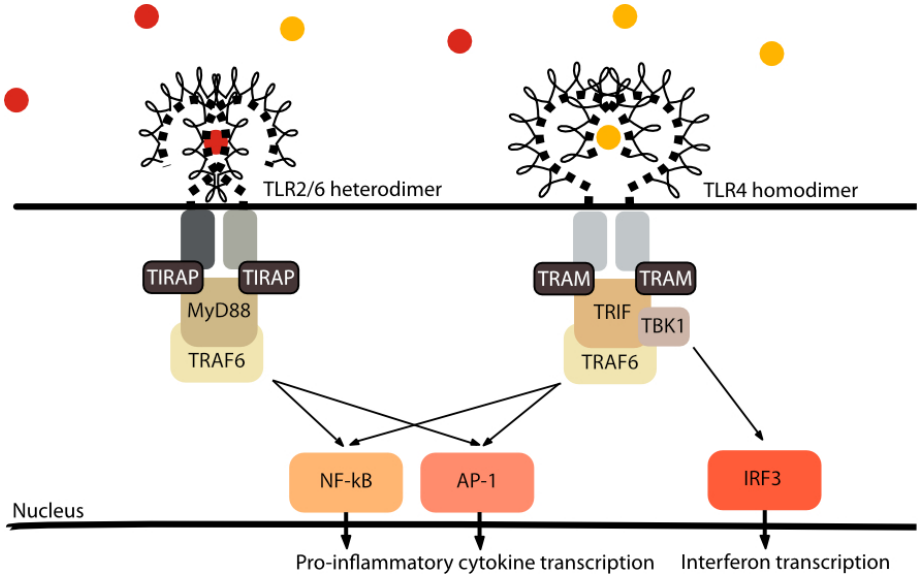

Figure 1: Schematic representation of Activation Pathways for TLR-2/6 vs. TLR-4 and downstream signalling pathways.[64]

2b.iii.ii Toll-like Receptors in Cartilage and Disc Degeneration

TLRs have recently begun to be studied in relation to disc degeneration. However, their significance in osteoarthritis and rheumatoid arthritis has previously been well examined. While there are significant differences between cartilage and discs, many commonalities may help explain TLRs' involvement in disc degeneration. Notably, earlier studies suggest that TLRs play a crucial role in the breakdown of connective tissues[60]. In osteoarthritis, like disc degeneration, the expression of TLR1-9 rises as cartilage degradation increases[65]. Several studies have demonstrated that activation of chondrocyte TLR by bacterial ligands such as lipopolysaccharide or peptidoglycan and endogenous ligands such as fragmented aggrecan or fibronectin enhances cytokine and protease production and inhibits matrix synthesis[66-69].

Literature on cartilage has also shown that non-immune cells contain TLRs and that chondrocytes respond to several alarmins, such as HMGB1, S100 proteins, fibronectin fragments, and low molecular weight hyaluronan[60,70]. These investigations demonstrate that TLR activation by endogenous ligands results in cartilage degradation. They have also described a role for TLRs and alarmins in non-immune cells, which provides a more pertinent framework for studying TLRs and alarmins in intervertebral discs. Less is known about the involvement of TLRs in disc degeneration compared to arthritis. Recent studies have shown TLR-1, -2, -3, -4, -5, -6, -9, and -10 gene expression in disc cells (NP and AF cells were not separated). TLRs-1, -2, -4, and -6 are constitutively expressed, and their expression corresponds with the severity of degeneration in postoperative low back pain patients' samples[71].

Treatment of cells with IL-1 or TNF, or TLR-2 activation enhances TLR-2 and -4 gene expression[20,71,72], but how and why TLR expression rises *in vivo* during degeneration remains unknown. The disc contains several alarmins that can activate TLRs, such as 30 kDa fibronectin fragments, aggrecan fragments, low molecular weight hyaluronic acid fragments, and HMGB1[12,72-74]. Human disc cells treated with low molecular weight hyaluronic acid express more IL-1, -6, and -8 genes via TLR-2. Injecting fibronectin fragments with a molecular weight of 30 kDa into rabbit discs produces degenerative alterations, although whether this effect is due to TLR activation has not been examined[75,76]. Other alarmins' effects have yet to be thoroughly described in the disc. TLR agonists, such as lipopolysaccharide and Pam2CSK4, are utilized more frequently to activate TLR-2 or TLR-4. TLR-2 and TLR-4 stimulate the production of MMP-1,-3,-13, ADAMTS-4 and -5, IL-6, IL-8, and iNOS in bovine or human disc cells (**Figure 2**)[67,71,72,77]. Previous research suggests that TLRs may play a role in

controlling disc degeneration. However, it is uncertain if TLRs are involved in degenerative pathways that contribute to pain, if TLR activation leads to degenerative changes in cultured human discs, and if TLRs are viable disease-modifying targets.

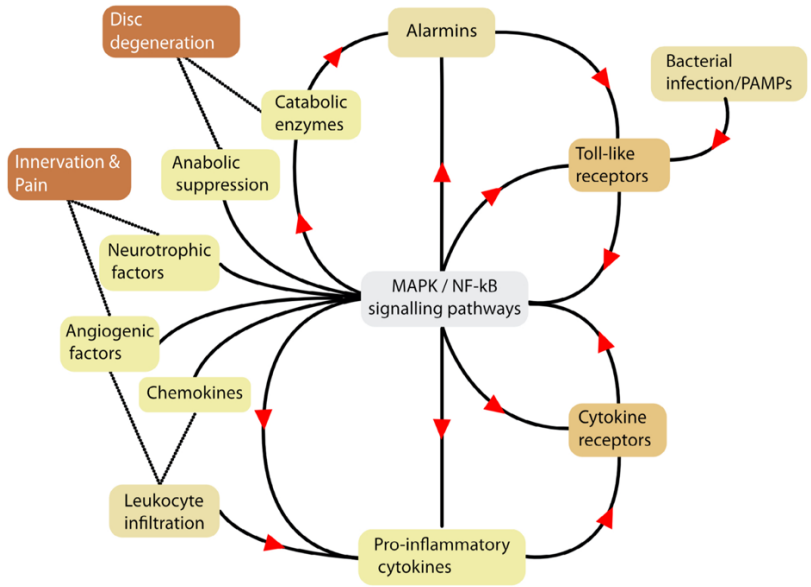

Figure 2: TLR and cytokine receptor-centric positive feedback loops contributing to IVD degeneration and back pain.[64]

2b.iv Cell Senescence

Senescent cells are viable cells that can no longer proliferate and accumulate in the body with age as a result of telomere lengthening during replicative cycles[78,79]. Stressors such as DNA-damaging agents, oxidative stress, mitochondrial dysfunction, load-induced injury, and disruption of epigenetic regulation can also prematurely induce senescence which is commonly referred to as stress-induced premature senescence (SIPS) (**Figure 3**)[80,81]. Increased presence of senescent cells is characteristically seen in degenerating IVDs as well as in younger individuals with painful IVD degeneration and/or herniation[82-84]. Senescent cells release a series of inflammatory cytokines, chemokines, and proteases known collectively as the senescence-associated secretory phenotype (SASP)[85]. As a result they trigger an inflammatory

environment which prevents adjacent cells from maintaining tissue homeostasis[86,87]. Studies have proposed that senescent cells induce senescence in a paracrine manner, thus exacerbating tissue deterioration[88]. In addition to their ability to induce an inflammatory environment senescent cells are resistant to apoptosis which allows them to accumulate. Of note, while all senescent cells share similar features, differences in the SASP factors released and the anti-apoptotic pathways used vary[89,90].

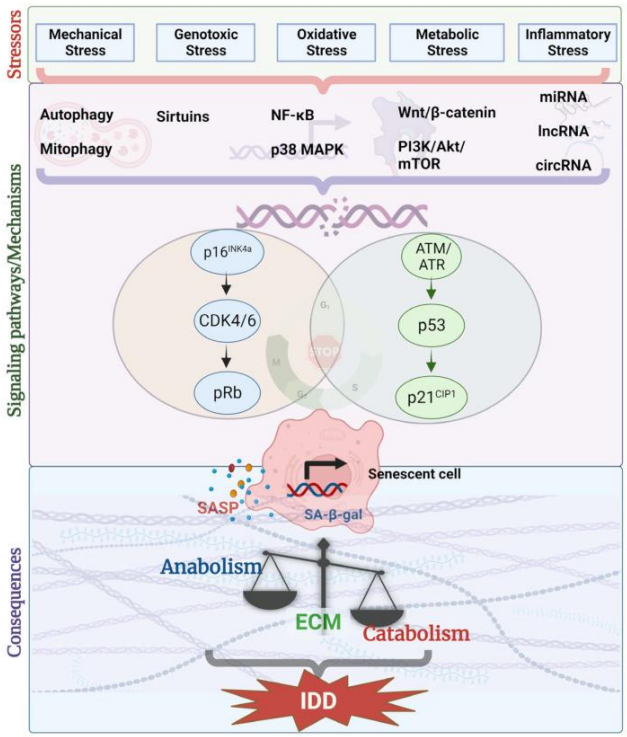

Figure 3: Overview of cellular senescence during intervertebral disc degeneration. The reported pathways of stressors, signaling molecules, and consequences of cellular senescence during intervertebral disc degeneration (IDD) are depicted.[91]

2b.iv.i Replicative Senescence

The process of ageing is characterized by the irreversible functional decline of an organism's tissues and organs, as well as the weakening of the control of internal environmental homeostasis[92-94]. According to research by Hayflick et al., human fibroblasts have a finite

capacity for cell division before experiencing replicating ageing or permanent growth arrest. As a result, they proposed the hypothesis that tissue ageing results from the gradual reduction of cell growth[79]. According to Wu et al. 2022, cellular senescence is a state of irreversible cell cycle stoppage in cells brought on by ongoing external and endogenous stress and injury[95]. One of the major contributors to defective tissue regeneration, chronic aging-related illnesses, and organismal ageing are senescent cells, which are a by-product of the cellular ageing process and have unique biological properties and functions. To date, no single marker can identify senescent cells with 100% certainty. To detect senescent cells, it is currently utilized to combine cytoplasmic (e.g., senescence-associated-galactosidase (SA-Gal), lipofuscin) and nuclear (e.g., $p16^{INK4A}$, p21WAF1/Cip1, Ki-67) markers[95,96]. Furthermore, the preferred technique for identifying ageing is microscopic imaging. Additionally, single-cell assays are utilized to identify senescent cells, including immunostaining, *in situ* hybridization, and multicolour (imaging) flow cytometry[97]. In a quantitative investigation of senescent cells, Biran et al. discovered that older mice had considerably more senescent cells than younger mice[98]. Since over time, the immune system is unable to eliminate senescent cells, their accumulation with age has a deleterious effect[99]. Long-term cellular senescence can induce inflammation and disease, whereas short-term cellular senescence is essentially harmless because the immune system will entirely eradicate it[100]. Cellular senescence is characterized by changes in chromatin, gene expression, organelles, and cell shape in addition to irreversible cell cycle arrest[97]. While this is going on, the extrinsic activity of senescent cells is closely linked to the activation of SASP, which intensifies the consequences of intrinsic cell proliferation arrest and promotes age-related chronic illnesses, delayed tissue regeneration, and organismal ageing[101].

2b.iv.ii Stress-Induced Senescence

SIPS is a phenomenon characterized by the accelerated aging and functional decline of cells due to chronic or excessive stress. It involves intricate biological mechanisms that impact cellular health and contribute to the development of age-related diseases. One of the key pathways involved in SIPS is the DNA damage response (DDR) (**Figure 3**). When cells are exposed to stress, whether it is physical, psychological, or environmental, they activate the DDR pathway to detect and repair damaged DNA. However, in cases of chronic stress, the burden of DNA damage can overwhelm the repair mechanisms, leading to persistent activation of the DDR and triggering cellular senescence. The activation of DDR in SIPS involves the upregulation of various signaling molecules, including tumour suppressor protein p53 and MAPK pathways, such as p38. These signaling pathways modulate gene expression and cellular processes to promote growth arrest and senescence-associated phenotypes.

Chronic stress also induces oxidative stress, which occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the cell's antioxidant defence mechanisms. Oxidative stress can lead to the accumulation of oxidative DNA damage, protein modifications, and lipid peroxidation, all of which contribute to cellular aging and senescence. Furthermore, chronic stress disrupts the balance of telomeres, the protective structures at the ends of chromosomes. Telomeres progressively shorten with each cell division and serve as a buffer to protect genomic integrity. However, stress-related factors, such as inflammation and oxidative stress, can accelerate telomere attrition. When telomeres become critically short, cells enter a state of replicative senescence, leading to impaired cell function and tissue dysfunction.

The consequences of SIPS extend beyond cellular aging and can impact overall health. Chronic stress-induced premature senescence has been associated with a wide range of age-related diseases, including cardiovascular disorders, neurodegenerative conditions, metabolic

765 dysfunction, and immune system dysregulation. The accumulation of senescent cells and the
766 secretion of SASPs, can contribute to chronic inflammation, tissue degeneration, and the
767 development of age-related pathologies.

768 **2b.v Possible Mechanisms Leading to Pain**

769 Only the outer few laminas of the AF are innervated in non-degenerating discs, which
770 are almost aneural[10]. Nonetheless, several investigations have demonstrated that degenerating
771 discs in patients with low back pain have increased innervation and innervation of typically
772 aneural locations. Some of the nerve fibres that innervate the disc also include neuropeptides
773 associated with pain, such as the substance P[102-104].

774 Provocative discography indicates that non-degenerating discs are painless, but
775 degenerating discs cause low back discomfort[6]. Small-diameter, nociceptive dorsal root ganglia
776 (DRG) neurons innervate discs predominantly[10]. Recent research identified neurofilament-200
777 immunoreactive fibres, showing that discs may also be innervated by myelinated neurons of
778 great diameter[54]. DRG neurons project onto the dorsal horn of the spinal cord, where pain-
779 causing nociceptive impulses can be sent. There are two different but overlapping explanations
780 for the development of persistent low back pain: mechanical/neuropathic damage and
781 inflammatory sensitization of nerve fibres.

782 **2b.v.i Mechanical Sources of Pain**

783 Neuropathic pain can result from mechanical damage to the nervous system. This can
784 occur when disc herniation, collapse, loss of height, or bulging compress the spinal cord, DRG,
785 or dorsal root[6]. These mechanical injuries may cause both radiating leg pain and low back
786 discomfort. Pain is caused by the first neuropathic insult, and the following sterile inflammation
787 will lead to the development of neuronal sensitization and chronic pain[21].

2b.v.ii Neurotrophic and Inflammatory Pain

Disc degeneration is characterized by sterile inflammation, as discussed previously. In addition to an increase in cytokines and chemokines, the inflammatory milieu of degenerating discs also exhibits an increase in the number of neurotrophins. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5 make up the neurotrophin family. Neurotrophins bind the high-affinity receptors TrkA (NGF), TrkB (BDNF/NT-3) and TrkC (NT-4/5) and the low-affinity receptor p75NTR[105]. Neurotrophins increase neuronal survival, growth, maturation, and innervation of target tissues throughout development. However, neurotrophins are also peripheral pain mediators, with NGF being the best understood. NGF is frequently detected in the inflammatory milieu following nerve damage or other pain-related causes of inflammation. Although the specific processes are unknown, NGF promotes fast sensitization of peripheral nociceptive fibres by binding to TrkA. TRPV1 (Transient Receptor Potential cation channel subfamily V member 1) is an ion channel implicated in heat and inflammatory pain. TrkA activation is believed to promote TRPV1 membrane insertion and sensitization, resulting in neuronal sensitization. In addition, retrograde transport of NGF to the DRG soma stimulates the expression of many pain-related proteins, including TRPV1, substance P, and calcitonin gene-related peptide (CGRP)[106,107]. According to preclinical investigations, NGF likely contributes to pain in degenerative disc degeneration. NGF gene and protein expression is elevated in degenerating human discs[54,102,108-110], and *in vivo* studies suggest that NGF regulates the production of neurochemical pain indicators such as CGRP[111].

Multiple functions are carried out by cytokines and chemokines in the genesis of pain. Cytokines stimulate the production of pronociceptive substances such as prostaglandins,

bradykinin, and NGF[112]. Moreover, subsets of DRG neurons carry cytokine receptors, including those for IL-1, IL-6, and TNF. Cytokines directly enhance neuronal activity and long-lasting sensitivity[107,113]. Chemokine receptors are also expressed on DRG neurons. DRG neurons contain receptors for CCL2 (CCR2), CXCL8/IL-8, and CXCL1 (CXCR1 and 2), and activation of these receptors results in enhanced activity, sensitization, and TRPV1 activity modulation. Evidence from preclinical and clinical studies suggests that cytokines and chemokines contribute directly to chronic musculoskeletal pain, such as low back pain or osteoarthritis[23,114]. Animal models of disc herniation and injury show, for example, that IL-6 and TNF play direct roles in pain[53,115,116]

2b.vi Treatments

2b.vi.i Disease Modifying Drugs

The definition of disease-modifying medication is a drug that modifies the course of an underlying pathology for the better. Regarding disc degeneration and persistent low back pain, a disease-modifying medicine should delay or prevent disc degeneration's progression. Ideally, a medication would promote disc regeneration; however, this is unlikely due to the low cell population, insufficient nourishment, and low pH values[25,117,118]. The effectiveness of the medication would be measured by its ability to delay or halt the course of deterioration. For instance, this may be described as a reduction in pro-inflammatory cytokines, proteases, and neurotrophins or a slower progression of degeneration as measured by MRI in comparison to a control group. Slowing or halting degeneration might potentially render the disc more amenable to tissue engineering procedures for disc reconstruction, such as intradiscal stem cell treatment. Pro-inflammatory cytokines such as TNF or IL-1, for instance, can function as nociceptive agents and control NGF. Consequently, a medication that addresses the pro-

inflammatory milieu of the disc may alleviate pain. However, as noted above, chronic low back pain might also have a mechanical component that a medication may not be able to treat. A disease-modifying medicine may exist as an inhibitor of a specific target or biological activity or as a biological factor that influences the progression of the illness. Link-N, an N-terminal portion of the Link protein that induces disc cells to create de novo ECM and reduces the negative effects of IL-1, is an example of the latter[119]. Several options for disease-modifying medication targets have been found, and a variety of data exists in favour of or against each.

2b.vi.ii Senolytics

Senolytic drugs are a class of pharmacological agents that exhibit selectivity in their ability to specifically target and eliminate senescent cells from the body[96,120]. While the mode of action and target of some senolytics have been extensively characterized the mechanisms of other senolytics remains to be elucidated. To date, four main groups have been reported which include 1) inhibitors of the Bcl-2 family of apoptosis regulatory proteins, 2) inhibitors of the p53/MDM2 complex that alleviate resistance to apoptosis, 3) HSP-90 and PI3K/Akt inhibitors, releasing pro-apoptotic transcription factors and 4) natural flavonoids with a less clear mode of action[96,120-123].

The drug UBX0101, which has completed both Phase I clinical trials and entered Phase II trials for the treatment of moderate to severely painful knee osteoarthritis (UNITY Biotech) is an inhibitor of the p53/MDM2 complex known to reduce senescent cell burden in cartilage[122,124,125]. Initials results released from the clinical trial suggest that the drug UBX0101 failed to demonstrate less pain than that the placebo control after short-term follow-up[124,125]. Like the drug UBX0101, RG-7112, a senolytic compound which will be discussed at length in this thesis is also a member of the p53/MDM2 complex inhibitors. To date, RG-7112 has been

857 FDA-approved for use in treating acute myeloid leukemia[126]. Given that at high doses, it causes
858 hematological toxicity and its lack of efficiency in treating other cancers, it is not routinely
859 used[127,128]. At low doses however, RG-7112 has been shown to remove senescent IVD cells *in*
860 *vitro* and *ex vivo*[84,129] which suggests that the administration of low doses of this senolytic drug
861 may mitigate the aforementioned adverse effects in individuals undergoing treatment for IVD
862 degeneration.

863 Natural flavonoids such as Fisetin, Quercetin, Piperlongumine, o-Vanillin and
864 Curcumin have been receiving increasing interest for their use as replacements to conventional
865 pharmacotherapy or as adjuncts to senolytics to help reduce the senescent cell burden[78]. This
866 is particularly due to the high costs and negative side effect profiles associated with
867 conventional pharmacotherapy. While many studies have investigated natural flavonoids
868 efficacy in a variety of inflammatory disorders[80,81,122,130] their senolytic activity is unclear. A
869 recent Phase I and Phase II clinical trial in 2020 and 2021 respectively is investigating Fisetin
870 in osteoarthritis, with results projected for 2023. Moreover, o-Vanillin has been shown to
871 effectively kill senescent IVD cells[129,131].

872 Given the promising results associated with senolytics, gaining a better understanding
873 of how senolytic drugs target and remove heterogeneous populations of senescent IVD cells
874 and affect non-senescent cells is required to proceed with the development of new and
875 improved compounds.

876 The failure to target various senescent anti-apoptotic pathways in the same cell type or
877 in different cell types within a target tissue is one disadvantage associated with utilizing a single
878 senolytic drug. Targeting numerous and indirectly linked anti-apoptotic mechanisms at the
879 same time may result in enhanced selectivity for senescent cells while avoiding damage to

normal growing or quiescent cells. The combination of Dasatinib and Quercetin[132] which targets antiapoptotic networks instead of a single target represents the benefits of combination therapy. Combinations enabled lower therapeutic dosages, which reduced negative effects associated with single drug treatment[132]. Phase II clinical trials consisting of Fisetin, Dasatinib and Querceptin commenced in 2020 and are expected to provide results in 2022 and 2023[133]. The benefits of combination therapy is further supported by literature surrounding combination chemotherapy with metformin, a known inhibitor of the SASP which provides prolonged tumor remission[134].

2b.vii Models of Disc Degeneration

Models of disc degeneration vary in species size (from bovine to mouse), ranging from *in vitro* cell culture to *in vivo* disc damage to genetic models. Most big animal models employ *ex vivo* intervertebral disc organ cultures, including bovine caudal discs, bovine discs, and porcine discs[135]. Laboratories with access to human tissue have developed a variety of human disc organ culture systems. Degeneration can be triggered enzymatically, physically, or by pro-inflammatory agents following disc resection. Several rodent models of disc degeneration exist. Although rabbits, rats, and mice have all been utilized, only mice and rats have been utilized extensively for pain and disc degeneration research. The development of degeneration in rat and mouse models may be classified into two groups: mechanical/surgical or genetic.

Several mechanical and surgical rodent models of degeneration exist. A popular approach to cause disc degeneration is by puncturing or stabbing the disc with a needle. There are various versions and procedures for this model, but they often simulate a herniation or annular rupture by producing a severe degeneration of the disc. Some researchers have discovered that a stab wound creates a strong pain phenotype[136,137]. A drawback of the stab or

puncture model is that it simulates an acute injury and thus does not represent the gradual evolution of disc degeneration but rather fast traumatic damage. Another technique is to cause disc degeneration mechanically. For instance, organizations have constructed a rat tail compression model employing a device of the Illizarov type[138]. Looping the tail of a mouse back onto itself for four to twelve weeks similarly produces disc degeneration by asymmetric loading[139]. These mechanical induction models led to a more gradual beginning of degeneration, akin to normal disc degeneration, as compared to puncture models. Rat nucloectomy models are frequently used to represent disc herniation and discomfort as a final surgical model. When the NP of tail discs is removed and positioned or squeezed over the sciatic nerve, a severe pain phenotype results[140,141]. This model is intended to simulate disc herniation and is utilized to investigate pain as opposed to deterioration.

These models each offer several pros and cons. Disc puncture models, for instance, allow researchers to examine patterns of cytokine production, innervation and vascularization, and animal pain response. However, they may not be suitable for studying techniques for tissue repair, progressive degenerative processes, or disease-modifying medications. In a similar manner, tail compression models may provide the investigation of progressive degeneration and tissue regeneration techniques, but they are unsuitable for examining the pain behaviour associated with lumbar disc degeneration. All of these models have unique positive and negative characteristics; consequently, models should be selected according to the study topic.

Several genetic mice models of disc degeneration, including the secreted protein acidic and rich in cysteine (SPARC)-null mouse model utilized in this research, have been produced. SPARC-null mice lack the gene encoding SPARC, also known as Osteonectin. The SPARC-null mice were first created to investigate cataractogensis, but it was subsequently revealed that

they also exhibit progressive disc degeneration[142]. Compared to wild-type mice, SPARC-null animals lose disc height, have histological alterations indicating proteoglycan loss, and develop disc herniations. They also acquire a severe axial and radiating pain phenotype[143-147]. Due to the gradual degeneration of SPARC-null discs, these mice provide a useful and representative model of human disc degeneration. In addition to disc degeneration and impaired gait, and enhanced mechanical sensitivity, Col9a1-null mice also exhibit disc degeneration. Nonetheless, osteoarthritis complicates the interpretation of functional and behavioural data[148]. Utilizing notochordal-cell-specific knockout mice is a tissue-specific method for creating genetic models of disc degeneration[149]. The fact that mouse NP cells are generated from notochordal cells enables NP-specific gene knockout. Utilizing notochordal-specific knockouts, such as CCN2 and HIF-1, in disc degeneration and development have been investigated[150,151]. Notochordal-specific knockout mice permit the study of pain behaviour and pharmacological treatments while attributing effects to the disc. Genetic models have benefits over surgically and mechanically generated degeneration models due to the absence of intrusive procedures. In addition, genetic models are more effective for longitudinal research because disc degeneration can proceed gradually, as in SPARC-null and CCN2 notochordal knockout animals. This may allow researchers to simulate the gradual, long-term nature of human disc degeneration more accurately. Despite their limitations, small rodent models are a helpful tool for advancing our understanding of disc degeneration, and low back pain causes.

3. Rationale

This project will investigate the potential of two different Senolytics (RG-7112 and O-Vanillin) as treatment options for patients suffering from painful IVD degeneration. It is known

that senescent cells accumulate over time in tissue. Previously it was believed that these cells were inactive bystanders, but many studies have shown that these cells secrete factors that drive the remodeling or destruction of the ECM, cause pain, induce fibrosis, and trigger cell death[82,83,121,152]. Moreover, studies for our lab have demonstrated that there is an overall increase in senescent cells in degenerate IVDs[83,84,129]. Senolytics could potentially be used therapeutically to treat painful discs, repair loss of disc height in already degenerate discs, or prophylactically to prevent future degeneration either in individuals at risk or following fusion for adjacent disc disease. Senolytics are not effective in all cell types and have not been evaluated in IVDs. This project will aim to shed light on whether these compounds are a valid treatment option with *in vitro* and *in vivo* models.

4. Hypothesis

We hypothesize that senescent cells contribute to painful IVD degeneration and that their presence prevents spontaneous and induced tissue repair. Moreover, we hypothesize that removing senescent cells will reduce TLR expression and will ultimately decrease the levels of cytokines and proteases.

5. Specific Aims

1. To quantify the potential of o-Vanillin and RG-7112 at reducing cytokines and pain mediators in cells from human IVDs.
2. To determine if there is a link between TLR expression and senescent cells.
3. To determine if RG-7112 and/or o-Vanillin treatment clears senescent IVD cells in SPARC-null mice degenerating IVDs and spinal cords.

4. To determine if senolytic drugs can reduce pain and improve tissue homeostasis in SPARC-null mice *in vivo* using behaviour, molecular and biochemical analyses of treated and non-treated animals.

Chapter 1 Preface:

There is a limited understanding of the early onset and progression of painful IVD degeneration, one of the most common disabilities in young and middle- aged individuals. Although so common and costly, very little is known about the mechanism leading to back pain, and no early treatment options are currently available. As there are no conservative treatment options available, patients presenting with advanced stages of the disease require invasive surgery to relieve pain. Current evidence suggests that changes in the biomechanical properties of degenerating discs are associated with matrix fragmentation, inflammation, and pain. It is, however, less clear how pain and degeneration are initiated and how they could be prevented. The high cost and potential negative side effects of conventional pharmacotherapy have stimulated interest in natural plant products with anti-inflammatory and regenerative properties as an alternative or adjunct to conventional therapy.

o-Vanillin interacts with a variety of cell surface receptors, including TLRs, Vanilloid, Chemokine and Opioid receptors and could potentially broadly reduce the levels of pro-inflammatory mediators and matrix degradation, thus alleviating spinal pain and possibly prevent IVD and facet joint degeneration. Expression of TLR-1, -2, -3, -4, -5, -6, -9 and -10 has been identified in human IVDs and expression of TLR -1, -2, -4, and -6 are increased with the degree of disc degeneration and pain. Overloading of primary chondrocytes and IVD cells can upregulate TLR-2/4 expression, and our own data demonstrate that activating TLR receptors induces IVD degeneration. In addition, we could use a synthetic TLR antagonist to reduce pain but not provide tissue regeneration in a mouse model of back pain. These studies provide a rationale for targeting TLRs to decrease inflammatory processes and modify spine disease. The **overall objective** of this chapter is to evaluate the potential of the senolytic o-

Vanillin in reducing inflammatory mediators and proteases in cells from painful degenerating human IVDs. Having both anti-inflammatory and antioxidant properties in chondrocytes, we **hypothesize** that o-Vanillin will reduce the level of cytokines and proteases present to reduce spinal pain and delay the need for surgical interventions in patients with low back pain. To address this hypothesis, we used cells from painful degenerating IVDs that were obtained from patients undergoing surgery for low back pain to; **1)** quantify the potential of o-Vanillin at reducing cytokines and pain mediators in cells from human IVDs and **2)** determine if there is a link between TLRs and senescent cells. This study was published in 2021 in the journal Arthritis Research and Therapy.

Chapter 1

Title: Toll-Like Receptor 2 Induced Senescence in Intervertebral Disc Cells of Patients with Back Pain can be Attenuated by o-Vanillin

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Abstract

Background: There are an increased level of senescent cells and Toll Like Receptor-1 -2 -4 and -6 (TLR) expression in degenerating intervertebral discs (IVDs) from back pain patients. However, it is currently not known if the increase in expression of TLRs is related to the senescent cells or if it is a more general increase on all cells. It is also not known if TLR activation in IVD cells will induce cell senescence.

Methods: Cells from non-degenerate human IVD were obtained from spine donors and cells from degenerate IVDs came from patients undergoing surgery for low back pain. Gene expression of TLR-1,2,4,6, senescence and senescence-associated secretory phenotype (SASP) markers was evaluated by RT-qPCR in isolated cells. Matrix synthesis was verified with safranin-O staining and Dimethyl-Methylene Blue Assay (DMMB) confirmed proteoglycan content. Protein expression of $p16^{INK4a}$, SASP factors and TLR-2 was evaluated by immunocytochemistry (ICC) and/or by enzyme-linked immunosorbent assay (ELISA).

Results: An increase in senescent cells was found following 48h induction with a TLR-2/6 agonist in cells from both non-degenerate and degenerating human IVDs. Higher levels of SASP factors, TLR-2 gene expression and protein expression was found following 48h induction with TLR-2/6 agonist. Treatment with o-Vanillin reduced the number of senescent cells, and increased matrix synthesis in IVD cells from back pain patients. Treatment with o-Vanillin after induction with TLR-2/6 agonist reduced gene and protein expression of SASP factors and TLR-2. Co-localized staining of $p16^{INK4a}$ and TLR-2 demonstrated that senescent cells have a high TLR-2 expression.

Conclusions: Taken together our data demonstrate that activation of TLR-2/6 induce senescence and increase TLR-2 and SASP expression in cells from non-degenerate IVDs of organ donors without degeneration and backpain and in cells from degenerating human IVD of patients with disc degeneration and backpain. The senescent cells showed high TLR-2 expression suggesting a link between TLR activation and cell senescence in human IVD cells. The reduction in senescence, SASP and TLR-2 expression suggest o-Vanillin as a potential disease modifying drug for patients with disc degeneration and backpain.

Key Words: intervertebral disc; senescence; toll-like receptor 2; o-Vanillin; degeneration; inflammation; senolytics; back pain

Background

Low back pain is a global health problem that has been associated with intervertebral disc (IVD) degeneration [153-155]. It is experienced by approximately 80% of individuals at some time in their lifespan [156]. Globally back pain is the number one cause of years lived with disability [156]. The personal costs in reduced quality of life, as well as the economic cost to healthcare systems are enormous and exceeds $100 billion per year in the US alone [157]. Current evidence suggests that changes in the biomechanical properties of degenerating discs is associated with matrix fragmentation, inflammation and pain [158]. However, it is less clear how pain and degeneration are initiated and how they could be prevented. There is a growing interest in the accumulation of senescent cells in degenerating and ageing tissues. These senescent cells are viable cells that can no longer divide. Senescence can be induced due to the successive shortening of telomere length during replicative cycles [78]. In addition, the number of senescent cells can also be increased by stressors including DNA damaging agents, oxidative stress, mitochondrial dysfunction, load induced injury and disruption of epigenetic regulation. This phenomenon is called stress-induced premature senescence and it is believed to be linked to the accumulation of senescent cell in degenerate IVDs [80,81]. Furthermore, senescent cells release an array of inflammatory cytokines, chemokines, and proteases known collectively as the senescence-associated secretory phenotype (SASP) [63].

All senescent cells have common features, but they also possess distinct characteristics which are linked to the different types of senescence (replicative & stress-induced senescence), cell and tissue types [89,90]. The inflammatory environment triggered by senescent cells prevents adjacent cells from maintaining tissue homeostasis [86,87] and it is proposed to induce senescence in a paracrine manner thus exacerbating tissue deterioration [88]. Currently, conventional

pharmacotherapy for IVD degeneration has both a high cost and many potential negative side effects, which has stimulated the interest in natural plant-based products with anti-inflammatory and regenerative properties, as an alternative or adjunct to conventional therapy. These products are being investigated for potential efficacy in a wide range of disorders with an inflammatory component, including osteoarthritis and cancer [159,160]. Recently, there have been a number of synthetic and natural drugs described with a specific mode of action to target and remove senescent cells, referred to as senolytics [84,129].

Senolytics target many different pathways such as interfering with the dependence receptors, which promote apoptosis when unoccupied by ligands. Targeting and blocking signaling pathways involved in cell survival regulation interferes with mitochondrial-dependent apoptosis [122]. One natural senolytic, o-Vanillin, a metabolite of a Curcumin, has anti-inflammatory properties and potent senolytic activity with a very wide non-toxic window for non-senescent IVD cells [84]. Treatment with o-Vanillin has previously been shown to increase proteoglycan production of nucleus pulposus (NP) cells pellet culture [84]. Furthermore, o-Vanillin interacts with a variety of cell surface receptors including Toll-like Receptors (TLR), Vanilloid, Chemokine and Opioid receptors and could broadly reduce the levels of pro-inflammatory mediators and reduce matrix degradation, possibly preventing IVD degeneration [161,162].

In human IVD's there is expression of TLR-1, -2, -3, -4, -5, -6, -9 and -10 and the expression of TLR-1, -2, -4, and -6 are increased with the degree of disc degeneration and pain [76]. Overloading of the intact disc and IVD cells can upregulate TLR-2 and -4 expression, and previous data from our lab demonstrates that activating TLR receptors with the synthetic agonists (PAM2csk4, TLR-2/6 agonist) induced IVD degeneration [19,76,163]. Furthermore,

studies using multiple cell lines proposed that TLR activation is associated with the induction of senescent cells and SASP factor release [164-166].

The present study investigates a possible link between the increase of TLRs and senescent cells in degenerate IVDs from patients undergoing surgery for low back pain. We show that a TLR-2/6 agonist increased the number of senescent cells from non-degenerate IVDs and in cells from degenerate IVDs. As well, we describe that TLR-2 has the highest expression and co-localization with senescent cells from degenerate IVDs from patients undergoing surgery for low back pain. Furthermore, treatment with o-Vanillin reduced the number of cells co-localized for TLR-2 and senescence markers. From this study, we propose that TLR-2 has a role in the increase of senescent cells found in degenerating IVDs and that o-Vanillin's senolytic and anti-inflammatory activity could be a disease modifying pharmaceutical for low back pain.

Methods:

Tissue collection and cell isolation

All procedures performed were approved by the ethical review board at McGill University (IRB#s A04-M53-08B and A10-M113-13B). Non-degenerate IVDs from humans with no history of back pain were obtained through a collaboration with Transplant Quebec. Degenerate IVDs were obtained from patients with chronic low back pain that received discectomies to alleviate pain. Donor information is presented in **Supplementary Table 1**. IVD cells were isolated, as previously described [20]. Briefly, samples were washed in phosphate-buffered saline solution (PBS, Sigma-Aldrich, Oakville, ON, Canada) and Hank's-buffered saline solution (HBSS, Sigma-Aldrich, Oakville, ON, Canada) supplemented with

Primocin™ (InvivoGen, San Diego, CA, USA) and Fungiozone (Sigma-Aldrich, Oakville, ON, Canada). Then, the matrix was minced and digested in 0.15% collagenase type II (Gibco) for 16 hours at 37°C. Cells were passed through both a 100-µm filter and 70-µm filter, before being re-suspended in Dulbecco's Modified Eagle Media (DMEM, Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% fetal bovine serum (FBS, Gibco), Primocin™, Glutamax (Oakville, ON, Canada), and maintained in a 5% CO_2 incubator at 37 °C.

***In Vitro* Cell Culture and Treatment**

Monolayer culture: Experiments were performed with NP cells from non-degenerate IVDs and degenerate IVDs (NP and AF cells) within passage 1 to 2. 20,000 cells were seeded in 8-well chamber slides (Nunc™ Lab-Tek™ II Chamber Slide™ System) for immunocytochemistry experiments following treatment. 300,000 cells were seeded in 6-well plates (Sarstedt, TC plate 6-well, Cell+, F) for ELISA and RNA extraction following treatment. All cells were left to adhere for 12 to 24 hours and then serum-starved in DMEM with 1X insulin-transferrin selenium (ITS, Thermo Fisher, Waltham, MA, USA) for 6 hours prior to treatment. To examine the effects of different treatments, healthy cells were treated with either 100 ng/ml Pam2CSK4 (TLR-2/6 agonist, Invivogen), 100 ng/mL Pam3CSK4 (TLR-1/2 agonist, Invivogen) or 5µg/mL lipopolysaccharide (LPS) (TLR-4 agonist, Invivogen) for 6, 12, 24 and 48 hours. Cells were either left untreated (negative control) or treated with 100 ng/mL of Pam2CSK4 for 48 hours of which treatment with 100 µM o-Vanillin (Sigma-Aldrich, Oakville, ON, Canada) was initiated in the last 6 hours of incubation [76,84,111].

Pellet culture: 300,000 cells/tube were collected by centrifugation at 1500 rpm for 5 minutes. Pellets were incubated in 1mL DMEM, 2.25g/L glucose (Sigma-Aldrich, Oakville, ON, Canada), 5% FBS, 5 µM ascorbic acid, 1% GlutaMAX, 0.5% Gentamicin (Thermo Fisher,

Waltham, MA, USA) at 37°C and 5% CO_2. Pellets were left in DMEM for four days to form and stabilize (in pretreatment media) and then treated with 100 μM o-Vanillin (Sigma-Aldrich, Oakville, ON, Canada) for four days, meanwhile pellets in the control group stayed in DMEM with vehicle 0.01% DMSO (Sigma-Aldrich, Oakville, ON, Canada). Following the treatment period, pellets from both groups were cultured for 21 days and their culture media was collected every 4 days and pooled as post-treatment media.

Immunofluorescence

Monolayer cultures (20,000 cells/well in 8-well chambered slide) were washed with PBS, fixed with 4% paraformaldehyde (Thermo Fisher, Waltham, MA, USA), and blocked in PBS with 1% BSA (Sigma-Aldrich, Oakville, ON, Canada), 1% goat serum, and 0.1% Triton X-100 (Sigma-Aldrich, Oakville, ON, Canada) for 1 hour. Pam2CSK4 treated cells were stained with primary antibodies specific to NGF (Santa Cruz, Dallas, Tx, USA), $p16^{INK4a}$ (Cintec-Roche, Laval, Qc, CAN), IL1β, TNF-α, IL8 and TLR-2 (Abcam, Cambridge, Ma, USA) overnight at 4 °C. Healthy cells were treated with $p16^{INK4a}$ and TLR-2 only. After washing, cells were incubated with the appropriate Alexa Fluor® 488 or 594-conjugated secondary antibody (Thermo Fisher, Waltham, MA, USA) for 2 hours at room temperature, and then counterstained with DAPI for nuclear staining. Photomicrographs were acquired with a fluorescent Olympus BX51 microscope equipped with an Olympus DP71 digital camera (Olympus, Tokyo, Japan). Ten images of each condition per donor were analyzed and positive cell percentage was quantified by Fiji ImageJ (version: 2.1.0/1.53c). Briefly, the number of cells stained positive for one of the target proteins (NGF, IL-1β, TNF-α, and IL-8) were counted and compared to the total number of cells positive for DAPI staining. For the double staining (TLR-2 and $p16^{INK4a}$) the percentage of positive cells represents the ratio of the number of cells

positively stained for either one of the 2 markers (TLR-2 and $p16^{INK4a}$) divided by the total number of cells positively stained for DAPI.

Immunohistochemistry

Safranin-O staining: Pellet culture samples were heated on an iron heater at 50°C for 30 minutes and rehydrated with PBS. Samples were stained with 0.1% Safranin-O (Sigma-Aldrich, Oakville, ON, Canada) for 5 minutes at room temperature and rinsed with water, 75% ethanol (15s), and 95% ethanol (15s). Coverslips were mounted with Permount™ Mounting Medium (Fisher Scientific). Samples were imaged with Olympus DP70 digital camera (Olympus) pre-fixed to a Leica microscope (Leica DMRB) under visible light.

$p16^{INK4a}$ staining: $p16^{INK4a}$ staining was performed for both monolayer cultures and pellet samples. Only the pellet samples were heated on an iron heater at 50°C for 30 minutes and rehydrated by PBS-T (0.1% Triton X-100) for 10 minutes. Both healthy monolayer cultures and pellet samples were blocked with hydrogen peroxide for 10 minutes, washed three times, and saturated with 1% BSA, 1% goat serum, and 0.1% Triton X-100 for 10 minutes. All samples were incubated at 4°C overnight for $p16^{INK4a}$ antibody (CINTec Kit, Roche) and PBS-T for negative control. The HRP/DAB Detection IHC Kit (Abcam, ab64264) was used for detection. Counting staining was applied with Meyer's hematoxylin (Sigma-Aldrich, Oakville, ON, Canada) for 2 minutes. Samples were rinsed with water (30s), 75% ethanol (15s), and 95% ethanol (15s) afterwards and coverslips were mounted with Permount™ Mounting Medium (Fisher Scientific). Images were captured as described [84] for Safranin-O staining, and analyzed with Fiji Image J (version 2.1.0/1.53c).

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA was extracted using the TRIzol chloroform extraction method previously described [167]. 500 ng of RNA was then reverse transcribed using a qScript cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA) with an Applied Biosystems Verti Thermocycler (Thermo Fisher, Waltham, MA, USA). RT-qPCR was performed using an Applied Biosystems StepOnePlus machine (Thermo Fisher, Waltham, MA, USA) with PerfecCTa SYBR Green Fast Mix (Quanta Biosciences, Beverly, MA, USA). Primer sequences for TLRs, senescent markers, pain and inflammatory markers (IL-6, IL-8, $p16^{INK4a}$, p21, TNF-α, CXCL-10, CXCL-1, GM-CSF, TGF-β, CCL-2, CCL-5, CCL-7, CCL-8, NGF, BDNF, IL-8, TLR-1,2,4,6) and the housekeeping gene (GAPDH) can be found in **Supplementary Table 2**. All reactions were conducted in technical triplicate, and fold-changes in gene expression were calculated by using the $2^{-\Delta\Delta Ct}$method, after normalizing to actin and non-treated samples [168].

Protein analysis

To determine the concentration of NGF, IVD cells were cultured in monolayer (250,000 cells/sample) and then lysed using 300 μL of Cell Lysis buffer (RayBiotech, Norcoss, GA, USA). Cell lysates were incubated for 48 hours at room temperature and protein concentrations were determined using ELISA kits, according to the manufacturer's instructions (RayBiotech, Norcoss, GA, USA). Cell culture media from degenerate IVD cells cultured in monolayer and in pellets was used to assess the concentrations of IL-6, IL-8, IL-1β and TNF-α. 150 μL of monolayer culture media and pellet pre-treated and pooled post-treated media was used. ELISAs were performed as per the manufacturer's instructions (RayBiotech, Norcoss, GA, USA). Colorimetric absorbance was measured with a Tecan Infinite M200 PRO (Tecan, Männedorf, Switzerland) spectrophotometer and analyzed with i-control 1.9 Magellan software

(Tecan, Männedorf, Switzerland). Protein levels of the treated conditions and controls were then compared.

Dimethylmethylene Blue Assay

Dimethylmethylene Blue (DMMB) assays were conducted as previously described [84] to quantify sulfated glycosaminoglycans (sGAG) in the conditioned media of IVD pellets with or without o-Vanillin treatment. Chondroitin sulfate was used to generate the standard curve. Pooled post-treatment media samples from treated and untreated pellets were used. All samples were ensured to fall into the linear portion of the standard curve. Each sample was placed in triplicate into clear 96-well plates (Costar, Corning, NY, USA). DMMB dye was then added to the wells. The absorbance was measured immediately at room temperature using Tecan Infinite T200 spectrophotometer (Mannedorf, Switzerland).

Statistical analysis:

Data were analyzed using Graph Prism 8 (Graph Pad, La Jolla, CA, USA). Analysis was performed using a two-tailed Student's t-test or Two-way ANOVA. Specific tests are indicated in the figure legends with the corrections. A p-value <0.05 was considered statistically significant. Data are presented as mean ± SD.

Results

Cell activation with a TLR-2/6 agonist, caused an increase in the number of senescent cells and SASP factor release in cells from non-degenerate human IVDs

We have previously reported that TLR expression and the number of senescent cells are positively correlated with level of IVD degeneration [76,84]. Moreover, we have seen that treating cells from degenerating human IVDs with TLR agonists PAM2CSK4 (TLR-2/6), PAM3csk4 (TLR-1/2) and LPS (TLR-4) increased expression of pain mediators and pro-inflammatory cytokines when compared to vehicle control [76]. Here we aimed to determine the effect and the relation of TLR activation and cell senescence in human IVD cells from non-degenerate IVDs. Monolayer cultures were treated with TLR agonists activating TLR-2/6, 1/2 and 4 for 6, 12, 24 and 48 hours. $p16^{INK4a}$ was used to identify senescent cells and the number of senescent cells were significantly increased following TLR-2/6 activation with a 11% ± 1.732 increase at 24 hours ($p < 0.001$) and a 22.67% ± 4.163 ($p < 0.0001$) increase at 48 hours (**Fig.1A-B**). Furthermore, using RT-qPCR the gene expression levels of TLRs, senescent markers and SASP factors were evaluated in the treated cell pellets. The expression of the TLR-1,2,4,6 in pellets treated with TLR agonists for 48 hours was investigated. A significant increase (7.24-fold ± 3.458, $p < 0.001$) in expression of the TLR-2 in the cells exposed to the TLR-2/6 agonist was observed when normalized to the untreated control. Significance was not reached for TLR-1 ($p = 0.9995$), TLR-4 ($p = 0.9974$) and TLR-6 ($p = 0.1080$) (**Fig. 1C**). Moreover, a significant increase (2.56-fold ± 0.288, $p < 0.001$) of $p16^{INK4a}$ gene expression only in cells exposed to the TLR-2/6 agonist when compared to control was observed. Of note, no significant difference was found in p21 gene expression following exposure to TLR-2/6 agonist ($p = 0.8557$) (**Fig. 1D**). When evaluating SASP factors after 48 hours of exposure to the TLR agonists, the most

significant increase was observed following TLR-2/6 exposure (**Fig. 1E**). Comparing to the control, an increase in CCL2 (12.70 -fold ± 2.541, $p < 0.05$), CCL5 (55.11- fold ± 2.696, $p < 0.01$), CCL7 (13.78-fold ± 2.440, $p < 0.05$), CCL8 (12.68-fold ± 1.534, $p < 0.05$), IL-6 (1079.12-fold ±43.135, $p < 0.01$), IL-8 (1890.28-fold ± 85.617, $p < 0.01$), TNF-α (5.30-fold ± 0.214, $p < 0.01$), NGF (3.49-fold ± 0.250, $p < 0.05$) and BDNF (3.31-fold ± 0.051, $p < 0.01$) was seen following TLR-2/6 activation (**Fig. 1E**). Altogether, these results validate that activation of TLR-2/6 increases both the number of senescent cells and SASP factors produced in cells from non-degenerate IVDs.

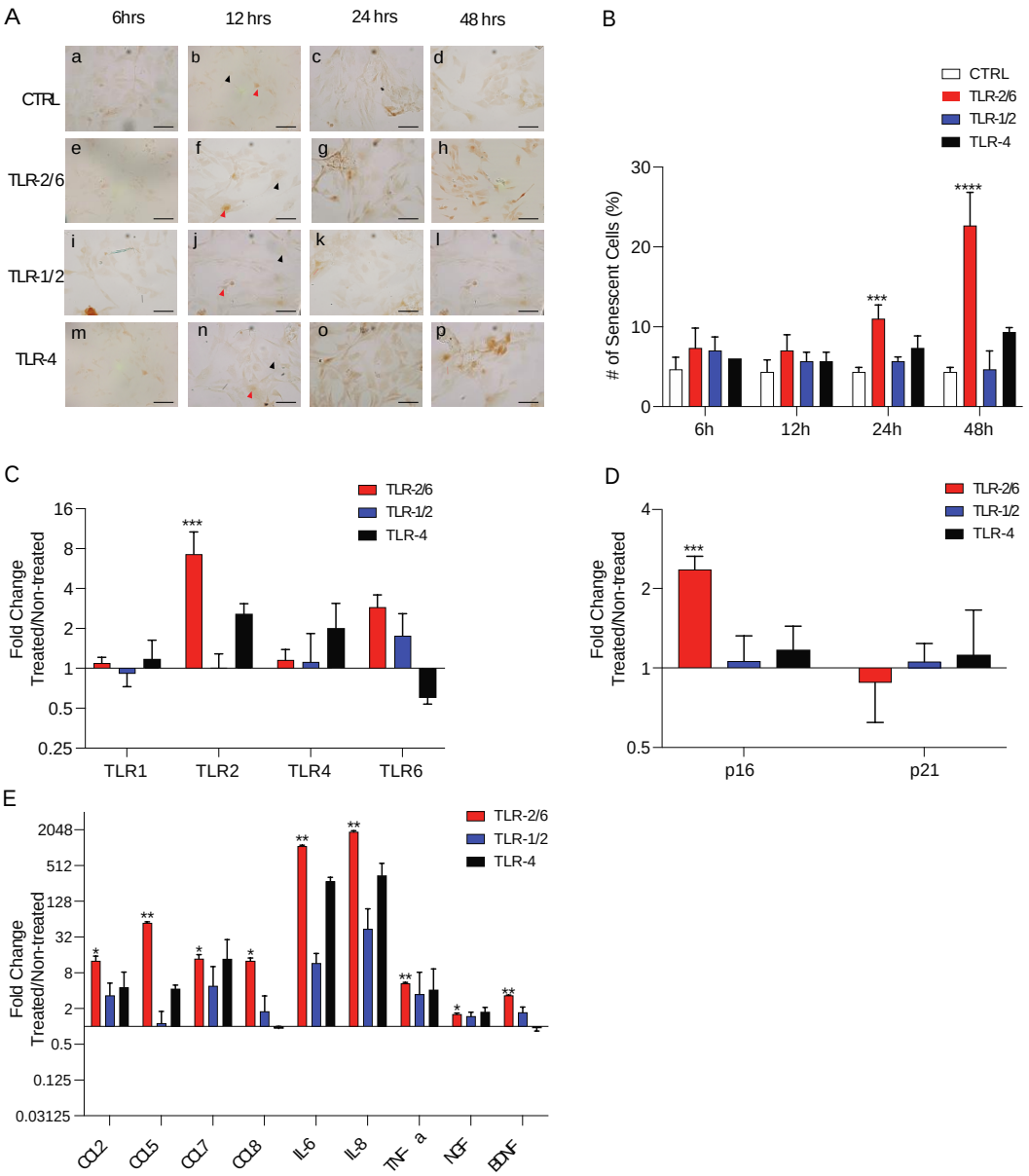


Figure 1: Cell activation with a TLR-2/6 agonist, caused an increase in the number of senescent cells and SASP factor release in cells from non-degenerate human IVDs. (A) $p16^{INK4a}$ immunostaining images of untreated cells (**a-d**) from healthy donor IVDs or treated with either TLR-2/6 agonist (**e-h**), TLR-1/2 agonist (**i-l**) or TLR-4 agonist (**m-p**) cultured for 6, 12, 24 or 48 hours. Scale Bars= 25µm. **(B)** Quantification of the percentage of $p16^{INK4a}$ positive cells in the control and treated cells for the four-time points. Examples of positive cells are indicated by the red arrow and negative cells by the black arrow. **(C, D, E)** qPCR was performed using cells from healthy donor IVDs cultured for 48 hours with TLR-2/6 agonist, TLR-1/2 agonist or TLR-4 agonist. Gene expression for **(C)** TLR-1, -2, -4 and -6), **(D)** senescence markers $p16^{INK4a}$ and p21 and **(E)** SASP factors (CCL2, CCL5, CCL7, CCL8, IL-6, IL-8, TNF-α, NGF and BDNF). Fold changes were normalized relative to the non-treated control (CTRL). **(B-E)** Two-way ANOVA with Tukey's multiple comparisons test, n = 5,

significance was evaluated between treated groups compared to the control group. Data shown as mean ± SD, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

O-Vanillin reduced the number of senescent cells and enhanced proteoglycan production in cell pellet cultures from degenerate IVDs

O-Vanillin senolytic activity and its effect on matrix production have never been assessed on cells from patients with back pain and degenerating IVDs. Here we evaluated o-Vanillin's senolytic activity in 3D pellet cultures of IVD cells in back pain patients. The pellet cultures were treated with o-Vanillin (100μM) or vehicle (DMSO 0.01%) for 4 days. At the end of the treatment period, the pellets were maintained in standard culture media for 21 days, with the post-treatment media collection occurring every 4 days. The senolytic activity was evaluated by immunostaining for the senescence marker $p16^{INK4a}$ **(Fig. 2A)**. The percentage of $p16^{INK4a}$ positive cells decreased significantly from 14.66% ± 2.758 in the untreated control to 6.38% ± 0.4973 in the o-Vanillin treated pellets ($p < 0.05$) (**Fig. 2B**). Safranin-O staining was used to evaluate proteoglycan content. A more intense red staining was observed, indicating higher proteoglycan content in the o-Vanillin treated IVD cell pellets compared to the control sample (**Fig. 2C**). Furthermore, a DMMB assay was performed to assess sGAG content in the culture media[169]. Pooled media from all post-treatment time points in the o-Vanillin treated cell pellets (1.62 μg/ml ± 0.4134) had significantly higher sGAG content than the untreated control pellets (0.33μg/ml ± 0.2876) ($p < 0.05$) (**Fig. 2D**). We then evaluated o-Vanillin's ability to reduce SASP factors (IL-1β, IL-8, IL-6 and TNF-α) that are commonly produced by senescent IVD cells [84]. Using ELISA immunoassay, we compared the percent difference of the pooled post-treated media over the pre-treated media. A significant decrease was observed in all evaluated SASP factors measured in the media of o-Vanillin treated compared to the untreated

controls. The percentage of difference in post compared to pre-treatment and measured in o-Vanillin and control groups were respectively for IL-1β (13.75 % ± 3.473 vs 30.63 % ± 3.279, $p < 0.01$), IL-8 (38.38 % ± 12.903 vs 61.5 % ± 18.821, $p < 0.05$), IL-6 (13.38 % ± 5.867 vs 25.75 % ± 1.652, $p < 0.05$) and TNF-α (19.38 % ± 0.408 vs 46 % ± 0.750, $p < 0.0001$) (**Fig.2E**).

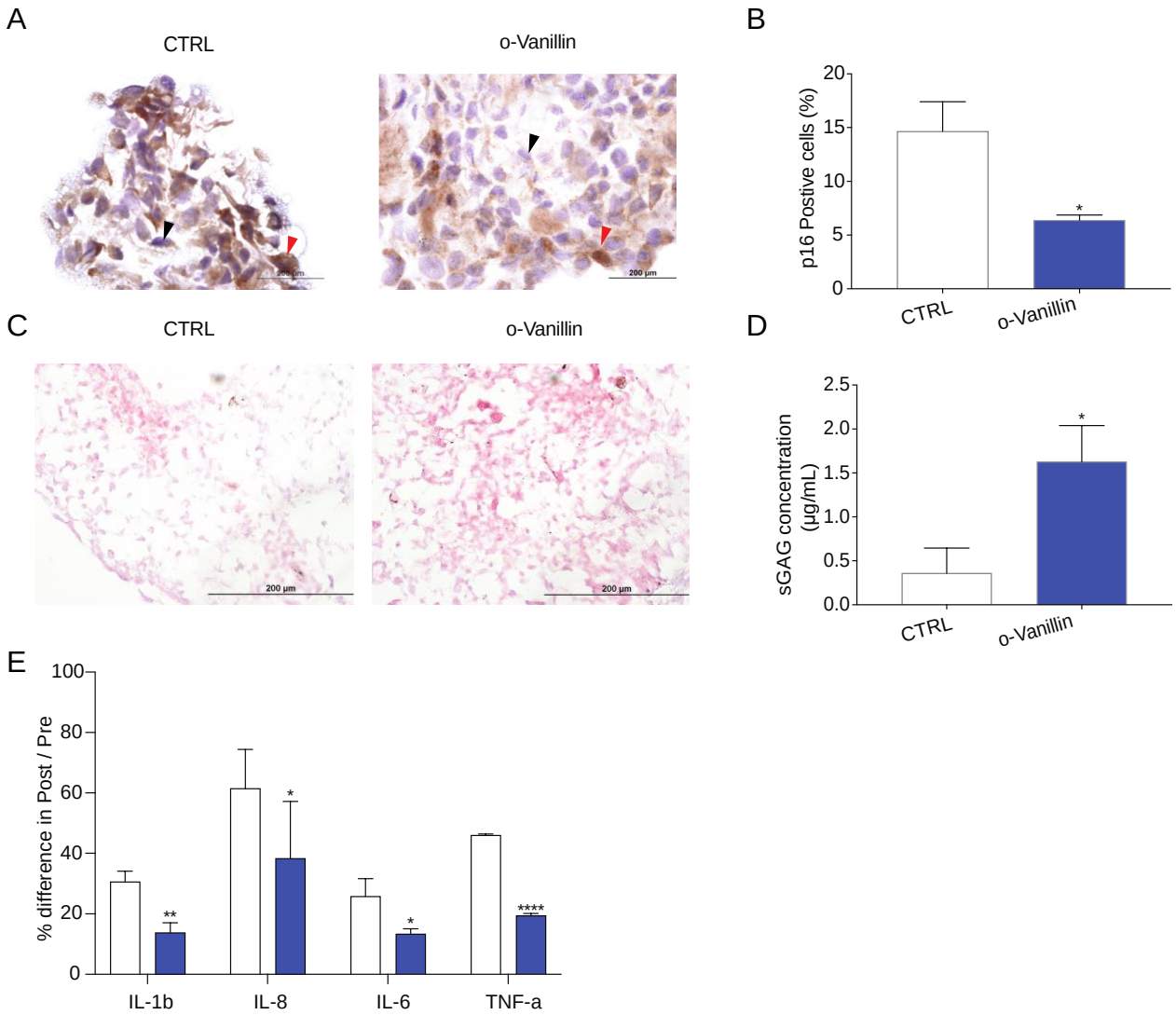


Figure 2: o-Vanillin reduced the number of senescent cells and enhanced proteoglycan production in cell pellet cultures from degenerate IVDs. All experiments were performed on day 21. **(A)** Representative photomicrographs of $p16^{INK4a}$ immunohistochemistry staining in pellet cultures from degenerate IVD cells either treated with o-Vanillin or not (CTRL). Examples of positive cell are indicated by the red arrow and for negative cells by the black arrow. **(B)** Quantification of the percentage of $p16^{INK4a}$ positive cells in the pellet cultures. n=3

(C) Images for Safranin O staining for proteoglycan content in CTRL and o-Vanillin cell pellet cultures from degenerate IVD. **(D)** sGAG concentration measured using DMMB assay in the culture media of CTRL and o-Vanillin cell pellets. n=5 **(E)** Percentage of change in concentration of IL-1β, IL-8, IL-6 and TNF-α in pellet culture media from untreated and o-Vanillin treated degenerate IVD cells measured by ELISAs. Percent difference was evaluated by normalizing the post-treated media to the pretreated media. n=5. *p<0.05, **p<0.01, ****p<0.0001. (B, D, E) Mean ± SD, Statistical analysis was done using paired t-test.

o-Vanillin reduced gene expression of p16, TLR-2 and SASP factors following TLR-2 activation in IVD cells from patients with back pain and IVD degeneration

IVD cells from patients with back pain and IVD degeneration were exposed the TLR-2/6 agonist for 48 hours in the presence or absence of o-Vanillin (100μM) during the last 6 hours of the treatment. We first assessed gene expression of senescence markers $p16^{INK4a}$ and p21. Similar to the effect observed in cells from non-degenerate IVDs, there was a significant increase in $p16^{INK4a}$ expression (3.83-fold ± 1.055, $p < 0.001$) and no significant difference in the expression of p21($p = 0.4279$) following TLR-2/6 exposure when compared to the untreated control (**Fig. 3A**). Interestingly, treatment with o-Vanillin significantly decreased $p16^{INK4a}$ expression (1.07-fold ± 0.308, $p < 0.001$) while no significant change was found for p21 expression ($p = 0.244$) (**Fig. 3A**). When assessing TLR expression in the cells from patients with back pain and IVD degeneration, a significant increase in TLR-2 gene expression (9.17-fold ± 1.594, $p < 0.001$) following TLR-2/6 exposure, compared to the control was seen. However, there was no significant increase in TLR-1 ($p = 0.2420$), TLR-4 ($p = 0.9985$) or TLR-6 ($p = 0.3491$) (**Fig. 3B**). These samples, when treated with o-Vanillin showed a significant decrease (1.67-fold ± 0.565, $p < 0.001$) in TLR-2 expression (**Fig. 3B**). Exposure of IVD cells from patients with back pain and IVD degeneration to TLR-2/6 agonist significantly increased the expression of SASP factors CCL2 (42.32-fold ± 11.337, $p < 0.001$), CCL5 (49.03-fold ±

11.487, $p < 0.001$), CCL7 (9.30-fold ± 1.430, $p < 0.01$), CCL8 (28.40-fold ± 4.936, $p < 0.001$), GM-CSF (118.55-fold ± 10.067, $p < 0.001$), BDNF (1.77-fold ± 0.126, $p < 0.01$), NGF (2.75-fold ± 0.586, $p < 0.01$), TNF-α (7.36-fold ± 2.361, $p < 0.001$), CLCX1 (594.16-fold ± 44.718, $p < 0.001$), IL-8 (594.5-fold ± 98.644, $p < 0.001$) and CLCX10 (745.23-fold ± 107.787, $p < 0.001$) when compared to the untreated control (**Fig. 3C**). o-Vanillin significantly reduced this increase, CCL2 (5.89-fold ± 2.075, $p < 0.001$), CCL5 (6.21-fold ± 2.156, $p < 0.001$), CCL7 (3.51-fold ± 1.521, 0.001), CCL8 (2.8-fold ± 2.281, $p < 0.01$), GM-CSF (6.33-fold ± 2.39, $p < 0.001$), BDNF (0.85-fold ± 0.368, $p < 0.01$), NGF (0.62-fold ± 0.135, $p < 0.01$), TNF-α (1.09-fold ± 0.656, $p < 0.001$), CLCX1 (24.67-fold ± 5.132, $p < 0.01$), IL-8 (75.49-fold ± 18.608, $p < 0.01$) and CLCX10 (10.8-fold ± 3.087, $p < 0.001$) (**Fig. 3C**).

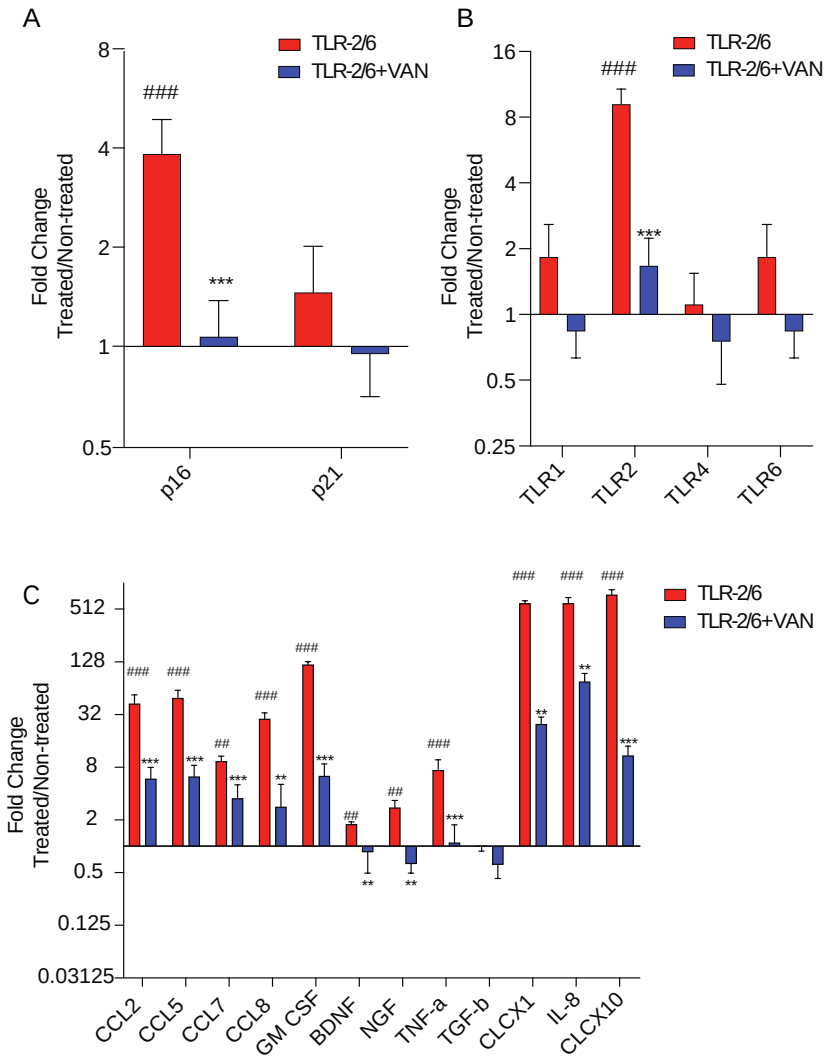
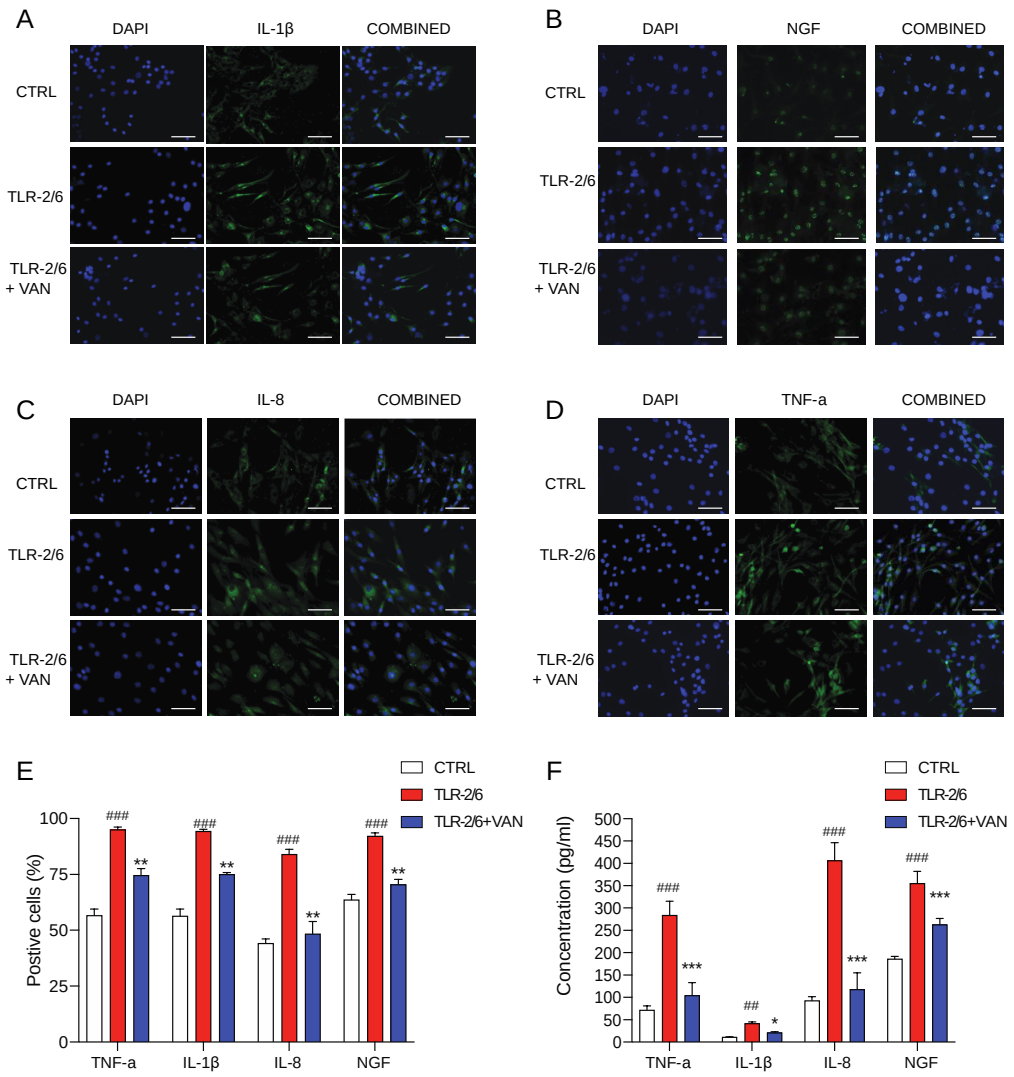


Figure 3: o-Vanillin reduced gene expression of p16, TLR-2 and SASP factors following TLR-2 activation in IVD cells from patients with back pain and IVD degeneration. (A-C) Gene expression of **(A)** senescence markers $p16^{INK4a}$ and p21, **(B)** TLR-1, -2, -4 and -6 and **(C)** SASP factors (CCL2, CCL5, CCL7, CCL8, GM CSF, BDNF, NGF, TNF-α, TGF-β, CLCX1, CLCX8, CLCX10) of disc cells from degenerate IVDs cultured for 48 hours with TLR-2/6 agonist with o-Vanillin (TLR-2/6 + VAN) or without o-Vanillin (TLR-2/6) treatment for 6h or no induction with TLR-2/6. Fold changes were normalized relative to non-induced control. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ indicate significant difference between the TLR-2/6 treated to the non-induced control and *p<0.05, **p<0.01, ***p<0.001 indicate significant difference between groups TLR-2/6 + VAN and TLR-2/6. **(A-C)** Mean ± SD, measured by Two-way ANOVA with Sidak's multiple comparisons test. Values are expressed in average fold change for n = 5.

o-Vanillin reduced the protein expression of SASP factors (IL-1β, NGF, IL-8 and TNF-α) following TLR-2/6 activation of IVD cells from patients with back pain and IVD degeneration

Protein expression of (TNF-α, IL-1β, IL-8 and NGF) was evaluated by immunohistochemistry following a 48 hrs exposure to TLR-2/6 agonist. (**Fig. 4A-D**). Quantification of the percentage of positive cells for each SASP factor was compared to untreated controls. A significant increase of TNF-α (56.6% ± 2.881 vs 95% ± 1.155, $p < 0.001$), IL-1β (56.4% ± 3.050 vs 94.25% ± 0.957, $p < 0.001$), IL-8 (44.2% ± 1.924 vs 84% ± 2.236, $p < 0.001$) and NGF (63.6% ± 2.408 vs 92.15% ± 1.388, $p < 0.001$) positive cells was observed (**Fig. 4E**). When evaluating the effect of o-Vanillin, a significant decrease was observed in protein expression in the treated samples for TNF-α (74.5% ± 3.109, $p < 0.01$), IL-1β (75% ± 0.816, $p < 0.01$), IL-8 (48.4% ± 5.550, $p < 0.01$) and NGF (70.46% ± 2.416, $p < 0.01$) (**Fig. 4E**). Additionally, to measure the concentrations of SASP factors affected by TLR-2/6 activation and o-Vanillin treatment we performed ELISAs immunoassay of the culture media for TNF-α, IL-1β, IL-8 and on the cell lysate for NGF. A significant increase was found in the SASP factors in the culture media following TLR-2/6 activation, TNF-α (72.03 pg/ml ± 9.044 vs 284.25pg/ml ± 30.972, $p < 0.001$), IL-1β (10.97 pg/ml ± 1.09 vs 42.11 pg/ml ± 3.022, $p < 0.01$), IL-8 (92.95 pg/ml ± 8.385 vs 406.25 pg/ml ± 39.891, $p < 0.001$) and in the cell lysate for NGF (186 pg/ml ± 5.957 vs 355.03 pg/ml ± 27.086, $p < 0.001$) when compared to the control and that this induction was significantly decreased for all evaluated SASP factors following treatment with o-Vanillin (TNF-α: 104.5 pg/ml ± 28.831($p < 0.001$), IL-1β: 21.15 pg/ml ± 2.123 ($p < 0.05$), IL-8: 117.8 pg/ml ± 36.944 ($p < 0.001$) and NGF: 262.83 pg/ml ± 5.208 ($p < 0.001$)) (**Fig. 4F**).



1445

1446 **Figure 4: o-Vanillin reduced the protein expression of SASP factors (IL-1β, NGF, IL-8**
1447 **and TNF-α) following TLR-2/6 activation of IVD cells from patients with back pain and**
1448 **IVD degeneration.** Disc cells from degenerate IVD were cultured for 48 hours with TLR-2/6
1449 with o-Vanillin (TLR-2/6 + VAN) or without o-Vanillin (TLR-2/6) treatment for 6h or no
1450 induction with TLR-2/6 (CTRL). **(A-D)** Using Immunocytochemistry, untreated and treated
1451 IVD cells were stained for DAPI and either **(A)** IL-1β, **(B)** NGF, **(C)** IL-8 **(D)** TNF-α
1452 respectively. Scale Bars= 25μm. **(E)** Quantification of the percentage of the cells that stained
1453 positive for IL-1β, NGF, IL-8 and TNF-α when non-induced, treated with TLR-2/6 agonist or
1454 treated with TLR-2/6 agonist and o-Vanillin. n=5 **(F)** ELISAs were performed to measure the
1455 concentration of TNF-α, IL-1β and IL-8 in cell media and NGF from cell lysate in non-induced,
1456 TLR-2/6 agonist treated or the combined treatment TLR-2/6 agonist and o-Vanillin treated
1457 samples. Percentage of positive cells in **(E)** were the average for n = 5. $^{\#}p<0.05$, $^{\#\#}p<0.01$,
1458 $^{\#\#\#}p<0.001$ indicate a significant difference between the TLR-2/6 agonist treated to the non-

induced control and *p<0.05, **p<0.01, ***p<0.001 indicate a significant difference between the tested groups (**E-F**) Mean ± SD, statistical analysis was done using paired t-test.

o-Vanillin reduced the number of cells co-expressing TLR-2 and $p16^{INK4a}$ in cells exposed to TLR-2/6 agonist

Based on our findings that exposure to TLR-2/6 agonist caused a significant increase in $p16^{INK4a}$ and TLR-2 gene expression in both cell and pellet cultures from non-degenerate and degenerate IVDs, we investigated the possibility that senescent IVD cells have an elevated TLR-2 expression. Protein expression of TLR-2 and $p16^{INK4a}$ was assessed by immunohistochemistry in IVD cells from patients with back pain and IVD degeneration following a 48 hrs exposure to TLR-2/6 agonist. (**Fig. 5A**). Quantification of TLR-2 and $p16^{INK4a}$ was done by measuring the percent of cells positive from the total cell population for the two markers. Following TLR-2/6 activation, it was found that there was a significant increase in the expression of TLR-2 (53.17% ± 8.684, $p < 0.001$) and $p16^{INK4a}$ (47.19% ± 7.951, $p < 0.001$) when compared to untreated controls; TLR-2 (29.92% ± 9.448) and $p16^{INK4a}$ (25.95% ± 6.071) (**Fig. 5B-C**). Furthermore, treatment with o-Vanillin for the final 6 hrs significantly reduced this increase for TLR-2 (36.3% ± 8.057, $p < 0.001$) and $p16^{INK4a}$ (31.07% ± 3.854, $p < 0.001$) (**Fig. 5B-C**). Finally, to verify the link between TLR-2 and cell senescence in IVD cells, we assessed the percentage of cells co-expressing $p16^{INK4a}$ and TLR-2 by determining the percentage of senescent cells ($p16^{INK4a}$ positive cell) that express TLR-2. In the untreated control, 26% ± 1.611 of the senescent cells expressed TLR-2 while following TLR-2/6 exposure, the percentage of senescent cells expressing TLR-2 increased significantly to 61.05% ± 6.946 ($p < 0.001$) (**Fig. 5D**). The most noteworthy finding was that o-Vanillin

significantly reduced the number of senescent cells expressing TLR-2 to 27.57% ± 2.509 ($p < 0.001$) when exposed to TLR-2/6 agonist. (**Fig. 5D**). These findings indicate a link between TLR-2 expression, cell senescence and SASP factor production that contribute to IVD degeneration and pain. This deleterious role of TLR-2 is blocked by the dual senolytic and anti-inflammatory effects of o-Vanillin.

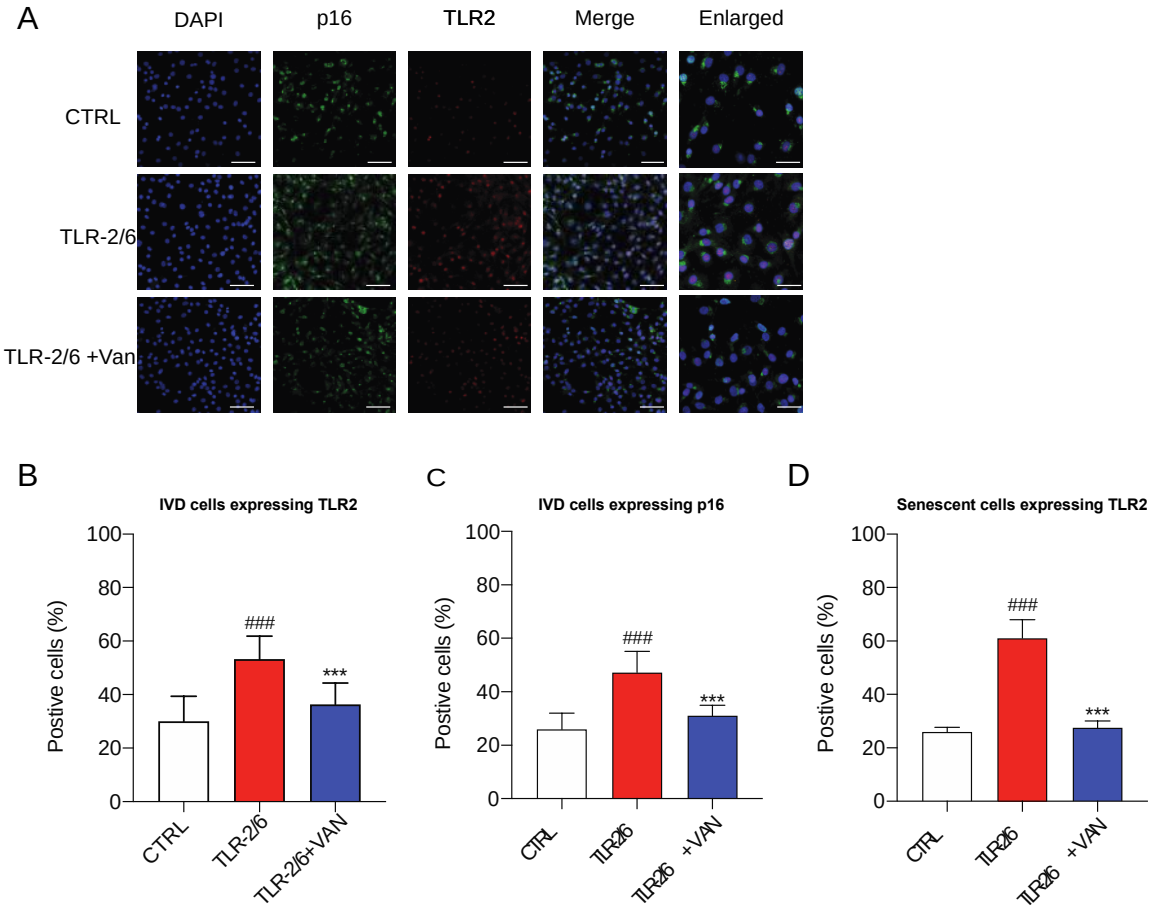


Figure 5: o-Vanillin reduced the number of cells co-expressing TLR-2 and $p16^{INK4a}$ in cells exposed to TLR-2/6 agonist. Disc cells from degenerate IVDs were induced with TLR-2/6 agonist for 48 hours with (TLR-2/6+VAN) or without (TLR-2/6) o-Vanillin treatment for 6 hours or no induction with TLR-2/6 agonist (CTRL). **(A)** Photomicrographs of IVD cells stained for DAPI (blue) and either $p16^{INK4a}$ (green), TLR-2 (red), or the merge ($p16^{INK4a}$ and TLR-2) as revealed by Immunocytochemistry. DAPI, $p16^{INK4a}$, TLR2, and merge images scale bars= 25µm. Enlarged images scale bar: 10µm. **(B-D)** Quantification of the percentage of IVD cells that stained positive for **(B)** TLR-2, **(C)** $p16^{INK4a}$ or **(D)** co-localized cells for TLR-2 and $p16^{INK4a}$. Percentage of positive cells in **(E)** were the average for n = 5. $^{\#}p<0.05$, $^{\#\#}p<0.01$,

###p<0.001 indicate significant difference between the TLR-2/6 agonist treated to the non-induced control and *p<0.05, **p<0.01, ***p<0.001 indicate significant difference between the TLR-2/6 agonist with o-Vanillin treated to the TLR-2/6 agonist treated. (**B-D**) Mean ± SD, statistical analysis was done using paired t-test.

Discussion:

Several studies, including our own, have demonstrated that senescent cells accumulate in degenerating IVDs and suggested that an elevated SASP factor release and increased expression of TLRs contribute to IVD degeneration[76,84]. Here we have shown a potential link between the accumulation of senescent cells and TLR activation. As well we show that o-Vanillin, a TLR antagonist and senolytic compound, has regenerative and anti-inflammatory effects on cells from degenerating IVDs[170].

In chondrocytes and IVD cells, TLRs are, in addition to molecules derived from pathogens, activated by exposure to intracellular proteins such as HSP60, HSP70, S100A8/9, HMGB1 released in response to stress and extracellular matrix fragments such as fibronectin, aggrecan, biglycan and other by-products of tissue degeneration [171]. As well it has been reported that synthetic TLR-2 and 4 agonists can induce IVD degeneration, increase inflammatory environment and increase in expression of TLRs [76,111]. The present study demonstrates that TLR-2 activation, in addition to inducing an inflammatory environment, caused IVD cells from non-degenerate IVDs to become senescent. We used cells of IVDs from organ donors with no signs of degeneration or history of back pain. These IVDs have a low number of senescent cells and low levels of SASP factor release compared to symptomatic degenerating IVDs [84]. Our results demonstrate that the synthetic TLR-2 agonist (Pam2CSK4) caused the greatest increase in senescent cell number, TLR-2 expression and SASP factor release in cells from non-degenerate IVDs after 48 h exposure. Our previous study using TLR-1, 2, and 4 agonists found

the cytokines (IL-1β, 6, 8), chemokines, proteases (MMP3, MMP13), and TLR-2 expression were greatest following exposure to the same TLR-2/6 agonist in NP cells of non-degenerate IVDs [76]. Other studies have shown that continuous stimulation of TLR-4 promotes cellular senescence in mesenchymal stem cells [172]. Moreover, TLR-2 and 10 have been found to be key mediators of senescence in IMR90 cells, a human diploid fibroblast cell line[164].

We then verified that these findings were also seen in cells isolated from degenerating IVDs of patients undergoing surgery to reduce low back pain[20,84]. TLR-2 activation of cells from symptomatic IVDs induced expression of SASP factors (CCL-2,5,7,8, IL-6, 8, GM-CSF, TNF-α, NGF, BNDF, CLCX-1, 10), a senescence marker ($p16^{INK4a}$) and of the TLR-2 receptor itself. Moreover, we confirmed that protein expression of SASP factors (NGF, IL-1β, TNF-α and IL-8) was higher in the TLR-2 activated cells. These proteins were chosen since they have been associated to be IVD degeneration and TLR-2 induction and have been reported to be highly expressed in degenerate human and mice IVDs [23,111,173]. Taken together, our results validate that IVD cells from patients with back pain and IVD degeneration at both gene and protein level respond to TLR-2 activation.

The use of synthetic antagonists aimed towards TLR-2 and TLR-4 has been evaluated in a variety of inflammatory diseases [174]. Antagonists such as TAK-242, a TLR-4 antagonist, have been shown to diminish LPS-induced TLR-4 signaling and inflammation in peritoneal macrophages [174]. Furthermore, our own previous study demonstrated that TAK-242 reduced pain but did not provide tissue regeneration in a mouse model of back pain[84]. Similar to our study, the anti-inflammatory properties of o-Vanillin were reported previously in NP cells from patients undergoing surgery for disc herniation or spinal stenosis following induction by high mobility group box-1[175]. Furthermore, the capability of o-Vanillin to reduce SASP factors has

been previously depicted in IVD cell pellet cultures [84,176]. o-Vanillin has also been shown to reduce cytokines, chemokines and proteases *in vitro* by in human HEK-TLR-2 and THP-1 cells and to reduce a tumor-promoting phenotype of microglia *in vivo* [170,177]. It has also previously been shown that o-Vanillin incorporated to Poly (Lactic-*co*-Glycolic Acid) scaffolds elicited more proteoglycan production and decreased inflammatory response of annulus fibrous cells compared to cells in un-supplemented scaffolds[178]. As well, o-Vanillin has been shown to significantly decrease the production of pro-inflammatory cytokines and significantly attenuate UVB irradiation-induced cytotoxicity in human keratinocyte stem cells[179].

Senolytic drugs target selective signaling pathways involved in cell survival and apoptosis[163]. These drugs could potentially be used therapeutically to treat disc degeneration, recover loss of disc height in already degenerate discs, or prophylactically to prevent future degeneration either in individuals at risk or following fusion for adjacent disc disease[96,120]. Our previous study demonstrated that o-Vanillin, reduced senescent cells and enhanced matrix production in cell pellet cultures generated from organ donor IVDs without a known history of back pain[84]. Here we show that o-Vanillin was able to reduce inflammation, remove senescent cells and enhance proteoglycan production in cell pellets from surgically removed symptomatic IVDs of patients with low back pain.

We further demonstrated that by targeting TLRs and senescent cells with o-Vanillin we can decrease inflammatory processes found in IVD cells from patients with back pain and IVD degeneration. Interestingly, our study demonstrates that both gene and protein expression of SASP factors (CCL2,5,7,8, GM-CSF, BDNF, NGF, TNF-α, CLCX1, CLCX8 and CLCX10, IL-1β, IL-8) were significantly reduced following TLR activation and o-Vanillin treatment.

The higher expression of TLR-2 in IVD cells from patients with back pain and IVD degeneration leads us to evaluate its expression level in senescent cells and investigate its role in disc cell senescence and associated SASP factors release. We found TLR-2 activation increased the expression of TLR-2 in the senescent cells. Also, treatment with o-Vanillin significantly reduced the number of senescent cells expressing TLR-2. One limitation of our study is that the degenerate cell population is a mix of NP and AF cells from patients suffering from chronic lower back pain. This is because of the difficulty in accurately distinguishing and separating NP and AF tissue from surgically removed IVD tissue. This limitation does not allow us to know whether the TLR-2/p-16 co-localization is in both cell types or in AF or NP cells specifically. To our knowledge, this is the first study to show a potential link between TLR-2 and cellular senescence in IVD cells. Further studies using genetically modified TLR-2 knock-out human IVD cell lines are needed to better decipher which mechanistic pathways are shared between o-Vanillin's senolytic activity and TLR-2's antagonistic effect.

Conclusions:

We showed that TLR-2/6 activation increased TLR-2 expression and senescent cells in IVD cells from both organ donors without degeneration and back pain and patients with disc degeneration and back pain. Further, o-Vanillin reduced the number of senescent IVD cells and the release of SASP factors. This data suggests a possible regulatory effect between TLR-2 and IVD cell senescence IVD. This phenomenon could be explained either by the induction of non-senescent neighbouring cells by senescent cells in a paracrine manner or alternatively, that senescent cells retain SASP factor production through TLR-2 activation in an autocrine manner. The detrimental effect of senescent cells can be inhibited by blocking TLR-2 activity with o-

Vanillin. These findings prompt the need to further understand the role of TLR-2 in IVD cell senescence and the mechanism by which o-Vanillin interferes in this pathway.

List of Abbreviations:

IVD = intervertebral disc

TLR = Toll-like receptors

SASP = senescence-associated secretory phenotype

Dimethyl-Methylene Blue Assay = DMMB

ICC = immunocytochemistry

ELISA = enzyme-linked immunosorbent assay

NP = nucleus pulposus

AF = annulus fibrosus

sGAG = sulfated glycosaminoglycans

Declarations

Ethics and Consent to participate:

IVD tissue used in this study was provided by Transplant Quebec or McGill Scoliosis and Spine Group. Tissues were collected during discectomies to alleviate pain or during organ harvesting. Participants provided written informed consent to participate in the study and to allow their biological samples to be tested. All procedures performed were approved by the ethical review board at RI-MUHC (IRB # Tissue Biobank 2019-4896, Extracellular Matrix 2020-564 and A08-M22-17B).

Consent for publication:

Not Applicable

Availability of data and materials:

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests

Funding:

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Authors' contributions

All authors read and approved the final manuscript. MM received a studentship from MITACS. MM and HC designed the study, conducted experiments, analysed data and wrote the manuscript. LL and KS conducted experiments. OR, JO, MW and PJ helped to design the study and extensively reviewed and revised manuscript. OR, JO, MW and PJ provided organ donor and surgical IVD samples. LH designed the study, wrote the manuscript and gave final approval of manuscript.

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Supplementary Table 1. Characteristics of the donors utilized for the study. (ICC): Immunocytochemistry including $p16^{INK4a}$, immunofluorescence for NGF, IL-1β, TNF-α, IL-8, TLR-2 and $p16^{INK4a}$ in a monolayer culture. (IHC): Immunohistochemistry for $p16^{INK4a}$ and Safranin-O in pellet culture sections. (RT-qPCR): Real-time Quantitative Polymerase Chain Reaction (ELISA): Enzyme-linked immunosorbent assays. (DMMB): Dimethyl methylene blue (DMMB) assays.

Donor	Age	Sex	Cause of death/ Reason For Surgery	ICC	IHC	RT-qPCR	ELISA	DMMB
Non-Degenerate IVD Donors								
1	17	M	Brain death	-		-	-	
2	20	F	Intoxication CVA	-		-	-	
3	21	M	Accident	-		-	-	
4	23	F	Accident	-		-	-	
5	18	M	Trauma	-		-	-	
Surgical samples								
6	32	M	Lower back pain and IVD Degeneration	-		-	-	
7	65	F	Lower back pain and IVD Degeneration	-	-	-	-	-
8	68	F	Lower back pain and IVD Degeneration	-		-	-	
9	47	F	Lower back pain and IVD Degeneration	-		-	-	
10	59	F	Lower back pain and IVD Degeneration		-		-	-
11	38	F	Lower back pain and IVD Degeneration	-	-	-	-	-
12	45	M	Lower back pain and IVD Degeneration		-		-	-
13	35	F	Lower back pain and IVD Degeneration		-		-	-

Supplementary Table 2. qRT-PCR Primer Sequences

Target	Forward Primer Sequence	Reverse Primer Sequence	Reference
BDNF	5'-TAACGGCGGCAGACAAAAAGA-3'	5'-GAAGTATTGCTTCAGTTGGCCT-3'	[111]
CCL-2	5'-GCATGAAAGTCTCTGCCG-3'	5'-GAGTGTTCAAGTCTTCGGA-3'	[180]
CCL-5	5'-GAAGGTCTCCGCGGCAGCC-3'	5'-CTGGGCCCTTCAAGGAGCGG-3'	[180]
CCL-7	5'-CACTTCTGTGTCTGCTGCTCAC-3'	5'-GTTTTCTTGTCCAGGTGCTTCATA-3'	[181]
CCL-8	5'-GCCTGCTGCTCATGGCAGCC-3'	5'-GCACAGACCTCCTTGCCCCG-3'	[180]
CXCL-10	5'-GTGGCATTCAAGGAGTACCTC-3'	5'-TGATGGCCTTCGATTCTGGATT-3'	[180]
IL-8	5'-TCCTGATTTCTGCAGCTCTG-3'	5'-GTCTTTATGCACTGACATCTAAGTTC-3'	[84]
GAPDH	5'-TCCCTGAGCTGAACGGGAAG-3'	5'-GGAGGAGTGGGTGTCGCTGT-3'	[84]
GM-CSF	5'-TCTCAGAAATGTTTGACCTCCA -3'	5'-GCCCTTGAGCTTGGTGAG-3'	[182]
CXCL-1	5'TGAAGGCAGGGGAATGTATGTG -3'	5'-AGCCCCTTTGTTCTAAGCCA-3'	[183]
IL-6	5'-TGAACCTTCCAAAGATGGCTG-3'	5'-CAAACTCCAAAAGACCAGTGATG-3'	[84]
NGF	5'-AAGTGCCGGGACCCAAAT-3'	5'-TGAGTTCCAGTGCTTTGAGTCAA-3'	[76]
$p16^{INK4a}$	5'-CTGCCCAACGCACCGAATA-3'	5'-GCTGCCCATCATCATGACCT-3'	[84]
p21	5'-GAGACTCTCAGGGTCGAAAAC -3'	5'-GGCGTTTGGAGTGGTAGAAA-3'	[84]
TGF-β	5'-TCCTGGCGATACCTCAGCAA-3'	5'-CTCAATTTCCCCTCCACGGC-3'	[184]
TLR-1	5'-CAGTGTCTGGTACACGCATGGT-3'	5'-TTTCAAAAACCGTGTCTGTTAAGAGA-3'	[76]
TLR-2	5'-GGCCAGCAAATTACCTGTGTG-3'	5'-AGGCGGACATCCTGAACCT-3'	[76]
TLR-4	5'-CAGAGTTTCCTGCAATGGATCA -3'	5'-GCTTATCTGAAGGTGTTGCACAT-3'	[76]
TLR-6	5'-GAAGAAGAACAACCCTTTAGGATAGC-3'	5'-AGGCAAACAAAATGGAAGCTT-3'	[76]
TNF-α	5'-ATGTTGTAGCAAACCCTCAAGC-3'	5'-TCTCTCAGCTCCACGCCATT-3'	[185]

Chapter 2 Preface:

IVD degeneration is a major factor contributing to lower back pain. The cellular pathogenesis of IVD degeneration and the mechanisms leading to pain are not fully understood. One novel approach to treating painful degeneration is to target cellular senescence, a state of irreversible growth arrest occurring in response to cellular stress. Stress-induced premature senescence is caused by factors such as oxidative and genotoxic stresses. Increasing evidence suggests that the accumulation of senescent cells during tissue degeneration contributes directly to the initiation and development of musculoskeletal degenerative diseases like osteoarthritis and IVD degeneration. Senescent cells secrete a range of cytokines, chemokines, growth factors, and proteases termed SASPs. These SASP factors are suggested to further induce senescence in a paracrine manner to promote matrix catabolism and sterile inflammation in IVDs, thereby accelerating the degenerative process. Elimination of senescent cells enhances disc tissue homeostasis in genetically modified progeroid $Ercc1^{-/\Delta 22}$ and p16-3MR mice, suggesting that senotherapeutic drugs have great potential to treat low back pain resulting from IVD degeneration.

The cell-cycle arrest of senescent disc cells is mainly mediated by two pathways: p53-p21-Rb and p16-Rb. During disc degeneration, both pathways are activated simultaneously to induce senescence. The FDA-approved drug RG-7112 (RO5045337) is a highly potent and selective MDM2 antagonist that restores the physiological activity of p53. RG-7112 is the first nutlin family member to be assessed clinically showing evidence of acceptable safety. RG-7112 was reported to selectively kill senescent lung fibroblasts (IMR90) where senescence was induced by ionizing radiation (IR). The natural compound o-Vanillin, known for its antioxidant

and anti-inflammatory effects, has recently been described for its senotherapeutic activity in human IVD cells.

One drawback of using a single senolytic agent is the failure to target multiple senescent anti-apoptotic pathways in the same cell type or different cell populations within a target tissue. Concurrently targeting multiple and indirectly related anti-apoptotic pathways may result in increased selectivity for senescent cells in the absence of toxicity for normal proliferating or quiescent cells. A successful combination therapy is exemplified by the combination of Dasatinib and Quercetin, which targets antiapoptotic networks instead of a single target. The lower therapeutic dosages enabled by combinations also decreased side effects associated with single drugs. Combined treatment at lower doses may allow the repurposing of drugs that were previously discarded due to undesirable side effects and increase success in clinical trials.

Here, we utilized a 3D pellet *in vitro* model to assess the senotherapeutic effects of RG-7112 and o-Vanillin on SIPS-occurring senescent cells in painful degenerating human IVD cells. We have previously shown that o-Vanillin has senolytic effects on senescent human IVD cells. o-Vanillin is a natural compound that, in addition to its senolytic effect, has antioxidant and anti-inflammatory properties. The overall objective of this chapter is to determine if the combination of two senolytic drugs, o-Vanillin and RG-7112, can selectively remove senescent cells, reduce inflammatory mediators, and relieve pain-sprouting from human IVD cells better than the drugs alone. This study was published in 2023 in Biomolecules.

Chapter 2

Title: Senolytic combination treatment is more potent than single drugs in reducing inflammatory and senescence burden in cells from painful degenerating IVDs

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Abstract:

Background: Low back pain is a global health problem directly related to intervertebral disc (IVD) degeneration. Senolytic drugs (RG-7112 & o-Vanillin) target and remove senescent cells from IVDs *in vitro*, improving tissue homeostasis. One drawback of using a single senolytic agent is the failure to target multiple senescent anti-apoptotic pathways. This study aimed to determine if combining the two senolytic drugs, o-Vanillin and RG-7112, could more efficiently remove senescent cells, and reduce the release of inflammatory factors and pain mediators in cells from degenerating human IVDs than either drug alone.

Methods: Preliminary data evaluating multiple concentrations of o-Vanillin and RG-7112 led to the selection of four treatment groups. Monolayer and pellet cultures of cells from painful degenerate IVDs were exposed to TLR-2/6 agonist. They were then treated with the senolytics o-Vanillin and RG-7112 alone or combined. $p16^{INK4a}$, Ki-67, caspase-3, inflammatory mediators and neuronal sprouting were assessed.

Results: Compared to the single treatments, the combination of o-Vanillin and RG-7112 significantly reduced the amount of senescent IVD cells, pro-inflammatory cytokines and neurotrophic factors. Moreover, both single and combination treatments significantly reduced neuronal sprouting in PC-12 cells.

Conclusion: Combining o-Vanillin and RG-7112 greatly enhanced the effect of either senolytic alone. Together, these results support the potential of senolytics as a promising treatment for IVD-related low back pain.

Keywords: low back pain; intervertebral disc degeneration; cellular senescence; senotherapeutics; senolytics; combination therapy

Introduction:

Low back pain is experienced by approximately 80% of individuals at some point in their lifetime. Globally, it is the number one cause of years lived with disability[186]. Despite its prevalence, little is known about the cellular and molecular mechanisms leading to painful intervertebral disc (IVD) degeneration, leaving surgical removal and vertebral fusion in end-stage disease as the mainstay of treatment. This age-related health problem is associated with IVD degeneration in many individuals[187].

Healthy, painless IVDs are largely avascular and aneural. Evidence shows that degenerating, painful IVDs are innervated[102], inferring that discogenic pain is associated with increased IVD innervation. Chronic pain induced by disc degeneration is speculated to stem from nerve damage and neuronal sensitization[105]. Moreover, inflammatory pathways involved in IVD degeneration have been linked to chronic back pain[105,188]. *In vitro*, increases in pro-inflammatory cytokines interleukins; IL-1β, IL-6 and tumor-necrosis-factor-alpha (TNF-α)) and pro-nociceptive factors (nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF)) have been observed in cells isolated from degenerating IVDs[40,108]. NGF and BDNF promote neuronal development, survival, and growth [102]. Moreover, it is known that degenerating human IVDs produce sufficient NGF and BDNF to promote neurite growth and nociceptive peptide production[167].

Targeting cellular senescence (i.e., viable cells which can no longer divide) is a novel venue currently being investigated to treat chronic lower back pain. To date, the literature

suggests that senescent cell accumulation during tissue degeneration contributes directly to the onset and progression of degenerative musculoskeletal illnesses such as osteoarthritis[122] and IVD degeneration.

The senescence-associated secretory phenotype (SASP) refers to the secretion of cytokines, chemokines, neurotrophins, growth factors, and proteases by senescent cells[189,190]. These SASP factors are believed to promote matrix catabolism, sterile inflammation and pain in IVDs, thereby speeding up the degenerative process in a paracrine manner[88].

In human IVD, there is an expression of TLR-1, -2, -3, -4, -5, -6, -9, and -10 and the expression of TLR--1, -2, -4, and -6 are increased with the degree of disc degeneration and pain[71,76]. Straining intact disc and IVD cells can upregulate TLR-2 and -4 expressions, and previous data from our lab demonstrate that activating TLR receptors with the synthetic agonists (PAM2CSK4, TLR-2/6 agonist) induced IVD degeneration, pain, and cellular senescence[76,191].

Synthetic drugs that selectively target and remove senescent cells (senolytic) have recently been identified[96,120]. Two compounds of interest are RG-7112 and o-Vanillin. The FDA-approved senolytic compound RG-7112 (RO5045337)[192] restores p53 physiological activity via MDM2[192,193]. RG-7112 has been shown to specifically kill senescent IVD cells[129,191]. O-Vanillin is a natural senolytic compound recognized for its antioxidant and anti-inflammatory properties, which have been reported to have senotherapeutic action on IVD cells from degenerating painful human IVDs and on IVD cells where senescence was induced by activation of TLR-2[129,175,191].

Senescent cells are heterogenous, and one drawback of using a single senolytic agent is the failure to target multiple senescent anti-apoptotic pathways in the same cell type or different

cell populations within a target tissue. Concurrently targeting multiple and indirectly related anti-apoptotic pathways may result in increased targeting of senescent cells in the absence of toxicity for normal proliferating or quiescent cells. Successful combination therapy is exemplified by the combination of Dasatinib and Quercetin that targets antiapoptotic networks instead of a single target[132]. The lower therapeutic dosages enabled by the combination also decreased side effects associated with single drugs [132].

The current manuscript used a 3D culture model of cells from painful degenerate IVD and stimulated them with a TLR-2 agonist to heighten the senescent phenotype. It was important to keep as many of the senescent cells from the tissue as possible in our experiments. As expansion will select for highly proliferative cells while TLR-2 expression is lost, we used cells in passages 1-2. The objectives were a) to determine if treatment with o-Vanillin and/or RG-7112 has the potential to alter factors that directly affect innervation, b) to determine if a combination treatment with o-Vanillin and RG-7112 was more potent than single treatment at reducing senescent phenotypes and SASP factors.

Materials and Methods:

Tissue collection and cell isolation

All procedures performed were approved by the ethical review board at McGill University (IRB# 2019-4896). Degenerate IVDs were obtained from chronic low back pain patients who received discectomies to alleviate pain. Donor information is presented in Supplemental Table 1. IVD cells were isolated, as previously described [191]. Briefly, samples were washed in phosphate-buffered saline solution (PBS, Sigma-Aldrich, Oakville, ON, Canada) and Hank's-buffered saline solution (HBSS, Sigma-Aldrich, Oakville, ON, Canada)

supplemented with Primocin™ (InvivoGen, San Diego, CA, USA) and Fungiozone (Sigma-Aldrich, Oakville, ON, Canada). Then, the matrix was minced and digested in 0.15% collagenase type II (Gibco) for 16 hours at 37°C. Cells were passed through both a 100-µm filter and 70-µm filter before being re-suspended in Dulbecco's Modified Eagle Media (DMEM, Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% fetal bovine serum (FBS, Gibco), Primocin™, Glutamax (Oakville, ON, Canada), and maintained in a 5% CO_2 incubator at 37 °C.

In Vitro Cell Culture and Treatment

Monolayer culture: Experiments were performed with degenerate IVDs (NP and AF cells) within passages 1 to 2. 20,000 cells were seeded in 8-well chamber slides (Nunc™ Lab-Tek™ II Chamber Slide™ System) for immunocytochemistry experiments following treatment. Three hundred thousand cells were seeded in 6-well plates (Sarstedt, TC plate 6-well, Cell+, F) for ELISA and RNA extraction following treatment. All cells were left to adhere for 12 to 24 hours and then serum-starved in DMEM with 1X insulin-transferrin selenium (ITS, Thermo Fisher, Waltham, MA, USA) for 6 hours prior to treatment. Induction of senescence was performed by exposing cells to 100 ng/mL of Pam2CSK4 (TLR-2/6 agonist, Invivogen) for 48 hours. Cells were treated with senolytic compounds for the last 6 hours of the incubation[191]. Serial dilutions with 0.25/0.5/2.5/5 µM of RG-7112 (Selleck Chemicals, Houston, TX, USA) or 5/10/50/100 µM of o-Vanillin (Sigma-Aldrich, Oakville, ON, Canada) was performed to identify concentrations which do not demonstrate senolytic activity. Combination treatments were as follows; 5 µM of RG-7112 and 100 µM of o-Vanillin, 2.5 µM of RG-7112 and 100 µM of o-Vanillin, 5 µM of RG-7112 and 50 µM of o-Vanillin or 2.5 µM of RG-7112 and 50 µM of o-Vanillin was performed. The concentration of both compounds is

based on previously published studies from our laboratory as well as work done by other groups working with o-Vanillin and RG-7112[84,129,175,191,194].

Pellet culture: 100,000 cells/tube were collected by centrifugation at 300g for 5 minutes. Pellets were incubated in 300 µL DMEM, 2.25g/L glucose (Sigma-Aldrich, Oakville, ON, Canada), 5% FBS, 5 µM ascorbic acid, 1% GlutaMAX, 0.5% Gentamicin (Thermo Fisher, Waltham, MA, USA) at 37°C and 5% CO_2. Pellets were left in DMEM for two days to form and stabilize, followed by two days of induction with 100 ng/mL of Pam2CSK4 and then treated with either 100 µM o-Vanillin, 5 µM RG-7112, 5 µM of RG-7112 and 100 µM of o-Vanillin, 2.5 µM of RG-7112 and 100 µM of o-Vanillin, 5 µM of RG-7112 and 50 µM of o-Vanillin or 2.5 µM of RG-7112 and 50 µM of o-Vanillin for four days; meanwhile, pellets in the control group stayed in DMEM (Sigma-Aldrich, Oakville, ON, Canada) (Figure 3A). Following the treatment period, pellets from all groups were cultured for 16 days, and their culture media was collected every 4 days and pooled as post-treatment media (Figure 3A). Cell pellets were washed in PBS, fixed with 4% paraformaldehyde (Thermo Fisher, Waltham, MA, USA), cryoprotected in 10–30% sucrose, and then transferred via spatula into a plastic mould for embedment in Optimum Cutting Temperature compound (OCT, Thermo Fisher, Waltham, MA, USA), and finally flash-frozen at -80°C and kept at -20°C. Sections were cut 5-µm-thick with a cryostat (Leica Microsystems, Richmond Hill, ON, Canada) and placed on slides for immunostaining.

PC-12 culture: Rat adrenal pheochromocytoma (PC-12) 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 20-22. PC-12 cells (ATCC, Manassas, VA, USA) in passages 2–7 were cultured on six-well plates (Nunc) or eight-

well chamber slides (Nunc) coated with 50 µg/ml rat tail collagen type I (Gibco) and 10 µg/ml Poly-l-Lysine (Sigma-Aldrich). 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). PC-12 and neuronal culture media was replaced after a 24-hour acclimatization period with day 12 media from non-treated and treated pellet cultures. PC-12 cells were exposed for 48 hours to the different media (n = 5 with no senolytic treatment, n = 5 treatment with 5 µM of RG-7112 and 100 µM of o-Vanillin, n = 5 with 2.5 µM of RG-7112 and 100 µM of o-Vanillin and n = 5 with 5 µM of RG-7112 and 50 µM of o-Vanillin).

Immunofluorescence

Pellet sections were heated to 60°C for 30 min, then washed and permeabilized with PBS 1% Tween and 0.1% Triton. Cells were blocked with 0.3% Triton X-100 in PBS, saturated with 1% BSA, 1% serum, and 0.1% Triton X-100 for 2 h and then incubated for 2 h at room temperature with $p16^{INK4a}$ (Roche, Ventana laboratories, Mississauga, ON, Canada), Ki-67 (Novus, Oakville, ON, Canada) and cleaved caspase-3 (C8487, recognizing the active 17 kDa fragment Sigma-Aldrich, Oakville, ON, Canada) primary antibodies. After washing, cells were incubated with the appropriate Alexa Fluor® 488 or 555-conjugated secondary antibody (Thermo Fisher, Waltham, MA, USA) for 2 hours at room temperature and then counterstained with DAPI for nuclear staining. Photomicrographs were acquired with a fluorescent Olympus BX51 microscope equipped with an Olympus DP71 digital camera (Olympus, Tokyo, Japan). Over 1000 cells were quantified for each pellet. Positive cell percentage was quantified by Fiji ImageJ (version: 2.1.0/1.53c). Briefly, the number of cells stained positive for one of the target proteins ($p16^{INK4a}$, Ki-67 or caspase-3) was counted and compared to the total number of cells positive for DAPI staining.

PC-12 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 (Toronto, ON, Canada) with a Canon PowerShot A640 camera and 52 mm Soligor adaptor tube (Mississauga, ON, Canada). The graph is generated by counting 10 images from 7 different donors per group. This would equal over 2000 cells being quantified for each condition. The percentage of cells with neurites was determined and then averaged for each experimental condition.

Immunocytochemistry

Monolayer cultures were washed with PBS, fixed with 4% paraformaldehyde (Thermo Fisher, Waltham, MA, USA), and blocked in PBS with 1% BSA (Sigma-Aldrich, Oakville, ON, Canada), 1% serum, and 0.1% Triton X-100 (Sigma-Aldrich, Oakville, ON, Canada) for 1 h. Slides were then incubated with the $p16^{INK4a}$ CINtec PLUS Kit (Roche, Ventana laboratories, Mississauga, ON, Canada) according to the manufacturer's instructions. Slides were also exposed to primary antibodies specific to Ki-67 (Novus, Oakville, ON, Canada) and caspase 3 (Sigma-Aldrich, Oakville, ON, Canada) overnight at 4°C. A mouse- and rabbit-specific HRP/DAB (ABC) Detection IHC Kit (ab64264, Abcam, Cambridge, Ma, USA) was used for $p16^{INK4a}$, caspase-3 and Ki-67 staining. Images were processed using AxioVision LE64 software (Zeiss, Oberkochen, Germany). Ten fields, randomly distributed across the well, were analyzed, total cells were counted, and the percentage of positive cells was calculated.

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA was extracted using the TRIzol chloroform extraction method previously described for surgical samples and PC-12 cells[167]. 500 ng of RNA was then reverse transcribed using a qScript cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA) with an Applied

Biosystems Verti Thermocycler (Thermo Fisher, Waltham, MA, USA). RT-qPCR was performed using an Applied Biosystems StepOnePlus machine (Thermo Fisher, Waltham, MA, USA) with PerfecCTa SYBR Green Fast Mix (Quanta Biosciences, Beverly, MA, USA). Primer sequences for TLRs, senescent markers, neurite growth markers, as well as pain and inflammatory markers ($p16^{INK4a}$, TNF-α, CXCL-10, CXCL-1, CXCL-8, GM-CSF, TGF-β, CCL-2, CCL-5, CCL-7, CCL-8, NGF, BDNF, TLR-1,2,4,6, neurofilament light chain (NF-L), plasminogen activator urokinase receptor (Plaur), Polo-like kinase 2 (Plk2), poliovirus receptor (PVR), vaccinia growth factor (VGF)) and the housekeeping gene (GAPDH), can be found in Supplemental Table 2. All reactions were conducted in technical triplicate, and fold-changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method after normalizing to GAPDH and non-treated samples[168].

ELISA

Cell culture media from degenerate IVD cells cultured in monolayer and pellets was used to assess the concentrations of IL-6, IL-8, IL-1β, TNF-α , NGF and BDNF. 150 µL of monolayer culture media and day 12 pellet culture media were used. ELISAs were performed per the manufacturer's instructions (RayBiotech, Norcoss, GA, USA). Colorimetric absorbance was measured with a Tecan Infinite M200 PRO (Tecan, Männedorf, Switzerland) spectrophotometer and analyzed with i-control 1.9 Magellan software (Tecan, Männedorf, Switzerland). Protein levels of the treated conditions and controls were then compared.

Dimethylmethylene Blue Assay

Dimethylmethylene Blue (DMMB) assays were conducted as previously described [84] to quantify sulfated glycosaminoglycans (sGAG) in the day 12 IVD pellet media. Chondroitin sulfate was used to generate the standard curve. All samples were ensured to fall into the linear

portion of the standard curve. Each sample was triplicated into clear 96-well plates (Costar, Corning, NY, USA). DMMB dye was then added to the wells. The absorbance was measured immediately at room temperature using a Tecan Infinite T200 spectrophotometer (Mannedorf, Switzerland).

Metabolic Activity

Metabolic activity was assessed by the Alamar Blue assay [84]. Briefly, on day 24, surgical sample cell pellets were exposed to 10% Alamar Blue reagent (Thermo Fisher, Waltham, MA, USA) in DMEM and incubated for 18h at 37°C. Fluorescence (Ex560/Em590) was measured by using a spectrophotometer (Tecan Infinite T200, Männedorf, Switzerland) equipped with Magellan software (Tecan, Männedorf, Switzerland). Results are presented as a percentage of metabolic activity compared to the control. Experiments were performed in triplicate wells for each pellet condition.

Statistical analysis:

Data were analyzed using Graph Prism 9 (Graph Pad, La Jolla, CA, USA). Analysis was performed using paired Student's t-test or one-way ANOVA. Specific tests are indicated in the figure legends with the corrections. A p-value <0.05 was considered statistically significant. Data are presented as mean ± SD.

Results

RG-7112 reduced the expression of $p16^{INK4a}$, TLR-2, and SASP factors in IVD cells from patients with back pain and IVD degeneration

IVD cells from patients with low back pain and IVD degeneration were exposed to a TLR-2/6 agonist for 48 hours in the presence or absence of RG-7112 (5μM) during the last 6 hours. We have previously observed that exposure of IVD cells from patients with low back

pain and IVD degeneration to a TLR-2/6 agonist significantly increases the expression of SASP factors, $p16^{INK4a}$ and TLR-2 [191]. We have also reported that o-Vanillin, a natural senolytic, can reduce senescence and SASP factors in this TLR-2/6-induced senescent human IVD cells[191]. Here, we sought to investigate if RG-7112 has a similar impact[191]. Like o-Vanillin, when compared to the control, RG-7112 significantly reduced the expression of CCL2 from 42.32 ± 11.34 to 2.3 ± 3.29 ($p = 0.004860$), CCL5 from 49.03 ± 11.49 to 15.00 ± 6.14 ($p = 0.015869$), CCL7 from 9.30 ± 1.43 to 4.96 ± 2.20 ($p = 0.015001$), CCL8 from 28.40 ± 4.94 to 2.91 ± 2.40 ($p = 0.003127$), GM-CSF from 118.55 ± 10.07 to 7.15 ± 4.83 ($p = 0.000239$), BDNF from 1.77 ± 0.13 to 1.07 ± 0.16 ($p = 0.002507$), NGF from 1.35 ± 0.11 to 0.61 ± 0.19 ($p = 0.000954$), TNF-α from 7.36 ± 2.36 to 1.77 ± 0.77 ($p = 0.022738$), CXCL1 from 594.16 ± 44.72 to 38.66 ± 36.54 ($p = 0.000002$), CXCL8 from 594.50 ± 98.64 to 209.32 ± 157.89 ($p = 0.022738$), and CXCL10 from 745.23 ± 107.79 to 74.22 ± 89.64 ($p = 0.001659$) (**Figure 1A**). In addition, treatment with RG-7112 significantly decreased $p16^{INK4a}$ expression from 3.83 ± 1.05 in control to 1.21 ± 0.22 in RG-7112 ($p = 0.004324$) (**Figure 1B**). Lastly, a significant decrease in TLR-2 expression ($p = 0.009338$) was also observed in RG-7112 (2.69 ± 0.98) treated cells compared with control (9.17 ± 1.29), as has previously been reported for o-Vanillin[191] (**Figure 1C**).

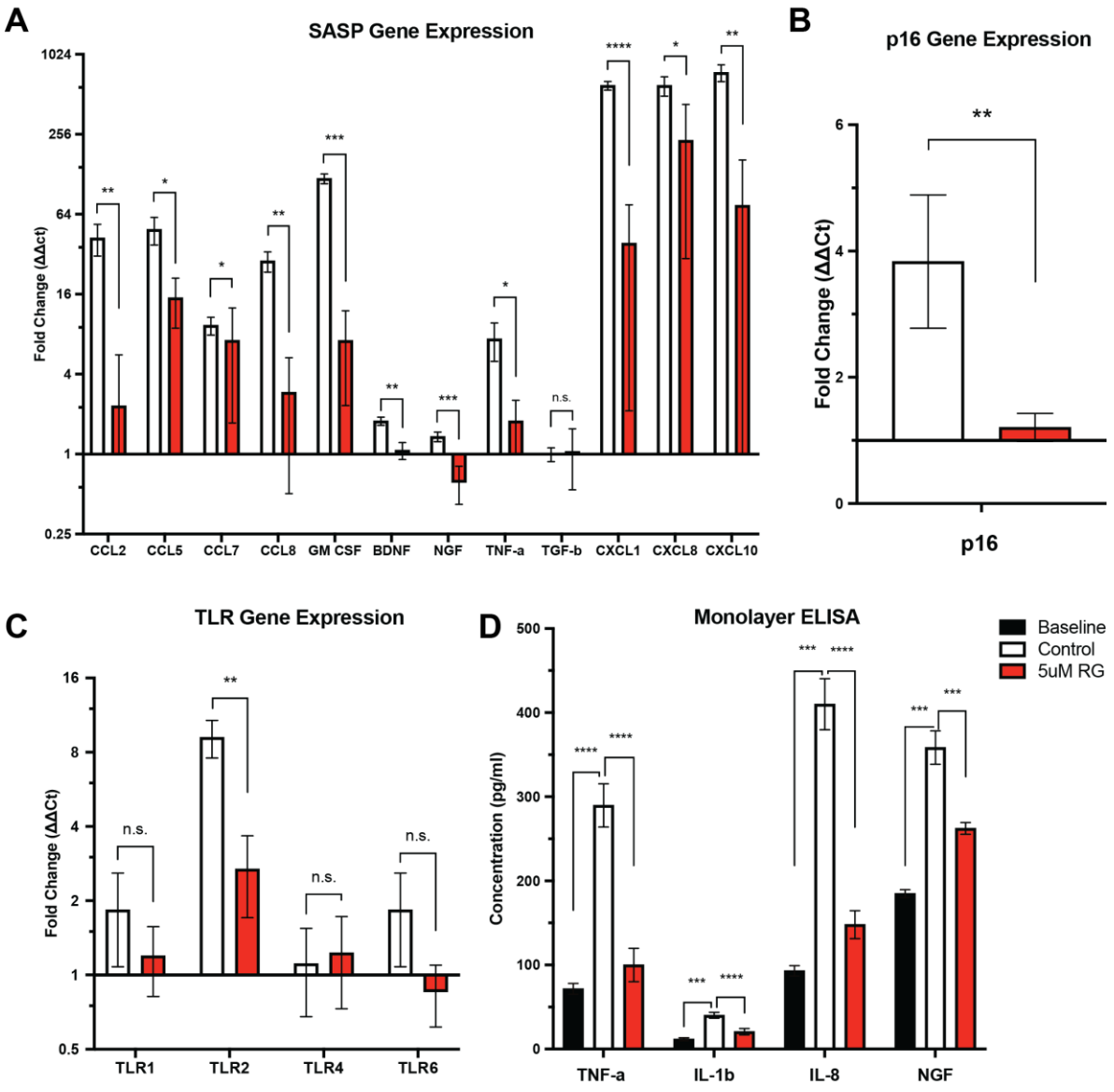

Figure 1. RG-7112 reduces the expression of $p16^{INK4a}$, TLR-2, and SASP factors following TLR-2 activation in IVD cells from patients with back pain and IVD degeneration. **(A-D)** Human IVD (replace IVD by senescent?) cells from painful degenerate IVDs in monolayer culture induced with Pam2CSK4 and treated with 5µM RG-7112. qRT-PCRs were normalized to the baseline (cells from painful degenerate IVD not induced with Pam2CSK4 and not treated with RG-7112). **(A)** Gene expression of SASP factors (CCL2, CCL5, CCL7, CCL8, GM CSF, BDNF, NGF, TNF-α, TGF-β, CXCL1, CXCL8, and CXCL10), **(B)** Gene expression of senescence marker $p16^{INK4a}$. **(C)** Gene expression of Toll-like receptors (TLR1, TLR-2, TLR-4, and TLR-6). **(D)** All monolayer culture media was analyzed by Raybio Human Cytokine Array. TNF-α, IL-1β, IL-8 and NGF protein concentration were evaluated. Values are presented as fold change ± SD in **(A-C)** and mean ± SD in **(D)**.* Indicates significance calculated using a one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$. **(A-D)** n = 5 donor samples per condition.

Additionally, SASP factor release into the culture media was assessed from cultures exposed to a TLR-2/6 agonist for 48 hours in the presence or absence of RG-7112 (5μM) during the last 6 hours of culture. As previously reported, [191] treatment with TLR-2/6 agonist Pam2CSK4 resulted in a significant increase in SASP factor expression (namely TNF-α, IL-1β, IL-8 and NGF) when compared to the control. Interestingly, this induction was significantly decreased for all evaluated SASP factors following treatment with RG-7112. TNF-α expression decreased from 289.67 pg/ml ± 25.69 in control to 99.99 pg/ml ± 19.91, p=0.000068 in RG-7112 treated cells, IL-1β expression decreased from 40.21 ± 3.53 pg/ml in control to 20.68 pg/ml ± 3.86, p=0.000098 in RG-7112 treated cells, IL-8 expression decreased from 410.01 pg/ml ± 30.47 in control to 147.92 pg/ml ± 16.56, p=0.000030 in RG-7112 treated cells and NGF expression decreased from 358.43 pg/ml ± 19.85 in control to 262.32 pg/ml ± 6.96, p=0.000227 in RG-7112 treated cells (**Figure 1D**).

Identifying the lowest effective concentration of RG-7112 and o-Vanillin at which senolytic activity is preserved in monolayer culture

Given the role of o-Vanillin in TLR-2/6-induced senescence and the findings above, which demonstrate a similar role to RG-7112, we investigated whether there is an additive effect of combining the two senolytic compounds. First, to determine the lowest concentration with senolytic activity, IVD cells from patients with low back pain and IVD degeneration were exposed to the TLR-2/6 agonist for 48 hours in the presence of serial dilutions of either RG-7112 or o-Vanillin during the last 6 hours of the treatment. Control samples only received induction with the TLR-2/6 agonist. A significant decrease in senescent cells (i.e., $p16^{INK4a}$ positive cells) was observed with all four dilutions of RG-7112 (0.25 μM, 0.5 μM, 2.5 μM, 5

μM) and three dilutions of o-Vanillin (10 μM, 50 μM, 100 μM). Representative images can be seen in **Figure 2A(a,d)**. Relative to the control (21.24% ± 1.23), the percent of $p16^{INK4a}$ positive senescent cells with RG-7112 was 18.50% ± 1.61 at 0.25 μM (p=0.0437), 14.58% ± 0.42 at 0.5 μM (p=0.004), 13.25% ± 0.46 at 2.5 μM (p<0.0001), 10.57% ± 0.48 at 5 μM (p<0.0001). With o-Vanillin, the remaining percentage of $p16^{INK4a}$ positive cells was 15.59% ± 1.42 at 10 μM (p=0.0076), 14.21% ± 1.41 at 50 μM (p=0.0013) and 11.17% ± 1.01 at 100 μM (p<0.0001) (**Figure 2B**). The number of Ki-67 positive proliferative cells was significantly (p=0.0149) increased to 21.50% ± 0.75 following treatment with 5μM RG-7112 treatment and to 21.61%±1.22 (p=0.0093) following 100μM o-Vanillin treatment as compared to the untreated control (18.36% ± 0.96) (**Figure 2A (b, e)**) and **Figure 2C**). Using an antibody to cleaved caspase-3, we found that both compounds also increased the number of caspase-3 positive apoptotic cells (**Figure 2A (c, f)** and **Figure 2D**). Caspase-3 positive cells increased to 11.74% ± 0.86 (p=0.0141) for 5μM RG-7112 and by 10.87% ± 1.03 (p=0.0144) for 100μM o-Vanillin, compared with the untreated control (9.09% ± 0.77).

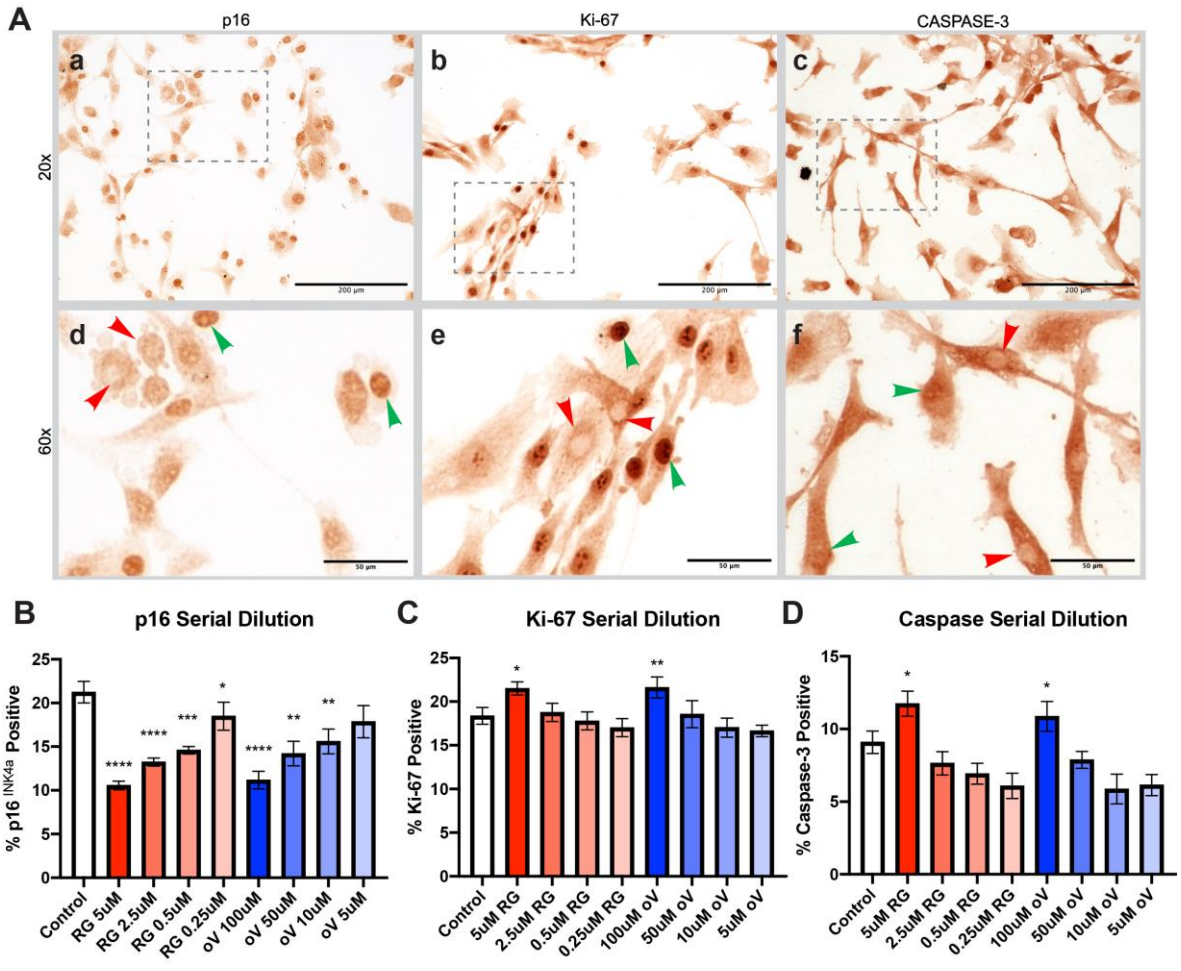

Figure 2. Identifying the lowest effective concentration of RG-7112 and o-Vanillin at which senolytic activity is preserved in monolayer culture. **(A)** Representative images of monolayer cultures stained with (a) $p16^{INK4a}$, (b) Ki-67 and (c) Caspase-3. (d-f) Magnified images of (a-c) with arrowheads representing positive (green) and negative (red) stained cells. Scale bars (A); (a-c) 200µm and (d-f) 50µm. Quantification of senolytic serial dilution experiments of **(B)** $p16^{INK4a}$, **(C)** Ki-67 and **(D)** Caspase-3 expression. Values are presented as mean ± SD in (**B-D**). * Indicates significance between treatment and control group. All analyses were performed using a one-way ANOVA, n = 7 donor samples. * or # $p < 0.05$; ** or ## $p < 0.01$; *** or ### $p< 0.001$, **** or #### $p < 0.0001$.

Given that 5µM of RG-7112 and 100µM of o-Vanillin resulted in the most significant decrease in senescence, combinations of these concentrations were selected for future experiments. Moreover, 2.5µM of RG-7112 and 50 µM of o-Vanillin were further investigated, given that they both resulted in a significant decrease in the expression of $p16^{INK4a}$. Four

combinations were assessed, 5 μM of RG-7112 and 100 μM of o-Vanillin; 2.5 μM of RG-7112 and 50 μM of o-Vanillin.

A combination of o-Vanillin and RG-7112 has additive apoptotic and proliferative activity in pellet cultures

To further assess the combination of RG-7112 and o-Vanillin, pellet cultures of IVD cells from patients with low back pain and IVD degeneration were exposed to the TLR-2/6 agonist for 48 hours, treated with senolytics (in combination or single drugs) for four days and then cultured for a total of 24 days (**Figure 3A**). The control group only received TLR-2/6 agonist for 48h but no senolytics. Pellet cultures of IVD cells were used as they have been suggested to mimic 3-dimensional *in vivo* conditions more closely, allowing for the evaluation of the matrix synthesis.

The pellet cultures' expression of $p16^{INK4a}$, apoptotic (Caspase-3), and proliferative (Ki-67) capacities were assessed. All treatments significantly reduced the number of senescent cells compared to the control. The largest decrease in $p16^{INK4a}$ positive cells was observed in pellet cultures treated with the combination of 5 μM of RG-7112 and 100 μM of o-Vanillin. Control cultures had 18.97% ± 1.89 while treated with combination cultures had 3.74% ± 1.52 ($p=0.0004$) senescent cells (**Figure 3B (b, g)** and **Figure 3D**). The second-most efficient treatment was with a combination of 2.5 μM of RG-7112 and 100 μM of o-Vanillin, which reduced senescent cells to 5.77% ± 1.38 ($p=0.0002$), followed by 5 μM of RG-7112 and 50 μM of o-Vanillin which reduced senescent cells to 9.26 ± 1.35 ($p=0.0092$) and 5 μM RG-7112 and 100 μM o-Vanillin applied as single drugs which reduced the number of senescent cells to 11.09% ± 0.98 ($p<0.0001$), and 10.31% ± 0.84 ($p<0.0001$), respectively (**Figure 3D**). Compared to RG-7112 and o-Vanillin alone, only 5 μM of RG-7112 and 100 μM of o-Vanillin

(relative to RG-7112 p=0066, relative to o-Vanillin p=0.0039) and 2.5 μM of RG-7112 and 100 μM of o-Vanillin (relative to RG-7112 p=0.0033, relative to o-Vanillin p=0.0071) significantly enhance the effect.

The effect on proliferation with the greatest increase in Ki-67 positive cells was observed when treating cells with 5 μM of RG-7112 and 100 μM of o-Vanillin relative to control. Proliferating cells increased from 15.24% ± 1.10 to 36.74% ± 3.25 (p=0.0011). Treatment with 2.5 μM of RG-7112 and 100 μM of o-Vanillin showed 29.27% ± 2.35 proliferating cells, and 5 μM of RG-7112 and 50 μM of o-Vanillin showed 20.93 ± 2.99 proliferating cells (p=0.0415). The percentage of proliferating cells was 22.52% ± 3.08 (p=0.0132) and 21.40% ± 4.17 (p=0.0588) in RG-7112 and o-Vanillin-treated cultures. Compared to RG-7112 and o-Vanillin alone, only the two combinations of 100 μM of o-Vanillin with either 5 μM (relative to RG-7112 p=0.0241, relative to o-Vanillin p=0.0026) or 2.5 μM (relative to RG-7112 p=0.0055, relative to o-Vanillin p=0.0257) of RG-7112 significantly enhance the effect (**Figure 3C (b, e)** and **Figure 3E**). Moreover, relative to the control (10.80% ± 1.43), the greatest increase in caspase-3 positive cells was observed when treating cells with a combination of 5 μM of RG-7112 and 100 μM of o-Vanillin (17.73% ± 1.54, p=0.0057) (**Figure 3B(c, h)** and **Figure 3F**). Interestingly, when assessing colocalization of caspase-3 and $p16^{INK4a}$, combination-treated pellets (i.e. 5μM RG-7112 and 100μM o-Vanillin) had a significantly greater number of senescent cells undergoing apoptosis relative to the RG-7112 and o-Vanillin single senolytic groups (the percentage of caspase-3 and $p16^{INK4a}$ positive cells colocalized in combination treatment was 54.57% ± 5.85 compared with RG-7112 35.96% ± 7.71 (p=0.036) and o-Vanillin 38.04% ± 7.52 (p=0.0357) (**Figure 3B(d, i)** and **Figure 3G**).

Furthermore, an Alamar Blue assay, which assesses metabolic activity, was performed to confirm that the RG-7112 and o-Vanillin combination treatment was not toxic to non-senescent cells in pellet cultures from degenerate tissue. No significant difference in metabolic activity was observed in any combination or single senolytic treatment groups (**Figure 3H**). Finally, to assess the effect of combination treatment on matrix synthesis, a DMMB assay was performed to measure sGAG release into the culture media. An increase in proteoglycan content was observed through a significant increase in sGAG release on day 12 of culture (**Figure 3I**). The combination of 5 μM of RG-7112 and 100 μM of o-Vanillin resulted in the greatest sGAG release relative to control (combination 9.74μg/mL ± 1.17 vs. control 3.14μg/mL ± 0.61, p=0.0002) (**Figure 3I**). Similarly, when compared to the single treatment of RG-7112 (combination 9.74μg/mL ± 1.17 vs. RG-7112 7.68μg/mL ± 0.68, p=0.0231) and the single treatment with o-Vanillin (combination 9.74μg/mL ± 1.17 vs. o-Vanillin 6.89μg/mL ± 1.27, p=0.0139), the combined treatment still had higher sGAG released into the culture media (**Figure 3I**).

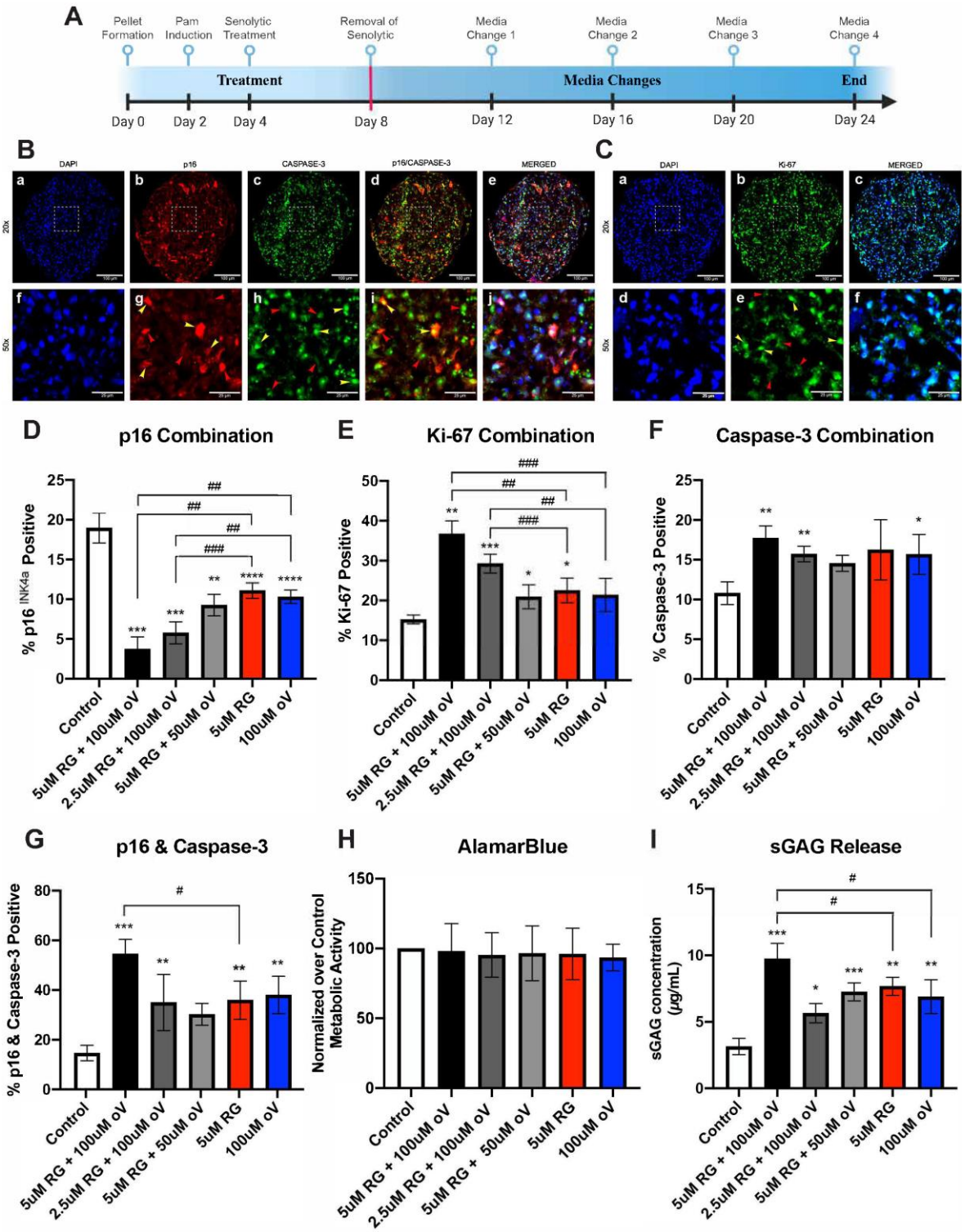

Figure 3. Combination treatment with o-Vanillin and RG-7112 results in additive apoptotic and proliferative activity in pellet cultures. **(A)** Timeline of pellet culture. **(B)** Representative images of pellets stained with (a) DAPI, (b) $p16^{INK4a}$, (c) Caspase-**3**, (d) $p16^{INK4a}$ and Caspase-3 merged staining, (e) $p16^{INK4a}$, Caspase-3 and DAPI merged staining. (f-j) Magnified images of (a-c) with arrowheads representing positive (yellow) and negative (red) stained cells. Scale

bars (a-e) 100µm and (f-j) 25µm. **(C)** Representative images of pellet cultures stained with (a) DAPI, (b) Ki-67, and (c) DAPI and Ki-67 merged. (d-f) Represent magnified images of (a-c) with arrowheads representing positive (yellow) and negative (red) stained cells. Scale bars (a-c) 100µm and (d-f) 25µm. Quantification of pellet fluorescent staining of **(D)** $p16^{INK4a}$, (E) Ki-67, (F) Caspase-3 and (G) $p16^{INK4a}$ and Caspase-3 expression. **(H)** AlamarBlue assessed the evaluation of cell viability. **(I)** sGAG concentration measured by DMMB assay using day 12 pellet culture media. Values are presented as mean ± SD in **(D-I)**. * Indicates significance between treatment (single or combination) and control group. # Indicates significance calculated when combination treatment is compared to single treatment groups. All analyses were performed using a one-way ANOVA, n = 7 donor samples. * or # $p < 0.05$; ** or ## $p < 0.01$; *** or ### $p < 0.001$, **** or #### $p < 0.0001$.

Combination treatment with RG-7112 and o-Vanillin results in a significant decrease of SASP factor release in pellet cultures

As has been shown, both RG-7112 and o-Vanillin each reduce SASP factor release in monolayer cultures [191] (**Figure 1**). Next was to evaluate whether combined treatment with these two senolytics has an additive effect on reducing SASP factor release. Pellet culture media was used to assess protein concentrations of cytokines (IL-6, IL-8, IL-1β, TNF-α) and neurotrophins (BDNF and NGF). The concentrations in control media were IL-6: 832.9pg/mL ± 50.39, IL-8: 639.7pg/mL ± 40.46, IL-1β: 120.9pg/mL ± 12.02, TNF-α: 268.8pg/mL ± 41.67, BDNF: 1777pg/mL ± 138.1 and NGF: 311.1pg/mL ± 23.57. The greatest reduction in cytokine and neurotrophin expression was observed using the combination of 5 µM of RG-7112 and 100 µM of o-Vanillin. IL6 decreased to 558.3 pg/mL ± 70.30 ($p=0.0034$), IL-8 to 400.2 pg/mL ±31.14 ($p=0.0003$), IL-1β to 70.86 pg/mL ± 4.399 ($p=0.0002$), TNF-α to 157.7 pg/mL ± 19.47 ($p=0.0109$), BDNF to 808.5 pg/mL ± 62.77 ($p<0.0001$) and NGF to 166.1 pg/mL ± 8.89 ($p=0.0001$) (**Figure 4A-F**). However, the combination treatment was only significantly better at reducing IL-6, IL-8, and IL-1β relative to the single-group treatments (**Figure 4A-C**). TNF-α protein expression reduction was only significantly reduced when comparing combination treatment to single treatment with RG-7112 and not with o-Vanillin (**Figure 4D**). Relative to

the single treatments, only the combination of 5 μM of RG-7112 and 100 μM of o-Vanillin significantly reduced BDNF (relative to RG-7112 p=0.0002, relative to o-Vanillin p=0.0013) and NGF protein expression (relative to RG-7112 p=0.0073, relative to o-Vanillin p=0.0083) (**Figure 4E** and **F**).

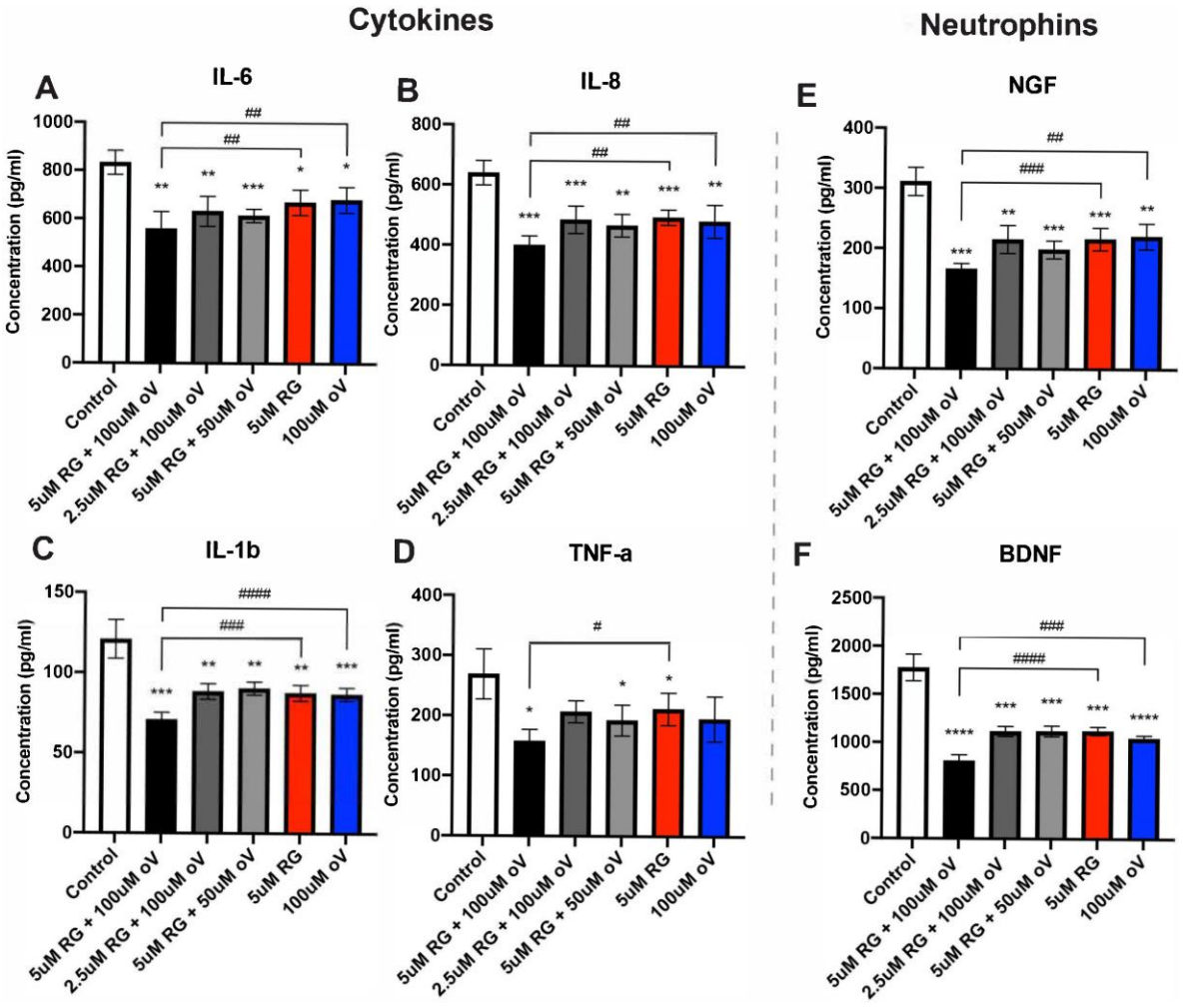

Figure 4. Combination treatment with RG-7112 and o-Vanillin significantly decreases SASP factor release in pellet cultures**.** Pellet culture media was analyzed by Raybio Human Cytokine Array. The cytokine concentration of **(A)** IL-6, **(B)** IL-8, **(C)** IL-1β, **(D)** TNF-α and neurotrophic factors **(E)** NGF and **(F)** BDNF were evaluated. Values are presented as mean ± SD in **(A-F)**. * Indicates significance between treatment (single or combination) and control group. # Indicates significance calculated when combination treatment is compared to single treatment groups. All analyses were performed using a one-way ANOVA, n = 7 donor samples. * or # $p < 0.05$; ** or ## $p < 0.01$; *** or ### $p < 0.001$, **** or #### $p < 0.0001$.

Pellet media from RG-7112 and o-Vanillin treated pellets induced less neurite sprouting and neurite growth gene expression in PC-12 cells

As seen from the ELISA data above, two of the SASP factors released after induction with TLR-2/6 agonist in cells from painful degenerate IVDs are NGF and BDNF. It has previously been shown that stimulation with NGF can cause neurite sprouting in PC-12 cells. Therefore, to assess the difference in levels of PC-12 sprouting, these cells were cultured for 48 hours in culture media of non-treated and senolytic-treated pellets. Gene expression analysis of common neuronal markers was performed to confirm that pellet culture media induces neuronal differentiation. After 48 hours of culture, NF-L, Plaur, Plk2, PVR, and VGF were significantly lower in the 5 μM of RG-7112 and 100 μM of o-Vanillin combination treated culture media samples than that of non-treated control (Control: NF-L: 12.06 ± 1.65, Plaur: 6.21 ± 0.39, Plk2: 12.40 ± 0.73, PVR: 11.88 ± 1.46 and VGF: 6.00 ± 0.48) and (Combination: NF-L: 4.54 ± 0.79, p=0.001669; Plaur: 2.10 ± 0.61, p=0.001669; Plk2: 5.05 ± 0.57, p=0.002624; PVR: 5.88 ± 0.42, p=0.001669; VGF: 2.07 ± 0.72, p=0.001669) (Figure 5A). Interestingly, for NF-L, Plaur and PVR, the combination treatment (5 μM of RG-7112 and 100 μM of o-Vanillin) was only better than the RG-7112 treatment groups (NF-L: RG-7112: 7.8 ± 1.08, p=0.008804; Plaur: RG-7112: 3.39 ± 0.39, p=0.003223; PVR: RG-7112: 8.63 ± 1.30, p=0.002073) (**Figure 5A**). Next was to observe the number of PC-12 cells that had neurite growth. The lowest number of neurite growth was found in the 5 μM of RG-7112 and 100 μM of o-Vanillin combination-treated group when compared to the non-treated group (combination 60.93% ± 4.00 vs. control 39.91% ± 2.92, p<0.0001) (**Figure 5B** and **C**). When compared to the single treatment groups, the combined treatment (5 μM of RG-7112 and 100 μM of o-Vanillin) had significantly decreased the number of cells with extended neurites from 60.93%

± 4.00 for the combination to 49.86% ± 3.84 for RG-7112, ($p<0.0001$) and to 47.74% ± 4.65 ($p<0.0001$) for o-Vanillin (**Figure 5B-C** and **Supplemental Figure 1**).

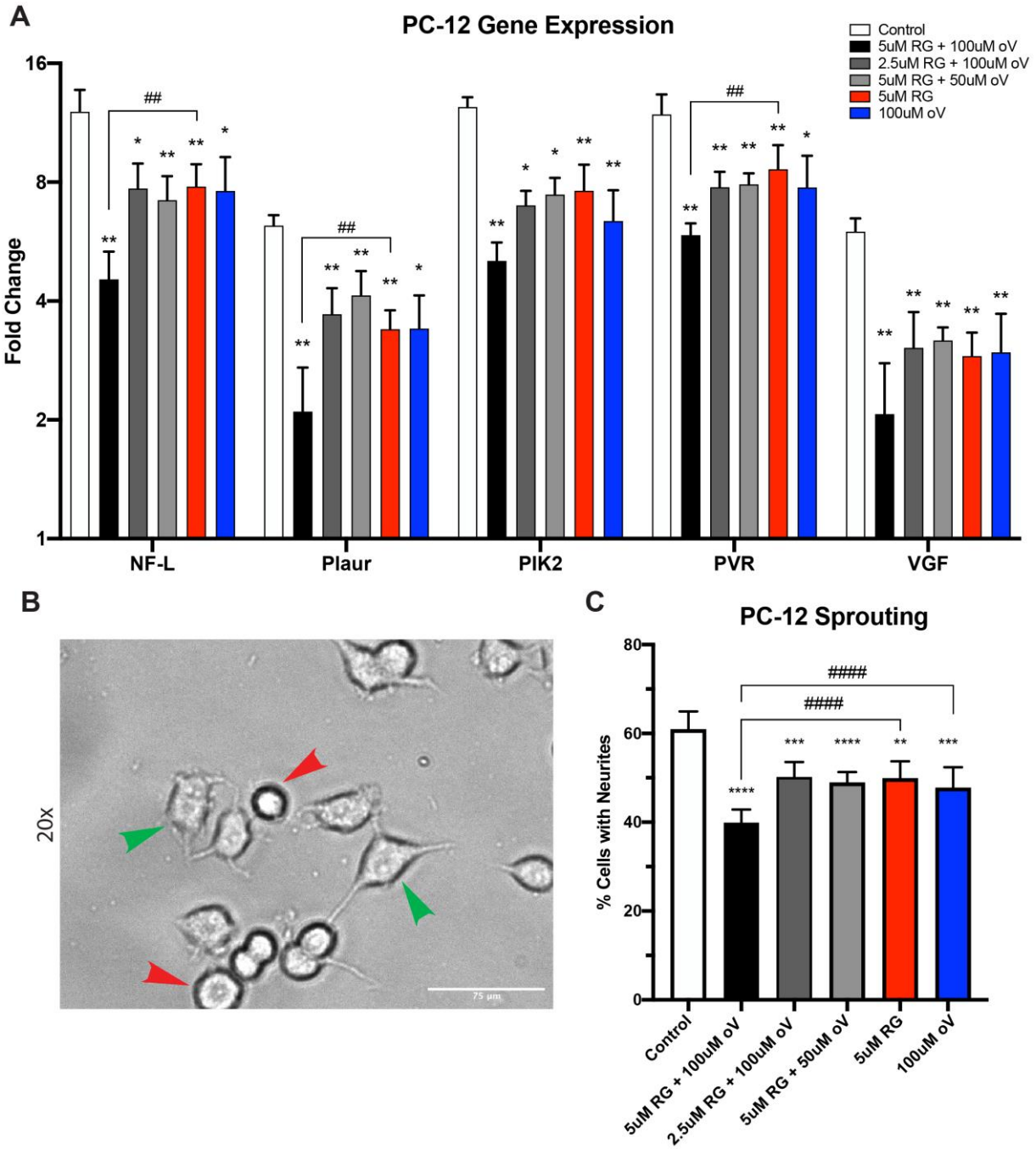

Figure 5. Pellet media from RG-7112 and o-Vanillin combined treated pellets have lower levels of neurite sprouting and neurite growth gene expression in PC-12 cells. **(A)** Gene expression of neurite growth factors (NF-L, Plaur, PIK-2, PVR and VGF) was evaluated in PC-12 cells cultured in day 12 pellet media for 48 hours. qRT-PCRs were normalized to culture media from pellets that were not induced with Pam2CSK4 and no senolytic treatment. **(B)** Representative

images of neurite sprouting with arrowheads indicating neurite sprouting (green) and no neurite sprouting (red). Scale bar 75μm. **(C)** Quantification of PC-12 neurite sprouting. Values are presented as **(A)** fold change ± SD or **(C)** mean ± SD. All analyses were performed using a one-way ANOVA, n = 7 donor samples. * or # $p < 0.05$; ** or ## $p < 0.01$; *** or ### $p < 0.001$, **** or #### $p < 0.0001$.

Discussion:

Previously, we demonstrated that TLR-2/6 activation induces cell senescence and SASP factor release in IVD cells[191]. We further illustrated that using o-Vanillin, a senolytic compound, senescence was attenuated in TLR-2/6 induced IVD cells[191]. Our lab has also shown that the senolytic compound RG-7112 has senotherapeutic activity on IVD cells[129]. The role of RG-7112 is further elucidated in this manuscript, where we demonstrated that, like o-Vanillin, RG-7112 could attenuate the effect of TLR-2/6 induction on cells from painful degenerate IVD. Since both compounds have similar effects but target different pathways, there is a potential for these compounds to work when used in combination. Therefore, one objective of the current study was to demonstrate that combining o-Vanillin and RG-7112 can supersede the effect of o-Vanillin or RG-7112 alone. Moreover, knowing that cells from painful degenerate IVD can drive neurite sprouting[167], we sought to determine if treating IVD cells with senolytic compounds alone or in combination can reduce neuronal sprouting, which has been reported as an indirect indicator for chronic pain.

Cellular senescence can be induced by replicative senescence or stress-induced premature senescence[195]. In low back pain patients, cellular senescence is thought to be majorly stress-induced. Using a model with a TLR-2/6 agonist, we can mimic a “stress-induced” environment often seen in degenerate IVDs[191]. Eliminating senescent cells from multiple tissues or even a single tissue will probably require combinations of multiple senotherapeutic drugs[132]. This model shows that 100 μM of o-Vanillin or 5 μM of RG-7112 each provide

2213 senolytic activity. One benefit of combining treatments is that each independent compound's
2214 concentration could be reduced, reducing the risk of side effects. To find the concentrations at
2215 which each drug maintained senolytic potential, dilutions of o-Vanillin and RG-7112 were
2216 evaluated in monolayer cultures. The senolytic capabilities (i.e., remove $p16^{INK4a}$ positive cells,
2217 increase proliferation and increase apoptosis) were reduced once the concentration of o-
2218 Vanillin is below 50 μM and the concentration of RG-7112 is below 2.5μM. Being that this is
2219 the first time that the combination of o-Vanillin and RG-7112 is being assessed, we decided
2220 only to use concentrations at which the senolytic compounds alone demonstrated senolytic
2221 activity, which is why we did not go below 50 μM for o-Vanillin and 2.5 μM for RG-7112 in
2222 the treatment groups (single or combined treatment).

2223 The assessment of monolayer culture at low passages is an acceptable model to
2224 determine if compounds have senolytic effects; however, to better understand if there is any
2225 physiological relevance to these treatments, a model that mimics the physiological environment
2226 of the human disc is required. As previously described, 3D pellet models are relevant models
2227 to simulate the physiological environment and matrix formation of the IVDs[84,129,196-199]. When
2228 studying senescent cells and senolytic drugs, it is also important to keep cell expansion to a
2229 minimum, as expansion is selected for highly proliferating cells. This limits the number of cells
2230 available, and choose to use 100,000 cells per 3D pellet. In this model, we further induced
2231 senescence with a TLR-2/6 agonist and treated the cells with senolytics. The combination of
2232 100μM of o-Vanillin + 5 μM RG-7112 had a greater effect than the single treatment in reducing
2233 senescence and SASP factor release. It also increased senescent cells undergoing apoptosis and
2234 the number of proliferating non-senescent cells. Proteoglycan production was also enhanced.
2235 This data concords with published data[200,201]. This potential additive effect between o-Vanillin

and RG-7112 is similar to that previously published in the literature for combination therapy with Fisetin, Dasatinib and Quercetin in the context of osteoarthritis[132]. Like o-Vanillin and RG-7112, Dasatinib and Quercetin target antiapoptotic networks instead of a single target. This was possible by leveraging natural and synthetic forms of senolytics. Moreover, with Dasatinib and Quercetin combination treatment, lower therapeutic dosages were used, and fewer side effects were observed than with the single treatment groups. Recently, it has been shown that multiple and long-term injections of a combination of Dasatinib and Quercetin in elderly mice reduce $p16^{INK4a}$ and SASP expression levels in the IVD[200]. Dasatinib and Quercetin combination treatment also increased the expression of extracellular matrix proteins, thereby restoring tissue homeostasis[200]. A phase II clinical trial initiated in 2020 is currently undergoing using a combination of the senolytic compounds Fisetin, Dasatinib, and Quercetin to target cellular senescence to improve skeletal health in older humans; data is set to be available in 2023[132]. Of note, it is difficult to compare the combination treatment of Dasatinib and Quercetin with the RG-7112 and o-Vanillin combination since the Dasatinib and Quercetin study did not provide a single drug control[200]. Collectively, this supports the need to investigate further the role of senolytics in managing cellular medical conditions.

With the growing body of evidence supporting the critical value of senolytic compounds in musculoskeletal diseases, there has been an increasing interest in evaluating the effect of senolytic compounds in chronic pain. An association between chronic pain and senescence has previously been suggested in mice subjected to experimental nerve damage. Here, reduced telomere length and p53-mediated cellular senescence in the spinal cord resulted in mice maintaining a high pain level[202]. Low back pain is often associated with IVD degeneration, but how disc degeneration is related to low back pain is not fully understood.

Pain is thought to develop 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. Among the factors identified in IVD-conditioned media, degenerating and painful IVDs show elevated levels of NGF and BDNF [76,167]. Surprisingly, the relatively low concentrations of these factors in the conditioned media significantly induced sprouting in the PC-12 cells[76,167]. In the literature, anti-NGF treatment has been shown to reduce neurite sprouting, demonstrating an important role for NGF in degenerating IVDs, even at low concentrations [76,167]. In the current study, combination and single treatment with o-Vanillin and/or RG-7112 significantly reduced both NGF and BDNF release compared to the untreated IVD cell pellets. Furthermore, we observed that the culture media from the treated pellets induced significantly less gene expression of markers associated with neurite sprouting and less neurite sprouting visualized in PC-12 cells. These observations are the first inference that senolytic treatment is linked to reducing pain markers in low back pain. Previously, the reduction of pain markers with a combination of senolytic drugs has been observed in an *in vivo* osteoarthritis model where they observed a decrease in NGF, calcitonin gene-related peptide (CGRP) and selectively removed senescent cells[203] further supporting our findings. Overall, we hypothesize that due to the removal of senescent cells, cells secreting neurotrophic factors are reduced, which in turn limits neurite sprouting, ultimately decreasing low back pain.

Future studies should use *in vivo* models of disc degeneration to better represent the physiological environment of the IVD and to utilize pain behaviour techniques to better understand if o-Vanillin and RG-7112 can lower pain levels in a live animal model. Moreover, a greater understanding of the molecular mechanisms of how o-Vanillin and RG-7112 work in

combination for IVD degeneration may lead to improved therapies and quality of life for individuals with discogenic pain.

In conclusion, the present study shows that combining o-Vanillin and RG-7112 greatly enhanced the effect of either drug alone in reducing the number of senescent cells. Additionally, we demonstrated that combination treatment reduced gene expression of markers involved in neurite sprouting and decreased sprouting in PC-12 cells. Our results support the potential of senolytics to reduce chronic pain.

Acknowledgments: None to disclose

Conflicts of interest: None to declare

Ethics: 2019-4896

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Author Contributions: Conceptualization M.M., O.W.M, H.C and L.H.; Methodology, M.M., O.W.M and L.H.; Validation, M.M., O.W.M and L.H.; Formal analysis, M.M., O.W.M, and

L.H.; Investigation, M.M., O.W.M., K.S., L.L.; Resources, R.N.R, P.J., J.A.O and L.H.; Data curation, M.M. and O.W.M Writing and initial draft preparation, M.M.; Writing review and editing, M.M., O.W.M, H.C and L.H.; Visualization, M.M., O.W.M, H.C and L.H.; Supervision, H.C. and L.H.; Project administration, L.H.; Funding acquisition, H.C. and L.H. All authors have read and agreed to the published version of the manuscript.

Data availability statement: The data supporting this study's findings are available from the corresponding author upon reasonable request.

Supplementary Table 1: Characteristics of the Painful Degenerate Donors Utilized in the study (-). (ICC): Immunocytochemistry of monolayer culture including $p16^{INK4a}$, Ki-67 and Caspase-3. Immunofluorescence of pellet culture for $p16^{INK4a}$, Ki-67 and Caspase-3. (RT-qPCR): Real-time Quantitative Polymerase Chain Reaction. (ELISA): Enzyme-linked immunosorbent assays, (DMMB): Dimethyl methylene blue (DMMB) assays.

Donor	Age	Sex	ICC	IHC	RT-qPCR	ELISA	DMMB
1	32	M	-		-	-	
2	65	F	-		-	-	
3	66	M	-		-	-	
4	47	F	-		-	-	
5	61	F	-	-	-	-	-
6	55	F	-	-	-	-	-
7	45	M	-	-	-	-	-
8	35	F		-	-	-	-
9	67	M		-	-	-	-
10	43	M		-	-	-	-

11	56	M		-	-	-	-

2320

2321

2322

2323 **Supplementary Table 2:** qRT-PCR Primer Sequences

2324

Target	Forward Primer Sequence	Reverse Primer Sequence	Reference
BDNF	5'-TAACGGCGGCAGACAAAAAGA-3'	5'-GAAGTATTGCTTCAGTTGGCCT-3'	[111]
CCL2	5'-GCATGAAAGTCTCTGCCG-3'	5'-GAGTGTTCAAGTCTTCGGA-3'	[180]
CCL5	5'-GAAGGTCTCCGCGGCAGCC-3'	5'-CTGGGCCCTTCAAGGAGCGG-3'	[180]
CCL7	5'-CACTTCTGTGTCTGCTGCTCAC-3'	5'-GTTTTCTTGTCCAGGTGCTTCATA-3'	[181]
CCL8	5'-GCCTGCTGCTCATGGCAGCC-3'	5'-GCACAGACCTCCTTGCCCCG-3'	[180]
CXCL-10	5'-GTGGCATTCAAGGAGTACCTC-3'	5'-TGATGGCCTTCGATTCTGGATT-3'	[180]
CXCL-8/IL-8	5'-TCCTGATTTCTGCAGCTCTG-3'	5'-GTCTTTATGCACTGACATCTAAGTTC-3'	[84]
G-CSF	5'-GAGCAAGTGAGGAAGATCCAG-3'	5'-CAGCTTGTAGGTGGCACACTC-3'	[182]
GAPDH	5'-TCCCTGAGCTGAACGGGAAG-3'	5'-GGAGGAGTGGGTGTCGCTGT-3'	[76,84]
GM-CSF	5'-TCTCAGAAATGTTTGACCTCCA-3'	5'-GCCCTTGAGCTTGGTGAG-3'	[182]
GRO/CXCL-1	5'-TGAAGGCAGGGGAATGTATGTG-3'	5'-AGCCCCTTTGTTCTAAGCCA-3'	[183]
IFN-γ	5'-AACTACTGATTTCAACTTCTT-3'	5'-ATTACTGGGATGCTCTT-3'	[204]
IL-1β	5'-ACAGATGAAGTGCTCCTTCCA-3'	5'-GTCGGAGATTCGTAGCTGGAT-3'	[52]
IL-6	5'-TGAACCTTCCAAAGATGGCTG-3'	5'-CAAACTCCAAAAGACCAGTGATG-3'	[84]
NGF	5'-AAGTGCCGGGACCCAAAT-3'	5'-TGAGTTCCAGTGCTTTGAGTCAA-3'	[52]
$p16^{INK4a}$	5'-CTGCCCAACGCACCGAATA-3'	5'-GCTGCCCATCATCATGACCT-3'	[84]
p21	5'-GAGACTCTCAGGGTCGAAAAC-3'	5'-GGCGTTTGGAGTGGTAGAAA-3'	[84]
TGF- β	5'-TCCTGGCGATACCTCAGCAA-3'	5'-CTCAATTTCCCCTCCACGGC-3'	[184]
TLR-1	5'-CAGTGTCTGGTACACGCATGGT-3'	5'-TTTCAAAAACCGTGTCTGTTAAGAGA-3'	[52]
TLR-2	5'-GGCCAGCAAATTACCTGTGTG -3'	5'-AGGCGGACATCCTGAACCT-3'	[52]
TLR-4	5'-CAGAGTTTCCTGCAATGGATCA-3'	5'-GCTTATCTGAAGGTGTTGCACAT-3'	[52]
TLR-6	5'-GAAGAAGAACAACCCTTTAGGATAGC-3'	5'-AGGCAAACAAAATGGAAGCTT-3'	[52]
TNF-α	5'-ATGTTGTAGCAAACCCTCAAGC-3'	5'-TCTCTCAGCTCCACGCCATT-3'	[205]
NF-L	5′-AGACATCAGCGCCATGCA-3′	5′-TTCGTGCTTCGCAGCTCAT-3′	[206]
VGF	5′-GCTCGAATGTCCGAAAACGT-3′	5′-ACACTCCTTCCCCGAACTGA-3′,	[206]
PVR	5′- ATGAGTGTCAGATTGCCACGTT-3′	5′- TCGGGCGAACACCTTCAG-3′	[206]
Plaur	5′- GGCTGGACCCAGGAACTTTT-3′	5′- CGCCTGTCCTCAAAGATGGA-3′	[206]
Plk2	5′- GCCCCACACCACCATCA-3′	5′-GGTCGACTATAATCCGCGAGAT-3′	[206]

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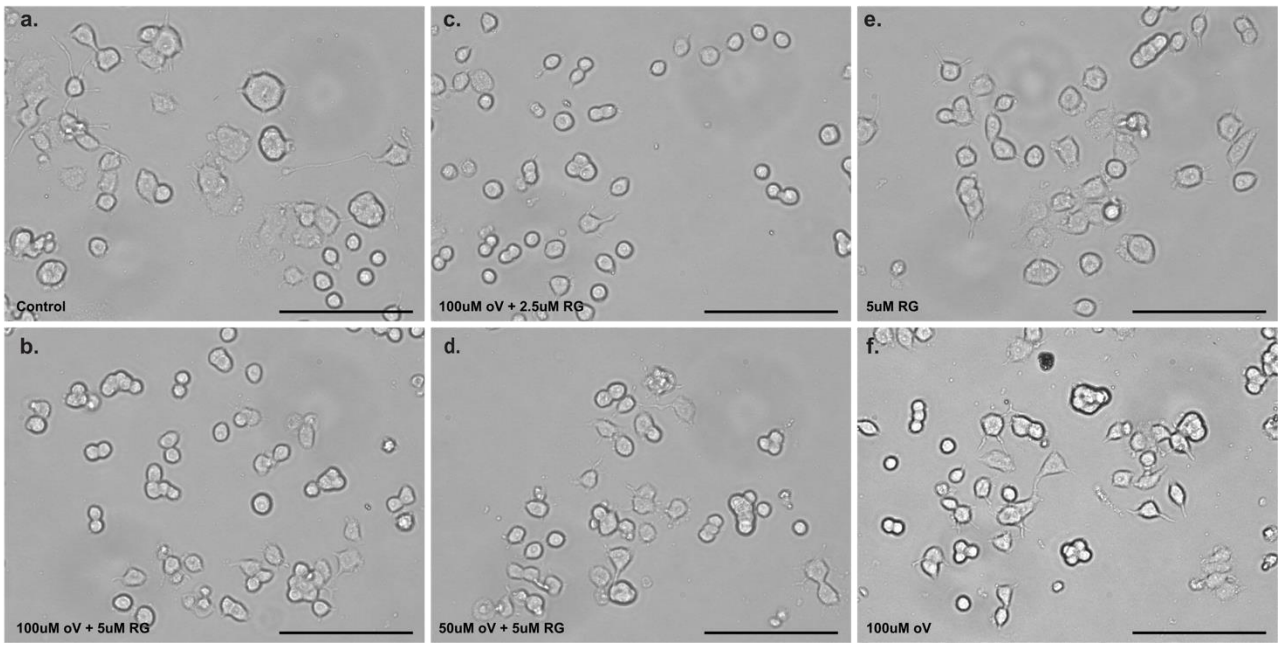

Supplemental Figure 1: PC-12 monolayer cultures treated with pellet media. (A) PC-12 cells were cultured for 48 hours in day 12 pellet media from the untreated and treated groups with either a single or a combination of the two senolytics (o-Vanillin and RG-7112). Over 2000 cells were quantified for each condition by counting 10 images from 7 different donors per group to determine the percentage of cells with neurites for each experimental condition. Representative image of PC-12 monolayer sprouting treated with (a) no senolytic treatment control, (b) 100µM o-Vanillin + 2.5µM RG-7112, (c) 5µM RG-7112, (d) 100µM o-Vanillin + 5µM RG-7112, (e) 50µM o-Vanillin + 5µM RG-7112 and (f) 100µM o-Vanillin. (a-f) Scale bar: 200µm.

Chapter 3 Preface:

Lower back pain is a global health problem that is directly related to IVD degeneration. It is experienced by approximately 80% of individuals at some time in their lifespan. Despite its prevalence, little is known about the mechanisms leading to painful IVD degeneration, leaving surgical removal and vertebral fusion in end-stage disease as the most common treatment. The personal costs in reduced quality of life, and the economic cost to the healthcare system are enormous. There is growing recognition that senescent cells accumulate during tissue degeneration, where they contribute directly to disorders like heart-disease, cancer, and osteoarthritis. In tissues with healthy homeostasis, anabolism and catabolism are in balance. In contrast, in ageing and degenerating IVDs, tissue homeostasis is disrupted by the accumulation of senescent cells producing inflammatory and nociceptive factors that cause pain and inflammation, along with proteases degrading the tissue. We have previously demonstrated that senolytic drugs (RG-7112 and o-Vanillin) target and remove senescent cells from the IVDs both *in vitro* and *in vivo,* improving tissue homeostasis and providing symptomatic pain relief. An obstacle associated with using the senolytic agents currently under investigation is their failure to target the same cell type in different species or different cell types within a species. It is, therefore, imperative to test novel agents for effectiveness against senescent cell burden, and for potential deleterious side effects, in pre-clinical models with clinically relevant cells. Mice with targeted deletion of the gene encoding SPARC have been characterized as a useful model of pain associated with IVD degeneration. SPARC-null mice have progressive, age-dependent, intervertebral disc degeneration and back pain, presenting a clinically relevant model of low back pain. The ability to prevent or even slightly delay the onset of low back pain would have a tremendous socio-economic impact. The overall objective of this chapter is to

determine if a single drug or combination treatment with o-Vanillin and RG-7112, can selectively remove senescent cells, reduce inflammatory mediators, and relieve pain behaviour in middle-aged SPARC-null mice with back pain *in vivo*. This manuscript is in preparation for submission.

Chapter 3

Title: Evaluation of RG-7112 and o-Vanillin Treatment for Intervertebral Disc Degeneration in SPARC-null Mice

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Ethics: The animal study was approved by the ethical review board at McGill University (AUP-5405).

ABSTRACT:

Purpose: Low back pain is a global health problem that is directly related to intervertebral disc (IVD) degeneration. Despite its prevalence, little is known about the mechanisms leading to painful IVD degeneration. In ageing and degenerating IVDs, tissue homeostasis is disrupted by the accumulation of senescent cells producing inflammatory and nociceptive factors that cause pain and inflammation, along with tissue degrading. The overall objective of this study is to determine if two senolytic drugs, o-Vanillin and RG-7112, can selectively remove senescent cells, reduce inflammatory mediators, and relieve pain in middle-aged SPARC null mice.

Methods: 7-month-old SPARC-null mice with signs of IVD degeneration and low back pain and age-matched wild-type C57BL6 female and male animals were used. The mice were randomized into seven treatment groups, and drugs were administered by oral gavage once a week for 8 weeks. Inflammatory mediators, pain behaviour, pain markers and senescence burden were assessed.

Results: Cold allodynia, radicular pain, and axial discomfort were significantly reduced as of 4 weeks of treatment in the SPARC-null mice treated with 100 mg/kg of o-Vanillin and/or 5 mg/kg of RG7112 alone and in combination. The drugs also significantly decreased inflammatory mediators, pain markers and senescence burden in IVDs and dorsal horns. Combining the drugs had a greater effect on all outcome measures.

Conclusion: The senolytic drugs RG-7112 and o-Vanillin reduced pain behaviour, pain mediators and improve tissue homeostasis in a clinically relevant mouse model of low back pain and IVD degeneration. A combination of the drugs provided an additive effect and could potentially serve as a treatment for back pain.

INTRODUCTION:

Low back pain is experienced by approximately 80% of individuals in their lifetime and is globally the number one cause of years lived with disability[186]. This age-related health problem is associated with intervertebral disc (IVD) degeneration in many individuals[207-209]. Despite its prevalence, little is known about the cellular and molecular mechanisms leading to painful IVD degeneration resulting in few early interventions, leaving surgical removal and vertebral fusion in end-stage disease as the most common treatment. The personal costs in reduced quality of life and the economic cost to healthcare systems are enormous[210], exceeding $100 billion per year in the US alone[211].

There is growing recognition that senescent cells accumulate with ageing and during tissue degeneration, where they contribute directly to disorders including heart disease, cancer and osteoarthritis[85,121,122,152,212,213]. Similarly, senescent cells accumulate as IVDs degenerate, contributing to disease progression and back pain[82,83,214]. Senescent cells accumulate due to successive shortening of telomere length during replicative cycles[79,215].

Senescence can also be induced prematurely by stressors, including DNA-damaging agents, oxidative stress, mitochondrial dysfunction, load-induced injury, and disruption of epigenetic regulation; this is termed stress-induced premature senescence (SIPS)[80,81]. Increases in senescent cells occur in degenerating IVDs, also in younger individuals with painful IVD degeneration and/or herniation[82-84]. Senescent cells are resistant to apoptosis and, in addition to changes in their replicative status, they release an array of inflammatory cytokines, chemokines, and proteases known collectively as the senescence-associated secretory phenotype (SASP)[85]. The inflammatory environment triggered by senescent cells prevents adjacent cells from maintaining tissue homeostasis, and it is proposed to induce senescence in

a paracrine manner, thus exacerbating tissue deterioration[86-88]. All senescent cells share these general features, but there are distinct differences in SASP, and anti-apoptotic pathways linked to cell type, species, and inducer of senescence[89,90].

As senescent cells are heterogenous and cell-type specific, testing novel agents for effectiveness in both pre-clinical models and with clinically relevant human target cells is crucial. However, the primary manifestation of IVD morbidity in humans is low back pain, which is impossible to evaluate in cell and tissue culture and difficult to assess in animals. Mice with targeted deletion of the gene encoding secreted protein acidic and rich in cysteine (SPARC), have been characterized as a valuable model of pain associated with IVD degeneration[143,145,147,216,217]. SPARC function is compensated by the protein HEVIN in most tissue. However, the lack of HEVIN expression in the IVD may explain the pronounced IVD phenotype found in SPARC-null mice[218]. Mutations in the SPARC genes are linked to back pain in human patients, and patients with back pain and IVD degeneration show reduced levels of SPARC in their IVDs[218,219]. SPARC-null mice display axial discomfort in response to stretching along the axis of the spine during tail suspension and grip force assays making these mice well-suited for the *in vivo* pre-clinical validation studies.

Drugs termed senolytics, selectively target and remove senescent cells in degenerate IVDs. The mode of action and target of some senolytics are well characterized, while the mechanism of others still needs to be clarified. There are four main groups described to date which include; a) inhibitors of the Bcl-2 family of apoptosis regulatory proteins, b) inhibitors of the p53/MDM2 complex that alleviate resistance to apoptosis, c) HSP-90 and PI3K/Akt inhibitors, releasing pro-apoptotic transcription factors and d) natural flavonoids with a less clear mode of action[120-123].

RG-7112 is a member of the p53/MDM2 complex inhibitors. It is FDA-approved for acute myeloid leukemia and underwent clinical trials to treat a range of cancers[126]. The drug lacked efficiency for cancer, and the high doses lead to hematological toxicity[127,220]. However, previously published studies demonstrate that using a low non-toxic concentration removed senescent IVD cells *in vitro* and *ex vivo*[129]. Low doses can potentially avoid the described side effects in patients treated for IVD degeneration. Furthermore, the high cost and potential adverse side effects, together with the inability of conventional pharmacotherapy to eliminate heterogeneous populations of senescent cells, have stimulated interest in natural flavonoids like Fisetin, Quercetin, and o-Vanillin as substitutes or adjuncts for senolytic pharmaceuticals[215]. These naturally derived agents are being investigated for potential efficacy in various disorders with an inflammatory component[80,81,122,130].

One drawback of using a single senolytic agent is the failure to target multiple senescent anti-apoptotic pathways in the same cell type or different cell populations within a target tissue. Concurrently targeting multiple and indirectly related anti-apoptotic pathways may increase selectivity for senescent cells in the absence of toxicity for normal proliferating or quiescent cells. Successful combination therapy is exemplified by the combination of Dasatinib and Quercetin[132], which target antiapoptotic networks instead of a single target. The lower therapeutic dose combinations also decreased side effects observed with single drugs[132].

The primary objective of this study is to determine if two senolytic drugs, o-Vanillin and RG-7112, can selectively remove senescent cells, reduce inflammatory mediators, and relieve pain in middle-aged SPARC null mice with IVD degeneration and back pain. The second objective is to evaluate if a combination of the drugs provides a better effect that either drug alone.

METHODS:

Animals:

All experiments were approved by the Animal Care Committee of McGill University following the Canadian Council of Animal Care guidelines. Age-matched male and female C57BL/6N (wild-type) and SPARC-null mice were used to carry out all experiments. SPARC-null mice were developed on a C57BL/6x129SVJ background as previously described[142,143]. Mice were housed in a temperature-controlled room with a 12-hour light/dark cycle, 2–5 per ventilated polycarbonate, cage (Allentown), and with corncob bedding (Envigo), cotton nesting squares, ad libitum access to food (Global Soy Protein-Free Extruded Rodent Diet, Irradiated) and water.

Ex Vivo Mouse Disc Culture:

9-month-old SPARC-null and wild-type mice were euthanized, and the L3–4, L4–5, L5–6, and L6–S1 discs with a cartilaginous endplate and no bony endplate were excised. SPARC-null discs were cultured for 48 hours in Dulbecco's Modified Eagle Medium (DMEM) with 1× GlutaMAX, 10 U/mL penicillin and 10 µg/mL streptomycin and one of the following senolytic treatment regimens; a) 100 µM of o-Vanillin only, b) 5 µM of RG-7112 only, c) 100 µM of o-Vanillin and 5 µM of RG-7112, d) 100 µM of o-Vanillin and 2.5 µM of RG-7112, e) 50 µM of o-Vanillin and 5 µM of RG-7112 or f) 50 µM of o-Vanillin and 2.5 of µM RG-7112. The disc media was changed after 48 hours of treatment and replaced with DMEM with 1× GlutaMAX, 10 U/mL penicillin and 10 µg/mL streptomycin, which was then collected and used for protein analysis. Wild-type discs were cultured for a total of 96 hours, with DMEM with 1× GlutaMAX, 10 U/mL penicillin and 10 µg/mL streptomycin with a media change at the 48-hour time point.

Mouse Treatments:

Experimenters were blinded to the treatment regimens for all experiments, and mice were randomized into treatment groups. 7-month-old SPARC-null and wild-type mice underwent oral gavage on a weekly basis for a 2-month period. SPARC-null mice received either a) 100mg/kg o-Vanillin only, b) 5mg/kg RG-7112 only, c) 100mg/kg o-Vanillin and 5mg/kg RG-7112, d) 100mg/kg o-Vanillin and 2.5mg/kg RG-7112, e) 50mg/kg o-Vanillin and 5mg/kg RG-7112, f) 50mg/kg o-vanillin and 2.5mg/kg RG-7112 or g) 0.01% Dimethyl sulfoxide (DMSO) in saline as a control. All wild-type mice underwent oral gavage with 0.01% DMSO in saline as a control.

Pain Behaviour Schedule:

Pain behaviour was performed as was previously described in our laboratory[173]. All mice were tested in a dedicated behavioural testing room with regular indoor lighting between 8:00 AM and 12:00 PM. Mice were habituated to the room for 1 hour and to Plexiglas testing boxes on a metal grid for another hour (when applicable). Grip strength (axial discomfort), acetone-evoked behaviour and mechanical sensitivity to von Frey filaments (radicular pain) were assessed on non-treatment days bi-weekly. The tail suspension test (axial discomfort and depression-like behaviour) and distance travelled in an open field (motor ability and anxiety-like behaviour) were measured every 4 weeks.

Grip Strength: Axial discomfort was measured with a Grip Strength Meter (Stoelting Co.) by allowing the mice to grip a bar with their forepaws and stretching them by pulling on their tail. The force at which they released was recorded in grams[145]. In a session, grip strength was

measured two to three times and then averaged. Mice were returned to their home cages for approximately 15 minutes between measurements.

Acetone-evoked Behaviour Test for Cold Sensitivity: Behavioral reaction to a cold stimulus was used as a measure for radiating leg pain. Acetone was applied to the left and right hind paw, and the total time of evoked behaviour (paw lifting, shaking and scratching) was recorded[145].

Von Frey Test for Mechanical Sensitivity: von Frey filaments (Stoelting Co.) were applied to the left and right hind paw plantar surfaces until withdrawal or for a total of five seconds, whichever came first[147]. Stimuli intensity ranged from 0.6 to 4.0 g. The 50% withdrawal threshold in grams was calculated using the up-down method[221].

Tail Suspension Assay Mice were individually suspended by the tail underneath a platform. Adhesive tape was used to attach the tail (0.5-1 cm from the base) to the platform and were videotaped for three minutes. The duration of time spent in a) immobility (not moving but stretched out), b) rearing (trying to reach the underside of the platform), c) full extension (actively reaching for the floor), and d) self-supported (holding either the base of its tail or the tape), was analyzed by a blinded observer using digital software (Labspy®, Montreal, QC) over the entire testing period.

Open Field: Mice were placed in a 24 cm by 24 cm Plexiglas enclosure for five minutes. Mice were video recorded from above, and the total distance travelled was analyzed using AnyMaze[145].

Histological analysis:

Sample preparation: Following the 2-months of senolytic treatment, SPARC-null and wild-type mice were deeply anesthetized by an intraperitoneally administered mixture of ketamine 100 mg/kg, xylazine 10 mg/kg, and acepromazine 3 mg/kg, and perfused through the left cardiac ventricle with vascular rinse followed by 200 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at room temperature, for 20 minutes. The T13–S1 spinal segment was collected post-perfusion and kept in fixative overnight at 4°C, and then decalcified by immersion in 4% ethylenediaminetetraacetic acid in phosphate-buffered saline at 4°C for 14 days. The samples were cryoprotected in 30% sucrose in phosphate-buffered saline for 4 days at 4°C and embedded in an optimal cutting temperature cutting medium (Tissue-Tek, Sakura Finetek, Torrance, CA, USA). Sixteen micrometre-thick cryostat (Leica CM3050S, Leica Microsystems, Inc, Concord, Ontario, Canada) sections were cut in the sagittal plane, thaw-mounted onto gelatin-coated slides and stored at −20°C.

FAST Staining: Staining was performed as described by Millecamps et al.[143]. This staining was adapted from the FAST method for colorimetric histologic staining described by Leung et al. for intervertebral discs[222]. The FAST profile identifies intervertebral disc compartments and detects matrix remodelling within the disc. The procedure consists of consecutive baths in 1) Acidic Alcian Blue (Sigma #A5268), 10 minutes; 2) Safranin-O (Sigma #S2255), 2.5 minutes; 3) 50% ethanol, 1 minute; 4) Tartrazine (Sigma #HT3028), 10 seconds; and 5) Fast Green (Sigma #F7252), 5 minutes. After drying, slides were mounted with Dibutylphthalate Polystyrene Xylene (DPX) (Sigma-Aldrich, USA). Disc degeneration severity grading scale:

Grade 0) Healthy IVDs display intact structure, a clear distinction between outer AF and inner NP and negatively charged proteoglycans; Grade 1) The changes in extracellular components and IVD integrity were identified as grade 0 - normal structure, but the loss of proteoglycans in inner NP; Grade 2) Internal disruption (loss of boundary) between NP and AF; Grade 3) Bulging of NP in dorsal aspect; Grade 4) Herniation.

Luminex Multiplex Assay of In Vivo and Ex Vivo Culture Media:

Ex vivo culture media (as described above) and the *in vivo* samples were used in the Luminex multiplex assay. *In vivo* samples were obtained following 2-months of treatment with senolytic compounds from SPARC-null and wild-type mice which were euthanized, and the L3–4, L4–5, L5–6, and L6–S1 discs with a cartilaginous endplate and no bony endplate were excised. SPARC-null discs were cultured for 48 hours in DMEM with 1× GlutaMAX, 10 U/mL penicillin and 10 μg/mL streptomycin. Fifteen proteins were selected for analysis by Luminex multiplex assay according to the manufacturer's instructions. A limitation in the number of factors we could measure was the incompatibility of some factors to be measured simultaneously, as indicated by the supplier. Concentrations (pg/mL) (INF-γ, TNF-α, IL-1β, IL2, IL6, IL10, CCL2, CCL7, CXCL1, CXCL5, CXCL9, CXCL10, M-CSF, RANKL, VEGF-A) were measured in 40 μL media. Median fluorescence intensity (MFI) from microspheres was acquired with a BD FACSCanto II and analyzed in FlowCytomix Pro2.2.1 software (eBioscience). The concentration of each analyte was obtained by interpolating fluorescence intensity to a seven-point dilution standard curve supplied by the manufacturer.

Immunofluorescent histochemistry

Spinal cords: Spinal cords were harvested following euthanasia and fixed in 4% paraformaldehyde for 24 hours at 4°C, followed by cryoprotection using 30% sucrose solution for 24 hours at 4 °C. Samples were embedded in blocks of six spinal cords in optimum cutting temperature medium (Tissue-Tek). 16 µm cryostat (Leica CM3050S) sections were thaw mounted on gel-coated slides and stored at 20°C until use. Three sections per animal were randomly selected, spanning the lumbar spinal cord for each antibody. Sections were incubated for 1 hour at room temperature in a blocking buffer containing 0.3% Triton X-100, 1% bovine albumin, 1% normal donkey serum and 0.1% sodium azide in phosphate-buffered saline. Slides were then incubated with either recombinant anti-CDKN2A/p16^{INK4a} antibody (1:100; Abcam, catalogue #ab211542), sheep anti-calcitonin gene-related peptide (CGRP) polyclonal antibody (1:1000; Enzo Life Sciences, catalogue# BML-CA11370100, lot# 0807B74), goat anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:1000; Sigma-Aldrich, catalogue# SAB2500462, lot #747852C2G2), or rat monoclonal anti-CD11b antibody (1:1000; BioRad, catalogue# MCA711G, lot# 0614) in blocking buffer overnight at 4°C, washed three times for five minutes in phosphate-buffered saline and incubated for 1.5 hours at room temperature with appropriate donkey-derived secondary antibodies from Jackson Immunoresearch; Donkey anti-Sheep Cy3, catalogue# 713-165-147; Donkey anti-Goat AlexaFlour 594, catalogue# 705-85-144; Donkey anti-Rat AlexaFluor 488, catalogue# 712-225-153, Donkey anti-Rabbit AlexaFluor 488, catalogue# 711-545-152, Donkey anti-Rabbit Cy3, catalogue# 711-165-152 in blocking buffer. DAPI (1:50000 in water, Sigma-Aldrich) was briefly applied, and slides were washed another three times for five minutes. Coverslips were mounted using Aqua Polymount (Polysciences Inc.). Images were taken at 10× magnification using an Olympus BX51 microscope equipped with an Olympus DP71 camera (Olympus). 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 percent area of the region of interest at, or above, the threshold was quantified to measure $p16^{INK4a}$-ir, CGRP-ir, GFAP-ir or CD11b-ir. The average percent area immunoreactivity across each animal was averaged and used as the value for that mouse. Image analysis was performed by an experimenter blind to strain and treatment group.

Spine: Samples were prepared as described above under the "histological analysis sample preparation" section. Slide-mounted sagittal sections of the lumbar spine were encircled with a DAKO pen (S2002; DAKO). Two to three discs per animal were selected, spanning the L4-S1 disc for each antibody. Sections were incubated for 1 hour at room temperature in a blocking buffer containing 0.3% Triton X-100, 1% bovine albumin, 1% normal donkey serum and 0.1% sodium azide in phosphate-buffered saline. Slides were then incubated with recombinant anti-CDKN2A/$p16^{INK4a}$ antibody (1:100; Abcam, catalogue #ab211542) in blocking buffer overnight at 4 °C, washed three times for five minutes in phosphate-buffered saline and incubated for 1.5 hours at room temperature with Donkey anti-Rabbit Cy3, catalogue# 711-165-152 in blocking buffer. DAPI (1:50000 in water, Sigma-Aldrich) was briefly applied, and slides were washed thrice for five minutes. Coverslips were mounted using Aqua Polymount (Polysciences Inc.). Images were taken at 10× magnification using an Olympus BX51 microscope equipped with an Olympus DP71 camera (Olympus). Using ImageJ, a percent of $p16^{INK4a}$ positive cells was obtained by counting the total number of $p16^{INK4a}$ positive cells over the total number of DAPI cells. An average of approximately 5000 cells were counted per

treatment group. Image analysis was performed by an experimenter blind to strain and treatment group.

Statistical Analyses:

Power analysis was set with a margin error alpha of 0.05, a confidence level of 95% and a 50% response distribution. Power analysis determined a sample size of 8-10 wild-type and 8-10 SPARC-null animals to observe significant differences (10-15 if sex differences become apparent). Data were analyzed using GraphPad Prism 9, with $P \leq 0.05$ being considered statistically different. Data are presented as mean ± standard deviation. Luminex Multiplex data was assessed by repeated measures of one-way ANOVA or two-tailed unpaired t-tests. Pain-like behaviour data was analyzed by repeated-measure 2-way ANOVA. For two-way ANOVA, the between-group variable was the treatment group, and the within-group variable was time. ANOVAs were followed by Tukey's post hoc test.

RESULTS:

Combination treatment with RG-7112 and o-Vanillin results in a significant decrease of SASP factor release from SPARC-null IVDs ex vivo

RG-7112 and o-Vanillin reduce SASP factor release from human IVD cells and intact human IVDs. To evaluate if the drugs are also able to reduce SASP factors in mouse IVDs, the L3–4, L4–5, L5–6, and L6–S1 discs were harvested from 9-month-old SPARC-null and wild-type mice. The IVDs were treated *ex vivo* with single or combined treatment of RG-7112 and o-Vanillin. IVDs were cultured in DMEM media for 48 hours with specific senolytic compounds; a) 100 μM of o-Vanillin only, b) 5 μM of RG-7112 only, c) 100 μM of o-Vanillin and 5 μM of RG-7112, d) 100 μM of o-Vanillin and 2.5 μM of RG-7112, e) 50 μM of o-Vanillin and 5 μM of RG-7112 or f) 50 μM of o-Vanillin and 2.5 of μM RG-7112, the drugs were removed after 48 hours, and fresh DMEM without drugs was added and the IVDs were cultured for another 48 hours. The media was recovered and a fifteen-protein multiplex assay was performed to assess SASP factor release. Ten of the fifteen proteins assessed showed a significant difference between the untreated wild-type and SPARC-null IVDs, demonstrating that SPARC-null IVDs release significantly more SASP factors than wild-type at baseline (**Figure 1**). Next, protein analysis was performed on senolytic-treated SPARC-null IVDs and SASP release was compared to untreated SPARC-null IVDs. The concentrations in SPARC-null untreated IVDs were; CXCL-1: 1360pg/mL ± 85.30, CXCL-5: 1611pg/mL ± 89.40, CXCL-9: 706.2pg/mL ± 63.11, CXCL-10: 590.1pg/mL ± 46.36, CCL-2: 11,005pg/mL ± 2960, CCL-7: 824.9pg/mL ± 63.51, IL-1β: 494.6pg/mL ± 53.46, IL-6: 21,043pg/mL ± 956, VEGF-α: 504.3pg/mL ± 68.87, and TNF-α: 782pg/mL ± 58.87 (**Figure 1A-J**). The greatest reduction in SASP factor release was observed using the combination of 100 μM of o-Vanillin + 5 μM of RG-7112; CXCL-1:

712.9pg/mL ± 65.62 p<0.0001, CXCL-5: 989.5pg/mL ± 44.13 p<0.0001, CXCL-9: 380.9pg/mL ± 46.87 p=0.0008, CXCL-10: 315.6pg/mL ± 52.24 p=0.0003, CCL-2: 3779pg/mL ± 354.8 p=0.0025, CCL-7: 485.1pg/mL ± 39.63 p=0.0002, IL-1β: 304.4pg/mL ± 68.68 p=0.0012, IL-6: 13127pg/mL ± 324.6 p<0.0001, VEGF-α: 414pg/mL ± 48.11 p=0.0153, and TNF-α: 513 pg/mL ± 53.26 p=0.0004 (Figure 1A-J). Relative to the single treatments, only the combination of 100 μM of o-Vanillin + 5 μM of RG-7112 significantly reduced CXCL-1 (relative to RG-7112 p=0.037), CXCL-5 (relative to RG-7112 p=0.008), CXCL-10 (relative to RG-7112 p=0.0143, relative to o-Vanillin p<0.0001), CCL-7 (relative to RG-7112 p=0.0110, relative to o-Vanillin p=0.0018) and TNF-α (relative to RG-7112 p=0.0297, relative to o-Vanillin p=0.0497).

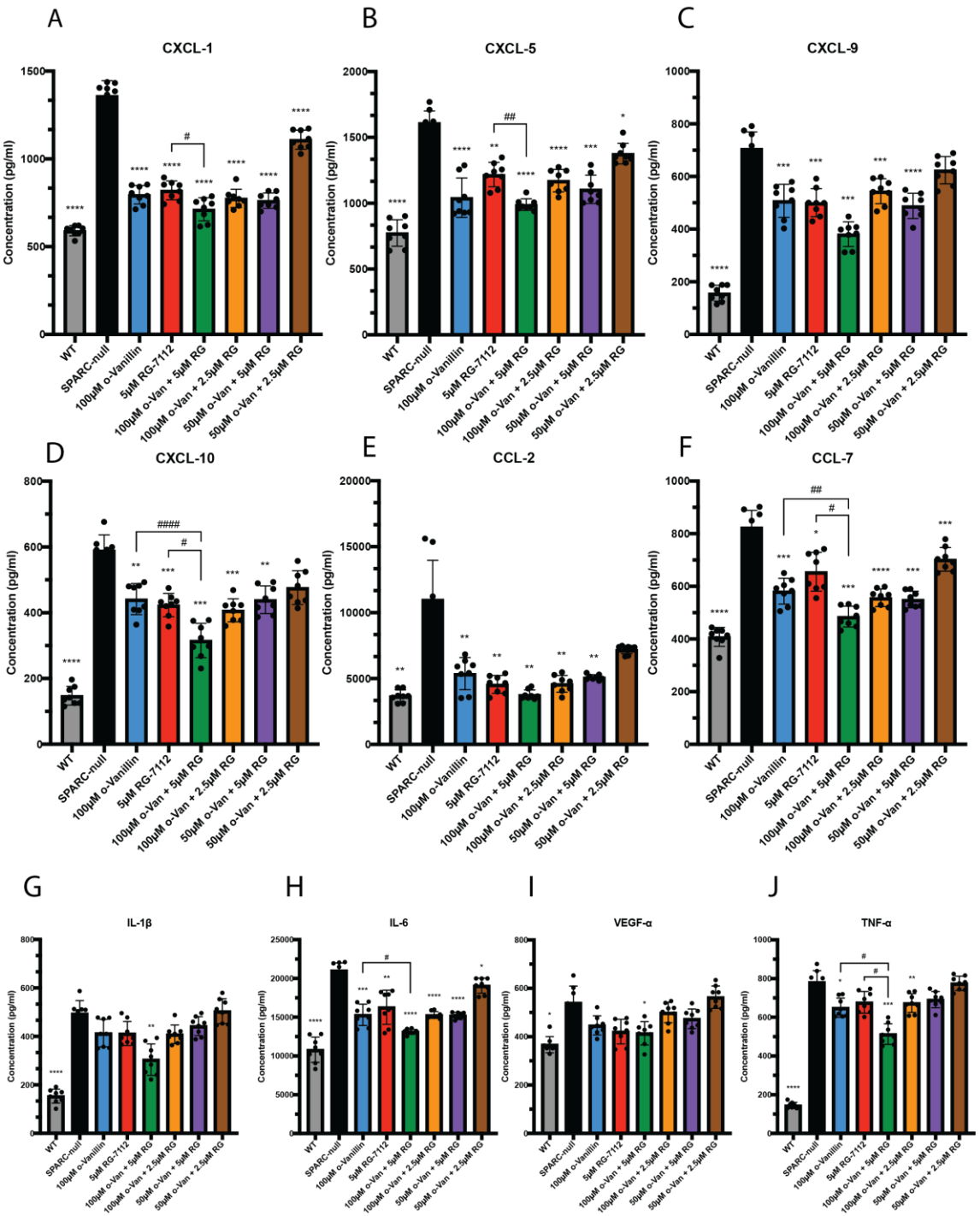


Figure 1: ***Combination treatment with RG-7112 and o-Vanillin results in a significant decrease of SASP factor release from SPARC-null IVDs ex vivo***

(A-J) Discs from 9-month-old SPARC-null mice were treated with senolytics or were left untreated (non-treated control) for an initial 48 hours. The media was then changed and replaced with fresh culture media containing no senolytics. This culture media was analyzed

on selected protein multiplex. Proteins analyzed include; **(A)** CXCL-1, **(B)** CXCL-5, **(C)** CXCL-9, **(D)** CXCL-10, **(E)** CCL-2, **(F)** CCL-7, **(G)** IL-1β, **(H)** IL-6, **(I)** VEGF-α, **(J)** TNF-α. **(A-J)** * Indicates significance between treatment (single or combination) and control group. # Indicates significance calculated when combination treatment is compared to single treatment groups. All analyses were performed using a one-way ANOVA. Data are presented as mean ± SD. * or # $p < 0.05$; ** or ## $p < 0.01$; *** or ### $p < 0.001$, **** or #### $p < 0.0001$. n=8 mice.

Oral gavage treatment of o-Vanillin and RG-7112 has no effect on body weight, activity level or mortality in SPARC-null mice

Potential adverse effects of o-Vanillin and RG-7112 were evaluated by assessing body weight, the distance travelled in an open field, and mortality over 8-weeks of weekly oral gavage treatments (**Figure 2**). The weight of both male and female SPARC-null (non-treated and treated) and wild-type mice (non-treated) were evaluated. No significant differences in weight were observed between the sexes, treated and untreated groups, and among treated groups over the 8-weeks (**Figure 2A-B**). In addition, when assessing the total distance travelled in open field as a measure of locomotion, no significant differences were observed between treated and untreated groups (Figure 2C). Regarding mortality, no significant differences were observed between treatment groups and when comparing treated to non-treated groups (**Figure 2D**). There were four mortalities by the end of the 8-week study period, and they were all due to natural causes (e.g., injury from mouse aggression) (**Figure 2D**).

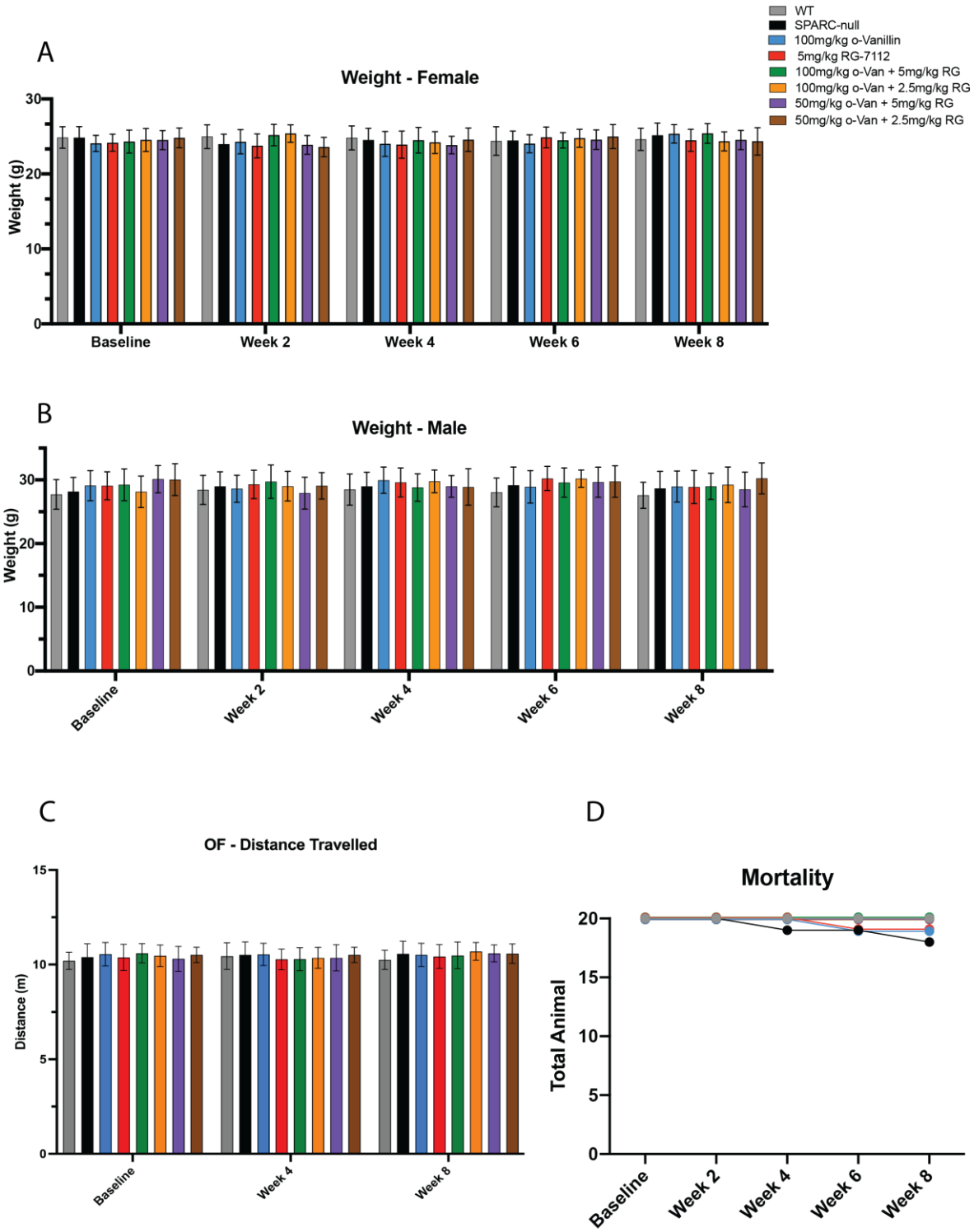


Figure 2: ***Oral gavage treatment of o-Vanillin and RG-7112 has no effect on body weight, activity level or mortality in SPARC-null mice***

To examine the effects of chronic administration of senolytic compounds on SPARC-null mice, **(A, B)** weight, **(C)** locomotion and **(D)** mortality were assessed. **(A-D)** Non-treated wild-type

and SPARC-null mice served as non-treatment controls. **(A, B)** Weight was assessed bi-weekly (i.e., baseline, 2-week, 4-week, 6-week and 8-week time points) for both **(A)** female and **(B)** male mice. **(C)** Distance was assessed in a 5-minute open field test bi-weekly (i.e., baseline, 4-week, and 8-week time points). **(D)** Number of mortalities are plotted bi-weekly (i.e., baseline, 2-week, 4-week, 6-week and 8-week time point). **(A-C)** Data are presented as mean ± SD. n=10-19 mice.

Oral gavage with o-Vanillin and RG-7112 of SPARC-null mice reduces behavioural indices of chronic back pain

Given that the chief complaint from patients with IVD degeneration is low back pain and the only currently available treatment options to relieve pain are unsatisfactory, we wanted to assess the impact of senolytics on indices of chronic back pain in the SPARC-null model. Mice were treated with varying concentrations of o-Vanillin and/or RG-7112 for 8-weeks and mechanical sensitivity to von Frey filaments, acetone-evoked behaviour, and grip strength were evaluated on non-treatment days during weeks 0 (baseline), 2, 4, 6, and 8. Tail suspension was evaluated at weeks 0 (baseline), 4 and 8. A significant difference was observed at baseline in all assessments between wild-type and SPARC-null mice, confirming that SPARC-null mice have established back pain at 7 months of age (**Figure 3A-H**).

Testing using von Frey filaments provides a measure of mechanical sensitivity indicative of radiating pain to the leg. As demonstrated by Figure 3A,B, no significant reduction of pain was observed prior to the 4-week time point; 4 weeks onward (i.e., at 6 and 8 weeks), significantly less mechanical sensitivity was observed between SPARC-null untreated and treated mice (**Figure 3A, B**). Reducing the drug concentrations to 50% (50mg/kg o-Vanillin + 2.5mg/kg of RG-7112) did not reduce sensitivity at any timepoint (**Figure 3A, B**). Only combination treatment with the higest doses (100mg/kg of o-Vanillin + 5mg/kg of RG-7112)

provided a significant improvement over single senolytic treatment at the highest concentrations (**Figure 3A, B**).

The acetone-evoked behaviour test uses principles of cold sensitivity to provide an index of radiating pain to the leg. Combination treatment of 100mg/kg of o-Vanillin + 5mg/kg of RG-7112 and 100mg/kg of o-Vanillin + 2.5mg/kg of RG-7112 demonstrated significant improvement when compared to the untreated SPARC-null animals as of the 2-week time point (**Figure 3C, D**). As of 8-weeks, significantly less acetone-evoked pain behaviour compared to the SPARC-null untreated group was detected in all the treatment groups except when drug concentrations were reduced to 50% (50mg/kg of o-Vanillin + 2.5mg/kg of RG-7112) (**Figure 3C, D**). From week 2, the combination treatments (100mg/kg of o-Vanillin + 5mg/kg of RG-7112, 100mg/kg of o-Vanillin + 2.5mg/kg of RG-7112 and 50mg/kg of o-Vanillin + 5mg/kg of RG-7112) displayed significantly less acetone-evoked pain than the single o-Vanillin and RG-7112 treatment groups.

Grip strength represents axial discomfort, where mice with back pain release the handle at a lower force. No significant differences were observed prior to the 4-week time point (**Figure 3E, F**). Four weeks onward, a significantly greater force was required to induce axial discomfort in SPARC-null-treated mice compared to untreated SPARC-null mice (**Figure 3E, F**). The only instance where no change was seen at any time point was using the drugs at 50% (50mg/kg of o-Vanillin + 2.5mg/kg of RG-7112) (**Figure 3E, F**). Combination treatment that provided a significant reduction in axial discomfort when compared to single senolytic treatment when combined at the highest concentration of o-Vanillin together with 100% and 50% of RG-7112 (100mg/kg of o-Vanillin + 5mg/kg of RG-7112 and 100mg/kg of o-Vanillin + 2.5mg/kg of RG-7112) as of 6-weeks (**Figure 3E, F**).

Similar to grip strength, tail suspension can be used to measure axial discomfort. At 4-weeks, less pain (i.e., greater time immobile) was observed in all treatment groups except the 50mg/kg o-Vanillin + 2.5mg/kg of RG-7112 (**Figure 3G, H**). As of 8-weeks, all SPARC-null treated groups had significantly less pain when compared to untreated mice (**Figure 3G, H**). Combining the drugs, at the highest doses, provided a significant reduction in pain at 4- and 8-weeks compared to either drug alone (**Figure 3G, H**).

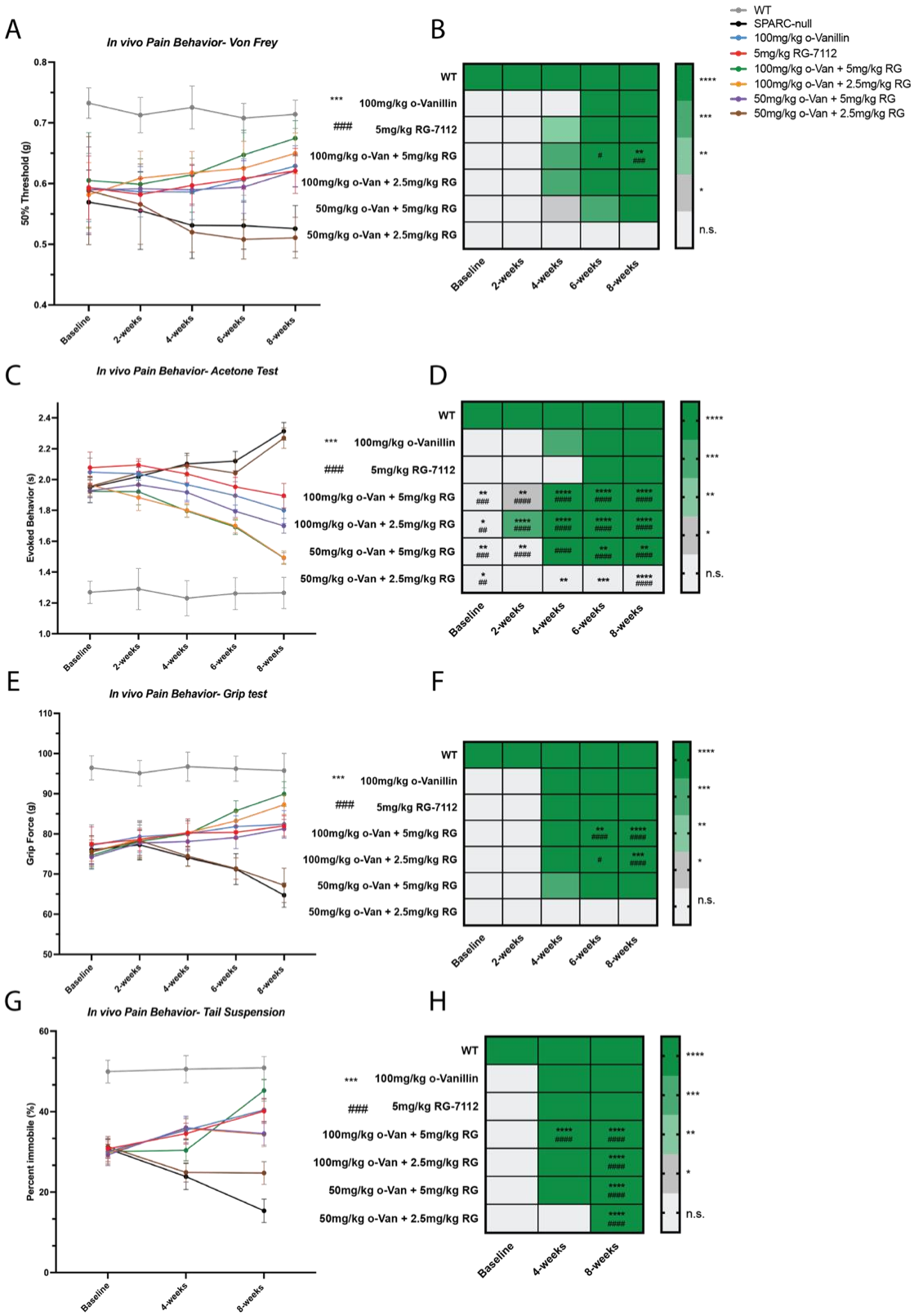


Figure 3: ***Oral gavage with o-Vanillin and RG-7112 of SPARC-null mice reduces behavioural indices of chronic back pain***

Senolytic treatments were administered by oral gavage 1 time/week for 8 weeks in 7–9-month-old SPARC-null and wild-type mice. **(A, B)** Mechanical sensitivity to von Frey filaments, **(C, D)** acetone-evoked behaviour (cold sensitivity; an index of radiating leg pain) and **(E, F)** Grip strength (axial pain) were assessed on non-treatment days during weeks 0 (baseline), 2, 4, 6 and 8. **(G, H)** Tail suspension (axial pain) was assessed on non-treatment days during weeks 0 (baseline), 4 and 8. **(A-D)** Data are presented as mean ± SD and analyzed by repeated measures of two-way ANOVA followed by Tukey's posthoc test. Color scale indicated in Figures **B, D, F, and H** indicates the treatment group and wild-type compared to non-treated SPARC-null mice. * Indicates significance between combination treatment and single group treatment with o-Vanillin. # Indicates significance between combination treatment and single group treatment with RG-7112. * or # $p < 0.05$; ** or ## $p < 0.01$; *** or ### $p < 0.001$, **** or #### $p < 0.0001$. n=14-20 mice.

Combination treatment with RG-7112 and o-Vanillin results in a significant decrease of SASP factor release from SPARC-null IVDs

Similar to the investigations performed using the *ex vivo* model, following 8-weeks of oral gavage treatment, mice were euthanized, and the the L3–4, L4–5, L5–6, and L6–S1 discs were harvested. The IVDs were kept viable in DMEM for 48 hours, and the release of SASP factors was then measured. Of the fifteen proteins assessed, protein concentrations were significantly greater in untreated SPARC-null IVDs when compared to untreated wild-type IVDs (**Figure 4A-J**). The concentrations in SPARC-null untreated IVDs were; CXCL-1: 1532pg/mL ± 131.1, CXCL-5: 466.6pg/mL ± 37.51, CXCL-9: 249.7pg/mL ± 18, CXCL-10: 251.2pg/mL ± 31.87, CCL-2: 8577pg/mL ± 424.8, CCL-7: 692.1pg/mL ± 72.1, IL-1β: 225.3pg/mL ± 16.93, IL-6: 19896pg/mL ± 1278, VEGF-α: 591.8pg/mL ± 45.11, and TNF-α: 111.7pg/mL ± 3.20 (**Figure 4A-J**). All treatments resulted in a significant reduction in SASP factor release except for combined treatment at 50% (50mg/kg of o-Vanillin + 2.5mg/kg of RG-7112), which did not result in a significant decrease in the release of CCL-7 (**Figure 4A-J**). The greatest reduction in SASP factor expression was observed using the combination of 100mg/kg of o-Vanillin + 5mg/kg of RG-7112; CXCL-1: 673.9pg/mL ± 55.13 $p<0.0001$,

CXCL-5: 124.3pg/mL ± 17.92 p<0.0001, CXCL-9: 144.3pg/mL ± 4.123 p<0.0001, CXCL-10: 73.66pg/mL ± 11.55 p<0.0001, CCL-2: 4732pg/mL ± 539 p<0.0001, CCL-7: 398.7pg/mL ± 12.59 p<0.0001, IL-1β: 62.21pg/mL ± 6.59 p<0.0001, IL-6: 14120pg/mL ± 612.3 p<0.0001, VEGF-α: 355.1pg/mL ± 39.56 p<0.0001, and TNF-α: 61.91pg/mL ± 4.61 p<0.0001 (**Figure 4A-J**). Relative to the single senolytic treatments, 100mg/kg of o-Vanillin + 5 mg/kg of RG-7112 significantly reduced all SASP factors (**Figure 4A-J**).

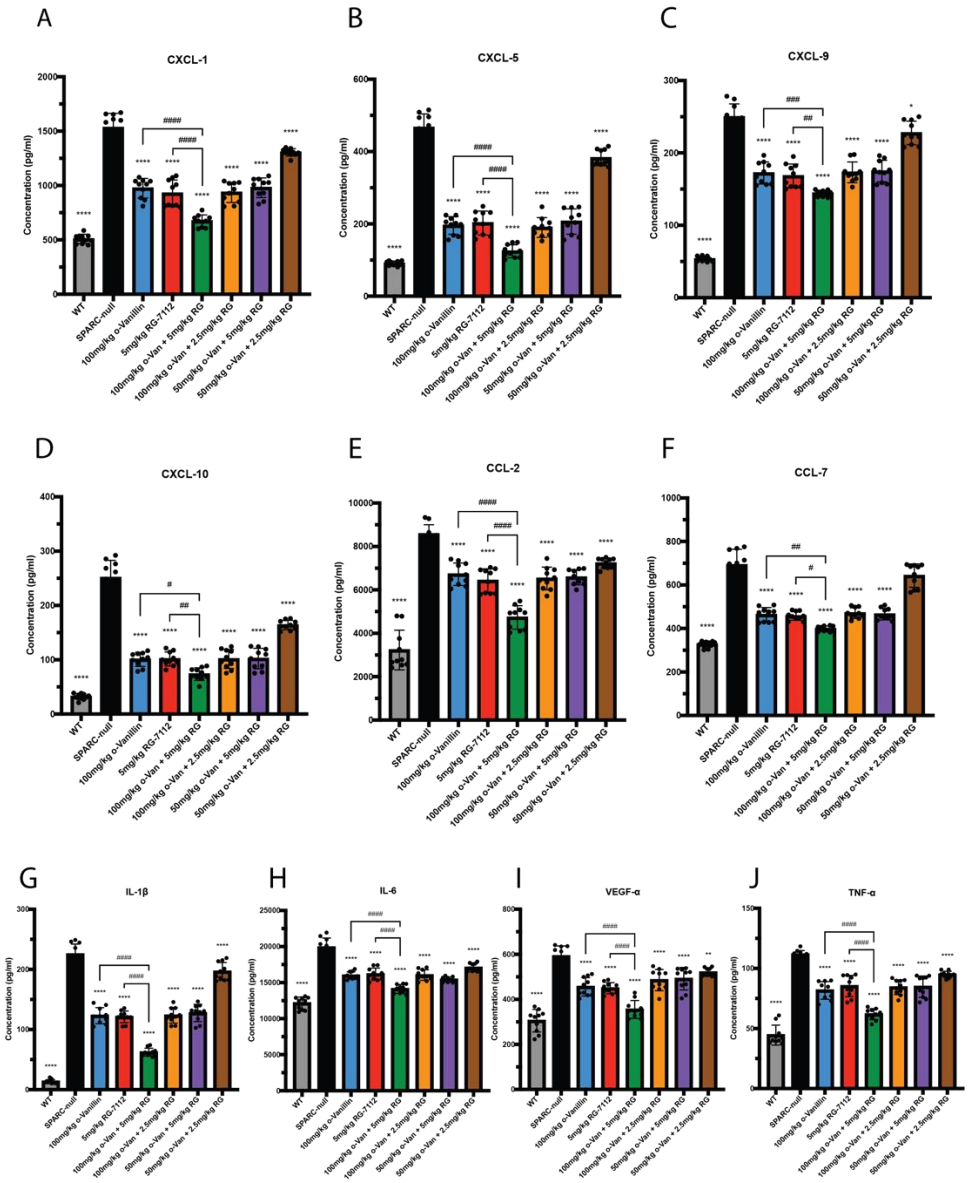


Figure 4: ***Combination treatment with RG-7112 and o-Vanillin results in a significant decrease of SASP factor release from SPARC-null IVDs***

(A-J) Following 8 weeks of treatment, discs from 9-month-old SPARC-null mice were isolated and cultured for 48 hours in culture media, and then this culture media was analyzed on selected protein multiplex. Proteins analyzed include; **(A)** CXCL-1, **(B)** CXCL-5, **(C)** CXCL-9, **(D)** CXCL-10, **(E)** CCL-2, **(F)** CCL-7, **(G)** IL-1β, **(H)** IL-6, **(I)** VEGF-α, **(J)** TNF-α. **(A-J)** * Indicates significance between treatment (single or combination) and control group. # Indicates significance calculated when combination treatment is compared to single treatment groups. All analyses were performed using a one-way ANOVA. Data are presented as mean ± SD. * or # $p < 0.05$; ** or ## $p < 0.01$; *** or ### $p < 0.001$, **** or #### $p < 0.0001$. n=10 mice.

Oral gavage treatment with o-Vanillin and RG-7112 for 8-weeks results in less severe disc degeneration in SPARC-null mice

IVDs from disc levels L3 to L6 were assessed for disc degeneration severity using a 0-4 point histological grading scale as previously described by Millecampes et al. 2015[143] (**Figure 5A, B**). As expected at 9-months of age, untreated SPARC-null mice displayed more severe disc degeneration that wild-type mice (**Figure 5A, B**). When comparing treated to untreated SPARC-null mice, all treatments except for the 50mg/kg of o-Vanillin + 2.5mg/kg of RG-7112 combination demonstrated significantly less severe disc degeneration (**Figure 5C**). Combination treatment at the highest doses (100mg/kg of o-Vanillin + 5mg/kg of RG-7112) further significantly reduced disc degeneration compared to either drug alone (**Figure 5C**).

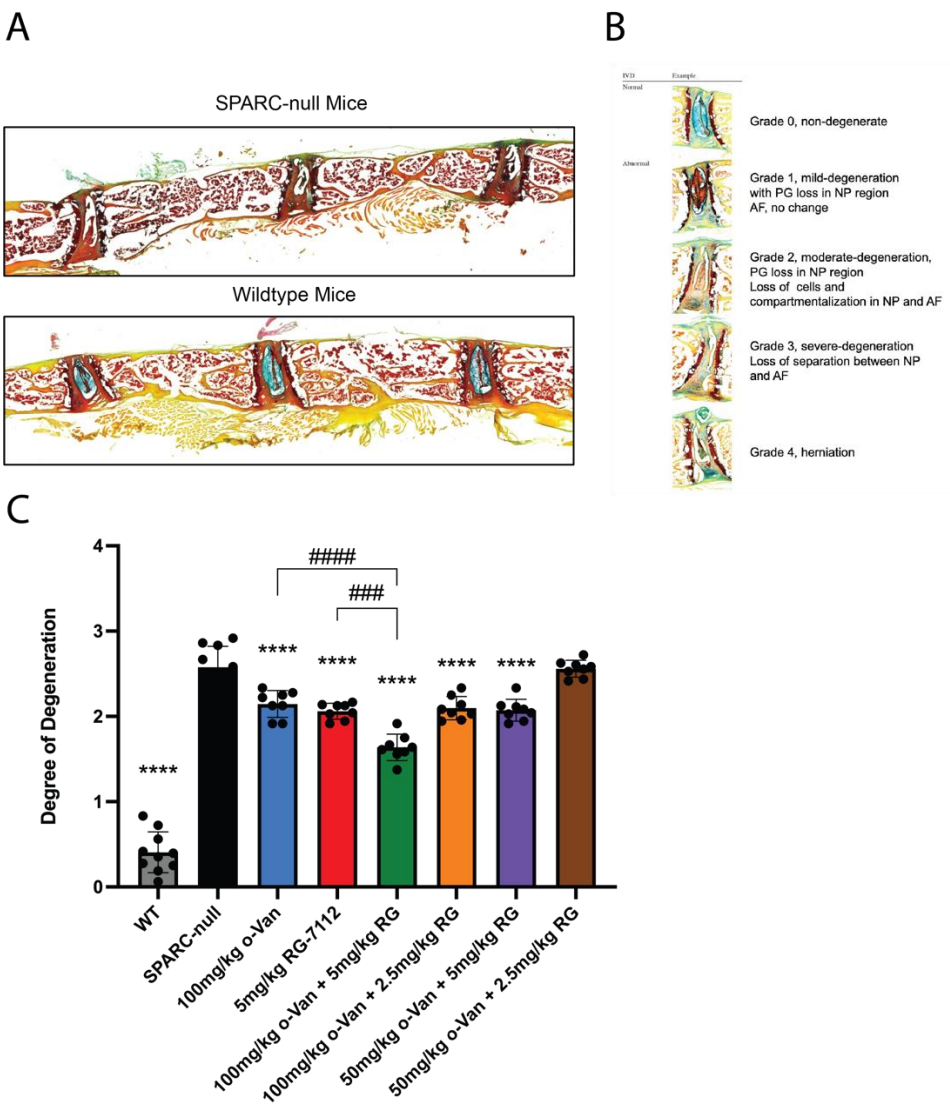


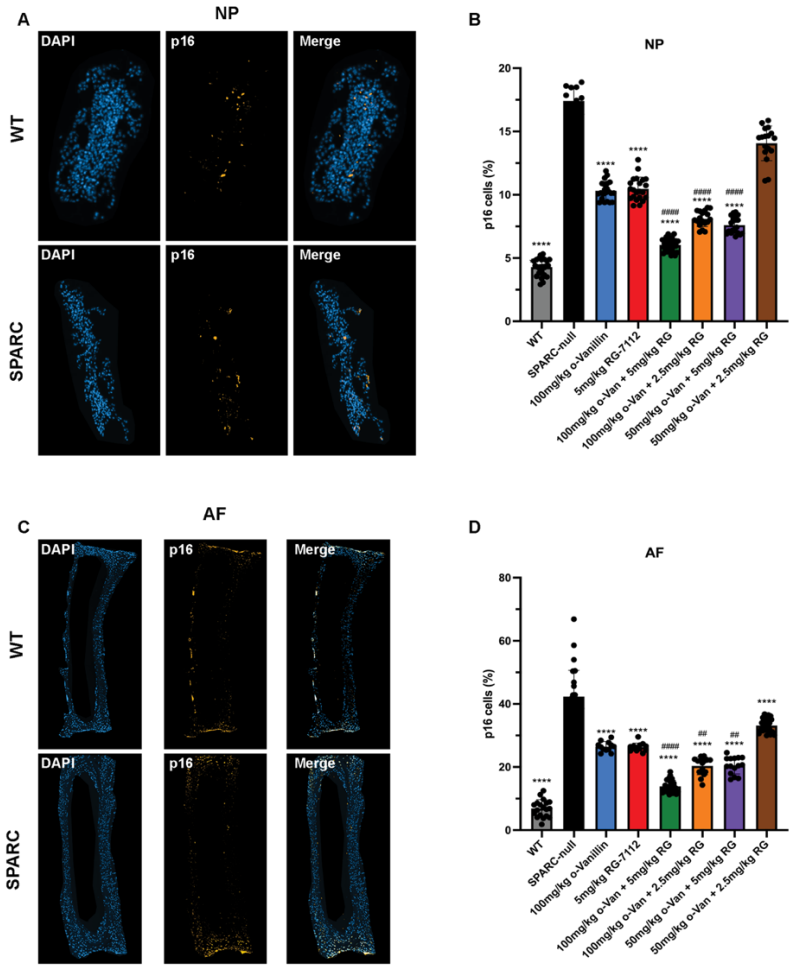
Figure 5: ***Oral gavage treatment with o-Vanillin and RG-7112 for 8-weeks results in less severe disc degeneration in SPARC-null mice***

(A) The average disc degeneration severity score of IVD levels L3-S1 using a 0–4-point scale. * Indicates significance between treatment (single or combination) and control group. # Indicates significance calculated when combination treatment is compared to single treatment groups. All analyses were performed using a one-way ANOVA. Data are presented as mean ± SD. * or # $p < 0.05$; ** or ## $p < 0.01$; *** or ### $p < 0.001$, **** or #### $p < 0.0001$. n= 8-10 mice. **(B)** Representative images of a SPARC-null and wild-type mouse spines. **(C)** description of the 4-point scale described in Millecampes et al. 2015[143].

Oral gavage with o-Vanillin and RG-7112 of SPARC-null mice reduces the expression of p16 ^{INK4a} positive cells in NP and AF regions of IVDs

Untreated SPARC-null mice had a significantly greater amount of p16^{INK4a} positive cells in the NP (NP 17.41% ± 1.08, p<0.0001) and in the AF regions (42.33% ± 8.32, p<0.0001), compared to wild-type (NP 4.28% ± 0.66, AF 6.89% ± 2.76) (**Figure 6A-D**). To evaluate if o-Vanillin and RG-7112 removed senescent cell burden in the IVD, p16^{INK4a} positive cells were quantified in the NP and AF regions.

All senolytic treatments demonstrated significantly lower levels of p16 ^{INK4a} relative to the untreated SPARC-null mice in both the NP and AF regions. Combination treatment at the highest doses had the greatest effect (p16^{INK4a} positive cells; NP: 6.04% ± 0.5, AF: 13.86% ± 1.97, p<0.0001 compared to single drugs), 100mg/kg of o-Vanillin + 2.5mg/kg of RG-7112 (p16 ^{INK4a} positive cells; NP: 8.10% ± 0.62, AF: 20.34% ± 2.79, relative to o-Vanillin and RG-7112; NP: p<0.0001, relative to o-Vanillin; AF: p=0.0039, relative to RG-7112; AF: p=0.0044) and 50mg/kg of o-Vanillin + 5mg/kg of RG-7112 (p16^{INK4a} positive cells; NP: 7.60% ± 0.65, AF: 20.47% ± 2.70, relative to o-Vanillin and RG-7112; NP: p<0.0001, relative to o-Vanillin; AF: p=0.0052, relative to RG-7112; AF: p=0.0058) resulted in a significantly larger reduction in p16 ^{INK4a} positive cells in the NP and AF regions when compared to single drug treatment with o-Vanillin (NP: 10.31% ± 0.73, AF: 26.46% ± 1.59) or RG-7112 (NP: 10.47% ± 0.92, AF: 26.28% ± 1.30) (**Figure 6A-D**).



2924

2925 **Figure 6**: ***Oral gavage with o-Vanillin and RG-7112 of SPARC-null mice reduces the***
2926 ***expression of p16^{INK4a} positive cells in NP and AF regions of IVDs***
2927 **(A-D)** Following 8 weeks of treatment, Discs from 9-month-old SPARC-null mice were
2928 isolated and were analyzed on the senescent burden in both NP and AF regions of discs L4-S1.
2929 **(A)** NP representative images of untreated SPARC-null mice and wildtype for p16^{INK4a} staining
2930 in the IVD **(C)** AF Representative images of untreated SPARC-null mice and wildtype for
2931 p16^{INK4a} staining in the IVD. **(B)** NP percentage of positive senescent cells. **(B)** AF percentage
2932 of positive senescent cells * Indicates significance between treatment (single or combination)

and control group. # Indicates significance calculated when combination treatment is compared to single treatment groups. All analyses were performed using a one-way ANOVA. Data are presented as mean ± SD. * or # $p < 0.05$; ** or ## $p < 0.01$; *** or ### $p< 0.001$, **** or #### $p < 0.0001$. n=12-29 lumbar discs.

Oral gavage with o-Vanillin and RG-7112 of SPARC-null mice reduces the expression of senescence and pain markers in SPARC-null mice dorsal horns

The last aim was to evaluate if o-Vanillin and RG-7112 also reduced the senescent burden and pain-related markers in the dorsal horn spinal cords of SPARC-null mice. Untreated SPARC-null mice showed a significantly larger intensity area for $p16^{INK4a}$ (16.46% ± 1.36, $p<0.0001$), CGRP (34.98% ± 1.70, $p<0.0001$), GFAP (23.29% ± 1.91, $p<0.0001$) and CD11b (3.47 ± 0.30, $p<0.0001$) compared to the wild-type control (**Figure 7 A-E**). All senolytic treatments except for 50mg/kg o-Vanillin + 2.5mg/kg of RG-7112 significantly decreased the expression of $p16^{INK4a}$, CGRP, GFAP and CD11b (**Figure 7A-E**). Interestingly, the therapeutic combination which provided the greatest reduction in all markers was 100mg/kg o-Vanillin + 5mg/kg of RG-7112 when compared to untreated SPARC-null mice. Specifically, the intensity areas were; $p16^{INK4a}$ (5.87% ± 0.29, $p<0.0001$), CGRP (13.67% ± 0.86, $p<0.0001$), GFAP (10.59% ± 0.92, $p<0.0001$), CD11b (1.32% ± 0.23, $p<0.0001$). Moreover, combination treatment at the highest doses further significantly reduced the area compared t the single drugs in all markers (**Figure 7A-E**).

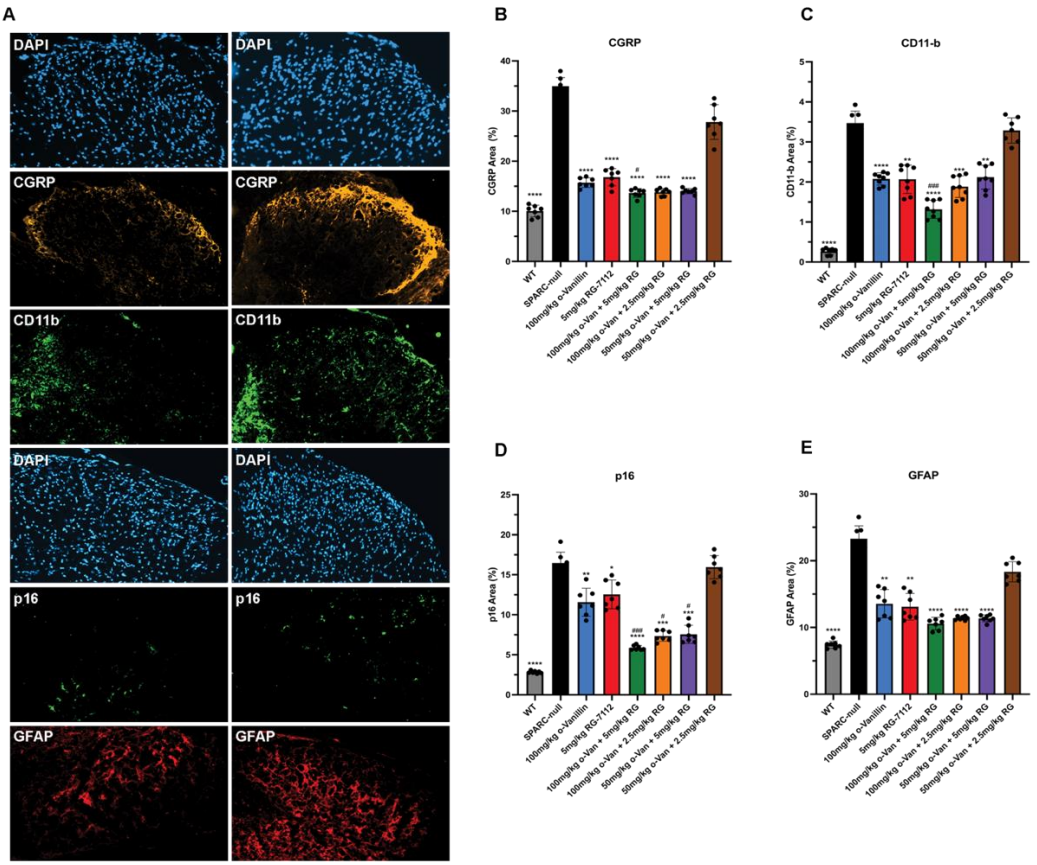


Figure 7: ***Oral gavage with o-Vanillin and RG-7112 of SPARC-null mice reduces the expression of senescence and pain markers in SPARC-null mice dorsal horns***
(A-E) Following 8 weeks of treatment, Spinal cords from 9-month-old SPARC-null mice were isolated and were analyzed on the senescent burden and pain markers in the dorsal horn. **(A)** Representative images of untreated SPARC-null mice and wildtype mice of each marker **(B)** Quantification percentage of area expressing CGRP. **(C)** Quantification percentage of area expressing CD11-b. **(D)** Quantification percentage of area expressing $p16^{INK4a}$. **(E)** Quantification percentage of area expressing GFAP. * Indicates significance between treatment (single or combination) and control group. # Indicates significance calculated when combination treatment is compared to single treatment groups. All analyses were performed using a one-way ANOVA. Data are presented as mean ± SD. * or # $p < 0.05$; ** or ## $p < 0.01$; *** or ### $p < 0.001$, **** or #### $p < 0.0001$. n= 7-8 mice.

DISCUSSION:

Globally, back pain is the number one cause of years lived with disability. Yet, there are no preventative or curative treatments available. The ability to improve, prevent or even slightly delay progression would have a tremendous socioeconomic impact. Surgeries aimed at providing symptomatic relief are not optimal. Recent research has indicated that senescence plays a significant role in the advancement of painful IVD degeneration[201]. In the present study, the therapeutic potential of the senolytic drugs RG-7112 and o-Vanillin, which have shown promising results to treat painful IVD degeneration in human *in vitro* and *ex vivo* models, were evaluated in the SPARC-null mouse model. SPARC-null mice have well-established disc degeneration and back pain at 4 months of age[143]. The data presented here demonstrate that pain behaviour and pain markers can be reduced through systemic treatment with o-Vanillin and RG-7112. The results also show that combining o-Vanillin and RG-7112 produces an additive effect in reducing senescent cells in the mouse IVD and dorsal horn. These results provide proof of principle that the senolytics o-Vanillin and RG-7112 may be useful in mitigating painful IVD degeneration by decreasing local senescence status and degeneration while lowering levels of inflammation.

Senolytics target senescent cells by disrupting their pro-survival signalling pathways. Treatment with o-Vanillin and RG-7112 has previously demonstrated significant efficacy in targeting senescent cells in human IVDs, as shown in[84,129,191]. It is worth noting that prior research has illustrated an increase in senescent cells during both disc degeneration and aging in both human and mouse subjects. Furthermore, the systematic eradication of $p16^{INK4a}$ positive senescent cells in aging mice resulted in an improvement of disc degeneration in aged mice. Notwithstanding the established causal association between senescence and disc pathology, the

utilization of extended senolytic therapy in preclinical models of lower back pain and disc degeneration has not been implemented. Consequently, SPARC-null mice were administered with o-Vanillin and/or RG-7112 on a weekly basis through oral gavage, commencing at 7 months until 9 months of age, when advanced disc degeneration becomes apparent. The findings indicate that the administration of o-Vanillin and/or RG-7112 during the initial stages of disc degeneration can effectively decelerate the progression of the disease, as evidenced by the preservation of tissue and cell morphology and lower grades of degeneration. Interestingly treatment with o-Vanillin and RG-7112 alleviated pain behaviour and decreased the expression of pain markers in SPARC-null mice spinal cords, thereby demonstrating that o-Vanillin and RG-7112 have localized effects in the disc and systemic effects. Further investigations are necessary to determine the molecular pathways targeted by o-Vanillin and RG-7112.

The secretion of various chemokines, pro-inflammatory cytokines, growth factors, and matrix remodelling enzymes by senescent cells is commonly referred to as SASP factors[191]. The proteins associated with the SASP phenotype, including but not limited to TNF-α, IL-6, MMPs, monocyte chemotactic protein-1, and insulin-like growth factor binding proteins, exhibit an upward trend with age in various tissues, as reported by Freund et al[223]. Previous studies have shown that the secretion of SASP factors from senescent cells fluctuates during senescence[224]. In addition, it has been observed that SASP factors can induce adjacent cells to undergo senescence and enhance SASP factor production through autocrine and paracrine mechanisms[100]. The autocrine and paracrine secretion of SASP factors has been recognized as a crucial factor in the pathogenesis of age-related disorders, including IVD degeneration, osteoarthritis, and osteoporosis[122].SASP factor secretion can be considered a two-sided phenomenon in select circumstances. On the one hand, paracrine secretion can expedite cellular

senescence by prompting neighbouring cells to undergo senescence. On the other hand, autocrine secretion can strengthen senescent growth arrest, including the suppression of senescent tumours[88]. When selecting a model to evaluate senolytic drug potential, it is imperative that the model has a SASP phenotype otherwise, it would not be a representative physiological model of cellular senescence. In the current study, using the non-treated SPARC-null mouse model, SASP factors were found to be significantly upregulated when compared to wild-type mice in both the *ex vivo* and *in vivo*. Furthermore, SASP factor expression was decreased when the SPARC-null mice were treated with o-Vanillin and RG-7112.

Recently, several studies have looked at the potential of combining senolytic compounds. One disadvantage of single-drug treatment includes the drug failing to target multiple senescent anti-apoptotic pathways in the same cell type or different cell populations within a target tissue. By targeting multiple and indirectly related anti-apoptotic pathways, increased selectivity for senescent cells may be achieved in the absence of toxicity for normal proliferating or quiescent cells. This theory is further supported by a recent study showing the benefit of combining Dasatinib and Quercetin, which target antiapoptotic networks instead of a single target[130,200]. Combining compounds could also potentially lower the therapeutic dosages required, which may decrease the side effects associated with single drugs. Furthermore, combined treatment at lower doses may allow the repurposing of drugs that were previously discarded due to undesirable side effects and increase success in clinical trials. One of the primary findings reported in the present study is that combining o-Vanillin and RG-7112 was significantly better at reducing the senescent cell burden, pain behaviour, and SASP factors. This may be explained by the fact that o-Vanillin is suggested to have a broad effect, targeting multiple different pathways involved in senolytic activity, one being the $p16^{INK4a}$/pRB

pathway, whereas RG-7112 is suggested to work by inhibiting MDM2 in the p21/p53 pathway, specifically[84,129]. Given that combining these two mechanistically different drugs appears to have greater therapeutic potential, the idea of senolytic compounds appears feasible and beneficial.

Although it has been demonstrated that RG-7112 and o-Vanillin have senolytic activity in IVD cells, it is important to determine whether they can attenuate perceived pain and degeneration in a clinically relevant animal model without causing adverse effects[84,129,173]. There are many shared mechanisms in SPARC-null mice and human patients with IVD degeneration and pain symptoms, including age-related disease onset, degeneration severity, reduced SPARC expression in degenerating IVDs, axial and radiating pain symptoms, nerve ingrowth and accumulation of senescent cells in degenerating IVDs[173]. Furthermore, the feasibility of this model is well documented in our previous studies, where IVD degeneration was measured, low back pain and pain-related neuroplasticity in the spinal cord in the same animals by measuring pain behaviour *in vivo* during treatment and expression profile and tissue homeostasis *ex vivo* after the treatment period[173].For all these reasons, it was believed that the use of the SPARC model was an ideal model to use to evaluate the potential of o-Vanillin and RG-7112. As demonstrated in this study IVDs of SPARC-null mice, like symptomatic human IVDs, release increased levels of inflammatory mediators. These factors directly activate pain-sensing nerve fibres in or surrounding the IVD[173]. SPARC-null mice with fibres exhibit signs of sensory neuroplasticity in the spinal cord associated with pain, including increased microglia and astrocyte numbers and increased calcitonin gene-related peptide (CGRP) immunoreactivity in the dorsal horn[173]. SPARC- null mice start to show signs of degeneration at 3-4 months of age, corresponding with the development of behavioral signs of back pain. By 6 months of age,

several lumbar IVDs are at least moderately degenerated and behavioural signs of pain are consistently observed. At 9 months of age, the SPARC-null mice show signs of severe degeneration and show clear signs of axial discomfort and radiating pain[143,145,147]. In the present manuscript, treatment of SPARC-null mice started at 7 months and ended at 9 months of age to observe if o-Vanillin or RG-7112 could slow down the progression of degeneration in SPARC-null mice and avoid the IVDs progressing to a more severe degenerative state.

This study is not without limitations. The present study is limited by the utilization of a global deletion of the SPARC gene (i.e., SPARC-null), which has the potential to modify other tissues. SPARC-null mice exhibit osteopenia reduced dermal strength, impaired wound healing, and early-onset cataractogenesis[142,225]. Moreover, SPARC-null mice do not exhibit knee arthritis, whereas arthritic cartilage demonstrates elevated levels of SPARC[225]. Although the possibility of involvement from other tissues cannot be completely dismissed, the behavioural phenotype resulting from SPARC deficiency exhibits regional specificity, as evidenced by a heightened sensitivity in the hind paw region but not in the facial area[145]. Nevertheless, it is imperative to evaluate the involvement of senolytics in other models of disc-related low back pain that possess supplementary mechanisms, such as the disc puncture models[226,227].

Here, studies were performed using middle-aged mice (age 7-9 months); where the lumbar discs of SPARC-null mice exhibit moderate degeneration in multiple instances, although disc rupture is infrequent. While this model simulates a clinical setting in which both pain and degenerative disc conditions are present, most intervertebral discs exhibit no significant compromise. Future studies should further explore the role and therapeutic value of the o-Vanillin and RG-7112 in earlier disc pathology as a means of prophylaxis. Moreover, the

use of larger animal models as well as using naturally occurring models of degeneration would be worthwhile to compare the data presented here.

CONCLUSION:

In summary, systemic administration of o-Vanillin and RG-7112 possesses an exciting therapeutic approach to treating IVD degeneration without the inherent risks associated with invasive surgical interventions. The potential benefits of o-Vanillin and RG-7112 treatment include alleviation of IVD degeneration, reduction in systemic inflammation, and improved pain behaviour. Furthermore, insights into the pain phenotype effects of o-Vanillin and RG-7112 treatment and provided. The issues presented here will be critical to consider during future pre-clinical studies in large animal models of disc degeneration.

Discussion and Conclusion:

Summary:

Although common and costly, very little is known about the mechanism leading to back pain, and no early treatment options are available. Given the lack of conservative/early treatment options, patients present with advanced stages of the disease and thus require invasive surgery to relieve pain. Current evidence suggests that changes in the biomechanical properties of degenerating discs are associated with matrix fragmentation, inflammation, and pain. It is, however, less clear how pain and degeneration are initiated and how they could be prevented. Growing evidence suggests that senescent cells accumulate during tissue degeneration, contributing directly to disorders like heart disease, cancer, and osteoarthritis. In ageing and degenerating IVDs, tissue homeostasis is disrupted by the accumulation of senescent cells producing inflammatory and nociceptive factors that cause pain and inflammation, along with proteases degrading the tissue. Whereas in tissues with healthy homeostasis, anabolism and catabolism are in balance.

By interacting with various cell surface receptors (e.g., TLRs, Vanilloid, chemokine and opioid receptors), o-Vanillin has the ability to reduce pro-inflammatory immune mediators and matrix degradation which in turn can alleviate spinal pain and prevent IVD and face degeneration. Eight TLRs (TLR-1, -2, -3, -4, -5, -6, -9 and -10) have been recognized for their role in human IVDs. Specifically, TLR-1, -2, -4, and -6 levels have been shown to increase with increasing disc degeneration and pain. For example, TLR-2/4 has been shown to be upregulated and activated and when primary chondrocytes and IVD cells are subjected to stress thereby inducing IVD degeneration. Synthetic TLR antagonists can also be used to reduce pain however they have not been shown to provide tissue regeneration in mouse models of back

pain. Using a TLR activation induction model in cells from painful degenerate human IVDs, the first chapter of this thesis focused on identifying a link between TLRs and cellular senescence in cells from painful degenerate human IVDs and determined if o-Vanillin could reduce inflammatory mediators and proteases from TLR-induced cells from painful degenerate human IVDs. These findings allowed us to continue utilizing our models to study RG-7112 and o-Vanillin.

It has been previously shown that the senolytic drugs RG-7112 and o-Vanillin target and remove senescent cells from IVDs both *in vitro* and *ex vivo* and as such, improve tissue homeostasis. One drawback of using a single senolytic agent is the failure to target multiple senescent anti-apoptotic pathways in the same cell type or different cell populations within a target tissue. Concurrently targeting multiple and indirectly related anti-apoptotic pathways may increase selectivity for senescent cells without toxicity for normal proliferating or quiescent cells. Successful combination therapy is exemplified by the combination of Dasatinib and Quercetin, which targets antiapoptotic networks instead of a single target. The lower therapeutic dosages enabled by combinations also decreased side effects associated with single drugs. Combined treatment at lower doses may allow the repurposing of previously discarded drugs due to undesirable side effects and increased success in clinical trials. By using a 3D cell culture model and further utilizing our model of TLR induction in cells from painful degenerate human IVDs, the second chapter of this thesis aimed to evaluate if the combination of RG-7112 and o-Vanillin could have a greater effect on removing senescent cells and decreasing SASP factors from cells in painful degenerate human IVDs. Furthermore, this study demonstrated that senolytic treatment could decrease neuron sprouting from PC-12 cells. This chapter also provided evidence that o-Vanillin and RG-7112 could relieve pain and reduce

senescence burden in an *in vivo* model.

An obstacle of the senolytic agents currently under investigation is their failure to target the same cell type in different species or different cell types within a species. It is, therefore, imperative to test novel agents for effectiveness against senescent cell burden, and for potential deleterious side effects, in pre-clinical models with clinically relevant cells. As noted above, mice with targeted deletion of the SPARC gene have been characterized as a valuable model of pain associated with IVD degeneration. The SPARC-null mice have progressive, age-dependent, intervertebral disc degeneration and back pain, presenting a clinically relevant model of low back pain. The ability to prevent or slightly delay the onset of low back pain would have a tremendous socio-economic impact. Chapter 3 demonstrated that the combination of two senolytic drugs, o-Vanillin and RG-7112, can selectively remove senescent cells, reduce inflammatory mediators, and relieve behaviour pain in middle-aged SPARC null mice with back pain *in vivo*.

Discussion

Sterile Inflammation and TLRs

This thesis frequently discussed how through the secretion of SASP factors from senescent cells or TLR-2 induction results in sterile inflammation. Sterile inflammation is characterized by an increase in proinflammatory substances and other inflammatory processes in the absence of a pathogenic infection. When disc degeneration occurs in intervertebral discs, the local cells produce a sterile inflammation[21]. Additionally, it is hypothesized that a range of immune cells, such as macrophages, neutrophils, and T-cells, infiltrate degenerating discs after disc herniation[23,228]. Numerous pro-inflammatory cytokines, proteases, and neurotrophins are increased by resident NP and AF cells alone as well as when combined with immune cells to

accelerate disc degradation, innervation, and vascularization. Previously, it has been shown that degenerating discs from individuals with low back pain emit more cytokines and neurotrophins than non-degenerating discs do[167]. In this thesis, it was demonstrated that there is a significant increase in inflammatory markers following TLR-2 induction in painful degenerate IVD cells when compared to the non-induced cells[191]. These findings further supplied us with a model to mimic this sterile inflammation *in vitro* to test the effectiveness of our compounds in hindering inflammatory processes.

Knowing whether certain components are released from the disc or not is a crucial consideration for creating new therapeutic targets, presuming these processes are connected to the course of degeneration and the development of pain. Once released from the disc, produced substances may directly affect nerve cells and cause various negative effects. Endplates and vertebral bodies may suffer deleterious consequences from secreted nociceptive and inflammatory substances. For instance, aberrant endplates are innervated and may be affected by nociceptive variables produced from the disc[229]. Chronic low back pain is connected to disc-derived inflammation and accompanied by modifiable alterations in the endplates and vertebral bodies[230,231]. Chronic pain may arise because of modic changes, pathological alterations in vertebral bodies, and released substances from ageing discs. Adult bone, such as a vertebra, is innervated by pain-sensing TrkA (NGF receptor) and CGRP-expressing fibres (A and C-fibers)[232]. Chronic pain may arise because of NGF and cytokine sensitization of these fibres. NGF, BDNF, IL-1, TNF, and many other cytokines and proteases are produced and secreted in greater amounts when TLR-2 is activated *in vitro*[191] (*Mannarino et al. in submission*). Furthermore, previous literature has shown that TLR-4 suppression in mice reduces the symptoms of sterile inflammation[233]. Studies conducted *in vitro* show that both NP and AF

cells express functional TLRs. Endogenous alarmins and TLRs may both be expressed by immune cells that invade ruptured discs. The findings of our research collectively show that TLRs are involved in the control of sterile disc inflammation. The hypothesis that TLRs may play a role in the sterile inflammation from cellular senescence and contribute to the emergence of a vicious cycle that results in degeneration and discomfort is the outcome of our research. Alarmins, cytokines, and chemokines can further increase after the initial spike. The cycle would go more quickly if receptor expression was increased. The disc's ECM would continue to deteriorate because of these circumstances, eventually leading to a structural breakdown. The ECM in degenerative discs can no longer resist nerve fibres, probably as a result of the diminished anionic charge[234]. Semaphorin expression changes during disc degeneration and is partially regulated by cytokines, including IL-1[235,236], which are guiding and repulsive cues for axon development. Sterile inflammation enhances vascularization factors like VEGF[136,237,238] and neurotrophic factors that support innervation like NGF and BDNF[102,108,111,167]. Additionally, there is an increase in the number of chemotactic substances such as CXCL1, CCL2, and CCL8, which draw and stimulate immune cells. Vascularization enables immune cell infiltration, which increases the production of cytokines[23,228]. Although this has not been researched, innervating DRG nerve fibres may also have proinflammatory neurogenic consequences in the disc. Therefore, the pro-inflammatory and pro-degenerative cycle may be accelerated by innervation and vascularization. It would be necessary to break the pro-inflammatory cycle to delay or stop the course of disc degeneration. For regenerative medicine techniques to be effective, sterile inflammation most likely needs to be decreased in addition to reducing or preventing the progression of disc degeneration[27]. NSAIDs and other current anti-inflammatory tactics do not seem to be enough. Our work demonstrated that both *in vitro*

and in vivo o-Vanillin decreased sterile inflammation when compared to non-treated controls. Moreover, these findings allowed us to observe the anti-inflammatory effect of both RG-7112 and o-Vanillin.

The risk factors for disc degeneration and chronic low back pain include unfavourable mechanical loads, traumatic events, and several lifestyle decisions. Similar to how a combination of risk factors may cause chronic low back pain[6,42,239], a combination of risk variables may cause disc degeneration. Because of this, the development and early phases of disc degeneration are not well understood, which makes it difficult to create medications that can slow or stop the illness. Regardless of the cause, ECM degradation is a fundamental aspect of disc degeneration. Rapid ECM deterioration is another side effect of trauma[19]. Physiological ECM degradation also takes place during regular ECM turnover in addition to pathological ECM degradation. Due to the inadequate waste exchange from disc[6], ECM turnover may increase alarmins slowly. Alarmins can activate TLRs as a result of increased normal and pathologic matrix turnover, which may contribute to the onset of disc degeneration. The main result of TLR activation is an increase in pro-inflammatory cytokine production. Additionally, it has been demonstrated that TLR activation in discs enhances NGF and BDNF production, lowers ECM production, and increases protease production[111]. Specifically, proteases MMP3 and MMP13 cleave the ECM components type II collagen, aggrecan, and fibronectin, potentially producing additional alarmins. It is probable that the initial activation of TLRs in a pathogenic state causes an increase in cytokines, proteases, TLRs, and cytokine receptors. It has been previously demonstrated that TLR-2 activation causes degenerative alterations in human discs that are not yet degenerating, suggesting that TLRs may play a role in the early phases of disc degeneration and cellular senescence. TLR-2 is thus a possible therapeutic target

for senolytics. The role of TLR-2 in cellular senescence and its induction was the main question of this thesis's research. Additional work focused on examining if o-Vanillin and RG-7112 could attenuate the TLR-2 induced cells. According to research on arthritis and cartilage, S100 proteins, HMGB1, annexins II, V, and VII, uric acid, fragments of fibronectin, low-molecular-weight hyaluronan, biglycan, and tenascin[60] all activate TLR-4. Additionally, many of these alarmins that can be considered SASP factors can stimulate TLR-2. By treating human disc cells with lipopolysaccharide, we evaluated TLR-4 briefly. We discovered that TLR-2 agonist PAM2CSK4 had a greater gene and protein expression of senescence, cytokine, and neurotrophins than that of TLR-4 induction with lipopolysaccharide. Despite being previously identified as a TLR-4 ligand, Quero et al. discovered alarmins like low molecular weight hyaluronan selectively activate TLR-2 on human disc cells[72].

There are a few potential causes for the differences in TLR-2 and TLR-4 between human disc studies and other investigations. There may be a species difference since studies have linked TLR-4 to the degeneration of rodent discs and TLR-2 to that of human discs. Another possibility is that discs express TLR co-receptors, which activate TLR-2 rather than TLR-4. A potential argument is that only a few alarmins' effects on disc cells have been studied, and it is possible that further alarmins will cause TLR-4 to become active. Although context and tissue specificity are probably crucial. Alarmins, for instance, affect synoviocytes differently than chondrocytes[60]. TLR-2 appears to play a significant part in human disc degeneration and be linked to cellular senescence, despite the need for more research on TLR-4's function. There are additional alarmin receptors in addition to TLR-2 and TLR-4. Recently, it was discovered that TLR-5 is an HMGB1 receptor[240]. In joint cells, the alarmin receptor for AGEs, S100 proteins, and HMGB1 is known as the receptor for advanced glycation end products (RAGE).

Depending on the situation, purinoceptors—such as ATP P2X receptors—can alternatively be referred to as alarmin receptors[60]. Alarmins found outside of the ECM or in non-physiological sites (such as soluble biglycan and extracellular HMGB1) share the property of being necessary for normal physiological function. Alarmin and alarmin receptors have not been well studied in intervertebral discs up to this point. Alarmins probably have a much larger impact on disc degeneration and cellular senescence than just TLR-2, which merits further study. Nevertheless, the research presented in this thesis demonstrates that inducing TLR-2 increases the number of senescent cells in cells from painful degenerate IVDs and the release of cytokines and neurotrophins. Furthermore, it was demonstrated that both RG-7112 and o-Vanillin could attenuate the effect of TLR induction and decrease the expression of TLR-2 in painful degenerate IVD cells. Together, these findings strongly support a potential role for TLRs in cellular senescence in disc degeneration and point to TLRs as prospective therapeutic targets for senolytic activity.

Senescence and Therapeutics

Cellular senescence is the irreversible loss of cell proliferation that takes place in response to developmental cues and the buildup of unrepaired DNA damage. It is a complicated, multi-step dynamic process, and new experimental findings continue to advance our understanding of it. In general, the early Cellular senescence step is characterized by prolonged activation of the $p16^{INK4a}$ and/or p53-p21 pathways, which causes cells to exit their cell cycle and enter the stable cell-cycle arrest phase. When early Cellular senescence cells subsequently experience morphological alterations, chromatin remodeling, and the production of SASP components, full Cellular senescence is the result. It is believed that other genetic and epigenetic alterations, including chromatin budding, histone proteolysis, and

retrotransposition, which encourage further transcriptional change and SASP heterogeneity, are what ultimately lead to late Cellular senescence[100,241]. Replicative senescence (RS), which is brought on by telomere shortening, and stress-induced premature senescence (SIPS), which is brought on by the buildup of DNA damage and other forms of cellular stress, are the two types of cellular senescence[242]. Cell-cycle withdrawal, macromolecular damage, SASP, and dysregulated metabolism are key characteristics of Cellular senescence and are interdependent on one another when senescence is activated[97]. Growth factors, MMPs, and pro-inflammatory cytokines and chemokines are all abundantly produced and secreted by cells expressing SASP[97]. Stress triggers such as telomere erosion, DNA damage, lysosomal stress, oncogene activation, and oxidative stress are among the many factors that cause senescence. Due to cell cycle arrest, resistance to mitogens, and neoplastic transformation brought on by these stresses, Cellular senescence results. Tissue injury and degeneration are brought on by the establishment of persistent senescence and the buildup of senescent cells in the neighbourhood microenvironment.

Multiple biomarkers, including increased expression of cell cycle regulatory kinases, including p53, p21CIP1, $p16^{INK4a}$, and phosphorylated retinoblastoma (Rb), are utilized to detect senescent cells or the senescence process. Telomere damage, elevated levels of SASP components, the activity of senescence-associated beta-galactosidase (SA-gal), and expression of anti-apoptotic markers such as Bax-1 are further indicators of cellular senescence. Due to the variability of senescent cells and the lack of a single reliable biomarker, senescence is difficult to characterize.

Age-related IVD degeneration has been the subject of research into the mechanics of senescence using cell culture models. SIPS, or acute cellular senescence as it is sometimes

called, is induced when initial cells are incubated with various stressors for several days in addition to replicative senescence[152,242]. In this thesis, the use of $p16^{INK4a}$ was the most common marker used since it has been previously demonstrated from work in our lab that $p16^{INK4a}$ gives the truest and most realistic value for senescence in degenerate IVDs.

One of the primary models that were utilized in this thesis was to induce TLR-2 activity to mimic SIPS by creating a large inflammatory response in our cell culture. External stimulation of disc cells activates SIPS, which, together with *in vivo* IVD degeneration investigations, is utilized to examine cellular senescence and IVD degeneration. The full senescence phenotype found in SIPS often takes cells grown *in vitro* two weeks to establish after exposure to genotoxic or oxidative stress. Despite not being explicitly stated, the stimulation of disc cells over 1-3 days with external stimuli, as described in much research, most likely activates the acute response to stress rather than the actual SIPS. To distinguish between replicative senescence, SIPS, and merely an acute reaction to stress in disc cells, more research is required.

Eliminating senescent cells can stop or postpone the alterations brought on by aging, as senescent cells multiply as we age[122,243,244]. The removal of senescent cells may be able to restore tissue homeostasis in aged tissue, as suggested by the reduction in age-associated IVD degeneration in aging mice following systemic clearance of $p16^{INK4a}$-positive cells via a genetic approach[201]. Senolytics are a group of medications that specifically target senescent cells to cause apoptosis by obstructing the pro-survival or anti-apoptotic pathways that are increased during senescence. In numerous pathological situations, including fibrotic pulmonary illness, bone loss, etc., a number of small compounds and medications have undergone substantial research as senolytics[245-247], But research on their therapeutic potential in relation to disc

degeneration is still in its early stages. Dasatinib, a kinase inhibitor, causes apoptosis in senescent cells by blocking Src-family tyrosine kinases, whereas Quercetin, a polyphenol, does so via blocking the anti-apoptotic protein Bcl-xL. In the Ercc1/ mouse model of accelerated aging, the inhibitors Dasatinib + Quercetin were the first senolytics to be shown to diminish IVD degeneration and to increase health span and lifespan in mice that were already old. Through suppression of the NF-B pathway and activation of the antioxidant Nrf2, quercetin has been shown to reduce disc degeneration in a puncture-induced rat model and prevent IL-1-induced cellular senescence[248]. In naturally aged mice, intermittent Dasatinib and Quercetin therapy can improve disc health[200]. According to transcriptome analysis, Dasatinib and Quercetin affects key genes linked to aging in NP cells. The senescence markers $p16^{INK4a}$, p19, and SASPs decreased together with the level of IVD degeneration after receiving the Dasatinib and Quercetin treatment for 6 and 14 months. In addition to degeneration prevention, Dasatinib and Quercetin treatment protected intact disc ECM and decreased aggrecan breakdown in mice. Additionally, this study's finding that sustained therapy is well-tolerated points to the therapeutic potential of Dasatinib + Quercetin therapy for IVD degeneration[200]. According to this research, quercetin, either alone or in combination with Dasatinib, may be a viable therapeutic drug for the treatment of IVD degeneration. The studies discussed gave us the premise to evaluate both natural and synthetic senolytic compounds. Furthermore, to evaluate their combination. In this thesis, we found similar results regarding the use of the combination of senolytic compounds RG-7112 and o-Vanillin both *in vitro* and *in vivo*.

In IVD degeneration models, natural substances have also been explored as senolytics. In both an H2O2-induced NP cellular senescence cell culture paradigm, morroniside, a main iridoid glycoside of the well-known traditional Chinese medication Fructus Corni, is helpful in

reducing IVD degeneration characteristics[249]. For protection against the advancement of IVD degeneration, morroniside inhibits the ROS-Hippo-p53 signalling pathway, reduces cellular senescence signalling, and stops matrix deterioration[249]. A natural active therapeutic substance called curcumin is utilized to treat a variety of human diseases, including illnesses connected to aging[250,251]. In TBHP-treated human NP cells, curcumin had anti-apoptotic and anti-senescence properties, and it prevented the progression of IVD degeneration in a rat model[252]. To prevent TBHP-induced apoptosis, senescence, ECM breakdown, oxidative stress, and mitochondrial dysfunction in human NP cells, curcumin restored the TBHP-induced blocking of autophagy flux[252]. In a monolayer and pellet culture, treatment of human NP cells with curcumin or its metabolite o-vanillin reduced the amount of $p16^{INK4a}$ positive cells and raised the number of Ki-67 and caspase-3 positive cells[84]. Curcumin and o-vanillin also reduced inflammatory signalling in NP cells, which was shown by lower phosphorylation of p65/RelA, Nrf2, JNK, and ERK, confirming their senolytic and anti-inflammatory characteristics[84]. O-vanillin and the synthetic substance RG-7112 were compared, and it was discovered that both of them trigger apoptotic pathways to kill senescent IVD degenerate cells. Contrarily, it has been noted that these substances stimulate pathways involved in proliferation in non-senescent disc cells[129]. In pellet culture and in intact human IVDs, both substances decreased inflammatory cytokines, chemokines, and growth and neutrophilic factors[129]. This thesis describes further work done with RG-7112 and o-Vanillin. We demonstrate that RG-7112 and o-Vanillin prevented TLR-2-induced senescence of disc cells isolated from individuals with back pain and IVD degeneration and decreased the number of senescent cells in cell pellet culture from deteriorated discs[191]. Moreover, *in vivo,* we have shown that both these compounds and their combination have decreased SASP factor release, expression of pain

markers in the spinal cord and decreased expression of senescent cells in the IVD and spinal cord.

Animal Models for Disc Degeneration and Pain

SPARC-null mice have been used to characterize the development of disc degeneration as assessed by x-ray, histology, and MRI, pain phenotype, pain-related spinal cord neuroplasticity, and pharmacological profiles[143-147]. SPARC-null mice's age-related, progressive disc degeneration serves as a model for human discs' age-related, progressive degeneration[6]. This knowledge gave us the ability to design our trials at a period when SPARC-null animals have been experiencing disc degeneration and discomfort for several months but have not yet reached end-stage degeneration, allowed us to study the effects of senolytic compounds RG-7112 and o-Vanillin.

To imitate human chronic low back pain and disc degeneration, where patients seek treatment after more than three months of discomfort but before surgery is necessary, we timed our intervention at this point. Other rodent models are available. Most frequently, an acute injury—such as a needle puncture or a sustained, harmful strain—causes disc degeneration. Injury models suffer from a few significant problems. First off, disorders that affect middle-aged and old adults are most frequently modelled using young rats[137,138,253]. Age-related changes in the neurological system and discomfort are a result of aging. Age-dependent alterations occur in nociceptor sensitization, and chronic pain behaviours in rodents are similarly influenced by age[254]. Grey matter and white matter alterations that occur in the human brain 270,271 may influence how chronic pain is perceived. Age also has an impact on human pain tolerance, pain ratings, and pain perception[255,256]. Therefore, when trying to draw therapeutically applicable conclusions from young rat models, using young mice poses a

possible complicating issue. The fact that injury models are acutely produced means they cannot accurately represent the gradual or age-related alterations to intervertebral discs that occur in humans. The following are some benefits of injury-induced models of disc degeneration: The onset of degeneration is controlled, the disc impacted by degeneration is known and can affect one or more discs, the disc affected by degeneration is known, and traumatic disc injuries, another prevalent cause of low back pain, can be modelled using puncture models. Injury models' temporal and anatomical control is advantageous when illuminating certain pathways or considering therapeutic options.

There are some restrictions on our investigation of cellular senescence utilizing the SPARC-null mice. First, because SPARC is expressed throughout the body, the loss may have an impact on other structures, which could change how we interpret our data. Mouse vision, bone density, epidermis, periodontal ligaments, and fat tissue are all impacted by SPARC deletion[257,258]. SPARC-null mice, however, do not seem to progress to osteoarthritis[143]. During human disc degeneration, SPARC also declines, indicating that it is connected to disease pathogenesis[196,259]. Senescent cells are expressed in a variety of cell types and senolytics were administered systemically through oral gavage. These senolytic compounds may therefore operate in other tissues to modify their phenotype of pain. We are convinced that RG-7112 and o-Vanillin did influence the disc in SPARC-null mice since we discovered reduced disc inflammation. Human disc herniation typically happens before end-stage degeneration because the NP needs enough hydrostatic pressure to burst through the AF or the endplate[6,260]. In contrast, herniation occurs later in the degenerative phase in SPARC-null mice. Since we employed SPARC-null mice at an age when their discs had not herniated in earlier investigations[143,145], it is likely that this change had no impact on our study. A gene could be

deleted in notochordal cells to have an effect solely in the NP as a way to avoid some of the potential side effects of employing a global gene deletion. This method has been used to precisely remove CCN2 in the NP, and it causes disc degeneration[150]. Concerns about impacts on other tissues in SPARC-null mice would be lessened by tissue-specific deletions of SPARC.

The cellular makeup of rodents' discs differs from that of humans, which is a limitation of all rodent models. While notochordal cells are present in the NP throughout the lifespan of rodents, this may not be the case in humans[13,261]. The NP cell density of mouse and rat discs is substantially higher than that of human discs. Rodent discs have a lower capacity for self-repair than human discs, perhaps as a result of differences in cell density and cell type. This may be the reason why some groups inject TNF into discs to ensure a robust degenerative effect[136] when using a puncture model. Although higher cell density may still result in variations in cytokine, protease, neurotrophin, and ECM synthesis relative to humans, SPARC-null mice exhibit well-characterized disc degeneration. Increased cell density might alter how discs react to treatment methods. No single animal model of disc degeneration is perfect, as was discussed here, but the ability to employ a variety of species to address particular issues will help us understand how disc degeneration and pain work.

When employing animal models, pain and loss of function—which are what prompt patients to seek medical assistance for disc degeneration—should be taken into account and assessed. Here, we employed a variety of behavioural assays, such as the Von Frey, acetone-evoked behaviour, and grip strength tests. These behavioural experiments have been extensively utilized by our team in SPARC-null mice to validate them[143-147]. In keeping with earlier research, we discovered that SPARC-null mice have greater axial, radiating pain and mechanical sensitivity. Acetone-evoked behaviour is thought to be a measure of radiating pain

caused by disc degeneration 117,119. We demonstrated here that degenerating discs from SPARC-null animals produce several inflammatory cytokines. It is interesting to note that inflammatory mediators can generate cold sensitivity[262]. Other structures might start to contribute to the pain phenotype as a result of the global ablation of SPARC. However, since models of osteoporosis exhibit non-localized heat and mechanical sensitivity, it is unlikely that decreasing bone density influences pain behaviour. There is, of course, a chance that other organs, such as ligaments, could be involved in the SPARC- deficient mice's pain phenotype. A mechanical sensitivity phenotype[253] has been noted in various injury-induced animal models of disc degeneration that have studied pain behaviour. These variations could be caused by various pain-causing processes in the various models. Using a variety of animal models will improve understanding of how disc degeneration and disc injury relate to pain, despite these variations and restrictions. The behavioural assays that we and others have utilized in disc degeneration mice are mostly evoked pain measurements. Von Frey and other evoked measures of pain are practical, repeatable techniques for studying pain in rodents and can shed light on neuronal sensitization and nervous system reorganization. However, induced pain assessments in rodents could not accurately capture spontaneous pain and functional changes that cause humans to seek medical assistance. Rodents' spontaneous pain and functional changes are measured using a variety of tests. For instance, the tail suspension and open field can be used to monitor changes in behaviour, such as scratching, spontaneous rearing, and changes in movement. Changes in function can provide information regarding an animal's pain state. Although not all chronic pain syndromes have a phenotype, different rodent strains exhibit similar behaviours in various ways. For instance, changes in pain severity, stress, and sex may all have an impact on nesting behaviours 283-285. By weighing the advantages and

disadvantages of spontaneous and evoked measurements of pain, it appears likely that a combination of evoked and spontaneous measures is the most effective strategy for studying animal pain behaviour.

The Use of Human Tissue and Animal Models for Translational Science

The biggest causes of disability worldwide are chronic low back pain and disc degeneration[1,2]. Therefore, translational research goals should be included in studies of disc degeneration. Finding and using basic scientific ideas to deepen our understanding of a clinical issue to better treat patients is the aim of translational, preclinical research. When creating the projects for this thesis, we kept this idea in mind. Examples include investigating senolytics potential in cells from symptomatic patient populations and using an animal model where disc degeneration and pain had developed months prior to beginning an intervention. The use of human *in vitro* cell culture, Human *in vitro* 3D cell culture, and *in vivo,* animal models was one of this thesis's strongest points. We were able to thoroughly validate the potential of senolytics compounds RG-7112 and o-Vanillin to treat disc degeneration using a combination of models that no one model could, which eventually improved our understanding of therapeutic targets for senescence and chronic low back pain. It's vital to emphasize that our team is extraordinarily privileged to work with a large group of spine surgeons, which offers us access to human intervertebral discs that are from patients that suffer from IVD degeneration and chronic lower back pain. This makes it possible for us to see if our compounds could potentially have an effect on a symptomatic patient population and examine the mechanisms that are affected by senolytic compounds in disc degeneration. In the future, it will be crucial to continue using various tissue sources, cell and organ cultures, and animal models to provide translational knowledge.

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Appendix:

1. Permission

Figures 1, 2 and 3 found within the introduction section of this thesis were obtained from the following two papers; 1) For whom the disc tolls: intervertebral disc degeneration, back pain and toll-like receptors and 2) Cellular Senescence in Intervertebral Disc Aging and Degeneration: Molecular Mechanisms and Potential Therapeutic Opportunities which were published in open access journals with Creative Commons Attribution Licences and therefore do not need permission for use.

2. Related Work

During my Ph.D. study, I contributed to three manuscripts in the field of intervertebral disc degeneration and pain as a co-author:

1. Cherif H, **Mannarino M**, Pacis AS, Ragoussis J, Rabau O, Ouellet JA, Haglund L. Single-Cell RNA-Seq Analysis of Cells from Degenerating and Non-Degenerating Intervertebral Discs from the Same Individual Reveals New Biomarkers for Intervertebral Disc Degeneration. Int J Mol Sci. 2022 Apr 3;23(7):3993. doi: 10.3390/ijms23073993. PMID: 35409356; PMCID: PMC8999935.

2. Li L, Sheng K, **Mannarino M**, Jarzem P, Cherif H, Haglund L. o-Vanillin Modulates Cell Phenotype and Extracellular Vesicles of Human Mesenchymal Stem Cells and Intervertebral Disc

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