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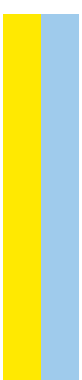
Modulation of inflammatory response associated with intervertebral disc degeneration

Graciosa Patrícia Quelhas Teixeira



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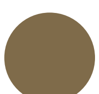

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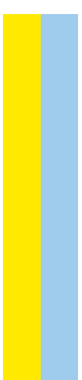

GRACIOSA PATRÍCIA QUELHAS TEIXEIRA

MODULATION OF INFLAMMATORY RESPONSE ASSOCIATED WITH INTERVERTEBRAL DISC DEGENERATION

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Doutora Raquel Madeira Gonçalves

Categoria – Investigador auxiliar

Afiliação – Instituto de Investigação e Inovação em Saúde/Instituto de Engenharia Biomédica da Universidade do Porto

Coorientador - Doutora Cornelia Neidlinger-Wilke

Categoria – Investigador auxiliar

Afiliação – Institute of Orthopaedic Research and Biomechanics, University of Ulm, Germany

Coorientador - Professor Doutor Mário Adolfo Monteiro da Rocha Barbosa

Categoria – Professor catedrático

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto; Instituto de Investigação e Inovação em Saúde/Instituto de Engenharia Biomédica da Universidade do Porto

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To my parents

My role models.

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ABSTRACT

Intervertebral disc (IVD) degeneration and associated inflammation often lead to low back pain, one of the major causes of disability worldwide. Although available clinical treatments decrease symptoms' progression, they fail to restore native IVD properties. Clinical trials using cell therapies are increasing, but the hostile pro-inflammatory environment of IVD may challenge their success, since may impair cell survival and matrix formation. Due to the high impact on population health and the lack of adequate solutions, novel therapies that modulate the inflammatory response can be a new hope to treat IVD degeneration.

This works opens new perspectives on alternative therapeutic approaches to modulate inflammation, while stimulating IVD regeneration, and on how the inflammatory environment of IVD can challenge the regenerative process.

Though a high number of animal models have been developed to mimic IVD degeneration, when this investigation commenced there was no model that simulated the inflammatory process of human progressive disc degeneration, while allowing its standardized control. Therefore, a standardized degenerative/pro-inflammatory *ex vivo* IVD model was first established and validated, using bovine caudal disc cultures, under static loading, stimulated by needle-puncture and IL-1β. From several conditions tested, these were shown to mimic more closely the human IVD degeneration and associated inflammatory response. Moreover, this model showed great potential for testing bioactive molecules and cell therapy approaches.

A nanotechnology-based therapy, based on chitosan (Ch) and poly(γ-glutamic acid) (γ-PGA) nanoparticles/nanocomplexes (NCs) with a non-steroidal anti-inflammatory drug, diclofenac (Df), were previously developed in the group. The anti-inflammatory NCs were injected into the established IVD *ex vivo* model and were shown to effectively down-regulate pro-inflammatory markers production, while promoting matrix remodelling by native cells. Df-NCs were then injected in degenerated discs *in vivo*, in a rat model of tail punctured IVD, already established in the lab. The preliminary results obtained indicate that 2 weeks' post-injury the intradiscal administration of Df-NCs did not seem to promote proteoglycan production in the IVD, contrasting with the promising *ex vivo* results previously obtained. Moreover, Df-NCs did not promote hernia regression. Nevertheless, in the group of animals receiving intradiscal Df injection, the hernia volume was reduced. Ongoing work is being performed to comprehend the results discrepancy.

Finally, due to the promisor but controversial results obtained with cell therapies, namely with mesenchymal stem/stromal cells (MSCs) for low back pain, the effect of pro-

inflammatory/degenerative environment of IVD on MSCs evaluated using the ex vivo model previously established. MSC revealed to have an immunomodulatory, but not pro-regenerative, role on degenerated IVD, decreasing the inflammatory response of IVD cells. The results obtained suggest that MSCs act through a feedback loop mechanism, producing other inflammatory factors, but how this impacts on low back pain has not been addressed so far.

In summary, this thesis contributed to advance knowledge on how the modulation of inflammation can affect IVD regeneration. The work developed in this thesis also opened new perspectives in the use intradiscal injection of anti-inflammatory drugs in IVD degeneration and how the degenerated IVD can influence the success of cell therapies for low back pain.

Resumo

A degeneração do disco intervertebral (IVD) e a inflamação associada contribuem frequentemente para a dor lombar, uma das principais causas mundiais de incapacidade. Embora os tratamentos clínicos disponíveis diminuam a progressão dos sintomas, não têm capacidade de restaurar as propriedades nativas do IVD. Os ensaios clínicos que utilizam terapias celulares estão a aumentar, mas o ambiente pró-inflamatório hostil do IVD pode desafiar o seu sucesso, pois pode impedir a sobrevivência celular e a formação da matriz.

Devido ao elevado impacto sobre a saúde da população e à falta de soluções adequadas, novas terapias que modelem a resposta inflamatória podem ser uma nova esperança para o tratamento da degeneração do IVD.

Este trabalho apresenta novas perspetivas sobre abordagens terapêuticas alternativas para modular a inflamação, e ao mesmo tempo estimular a regeneração do IVD, e sobre como o ambiente inflamatório do IVD pode desafiar o processo regenerativo.

Embora haja um elevado número de modelos animais desenvolvidos para mimetizar a degeneração do IVD, quando esta investigação começou, não existia um modelo que simulasse o processo inflamatório da degeneração progressiva do disco humano, permitindo um controlo padronizado. Por conseguinte, um modelo *ex vivo* degenerativo/pró-inflamatório de IVD padronizado foi primeiro estabelecido e validado, utilizando culturas de disco da cauda de bovino, sob carga estática, estimuladas por punção com agulha e IL-1β. Das várias condições testadas, estas foram mostradas as mais próximas em mimetizar a degeneração do IVD humano e a resposta inflamatória associada. Além disso, este modelo mostrou grande potencial para testar abordagens que incluam moléculas bioativas e terapia celular.

Uma terapia nanotecnológica baseada em nanopartículas/nanocomplexos (NCs) de quitosano (Ch) e ácido poli(γ-glutâmico) com um fármaco anti-inflamatório não esteróide, diclofenac (Df), foi anteriormente desenvolvido no grupo. Estes NCs anti-inflamatórios foram injetadas no modelo *ex vivo* de IVD estabelecido e demonstraram efetivamente diminuir a produção de marcadores pró-inflamatórios, enquanto promoveram a remodelação da matriz por células nativas. Os Df-NCs foram então injetados *in vivo*, em discos degenerados, num modelo de IVD da cauda de rato puncionado, já estabelecido no laboratório. Os resultados preliminares obtidos indicam que, 2 semanas após a lesão, a administração intradiscal de Df-NCs não pareceu promover a produção de proteoglicanos no IVD, contrastando com os resultados promissores *ex vivo* anteriormente obtidos. Além disso, os Df-NCs não promoveram a regressão da hérnia. No entanto, no grupo de animais que receberam injeção

intradiscal de Df, o volume da hérnia foi reduzido. Está em curso a continuação deste trabalho no sentido de compreender a discrepância dos resultados.

Por fim, devido aos resultados promissores, mas controversos, obtidos com as terapias celulares, nomeadamente com células estaminais/estromais mesenquimais (MSCs) para a dor lombar, o efeito do ambiente pró-inflamatório/degenerativo do IVD nas MSCs foi avaliado, utilizando o modelo *ex vivo* anteriormente estabelecido. As MSCs revelaram ter um papel imunomodulador, mas não pró-regenerativo, sobre o IVD degenerado, diminuindo a resposta inflamatória das células do IVD. Os resultados obtidos sugerem que as MSCs atuam através de um mecanismo de autorregulação, produzindo outros fatores inflamatórios, mas como isto afeta a dor lombar não tem sido abordado até agora.

Em suma, esta tese contribuiu para o avanço do conhecimento em como a modulação da inflamação pode afetar a regeneração IVD. O trabalho desenvolvido nesta tese também abriu novas perspetivas no uso de injeção intradiscal de drogas anti-inflamatórias na degeneração do IVD, e como o IVD degenerado pode influenciar o sucesso de terapias celulares para a dor lombar.

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List of Abbreviations

γ-PGA	Poly-γ-glutamic acid
ACAN	Aggrecan
ACs	Articular chondrocytes
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AF	Annulus fibrosus
Akt	Serine/threonine-protein kinase
ANG	Angiopoietin
AnxV	Annexin V
AP	Activating protein
AQP	Aquaporin
ASCs	Adipose-derived stem cells
ASIC3	Acid-sensing ion channel 3
BASP1	Brain abundant membrane attached signal protein 1
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BM	Basal medium
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CA	Carbonic anhydrase
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CDH2	Cadherin 2
CDMP	Cartilage-derived morphogenetic protein
cDNA	Complementary DNA
CEP	Cartilaginous endplate
Ch	Chitosan
C-KIT	Mast/stem cell growth factor receptor Kit
CLSM	Confocal laser scanning microscopy
COL	Collagen
COMP	Cartilage oligomeric matrix protein
COX	Cyclo-oxygenase
CRDs	Cysteine-rich domains

CTB	CellTracker Blue
CTGF	Connective tissue growth factor
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
CYTL	Cytokine like
DA	Degree of acetylation
Df	Diclofenac
DLL	Delta-like
DMEM	Dulbecco’s modified Eagle’s medium
DMMB	1,9-dimethyl-methylene blue
DR	Death receptor
DRG	Dorsal root ganglion
DSC	Desmocollin
dsDNA	Double standard DNA
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EthD-1	Ethidium homodimer-1
FADD	Fas-associated protein with death domain
FasL	Fas ligand
FasR	Fas receptor
FBLN	Fibulin
FBS	Fetal bovine serum
FGFR	Fibroblast growth factor receptor
FITC	Fuorescein isothiocyanate
FOX	Forkhead box
ftCh	Fuorescein isothiocyanate labeled chitosan
FTIR	Fourier transform infrared spectroscopy
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS1	Growth arrest specific gene/protein 1
G-CSF	Granulocyte colony-stimulating factor
GD	Ganglioside
GDF	Growth differentiation factor
GGT	γ-glutamyltransferase
GLUT	Glucose transporter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp130	Glycoprotein 130

GPC3	Glypican 3
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigen
HO	Hemeoxygenase
HOXD10	Homeobox D10
HPAN	Hydrolyzed polyacrylonitrile
HSA-HA	Human serum albumin-hyaluronan
IBSP	Integrin binding sialoprotein
ICAM	Intracellular adhesion molecule
IDO	Indoleamine-2,3-dioxygenase
IF	Immunofluorescence
IFN	Interferon
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
IL-1R1	Interleukin-1 receptor, type 1
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-6 sR	Interleukin-6 soluble receptor
IL-6Rα	Interleukin 6 receptor, subunit alpha
iNOS	Inducible nitric oxide synthase
IP-10	Interferon-gamma-induced protein 10
IQR	Interquartile range
IRAK	Interleukin-1 receptor-activated protein kinase
IVD	Intervertebral disc
IκB	Inhibitor of kappa B
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KRT	Keratin
LBP	Low back pain
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
M-CSF	Macrophage colony-stimulating factor
MIG	Monokine induced by interferon-gamma

mIL-1	Membrane-bound interleukin-1
mIL-6R	Membrane-bound interleukin-6 receptor
MIP	Macrophage inflammatory protein
miRNA	microRNA
MKP	Mitogen-activated protein kinase phosphatase
MMPs	Metalloproteinases
mRNA	Messenger RNA
MSCs	Mesenchymal stem/stromal cells
mTNF	Membrane-bound tumor necrosis factor
MYD88	Myeloid differentiation primary response gene 88
NCAM	Neural cell adhesion molecule
NCs	Nanoparticles/nanocomplexes
NF1	Nuclear factor 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NO	Nitric oxide
NOTCH	Neurogenic locus notch homolog protein
NP	Nucleus pulposus
NSAIDs	Non-steroidal anti-inflammatory drugs
O_2-O_3	Oxygen-ozone
OCT	Octamer-binding transcription factor
OD	Optical density
PAX	Paired box
PDGF-BB	Platelet-derived growth factor-BB
PdI	Polydispersion index
PFA	Paraformaldehyde
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PI	Propidium iodide
PI3K	Phosphoinositide-3 kinase
PIK3CD	Phosphoinositide-3 kinases catalytic subunit delta
PLA_2	Phospholipase A_2
pri-miRNA	Primary microRNA
PRP	Platelet-rich plasma
PTEN	Phosphatase and tensin homolog
PTN	Pleiotrophin
qRT-PCR	Quantitative real-time reverse transcription-polymerase chain reaction

RANTES	Regulated on activation, normal T-cell expressed, and secreted
RhoC	Ras homolog gene family, member C
RIP1	Receptor-interacting protein 1
sGAG	Sulphated glycosaminoglycan
Shh	Sonic hedgehog
sIL-6R	Soluble interleukin-6 receptor
SMAD3	SMAD family member 3
SNAP	Synaptosomal-associated protein
SOCS	Suppressor of cytokine signaling
SOSTDC	Sclerostin domain containing
Sox-9	Transcription factor Sox-9
STAT	Signal transducers and activators of transcription
sTNF	Soluble tumor necrosis factor
sTNFR	Soluble tumor necrosis factor receptor
STRO	Stromal precursor antigen
TACE	Tumor necrosis factor-alpha-converting enzyme
T_C cells	Cytotoxic T cells
TGF	Transforming growth factor
T_H cells	T helper cells
THD	Tumor necrosis factor homology domain
TIE2	Transmembrane tyrosine-protein kinase receptor 2
TIMP	Tissue inhibitor of metalloproteinase
TLBN	Translamellar bridging network
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TNFAIP	Tumor necrosis factor-alpha induced protein
TNFR	Tumor necrosis factor receptor
TNMD	Tenomodulin
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TRAF2	Tumor necrosis factor-receptor-associated factor 2
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Trpv1	Transient receptor potential cation channel, subfamily V, member 1
TSG	Tumor necrosis factor-alpha stimulated gene/protein
VCAN	Versican
VEGF	Vascular endothelial growth factor
VEP	Vertebral endplate
YLDs	Years lived with disability

CHAPTER I

General introduction

1. Social impact of low back pain

Low back pain (LBP) has been described to affect approximately two thirds of the word population at some point in their life (Andersson 1999, Deyo and Weinstein 2001) and it is considered the number one disease regarding global years lived with disability (YLDs) (Vos et al. 2012). Recent findings showed that both in 1990 and 2010, LBP was contributing to about 10.7% of total YLDs (Vos et al. 2012). Over the past two decades, this musculoskeletal condition has been common cause of activity limitations in people younger than 45 years, absence from work, seeking for primary care, admission to hospital, and surgical procedures (Andersson 1999). Nonetheless, there is also a possibility that psychosocial factors may influence the prevalence of the disease, namely stress, anxiety, and depression (Andersson 1999). The population awareness of the symptoms and their reporting may also contribute to the high LBP prevalence observed (Croft 2000, Weiner 2008).
Although it is estimated that about 90% of the patients recover from LBP within a few months after receiving primary care (Shekelle et al. 1995), the remaining patients may develop chronic LBP (described as pain lasting for 12 weeks or longer) or suffer from recurrent pain episodes (corresponding to around 20% to 44% within one year after the initial episode and may reach up to 85% along life) (Andersson 1999, van Tulder et al. 2002). Reports estimate that the total costs associated with back problems corresponded to about $85.9 billion per year, in the USA (Martin, Deyo et al. 2008) and £12.3 billion in the UK (Maniadakis and Gray 2000, Hong et al. 2013).

2. Low back pain generators

Acute LBP (defined as lasting less than 4 weeks) is most commonly caused by muscle strains, ligaments sprains, and tendonitis (Cooper 2015). It may also be caused by traumatic injury, intervertebral disc (IVD) degeneration, disc herniation or rupture, radiculopathy (compression, inflammation and/or injury to a spinal nerve root), skeletal irregularities (e.g. scoliosis and lordosis), spinal stenosis (spine narrowing, which pressures the spinal cord and nerves) or spondylolisthesis (displacement of the vertebra and pinching of the nerves) (Cooper 2015). Although rarer, LBP may also be related to underlying conditions such as compression fracture, cancer, cauda equine syndrome or spinal infection (Chou et al. 2007).
Degeneration of the IVD has been perceived as the major cause of functional alterations and spinal instability (Iatridis et al. 2009, Galbusera et al. 2014). In young individuals, it has been described that discogenic LBP (with absence of disc herniation and nerve root compression) accounts for about 40% of chronic LBP (Cheung et al. 2009, Verrills et al. 2015). Sciatica cases (with disc herniation and nerve root compression) represent about 20 to 30% (Koes et al. 2007), and zygapophysial joint pain is estimated to account between 5% to 15% of the chronic

LBP cases (van Kleef et al. 2010, Cooper 2015).

The neurological symptoms of LBP are treated depending on whether the pain is acute or chronic. Frequently, the doctors struggle about the most effective option. Commonly, surgery is only recommended when diagnostics indicate worsening nerve damage or spinal structural changes that can be corrected with surgery. Conventionally, treatments with non-surgical methods consist on alleviating pain through resting, physical therapy and/or pain medication, including administration of analgesics, corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), opioids and antidepressants (Shen et al. 2006). If these treatments fail, surgical interventions such as discectomy, spinal arthroplasty, and arthrodesis may be considered (Leahy et al. 2008, Wei et al. 2013a). However, these treatments are transient in time, and may cause neurological alterations, affecting patients' mobility, and potentially altering spine biomechanics, leading to degeneration of adjacent discs (Lund and Oxland 2011, Natarajan and Andersson 2017).

Several proposals converging into regenerative medicine, as cell-based therapies, growth factor injection, gene therapy and tissue engineering, have been focusing on IVD's mechanobiology and function reestablishment (Hughes et al. 2012, Molinos et al. 2015a). These alternatives look for less invasive, long-term effective and safe treatment options which could bring greater consensus among the medical community. Furthermore, by integrating these strategies, it would be possible to target different discogenic disease features such as to modulate inflammation and decrease pain, as well as to promote native tissue regeneration.

3. The healthy intervertebral disc

The IVD's complex structure has a major biomechanical role, despite the differences among humans and other species in the disc size, mechanics, biochemistry and nutrition (Alini et al. 2008). In humans, IVD enables stability, absorption and dispersion of loads, while allowing spine's multiaxial motions, such as flexion-extension, rotation, and lateral bending (Stokes and Iatridis 2004). The IVD is the main spinal joint (occupying about one third of its length), and the largest avascular and aneural tissue in the body of a healthy adult (Urban and Roberts 2003, Raj 2008, Huang et al. 2014). The biomechanical role of the disc is conditioned by the synthesis of macromolecules by a small population of resident cells (Huang et al. 2014). It is known since the 70's that the IVD is mainly composed by water, proteoglycans, and collagen (Adams and Muir 1976, Eyre 1979), with their relative proportions varying between its different constitutive regions. The IVD comprises a highly hydrated central structure, the nucleus pulposus (NP), surrounded by a concentric annular lamellar structure, the annulus fibrosus (AF), delimited above and below by cartilaginous endplate (CEP) that connect an IVD to the adjacent vertebrae (Raj 2008). A schematic representation of the IVD is depicted in Figure 1.

In Table 1, it is summarized the differences in extracellular matrix (ECM) composition within the different areas of the IVD.

The disc size varies along the spinal region and the cellular content also varies between the regions. Of notice, in the human lumbar disc the NP cellular content is only about $4x10^6$ cells/cm^3, while the AF has around $9x10^6$ cells/cm^3, corresponding to approximately 1% of the IVD volume (Roughley 2004, Anderson et al. 2005). The specific cells within each region of the IVD are affected by a variety of physical and biochemical cues from the microenvironment. The cells are crucial for producing IVD ECM components and maintain its homeostasis (Hwang et al. 2014). Overall, the low cell concentration is described as an adaptive response to the IVD microenvironment, which has a limited nutrient supply to support cell proliferation (Anderson et al. 2005).

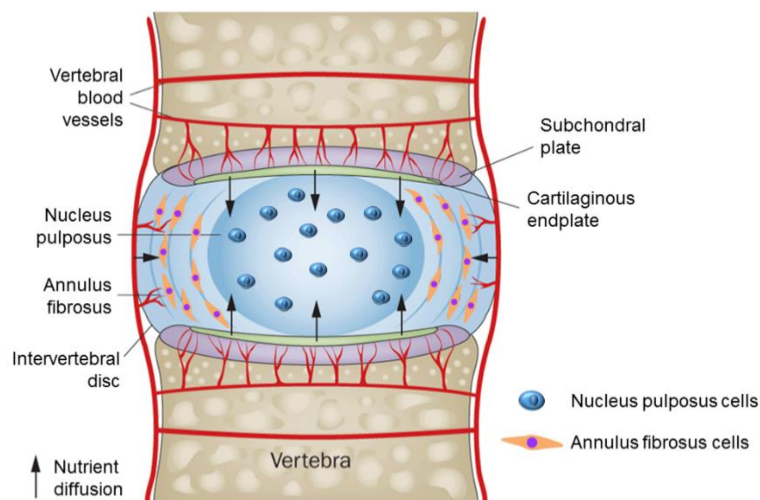

Figure 1. Schematic representation of healthy intervertebral disc. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Rheumatology (Huang et al. 2014), copyright (2014).

3.1. Cartilaginous endplate

In adults, the CEP consists of relatively thin layers of hyaline cartilage (of about 0.5 - 1 mm) adjacent on one side to the vertebral endplate (VEP), the region from the subchondral bone to a depth of 2 mm, and are continuous with the AF and NP on the other side (Donisch and Trapp 1971, Rodriguez et al. 2012). The CEP ECM consists largely of proteoglycans and collagen fibers, namely collagen type II (COL2), with a water content lower than that of the NP and AF (Roberts et al. 1989). Cells resemble chondrocytes, but present a slightly different gene expression signature than the IVD cells (Minogue et al. 2010a). CEP and vertebrae are derived from the sclerotome (Risbud and Shapiro 2011). In babies (younger than approximately 12 months old), blood vessel capillaries and nerves arising from multiple superior and inferior dorsal root ganglia are still present in the endplate and IVD (Raj 2008).

Table 1. IVD anatomy and physiology.

IVD tissue	Composition (% to wet weight)			Biomechanical characteristics	References
	water	**proteoglycans**	**collagen**		
CEP	55%	8%	25% ↑ COL2	dispersion of loads	(Raj 2008, Ochia et al. 2003)
AF	60-70% (no change with age)	5% (decrease with age)	15% (little change with age)	↑ elasticity ↑ tensile loading capacity	(Raj 2008, Smith and Fazzalari 2009, Shapiro and Risbud 2014, Le Maitre et al. 2007a)
Outer AF			↑ COL1		
Inner AF			COL1 to COL2 transition		
NP	90% at birth 80% at age 20 70% at older age	15% (decrease with age)	4% ↑ COL2 (little change with age)	↑ hydrostatic pressure ↑ absorption and dispersion of compressive loads	(Raj 2008, Stokes and Iatridis 2004, Shapiro and Risbud 2014, Le Maitre et al. 2007a)

↑ higher

During spine development, the IVD becomes practically avascular, the vertebrae ossify and the CEPs undergo changes of shape, circumference, thickness and maturation (Donisch and Trapp 1971). The CEP has been considered the main diffusional route for oxygen, nutrients and residues, which occurs predominantly through passive diffusion (Holm et al. 1981, Ogata and Whiteside 1981, Huang et al. 2014). Therefore, the concentration gradients are determined by the balance in cells consumption and nutrients supply (Urban et al. 1982, Huang et al. 2014). Nonetheless, due to the scarcity of blood vessels, a hypoxic and limited nutrition environment is generated. While the cells present in the outer AF can eliminate their metabolites and have access to nutrients through the capillaries present in the surrounding soft tissues, the remaining cells within the IVD only have access to a scarcity of capillaries that enter the subchondral plate and terminate adjacent to the CEP, feature which may impair IVD's regenerative capacity (Raj 2008, Huang et al. 2014).

3.2. Annulus fibrosus

The AF is derived from the sclerotome and formed by a concentric lamellar structure, with around 70% of water, and rich in COL1 fibers, lying parallel within each lamella (Raj 2008, Risbud and Shapiro 2011, Huang et al. 2014). The concentric lamellae of regularly arranged collagen fibers, which are interconnected by a network of elastin and fibrillin, form the translamellar bridging network (TLBN) (Yu et al. 2007, Schollum et al. 2009, Yu et al. 2015). The presence of elastin fibers, crossing radially the collagen ones, contribute to tissue mechanical support and elasticity (Raj 2008). The AF provides lateral NP confinement and resistance to tensile and compressive stresses during physiological loading, with changes in the loading environment from more tension in the outer AF, to more compression towards the

NP (Eyre 1979). The outer AF fibers are directly inserted into the cortical bone of the vertebrae, whereas the inner tissue connects to the endplate, this probably to support the higher tensile loads present in the outer AF (Eyre 1979). A transitional region from COL2 to COL1, poorly organized and interspersed with aggregated proteoglycans (corresponding to approximately 5% wet weight) and elastin fibers, characterizes the inner part of the AF (Raj 2008, Rodrigues-Pinto et al. 2014). The AF cells are elongated and fibroblastic in appearance, and are orientated in the same axis as the collagen fibrils (Raj 2008).

3.3. Nucleus pulposus

The NP is rich in aggrecan (ACAN), the major proteoglycan of the IVD, and interspersed with COL2 fibers, randomly arranged, and with elastin fibers radially distributed (Raj 2008, Huang et al. 2014, Rodrigues-Pinto et al. 2014). Of notice, ACAN is responsible for mediating the osmotic pressure within the NP, and the resistance to compressive loads. ACAN forms large aggregates by binding to hyaluronan, and this mesh limits ACAN diffusion within the matrix. ACAN osmotic properties and water binding capacity are due to its substitution by other glycosaminoglycan (GAG) chains of chondroitin sulfate and keratan sulfate (Urban et al. 1979, Lotz and Hsieh 2014). Water content represents about 80% of the wet weight of the NP, having a mechanical behavior characteristic of a viscoelastic material (Iatridis et al. 1997, Raj 2008).
In the mature NP, it is possible to identify mostly NP cells, which are small (approximately 10 µm diameter) and resembling chondrocytes in morphology (Sive et al. 2002). Nonetheless, it is recognized a morphological heterogeneity of cells within the NP. The NP derives from the notochord, and notochordal cells can still be found in the NP in immature and young IVDs in humans (Risbud and Shapiro 2011, Risbud et al. 2015, Sakai and Andersson 2015). Notochordal cells have a distinct morphology from NP cells. They are larger (around 25 - 85 µm diameter), commonly appear in clusters, and contain intracellular vacuoles that occupy at least 25% of the cell area (Trout et al. 1982, Hunter et al. 2003, Risbud et al. 2015, Sakai and Andersson 2015). After birth, the number of notochordal cells decreases very rapidly. Nonetheless, it has been described that the human and bovine NP tissue still retains some notochordal cells throughout life, even if in low number (Gilson et al. 2010). In other species, such as mouse, rat, cat, mink, dog, pig and rabbit, the number of notochordal cells found in adults is higher (Alini et al. 2008, Weiler et al. 2010, Sakai and Andersson 2015). Henriksson and colleagues' studies in rabbit raised the possibility of stem/progenitor cell niches within the IVD, namely present in the epiphyseal plate and inner parts of the IVD (Henriksson et al. 2009, Henriksson et al. 2012). Notochordal cells have been shown to shift into NP cells, under standard *in vitro* culture (Kim et al. 2009a) or dynamic loading (Purmessur et al. 2013a), as well as after injury stimulus *in vivo* (Yang et al. 2009). Notochordal cells, as well as a progenitor

cell population, which can differentiate along the mesengenic pathway, present higher expression of NP-phonotypic markers, among other markers, as discussed below and summarized in Table 2 (Minogue et al. 2010a, Risbud et al. 2010, Risbud and Shapiro 2011, Sakai et al. 2012). Furthermore, while previous studies from Kim and colleagues suggested chondrocyte migration from the CEP and the inner AF into the NP as source of NP cells in mature IVDs of rat and rabbit (Kim et al. 2003, Kim et al. 2005a), Henriksson et al. (2009) proposed the existence of stem/progenitor cell niches within the IVD.

4. Phenotypic markers of the different IVD cell populations

Gene expression and phenotypical differences between notochordal, NP, AF, CEP cells and articular chondrocytes (ACs) have been pursued to trace a distinctive phenotypic profile for these cells. In the adult IVD, cells share typical markers with articular chondrocytes (ACs), namely regarding the production of ECM components such as COL2, ACAN and versican (VCAN) (Sive et al. 2002). Recently, Molinos et al. (2015b) identified three phenotypically distinct cell subpopulations in the young bovine NP, corroborating the heterogeneity previously observed. Yet, the distinctive function of the tissues is determined by the exact amount and composition of the proteins synthesized (Sive et al. 2002, Minogue et al. 2010a, Minogue et al. 2010b).

Finding distinct markers of IVD cells may play an important role in the development of regenerative strategies for IVD degeneration, in addition to providing further knowledge of its biology (Minogue et al. 2010a). Studies struggle with identifying markers that are both cell- and species-specific. Up to date, it has not been identified an exclusive NP marker. Henriksson and Brisby (2013) reviewed the differences in marker profiles between NP cells and ACs. However, studies have been focusing on differentiating AF cells, NP cells and ACs mostly by comparing the fold-change in expression of IVD markers. Taking this into account, it was defined in 2014 a series of recommendations by the Spine Research Interest Group at the Annual ORS Meeting, to characterize the healthy NP phenotypic markers. A ratio of ACAN/COL2 > 20 seen in healthy human juvenile and young adult NP, and the expression of hypoxia inducible factor (HIF)-1α, glucose transporter (GLUT)-1, sonic hedgehog (Shh), Brachyury (*T*), keratin (KRT)18 and KRT19, carbonic anhydrase (CA)12, and CD24 were the proposed primary markers (Risbud et al. 2015). However, other secondary markers are also presented in Table 3.

HIF-1α is responsible for the up-regulation of many pro-survival genes in NP, namely GLUT-1 and -3, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ACAN, β-1,3-glucuronyltransferase, galectin-3 and vascular endothelial growth factor (VEGF)-A (Risbud et al. 2015). GLUT-1 is expressed in hypoxic tissues and CA12 promotes acid-base balance

(Richardson et al. 2008a). Moreover, genes expressed during notochord development, such as Shh, a ligand of the hedgehog family (Dahia et al. 2009, Dahia et al. 2012, Winkler et al. 2014), and Brachyury (Minogue et al. 2010b, Risbud and Shapiro 2011, Smolders et al. 2012, Maier et al. 2013) have been reported to remain active in the postnatal and mature human, bovine, canine and murine NP cells, being crucial for the growth, differentiation and function of NP cells (Risbud et al. 2015). KRT18 and KRT19, also identified in human notochordal cells during development, are important for cell integrity, and are possibly involved in signaling pathways, although not yet fully elucidated (Risbud et al. 2015). CD24 is a cell adhesion molecule, yet its relevance to NP physiology is still unknown (Fujita et al. 2005, Rutges et al. 2010, Risbud et al. 2015). Also of notice is the presence of $CD68^{+}$ cells in human nonherniated disc (which are not invading monocytes or macrophages), indicating phagocytic activity of NP cells (Nerlich et al. 2002).

Nonetheless, it is noteworthy that, when comparing humans to other animals, there are differences in the markers expressed, as well as in their amount. It is hypothesized that this may be due to differences in age, size, native cell composition, and environment in the IVD among species (Minogue et al. 2010a). For example, the expression of glypican 3 (GPC3) and KRT19 from murine (Lee et al. 2007), or KRT18 and cadherin 2 (CDH2) from bovine studies did not translate directly into similar expression levels by human IVD cells (Minogue et al. 2010b). Also, HIF-1α (Risbud et al. 2006), GLUT-1 (Richardson et al. 2008a) and VEGF-A (Rajpurohit et al. 2002, Fujita et al. 2008) characterized in human did not appear to change significantly in bovine NP cells, when compared with ACs (Minogue et al. 2010a). Moreover, CD24 was identified as a specific cell surface marker for NP cells in the rat (Fujita et al. 2005), and neural cell adhesion molecule CD56 was found to be expressed in canine NP cells (Sakai et al. 2009), but gene expression analysis of human disc cells revealed that CD24 was not specific for NP cells, whereas CD56 was expressed only at low levels (Rutges et al. 2010).

Regarding progeny, compared with NP cells, notochordal cells were shown to display significantly higher expression of KRT8, KRT18, KRT19, CDH2, sclerostin domain containing 1 (SOSTDC1) (Gilson et al. 2010, Minogue et al. 2010a), integrin subunits α3, α6 and β1 (Chen et al. 2006), as well as Brachyury (Minogue et al. 2010b, Risbud and Shapiro 2011).

Several works have shown that, when culturing cells derived from CEP, AF or NP *in vitro*, these also express mesenchymal stem/stromal cells (MSCs) markers, namely CD73, CD90, CD105 and stromal precursor antigen (STRO)-1, being negative for the pan-macrophage marker CD11b, the pan-monocytic antigen CD14, the pan-B-cell markers CD19, CD79, the hematopoietic stem cell marker CD34, the pan-hematopoietic marker CD45 and the class II human leukocyte antigen (HLA) antigen HLA-DR (Risbud et al. 2007, Liu et al. 2011, Brisby et al. 2013, Sakai and Andersson 2015, Chen et al. 2016). It was also observed that both NP and

Table 2. Characterization of the phenotypic markers of the IVD cell types in different species. The table's information was partially adapted from Risbud et al. (2015) and Sakai and Anderson (2015).

IVD cell type	Phenotypic markers				References
	human	**bovine**	**canine**	**murine**	
AF cells	CDH2 COL1 COMP FOXF1 GPC3 KRT8 KRT18 SNAP25 TNMD VCAN	AQP1 COL1 FOXF1 FOXF2 IBSP PTN TNFAIP6 TNMD FBLN1^{-}			(Minogue et al. 2010b, Rutges et al. 2010)
NP cells	ACAN/COL2 ratio > 20 Brachyury CA12 CD24 CD68 CDH2 FOXF1 GLUT-1 Hemoglobin β-chain HIF-1α Integrin α3, α6, β4 KTR8 KTR18 KTR19 Lubricin NCAM-1 Ovostatin PAX1 Shh SNAP25 VCAN VEGF-A α2-macroglobulin CYTL1^{-} FBLN1^{-} GDF-10^{-} IBSP^{-}	BASP1 Brachyury CDH2 FOXF1 KRT8 KRT18 KRT19 SNAP25 SOSTDC1 FBLN1^{-} IBSP^{-}	Brachyury CD56 DSC-2 KRT18 α2-macroglobulin	Annexin A3 BASP1 Brachyury CA3 CA12 CD24 CD56 CD155 CD221 GLUT-1 GPC3 HIF-1α KRT19 Neurochondrin Neuropilin-1 PTN Shh	(Lyons et al. 1991, Buckwalter 1995, Nerlich et al. 2002, Rajpurohit et al. 2002, Sive et al. 2002, Mwale et al. 2004, Nettles et al. 2004, Fujita et al. 2005, Risbud et al. 2006, Agrawal et al. 2007, Lee et al. 2007, Le Maitre et al. 2007b, Risbud et al. 2007, Agrawal et al. 2008, Fujita et al. 2008, Richardson et al. 2008a, Shine et al. 2009, Chen et al. 2009, Dahia et al. 2009, Sakai et al. 2009, Gilson et al. 2010, Minogue et al. 2010a, Minogue et al. 2010b, Rutges et al. 2010, Power et al. 2011, Risbud and Shapiro 2011, Dahia et al. 2012, Smolders et al. 2012, Önnerfjord et al. 2012, Sakai et al. 2012, Tang et al. 2012, Maier et al. 2013, van den Akker et al. 2014, Winkler et al. 2014)

Notochordal cells	Brachyury CD24 Galectin-3 Integrin α3, α6, β1 KRT8 KRT18 KRT19	Brachyury CDH2 KRT8 KRT18 KRT19 SOSTDC1		CA3	(Lyons et al. 1991, Chen et al. 2006, Minogue et al. 2010b, Weiler et al. 2010, Risbud and Shapiro 2011, Smolders et al. 2012)
Stem/progenitor cells	CD73 CD90 CD105 C-KIT DLL4 GD2 Jagged-1 Ki-67 NOTCH1 OCT3/4 STRO-1 TIE2 CD11b$^-$ CD14$^-$ CD19$^-$ CD34$^-$ CD45$^-$ CD79$^-$ HLA-DR$^-$			C-KIT GD2 Jagged-1 Ki-67 NOTCH1 STRO-1 TIE2	(Risbud et al. 2007, Henriksson et al. 2009, Blanco et al. 2010, Feng et al. 2010, Liu et al. 2011, Sakai et al. 2012, Brisby et al. 2013, Chen et al. 2016)

AF cells of rabbit, rat, minipig and human degenerated IVD tissue expressed progenitor markers such as the multipotency marker octamer-binding transcription factor (OCT)3/4, delta-like (DLL)4, neurogenic locus notch homolog protein (NOTCH)1, Jagged-1, mast/stem cell growth factor receptor Kit (C-KIT) and Ki-67 (Henriksson et al. 2009, Brisby et al. 2013). Sakai and colleagues observed, in mouse and human NP, progenitor cell populations expressing transmembrane tyrosine protein kinase receptor TIE2 (also named angiopoietin-1 receptor) and ganglioside GD2 (Sakai et al. 2012). These cells were more proliferative, could form spheroids with multipotent differentiation capacity, and were capable of differentiating towards the chondrogenic lineage, expressing COL2 and ACAN (Sakai et al. 2012, Sakai and Andersson 2015).

These findings show great potential for the development of therapies that may stimulate degenerated IVD native stem cells differentiation into functional NP and AF cells to reestablish the balance between anabolic and catabolic events and promote tissue regeneration. For such, it is important the understand of the morphological and biochemical changes that occur during aging and in premature degenerative diseases.

5. Aging and degeneration

Disc degeneration is linked with aging (Roberts et al. 2006), as recently reviewed by Vo et al. (2016). Nonetheless, it has been also observed in young children (11 to 16 years old) (Boos et al. 2002). IVD's well-defined microstructural organization and biochemical composition is affected by aging molecular mechanisms, and can ultimately lead to a cell-mediated structural failure (Iatridis et al. 2009, Vo et al. 2016). With age, variations in abundance and structure of IVD's ECM macromolecules may be a consequence of catabolism and anabolism imbalance (Roughley 2004), but in cases of early degeneration, abnormal age-related changes also occur (Iatridis et al. 2009).

Degenerated IVD pathogenesis might be affected by multiple factors such as gene polymorphisms, as recently reviewed by Martirosyan et al. (2016), which include genes that mediate apoptosis, contribute to structural proteins, and encode molecules involved in inflammatory pathways (Martirosyan et al. 2016).

5.1. The aging/degeneration mechanism

The aging/degenerative process of IVD is characterized by an initial increase in cell proliferation and formation of cell clusters, as well as alterations in cell cycle and an increase in cell senescence and apoptosis, with increased production of pro-apoptotic (Fas ligand [FasL], caspase-3) proteins, and death (Roberts et al. 2006, Richardson et al. 2007, Gruber et al. 2009, Bertolo et al. 2011).

The NP changes from gelatinous to a more fibrous structure, cracks and fissures often occur, namely in the AF, and there is a decrease in IVD water content. This is commonly due to a turnover of ECM components (shift from COL2 to COL1 production by NP cells, and a decrease in ACAN synthesis), schematically depicted in Figure 2 (Richardson et al. 2007, Bertolo et al. 2011). An up-regulation of specific metalloproteinases (MMPs), such as MMP-1, -2, -3, -7, -8, -10, and -13, a disintegrin and MMP with thrombospondin motifs (ADAMTS)-1, -4, -5, -9 and -15, and tissue inhibitors of MMPs (TIMPs)-1 and -2 were observed (Doita et al. 2001, Le Maitre et al. 2007b, Bachmeier et al. 2009, Pockert et al. 2009, Vo et al. 2013).
During degeneration, several changes may occur in the capillaries arising from the vertebral bodies, namely atherosclerosis, reduced capillary density, occlusion of the marrow spaces and CEP obstruction due to calcification/increased mineralization (Huang et al. 2014, Grant et al. 2016a). It has been hypothesized that an increase in free calcium ions (Ca^{2+}) may impair CEP homeostasis, compromising nutrient diffusion and availability to the cells, consequently leading to alterations in cell metabolism and viability (Huang et al. 2014, Grant et al. 2016a). It has also been reported that ECM components degradation may promote obstruction of the CEP, contributing to the drastic decrease of oxygen and nutrients diffusion into the disc (Ogata and Whiteside 1981, Huang et al. 2014). Particularly the NP, it is subjected to high mechanical and osmotic pressures, severe hypoxia and limited nutrients supply (Mehrkens et al. 2012). Additionally, blood vessels begin to grow into the disc from the outer areas of the AF (Roberts et al. 2006).

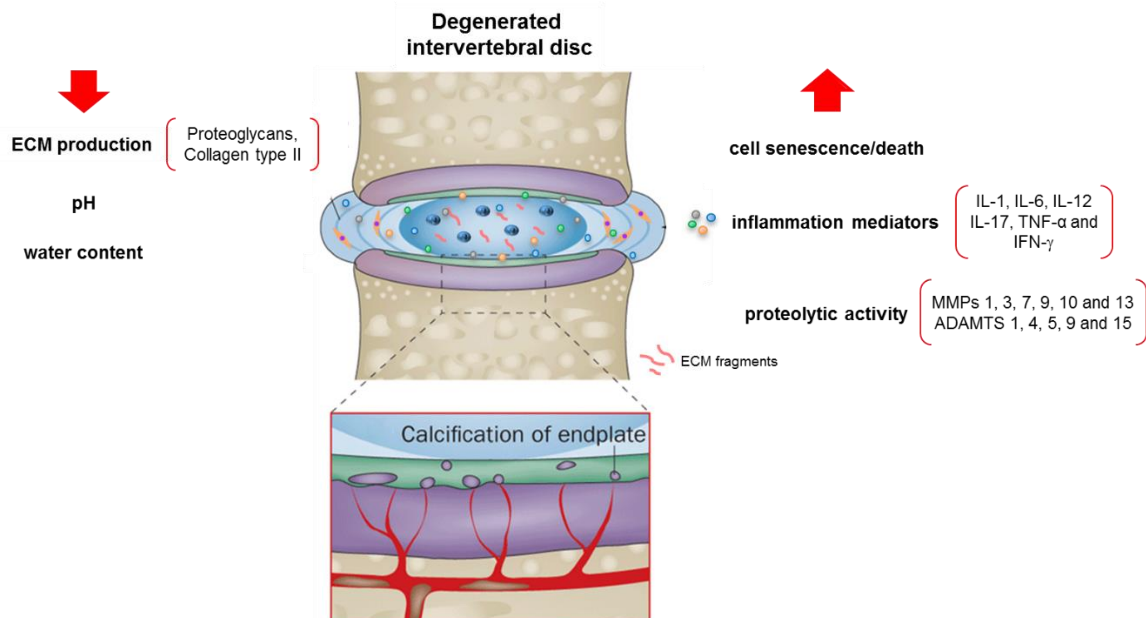

Figure 2. Schematic representation of intervertebral disc degeneration. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Rheumatology (Huang et al. 2014), copyright (2014).

Moreover, a wide number of inflammatory mediators, including prostaglandins, namely PGE_2, interleukins (IL-1, -6, -8, -12 and -17), tumor necrosis factor (TNF)-α and interferon (IFN)-γ have been described as crucial players in the catabolic processes in human NP and AF, nerve ingrowth and pain (Le Maitre et al. 2007a, Cuellar et al. 2010, Shamji et al. 2010, Purmessur et al. 2013b, Risbud and Shapiro 2014). Furthermore, increased amounts of nitric oxide (NO) have also been detected (Saal et al. 1990, Kang et al. 1996, O'Donnell and O'Donnell 1996). As the latest reviews point out, inflammation is an important contributor to the pathogenesis of IVD degeneration (Wuertz and Haglund 2013, Risbud and Shapiro 2014, Gorth et al. 2015). A balance between inflammatory response and tissue resorption may be achieved by controlling the levels of pro-inflammatory cytokines known to be involved in enzymatic degrading activity (Le Maitre et al. 2007c).

6. Study models of IVD degeneration and inflammation

Several *ex vivo* and *in vivo* models have focused on mechanical injury to simulate IVD degeneration (Anderson et al. 2002, Sobajima et al. 2005a, Sobajima et al. 2005b, Iatridis et al. 2009). Mechanical injury often comprises stab or needle puncture, and may include partial mechanical or chemical tissue removal with collagenase (Stern and Coulson 1976), papain (Roberts et al. 2008, Chan et al. 2013, Malonzo et al. 2015), trypsin (Jim et al. 2011, AlGarni et al. 2016) or chondroitinase ABC (Yamada et al. 2001, Ghosh et al. 2012, Krupkova et al. 2016). Several works showed, depending on needle gauge size, that needle puncture may induce AF disruption, while causing depressurization of the NP (Masuda et al. 2005, Iatridis et al. 2009). Needle calibers varying between 16 to 22G often lead to significant pressure failure, decreased cell viability, expression of pro-inflammatory and degenerative factors and alterations in ECM composition in IVDs from both large animals, such as bovine (Illien-Junger et al. 2012, Pattappa et al. 2014), and small animals, namely rabbit (Yang et al. 2015) and rat (Masuda et al. 2005). Of notice, as reviewed by Elliott and colleagues (2008), in animal models in which degeneration was stimulated with needle puncture or sham injection, with needle diameter/disc height ratios up to 25%, no significant disc changes were observed. For ratios between 25-40%, effects were variable with some minor nonsignificant changes (Elliott et al. 2008). However, when needle diameter/disc height ratios were over 40%, degenerative changes were observed in all animal modes reviewed (rat, rabbit, dog, minipig, and sheep) (Elliott et al. 2008). Disc height, area and NP ratio (normalized to human IVD) are summarized for different animals in Table 3.

Due to the defects induced by needle puncture injury, AF and NP mechanical integrity can be compromised and lead to degeneration, allowing the recreation of an injury scenario to be studied (Korecki et al. 2008). Overall, for needles with caliber higher that 29G, no apparent

degenerative changes were induced in small animals as rabbit (Henderson et al. 1991) or rat (Crevensten et al. 2004). In mouse, 33 and 35G needles did not lead to significant degenerative changes between punctured and non-punctured groups (Ohnishi et al. 2016).

Table 3. IVD size and cell content in different species. Adapted from O'Connell et al. (2007).

Species	IVD height (mm)	IVD area (mm^2)	NP area (mm^2)	NP/IVD ratio	NP/IVD fold change (Human)
Human (lumbar)	11.30±0.30	1727±550	479±110	0.28	-
Bovine (tail)	6.90±0.35	622±71	176±22	0.28	1.02
Baboon (lumbar)	4.45±1.39	749±82	242±50	0.32	1.16
Sheep (lumbar)	3.93±0.07	676±122	267±79	0.39	1.42
Rabbit (lumbar)	1.42±0.39	73.4±6.1	18.0±1.6	0.25	0.88
Rat (lumbar)	0.93±0.24	20.4±2.1	5.00±2.06	0.25	0.88
Rat (tail)	0.94±0.09	8.86±3.54	3.30±1.55	0.37	1.34
Mouse (lumbar)	0.31±0.03	1.81±0.14	0.33±0.07	0.18	0.66
Mouse (tail)	0.24±0.06	1.19±0.51	0.35±0.09	0.29	1.06

6.1. *Ex vivo*

Several authors have pointed out the importance of developing *in vivo*-mimicking *ex vivo* organ cultures to translate the degenerative events that occur in humans (Korecki et al. 2007, Teixeira et al. 2015, Krupkova et al. 2016). IVD organ cultures are one step further in complexity than *in vitro* studies, allowing the introduction of more variables in a mechanically and biochemically controlled environment, maintaining several microenvironment cues and the tissue structure (Korecki et al. 2007, Korecki et al. 2008). However, most existing organ culture systems induce severe tissue degradation with only limited representation of the *in vivo* processes (Krupkova et al. 2016), low oxygenation and nutrition (Rinkler et al. 2010, Neidlinger-Wilke et al. 2012), or pro-inflammatory cues (Teixeira et al. 2015).

Ex vivo studies use a variety of tissue sources, including human, bovine, sheep, rabbit, rat and mouse (as briefed in Table 4), among others. Commonly safe and easy to manipulate, organ cultures can be used to screen several experimental conditions, while reducing the number of animals in further *in vivo* trials (Teixeira et al. 2015). They allow detailed analysis of ECM composition, cellular mechanisms, metabolism and related pathways in health and disease (Korecki et al. 2007, Korecki et al. 2008). These models have also the advantage of being combined with loading systems (0.1 MPa to 0.6 MPa) simulating physiological forces applied on the spine (Korecki et al. 2007, Illien-Junger et al. 2010, Illien-Junger et al. 2012, Pirvu et al. 2015).

Ex vivo models are promising alternatives to examine the effect of different treatments (Alini et al. 2008). An IVD organ culture system, namely of animal origin, offers a simpler and inexpensive alternative, when compared to humans, due to the difficulty of obtaining human material, particularly "normal" human tissue (Alini et al. 2008), and facilitates the design of

Table 4. *Ex vivo* models for studying intervertebral disc degeneration and inflammation. Adapted from Gantenbein et al. (2015) (Gantenbein, Illien-Junger et al. 2015).

Model	Species	Degenerative stimulus					References
		Spontaneous	Mechanical	Chemical	Biochemical	Genetic	
IVD explant (NP and AF)	Human	Degenerative disc disease, trauma					(Bertolo et al. 2011)
		Herniation (protrusion, extrusion, sequestration), scoliosis					(Burke et al. 2002a)
		Degenerative disc disease					(Le Maitre et al. 2004)
	Bovine		Needle puncture (27 G)	Chondroitinase ABC (1-20 U/mL)			(Krupkova et al. 2016)
					IL-1β (100 ng/mL) plus TNF-α (100 ng/mL)		(Krupkova et al. 2016)
			Static loading, needle puncture (21G)	LPS (10 µg/mL)	Low glucose, hypoxia		(Teixeira et al. 2015)
			Static loading, needle puncture (21G)		Low glucose, hypoxia, IL-1β (10-100 ng/mL)		(Teixeira et al. 2015, Teixeira et al. 2016)
	Rabbit		Annular stab				(Feng et al. 2009)
IVD without endplate	Bovine		Complex loading				(Walter et al. 2011)
			Static loading		TNF-α (200 ng/mL)		(Purmessur et al. 2013b)
IVD with endplate	Human	Degenerative disc disease					(Krock et al. 2014)
			Endplate trauma				(Alkhatib et al. 2014)
	Bovine		Needle puncture (14, 25 G)				(Korecki et al. 2008)
			Free swelling, static loading				(Pirvu et al. 2015)
			Static loading, diurnal loading				(Korecki et al. 2007)
			High-frequency loading, needle puncture (22 G)				(Illien-Junger et al. 2012, Pattappa et al. 2014)
			Partial nucleotomy				(Pereira et al. 2014)
			Needle puncture (25 G)	Papain (30-150 U/mL)			(Chan et al. 2013)

		Needle puncture (22, 25 G)	Papain (60 U/mL)			(Malonzo et al. 2015, Bucher et al. 2013)
			Papain (360 U/mL), Trypsin (12,400-248,000 U/mL)			(Roberts et al. 2008)
		Needle puncture (28 G)	Trypsin (0.05 µg/µL) (1.3 µg/µL) (0.2, 2 µg/µL)			 (Jim et al. 2011) (Mwale et al. 2014, AlGarni et al. 2016) (Gawri et al. 2014a)
				HTRA1, MMP3, ADAMTS-4 (10 µg/mL each)		(Furtwangler et al. 2013)
		Partial nucleotomy		IFN-$\alpha_2\beta$ (100 U/mL)		(Kazezian et al. 2016)
		Dynamic loading		TNF-α (100 ng/mL)		(Walter et al. 2015, Walter et al. 2016)
	Sheep	Dynamic loading				(Gantenbein et al. 2006)
		High frequency loading		Low glucose		(Jünger et al. 2009, Illien-Junger et al. 2010)
	Rabbit	Burst fracture				(Haschtmann et al. 2008, Dudli et al. 2012, Dudli et al. 2014, Dudli et al. 2015)
		Needle puncture (18 G)				(Dudli et al. 2014)
	Rat	CEP fracture				(Kim et al. 2005a)
		Needle puncture (21, 25, 30 G)				(Michalek et al. 2010)
			LPS (10 µg/mL)			(Li et al. 2015a, Li et al. 2016a, Li et al. 2016b)
			Chondroitinase ABC (25 U/mL)			(Yerramalli et al. 2007)
				IL-1β (10 ng/mL) plus TNF-α (100 ng/mL)		(Ponnappan et al. 2011, Markova et al. 2013)
	Mouse	Static loading				(Ariga et al. 2003)
		Stab			NF-κβ-luciferase	(Abraham et al. 2016)
				IL-1β (10 ng/mL)	*NF1*$^{fl/fl}$	(Pelle et al. 2014)

experiments with more replicates. Nonetheless, due to the IVD's great swelling potential and inhomogeneity (Urban et al. 1979), it is a complex task to establish an adequate model. Degenerated discs often present a low proteoglycan to collagen ratio, as well as low hydration (Urban et al. 1979). By maintaining the IVD endplates (Gantenbein et al. 2006, Haschtmann et al. 2008, Parolin et al. 2010, Alkhatib et al. 2014, Krock et al. 2014, Pereira et al. 2014, Grant et al. 2016b), or by culturing NP (Teixeira et al. 2015) and IVD tissue (Walter et al. 2011, Purmessur et al. 2013b) under constrained conditions, the swelling may be limited (Iatridis et al. 2009). To avoid swelling, but also obstructed transport of nutrients and residues and a decrease in cell viability, bovine IVD cultures are commonly done with CEP (Parolin et al. 2010, Pereira et al. 2014, Grant et al. 2016b). Nonetheless, for instance, Gantenbein et al. (2006) developed an IVD model maintaining the VEPs, which requires a systemically anticoagulant administration before killing the animals.

Another relevant limitation is that human and animal explants can only be kept in culture for a limited time to ensure cell viability (Korecki et al. 2008, Bertolo et al. 2011, Pereira et al. 2016), commonly up to 28 days (Dudli et al. 2014). Bioreactors were proposed as alternatives to culture IVD explants from large animals and human cadavers, providing a defined nutritional and mechanical environment, essential for maintaining cell viability and matrix biology (Gantenbein et al. 2015). However, the culture periods reported are also only up to 21-22 days (Paul et al. 2012, Castro et al. 2014).

Several *ex vivo* models using bovine caudal IVDs have been developed to study degeneration mechanisms and biology, which allow the outlining of *ex vivo* trials (Table 4). Bovine coccygeal discs are described as the most suitable alternative candidates for *ex vivo* studies (Roberts et al. 2008), due to the commonly easy availability of bovine tails, and given their large size (area and volume around 622 mm^2 and 4291 mm^3, respectively), and similar NP aspect ratio (1.02), diffusion distance and resting pressure (0.2-0.3 MPa) to human lumbar IVDs (Oshima et al. 1993, O'Connell et al. 2007). Besides, cellular and ECM composition similarities with human lumbar discs are also high, namely the fast decrease of notochordal cells after birth, the rate of proteoglycan synthesis and the composition profile: collagen content lower in the NP and higher in the outer AF, with higher hydration and proteoglycan content in the NP (Oshima et al. 1993, Demers et al. 2004, Alini et al. 2008, Roberts et al. 2008). However, it was described by Demers et al. (2004) some noticeable differences with age. For instance, they observed that water content does not drop as abruptly in bovine IVDs as in humans, and that the denaturated COL2 content may vary with age and location in both bovine and human IVDs (Demers et al. 2004). For bovine, as for other models, caution must always be present when interpreting the results.

If the low degree of complexity can be an advantage, it is also a limitation of organ cultures, which may lack vascularization, innervation and the multiple interactions with adjacent tissues

and infiltrating immune cells, characteristic of several degraded and pro-inflammatory environments (Molinos et al. 2015a, Sakai and Andersson 2015). The establishment of models using human IVD tissue (Burke et al. 2002a, Le Maitre et al. 2004, Bertolo et al. 2011) or whole IVD, initially developed by Parolin et al. (2010) with healthy discs, being later analyzed explants from donners suffering from degenerative disc disease (Alkhatib et al. 2014, Krock et al. 2014) is growing, facilitating IVD co-culture with different allograft cell types, namely MSCs and lymphocytes (Bertolo et al. 2011).

Nevertheless, although the most common degeneration models are established with mechanical injury (Anderson et al. 2002, Sobajima et al. 2005a, Sobajima et al. 2005b, Iatridis et al. 2009), few studies defined standardized parameters and outcome measurements of inflammation (as shown in Table 4). For simulation of the pro-inflammatory environment associated with disc degeneration, stimulation of organ cultures with chemical factors such as lipopolysaccharide (LPS) (Burke et al. 2003, Rajan et al. 2013), IL-1β (Ponnappan et al. 2011, Kepler et al. 2013) and/or TNF-α (Ponnappan et al. 2011, Purmessur et al. 2013b) can be used to up-regulate inflammatory factors and matrix degrading enzymes, and therefore impair matrix production (Aota et al. 2006, Gorth et al. 2012, Kim et al. 2013a, Rajan et al. 2013). LPS, although not physiological, was previously used as pro-inflammatory stimulus (Burke et al. 2003, Li et al. 2015a, Li et al. 2016a). TNF-α was shown by Purmessur et al. (2013b) to have an important role in the pathologic processes of IVD degeneration. Nonetheless, studies performed by Le Maitre and colleagues showed that IL-1, namely IL-1β, might have a more prominent role than TNF-α, being expressed at higher levels and in a larger proportion of samples (Le Maitre et al. 2005, Le Maitre et al. 2007a, Hoyland et al. 2008). Additionally, we have shown that IL-1β stimulation induces a degenerative and pro-inflammatory response, with expression of several factors identified in humans (Teixeira et al. 2015).

6.2. *In vivo*

The suitability of different animal models to study IVD degeneration has been extensively reviewed (Lotz 2004, Singh et al. 2005, Alini et al. 2008, Daly et al. 2016) and is briefed in Table 5.

Animal models are one step ahead organ cultures, being widely used to study IVD degeneration and to evaluate disc treatment methods, given their high biomechanical complicity, the feasibility of *in vivo* experiments and the possibility to include significant number of subjects to follow over time, when compared to human trials (Alini et al. 2008, Shapiro and Risbud 2014). Nonetheless, namely due to differences in IVD size, one important issue is the scaling up of specific parameters for the interpretation of the experimental findings from animal models (Alini et al. 2008).

Table 5. Animal models for studying intervertebral disc degeneration and inflammation. Adapted from Alini et al. (2008) and Daly et al. (2016).

Species	Degenerative stimulus					References
	Spontaneous	Mechanical	Chemical	Biochemical	Genetic	
Baboon	Natural aging					(Lauerman et al. 1992, Platenberg et al. 2001)
Rhesus monkey		Nucleotomy	Collagenase (5 mg/mL)			(Stern and Coulson 1976)
			Bleomycin (1.5 mg/mL)			(Wei et al. 2014)
			Pingyangmycin (1.5 mg/mL)			(Wei et al. 2015)
Non-chondrodystrophic dog	Aging					(Bergknut et al. 2012)
		Nucleotomy				(Hohaus et al. 2008)
Chondrodystrophic dog	Accelerated aging					(Gillett et al. 1988, Bergknut et al. 2012)
	Hyperactivity (running)					(Puustjarvi et al. 1993, Saamanen et al. 1993, Puustjarvi et al. 1994)
		Nucleotomy				(Hiyama et al. 2008, Serigano et al. 2010)
			Krill proteases (5.4 mg/mL)			(Melrose et al. 1995)
			Chymopapain (2-8 mU/disc)			(Melrose et al. 1996)
			Chondroitinase ABC (250 U/mL)			(Yamada, Tanabe et al. 2001)
Sheep		Annular lesion				(Osti et al. 1990, Melrose et al. 1997a, Melrose et al. 1997b, Fazzalari et al. 2001, Melrose et al. 2002a, Melrose et al. 2002b, Thompson et al. 2004)
		Needle puncture (29G)	Chondroitinase ABC (1.0 IU)			(Ghosh et al. 2012)
Goat		Stab/drill injury, annulotomy				(Zhang et al. 2011a)
			Chondroitinase ABC (0.25 U/mL)			(Hoogendoorn et al. 2007)
Minipig		Annular stab				(Bendtsen et al. 2011)
		Needle puncture (18G)				(Wang et al. 2007a)

		Nucleotomy				(Acosta et al. 2011, Omlor et al. 2012)
Rabbit		Annular stab				(Anderson et al. 2002, Sobajima et al. 2005b)
		Needle puncture (16, 18, 21G)				(Masuda et al. 2005, Moss et al. 2013, Yang et al. 2015)
		Nucleotomy				(Sakai et al. 2003, Kim et al. 2005b)
		Needle puncture (23G)	Camptothecin (1 mmol/L)			(Kim et al. 2005b)
			Chondroitinase ABC			(Kiester et al. 1994, Ando et al. 1995)
		Needle puncture (32G)	Fibronectin fragments (1 µmol/L)			(Greg Anderson et al. 2003)
Sand Rat	Accelerated ageing, obesity					(Silberberg et al. 1979, Moskowitz et al. 1990, Gruber et al. 2002, Gruber et al. 2007, Gruber et al. 2008, Gruber et al. 2014a)
Rat	Natural aging					(Laing et al. 2011)
		Needle puncture (33G)	LPS (1 µg/mL)			(Rajan et al. 2013)
					HLA-B27 and human β_2m transgenic	(Hammer et al. 1990, Taurog et al. 1999)
Rat (caudal spine)		Annular stab				(Ulrich et al. 2007, Jeong et al. 2009)
		Needle puncture (18, 20, 21G)				(Han et al. 2008, Zhang et al. 2009a, Zhang et al. 2011b, Cunha et al. 2015)
		Drill injury				(Kim et al. 2011a)
		Tail static bending				(Court et al. 2007)
		Loading, NP compression				(Ching et al. 2003, Chubinskaya et al. 2007)
		Needle puncture (24 G) plus compression				(Miyagi et al. 2011, Miyagi et al. 2012)
		Application of NP to DRG				(Olmarker et al. 2003, Ito et al. 2007, Sasaki et al. 2007, Kim et al. 2011b, Li

						et al. 2015b, Miao et al. 2015, Wang et al. 2015a, Song et al. 2016)
			Chondroitinase ABC (0.25 U/mL)			(Norcross et al. 2003)
Mouse	Natural aging					(Holguin et al. 2014)
		Needle puncture (26, 29, 31, 33, 35)				(Yang et al. 2009, Martin et al. 2013, Ohnishi et al. 2016)
		Tail static bending				(Court et al. 2001)
		Bipedal mice				(Higuchi et al. 1983)
				proteoglycan-induced spondylitis	*IL-4*$^{-/-}$	(Haynes et al. 2012, Tseng et al. 2016)
					Bmal1 deficiency	(Kondratov et al. 2006, Dudek et al. 2016)
					Ercc1 deficiency	(Vo et al. 2010, Nasto et al. 2012)
					Dystrophin-utrophin double knockout	(Isaac et al. 2013)
					CTGF knockout	(Bedore et al. 2013)
					Biglycan deficiency	(Furukawa et al. 2009)
					Cartilage matrix deficiency	(Watanabe et al. 1997, Watanabe and Yamada 2002)
					Myostatin knockout	(Hamrick et al. 2003)
					COL2 mutation	(Sahlman et al. 2001)
					COL9 mutation	(Kimura et al. 1996)
					Sickle tail mutation	(Semba et al. 2006)
					Ankylosis mutation	(Sweet and Green 1981)
					HLA-B27 transgenic	(Weinreich et al. 1995)

As for *ex vivo* models, there is not fully recognized consensus regarding an ideal *in vivo* model that mimics human disc degeneration (Drazin et al. 2012, Sun et al. 2013a). When considering the use of an animal model, several features should be taken into account, namely the development, anatomy (size and geometry of the discs), biochemistry and the mechanical forces that act on the spine. There are changes not only between different species, but also with age and spinal level (Alini et al. 2008, Daly et al. 2016). As previously mentioned, there are differences regarding, for instance, IVD's notochordal cell content. Species including mouse, rat, cat, dog, pig and rabbit retain them throughout their adult life, while in humans, cows and sheep they rapidly decrease after birth (Alini et al. 2008). When designing an *in vivo* model, and the experimental hypothesis, it should be considered that notochordal cells might be potential NP progenitor cells (Smolders et al. 2012), and that they can be involved in the regulation, for instance, of ECM components synthesis (Aguiar et al. 1999). Nonetheless, some authors argue that the apoptotic processes caused by induced degeneration play a role in notochordal cells loss, as in aged and degenerated human discs (Roberts et al. 2006, Gruber et al. 2009, Yurube et al. 2014), and therefore, the results obtained with these models have relevance after notochordal cells loss (Daly et al. 2016).

Spontaneous disc degeneration models are considered useful for studying the natural evolution of degeneration (Singh et al. 2005, Alini et al. 2008, Daly et al. 2016). Baboons, although quadruped, have been used for spontaneous disc degeneration models (Lauerman et al. 1992, Platenberg et al. 2001). They can spend much time in semi-erect and erect positions, conducting forces through the spine similarly to humans (Lauerman et al. 1992), are relatively large (adult males 20-26 kg, adult females 12-17 kg), have a long-life expectancy (30-45 years) and are closely related to humans (Lauerman et al. 1992, Platenberg et al. 2001). Nonetheless, these animals need space and time to develop a condition that cannot be fully controlled. Other animal models, such as the chondrodystrophic dog (Gillett et al. 1988, Bergknut et al. 2012) and sand rat (Silberberg et al. 1979, Moskowitz et al. 1990, Gruber et al. 2002, Gruber et al. 2007, Gruber et al. 2008, Gruber et al. 2014a) are also used, since they often develop disc related pathologies. Nevertheless, Singh et al. (2005) considered that naturally occurring animal models present several drawbacks, namely the lack of knowledge regarding the high rate of disc degeneration and impossibility to control the progressive structural failure. For instance, in chondrodystrophic canine models, the NP matrix contains higher collagen content, decreased proteoglycan and water content, and calcifications, in contrast with human discs (Singh et al. 2005).

Experimentally induced animal models have been extensively described in the literature, and when established in a control environment, can preset high reproducibility (Singh et al. 2005, Alini et al. 2008). Large animal models have been developed in rhesus monkey, dog, sheep, goat or minipig (Table 5). Sheep and goat present several advantages. Both species, in

comparison to humans, suffer a loss of notochordal cells in early adulthood, present similar lumbar disc size and are exposed to similar mechanical loadings, although being quadruped (Alini et al. 2008, Daly et al. 2016). Moreover, they are animals that commonly tolerate surgical interventions well (Daly et al. 2016).
Small animal models such as rabbit, rat and mouse are relatively simple to manipulate and present cost-effectiveness as a model, when compared to large animals (Daly et al. 2016). They are commonly used for developing models of mechanical injury and tissue enzymatic degradation, as shown in Table 5. Also, very important are the genetic knockout (Bedore et al. 2013, Isaac et al. 2013) and mutation (Sweet and Green 1981, Kimura et al. 1996, Sahlman et al. 2001, Semba et al. 2006) mice models, which allow to investigate the role that certain genes may play in disc degeneration (Singh et al. 2005, Daly et al. 2016). Nevertheless, small animal models have limitations regarding the injection of relevant volumes of therapeutics or implantation of engineered tissue constructs (Zhang et al. 2011a).

This chapter covered numerous works on the healthy and degenerated IVD anatomy and physiology, microenvironment, cell content, molecular key players and the pathomechanisms associated with degeneration. Nonetheless, to study degeneration, inflammation and how this correlates with pain, it is important not to look only to the IVD itself. Analysis at systemic level are also important to further understand questions such as, for instance, the interplay with the immune system. Several models might be chosen for IVD degeneration studies; however, the choice of a model should be clarified regarding the scientific question proposed and the outcomes to be analyzed. In general, animal models are highly focused in assessing outcomes at the IVD level, while often disregarding the neurological morphology and functions that may simulate the clinical symptoms (Alini et al. 2008).

CHAPTER II

Immunomodulation in degenerated intervertebral disc

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1. Immunogenic phenotype of IVD cell populations and induced immune cell response

An association between disc degeneration, herniation and inflammation has been established over time (Johnson et al. 2015, Molinos et al. 2015a). IVD cells can secrete pro-inflammatory cytokines to induce and enhance inflammation (Le Maitre et al. 2007a) and an inflammatory response occurs not only in the IVD, but also in the surrounding tissues (Risbud and Shapiro 2014). Therefore, an in-depth characterization of the synergic interplay between degeneration, inflammation and pain could promote the development of more advanced and targeted therapies for IVD degeneration and LBP (Teixeira et al. 2015, Molinos et al. 2015a, Teixeira et al. 2016). In this section, we discuss the contributions of different factors to cellular and tissue level changes seen during disc degeneration (schematically summarized in Figure 1).

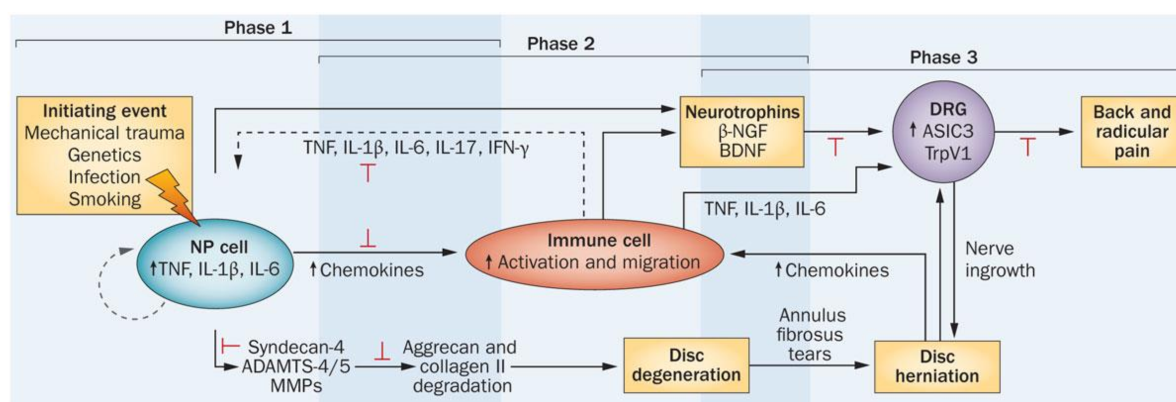

Figure 1. Role of the cytokines involved in different phases of intervertebral disc degeneration and herniation, leading to back and radicular pain. In the first phase of degeneration, IVD cells express several catabolic molecules in the inflammatory environment, promoting ACAN and COL2 degradation, which leads to mechanical instability and ECM breakdown. In many cases, AF tearing and herniation occur. Secondly, the release of cytokines and chemokines by the IVD cells enhances activation and infiltration of immune cells, which also produce pro-inflammatory factors by themselves, further amplifying the inflammatory response. Of notice, together with the infiltration of immune cells, there is also microvascularization and innervation by nociceptive nerve fibers arising from the dorsal root ganglion (DRG). In the third phase, neurogenic factors, particularly nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), produced by the herniated disc and immune cells, induce the expression of the pain associated cation channels like acid-sensing ion channel 3 (ASIC3) and transient receptor potential cation channel, subfamily V, member 1 (Trpv1) in the DRGs, promoting discogenic pain and enhancing the cytokine mediated disc degeneration. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Rheumatology (Risbud and Shapiro, 2013), copyright (2013).

IVD cells express several inflammatory factors already during homeostasis (Molinos et al. 2015a). However, an initial insult, related with aging and degeneration, leads to an upregulation of inflammation mediators such as key pro-inflammatory cytokines, namely IL-1β and TNF-α, but also IL-6, IL-17, IFN-γ and chemokines, among others (Takahashi et al. 1996, Kang et al. 1997, Burke et al. 2002b, Park et al. 2002, Specchia et al. 2002, Le Maitre et al. 2005, Weiler et al. 2005, Le Maitre et al. 2007a, Huang et al. 2008, Kokubo et al. 2008, Shamji

et al. 2010, Risbud and Shapiro 2014). These are initiating events of the IVD degenerative cascade (Risbud and Shapiro 2014, Walter et al. 2015). They contribute to the increase of cell senescence, unbalanced anabolism and catabolism in ECM synthesis (Le Maitre et al. 2005, Seguin et al. 2005, Shamji et al. 2010, Cuellar et al. 2013, Purmessur et al. 2013b). Herniated discs are known to induce a specific autoimmune response (Sun et al. 2013b). Macrophages, leucocytes, neutrophils and T cells were found in extruded tissues (Kokubo et al. 2008, Shamji et al. 2010, Risbud and Shapiro 2014), surrounded by granulation tissue, neovascularization and innervation (Burke et al. 2002a, Freemont et al. 2002a). Vascular and nerve ingrowth into the avascular IVD occurs from the outer layers of the AF into the NP (Sakai and Andersson 2015).

The factors produced by both IVD and immune cells, as well as their effect in degeneration, inflammatory state and associated pain will be discussed ahead. As Molinos et al. (2015a) highlighted, there are numerous inflammatory mediators found in the human IVD, which may be produced by NP, AF and/or infiltrating inflammatory cells, as summarized in Table 1.

1.1. Key pro-inflammatory molecules in IVD degeneration and associated inflammation

Both IVD cells and leucocytes secrete IL-1β and TNF-α (Le Maitre et al. 2005, Le Maitre et al. 2007a). IL-1 and TNF-α have been identified in painful hernia samples, being associated with the mechanism of sensory nerves ingrowth into the NP (Hayashi et al. 2008), damage of the dorsal root ganglion (DRG) and neuropathic pain (Olmarker and Larsson 1998, Igarashi et al. 2000, Murata et al. 2006, Leung and Cahill 2010). Expression of TNF-α and IL-1β was shown to increase with age and severity of degeneration, as observed by analysis of human hernia samples from donors with different ages, being these cytokines by themselves degeneration precursors (Le Maitre et al. 2005, Bachmeier et al. 2007, Le Maitre et al. 2007a, Wang et al. 2014, Johnson et al. 2015).

1.1.1. TNF-α

TNF-α was shown to be one of the first cytokines highly produced by human IVD cells in both IVD degeneration and herniation scenarios (Weiler et al. 2005, Le Maitre et al. 2007a, Ulrich et al. 2007, Dudli et al. 2012). It was shown that by exposing bovine organ cultures to TNF-α, as it may occur from injured surrounding tissues, it can penetrate in healthy intact IVDs, induce expression of additional pro-inflammatory cytokines and alter the tissue mechanical behavior (Millward-Sadler et al. 2009, Walter et al. 2015). NP cells stimulated with TNF-α and IL-1β showed a strong induction of ECM degrading enzymes expression, namely ADAMTS-4 and -5, MMPs 1, 2, 3 and 13, (Shen et al. 2003, Jimbo, Park et al. 2005, Le Maitre et al. 2005, Seguin et al. 2005, Wang et al. 2011a, Wang et al. 2014, Krupkova et al. 2016), and other pro-

Table 1. Inflammation mediators expressed with degeneration by IVD cells and infiltrating cells in painful human intervertebral discs. Table adapted from Wuertz and Haglund (2013) and Molinos et al. (2015a).

Mediators	Tissue	Tissue collection	Disorder	References
TNF-α	AF	Autopsy, biopsy	Degeneration	(Dongfeng et al. 2011)
	AF + NP	Autopsy, biopsy	Degeneration, herniation (protrusion, extrusion, sequestration)	(Weiler et al. 2005, Le Maitre et al. 2007a, Bachmeier et al. 2007)
	AF + NP	Autopsy, biopsy	Degeneration, herniation	(Weiler et al. 2005, Le Maitre et al. 2007a)
	AF + NP + CEP	Biopsy	Herniation, spondylosis	(Kokubo et al. 2008)
	NP	Autopsy, biopsy	Degeneration	(Richardson et al. 2009)
	NP	Biopsy	Herniation	(Park et al. 2011)
	NP	Biopsy	Herniation (protrusion, extrusion, sequestration)	(Chen et al. 2017)
	IVD	Autopsy, biopsy	Degeneration, herniation	(Akyol et al. 2010)
	IVD	Biopsy	Degeneration, herniation	(Lee et al. 2009a)
	IVD	Biopsy	Herniation (protrusion, extrusion, sequestration)	(Takahashi et al. 1996)
	IVD	Biopsy	Herniation (extrusion, sequestration)	(Miyamoto et al. 2000)
	IVD	Biopsy	Herniation (subligamentous extensions, transligamentous extensions including sequestration)	(Ahn et al. 2002)
TNFR1	AF + NP	Autopsy, biopsy	Degeneration, herniation (protrusion, extrusion, sequestration)	(Le Maitre et al. 2007a, Bachmeier et al. 2007)
TNFR2, TACE	AF + NP	Autopsy, biopsy	Degeneration, herniation (protrusion, extrusion, sequestration)	(Bachmeier et al. 2007)
IL-1α	AF + NP	Autopsy, biopsy	Degeneration	(Le Maitre et al. 2005)
	IVD	Biopsy	Herniation (protrusion, extrusion, sequestration)	(Takahashi et al. 1996)
	IVD	Biopsy	Herniation (subligamentous extensions, transligamentous extensions including sequestration)	(Ahn et al. 2002)
IL-1β	AF + NP	Autopsy, biopsy	Degeneration, herniation	(Le Maitre et al. 2007a)
	AF + NP	Autopsy, biopsy	Degeneration	(Le Maitre et al. 2005)
	NP	Autopsy, biopsy	Degeneration	(Richardson et al. 2009)
	NP	Autopsy, biopsy	Herniation	(Gronblad et al. 1994)
	NP	Biopsy	Herniation	(Park et al. 2011)
	IVD	Autopsy, biopsy	Degeneration, herniation	(Akyol et al. 2010)
	IVD	Biopsy	Degeneration, herniation	(Lee et al. 2009a)
	IVD	Biopsy	Herniation (protrusion, extrusion, sequestration)	(Takahashi et al. 1996)
	IVD	Biopsy	Herniation (extrusion, sequestration)	(Miyamoto et al. 2000)
IL-1Ra	AF + NP	Autopsy, biopsy	Degeneration	(Le Maitre et al. 2005)
IL-1R1	AF + NP	Autopsy, biopsy	Degeneration, herniation	(Le Maitre et al. 2007a)
	AF + NP	Autopsy, biopsy	Degeneration	(Le Maitre et al. 2005)
IL-2	IVD	Autopsy, biopsy	Degeneration, herniation	(Akyol et al. 2010)
IL-4	AF + NP	Autopsy, biopsy	Degeneration, herniation	(Shamji et al. 2010)

	NP	Biopsy	Herniation (subligamentous extrusion and protrusion, sequestration and transligamentous extrusion)	(Park et al. 2002)
	IVD	Autopsy, biopsy	Degeneration, herniation	(Akyol et al. 2010)
IL-6	AF + NP	Autopsy, biopsy	Degeneration, herniation	(Shamji et al. 2010)
	IVD	Biopsy	Degeneration, herniation	(Lee et al. 2009a)
	IVD	Biopsy	Degeneration, herniation (protrusion, extrusion, sequestration)	(Burke et al. 2002b)
	IVD	Biopsy	Herniation	(Kang et al. 1996)
	IVD	Biopsy	Herniation (extrusion, sequestration, protrusion)	(Takahashi et al. 1996)
	Lavage fluid from disc space	Biopsy	Herniation	(Gajendran et al. 2011)
IL-8	IVD	Biopsy	Degeneration, herniation	(Lee et al. 2009a)
	IVD	Biopsy	Degeneration, herniation (protrusion, extrusion, sequestration)	(Burke et al. 2002b)
	IVD	Biopsy	Herniation (subligamentous extensions, transligamentous extensions including sequestration)	(Ahn et al. 2002)
	IVD	Biopsy	Herniation (protrusion, extrusion, sequestration), scoliosis	(Burke et al. 2002a)
IL-10	IVD	Autopsy, biopsy	Degeneration, herniation	(Akyol et al. 2010)
	IVD	Biopsy	Herniation (subligamentous extensions, transligamentous extensions including sequestration)	(Ahn et al. 2002)
IL-12	AF + NP	Autopsy, biopsy	Degeneration, herniation	(Shamji et al. 2010)
	NP	Biopsy	Herniation (subligamentous extrusion and protrusion, sequestration and transligamentous extrusion)	(Park et al. 2002)
	IVD	Autopsy, biopsy	Degeneration, herniation	(Akyol et al. 2010)
	IVD	Biopsy	Degeneration, herniation	(Lee et al. 2009a)
IL-16	NP	Autopsy, biopsy	Degeneration, prolapse, herniation (protrusion, extrusion, sequestration)	(Phillips et al. 2013, Phillips et al. 2015)
IL-17	AF + NP	Autopsy, biopsy	Degeneration, herniation	(Shamji et al. 2010)
	AF + NP	Biopsy	Degeneration, herniation	(Gruber et al. 2013)
IL-20 (and its receptor subunits)	IVD	Biopsy	Herniation (extrusion, sequestration)	(Huang et al. 2008)
IL-21	NP	Biopsy	Herniation (protrusion, extrusion, sequestration)	(Chen et al. 2017)
CCL2, CCL7, CXCL8	NP	Autopsy, biopsy	Degeneration, prolapse, herniation (protrusion, extrusion, sequestration)	(Phillips et al. 2013)
CCR1, CXCR1, CXCR2	NP	Autopsy, biopsy	Degeneration, prolapse, herniation (protrusion, extrusion, sequestration)	(Phillips et al. 2015)
IFN-γ	AF + NP	Autopsy, biopsy	Degeneration, herniation	(Shamji et al. 2010)
	NP	Biopsy	Herniation (subligamentous extrusion and protrusion, sequestration and transligamentous extrusion)	(Park et al. 2002)
	Lavage fluid from disc space	Biopsy	Herniation	(Gajendran et al. 2011)

	Lavage fluid from disc space	Biopsy	Degeneration, scoliosis	(Cuellar et al. 2010)
RANTES	AF + NP	Biopsy	Degeneration, herniation	(Gruber et al. 2014b)
	IVD	Biopsy	Herniation (subligamentous extensions, transligamentous extensions including sequestration)	(Ahn et al. 2002)
TGF-β	IVD	Biopsy	Degeneration, herniation	(Lee et al. 2009a)
TGF-β1	IVD	Biopsy	Herniation (subligamentous extensions, transligamentous extensions including sequestration)	(Ahn et al. 2002)
Substance P	AF + NP + CEP	Biopsy	Herniation, spondylosis	(Kokubo et al. 2008)
	NP	Autopsy, biopsy	Degeneration	(Richardson et al. 2009)
MCP-1	IVD	Biopsy	Herniation (protrusion, extrusion, sequestration), scoliosis	(Burke et al. 2002a)
	Lavage fluid from disc space	Biopsy	Herniation	(Gajendran et al. 2011)
MIP-1β	Lavage fluid from disc space	Biopsy	Herniation	(Gajendran et al. 2011)
NGF	AF + NP + CEP	Biopsy	Herniation, spondylosis	(Kokubo et al. 2008)
	NP	Autopsy, biopsy	Degeneration	(Richardson et al. 2009)
	IVD	Biopsy	Degeneration, herniation	(Lee et al. 2009a)
bFGF	AF + NP + CEP	Biopsy	Herniation, spondylosis	(Kokubo et al. 2008)
VEGF	AF + NP + CEP	Biopsy	Herniation, spondylosis	(Kokubo et al. 2008)
	IVD	Biopsy	Degeneration, herniation	(Lee et al. 2009a)
GDF-5	AF	Biopsy	Degeneration, herniation	(Gruber et al. 2014c)
GM-CSF	IVD	Biopsy	Herniation: extrusion, sequestration, protrusion	(Takahashi et al. 1996)
MMPs	AF + NP	Biopsy	Degeneration, herniation (protrusion, extrusion, sequestration)	(Bachmeier et al. 2009)
	AF + NP + CEP	Biopsy	Herniation, spondylosis	(Kokubo et al. 2008)
	NP	Autopsy, biopsy	Degeneration	(Richardson et al. 2009)
	NP	Biopsy	Herniation (protrusion, subligamentous extrusion, transligamentous extrusion, sequestration)	(Matsui et al. 1998)
	IVD	Biopsy	Herniation	(Kang et al. 1996)
FasL	NP	Biopsy	Herniation (subligamentous extrusion and protrusion, sequestration and transligamentous extrusion)	(Park et al. 2001a)
FasR	NP	Biopsy	Herniation (subligamentous extrusion and protrusion, sequestration and transligamentous extrusion)	(Park et al. 2001b)
CDMP	AF + NP	Autopsy, biopsy	Degeneration	(Le Maitre et al. 2009)
COX-2	IVD	Biopsy	Herniation (extrusion, sequestration)	(Miyamoto et al. 2000)
PGE_2	NP	Biopsy	Herniation (protrusion, extrusion, sequestration)	(O'Donnell and O'Donnell 1996)
	IVD	Biopsy	Degeneration, herniation (protrusion, extrusion, sequestration)	(Burke et al. 2002b)
	IVD	Biopsy	Herniation	(Kang et al. 1996)
	IVD	Biopsy	Herniation (extrusion, sequestration)	(Miyamoto et al. 2000)

NO	IVD	Biopsy	Herniation	(Kang et al. 1996)
ADAMTS-1, -4, -5, -9, -15	AF + NP	Autopsy, biopsy	Degeneration	(Pockert et al. 2009)
ADAMTS-7	NP	Biopsy	Herniation (protrusion, extrusion, sequestration)	(Chen et al. 2017)
TIMP-1, TIMP-2	AF + NP	Biopsy	Degeneration, herniation (protrusion, extrusion, sequestration)	(Bachmeier et al. 2009)
TIMP-3	AF + NP	Autopsy, biopsy	Degeneration	(Pockert et al. 2009)
PLA_2	AF + NP	Autopsy, biopsy	Herniation, spondylosis, spondylolisthesis (among others)	(Miyahara et al. 1996)

inflammatory factors, as IL-6 or COX-2 (Jimbo et al. 2005, Fujita et al. 2012), previously identified in human IVD degenerated samples (Bachmeier et al. 2009, Pockert et al. 2009).
TNF-α belongs to a superfamily of ligand/receptor proteins designated TNF/TNFR superfamily proteins. Human TNF is synthesized as a type II transmembrane protein (membrane-bound TNF, mTNF), forming stable homotrimers. mTNF is processed by TNF-α-converting enzyme (TACE) into soluble TNF (sTNF) (Black et al. 1997, Risbud and Shapiro 2014, Johnson et al. 2015). Both sTNF-α and mTNF-α can bind through the TNF homology domain (THD) to the cysteine-rich domains (CRDs) of its receptors (TNFRs), TNFR1 or TNFR2, which act as TNF antagonists (Leung and Cahill 2010). TACE, TNFR1 and TNFR2 are expressed in human NP tissue (Johnson et al. 2015). Binding promotes the recruitment of several factors such as TNFR1-assoicated death domain protein (TRADD), receptor-interacting protein 1 (RIP1), TNF-receptor-associated factor 2 (TRAF2) and baculoviral IAP repeat containing 1 and 2, resulting in formation of Complex I signaling (Johnson et al. 2015). Downstream signaling is mediated by nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPK) pathways (Silke 2011, Risbud and Shapiro 2014).
NF-κB controls the expression of several inflammatory and catabolic genes, playing an important role in the regulation of inflammatory response (Risbud and Shapiro 2014). NF-κB is one of the most important regulators of the synthesis of cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, as of the expression of COX-2 (Tak and Firestein 2001). It is a direct modulator of HIF-1α expression, which is an important transcription factor in cells under hypoxia and vital to chondrocyte survival (Dudli et al. 2012). NF-κB activation may also be involved in cell apoptosis (Tak and Firestein 2001). Regarding the MAPK pathways, they not only control inflammation, but have several other functions as cell growth and differentiation, among others (Li et al. 2015a).
Moreover, it was shown that TNF-α can activate the Wnt/β-catenin signaling pathway in NP cells, increasing the expression of MMP13 (Ye et al. 2011), and that the Wnt/β-catenin signaling can also induce TNF-α expression in NP cells (Hiyama et al. 2013). It is hypothesized that this may lead to a pro-degenerative feed-forward loop between the two signaling pathways (Hiyama et al. 2013).

1.1.2. IL-1β

Regarding IL-1 family, among 9 other cytokines are IL-1α and IL-1β. Although TNF-α seems to be the first cytokines produced in a degeneration scenario by human IVD cells, IL-1 appears to be the predominant cytokine (Le Maitre et al. 2005, Weiler et al. 2005, Le Maitre et al. 2007a, Dudli et al. 2012). Both proteins are encoded by two separate genes and synthesized as pro-peptide precursors (pro-IL-1α and pro-IL-1β), and then activated through intracellular

proteolytic cleavage (IL-1α is cleaved by calpain and IL-1β by caspase-1), forming membrane-bound mIL-1α and mIL-1β (Gabay et al. 2010, Risbud and Shapiro 2014, Johnson et al. 2015). Although pro-IL-1β requires extracellular activation by neutrophil proteases, membrane associated pro-IL-1α is biologically active and can exert both intracellular and extracellular effects (Gabay et al. 2010). Pro-IL-1α can signal adjacent cells through the IL-1 receptor, type 1 (IL-1R1), which was identified by Le Maitre et al. (2005) in non-degenerate and degenerate human IVDs. Moreover, pro-IL-1α retains a nuclear localization sequence, working as transcriptional modulator (Risbud and Shapiro 2014, Johnson et al. 2015). Pro-IL-1α, mIL-1α and mIL-1β can bind to IL-1R1, recruit the IL-1 receptor accessory protein (IL-1RAcP) and create a complex, which then recruits two adaptor proteins, the myeloid differentiation primary response gene 88 (MYD88) and the IL-1 receptor-activated protein kinase (IRAK) (Risbud and Shapiro 2014, Johnson et al. 2015). This leads to downstream activation of numerous signaling proteins, such as c-Jun N-terminal kinase (JNK), p38 and MAPK, and transcription factors, like NF-κB and activating protein (AP)-1, controlling the expression of several inflammatory and catabolic genes (Risbud and Shapiro 2014, Johnson et al. 2015).

In organ culture models, stimulation with TNF-α and IL-1β down-regulated the expression of ECM components, increased the expression of ECM degrading enzymes, pro-inflammatory cytokines and PGE_2, and pain-associated molecule nerve growth factor (NGF) (Abe et al. 2007, Ponnappan et al. 2011, Markova et al. 2013, Purmessur et al. 2013b, Teixeira et al. 2015, Walter et al. 2015, Krupkova et al. 2016, Walter et al. 2016), and compromised disc biomechanics (Walter et al. 2015). *In vitro*, human disc cells, upon stimulation with IL-1β and TNF-α, produced high levels of regulated upon activation, normal T-cell expressed, and secreted (RANTES, also named CC chemokine ligand [CCL]5), which was also observed in lumbar disc AF tissue with higher degree of degeneration (Gruber et al. 2014b). Additionally, TNF-α and IL-1β treatment of NP cells also seems to mediate IVD cell proliferation, affecting the NOTCH signaling pathway (Wang et al. 2013).

1.1.3. IL-6

IL-6 is also a cytokine with impact in promoting IL-1 and TNF-α mediated catabolism in IVD cells (Risbud and Shapiro 2014). Similarly to the effect of TNF-α (Murata et al. 2008), IL-6 was also shown to induce DRG neurons apoptosis (Murata et al. 2011), and to contribute to neuropathic pain (Wei et al. 2013b). Secreted by T cells, macrophages and IVD cells (Rand et al. 1997), IL-6 has been characterized as a pro-inflammatory cytokine in the context of IVD degeneration, but it is also involved in regenerative or anti-inflammatory events (Scheller et al. 2011). IL-6 forms monomers and dimers and it can signal through a type I cytokine receptor complex, which includes the ligand-binding IL-6Rα chain and the membrane glycoprotein

gp130, a receptor and signal-transducing subunit, leading to the activation of intracellular signaling cascades via gp130 (Rose-John et al. 2007, Scheller et al. 2011). This pathway is limited to cells that express IL-6R on their surface (Rose-John et al. 2007). It signals through Janus kinase/signal transducers and activators of transcription (JAK/STAT), MAPK and phosphoinositide-3 kinase (PI3K) signal transduction pathways (Scheller et al. 2011), promoting functions include B- and T- cells growth and differentiation, as well as acute-phase protein induction, among others (Risbud and Shapiro 2014). On the other hand, soluble IL-6R (sIL-6R) can be formed by proteolytic cleavage of the mIL-6R protein or translation from alternatively spliced mRNA (Rose-John et al. 2007). sIL-6R amplifies IL-6-mediated signaling by the activation of cell that express the signal transducer protein gp130 but lack trans-membrane IL-6R, working as paracrine factor (Scheller et al. 2011, Risbud and Shapiro 2014).

1.2. TLRs

Toll-like receptors (TLRs) are plasma- and endolysosomal-bound pattern recognition receptors implicated in innate immunity and inflammation (Klawitter et al. 2014, De Nardo 2015). TLRs are usually expressed by immune cells, namely dendritic cells, macrophages, neutrophils, monocytes, T and B cells but can also be expressed by other cell types as synovial fibroblasts, chondrocytes and IVD cells (Klawitter et al. 2014, De Nardo 2015). Klawitter et al. (2014) detected also the expression of TLRs 1, 3, 5, 6, 9 and 10 in human cells isolated from degenerated discs, and observed that TLRs 1, 2, 4 and 6 expression was dependent on the IVD's degree of degeneration. While TLRs 1, 2, 4, 5 and 6 are located on the cell surface, TLRs 3, 7, 8 and 9 are in the endosomal/lysosomal compartment (Klawitter et al. 2014). Namely TLRs 2 and 4 have been described to be expressed by human (Ellman et al. 2012, Klawitter et al. 2012a, Klawitter et al. 2012b, Gawri et al. 2014b, Klawitter et al. 2014) and bovine IVD cells (Rajan et al. 2013). TLR2 and TLR4 are known to mediate the innate immunity, being highly specific in their pathogen recognition. They activate NF-κB, JNK, and p38 signaling pathways, leading to increased expression of TNF-α, IL-1α, IL-1β, IL-6, IL-8, COX-2, IκBα (an inhibitor of NF-κB transcription factor), MMP1, MMP13, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-2 and mitogen-activated protein kinase phosphatase (MKP)-1 (Schaefer et al. 2005, Gabay et al. 2010, Quero et al. 2013). JNK, p38 and MAPK, as well as NF-κB (Risbud and Shapiro 2014).

Furthermore, as Johnson et al. (2015) discussed, several studies have shown that ECM degradation products may act as signaling molecules, as TLRs endogenous ligands, playing a relevant role in the enhancement of the inflammatory state. For instance, proteolytically-cleaved biglycan activated pro-inflammatory cascades through binding to TLR2 and TLR4 in macrophages (Schaefer et al. 2005), hyaluronic acid fragments activated the TLR2 signaling

pathway in resident IVD cells (Quero et al. 2013), fibronectin fragments worked as endogenous ligands for TLR4 (Okamura et al. 2001), and VCAN aggregates activated TLR2 in carcinoma (Kim et al. 2009b). Moreover, it was observed that excessive mechanical loading of IVD cells may upregulate TLR2 and TLR4 expression (Gawri et al. 2014b). Also, it was seen a significant increase in TLR2 mRNA expression and production by stimulating human disc cells with IL-1β or TNF-α, which was linked to the NF-κB pathway activation (Klawitter et al. 2014).

1.3. microRNAs

The role of microRNAs (miRNAs) and their potential as biomarkers for early diagnosis of IVD degeneration has lately drawn great attention (Li et al. 2015c, Zhou et al. 2017). To date, the precise role of miRNAs in the pathogenesis of degeneration is not yet elucidated (Liu et al. 2014, Zhou et al. 2017).

miRNAs are small non-coding RNA molecules with about 18 to 22 nucleotides (Li et al. 2015c), transcribed from their respective gene loci as primary miRNAs (pri-miRNAs) (Papagiannakopoulos and Kosik 2008), followed by a series of maturation steps (Sato et al. 2011). pri-miRNAs can be transcribed from specific miRNA-encoding regions of the genome or derive from mRNA intronic sequences (Li et al. 2015c). miRNAs work by selectively binding to the 3'-untranslated region of their target mRNAs through complementary base pairing, leading to mRNA degradation or suppression of protein translation (Wang et al. 2011b, Ying et al. 2013).

As components of several gene regulatory networks, miRNAs are involved in cell proliferation, differentiation and apoptosis (Luo et al. 2013, Mathieu and Ruohola-Baker 2013, Cao et al. 2014), tissue development (Joglekar et al. 2009, Bae et al. 2012, Khoshgoo et al. 2013, Ying et al. 2013), homeostasis, metabolism and tumorigenesis (Majid et al. 2012, Xie et al. 2013). Defective expression or alterations in miRNAs combination with their target genes can contribute, for instance, to different cancers, including gastrointestinal (Bandres et al. 2009), osteosarcoma (Duan et al. 2011) and hepatocellular carcinoma (Furuta et al. 2010), autoimmune diseases, such as rheumatoid arthritis and osteoarthritis (Buckland 2010), and IVD degeneration (Wang et al. 2011c, Tsirimonaki et al. 2013, Zhao et al. 2014). Bioinformatics analysis are commonly used to investigate miRNA target genes and predict possible signaling pathways (Zhou et al. 2017). Several authors identified miRNAs which were differentially expressed by human NP cells in degenerative samples, compared to controls (Wang et al. 2011c, Zhao et al. 2014, Ji et al. 2016, Li et al. 2016c, Xu et al. 2016). miRNAs involved in the mechanisms associated with disc degeneration have been recently revised by Li et al. (2015c) and Zhou et al. (2017), and are summarized in Table 2.

Table 2. miRNAs reported to be involved in human degenerative NP. Adapted from Li et al. (2015c) and Zhou et al. (2017).

miRNA	Expression	Target	Function	References
Apoptosis mediators				
miR-27a	↑	PIK3CD	Regulates the PI3K/Akt signaling pathway	(Liu et al. 2013a)
miR-155	↓	FADD, caspase-3	Involved in the FasL-Fas signaling pathway	(Wang et al. 2011c)
miR-494	↑	JunD	Mediates TNF-α-induced cell apoptosis	(Wang et al. 2015b)
Cell proliferation mediators				
miR-10b	↑	HOXD10	Targets the RhoC-Akt signaling pathway	(Yu et al. 2013)
miR-15a	↑	MAP3K9	Inhibits NP cells proliferation and induced cells apoptosis by targeting MAP3K9. Involved in MAPKs signal pathway.	(Cai et al. 2017)
miR-21	↑	PTEN	Targets the PTEN/Akt signaling pathway	(Liu et al. 2014)
miR-27b	↓	MMP13	Induces type II collagen loss by directly targeting MMP13	(Li et al. 2016c)
miR-184	↑	GAS1	Negatively regulates the GAS1/Akt signaling pathway	(Li et al. 2017a)
Degeneration and inflammation mediators				
miR-7	↑	GDF-5	Mediates IL-1β-induced ECM degradation	(Liu et al. 2016a)
miR-15b	↑	SMAD3	Mediates IL-1β-induced ECM degradation	(Kang et al. 2017)
miR-34a	↑	GDF-5	Mediates IL-1β-induced ECM degradation	(Liu et al. 2016b)
miR-93	↓	MMP3	Positively regulates COL2 loss by directly targeting MMP3	(Jing and Jiang 2015)
miR-98	↓	STAT3	Promotes ECM degradation by targeting IL-6/STAT3 signaling pathway	(Ji et al. 2016a)
miR-100	↑	FGFR1, FGFR3	Activates MMP13 through suppression of FGFR3 via imbalance of FGFR1 and FGFR3 levels	(Yan et al. 2015)
miR-133a	↓	MMP9	Mediates COL2 loss by directly targeting MMP9	(Xu et al. 2016)
miR-146a	↓	FADD, IL-1β, IL-6, TNF, MMP16	Involved in IL-1 induced IVD degeneration and inflammation	(Gu et al. 2015)
miR-193a-3p	↓	MMP14	Positively regulates COL2 expression by directly targeting MMP14	(Ji et al. 2016b)
miR-377	↓	ADAMTS5	Negatively regulates ACAN degradation by ADAMTS5	(Tsirimonaki et al. 2013)

↓ Down-regulated. ↑ Up-regulated.

1.4. Immune cell activation

The IVD has been defined as an immune-privileged organ (Wang et al. 2007b, Sun et al. 2013b). A study by Sheikh et al. (2009) did not observed immune response to a xenograft of mouse cells in an immunocompetent rabbit model, which suggests the hypothesis of existence of immune-privileged sites within the IVD. The immunological privilege was shown to be maintained by FasL (predominantly expressed in activated T lymphocytes and stromal cells of immune-privileged sites) and the physiological barrier together in rat (Takada et al. 2002) and rabbit (Wang et al. 2007b, Wang et al. 2011c) models (Kaneyama et al. 2008). In human

samples, FasL expression was observed to decrease with degeneration (Kaneyama et al. 2008). FasL belongs to the TNF family and when binding to its receptor Fas, Fas-FasL pathway activation induces cell apoptosis of T lymphocytes (Bellgrau et al. 1995, Griffith et al. 1995, Greil et al. 1998) and of IVD cells (Park et al. 2001a, Park et al. 2001b, Wang et al. 2011d), and contributes to pro-inflammatory cytokines production (Yamamoto et al. 2013).
AF tear and NP leakage is recognizable to the immune system as a foreign body (Sun et al. 2013b). This may induce antigen capture, activation of B cells with the production of auto-antibodies and $CD8^+$ cytotoxic T (T_C) cells (Sun et al. 2013b). Antibodies/immunoglobulins have been detected in human herniated IVD tissue (Marshall et al. 1977, Pennington et al. 1988, Takahashi et al. 1996, Szymczak-Workman et al. 2009, Shamji et al. 2010). The immune system downstream cascades promote migration and infiltration, in the region, of specific and nonspecific immune cells, which together with the cytokines they and IVD cells secret, intensify the inflammatory response and cause pain (Risbud and Shapiro 2014). Takahashi and colleagues (1996) identified that most of the cytokine-producing cells, in protrusions, are IVD cells, but also histiocytes, fibroblasts, or endothelial cells, in extruded and sequestrated tissues.

Risbud and Shapiro (2014) reviewed the role of different immune cells infiltrating into the IVD, commonly in herniation and back and radicular pain scenarios, which is schematically presented in Figure 2 and described in the following sections.

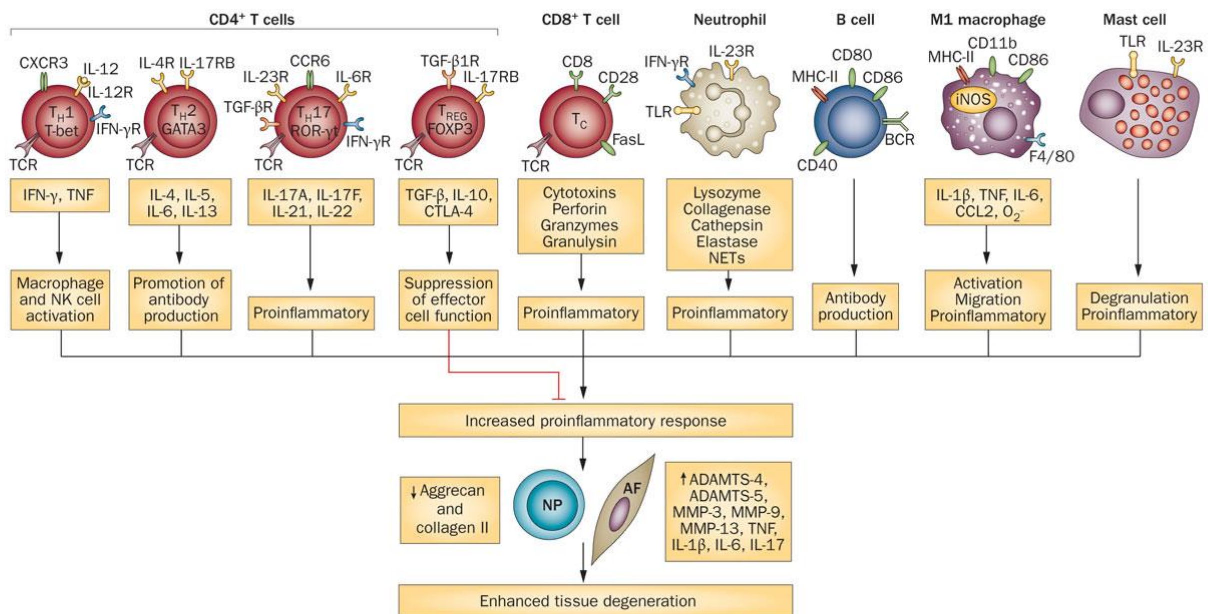

Figure 2. Role of the different classes of immune cells in amplifying the inflammatory response by disc cells during IVD degeneration. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Rheumatology (Risbud and Shapiro, 2013), copyright (2013).

1.4.1. T cells

The presence of inflammatory cells, predominantly macrophages, but also mast cells, subtypes of $CD4^{+}$ T helper (T_H) cells, and neutrophils was observed in painful herniated lumbar discs (Gronblad et al. 1994, Doita et al. 1996, Habtemariam et al. 1998, Matsui et al. 1998, Burke et al. 2002b, Peng et al. 2006, Shamji et al. 2010, Risbud and Shapiro 2014), with significant vascular invasion in non-contained/extruded tissues (Kokubo et al. 2008).
IL-12 and IFN-γ were shown to be highly expressed in herniated disc fragments, compared with bulging discs, which may suggest activation of T_H1 $CD4^{+}$ lymphocytes upon NP exposure to systemic circulation (Park et al. 2002, Cuellar et al. 2010, Shamji et al. 2010). IL-12, known to be produced mainly by macrophages, leads T_H1 cells to produce high amounts of IFN-γ, as well as TNF (Trinchieri 1994, Risbud and Shapiro 2014). Of notice, IFN-γ was found to be most commonly elevated in LBP symptomatic patients, but absent in asymptomatic controls (Cuellar et al. 2010).
On the other hand, increased levels of IL-4 were found in herniated IVD tissue (Shamji et al. 2010), which suggest the involvement of T_H2 $CD4^{+}$ cells (Risbud and Shapiro 2014). Moreover, higher levels of IL-4 had already been detected in contained discs, when compared to non-contained ones (Park et al. 2002).
The presence of IL-17 was also implicated in IVD degeneration, being identified $CD4^{+}IL\text{-}17A^{+}$ and $CD4^{+}CCR6^{+}$ IL-17-producing cells and high levels of IL-17 in degenerated and herniated tissues, in contrast with low level observed in control tissues obtained from autopsies (Shamji et al. 2010, Zhang et al. 2013a, Liu et al. 2016c). IL-17 is secreted by T_H17 cells, neutrophils, mast cells (Gaffen 2011, Gruber et al. 2013, Kenna and Brown 2013), was well as by IVD resident cells (Liu et al. 2016c). IL-17 is known to induce the activation and mobilization of neutrophils, triggering the production of chemokines and pro-inflammatory cytokines (Gaffen 2011, Gruber et al. 2013, Kenna and Brown 2013). IL-17 supplementation promoted the production of inflammatory mediators, such as NO, PEG_2 and IL-6, and the expression of Intercellular adhesion molecule (ICAM)-1 by IVD cells (Gabr et al. 2011). Moreover, co-stimulation with IL-12 and IFN-γ or TNF-α showed a synergistic increase of the inflammatory mediators and ICAM-1, suggesting an impact of IL-17 at different levels and an important role of T_H17 lymphocytes in the pathology of IVD disease (Gabr et al. 2011). Moreover, IVD cells might recruit additional lymphocytes and immune cells to the IVD (Gabr et al. 2011). IL-17 receptors may signal through JAK/STAT1, MAPK or NF-κB pathways, correlated with IFN-γ and TNF-α signaling pathways (Albanesi et al. 1999, Miljkovic and Trajkovic 2004, Weaver et al. 2007).
IL-21, also known to be a cytokine secreted by T_H17 cells (Wei et al. 2007, Liu et al. 2012), was recently found in human degenerated IVD (Chen et al. 2017). IL-21 production was shown

to contribute to the enhancement of IVD degeneration by stimulation of TNF-α through the JAK/STAT signaling pathway (Chen et al. 2017). It has also been previously shown that IL-21 produced by T_H17 cells leads to IL-17 production in a STAT3-dependent manner to promote/sustain T_H17 lineage commitment (Wei et al. 2007).

1.4.2. Macrophages

In herniated tissues, it was shown that aside degenerated IVD cells, also invading monocytes or macrophages ($CD68^+$ cells) may secrete cytokines in the IVD tissue (Peng et al. 2006, Kokubo et al. 2008, Shamji et al. 2010, Wuertz and Haglund 2013). Co-culture studies showed that the interaction between IVD cells and macrophages may lead to the production of IL-6, IL-8, inducible nitric oxide synthase (iNOS), and PGE_2 (Takada et al. 2004, Kim et al. 2008, Kim et al. 2009, Hamamoto et al. 2012, Kim et al. 2012, Takada et al. 2012, Yamamoto et al. 2013). After tissue injury or infection, monocytes can be recruited to the site as effectors and differentiate into macrophages and dendritic cells (Shi and Pamer 2011). Macrophages are important innate immunity participants, with heterogeneous functions dependent on the microenvironmental cues. Inflammatory macrophages (M1) are described as the "classically activated" subset (Mantovani et al. 2004, Ogle et al. 2016). M1-activated macrophages are part of polarized T_H1 response (i.e. stimulation with IFN-γ, LPS and/or inflammatory cytokines, such as TNF-α), producing numerous inflammatory cytokines (IL-1β, TNF-α, IL-6), reactive oxygen species, and growth factors, such as VEGF (Mills et al. 2000, Gordon 2003, Mantovani et al. 2004, Spiller et al. 2014). On the other hand, macrophages can also be polarized towards an anti-inflammatory phenotype (M2), which can further be subdivided into M2a, M2b and M2c, based on activation signals, cell surface receptors, and functional diversity (Mantovani et al. 2004). Naïve macrophages can be polarized, *in vitro*, by stimulation with IL-4 and/or IL-13 to M2a, with TLR or IL-1R ligands to M2b, or with IL-10 to an M2c phenotype (Mantovani et al. 2004). While M2a macrophages contribute to wound healing, M2b and M2c promote the resolution of inflammation through secretion of IL-10 (Mosser and Edwards 2008). Nonetheless, since macrophages polarization may depend, among other cues, on the amounts of factors present in the area where they migrate to (Mantovani et al. 2009), findings from Shamji et al. (2010) from herniated human disc fragments point out to immune lymphocyte activation of the T_H1 lineage, hence macrophages that migrate to herniated IVD tissues will most probably polarize towards an M1 phenotype.

Moreover, infiltrating macrophages, fibroblasts, and endothelial cells, together with native IVD cells, were shown to spontaneously produce MCP-1, MIP-1α, which together with IL-8 work as chemotactic molecules for macrophages and other immune cells (Gronblad et al. 1994, Burke et al. 2002a). Several studies hypothesize that the mechanism of spontaneous disc herniation

regression may include tissue retraction and dehydration, inflammatory response, and the recruitment, infiltration and activity of phagocytic cells, among which are neutrophils, monocytes, macrophages and mast cells (Ikeda et al. 1996, Ito et al. 1996, Haro et al. 1997, Burke et al. 2002a, Kim et al. 2013b). Peng and colleagues (2006) detected high numbers of macrophages and mast cells in painful IVDs. Macrophages and mast cells were similarly distributed around blood vessels and among collagenous fibers of scar/granulation tissue, while being absent in non-degenerated controls or aging discs (Peng et al. 2006). Mast cells are highly specialized mononuclear cells, which contribute to disc tissue inflammation, neovascularization, fibrosis, degradation and secretion of NGF, with a possible causative role in chronic LBP (Freemont et al. 2002b, Peng et al. 2006). Nonetheless, for instance, Nerlich et al. (2002) also observed that non-herniated NP tissue collected during surgery also presented high number of resident $CD68^+$ cells. Moreover, Jones et al. (2008) identified, *in vitro*, that IVD cells can undergo phagocytosis, by ingesting latex beads, indicating that endogenous inflammatory-like cells are comprised in the IVD.

Additionally, alterations at systemic level have also been reported, namely a significant increase in $CD3^+$, $CD4^+$, $CD4^+/CD8^+$ lymphocytes in the peripheral blood of patients with lumbar disc herniation, and with (Ma et al. 2010) or without (Tian et al. 2009) AF rupture. A positive correlation between the percentage of $CD4^+$ T lymphocytes or the ratio $CD4^+/CD8^+$ and pain was also observed (Tian et al. 2009, Ma et al. 2010).

1.5. Other factors involved in innervation, vascularization and pain

In human extruded or sequestrated discs, other factors have been identified, namely anti-IL-1, lymphocyte function-associated antigen (LFA)-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), basic fibroblast growth factor (bFGF) and VEGF, which suggests an active role of those factors in angiogenesis and neovascularization associated with IVD degeneration (Tolonen et al. 1995, Doita et al. 1996). Peng et al. (2006) reported strong expressions of bFGF, transforming growth factor (TGF)-β1 and their receptors, as well as cell proliferation, in granulation tissue from painful lumbar IVDs.

Furthermore, substance P and neurotrophins such as NGF and brain-derived neurotrophic factor (BDNF) have been implicated in the mechanisms associated with an enhancement of innervation and neuropathic pain in some cases of IVD degeneration (Freemont et al. 2002a, Freemont et al. 2002b, Purmessur et al. 2008, Ponnappan et al. 2011, Purmessur et al. 2013b). A study by Freemont and colleagues observed production of NGF in painful IVDs with ingrowth of blood vessels and nociceptive nerve fibers. Of notice, NGF expression was not identified in non-painful or control IVDs (Freemont et al. 2002a).

The production of neurotrophins induces DRGs pain associated cation channels depolarization

(Risbud and Shapiro 2014). The increased expression of transient receptor potential cation channel, subfamily V, member 1 (Trpv1) and the acid-sensing ion channel 3 (ASIC3) induce discogenic pain and further cytokine mediated disc degeneration (Zhang et al. 2005, Ohtori et al. 2006, Risbud and Shapiro 2014).

2. Strategies for immunomodulation of degenerated intervertebral disc

Some regenerative medicine- and tissue engineering-based strategies for degenerated IVD have considered the interplay between IVD degeneration, immune cell response and inflammation, when focused in promoting the production of healthy ECM by native IVD cells, while reducing discogenic pain (Molinos et al. 2015a). Well-balanced approaches targeting not only regeneration, but also the modulation of inflammation mediators have been presented as the most promising therapies in reducing IVD-associated pain (Molinos et al. 2015a). These include biological approaches (using different molecules such as growth factors), gene therapy, and cell therapies, ranging from autologous/exogenous cell transplantation to endogenous cell stimulation and recruitment (Figure 3), that are under different development levels (clinical trials, *in vivo* trials, *ex vivo* and *in vitro* studies) and have been reviewed over time (Hughes et al. 2012, Molinos et al. 2015a, Sakai and Andersson 2015, Richardson et al. 2016).

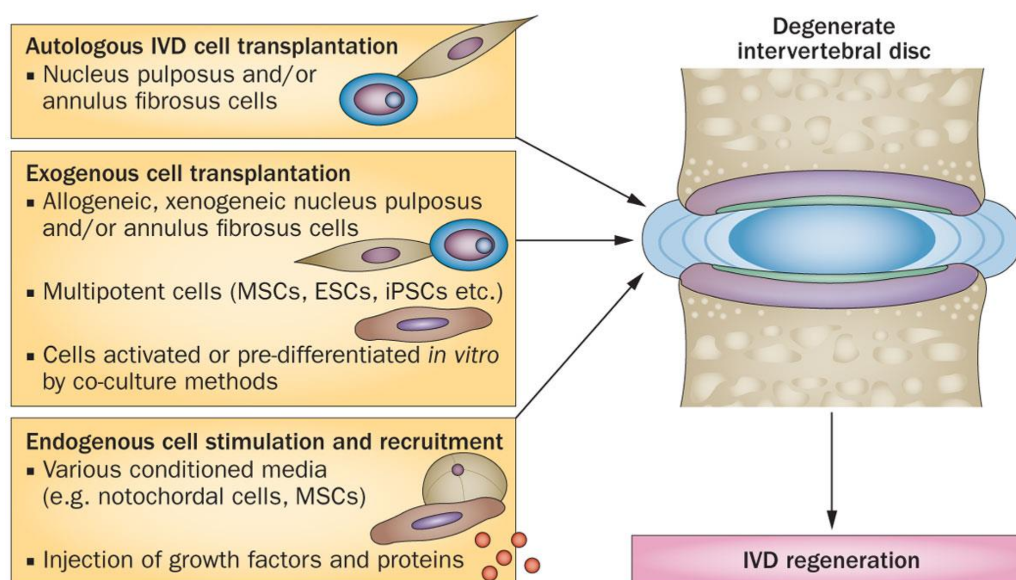

Figure 3. Cell sources for intervertebral disc regeneration. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Rheumatology (Sakai and Andersson, 2015), copyright (2015).

2.1. Molecular therapy: clinical trials

The modulation and balance of anabolic and anti-catabolic responses of IVD cells addressing the aberrant cytokine-rich/pro-inflammatory degenerative IVD environment are the main target

of the molecular therapies proposed so far (Vadala et al. 2015). An overview of these therapies is summarized in Table 3. Cocktails or single drug administrations of steroids, corticosteroids and anesthetics through epidural delivery or nerve root infiltration, as well as oxygen-ozone (O_2-O_3) gas infiltrations are routine treatments of discogenic diseases (Bonetti et al. 2005, Burgher et al. 2011). Recently, epidural injection of clonidine, an alpha-2 adrenergic receptor agonist, has also shown potential in patients' pain improvement (Burgher et al. 2011). Clonidine has been previously shown to have anti-inflammatory effects in preclinical studies of nerve injury and may also indirectly influence pain (Romero-Sandoval et al. 2005).

Nonetheless, several clinical trials have been evaluating the safety and efficacy of single-dose injections into the NP: for chronic LBP and degenerative disc disease, clinical trials are currently focusing on intradiscal injection of jellified ethanol (NCT02343484), hydrolyzed polyacrylonitrile (HPAN)-based hydrogels (NCT02763956), autologous platelet-rich plasma (PRP), combined with NSAID oral medication (NCT02983747), or recombinant human GDF-5 (NCT00813813, NCT01124006, NCT01158924), a member of the TGF-β superfamily and the bone morphogenetic protein (BMP) subfamily, which is known to influence the growth and differentiation of various tissues, including the intervertebral disc (Feng et al. 2015). Furthermore, in patients with inflammatory discopathy, intradiscal injection of steroids (NCT00804531) or corticoids (NCT01694134) have been compared. Drugs with TGF-β antagonist active ingredients (NCT02320019) have also been tested.

In patients suffering from IVD herniation, intradiscal injection of condoliase, a GAG-decomposing enzyme do degrade the herniated tissue, with high substrate specificity for chondroitin sulfate, dermatan sulfate and hyaluronic acid (NCT01282606), a recombinant human MMP (NCT01978912), or a fibrin sealant (Yin et al. 2014) have been proposed as potential alternatives. While the MMP and the condoliase studies are currently still in phases II and III of clinical trial, respectively, their selective activity on the hernia, leading to its degradation and regression without risk of side effects are expected to be low (NCT01978912, NCT01282606). On the other hand, the fibrin sealant was considered to improve pain and function in selected patients with discogenic pain, although neurological assessments, X-ray, and MRI showed no significant changes (Yin et al. 2014).

O_2-O_3, although promoting tissue stabilization through nucleolysis, is being considered by some clinicians a successful pain relief approach in some herniated disc patients who failed to respond to conservative therapy (Paoloni et al. 2009, Melchionda et al. 2012, Zhang et al. 2013b), although this is not consensual since many specialists point the absence of functional results in the patients.

TNF-α inhibition and antagonism of TNF-α receptors was early shown to reduce pain-related symptoms in a chronic constriction injury of rat nerve (Sommer et al. 1998). In human trials, a short course of TNF-α inhibitors, such as infliximab, adalimumab and etanercept, have shown

Table 3. Bioactive molecules to target IVD degeneration, inflammation and discogenic pain. Adapted from Molinos et al. (2015a).

Therapy	Administration route	Condition / Model	Post-treatment follow-up	Outcomes	References
Clinical trials					
Clonidine, triamcinolone	Transforaminal epidural injection	Acute lumbosacral radiculopathy related to disk herniation	Up to 1 month	Radicular pain improved rapidly with clonidine or triamcinolone, compared to a corticosteroid; corticosteroid injections led to greater functional improvement, without differences in analgesia.	(Burgher et al. 2011)
Corticosteroid	Peri-radicular infiltration	Radicular pain	Up to 1 year	No additional benefit, when compared to local anesthetic injection alone; corticosteroids did not avoid subsequent interventions such as additional root blocks or surgery.	(Tafazal et al. 2009)
	Intradiscal injection	Discogenic LBP	Up to 6 months	Potential as short-term alternative for LBP patients unwilling to accept surgery when conservative treatments failed.	(Cao et al. 2011)
Local anesthetic	Transforaminal epidural injection	Disc herniation and radiculitis	Up to 2 years	Significant improvement in all participants who received local anesthetic alone and who received local anesthetic and steroid.	(Manchikanti et al. 2014)
Oxygen-ozone (O_2-O_3)	Paravertebral injections	LBP due to lumbar disc herniation	Up to 6 months	Minimally invasive; seemed to be safe and effective in reducing root inflammation with a corresponding reduction of pain; reduced disability and intake of analgesic drugs.	(Paoloni et al. 2009, Melchionda et al. 2012)
	Intradiscal and intraforaminal injection	LBP pain and radicular pain	Up to 1 year	O_2-O_3 nucleolysis provided pain relief in most patients who failed to respond to conservative therapy; no significant differences between O_2-O_3 injection only or combined with steroid.	(Zhang et al. 2013b)
Pamidronate	Intravenous infusion	Erosive degenerative disc disease; patients fail to respond to NSAIDs	Up to 1 year	Significant improvements in pain and in mean disability scores. The pain no longer showed an inflammatory pattern in 9 of 10 patients.	(Poujol et al. 2007)
Steroid and O_2-O_3	Intraforaminal and intradiscal injections	Radicular pain related to acute lumbar disk herniation	Up to 6 months	Intraforaminal and intradiscal injections of steroid and O_2-O_3 were more effective than injections of a steroid alone.	(Gallucci et al. 2007)
Steroid	Oral administration	Acute sciatica due to herniated disc	Up to 13 months	Modest improvement in function and no improvement in pain after a short course of oral steroids administration, compared with placebo.	(Goldberg et al. 2015)
	Epidural injection	Sciatica caused by lumbosacral disc prolapse	Up to 3 months	Short-term management of painful sciatica, but no additional long-term improvement over placebo.	(Nandi and Chowdhery 2017)
TNF-α blocker - adalimumab	Subcutaneous injection	lumbar disc herniation, sciatica	Up to 6 months, 3 years	Small decrease in leg pain; significantly fewer surgical procedures.	(Genevay et al. 2010, Genevay et al. 2012)

TNF-α blocker - etanercept	Perispinal administration	Degenerative disc disease, disc herniation, sciatica	Up to 1 month, 230 days	Significant clinical improvement in selected patients with chronic, treatment-resistant disc-related pain.	(Tobinick and Britschgi-Davoodifar 2003, Tobinick and Davoodifar 2004)
	Subcutaneous injection	Sciatica	Up to 6 weeks	Patients with severe sciatica had sustained improvement after a short treatment with etanercept, compared with standard care plus a short course of methylprednisolone.	(Genevay et al. 2004)
	Transforaminal epidural injection	Persistent lumbosacral radicular pain secondary to lumbar disc herniation	Up to 26 weeks	Clinically significant reductions in mean daily worst leg pain and worst back pain compared to placebo.	(Freeman et al. 2013)
	Single intradiscal injection	Discogenic LBP	Up to 2 months	Discogenic LBP alleviation.	(Sainoh et al. 2016)
TNF-α blocker - infliximab	Intravenous infusion	Sciatica, disc herniation	Up to 6 months	Infliximab was superior in terms of leg pain and back-related disability decrease compared to control; but, did not appear to interfere with disc herniation resorption.	(Karppinen et al. 2003) (Autio et al. 2006)
	Intravenous infusion	Acute/subacute sciatica secondary to herniated disc	Up to 1 year	Short-term pain reduction; but, long-term results did not show differences between infliximab and placebo.	(Korhonen et al. 2006)
***In vivo* studies**					
BMP-7	Intradiscal injection	Rabbit, *in vivo* disc degeneration	Up to 2 months	Increased disc height up to the 8-week timepoint, and increased NP proteoglycan content at 2 weeks.	(An et al. 2005)
	Intradiscal injection	Rabbit, *in vivo* disc degeneration	Up to 4 months	A single BMP-7 injection dramatically reversed the decrease in disc height induced by chondroitinase ABC chemonucleolysis.	(Imai et al. 2007)
BMP-13	Intradiscal injection	Sheep, *in vivo* disc degeneration	Up to 4 months	BMP-13 injected at the time of injury reversed or arrested loss of matrix proteins.	(Wei et al. 2009)
Corticosteroid	Corticosteroid-loaded ceramic capsule placed adjacent to the punctured disc	Rat, *in vivo* disc degeneration	4 weeks	Continuous sustained release of corticosterone tricalcium phosphate from ceramic capsules could slow the process of degeneration within the traumatized disc in the rat model.	(Ragab et al. 2009)
COX-2 inhibitor	Epidural injection	Rat, *in vivo* disc degeneration	Up to 1 week	Decrease in mechanical hyperalgesia 1 hour, 3 and 7 days after the epidural injection of COX-2 inhibitor.	(Kawakami et al. 2002)
Epoxyeicosatrienoic acids	Intradiscal injection	Rat, *in vivo* disc degeneration	1 month	Enhanced the survival of NP cells and inhibited IVD degeneration.	(Li et al. 2017b)
GDF-5, TGF-β1	Intradiscal injection	Mouse, *in vivo* disc degeneration	Up to 4 weeks	Early intervention avoided or slowed the degenerative process.	(Walsh et al. 2004)

IκB kinase-β inhibitor	Intradiscal injection	Rat, *in vivo* disc degeneration	Up to 2 weeks	Injury-induced up-regulation of inflammatory cytokines within IVD, and increased levels of neuropeptides within DRG neurons could be suppressed by inhibiting IκB kinase-β.	(Kobori et al. 2014)
p38 MAP kinase inhibitor	Intradiscal injection	Rat, *in vivo* disc degeneration	Up to 2 weeks	A direct single application of p38 inhibitor did not suppress calcitonin gene-related peptide expression in DRGs innervating punctured discs.	(Hayashi et al. 2009)
Phosphodiesterase-2A inhibitor	Intrathecal administration	Rat, *in vivo* non-compressive lumbar disc herniation	Up to 1 week	Alleviates radicular inflammation and mechanical allodynia.	(Wang et al. 2017)
Platelet-rich plasma (PRP)	Intradiscal injection	Rabbit, *in vivo* disc degeneration	8 weeks	Suppression of degeneration progress.	(Nagae et al. 2007)
	Injection into and around the IVD	Rat, *in vivo* disc degeneration	Up to 6 weeks	PRP-treated groups retained more normal morphologic features, contained fewer inflammatory cells, and showed higher hydration on MRI.	(Gullung et al. 2011)
Resveratrol	Local application	Rodent, *in vivo* disc degeneration	Up to 2 weeks	Significant pain behavior reduction (it was also seen *in vitro*, in human NP tissue, that resveratrol exhibited an anti-inflammatory and anti-catabolic effect).	(Wuertz et al. 2011)
Simvastatin	Intradiscal injection	Rat, *in vivo* disc degeneration	Up to 4 weeks	A single injection of simvastatin loaded in a gel had the potential to retard or regenerate the degenerative disc.	(Zhang et al. 2009b)
Thalidomide	Injection in the epineurium (distal to the NP)	Rat, *in vivo* disc degeneration	Up to 28 days	Significantly inhibited radiculopathic pain *in vivo* (and the expression of pro-inflammatory mediators and MMPs *in vitro*)	(Song et al. 2016)
***Ex vivo* studies**					
Crocin	Culture medium supplementation	Rat, *ex vivo* disc degeneration	1 week	Effectively suppressed the degeneration-related inflammation and catabolism in rat IVDs, suggesting a potential use as a therapeutic strategy in the treatment of LBP.	(Li et al. 2015d)
Diclofenac	Intradiscal injection	Bovine, *ex vivo* disc degeneration	Up to 8 days	Df decreased the expression of pro-inflammatory factors; Df-loaded nanoparticles promoted an up-regulation of extracellular matrix proteins, namely COL2 and ACAN.	(Teixeira et al. 2015, Teixeira et al. 2016)
Epigallocatechin 3-gallate	Culture medium supplementation	Bovine, *ex vivo* disc degeneration	Up to 21 days	The anti-inflammatory and anti-catabolic compound epigallocatechin 3-gallate down-regulated the expression of inflammatory and catabolic genes in the NP.	(Krupkova et al. 2016)

clinical improvement in reducing initial pain in patients with acute or severe sciatica (Karppinen et al. 2003, Tobinick and Davoodifar 2004, Goupille et al. 2007, Genevay et al. 2010, Genevay et al. 2012), and the number of patients undergoing surgical procedures. Adalimumab subcutaneous injection, although showing after 3 years only a small decrease in leg pain, significantly reduced the need for back surgery (Genevay et al. 2012). In the case of sciatica, it was shown that intravenous or subcutaneous injection of anti-TNF therapy is short lived and, although lower, might have an associated risk of infection (Goupille et al. 2007). Nonetheless, a single etanercept intradiscal injection was recently shown to alleviate discogenic pain up to 2 months (Sainoh et al. 2016).

2.2. Molecular therapy: *in vivo* and *ex vivo* studies

Growth factors have been showing overall to enhance ECM production and to stimulate IVD cells proliferation (Masuda 2008). PRP injections into IVD injury models, in rat (Gullung et al. 2011) and rabbit (Nagae et al. 2007), pointed out a maintenance of tissue features, with fewer inflammatory cells, higher fluid content correlated with a more intense signal on MRI (Gullung et al. 2011), and therefore, a delay in the progression of degeneration (Nagae et al. 2007). BMP-7 (An et al. 2005, Masuda et al. 2006, Imai et al. 2007), BMP-13 (Wei et al. 2009), TGF-β1 (Walsh et al. 2004, Matta et al. 2017) and GDF-5 (Walsh et al. 2004, Chujo et al. 2006) have been shown to promote matrix synthesis *in vivo*. While very important, these studies are frequently limited in understanding the effect of the factors injected in native tissue production, disregarding inflammation and pain outputs. Exogenous growth factors were shown to promote matrix synthesis; however, have the disadvantage of a short biological half-life, ranging from hours to days, and a high cost (Winn et al. 1999, Richardson et al. 2016). Moreover, other works also raise questions about supra-physiologic doses administration for effectiveness and undesired blood vessel ingrowth into the IVD (Zhang et al. 2009b).

In vitro tests have also shown great potential of other factors. IL-1Ra released from poly(lactic-co-glycolic acid) microspheres attenuated IL-1β-mediated NP degradation up to 20 days in bovine NP cultures (Gorth et al. 2012). Fullerol nanoparticles were shown to suppress the catabolic activity and adipogenesis of vertebral bone marrow stromal cells under inflammatory stimulus (Liu et al. 2013b). Cobalt protoporphyrin IX treatment of human NP cells from patients with IVD degeneration induced hemeoxygenase (HO)-1 expression, which seemed to reverse the effect of IL-1β on expression of catabolic markers and matrix MMPs (Hu et al. 2016). Natural compounds such as curcumin (Klawitter et al. 2012a) and triptolide (Klawitter et al. 2012b) also exhibited anti-inflammatory, anti-catabolic and anti-oxidant activity in disc cells.

Other molecules have been successfully tested *ex vivo*: crocin, a bioactive component of saffron (Li et al. 2015d), diclofenac, a NSAID (Teixeira et al. 2015, Teixeira et al. 2016), and

epigallocatechin 3-gallate (Krupkova et al. 2016). All these molecules have shown potential to suppress the degeneration-related inflammation and catabolism in degenerated IVD tissue, suggesting they can be potentially used as therapeutic drugs in the treatment of LBP.

2.3. Gene therapy

The degenerative disc disease is a chronic condition. Therefore, high and long-lasting local levels of different molecules are necessary for a continuous effect of the regenerative therapies (Vadala et al. 2015). Gene therapy has gained significant attention since it promises more prolonged effects in the treatment of IVD degeneration and mediation of inflammation, and provides the possibility to locally modulate the expression of a specific gene and the consequent production of its protein (Vadala et al. 2007, Vadala et al. 2015).

IL-1Ra transfected cells have been suggested as a therapy to inhibit IVD matrix degradation (Muller-Ladner et al. 1997, Le Maitre et al. 2006, Le Maitre et al. 2007c). TGF-β1 transfection of IVD cells through an adenoviral vector was shown to enhance cell activity and proteoglycan synthesis in a rabbit model *in vivo* (Nishida et al. 1999), and in human NP and AF cells *in vitro* (Tan et al. 2003). Also, the transfection of BMP-2, insulin-like growth factor (IGF)-1 (Li et al. 2004) and their combination with TGF-β1 also promoted an increase in proteoglycan synthesis, namely the combined therapy showed a more promising effect in comparison with the individual transfection treatment (Moon et al. 2008). Moreover, rabbit intradiscal injection of adeno-associated virus serotype 2 vector carrying genes for BMP-2 and TIMP-1 demonstrated an IVD degeneration delay by 12 weeks (Leckie et al. 2012).

Though it has been successfully identified several therapeutic genes, the safety of the delivery systems, associated morbidity and cell irreversible alterations may limit the use of gene transfer vectors in clinics (Woods et al. 2011, Molinos et al. 2015a).

Also with great novel therapeutic targeting potential is the mRNA expression of cytokines and chemokines in degenerated IVDs (Ahn et al. 2002). For example, the inhibition of miR-494 protected NP cells from TNF-alpha-induced apoptosis by targeting JunD (Wang et al. 2015b), and the inhibition of miR-34a in NP cells prevented IL-1β-induced ECM degradation by increasing GDF-5 expression (Liu et al. 2016b).

Nonetheless, there is still a long way for the new therapies to go through. Extensive processes of *in vivo* tests and clinical trials are essential to guarantee their safety and long-term effectiveness before a widespread use.

2.4. Cell-based therapies

Cell-based therapies aim to colonize the IVD with cells capable of differentiating and of stimulating endogenous IVD cells' function (Sakai and Andersson 2015). Different cell types

have been transplanted over time. NP cells alone (Nishimura and Mochida 1998, Watanabe et al. 2003, Huang et al. 2011), in combination with AF cells (Gruber et al. 2002, Ganey et al. 2003), elastic cartilage derived chondrocytes (Gorensek et al. 2004), articular chondrocytes (Acosta et al. 2011), or MSCs have been widely reviewed in the literature (Molinos et al. 2015a, Sakai and Andersson 2015, Richardson et al. 2016, Vadalà et al. 2016), reporting that cells remain viable thought the studies time course and that a delayed IVD degeneration is observed. Moreover, a clinical trial using autologous cultured disc-derived chondrocytes transplantation, after discectomy, significantly reduced LBP and allowed retention of hydration in adjacent IVD segments at 2 years, when compared to operated patents without cell intervention (Meisel et al. 2007). Allogenic juvenile chondrocytes (NC01771471) and autologous disc chondrocytes (NCT01640457) are currently being tested in phase II clinical trials.

2.4.1. Endogenous therapies

Progenitor cell populations, as previously discussed, have been pointed out to be present within animal and human IVDs (Risbud et al. 2007, Henriksson et al. 2009, Sakai et al. 2012, Brisby et al. 2013). IVD-derived stem cells were shown to differentiate into chondrogenic and neurogenic lineages, suggesting potential for IVD regeneration (Erwin et al. 2013). They were also shown to play a protective role by modulating IVD inflammatory environment since, for instance, rabbit notochordal cells reduced the expression levels of IL-6, IL-8 and iNOS by human macrophage-exposed AF pellets (Kim et al. 2012). Although these promising results, Sakai et al. (2012) observed that a population of progenitor cells identified within the human IVD decreases with both age and degeneration, indicating that the isolation of sufficient cell numbers in the NP may be an obstacle when thinking of a clinical application.

It has also been proposed endogenous progenitor cell recruitment/homing to the degenerated disc, as an alternative therapeutic approach (Grad et al. 2015). MSCs migration was enhanced by degenerative cues and chemoattractor-delivery systems *ex vivo*, in bovine organ culture models (Illien-Junger et al. 2012, Pereira et al. 2014) and *in vivo*, in a mouse tail-looping disc degeneration model (Sakai et al. 2015). *In vivo*, cell homing by the degenerated environment alone is challenging, since it might be widely determined by the degree of neovascularization of the degenerated tissue and of the potential of circulating or bone marrow-derived MSCs to migrate into the NP (Grad et al. 2015, Sakai et al. 2015). Nonetheless, these results provide important data for the development of novel molecular therapies (Sakai et al. 2015), as discussed in the previous section.

On the other hand, MSCs transplantation potential has been linked to their ability to differentiate into an NP cell phenotype, possibly acquiring NP cell-like function, producing IVD

native ECM components, or promoting stimulation of endogenous IVD cells, thus enabling anticatabolic and anti-inflammatory effects, as reviewed by Sakai and Anderson (2015). Moreover, MSCs are also described to have an immunomodulatory role (Yoo et al. 2009, Prockop and Oh 2012).

2.4.2. Exogenous stem cell delivery: clinical trials

MSCs-based therapies have been tested in a few clinical scenarios of degenerative disc disease and LBP (Yoshikawa et al. 2010, Orozco et al. 2011, Pettine et al. 2015). Yoshikawa and colleagues (2010) reported two case studies, in which patients underwent hernia fenestration surgery and degenerated IVD percutaneous engraftment of a collagen sponge containing autologous MSCs. Two years after surgery, it was observed an increase in MRI signal intensity of IVDs with cell grafts, suggesting higher hydration. Disc instability and pain symptons also seemed to have improved (Yoshikawa, Ueda et al. 2010). Orozco et al. (2011) also showed safety and feasibility of autologous bone marrow-derived MSCs intradiscal inject. Patients exhibited rapid improvement of pain and disability (85% of maximum in 3 months) that approached 71% of optimal efficacy, described to be comparable with the results of procedures such as spinal fusion or total disc replacement. Although disc height was not recovered, water content was significantly elevated at 12 months (Orozco et al. 2011). Moreover, it was recently reported that percutaneous injection of autologous bone marrow concentrate cells significantly reduced lumbar discogenic pain over 12 months (Pettine et al. 2014).
These results encouraged other trials that are currently ongoing, addressing the use of allogenic (NCT02097862) or autologous cell transplantation (NCT02338271, NCT02529566) and implantation of cell-seeded scaffolds in degenerated IVD (NCT01290367, NCT01513694, NCT01643681, NCT02412735).

2.4.3. Exogenous stem cell delivery: *in vivo* and *in vitro* studies

Sakai and Anderson (2015) reviewed several preclinical studies investigating transplantation of stem cells derived from bone marrow, adipose, synovial and umbilical cord tissues, as well as from CEP, AF and NP for IVD regeneration. Overall, it was reported improvement in MRI signaling, disc height maintenance, or up-regulation of IVD ECM components expression (Sakai and Anderson 2015).
The immunomodulatory role of MSCs has been previously addressed in several contexts. *In vitro*, co-culture of human adipose-derived MSCs and osteoarthritic chondrocytes induced down-regulation of inflammatory factors such as IL-6, IL-8, IL-1β, MCP-1, MIP-1α and RANTES expression by MSCs (Manferdini et al. 2013). Pro-inflammatory cytokines, NO, and other damage-associated molecules from injured tissues have also been shown to activate

MSCs to secrete PGE_2, which binds to macrophages and polarizes them to an M2 phenotype that secretes IL-10 (Nemeth et al. 2009).

Ex vivo, synovial explants exposed to MSC-conditioned medium showed down-regulation of IL-1β, MMPs 1 and 13, and up-regulation of suppressor of cytokine signaling (SOCS)1 (van Buul et al. 2012). In cartilage, expression of IL-1Ra was upregulated, while ADAMTS-5 and COL2 were down-regulated. MSC-conditioned medium reduced NO production in cartilage explants and the presence of the NF-κB inhibitor, IκBα, was increased in synoviocytes and chondrocytes treated with MSC-conditioned medium (van Buul et al. 2012). MSCs administered systemically were shown to secrete anti-inflammatory TNF-α stimulated gene/protein (TSG)-6 in myocardial infarction in mice (Lee et al. 2009b), and in rat injured cornea (Roddy et al. 2011).

In the IVD context, FasL protein (found in other immune privileged sites) was shown to be expressed in the NP region after MSCs intradiscal administration into beagle nucleotomized IVDs, indicating that either MSCs differentiated into cells expressing FasL, or stimulated the few remaining NP cells to express it (Hiyama et al. 2008). Moreover, IL-1Ra was shown to mediate the anti-inflammatory and antifibrotic effects of MSCs in a mouse model of lung injury (Ortiz et al. 2007). However, MSCs mechanism of action in the IVD and their impact on inflammation mediators is often disregarded in the multiple studies across the literature (Molinos et al. 2015a).

This chapter covered numerous works on immunomodulatory and therapeutic approaches that have potential to promote a pro-regenerative milieu in the IVD. Although the inherent variability and contradictions arising from different studies, as suggested in Molinos et al. (2015a) review work, integrated strategies contemplating the different features of IVD degeneration may contribute to a better translation of *ex vivo* and *in vivo* results and therapeutics to humans.

CHAPTER III

Aim of the thesis

The main aim of this thesis was to enhance the knowledge regarding the inflammatory response of degenerated IVD, and propose potential immunomodulatory therapies with the final goal of regenerating the degenerated IVD.

For such, the work was divided in four main parts:

1. The establishment of a standardized degenerative/pro-inflammatory *ex vivo* IVD organ culture model. Different stimulation methods were compared to induce a pro-inflammatory/degenerative environment in bovine IVD organ cultures and inflammatory markers, MMPs and ECM components were analyzed by gene expression upon different stimuli.

2. The evaluation of the feasibility of an intradiscal application of anti-inflammatory nanoparticles/nanocomplexes (NCs), previously developed in our group (Gonçalves et al. 2015), to treat inflammation in degenerated IVD. This was performed in the degenerative/pro-inflammatory *ex vivo* IVD organ culture model previously established.

3. The assessment of the therapeutic potential of an intradiscal administration of anti-inflammatory NCs *in vivo*, in a rat model of degenerated/herniated IVD, established in our lab (Cunha et al. 2015, Cunha et al. 2016).

4. The analysis of the immunomodulatory potential of MSCs in degenerated IVD and the influence of the pro-inflammatory/degenerative IVD environment in their immunomodulatory/ regenerative role. This was conducted *ex vivo*, in the model established in the first part.

Overall, the work presented here opens new perspectives to immunomodulatory therapies in degenerated IVD, namely on the use of nanotechnology-based knowhow to improve intradiscal treatments. Furthermore, the work highlights the importance of taking into account the inflammatory environment when evaluating the potential of MSC-based therapies to treat IVD degeneration and associated LBP.

CHAPTER IV

A degenerative/pro-inflammatory intervertebral disc organ culture: an *ex vivo* model for anti-inflammatory drug and cell therapy

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IVD degeneration and associated inflammation often lead to low back pain, one of the major causes of disability worldwide (Johnson et al. 2015). Although broadly available, up-to-date treatments, ranging from more conservative approaches (e.g. physical therapy, drug prescription) to more invasive approaches (ultimately disc replacement) have proven to be, in many clinical scenarios, transient solutions, often leading to restrained patient mobility or adjacent disc degeneration (Lund and Oxland 2011, Natarajan and Andersson 2017). Therefore, there's a need for alternative treatments.

Although, the motivation of our work is, ultimately, the development of new therapies for intervertebral disc regeneration, we considered at the point of work developed in this chapter, that other models and studies focus on the degeneration, while poorly considering the interplay with inflammation (Molinos et al. 2015a), and its contribution to the challenges which are the novel cell therapies for degeneration and LBP. Taking this into account, the present manuscript describes the establishment of a new *ex vivo* degenerative/pro-inflammatory disc organ culture model.

IL-1β-treated discs in an organ culture showed increased levels of PGE_2, pro-inflammatory cytokines and MMPs, while ECM proteins were significantly down-regulated in the model. This is a standardized model that provides a mean for understanding the mechanobiology of the healthy and degenerated IVD and its link with inflammation, as well as it is suitable for testing intradiscal therapeutic approaches.

To validate this model, we have injected a non-steroidal anti-inflammatory drug, commonly used for back pain but administered orally (Df). Df intradiscal injection revealed to be an adequate therapy to reduce disc inflammation, while delaying/decreasing ECM degradation.

We also evaluated the suitability of MSCs injection to modulate the inflammatory response in the degenerated disc, since stem cells rather support matrix degradation and the hostile IVD environment impairs matrix formation (Huang et al. 2014). Although this approach could be tested in the model developed, our findings were not uniform among different MSCs donors, suggesting that MSCs-based therapy to degenerated disc requires further investigation. However, a treatment of inflammation prior to cell therapy might improve the conditions for a cell therapy approach.

Overall, we considered that this degenerative/pro-inflammatory organ culture can be a suitable approach for the continuation of our work, namely in vitro testing of intradiscal/anti-inflammatory therapeutic strategies for disc regeneration. This in more physiological conditions that in vitro cell culture, and able to reduce the number of animals in animal in vivo experimentation. Furthermore, intradiscal controlled release of anti-inflammatory drugs may be a promising disc therapy as this treatment might reduce inflammation, delay and/or decrease matrix protein degradation, further promoting MSCs effect, their integration and adaptation on IVD degenerative environment.

A degenerative/pro-inflammatory intervertebral disc organ culture: an *ex vivo* model for anti-inflammatory drug and cell therapy

Graciosa Q. Teixeira,[a-d] Antje Boldt,[a] Ines Nagl,[a] Catarina Leite Pereira,[b-d] Karin Benz,[e] Hans-Joachim Wilke,[a] Anita Ignatius,[a] Mário A. Barbosa,[b-d] Raquel M. Gonçalves,[b,c] * Cornelia Neidlinger-Wilke[a] *

[a]Institute of Orthopaedic Research and Biomechanics, Center for Musculoskeletal Research, University of Ulm, Ulm, Germany
[b]Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal
[c]Instituto de Engenharia Biomédica (INEB), Universidade do Porto, Porto, Portugal
[d]Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal
[e]Natural and Medical Sciences Institute (NMI) at the University of Tuebingen, Reutlingen, Germany
*equal contribution

Abstract

Resolution of intervertebral disc (IVD) degeneration-associated inflammation is a prerequisite for tissue regeneration and could possibly be achieved by strategies ranging from pharmacological to cell-based therapies. In this study, a pro-inflammatory disc organ culture model was established. Bovine caudal disc punches were needle punctured and additionally stimulated with lipopolysaccharide (10 μg/mL) or interleukin-1β (IL-1β, 10–100 ng/mL) for 48 h. Two intradiscal therapeutic approaches were tested: (i) a nonsteroidal anti-inflammatory drug, diclofenac (Df) and (ii) human mesenchymal stem/stromal cells (MSCs) embedded in an albumin/hyaluronan hydrogel. IL-1β-treated disc organ cultures showed a statistically significant up-regulation of pro-inflammatory markers (*IL-6*, *IL-8*, prostaglandin E_2 [PGE_2]) and metalloproteases (*MMP1*, *MMP3*) expression, while extracellular matrix (ECM) proteins (collagen type II, aggrecan) were significantly down-regulated. The injection of the anti-inflammatory drug, Df, was able to reduce the levels of pro-inflammatory cytokines and MMPs and surprisingly increase ECM protein levels. These results point the intradiscal application of anti-inflammatory drugs as promising therapeutics for disc degeneration. In parallel, the immunomodulatory role of MSCs on this model was also evaluated. Although a slight down-regulation of *IL-6* and *IL-8* expression could be found, the variability among the five donors tested was high, suggesting that the beneficial effect of these cells on disc degeneration needs to be further evaluated. The pro-inflammatory/degenerative IVD organ culture model established can be considered a suitable approach for testing novel therapeutic drugs, thus reducing the number of animals in *in vivo* experimentation. Moreover, this model can be used to address the cellular and molecular mechanisms that regulate inflammation in the IVD and their implications in tissue degeneration.

1. Introduction

Degeneration of intervertebral disc (IVD) is an age-related progressive process considered to be the major cause of spine disorders.[1] The current treatments either conservative (exercise, oral medication, physical therapy), surgical (discectomy, spinal fusion, disc replacement by mechanical prostheses) or analgesic (intradiscal injection of steroids or glucocorticoids) are transient solutions, often leading to restrained patient's mobility or adjacent disc degeneration.[2-6]

The degenerated IVD is characterized by cell death and changes in matrix composition, being the nucleus pulposus (NP) subjected to high mechanical and osmotic pressures, severe hypoxia and limited nutrient supply.[7-9] In this process, increased levels of nitric oxide and prostaglandin E_2 (PGE_2), as well as up-regulation of metalloproteases (MMPs) and a wide number of inflammatory mediators (e.g. tumor necrosis factor-α (TNF-α), interleukin-1β [IL-1β], IL-6) have also been observed.[3, 5, 10-13] Ultimately, structural damage of the outer annulus provides an opportunity for blood vessels and nerves to invade the disc and cause pain.[10, 14]

The high impact on population health and the lack of adequate solutions in the clinics stimulates the development of novel IVD biological therapies,[15] with the goal to promote IVD regeneration and/or control inflammation-associated pain.[8, 16, 17] But the question of how inflammation can be related with IVD degeneration is still controversial and has been described differently from *in vitro* experiments to *in vivo* animal models.[18]

Nevertheless, there is a lack of adequate models to study inflammation within IVD degeneration. IVD cells *in vitro* lose their ability to produce IVD native ECM.[19] *In vivo* models of disc injury by puncture alone or NP digestion do not mimic the natural process of human IVD degeneration.[20, 21] Thus, *ex vivo* organ culture models using disc explants from different species have been established for studying disc degeneration in a more physiologically relevant environment.[21] Explant cultures of bovine discs have the advantage of easy availability and they are assumed to be suitable *ex vivo* models for studying therapies of disc degeneration as they allow well controlled environmental conditions[21, 22] and they show high similarities with human samples regarding the induction of a degenerative environment.[23]

The aim of the present study was to establish a bovine organ culture model in a pro-inflammatory environment.

The control of inflammation by intradiscal injection of a nonsteroidal anti-inflammatory drug (NSAID), Diclofenac (Df), was then tested and used to validate this model. NSAIDs are known as the most effective anti-inflammatory agents in the market[24] but their systemic administration presents drawbacks such as short biological half-life, rapid metabolism and high percentage of protein binding. These characteristics lead to the use of high doses in patients, which causes

side effects in gastrointestinal, hepatorenal and cardiac systems.[25, 26] Intradiscal administration of these drugs could overcome some of the drug systemic side effects.

Furthermore, to analyze if mesenchymal stem/stromal cells (MSCs)-based therapy could have an impact in the reduction of inflammation in degenerated IVD, MSCs embedded in a human serum albumin/hyaluronan (HSA-HA) hydrogel[27] were injected in the same model. MSCs have been suggested to be an adequate cell source, being capable of differentiating towards an NP-like phenotype in co-culture with NP cells[28-31] and promoting the production of healthy ECM in NP *in vivo*.[32, 33] However, although animal model studies have reported that MSCs can promote IVD regeneration,[34, 20] less is known about possible beneficial effect of MSCs in the modulation/resolution of inflammation in IVD. The expression of Fas ligand (*FasL*), a protein found in immune privileged sites, has been restored in the IVD upon implantation of MSCs.[35] Human MSCs were able to down-regulate gene expression of pro-inflammatory cytokines (*IL-3*, *IL-6*, *IL-11*, *IL-15*, *TNF-α*) and MMPs produced by rat NP cells[36] and also reduce IgG production from human NP fragments.[14] In human trials, patients referred reduction of pain,[37, 38] but the influence of MSCs on inflammation was poorly investigated in the context of disc degeneration. Therefore, we investigated in our organ culture approach if MSCs injection into a pro-inflammatory disc environment has the capacity to control the inflammation response.

2. Materials and Methods

2.1. Establishment of a bovine organ culture model

Bovine IVD tissue was isolated from tails of young adult animals (age < 48 months old) from a local slaughterhouse (in Germany by the local abattoir Ulmer Fleisch GmbH, in Portugal with the ethical approval of the Portuguese National Authority for Animal Health). Within 3 hours after slaughter, up to six caudal discs from each specimen were isolated by removal of skin, muscles and ligaments. Discs were dissected from the adjacent vertebral bodies as close as possible to the upper and lower cartilaginous endplate (Fig. 1A, B).[39] Standardized punches (diameter of 13 mm) were prepared from each disc with the NP in the center and few surrounding annulus lamellae (Fig. 1C, D).

In pre-experiments, constrained and unconstrained conditions were compared (Supplementary Materials and Methods). Disc explants were cultured in 6-well cell culture plates (Nunc) (Fig. 1E) with membrane filter inserts and 0.46 MPa static loading (constrained conditions) (Fig. 1F). Parallel cultures without the inserts and the extra weight were used as unconstrained controls. Samples were maintained for 6 days in BM: Dulbecco's Modified Eagle's Medium with low glucose (DMEM, Biochrom), supplemented with 5% v/v fetal bovine serum (FBS, PAA), 1% v/v L-glutamine, 1% v/v non-essential amino acids, 1% v/v penicillin/streptomycin (10.000 U/mL/10.000 µg/mL), 0.5% v/v fungizone (all from Biochrom)

and with the osmolarity adjusted to IVD-physiological 400 mOsm by addition of 1.5% v/v of a 5 M NaCl/0.4 M KCl solution. Samples were incubated at reduced oxygen atmosphere (37 °C, 6% O_2 and 8.5% CO_2) and saturated humidity as described previously.[9] Culture medium was replaced every second day.

To compare constrained and unconstrained conditions, macroscopic parameters of disc punches were determined by measurement of disc height, diameter, and wet-weight after 14 days of culture in BM. Biochemical characterization was performed by quantification of glycosaminoglycans (GAG) release over a culture period of 5 weeks. Also, alcian blue staining of IVD sections was performed at different time points. For all the following experiments constrained conditions were used.

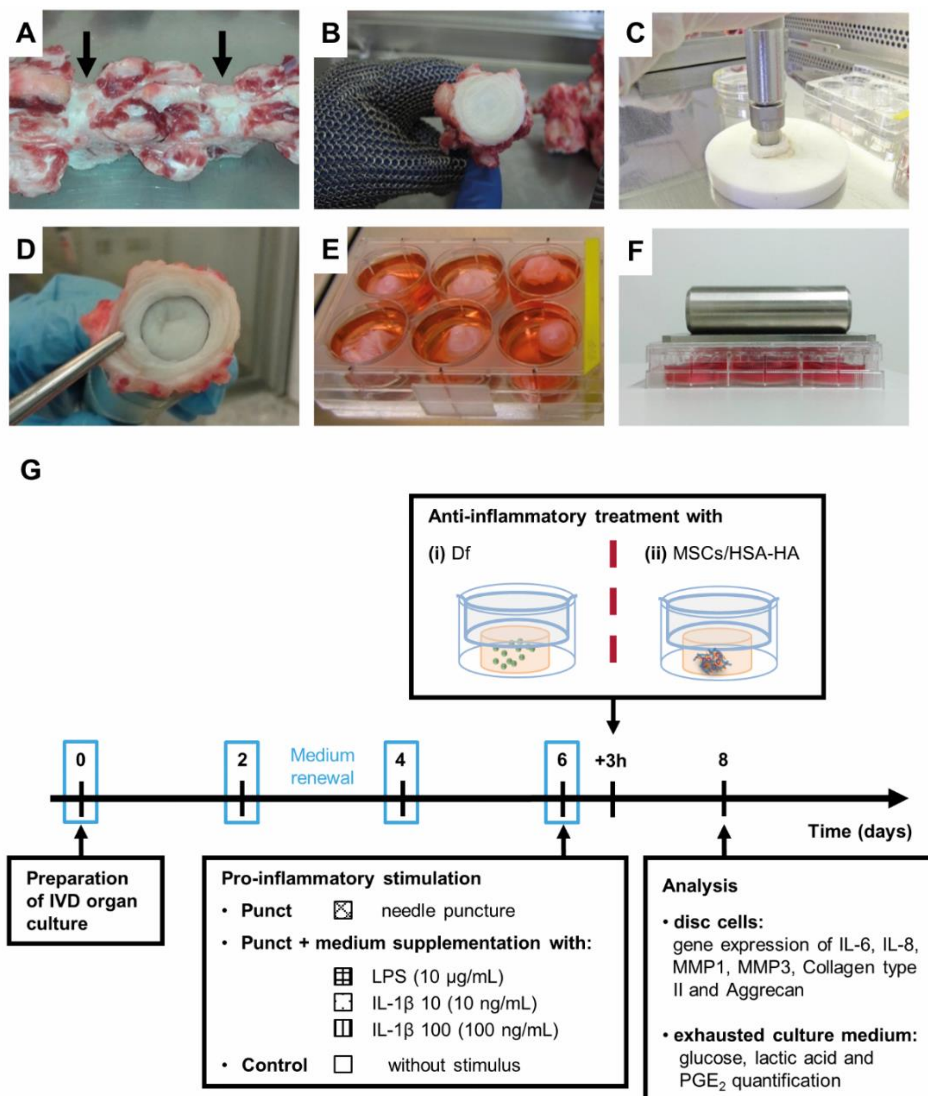

Fig. 1. Organ culture preparation. (**A**) Bovine tail segment with intervertebral disc (IVD; *arrows*). (**B**) IVD isolation. (**C**) IVD punch (diameter of 13 mm). (**D**) Collection of the nucleus pulposus with few surrounding annulus fibrosus. (**E**) Design of the organ culture system with IVD punches cultured in a six-well tissue culture plate. (**F**) 4.9 N weight placed on top of the plate with inserts to prevent punches from swelling over time in culture and generating a 0.46 MPa static loading. (**G**) Experimental timeline.

2.2. Simulation of pro-inflammatory environment

For simulation of the pro-inflammatory/degenerative environment, after 6 days of culture in BM, IVD organ cultures were injured by needle-puncture alone[40] (Punct) or also stimulated with pro-inflammatory factors: lipopolysaccharide (LPS)[41, 42] or IL-1β.[43, 44] Briefly, samples were punctured with a sterile 21G needle; discs were oriented with sterile forceps, while the needle was inserted laterally throughout the remaining AF into the NP and rotated clockwise for 30 seconds. Discs were cultured in BM supplemented with bacterial (*Escherichia coli*) LPS (10 µg/mL, Sigma-Aldrich) or recombinant human IL-1β (10 or 100 ng/mL, R&D Systems) according to Fig. 1G. Nonpunctured untreated discs (only cultured in BM) were used as controls. Tissue samples were collected after 48 h of pro-inflammatory treatment for cell viability and gene expression analysis. Metabolic activity analysis and PGE_2 quantification were performed in culture supernatants.

2.3. Evaluation of diclofenac injection in the pro-inflammatory IVD organ culture

Three hours after pro-inflammatory stimulus, discs were treated with injection of 500 µL of Df in solution (Sigma-Aldrich, 19 µM) using a microsyringe and a 33G needle (Hamilton). The time point for Df treatment was selected based on our previous work with in vitro studies of an anti-inflammatory treatment of activated macrophages.[45] Non-manipulated samples cultured only in BM were used as controls. The effects were evaluated 2 days later by gene expression and by PGE_2 production. The experimental scheme is represented in Fig. 1G.

2.4. Culture of human MSCs

Human MSCs harvested from bone marrow were obtained from 5 different donors who underwent knee-joint surgery with informed consent and according to the rules of the ethical commission of the University of Ulm (Ulm, Germany). One patient was a healthy young donor for bone marrow transplantation. MSCs phenotype was tested immunohistochemically by CD9, CD90, CD105, CD44 and Stro-1 staining. In addition, the cells were confirmed to be able to differentiate into osteogenic, chondrogenic, and adipogenic lineages as described.[46] All cells were expanded in DMEM (Biochrom) supplemented with 10% v/v FBS (HyClone, Thermo Scientific), 1% v/v penicillin/ streptomycin (10000 U/mL/10000 µg/mL, Biochrom) and 0.5% v/v fungizone (Biochrom). Cells were seeded at a concentration of 3000 cells/cm^2 and expanded in T-flasks at 37°C, under a humidified atmosphere of 5% v/v CO_2 in air, with culture medium being changed twice a week and trypsinized when reaching 70% confluence. Experiments were performed with MSCs in passage two.

2.5. MSCs injection in the pro-inflammatory IVD organ culture

A hydrogel of albumin-hyaluronan was selected as a carrier system for MSCs.[27, 47] MSCs/HSA–HA (500 µL) with a cell concentration of $2x10^6$ MSCs/mL of hydrogel was injected in the IVD in culture using a double-chamber syringe (Medmix Systems AG) and a 21G needle. The cell-containing gel mixture was filled in the larger chamber of a two-chamber syringe, the cross-linker in the smaller one. The gel was polymerized in situ.

2.6. Sample preparation for quantitative real-time reverse transcription polymerase chain reaction

Gene expression levels were determined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), performed in triplicate on cDNA derived from disc samples. NP samples were digested enzymatically, cell pellets were recovered, total RNA isolated, quantified and transcribed into cDNA.

Briefly, each tissue sample was dissected into 2 to 3 mm^3 fragments and enzymatically digested for 2 hours in 0.8 mg/mL collagenase type I (Sigma-Aldrich) in DMEM, under agitation (50 rpm), reduced oxygen atmosphere (37 °C, 6% O_2 and 8.5% CO_2) and saturated humidity. Supernatant was passed through a 40 µm filter (BD Falcon) to remove tissue debris. Cells were collected by centrifugation at 400g for 7 minutes. Total RNA was extracted from disc punch cells, using ReliaPrep RNA Cell Miniprep System (Promega), according to manufacturer´s instructions. RNA was quantified using a NanoQuant spectrophotometer (Infinite M200, Tecan). Quality was checked by means of RNA ratio, pooled from two disc samples for each condition. Of each RNA pool, 2 µg was reversed transcribed into cDNA using Omniscript RT Kit (Qiagen) completed with oligo-deoxythymidine primers (5 µM), random hexamer primers (50 µM) and RNase inhibitor (10 units) in a total volume of 20 µL. The obtained cDNA was diluted at a ratio of 1:4 in RNase free water (Qiagen) and used for qRT-PCR.

2.7. Quantitative real-time reverse transcription-polymerase chain reaction

Specific primer pairs were designed using published gene sequences (PubMed, NCBI Entrez Nucleotide Database) and Primer 3 software[48] for bovine *IL-6*, *IL-8*, *MMP1*, *MMP3*, *collagen type II (COL2)*, *Aggrecan (ACAN)* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Table 1) and synthesized by Thermo Fisher Scientific (Ulm, Germany). The analysis was carried out using SYBR® Green method. Reactions were conducted on StepOnePlus Real-TimePCR System (Applied Biosystems), in triplicate, in PCR 96-well TW-MT-Plates (Biozym Scientific), under standard conditions. Reaction mixes contained 12.5 µL of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) master mix, 0.25 µL ROX Reference Dye

(Invitrogen), 1 µL (0.4 µM) forward primer, 1 µL (0.4 µM) reverse primer, 8.25 µL RNase free water and 2 µL cDNA. For the analysis of the mRNA expression, cloned amplification products were provided and used as standards for qRT-PCR. Statistical analysis was performed on ΔC_t values according to a modified method described by MacLean et al.[49] Fold changes in gene expression were presented as $2^{-(\text{average}\Delta\Delta Ct)}$. The average Ct value of each triplicate measurement of each sample was normalized to the house-keeping gene GAPDH in each sample ($\Delta Ct = Ct(_{\text{gene of interest}}) - Ct(_{\text{GAPDH}})$). The ΔCt of each stimulated sample was related to the respective ΔCt of each control sample. Normalized values of samples collected at the end of the experiments were compared with the control and between the different experimental groups.

2.8. Statistical analysis

Since the data follows a non-parametric distribution, for qRT-PCR, Mann-Whitney and Kruskal-Wallis tests were used to compare two or several groups, respectively. Statistical analysis was performed using GraphPad Prism vs. 6.0 (La Jolla) for Windows (vs. 6.01), with values of $p<0.05$ considered significant.

Table 1. Bovine Oligonucleotide Primers

Gene	Sequence (forward and reverse primer)	Product length (bp)	NCBI Reference Sequence
GAPDH	5´-ACC CAG AAG ACT GTG GAT GG-3´ 5´-CAA CAG ACA CGT TGG GAG TG-3´	178	XM_001252511
IL-6	5´-ACC CCA GGC AGA CTA CTT CT-3´ 5´-GCA TCC GTC CTT TTC CTC CA-3´	183	EU276071
IL-8	5´-ATT CCA CAC CTT TCC ACC CC-3´ 5´-ACA ACC TTC TGC ACC CAC TT-3´	148	AF232704
MMP1	5´-ATG CTG TTT TCC AGA AAG GTG G-3´ 5´-TCA GGA AAC ACC TTC CAC AGA C-3´	193	NM_174112.1
MMP3	5´-AAT CAG TTC TGG GCC ATC AG-3´ 5´-CTC TGA TTC AAC CCC TGG AA-3´	237	AF069642
Collagen type II	5´-CCT GTA GGA CCT TTG GGT CA-3´ 5´-ATA GCG CCG TTG TGT AGG AC-3´	145	X02420
Aggrecan	5´-ACA GCG CCT ACC AAG ACA AG-3´ 5´-ACG ATG CCT TTT ACC ACG AC-3´	155	NM_173981

Bovine oligonucleotide primers used for qRT-PCR.

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; MMP, metalloprotease; qRT-PCR, quantitative real-time reverse transcription–polymerase chain reaction.

3. Results

3.1. Cell viability and metabolic activity in a bovine IVD organ culture model in pro-inflammatory conditions

Disc punches were prepared as described and cultured at 5 mM glucose, 400 mOsm, 8.5% CO_2 and 6% O_2 under constrained conditions. Discs were constrained with membrane-inserts posed on top of each disc punch covered by a weight on the top of the culture plate (simulating 0.5 MPa static loading, which corresponds to physiological loads during standing phase[50] and allowed the prevention of disc swelling and tissue deformation (Supplementary Results) that occurred without endplates (supplementary Fig. S1). In these conditions tissue GAG slightly decrease (supplementary Fig. S2).

To induce a degenerative and pro-inflammatory environment, cultures were stimulated at day 6 after isolation with Punct or puncture with supplements: LPS, 10 or 100 ng/mL IL-1β (IL-1β 10 or IL-1β 100, respectively). A microscopic evaluation of cell viability by LIVE/DEAD assay showed that cells remained viable at day 8 of culture, with no apparent differences between the tested conditions, indicating that neither the low oxygen tension nor the puncture and pro-inflammatory stimulus lead to a significant loss of cell viability (Fig. 2A). In addition, glucose and lactic acid levels were quantified in the collected supernatants during culture (Fig. 2B, C). Results showed similar glucose consumption and lactic acid production for all stimulated groups and the control, corroborating cell viability results.

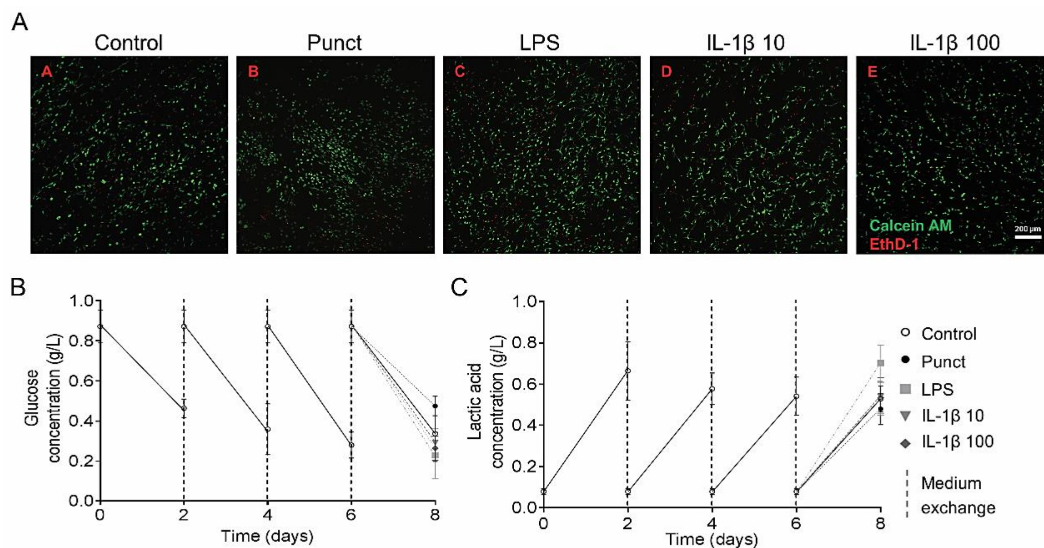

Fig. 2. Cell viability and metabolic activity over the culture period of IVD punches cultured under constrained conditions. (**A**) Representative CLSM images (z-stacks) from LIVE/DEAD cytotoxicity/viability assay acquired at day 8, after 2 days of stimulation, for the conditions tested: control, puncture alone (Punct), puncture plus culture medium supplementation with 10 μg/mL lipopolysaccharide (LPS), 10 and 100 ng/mL interleukin-1β (IL-1β 10 and IL-1β 100, respectively). Calcein AM stains live cells in green; ethidium homodimer-1 (EthD-1) stains dead cells in red (scale bar, 200 μm). (**B**) Glucose and (**C**) lactic acid concentration (g/L) in supernatants collected during time in culture. Results are shown as mean ± standard deviation (n=14-23).

3.2. Analysis of pro-inflammatory markers, MMPs and ECM proteins of the pro-inflammatory IVD organ culture model

PGE_2 production was quantified in culture supernatants over time. No statistically significant differences were found between time points during culture in basal medium (BM). After 2 days of stimulation with different conditions (day 8), PGE_2 production significantly increased for all the groups tested, in comparison to the control (Fig. 3A). LPS group showed the highest PGE_2 fold increase (11.5±1.2-fold, $p<0.0001$), while Punct presented only 1.8±1.2-fold ($p<0.01$). IL-1β 10 and 100 stimulated groups achieved respectively 3.7±1.2-fold and 5.8±1.2-fold (both with $p<0.0001$). Thus, IL-1β 100 increased 1.6±0.7-fold ($p<0.05$) compared with IL-1β 10.

Gene expression of pro-inflammatory markers and MMPs was also analyzed at day 8 of culture. IL-1β 10 and 100 groups showed statistically significant up-regulation of IL-6, *IL-8*, *MMP1* and *MMP3* expression compared with unstimulated discs ($p<0.05$, Fig. 3B, C). For *IL-8*, IL-1β 100 was also significantly up-regulated in comparison to IL-1β 10 ($p<0.05$).

Concerning gene expression of ECM proteins, *COL2* and *ACAN* was down-regulated in the presence of IL-1β (Fig. 3D). While a statistically significant down-regulation was observed in

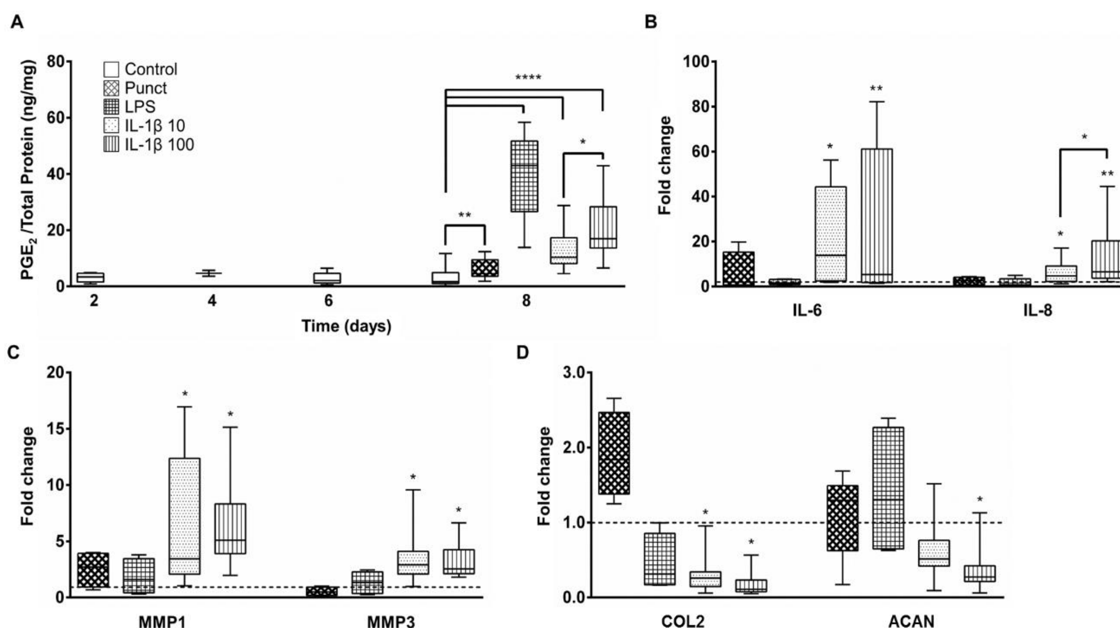

Fig. 3. Quantitative analysis of pro-inflammatory markers, matrix degrading enzymes, and extracellular matrix (ECM) components in IVD organ cultures. The IVD organ cultures were stimulated with Punct, puncture plus culture medium supplementation with 10 µg/mL LPS (LPS), 10 and 100 ng/mL IL-1β (IL-1β 10 and IL-1β 100, respectively), and compared with unstimulated control. (**A**) Prostaglandin E_2 (PGE_2) concentration normalized to total protein (ng/mg) in culture supernatants. The kinetics of PGE_2 production was traced by ELISA over an 8-day culture. (**B**) mRNA expression of *IL-6*, *IL-8*, (**C**) *MMP1*, *MMP3*, and (D) collagen type II (*COL2*) and aggrecan (*ACAN*). Levels of mRNA were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The ratio of stimulation to control indicates the fold change of induction after stimulation (control level=1; dashed line). Results are presented as box and whiskers plots (n=12-36 for PGE_2 and n=4-17 for mRNA expression). *$p<0.05$; **$p<0.01$; ****$p<0.0001$ (Kruskal–Wallis test).

COL2 expression for IL-1β 10 and 100 groups, *ACAN* expression was only down-regulated for IL-1β 100 ($p<0.05$).

Since IL-1β stimulation, in addition to puncture, appears to induce a pro-inflammatory/ degenerated IVD environment, with an up-regulation of pro-inflammatory markers, as well as a down-regulation of ECM proteins, and moreover, since it is a more physiological method to induce this inflammatory milieu than LPS, the pro-inflammatory model with IL-1β was selected for the following experiments.

3.3. Evaluation of an anti-inflammatory (diclofenac) injection in the pro-inflammatory IVD organ culture

To validate the pro-inflammatory IVD organ culture model, an injection of a commonly used anti-inflammatory drug (Df) was tested (Fig. 4). For that, soluble Df (19 μM) was injected in the NP, 3 hours after pro-inflammatory stimulus. The concentration and time point of addition was selected based on previous work from our group with Df treatment in human macrophages.[45]

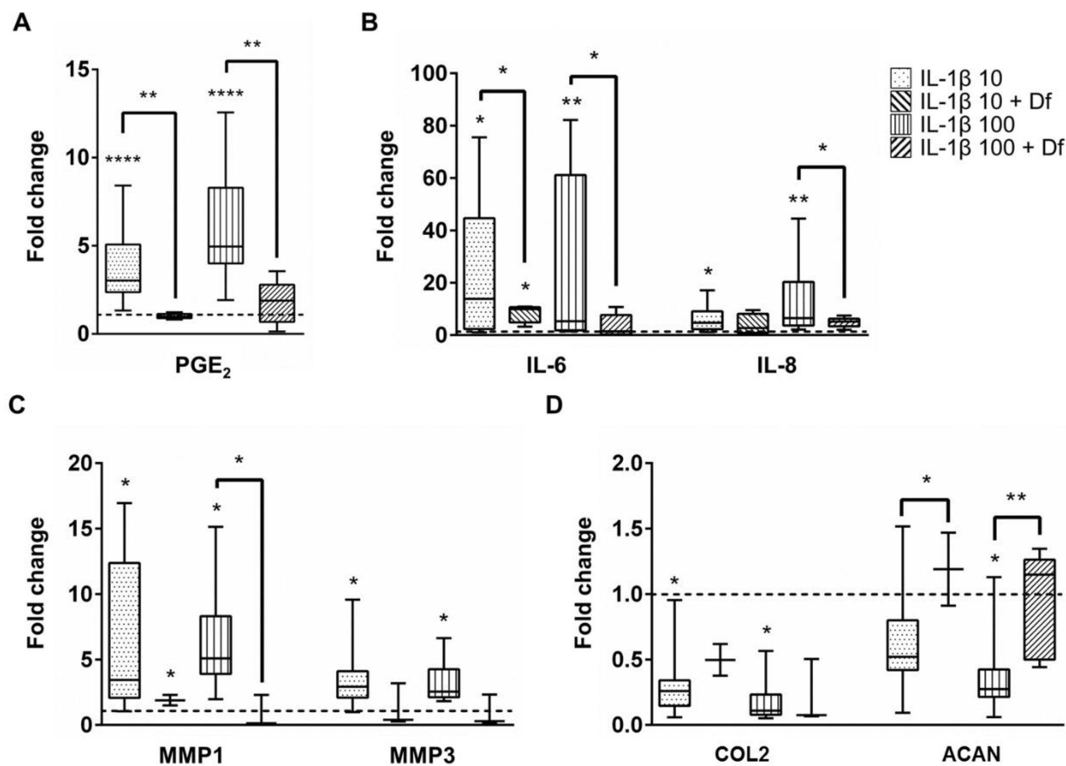

Fig. 4. Effect of Diclofenac injection in IVD organ cultures. Quantitative analysis of pro-inflammatory markers, matrix degrading enzymes and ECM components in IVD organ cultures after pro-inflammatory stimulus with puncture plus culture medium supplementation with 10 and 100 ng/mL IL-1β (IL-1β 10 and IL-1β 100, respectively), and treatment with 19 μM Df for 2 days (IL-1β 10 + Df and IL-1β 100 + Df). (**A**) PGE_2 fold change in culture supernatants. (**B**) mRNA expression of *IL-6*, *IL-8*, (**C**) *MMP1*, *MMP3*, (**D**) *COL2* and *ACAN*. Levels of mRNA were normalized to *GAPDH*. The ratio of stimulation to control indicates the fold change of induction after stimulation (control level=1; dashed line). Results are presented as box and whiskers plots (n=6-36 for PGE_2 and n=4-17 for mRNA expression). *$p<0.05$; **$p<0.01$; ****$p<0.0001$ (Mann–Whitney test).

Two days after injection, PGE_2 production significantly decreased in the groups supplemented by Df (IL-1β 10 + Df and IL-1β 100 + Df, $p<0.05$) in comparison to the respective IL-1β-stimulated group alone (IL-1β 10 and IL-1β 100, Fig. 4A). Expression of *IL-6* was significantly down-regulated and *MMP3* was slightly down-regulated with Df injection for both IL-1β concentrations ($p<0.05$, Fig. 4B, C). For the higher IL-1β concentration used, *IL-8* and *MMP1* were significantly down-regulated after Df injection ($p<0.05$, Fig. 4B, C). Concerning the ECM proteins, *COL2* did not show significant differences after Df injection. On the other hand, an up-regulation of *ACAN* was observed after Df injection for both IL-1β concentrations ($p<0.05$, Fig. 4D).

3.4. Evaluation of MSCs injection in the pro-inflammatory IVD organ culture

MSCs anti-inflammatory effect was evaluated in the IVD pro-inflammatory model stimulated with IL-1β 10. For this, MSCs were injected in the IVD using an HSA-HA hydrogel as vehicle. Injection of either MSCs embedded in HSA-HA (MSCs/HSA-HA) or HSA-HA alone did not alter PGE_2 production (Fig. 5A). Regarding the pro-inflammatory cytokines, there was an app arent

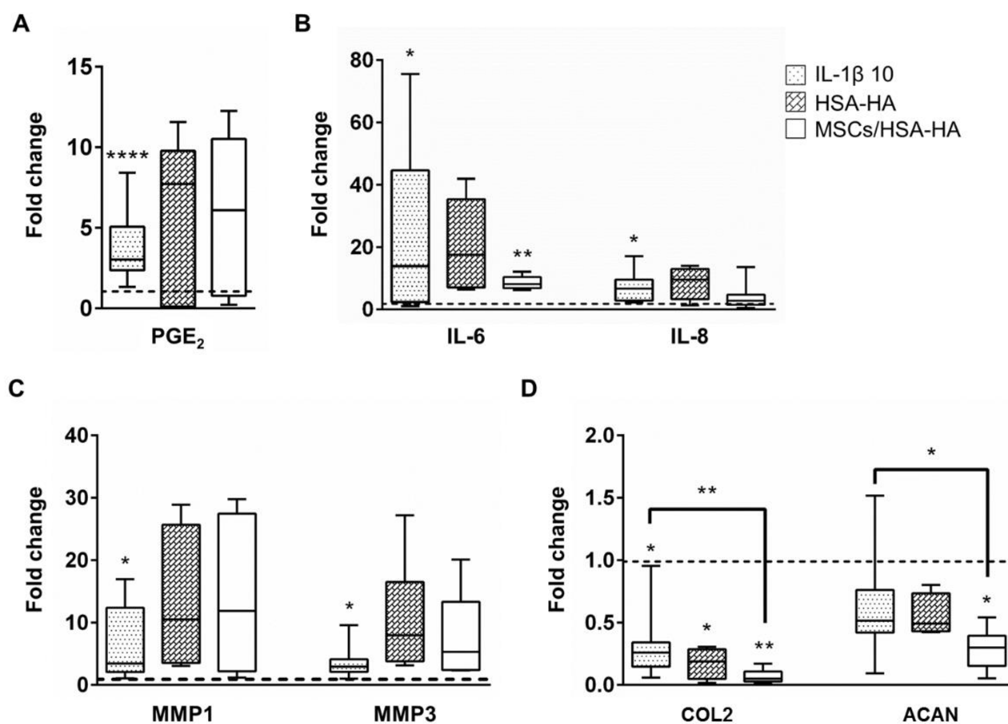

Fig. 5. Effect of MSCs injection in HSA-HA hydrogel in IVD organ cultures. Quantitative analysis of pro-inflammatory markers, matrix degrading enzymes and ECM components in IVD organ cultures after pro-inflammatory stimulus with puncture plus culture medium supplementation with 10 ng/mL IL-1β (IL-1β 10), and treatment with HSA-HA or MSCs/HSA-HA for 2 days. (**A**) PGE_2 fold change in culture supernatants. (**B**) mRNA expression of *IL-6*, *IL-8*, (**C**) *MMP1*, *MMP3*, (**D**) *COL2* and *ACAN*. Levels of mRNA were normalized to *GAPDH*. The ratio of stimulation to control indicates the fold change of induction after stimulation (control level=1; dashed line). Results are presented as box and whiskers plots (n=5-36 for PGE_2 and n=5-17 for mRNA expression). *p<0.05; **p<0.01; ****p<0.0001 (Kruskal–Wallis test).

slight down-regulation of *IL-6* and *IL-8* expression in the MSCs/HSA-HA injection group, when compared to IL-1β 10 stimulus and to the injection of HSA-HA alone (Fig. 5B). *MMP1* and *MMP3* did not appear to have been differently expressed between injections of MSCs/HSA-HA or HSA-HA alone, however they seem to be up-regulated in those two groups in comparison to IL-1β 10 (Fig. 5C). Gene expression of *COL2* and *ACAN* was down-regulated at all conditions with pro-inflammatory stimulation compared to the unstimulated controls, and HSA-HA scaffold alone or combined with the MSCs further decreased matrix protein expression by IVD cells ($p<0.05$, Fig. 5D).

4. Discussion

In the present study, we established a standardized bovine organ culture system that simulates pro-inflammatory conditions. The approach used tissue punches that allowed the standardization of explants size. The physical culture conditions in culture plates constrained by inserts and compressed by a constant static loading prevented tissue swelling and tissue deformation that usually occurs without endplates, as reported in the literature.[23, 51-53] Moreover, the tissue is more easily collected using a less complex protocol (e.g. it does not require sawing the bone or jet-lavage).[52, 54, 55] In our approach, we have used constrained conditions in all experimental groups for standardisation of the samples (prevention of swelling) but we did not simulate complex loading protocols, though we are aware of the importance of complex loading on the initiation and progression of disc degeneration.[23, 56] Nevertheless, GAG loss in these conditions was already reported to naturally occur in culture.[52] Although organ cultures are accepted as more reliable models than 2D in vitro cultures, since they constitute a step further in complexity before *in vivo* studies, there are also some limitations that all should be aware. Namely, an organ culture model cannot simulate complex interactions with adjacent tissues and can only be kept limited time in culture[14, 40, 57] to guarantee tissue viability. Another limitation is the lack of vascularization and immune cells. In our approach, tissue viability was assured by controlling metabolites production/consumption levels during culture time and the culture period was rather short. Concerning vascularization, since mature native IVD lacks vasculature, this limitation is of minor relevance in disc organ culture models.

To promote a pro-inflammatory environment, we tested different approaches. Some commonly methods in the literature use mechanical injury by needle-puncture,[58, 59] to stimulate disc degeneration, or medium supplementation with LPS.[60] However, although LPS is a known stimulus of inflammation,[60, 61] it is not really a physiological approach. By the contrary, IL-1β is an interesting candidate, since it is expressed at high levels in the pathogenesis of disc disease[13, 62, 63] and it has been demonstrated that exposure of NP cells to IL-1β leads to altered mechanical function, primarily due to loss of GAG.[64]

In general, none of the conditions that induce a pro-inflammatory environment impaired cell viability or altered glucose consumption/lactic acid production, thus they did not substantially alter cell metabolism in our approach. This is in accordance with other studies that observed a cell survival rate (after 14 days of rabbit IVD organ culture) higher than 90% in pro-inflammatory conditions (LPS and IL-1β).[60] However, Korecki and colleagues showed that at the needle insertion site cell damage may occur,[40] while results from Ponnappan and co-workers revealed an increase in apoptosis after IL-1β treatment.[44]
LPS stimulation is known to induce *COX-2* expression,[65] and consequently generates different prostanoids, including PGE_2.[66] PGE_2 is excessively produced in response to pro-inflammatory cytokines signaling, particularly IL-1 and TNF-α.[12, 67] LPS was also shown to stimulate pro-inflammatory cytokines production (IL-1β, IL-6 and IL-8) by chondrocytes[68] and by murine disc cells.[69] In the present study, LPS stimulus significantly increased PGE_2 production but no obvious effect was observed in other pro-inflammatory cytokines analyzed or in matrix remodeling.
In contrast, only (100 ng/mL) IL-1β-treated discs showed a significant up-regulation of pro-inflammatory cytokines and MMP expression accompanied by a significant down-regulation of ECM proteins, in accordance to disc degeneration description.[43, 44, 63] The other study by Burke *et al.* reported that disc cells from patients with scoliosis or degenerated IVDs respond to an exogenous pro-inflammatory stimulus with an increased secretion of pro-inflammatory markers IL-6, IL-8, and PGE_2.[41, 67] Therefore, a pro-inflammatory IVD organ culture under loaded conditions, punctured and stimulated with IL-1β (100 ng/mL) was selected as the most reliable model for further studies.
An anti-inflammatory drug (Df) was injected in discs stimulated by IL-1β and its effect on pro-inflammatory/degenerative IVD was evaluated. As expected, Df injection was able to decrease PGE_2 production, since Df is a *COX-2* inhibitor. But, besides this known effect, Df also down-regulated *IL-6*, *IL-8* and *MMP1* expression, while stimulating *ACAN* synthesis, suggesting that this treatment might not only reduce inflammation, but could also delay matrix proteins degradation and/or increase ECM proteins level. Intradiscal injection of steroids or glucocorticoids to control inflammation in IVD has been used in clinics. However, their influence on deregulation of matrix turnover leading to further disc degeneration is also known.[70] Df direct injection into the IVD may have a limited clinical use as it has a short biologic half-life and may require repeated administrations. Therefore, a sustained delivery system that prolongs Df release in the disc may be a promising alternative. In fact, Df intraperitoneal injection in a rat lumbar disc herniation model showed a reduced analgesic effect with time.[71] Nevertheless, in a pig model of NP-induced nerve root injury, Df reduced NP-induced nerve root dysfunction, showing good prognosis after Df treatment.[72] Nonetheless, most of these

studies are focused on pain by analysis of change in disability and pain scores, and not biological effects on matrix turnover and inflammation.[73-75]

In parallel, the effect of MSCs transplantation on the pro-inflammatory/degenerated IVD organ culture was here investigated. MSCs are capable to differentiate into NP-like cells,[38] increasing expression of non-specific markers as *ACAN* or *COL2*,[30] and were reported to induce less pain in IVD degeneration human clinical trials.[37] The cell density was used based on the work by Serigano *et al.*, which showed that 10^6 MSCs per transplanted disc was the ideal number of cells, since less viable cells were detected when 10^5 MSCs were transplanted and more apoptotic cells were found in 10^7 MSCs transplanted discs.[76]

The vehicle used to inject MSCs was based on a HSA-HA hydrogel. This hydrogel was shown to be adequate for MSCs differentiation in chondrogenic lineage, in vitro,[27] and to enhance disc endogenous repair after 6 months, in an *in vivo* nucleotomized sheep model.[47] *In vivo* studies using this hydrogel showed good integration with the host without reporting associated inflammatory response.[77,78] Only low levels of the pro-inflammatory cytokine IL-1β were reported in the nucleotomized sheep model, 6 months after implantation.[47] Nevertheless, the results from our model demonstrate that this carrier by itself activates the production of pro-inflammatory mediators PGE_2, *IL-6* and *IL-8* by disc cells, when compared to control discs. This suggests a "foreign-body"-type reaction, similar to what happens with biomaterial recognition by immune cells. Interestingly, upon implantation, MSCs were able to reduce this activation status, demonstrating their immunomodulatory effects in this model also.

The literature reports that MSCs are able to increase the expression of the immunosuppressive ligand *FasL* in IVD,[35] as well as down-regulate gene expression of pro-inflammatory cytokines (*IL-3*, *IL-6*, *IL-11*, *IL-15*, *TNF-α*) and MMPs produced by rat NP cells.[36] Bertolo *et al.* also showed that MSCs are able to reduce IgG production by human NP fragments and slightly reduce *TNF-α* expression, although no influence was observed on *IL-1β*.[14] Nevertheless, in the present study, MSCs effect on pro-inflammatory markers appeared to be highly donor dependent. PGE_2 revealed either an up-regulation or a decrease, depending on the donor, and only a slight down-regulation of *IL-6* and *IL-8* expression was observed after MSCs/HSA-HA injection. The preliminary short-term findings at gene expression level rather suggest impaired matrix formation and increased matrix degradation. Although two days after injection appears to be an adequate time point to address the inflammatory markers, we cannot exclude that it may be a too early time point to analyze matrix formation. Other aspect that we cannot exclude is the use of human MSCs on a bovine disc organ culture, although human MSCs are known by their immunomodulatory capacity[79, 80] and have frequently been used in animal studies from other species, without immune suppression.[81,82] In those cases, no exacerbated immune response was observed. New experiments with MSCs are currently in progress to clarify the open question about the immunomodulatory role of MSCs in the degenerated IVD.

In conclusion, we have developed and validated a reproducible pro-inflammatory/ degenerative organ culture model. This model is suitable to investigate inflammation-associated mechanisms and other possible pathways that cause disc degeneration. Moreover, this *ex vivo* model could be used to assess cytotoxic effects of novel pharmaceutical strategies for IVD, prior to animal experimentation. Particularly, Df intradiscal injection seems to be a promising approach to control inflammation while delay and/or decrease matrix protein degradation. Regarding the suitability of MSCs injection to modulate the inflammatory response in the degenerated disc, although this approach could be tested in the model developed, our findings were not uniform among different MSCs donors, suggesting that MSCs-based therapy with regard to control the inflammatory response in the IVD requires further investigation.

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Disclosure Statement

The authors declare no competing financial interests.

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Supplementary Data

Materials and Methods

Alcian blue staining

Organ culture samples were analyzed histologically in paraffin sections for proteoglycans. IVD punches were washed with PBS, fixed with 4% formalin solution for 2 days, dehydrated in a gradient series of EtOH and then embedded in paraffin blocks. Sections of 7 µm thickness were sequentially recovered, deparaffinised, diafanized and rehydrated. Sections were acidified with 3% acetic acid for 5 minutes, stained with 1% Alcian Blue 8GX (Sigma-Aldrich) in 3% acetic acid for 30 minutes and rinsed in distilled water. Counterstaining was carried out with nuclear fast red (Merck) for 3 minutes. Sections were rinsed in distilled water, dehydrated in absolute ethanol, diafanized in xylene and mounted with Vitro-Clud (Merck). Sections were imaged with brightfield microscopy (DMI6000 B, Leica).

Evaluation of glycosaminoglycan content in disc tissue

The amount of glycosaminoglycans (GAG) in the IVD was determined by Dimethylmethylene blue (DMMB) assay, based on the binding of DMMB to GAG.[1] Briefly, disc tissue samples were dissected into 2 to 3 mm^3 fragments and enzymatically digested overnight with proteinase K (0.5 mg/mL, Sigma-Aldrich) at 58°C. Afterwards, samples were vortexed to a clear solution, indicating that the digestion was complete after the incubation. A dilution series of 20 µg/mL to 0.625 µg/mL chondroitin 4-sulfate standards was prepared. Subsequently, 50 µL of standards and samples were transferred to a 96-well plate, 200 µL of DMMB reagent solution was added to each well and absorbance was measured at 656 nm. Each sample was measured in triplicate.

Analysis of cell viability

Cell viability was qualitatively assessed at day 8 through fluorescence-based LIVE/DEAD Cell Viability/Cytotoxicity kit (Invitrogen), by confocal laser scanning microscopy (CLSM), according to Teixeira et al.[2] Briefly, organ culture samples were rinsed first in PBS and then in phenol-red/serum-free DMEM (Gibco), three times each, to remove traces of esterases and to avoid phenol red interference with fluorescence readings. Samples were then simultaneously stained with a solution of 1 µM calcein acetoxymethyl ester (Calcein AM, Invitrogen) and 2.5 µM ethidium homodimer-1 (EthD-1, Invitrogen) for 45 minutes at 37ºC, protected from light. After discarding supernatant and adding new phenol red/serum free DMEM samples were imaged by CLSM (Leica SP2 AOBS SE, Leica Microsystems), using LCS Software (Leica Microsystems). Calcein AM (Ex 485 nm/Em 530 nm) stains live cells green, indicating

intracellular esterase activity, while EthD-1 (Ex 530 nm /Em 645 nm) stains dead cells red, indicating loss of plasma membrane integrity. Images were analyzed using ImageJ 1.43u software (Wayne Rasband).

Cell metabolic activity analysis

In order to follow cell metabolic profile in the different culture conditions, samples of 0.5 mL of exhausted culture medium were collected at time points 0, 2, 4, 6 and 8 days of culture. After collection, supernatants were incubated at 80ºC for 15 minutes, to inactivate most enzymes, and centrifuged at 10000 rpm for 5 minutes. The supernatant samples were kept at -20ºC until posterior analysis. D-glucose consumption and L-lactic acid production were quantified by UV-method kits (Boehringer Mannheim/R-Biopharm, Roche), adapted for 96-well microplates according to manufacturer's instructions. Each sample was measured in triplicate.

Evaluation of Prostaglandin E_2 concentration in conditioned media

Conditioned medium was collected at days 0, 2, 6 and 8, centrifuged (3000 rpm, 5 minutes) and the supernatant kept at -20ºC for posterior analysis. PGE_2 was quantified by ELISA (Arbor Assays) according to manufacturer's instructions and normalized by total protein.

Total protein quantification

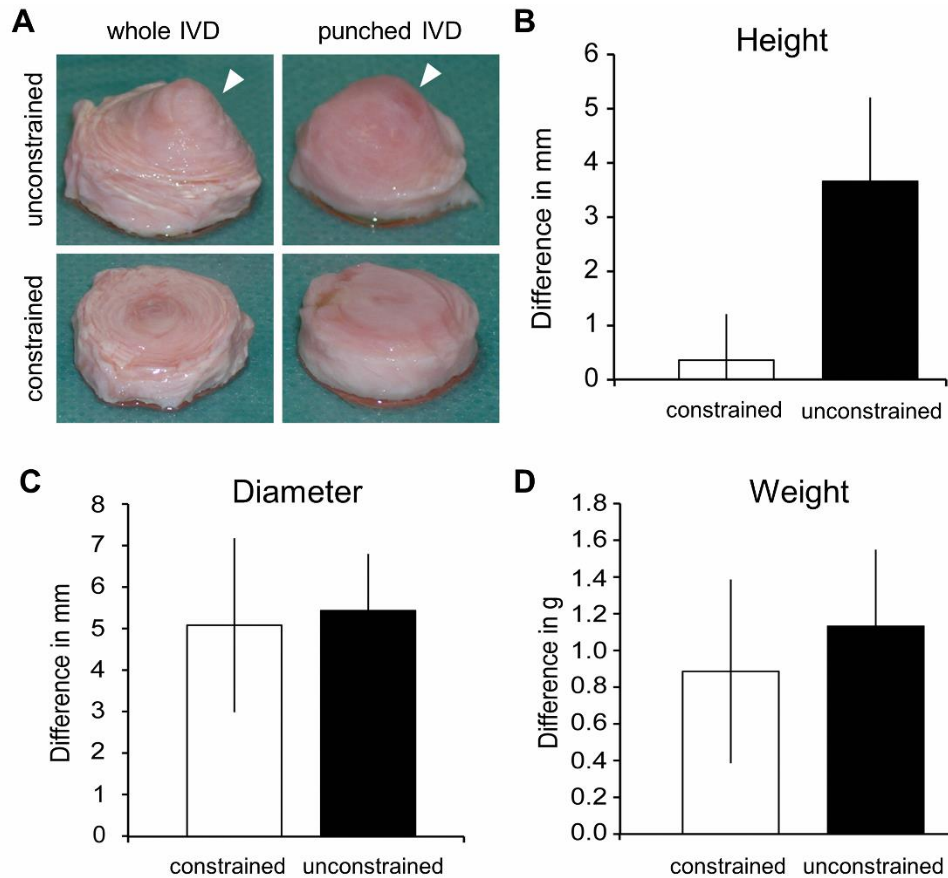
The bicinchoninic acid (BCA) colorimetric protein assay was performed in cell lysates according to the manufacturer's instructions (Bio-Rad), adapted to a microplate format. Briefly, 5 µL of lysed samples were transferred to a 96-well microplate (Greiner). 25 µL of working reagent (A) were added to the samples followed by 200 µL of solution B. Samples were incubated for 15 min at room temperature, protected from light. Absorbance was measured at 750 nm, in a microplate reader (PowerWave XS, Biotek). Total protein values were converted into mg/mL using a standard curve of bovine serum albumin (BSA) in the range of 0.03125 to 1 mg/mL. Each sample was measured in triplicate.

Results

Evaluation of constrained conditions in the establishment of a bovine IVD organ culture model

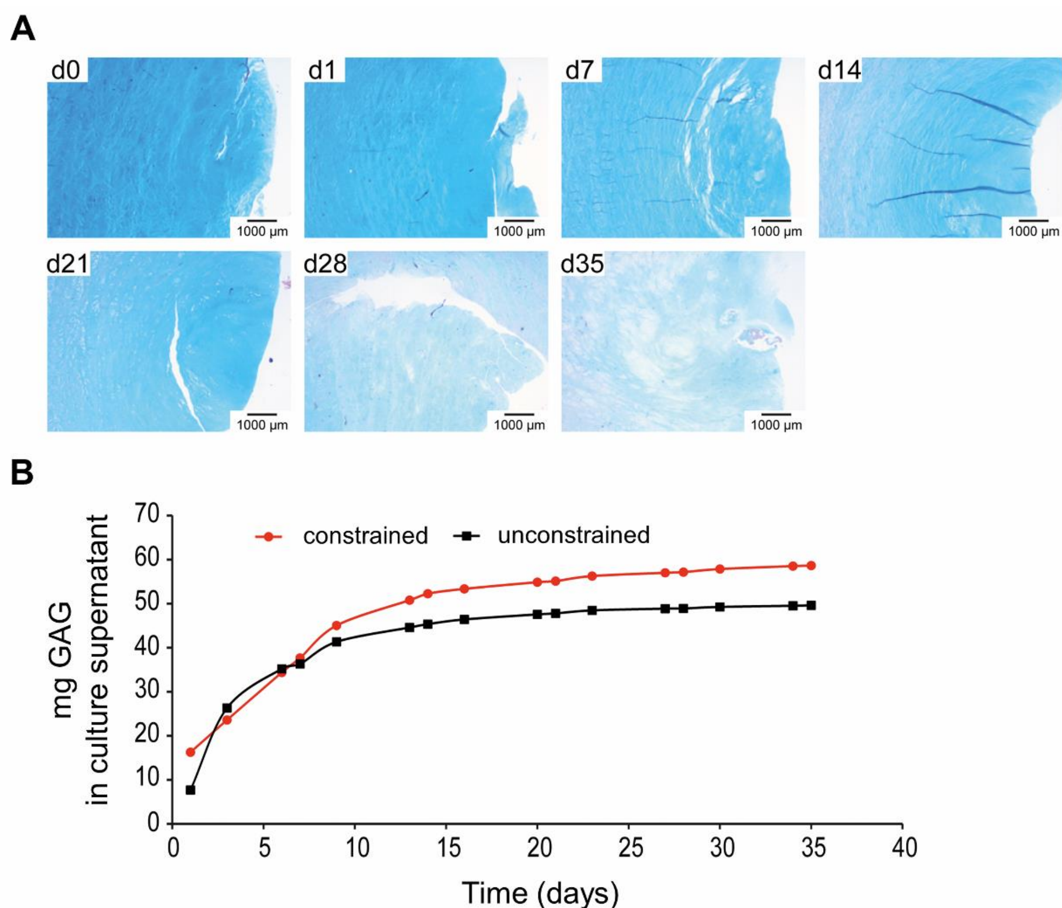
As caudal bovine discs differ in diameter, preparation of disc punches allowed a standardization of the organ culture explants with regard to disc diameter and disc size compared to complete discs (Fig. S1A). In the constrained group, IVD punches height was strongly reduced by covering the samples with inserts and by adding a weight on top to fixate the dish plate cover as described (Fig. 1B). Due to the differences in swelling behaviour between NP and AF, the NP was extruded in direction of lowest resistance under

unconstrained conditions and hence the height of the IVD increased (Fig. S1A, B). Determination of disc diameter (Fig. S1C) and disc weight (Fig. S1D) between constrained and unconstrained groups did not seem to reveal differences.



Supplementary Fig. S1. Macroscopic parameters of IVD. (**A**) Whole IVD were compared macroscopically to punched IVD under constrained (*white columns*) and unconstrained (*black columns*) culture conditions (*arrowhead* indicates swollen nucleus pulposus). IVD punches were analyzed concerning physical parameters: (**B**) height, (**C**) diameter and (**D**) weight. Parameters of the IVD were compared before and after cultivation and differences are shown as mean ± standard deviation (n=7 donors).

With ongoing culture time, the disc punches underwent a loosening of proteoglycans that were released into the culture medium. Histological analysis of organ culture samples revealed a decreased staining intensity for disc matrix proteoglycans as shown by alcian blue staining (Fig. S2A). This finding was confirmed by an increased concentration of GAG in the conditioned medium collected during medium exchange, as shown in Fig. S2B. Constrained and unconstrained groups presented only small differences between each other, namely when reaching a plateau stage. However, constrained samples seemed to have suffered higher GAG loss.



Supplementary Fig. S2. Release of glycosaminoglycans (GAG) over a culture period of 5 weeks. (**A**) Representative images of alcian blue staining of IVD punches of one donor at different time points (days 0, 1, 7, 14, 21, 28 and 35), under constrained conditions (strongly acidic mucosubstances are stained *blue*, cell nuclei are stained *pink* to *red*, and cytoplasm is stained *pale pink*; scale bars, 1000 µm). (**B**) Release of GAG to the supernatant. IVD punches were cultured under constrained (*red line*) and unconstrained (*black line*) conditions, and results were plotted cumulatively along the culture period.

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CHAPTER V

Anti-inflammatory Chitosan/Poly-γ-glutamic acid nanoparticles control inflammation while remodeling extracellular matrix in degenerated intervertebral disc

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IVD degeneration associated diseases have been focus of different treatment approaches. The regenerative potential of hydrogels for nucleus pulposus replacement, cell-based therapies, growth factors injection or gene therapy has been investigated (Sakai and Andersson 2015). However, as we have defended in the previous chapters, the modulation and control of inflammation are crucial for tissue regeneration.

Facing this, we consider immunomodulatory biomaterials of great interest for IVD applications. γ-PGA is one of the most appealing natural polymers, mainly due to its biodegradability into glutamate residues, as due to its potential in promoting chondrogenic differentiation of human MSCs (Antunes et al. 2015). Since γ-PGA is anionic (pka 2.19), it can be combined by electrostatic interaction with cationic polymers as Ch (Antunes et al. 2011), forming polyelectrolyte complexes with great potential as delivery systems. Our group has previously reported the production of a low molecular weight and highly pure γ-PGA (Pereira et al. 2012). The novelty of this work is the intradiscal injection of an anti-inflammatory therapy based on Ch/γ-PGA NCs with an anti-inflammatory drug (Df), previously developed by our team (Gonçalves et al. 2015).

Ch/Df/γ-PGA NCs were produced by co-acervation method (Pereira et al. 2012). This drug delivery system was tested in the pro-inflammatory/degenerative intervertebral disc *ex vivo* model presented in the previous chapter (Teixeira et al. 2015). Given the NCs liquid consistency, it is possible to inject them directly into the IVD tissue using a microsyringe and a 33G needle, reducing the challenges of further disc degeneration due to puncture.

The main findings support the success of an anti-inflammatory therapy for degenerated IVD that not only reduces inflammation but also promotes native IVD matrix production.

Furthermore, although the potential of this soluble intradiscal therapy to be used alone, it shows great prospective to be combined with other therapies, potentiating their effect.

Anti-inflammatory Chitosan/Poly-γ-glutamic acid nanoparticles control inflammation while remodeling extracellular matrix in degenerated intervertebral disc

Graciosa Q. Teixeira[a-d], Catarina Leite Pereira[a,b,d], Flávia Castro[a,b,d], Joana R. Ferreira[a,b,d], Maria Gomez-Lazaro[a,b], Paulo Aguiar[a,b], Mário A. Barbosa[a,b,d], Cornelia Neidlinger-Wilke[c], Raquel M. Goncalves[a,b,d]

[a]Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Rua Alfredo Allen 208, 4200-135 Porto, Portugal

[b]INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Rua Alfredo Allen 208, 4200-135 Porto, Portugal

[c]Institute of Orthopaedic Research and Biomechanics, University of Ulm, Helmholtzstrasse 14, 89081 Ulm, Germany

[d]ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal

Abstract

Intervertebral disc (IVD) degeneration is one of the most common causes of low back pain (LBP), the leading disorder in terms of years lived with disability. Inflammation can play a role in LPB, while impairs IVD regeneration. In spite of this, different inflammatory targets have been purposed in the context of IVD regeneration.

Anti-inflammatory nanoparticles/nanocomplexes (NCs) of Chitosan and Poly-(γ-glutamic acid) with a non-steroidal anti-inflammatory drug, Diclofenac (Df), were previously shown to counteract a pro-inflammatory response of human macrophages. Here, the effect of intradiscal injection of Df-NCs in degenerated IVD was evaluated. For that, Df-NCs were injected in a bovine IVD organ culture in pro-inflammatory/degenerative conditions, upon stimulation with needle-puncture and interleukin (IL)-1β. Df-NCs were internalized by IVD cells, down-regulating *IL-6*, *IL-8*, *MMP1* and *MMP3,* and decreasing PGE_2 production, compared with IL-1β-stimulated IVD punches. Interestingly, at the same time, Df-NCs promoted an up-regulation of extracellular matrix (ECM) proteins, namely collagen type II and aggrecan. Allover, this study suggests that IVD treatment with Df-NCs not only reduces inflammation, but also delays and/or decreases ECM degradation, opening perspectives to new intradiscal therapies for IVD degeneration, based on the modulation of inflammation.

1. Introduction

Pathologies of intervertebral disc (IVD) such as disc degeneration, herniation or cervical radiculopathology are strongly associated with low back pain (LBP).[1,2] This might be caused by herniation-induced pressure on over-sensitized nerve roots, due to mechanical stimuli, and by molecules arising from the inflammatory cascade.[1,3]

In degenerated discs, an up-regulation of metalloproteinases (MMPs) and an over-expression of a wide number of inflammatory mediators (tumor necrosis factor [TNF]-α, interleukin [IL]-1β, IL-6) have been observed. A balance between inflammatory mediators and their counter-regulatory molecules may be important for determining the immune and regenerative outcome of IVD pathologies.[4,5]

Current therapeutic interventions for degenerated IVD are determined by the degree, severity and persistence of pain: conservative approaches, involving rest, pain medication or physiotherapy, in contrast to non-conservative treatments that include microdiscectomy, spinal fusion of two or more disc levels, or disc replacement by prostheses. However, these approaches are only transient and may affect patients' mobility or induce adjacent-level IVD degeneration within few years, leading to chronic low back pain symptoms.[2,6]

Therefore, new therapies for degenerative disc disease have been encouraged and intradiscal injection of different molecules has been one of the most appealing strategies. In this context, inflammatory players (including TNF, IL-1 and IL-6) have been suggested as possible targets.[5,7] For example, intradiscal injections of steroids or glucocorticoids are currently performed to decrease intradiscal inflammation in patients with nucleus pulposus (NP)-induced spinal nerve root injury.[8,9] Nonetheless, only 25% of success rate has been shown with intradiscal steroid injection at short-term follow-up.[10] At long-term, in patients with chronic LBP, this strategy did not show clinical benefits.[11] Furthermore, intradiscal steroids are thought to promote spinal segment stabilization via further disc degeneration.[12]

Non-steroidal anti-inflammatory drugs (NSAIDs), as diclofenac (Df), are considered the most effective anti-inflammatory drugs.[13] These drugs affect the arachidonic acid cascade, inhibiting particularly the cyclo-oxygenase (COX) and lipoxygenase pathways, decreasing inflammation and pain,[14,15] and have been widely used in osteoarticular disorders.[16] A local NSAIDs-based therapy would increase drug targeting and bioactivity, while minimizing drug bio-distribution through the organism and the risk of side effects.[17]

Different strategies are being investigated to treat degenerated IVD, such as hydrogels for NP replacement, cell-based therapies, growth factors injection or gene therapy[18]. Inflammation is an important aspect of this disorder that is frequently neglected, but its control in the degenerated IVD scenario is crucial for tissue regeneration. Recently, IL-10 and transforming growth factor (TGF)-β anti-inflammatory molecules were described as potential successful

therapeutic approaches for the treatment of LBP mediated by IVD degeneration, not only inhibiting inflammation but also, in the case of TGF-β, promoting ECM production.[19,20] Anti-inflammatory NCs have been previously investigated by our group: Chitosan (Ch)/Df/Poly-γ-glutamic acid (γ-PGA) NCs were able to inhibit and revert prostaglandin E_2 (PGE_2) production by activated macrophages *in vitro*, while decreasing IL-6 and partially TNF-α production.[17] So, here we address the effect of these NCs to control inflammation in degenerated IVD. These NCs revealed to be an effective drug-delivery system that can be combined with other strategies as hydrogels to control local inflammation.

Chitosan (Ch) is a natural biodegradable polysaccharide that has been widely used in biomedical applications, mainly in drug delivery systems, gene therapy and tissue engineering[21]. Ch is biochemically active, biocompatible and non-toxic.[22] Previous studies from our group have shown that Ch ultra-thin surfaces polarized macrophages into an M2c phenotype and stimulated dendritic cells, without leading to significant T-cell proliferation.[23] *In vivo*, Ch implants with higher degree of acetylation (DA, 15%) induced a stronger inflammatory reaction, with more extended fibrous capsule and higher number of infiltrated cells.[24] Nevertheless, when Fibrinogen was adsorbed in Ch films, most inflammatory cytokines produced by monocytes/macrophages were down-regulated.[25] Also, when Resolvin D1, a lipid inflammatory mediator, was incorporated into Ch implants, the immune response was almost shut down.[26] Overall, Ch is a versatile biomaterial that can be tuned by its chemistry or protein incorporation to be immunomodulatory.[27] On the other hand, γ-PGA is a naturally occurring peptide that consists of D- and L-glutamic acids polymerized through γ-glutamyl bonds. Contrarily to α-PGA, a counterpart chemically synthetized, γ-PGA is microbially produced by certain Bacillus strains as a capsular or extra-cellular viscous material, is water-soluble, biochemically degraded into glutamate residues and non-toxic.[28] Also, by forming a ternary complex, γ-PGA can be recognized by an intrinsic membrane protein, γ-glutamyl transpeptidase (GGT), resulting in a significant increase in its cellular uptake.[28,29]

Ch and γ-PGA are ions with opposite charges that spontaneously self-assemble in a controlled pH environment. The electrostatic interactions between Ch and γ-PGA have been previously explored by our group.[22] Ch/γ-PGA polyelectrolytes are stable at pH 5.0 and have been proposed as delivery systems for different proteins/molecules in different contexts: stromal derived factor-1,[30] interferon-γ[31] and Df.[17] Ch/γ-PGA nanoparticles/nanocomplexes (NCs) with Df were previously demonstrated to be an effective anti-inflammatory drug delivery system *in vitro.*[17]

Therefore, we propose the intradiscal injection of an anti-inflammatory drug delivery system based on Ch/Df/γ-PGA NCs to locally control the inflammatory response in degenerated IVD. For that, a pro-inflammatory/degenerated bovine IVD organ culture model recently established

was used.[32] The effect of Ch/Df/γ-PGA NCs specifically on IVD inflammatory markers was here addressed, and also ECM remodeling upon this therapy was evaluated.

2. Materials and Methods

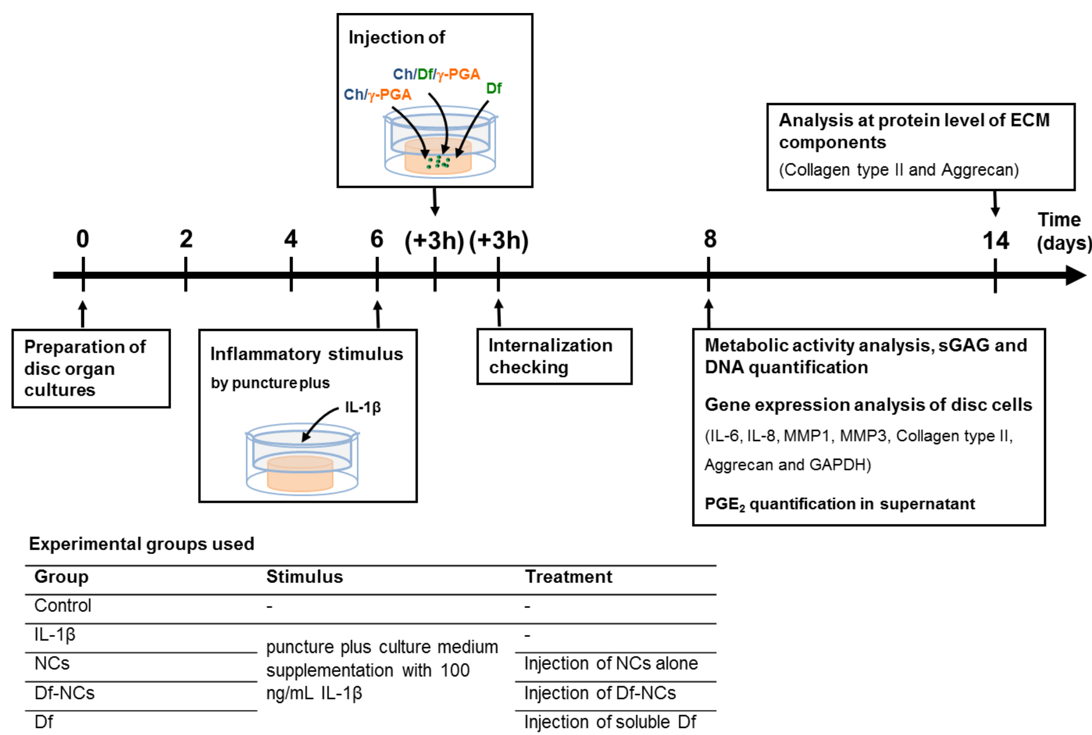
2.1. Pro-inflammatory IVD organ culture model and intradiscal anti-inflammatory treatment

Bovine IVDs were isolated from young adult animals' tails (age<48 months old) within 3 hours' post-slaughter in a local slaughterhouse, with the ethical approval of the Portuguese National Authority for Animal Health. Caudal discs were isolated and cultured according to Teixeira et al.[32] Briefly, standardized disc punches (with diameter of 9 mm) were collected with NP in the center and few surrounding annulus fibrosus (AF) and maintained for 6 days in 6-well tissue culture plates, with membrane filter inserts and 0.46 MPa static loading. Basal medium (BM) was Dulbecco's Modified Eagle's Medium with low glucose (DMEM, Biochrom), supplemented with 5% v/v fetal bovine serum (FBS, HyClone), 1% v/v penicillin/streptomycin (10.000 U/mL/10.000 µg/mL, Biowest), 0.5% v/v amphotericin B (Capricorn) and with the osmolarity adjusted to IVD-physiological 400 mOsm by addition of 1.5% v/v of a 5 M NaCl/0.4 M KCl solution. Samples were incubated at reduced oxygen atmosphere (37°C, 6% O_2 and 8.5% CO_2) and saturated humidity. Culture medium was replaced every second day.
Pro-inflammatory/degenerative stimulation was induced as optimized by Teixeira et al.[32] Briefly, after 6 days of culture in BM, IVD organ cultures were injured by needle-puncture with a sterile 21-gauge needle and stimulated with pro-inflammatory factor IL-1β (100 ng/mL, PeproTech). Three hours after pro-inflammatory stimulus, discs were treated with injection of 500 µL (corresponding to 10% v/v in solution) of Ch/γ-PGA NCs (0.7 mg/mL), Ch/Df/γ-PGA NCs (0.7 mg/mL) (Df, Sigma-Aldrich) using a microsyringe and a 33-gauge needle (Hamilton). The time point for treatment was selected based on previous work from our team.[17,32] Non-manipulated samples kept in BM were used as controls. The effects were evaluated 2 days later by gene expression and PGE_2 production quantification. Metabolic activity of the disc cells, tissue DNA and sGAG content and pH of culture supernatants were also analyzed at this time point. For analysis of ECM components at protein level, organ cultures were maintained for 14 days and samples collected for histology. The experimental scheme and groups are represented in Fig. 1.

2.2. Mitochondrial metabolic activity of IVD cells in the organ culture model

To assess cell mitochondrial metabolic activity, resazurin assay was performed. Resazurin (Sigma-Aldrich) stock solution (0.1 mg/mL) was added to IVD culture medium at a final concentration of 10% v/v. Samples were incubated for 3 hours at 37ºC. Fluorescence intensity was measured in a spectrophotometer microplate reader (BioTek Synergy HT), with 530 nm

excitation filters and 590 nm emission filters. A calibration curve was previously designed to exclude saturated values.



Experimental groups used

Group	Stimulus	Treatment
Control	-	-
IL-1β	puncture plus culture medium supplementation with 100 ng/mL IL-1β	-
NCs		Injection of NCs alone
Df-NCs		Injection of Df-NCs
Df		Injection of soluble Df

Fig. 1. Scheme of the experimental timeline and experimental groups.

2.3. DNA quantification

DNA content of IVD punches was quantified using Quant-iT PicoGreen double standard DNA (dsDNA) kit (Invitrogen), according to manufacturer's instructions, and normalized to the wet weight of the digested tissue. Tissue digests were obtained by previous incubation of the IVD minced samples with proteinase K (Sigma-Aldrich) solution (0.5 mg/mL in phosphate buffer containing 10.68 g/L $NaH_2PO_4.2H_2O$, 8.45 g/L $Na_2HPO_4.7H_2O$ and 3.36 g/L Disodium-EDTA in ultrapure water, pH 6.5) overnight at 56ºC.

2.4. Ch/γ-PGA nanocomplexes preparation and incorporation of diclofenac

Ch/γ-PGA and Ch/Df/γ-PGA NCs were prepared by co-acervation as previously described by our team.[17,33] Briefly, Ch (France-Chitine) was purified and characterized after purification according to Antunes et al.[22] Ch with DA of 10.4±1.6% (degree of deacetylation of approximately 89.6%), determined by Fourier transform infrared spectrometry using KBr pellets (FTIR-KBr), and molecular weight (Mw) of 324±27 kDa, determined by size-exclusion chromatography, was used. γ-PGA (Mw of 10-50 kDa; purity level of 99.5%) was microbially produced by *Bacillus subtilis* as described by Pereira et al.[33] Ch/γ-PGA NPs were prepared at

a molar ratio of 1:1.5 (mol Ch:mol γ-PGA).[17] Solutions of Ch (0.2 mg/mL in 0.2 M AcOH) and γ-PGA (0.2 mg/mL in 0.05 M Tris-HCl buffer with 0.15 M NaCl) were combined by co-acervation method, in which γ-PGA solution was added dropwise to Ch solution, using a 1 mL syringe in a syringe pump (KD Scientific Inc., Holliston, MA), at constant speed (3.6 µL/s) and high stirring at room temperature. The solution's pH was adjusted to 5.0. Df sodium salt (Sigma-Aldrich) solution (10 mg/mL in distilled water) was incorporated in Ch/γ-PGA NPs at a molar ratio of 2:0.35:1.5 (mol Ch:mol Df:mol γ-PGA), according to Gonçalves et al.[17]

2.5. Characterization of Ch/Df/γ-PGA nanocomplexes

NCs were characterized concerning their size and polydispersion index (PdI) by dynamic light scattering (DLS, ZetaSizer Nano Zs, Malvern Instruments) as described elsewhere.[35] The calculation used as dispersants the original solutions of γ-PGA (γ-PGA at 0.2 mg/mL in 0.05 M Tris-HCl buffer with 0.15 M NaCl) and Ch (0.2 mg/mL in 0.2 M AcOH), i.e. the solutions where the NCs were formed.

2.6. Preparation of fluorescent Ch and fluorescent Ch/γ-PGA nanocomplexes with and without Df

Fluorescent NCs were prepared according to Gonçalves et al.[17] Briefly, Ch was labeled with fluorescein isothiocyanate (FITC) with 5% of modification (5% of amine groups with FITC), 100 mg of dried Ch were dissolved in 100 mL of 1% v/v AcOH at 4ºC until complete dissolution. FITC (11 mg to achieve 5% modification) was dissolved in 100 mL of methanol. Both solutions (Ch and FITC) were mixed at constant stirring, protected from light, for 3 hours. The FITC-labeled Ch (ftCh) was then precipitated with 0.5 M NaOH and washed with ultrapure water until no fluorescence was seen in the supernatant. ftCh was lyophilized, dried and weighted. ftCh/Df/γ-PGA NCs (Df-ftNCs) were prepared as described above.

2.7. Analysis of internalization of ftCh/Df/γ-PGA nanocomplexes by IVD cells using confocal microscopy

Df-ftNCs internalization by IVD cells in the tissue organ culture was analyzed by confocal laser scanning microscopy (CLSM). The Df-ftNCs were injected in disc punches (0.7 mg/mL) as described above. After 1 hour of incubation at 37ºC, approximately a 1 mm thickness slice was collected from the center of the disc punch and fixed with 4% v/v paraformaldehyde (PFA). Cells cytoskeleton was stained with Alexa Fluor 594-conjugated Phalloidin (Invitrogen), while cell nuclei were stained with Vectashield with DAPI. The tissue was imaged by CLSM (Leica

TCS SP5 AOBS, Leica Microsystems). Z-stacks and orthogonal projections (in XZ and YZ) of single images were analyzed using ImageJ 1.43u software (Wayne Rasband).

2.8. Quantification of ftCh/Df/γ-PGA nanocomplexes internalization by IVD cells in the organ culture model

The internalization of Df-ftNCs was quantified by imaging flow cytometry. Disc punches were incubated with Df-ftNCs (0.7 mg/mL) for 3 hours. Afterwards, tissue samples were dissected into 2 to 3 mm^3 fragments and enzymatically digested for 2 hours in 1 mg/mL collagenase type I (Sigma-Aldrich) in DMEM, under agitation (50 rpm), reduced oxygen atmosphere (37 °C, 6% O_2 and 8.5% CO_2) and saturated humidity. The supernatant was passed through a 100 μm filter (BD Falcon) to remove tissue debris. Cells were collected by centrifugation at 400 g for 7 minutes. The cell suspension was washed once with PBS and fixed in 1% v/v PFA. For imaging flow cytometry (ImagestreamX, Amnis, EDM Millipore), only single cells were used in the analysis, ftCh fluorescence was assessed in Channel 2 (505-560 nm) and at least 2000 events were collected. Image analysis was performed using IDEAS® data analysis software (Amnis). Internalization quantification is described in Supplementary Materials and Methods (S1.1). Briefly, internalization score was calculated based on the ratio of the FITC fluorescence intensity inside the cell and the intensity of the entire cell.[34,35] Higher scores denote larger NCs concentration in the cell cytoplasm, while negative scores denote cells with little internalization.

2.9. Quantitative real-time reverse transcription polymerase chain reaction

Gene expression levels were determined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) on cDNA derived from disc samples. Specific primer pairs were designed using published gene sequences (PubMed, NCBI Entrez Nucleotide Database) and Primer 3 software48 for bovine *IL-6*, *IL-8*, *MMP1*, *MMP3*, collagen type II (*COL2*), aggrecan (*ACAN*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*),[32] and synthesized by Alfagene. The analysis was carried out using SYBR Green method. Briefly, IVD punches were digested enzymatically as described above, cell pellets were recovered and total RNA was extracted with ReliaPrep RNA Cell Miniprep System (Promega) according to the manufacturer's instructions. Total RNA was quantified by a NanoDrop spectrophotometer (ND-1000, Thermo) and RNA quality was assessed by means of RNA ratio. Total RNA was reverse transcribed into cDNA using SuperScript® III Reverse Transcriptase kit (Invitrogen). Gene expression levels were determined by qRT-PCR conducted on iQ5 Real-Time PCR Detection System (Bio-Rad), and using iQ™ SYBR® Green Supermix (Bio-Rad). Statistical analysis was performed on ΔC_t values according to a modified method described by MacLean et al.[36] Fold changes in gene expression were presented as $2^{-(\text{average}\Delta\Delta Ct)}$. The average Ct value

of each triplicate measurement of each sample was normalized to the house-keeping gene *GAPDH* in each sample ($\Delta Ct = Ct_{(gene\ of\ interest)} - Ct_{(GAPDH)}$). The ΔCt of each stimulated sample was related to the respective ΔCt of each control sample. Normalized values of samples collected at the end of the experiments were compared with the control and between the different experimental groups.

2.10. Prostaglandin E_2 quantification in culture supernatants

Culture medium collected at day 8 was centrifuged (3000 rpm, 5 minutes) and kept at -20ºC for posterior analysis. PGE_2 was quantified by ELISA (Arbor Assays) according to manufacturer's instructions and normalized by total protein. The bicinchoninic acid colorimetric protein assay was performed according to the manufacturer's instructions (Bio-Rad).

2.11. Sulphated glycosaminoglycans quantification

Sulphated glycosaminoglycan (sGAG) content of IVD punches was assessed at day 8 by reaction with 1,9-dimethyl-methylene blue zinc chloride double salt (DMMB, Sigma-Aldrich) dye reagent solution, containing 40 mM sodium chloride (NaCl, Roth), 40 mM Glycine (Roth) and 46 µM DMMB, previously adjusted to pH 3.0. Chondroitin sulphate A sodium salt from bovine trachea (Sigma) was used as standard. Results were normalized by DNA content.

2.12. Detection of proteoglycans by safranin O/light green staining

IVD punches collected at day 14 of culture were fixed with 4% v/v PFA, processed and embedded in paraffin. Sections of 7 µm thickness were sequentially recovered and stained for safranin O/light green (Saf. O/L. Green, 0.1% v/v Saf. O [Sigma]/0.4% v/v L. Green [Sigma]). Sections were imaged using an Olympus CX31 light microscope equipped with a DP-25 camera (Imaging Software CellˆB, Olympus) using the 20x objective.

2.13. Detection of collagen type II and aggrecan in the IVD

COL2 distribution was analyzed by immunofluorescence (IF) staining. ACAN production and distribution was analyzed by immunohistochemistry (IHC). For IHC, Novolink™ Polymer Detection Kit (Leica Biosystems) was used, following the manufacturer's instructions. For both, antigen retrieval was performed in paraffin sections through incubation with 20 µg/mL proteinase K (Sigma-Aldrich) solution for 15 minutes at 37ºC. For COL2 staining, after a blocking step, sections were then incubated for 2 hours at 37ºC with anti-collagen II-II6B3 (Developmental Studies Hybridoma Bank) at a 1:50 dilution. Alexa Fluor 594-labeled goat anti-mouse (Invitrogen-Molecular Probes, 1:1000) was used as secondary antibody. For ACAN,

sections were incubated overnight with ACAN primary antibody (H-300) sc-25674 (Santa Cruz Biotechnology) to a 1:50 dilution.

All sections were mounted in Fluorshield with DAPI (Sigma). Control sections for each labeling excluded primary antibody staining. Representative images of the slides were taken using an inverted fluorescence microscope (Axiovert 200 M, Zeiss) and the 20x objective, for COL2 staining. COL2 intensity was quantified using a custom-made MATLAB (The MathWorks Inc., Natick MA, USA) script, the IntensityStatisticsMask Software (described in Supplementary Materials and Methods). ACAN stained sections were imaged with light microscopy, the 20x objective for counting and the 100x oil objective for detailed imaging.

2.14. Statistical Analysis

Results are presented as Median±Interquartile Range (IQR) in box and whiskers plots. Data normality was first analyzed by D'Agostino and Pearson Normality Test. Statistical analysis was performed with non-parametric Kruskal-Wallis test and Dunns multiple comparison test as post hoc test in Graph Pad v6.02 for Windows. A confidence level of at least 95% (*$p<0.05$) was used.

3. Results

3.1. Viability of IVD organ culture model upon Ch/Df/γ-PGA nanocomplexes injection

In the present study, we investigated the ability of Df-NCs to revert IL-1β-induced pro-inflammatory stimulus, using an IVD organ culture model previously established.[32] The model closely mimics the IVD inflammatory/degenerative process *in vivo*, for which IL-1β is known to be one of the key mediators.[5] Df-NCs, previously optimized by our group, were able to decrease PGE_2, IL-6 and partially TNF-α production in LPS-activated macrophages,[17] thus suggesting that they might be potentially used in other inflammatory scenarios, as in degenerated IVD.

To produce Df-NCs, Df was incorporated in Ch/γ-PGA NCs at a molar ratio of 2.0:0.35:1.5 (Ch:Df:γ-PGA) at pH 5.0, as previously reported.[17] Particle size and PdI of obtained NCs and Df-NCs are summarized in Table 1. The molar ratio, polymer concentration and pH of interaction were first optimized to obtain a low poly-disperse solution with nano-size particles of Ch and γ-PGA.[33] Df concentration and its order of addition to those particles were then optimized to guarantee the maximum amount of drug incorporated in the NCs with nano-size and the lowest PdI.[17]

Table 1. Particle size and polydispersion index of Ch/γ-PGA nanocomplexes alone (NCs) and Ch/Df/γ-PGA nanocomplexes (Df-NCs).

	Particle size (nm)	Polydispersion index (PdI)	Zeta potential (mV)
NCs	166±32	0.24±0.02	20.8±1.6
Df-NCs	175±32	0.26±0.02	20.5±1.9

The concentration of Df in the NCs was confirmed as previously described, by UV/Vis absorbance (275 nm) of NCs supernatant obtained after NCs centrifugation: about 75% of the initial amount of Df (0.06 mg/mL) was incorporated in Ch/γ-PGA NCs, 1 hour after preparation, i.e. Df concentration estimated in the NCs is about 0.045 mg/mL. First, IVD cultures were evaluated concerning their mitochondrial metabolic activity and DNA content, after IL-1β stimulation and treatment with NCs or Df-NCs, to discard possible cytotoxic effects (Fig. 2A and B). Results of the ratio between metabolic activity of disc punches in different conditions and controls showed that IL-1β stimulation slightly increased IVD metabolic activity, which was posteriorly significantly reduced when NCs were injected. In addition, DNA content of IL-1β-treated IVD punches increased significantly, when compared to control discs (1.5±0.5-fold increase, $p<0.05$). NCs and Df-NCs-treated IVD punches presented similar DNA content to the control (ratios of 0.9±0.4 and 1.0±0.5-fold for NCs-treated/control IVD punches and Df-NCs-treated/control IVD punches in comparison, respectively). Regarding the pH alteration of the cell culture medium upon injection of acidic solutions (NCs and Df-NCs), which might create a toxic or inhibitory environment for the cells, no significant alterations were detected, indicating that the injection of NCs or Df-NCs at pH 5.0 did not significantly acidify the cell culture medium (Fig. 2C).

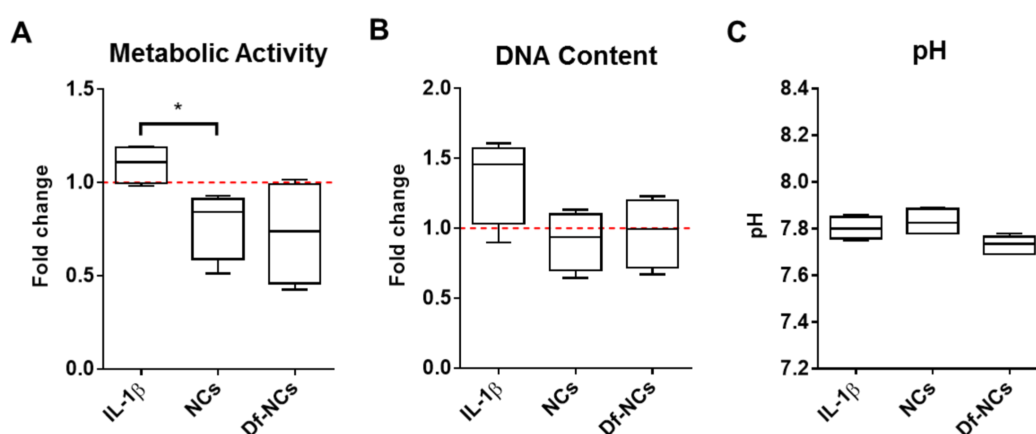


Fig. 2. Viability of the organ culture model, 2 days after pro-inflammatory stimulus with puncture plus IL-1β supplementation (IL-1β), and treatment with injection of Ch/γ-PGA NCs (NCs) or Df/Ch/γ-PGA NCs (Df-NCs). (**A**) Mitochondrial metabolic activity and (**B**) DNA content of disc punches. Results were compared with unstimulated IVD organ cultures (control=1; dashed line). (**C**) pH of organ culture supernatants for the different conditions. Results are shown as box and whiskers plots (n=4). *$p<0.05$

3.2. Evaluation of Ch/Df/γ-PGA NCs internalization in IVD organ culture

Imaging of Df-NCs in IVD was performed in inner slices of tissue, as schematically described in Fig. 3A. The fate of Df-NCs in IVD organ culture was evaluated 3 hours after Df-NCs injection, in the tissue, by CLSM imaging (Fig. 3B). Df-ftNCs were prepared by previously labeling Ch with FITC, as described by Gonçalves et al.[17] A broad distribution of NCs within the IVD tissue and partial NCs internalization by IVD cells was observed in randomly selected IVD regions (n=17 stacks from 3 discs) (Fig. 3B, images a and b). Orthogonal projections in XZ and YZ were performed to evaluate NCs internalization in IVD cells. In those images, we observed that some of Df-ftNCs aggregates were located outside the Phalloidin-stained cell membrane (Fig. 3C, image a, white arrow points Df-ftNCs aggregates), while other Df-ftNCs aggregates were effectively inside the cell (as in Fig. 3C, image b, white arrow points Df-ftNCs aggregates). Therefore, Df-ftNCs internalization was assessed in a high-throughput manner using imaging flow cytometry. First it was determined a viable cell population positive for FITC signal (as shown in Fig. 3D for one donor). By applying a cell mask (Fig. 3E, in blue) and a cytoplasm mask (Fig. 3F, in blue) in the FITC positive cell population, it was possible to determine the internalization ratio between FITC fluorescence intensity inside the cell cytoplasm and FITC fluorescence intensity of the whole cell. This result is depicted for one representative donor in Fig. 3G. Overall, about 92±1% of viable cells presented higher FITC fluorescence in the cytoplasm (Fig. 3H), represented by a positive value of the internalization score, thus being Df-ftNCs internalization^{+} cells. On the other hand, 6±1% of viable cells presented higher fluorescence intensity in the cell membrane (Fig. 3I), represented by a negative value of the internalization score, meaning that in these cells Df-NCs were mostly not internalized (Df-ftNCs membrane^{+} cells).

3.3. Anti-inflammatory potential of Ch/Df/γ-PGA nanocomplexes injection in pro-inflammatory/degenerative IVD organ culture model and evaluation of ECM remodeling

In the IVD organ culture model previously established, the up-regulation of the inflammatory markers *IL-6*, *IL-8* and PGE_2 obtained in pro-inflammatory conditions was reverted by intradiscal injection of Df.[32] Therefore, the efficacy of Df-NCs was first evaluated by assessing the expression of *IL-6* and *IL-8* by IVD cells, and by quantification of PGE_2 in culture medium, 2 days after treatment. MMPs and main ECM proteins of the pro-inflammatory/degenerated IVD *ex vivo* model were also analyzed 2 days' post-treatment with Df-NCs. The results are presented as the Median±IQR fold change to unstimulated IVD punches (Fig. 4).

In the present work, the injection of Df-NCs was able to significantly decrease PGE_2 (**, $p<0.01$) and down-regulate *IL-6* (*, $p<0.05$) when compared to IL-1β-stimulated group (Fig. 4A and B). Df-NCs also seemed to decrease *IL-8* of IL-1β group (from 19±25-fold to 4±7-fold).

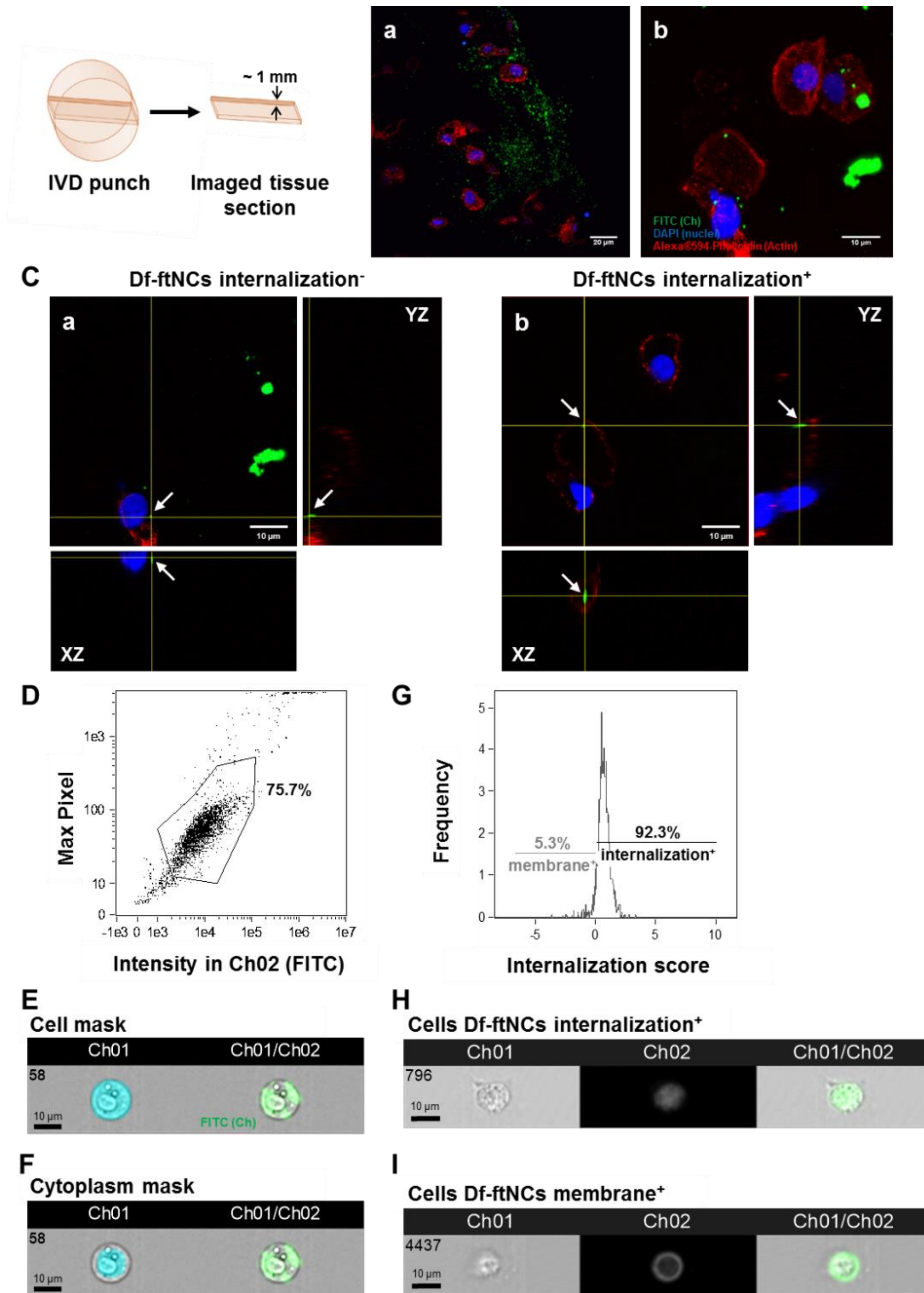


Fig. 3. Internalization of Df-ftNCs by disc cells, after injection treatment of IVD organ cultures, previously stimulated with puncture and IL-1β supplementation. (**A**) Scheme of tissue collection for image acquisition. (**B**) Representative CLSM z-projection images of IVD tissue with Df-ftNCs (images a and b; scale bars, 20 µm and 10 µm), acquired 3 hours after injection treatment. (**C**) Orthogonal projections of single images from A (image b) z-projection, showing negative (image a) and positive (image b) cells for Df-ftNCs internalization (white arrow points Df-ftNCs aggregates). Scale bars represent 10 µm. FITC stains the Ch from the Df-ftNCs in green, Alexa®594-Phalloidin stains F-actin in red and DAPI stains nuclei in blue. (**D**) Representative dot plot profile for the Df-ftNCs internalization analysis. Internalization was assessed by FITC fluorescence in Channel 2 (Ch02). (**E**) Cell mask (in blue). (**F**) Cytoplasm mask (in blue). (**G**) Representative internalization score histogram, after application of an internalization mask in the population of positive cells for Df-ftNCs (Df-ftNCs+). (**H**) Positive cells for Df-ftNCs internalization (internalization+). (**I**) Cells with higher fluorescence on the cell membrane compared to the cytoplasm (membrane+). Each cell is represented by a row of three images acquired simultaneously in flow, from left to right: brightfield (gray), FITC fluorescence (green) from the Df-ftNCs, merged image (scale bars, 10 µm) (n=4).

These values correspond to a reduction of about 73%, 61% and 78% for *IL-6*, PGE_2 and *IL-8*, in relation to IL-1β-stimulated discs. In parallel, control injections with NCs (without anti-inflammatory drug) were also performed. The injection of NCs by itself also reduced *IL-8* expression and PGE_2 production, although no significant differences were observed (reduction to 12±16-fold and 4±2-fold for *IL*-8 and PGE_2, respectively), representing approximately a reduction of 35% and 61% when compared to IL-1β group.

Df intradiscal injection was previously shown to down-regulate MMP1 and up-regulate ACAN gene expression levels, but no significant effects in MMP3 and COL2 levels were observed.[32] In this study, Df-NCs were able to significantly down-regulate both *MMP1* and *MMP3* gene expression (**, p<0.01, Fig. 4C). In addition, NCs alone significantly decreased *MMP3* (*, p<0.05) and slightly down-regulated *MMP1* (from 5±4-fold, for IL-1β group, to 2±4-fold). These results represent a down-regulation of approximately 63% and 40% for *MMP1* and *MMP3* genes, respectively. Concerning ECM proteins (Fig. 4D), *COL2* and *ACAN* were significantly

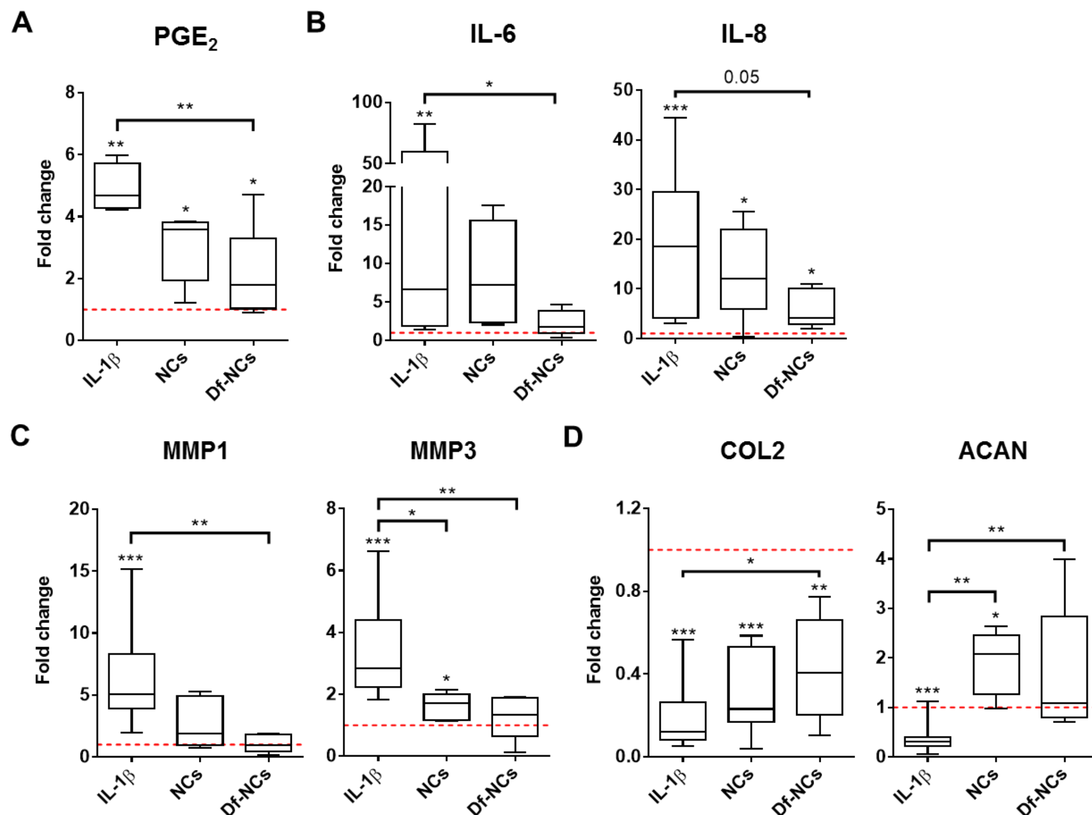


Fig. 4. Effect of different injectable treatments in the inflammatory response and in the ECM remodeling of IVD organ culture pro-inflammatory/degenerative model, 2 days after injection. Quantitative analysis of pro-inflammatory markers of IVD organ cultures stimulated with puncture and IL-1β supplementation (IL-1β), and treated with injection of NCs or Df-NCs. (**A**) PGE_2 fold change in culture supernatants. (**B**) mRNA expression of *IL-6*, *IL-8*, (**C**) matrix degrading enzymes *MMP1* and *MMP3*, and (**D**) ECM components *COL2* and *ACAN*. mRNA levels were normalized to *GAPDH* control gene and to the unstimulated discs (control level=1; dashed line). Results are presented as box and whiskers plots (n=6-19). *p<0.05; **p<0.01; ***p<0.001

up-regulated upon Df-NCs treatment (*, $p<0.05$ and **, $p<0.01$, for COL2 and ACAN, respectively) in IL-1β-treated IVD punches. Interestingly, NCs group by itself also up-regulated *ACAN* expression (**, $p<0.01$) and increased *COL2* (from 0.1±0.2 of IL-1β-IVDs to 0.2±0.4 of NCs-IVDs). These results represent an increase of about 45% and 85% for *COL2* and *ACAN* gene expression, in relation to IL-1β.

3.4. Evaluation at protein level of ECM remodeling in longer-term pro-inflammatory IVD organ culture upon treatment with Ch/Df/γ-PGA NCs

To confirm the effects of these different treatments at protein level, the IVD organ cultures were maintained for 14 days, after which COL2 and ACAN deposition were analyzed by histology/IHC. A group injected with soluble Df (19 μm) was added, since previous results only showed the effect of soluble Df at gene expression level.[32] Fig. 5A (images a-e) shows Saf. O/L. Green-stained sections of IVD NP for all conditions tested. The control group seemed to present a compact matrix, with cells and the respective lacunae perfectly contained within the matrix, while in the remaining conditions a higher disorganization in the fibers arrangement was observed, namely in IL-1β condition (Fig. 5A, image b, arrow). Quantification of disc punches (NP containing few surrounding AF) sGAG content was performed at day 8 of culture. IL-1β and Df presented a lower concentration of sGAG in tissue, when compared to the control, as shown in Fig. 6B. On the other hand, NCs-treated group presented a significantly higher sGAG content relatively to IL-1β-stimulated group (*, $p<0.05$).

COL2 and ACAN deposition were assessed by IHC. Fig. 5A (images f-j) shows images of COL2 staining of all the conditions tested. There were collected 65 to 98 images from randomly selected areas of each section and samples were collected from 4 different donors. COL2 fluorescence intensity was quantified in the IntensityStatisticsMask Software and is depicted as fold change to unstimulated IVD punches in Fig. 5C. The results obtained display significantly higher COL2 deposition in Df-NCs group, in comparison with the IL-1β-stimulated samples (****, $p<0.0001$). This was not observed in NCs and in Df groups.

In Fig. 5A (images k-o) it is also shown ACAN deposition (brown) for the different conditions analyzed (images p and q portray in higher magnification cells negative ($ACAN^-$, Δ) and positive ($ACAN^+$, +) for ACAN production). Since ACAN deposition was located only around the cells, the numbers of $ACAN^+$ and $ACAN^-$ cells were quantified (Fig. 5D). In Fig. 5D it is depicted the fold change of the % of $ACAN^+$ cells (normalized to control group), for 4 different donors. The results obtained show that IVD treatments with NCs and Df-NCs significantly increased the percentage of $ACAN^+$ cells, compared to IL-1β-stimulated discs, when normalized to control group (*, $p<0.05$).

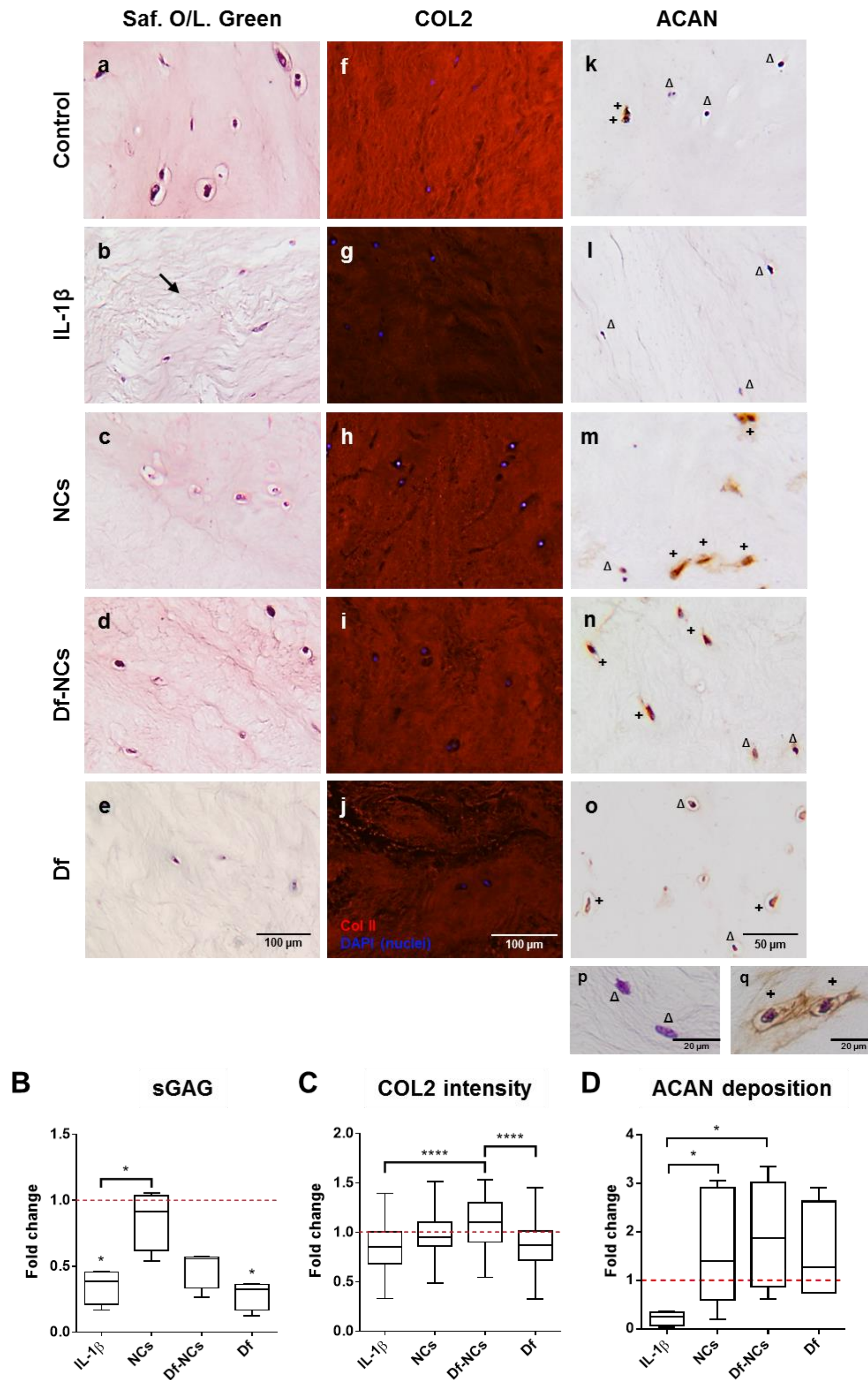


Fig. 5. Effect of different injectable treatments in the ECM of IVD pro-inflammatory/degenerative organ culture model, at the protein level. (**A**) Sagittal sections of disc punches stained for proteoglycans (a-e; scale bar, 100 µm), COL2 (f-j; scale bar, 200 µm), ACAN (k-o; scale bar, 100 µm) and higher magnification of ACAN negative (p, $ACAN^-$, Δ) and positive (q, $ACAN^+$, +) cells (scale bars, 10 µm). Samples were collected after a 14-days culture. One representative experiment of 3-4 different donors is presented here. (**B**) Biochemical analysis of sGAG content of IVD punches, at day 8 of organ culture, normalized to control (n=4 donors). (**C**) COL2 fluorescence intensity normalized to control (dashed line; n=65-98 from 4 donors), at day 14 of culture. (**D**) Fold change in % of $ACAN^+$ cells normalized to control group (n=4 donors), at day 14 of culture. Results are shown as box and whiskers plots (B-D). *p<0.05; ****p<0.0001

4. Discussion

This study hypothesizes that local control of inflammation in degenerated IVD could improve ECM remodeling, which would improve LBP symptoms, constituting a more effective intradiscal therapy. Pro-inflammatory cytokines, TNF-α and IL-1β, are known to induce the expression of genes coding for MMPs, and also induce NP cells to secrete innervation and angiogenic growth factors when the balance between human IVD anabolism and catabolism is disrupted. MMPs degrade both collagen and proteoglycans, leading to tissue dehydration and progressive ECM disorganization. With increasing time, COL2 in the NP is replaced by COL1, and the anatomical border between NP and AF becomes less defined, with the nucleus becoming fibrous.[37]

For that, a pro-inflammatory/degenerative organ culture model, with 0.46 MPa static loading, tissue needle punctured and stimulated with IL-1β (100 ng/mL) was used upon previous validation.[32] This model mimics human IVD degeneration, in which pro-inflammatory cytokines TNF-α and IL-1β are key mediators.[5,38] In this model, an up-regulation of pro-inflammatory markers (*IL-6*, *IL-8*, *MMP1* and *MMP3*), as well as a down-regulation of ECM proteins (*COL2* and *ACAN*) was observed,[32] in accordance to findings reported during human disc degeneration.[5,38]

Although organ cultures as this model are accepted as more reliable models than 2D *in vitro* cultures, constituting an important step before animal experimentation, they have some limitations. One of these include time in culture to guarantee tissue viability (usually up to 1 week).[39-41] Here, tissue viability maintenance was monitored by mitochondrial metabolic activity and DNA content. Other studies had already reported high cell viability in IVD organ cultures in pro-inflammatory conditions after 7[32] and 14 days.[42] Nonetheless, needle insertion can cause cell damage,[40] as well as an increase in cell apoptosis after IL-1β treatment.[43] Also, cell viability upon intradiscal injection in acidic conditions (with NCs) was not significantly affected. Another limitation of the organ culture model is the lack of vasculatization/innervation (and immune cells) in the disc surroundings. Nevertheless, in this case the lack of complexicity constitutes an important advantage when analyzing the direct effect of an intradiscal therapy on IVD cells, without the complex cell crosstalk that we find *in vivo*.

As previously discussed, intradiscal steroid therapy has been adopted by patients with symptomatic disc degeneration and low back pain, unwilling to accept surgical procedures.[10,44] However, placebo-controlled studies about intradiscal steroid injections for discogenic pain have reported either no clinical improvement compared to placebo[11] or short-term improvement.[10] Furthermore, their influence on deregulation of matrix turnover promotes disc degeneration.[8] Also, an increase in the frequency and dosage of intradiscal steroid injections may further accelerate it through puncture injury.[44]

Df reduced NP-induced nerve root dysfunction after 7 days of treatment.[15] In the pro-inflammatory/degenerated IVD *ex vivo* model previously established by us, Df injection down-regulated *IL-6*, *IL-8* and decreased PGE_2 production, and also seemed to have an effect in IVD ECM remodeling by down-regulating *MMP1*, while up-regulating *ACAN* expression.[32]
Nonetheless, Df intraperitoneal injection in a rat lumbar disc herniation model showed a reduced analgesic effect with time.[45] Therefore, Df direct injection into the IVD may have a limited long-term clinical use as it has a short biologic half-life and may require repeated administrations.[32]
In this study, we hypothesize that a Df delivery system based on Ch/γ-PGA NCs could extend Df action, controlling inflammation while contributing to ECM remodeling in degenerated IVD. Therefore, Df-NCs were tested as an anti-inflammatory therapy for degenerated IVD.
Small-scale particles are emerging as delivery systems for IVD regeneration. NCs size enables them to pass through biological barriers, having the possibility to be internalized into target cells.[46] Moreover, NCs can be easily combined with hydrogels for cell delivery or NP regeneration, increasing the functionality of biomaterials for IVD.[47, 48] Examples of NCs are fullerol NCs (approximately 25-50 nm size) that decreased IVD degeneration in human cells and rabbits.[49] NCs are low viscous vehicle, thus easy to inject into the IVD. Moreover, Ch/γ-PGA NCs were recently shown to promote COL2 production in nucleotomized IVD model.[50]
Df-NCs are a monodisperse population of NCs that release Df within 2 hours in physiological pH (maximum of 80%).[17,33] In this study, Df-NCs were injected in IVD organ cultures 3 hours after the pro-inflammatory stimulus. This time point was selected based in previous work using LPS-activated macrophages, after which PGE_2, the Df target, started to be released to the culture medium.[17] NCs internalization by IVD cells was verified, showing that about 65±6% of tissue total cells contained NCs, corroborating the phagocytic activity of NP cells previously suggested.[50,51] The mechanism of intracellular trafficking of these NCs was not specifically addressed, but others have already investigated the intracellular fate of Ch/γ-PGA nanoparticles. Peng et al. showed that Ch/DNA/γ-PGA nanoparticles can be internalized by specific trypsin-cleavable proteins,[52] and by a lipid raft-mediated route, and via macropinocytosis, in a minor extent.[29] Moreover, these authors have shown that when γ-PGA is present in Ch/DNA nanoparticles, less percentage of nanoparticles co-localization with lysosomes, is observed, suggesting that γ-PGA can escape this defense mechanism.[29]
Df-NCs decreased *IL-6*, *IL-8* and PGE_2 production, indicating that Df released from the NCs maintained its ability to inhibit COX-2 pathway, as expected,[15] and similarly to what was observed with Df injection.[32] NCs, with and without Df, were shown to affect macrophage functional behavior *in vitro*, by stimulating the production of IL-6, IL-10, TNF-α but not IL-12/23,

while PGE_2 was only stimulated by NCs without Df.[17] Weather Df-NCs influence macrophage behavior in degenerated IVD will be addressed in the future.

The promisor results of Df-NCs in the reduction of pro-inflammatory markers in the *ex vivo* model do not exclude the need to perform more studies in order to conclude about the feasibility of this therapy, namely testing *in vivo* different times and dosages of NCs administration before moving to pre-clinical research. Although this model aims to mimic human IVD degeneration by up-regulating pro-inflammatory mediators, MMPs and down-regulating ECM proteins,[32] it cannot be considered as mimicking the process of chronic IVD degeneration, as naturally occurs in humans and in other species as chondrodystrophic dogs[53] or the sand rat.[54] Nevertheless, these models also present drawbacks: first, the rat/dog IVD contains notochordal cells, which does not happen in human adults or bovine IVD; second, the long waiting time to observe spontaneous IVD degeneration; and third, the lack of control of this process, which discourages the use of these models.[53,54]

Concerning ECM remodeling, previous studies have demonstrated that Df injection was able to decrease *MMP1* and increase *ACAN* expression.[32] Interestingly, NCs by itself down-regulated *MMP3* (but not *MMP1*) expression, while Df-NCs down-regulated the expression of both *MMP1* and *MMP3* compared to IL-1β-stimulated discs. In the case of ECM proteins, NCs alone significantly up-regulate *ACAN* expression, while Df-NCs significantly increase both *COL2* and *ACAN* gene expression levels. This result was confirmed at the protein level.

These results support previous evidences from our group, demonstrating that γ-PGA promotes chondrogenesis of MSCs *in vitro*, enhancing COL2, ACAN and Sox-9 early expression.[55] This effect was partially observed in IVD organ cultures.[50] In addition, γ-PGA injections have already been patented for treating joint pain.[56] Nevertheless, Ch/γ-PGA NCs mechanism behind chondrogenesis/cartilage formation remains to be explored. The concentration of γ-PGA used in this study was in accordance with previous work from our group.[17,22,30,31,33,50] Nevertheless, in the literature several studies have been used γ-PGA to elicit immune response: about 2.7 mg/mL of γ-PGA with different Mw (from 10 to 2000 kDa) were orally administered inducing significant NK-cell-mediated anti-tumor immunity in mice.[57] Other study administered 5 mg/mL of γ-PGA in mice, inducing antiviral activity and protective immune responses against H1N1 influenza-A virus infection.[58] Another frequent use of γ-PGA is as adjuvant in cancer treatment, by combination with chemotherapeutic agents. γ-PGA nanoparticles were shown to activate dendritic cells usually in high concentrations (10 mg/mL).[59,60] To the best of our knowledge, the concentrations administered are slightly higher than ours.

Previous studies demonstrate that Ch/γ-PGA NCs are able to infiltrate cell-cell junctions[61,62] and that internalization of Ch/γ-PGA NCs might occur mainly via non-specific charge-mediated interaction (NCs positive charge vs negative charged cell membrane).[52] Further studies found

that Ch/DNA/γ-PGA internalization take place via macropinocytosis and caveolae-mediated pathway, with the latter playing a major role.[29] On the other hand, γ-PGA-coated complexes can be internalized via a specific γ-glutamyltransferase (GGT)-mediated pathway.[28,63] The results obtained in this study suggest the involvement of MMP3, but not MMP1, in the IVD ECM remodeling mediated by Ch/γ-PGA complexes. Moreover, the synergy between Df and Ch/γ-PGA NCs suggests that control of inflammation in degenerated IVD is essential for COL2, but not for ACAN production. In fact, this observation was observed in other models across the literature. For example, in PGE_2 (10 pg/mL)-stimulated osteoarthritic cartilage-explant cultures cleavage of COL2 was down-regulated, while no effect was observed in ACAN production.[64] In MSCs/NP cells co-cultures, inhibition of TGF-β1 profoundly constrained COL2 production, while ACAN synthesis was only slightly inhibited, suggesting a crucial role of TGF-β in COL2 production in the NP.[20] Nevertheless, to our knowledge this relation is not straightforward, since other authors have suggested that ACAN production is also dependent on inflammation control and that TGF-β1 may be also involved. Treatment of (TNF-α+IL-1β)-stimulated AF cells with TGF-β1 and bone morphogenic protein (BMP)-2 showed a synergic action of both proteins in recovering degenerated IVD ECM: a high increase in ACAN gene expression was observed after TGF-β1-treatment, while a high increase in COL2 was observed with BMP-2 treatment. Overall, when treated with TGF-β1+BMP-2, an increase in both ACAN and COL2 was observed.[65]

Nevertheless, future studies will be necessary to highlight the molecular mechanisms behind Ch/Df/γ-PGA NCs-driving effect in degenerated IVD and confirm the hypothesis of a MMP3-mediated stimulation of ECM production in the disc.

5. Conclusions

Intradiscal injection of Ch/Df/γ-PGA NCs reduced pro-inflammatory mediators (*IL-6*, *IL-8* and PGE_2) in a pro-inflammatory/degenerative IVD organ culture model. This anti-inflammatory delivery system also down-regulated the expression of both *MMPs 1* and *3*, while up-regulated COL2 and ACAN production. Overall, this study suggests that Ch/Df/γ-PGA NCs injection is a promisor intradiscal therapy for degenerated IVD repair/regeneration. This work provides a solid base for testing intradiscal injection of Ch/Df/γ-PGA NCs *in vivo* in an animal model. Although Df has a limited long-term clinical use, as it has a short biologic half-life, we hope to decrease Df administration rates with this strategy and contribute to sustain the native ECM production in patients with discogenic pain. Moreover, the versatility of Ch/γ-PGA NCs allows its combination with other therapies.

Disclosure

The authors declare no competing financial interests.

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Supplementary Data

Materials and Methods

S1.1. Quantification of Ch/Df/γ-PGA nanocomplexes internalization

For the quantification of the particles internalization, the “internalization score” was measured for every cell. For each cell image two masks were created: the cell mask that defines the total area of the cell and the corresponding cytoplasm mask performed by eroding the cell membrane from the cell mask (obtained in brightfield images in Channel 1). NCs internalization was then assessed by FITC fluorescence in Channel 2. To define the positive FITC signal, IVD cells with Df-NCs, without previous FITC labeling, were run in the same conditions in the imaging flow cytometer. Internalization score was calculated based on the ratio of the FITC fluorescence intensity inside the cell and the intensity of the entire cell. Higher scores denote larger NCs concentration in the cell cytoplasm, while negative scores denote cells with little internalization.

S1.2. Collagen type II quantification

IF was performed in IVD section and COL2 expression intensity was quantified. Images were captured using an inverted microscope (Axiovert 200 M, Zeiss) with the 20x objective. The intensity of COL2 expression in the tissue was determined using an additional custom-made MATLAB script, the IntensityStatisticsMask Software.

Software design

The purpose behind the construction of this script was to obtain a faster and unbiased method for color intensity quantification. The script performs image segmentation, based on a user-defined threshold level, to create a mask for the tissue regions expressing collagen. Intensity measurements, such as mean values and standard deviation, are then calculated only for the pixels belonging to the collagen mask. Unless otherwise stated, the intensity threshold value used for the segmentation was 10.

CHAPTER VI

Evaluation of anti-inflammatory chitosan/poly-γ-glutamic acid nanocomplexes *in vivo*: insights from a rat caudal degenerated/herniated intervertebral disc model

As previously discussed, intradiscal therapies that not only promote IVD matrix synthesis, but also modulate the inflammatory response can have a major impact in IVD regeneration.
Ch/Df/γ-PGA NCs that previously presented promising results, upon intradiscal administration in *ex vivo* IVD punches under pro-inflammatory/degenerative conditions (Teixeira et al. 2016), were here evaluated *in vivo*. In this context, an IVD herniation and degeneration model by rat caudal needle puncture using a 21G needle, previously established in our team, was used (Cunha et al. 2015, Cunha et al. 2016). This model leads to an increase in cell death in the IVD, hernia formation and its infiltration by $CD68^+$ macrophages; however, the degeneration features were observed to spontaneously regress between 2 to 6 weeks (Cunha et al. 2015).
In this work, 10 μL of soluble Df and Df-NCs were injected into the lesioned rat IVDs, with hernia formation, 24 hours after injury, using a 33G needle. Two weeks' post IVD injury, Df-NCs did not promote NP-like matrix production, which contrasts with the *ex vivo* results (Teixeira et al. 2016). Furthermore, Df-NCs did not seem to influence hernia reduction at 2 weeks timepoint. On the other hand, the Df intradiscal injection seemed to slightly contribute to the decrease of hernia volume.
We are currently performing further experiments to better understand the behavior of the injected NCs and the rat physiological response to the treatment.

Evaluation of anti-inflammatory chitosan/poly-γ-glutamic acid nanocomplexes *in vivo*: insights from a rat caudal degenerated/herniated intervertebral disc model

Graciosa Q. Teixeira[a-c]*, Carla Cunha[a,b] *, Cláudia Ribeiro-Machado[a,b], Catarina Leite Pereira[a-c], Joana R. Ferreira[a-c], Maria Molinos[a-c], Susana G. Santos[a,b], Mário A. Barbosa[a-c], Raquel M. Goncalves[a-c]

[a]Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal
[b]INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Portugal
[c]ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal
*equal contribution

Abstract

Low back pain (LBP) is often associated with nucleus pulposus (NP) extrusion and herniation-induced pressure that promote over-sensitized nerve roots, due to mechanical stimuli and by molecules arising from the inflammatory cascade. Inflammation plays an important role in LPB and IVD degeneration. Hence, different inflammatory targets have been purposed in the context of regeneration. Non-steroidal anti-inflammatory drugs, as diclofenac (Df), are commonly prescribed for LBP symptoms, with oral, intravenous or percutaneous (in some cases, epidural) administration presenting moderate success. If administered through intradiscal injection and using a long-term release delivery system, several drawbacks of these drugs could possibly be overcome, increasing their success rate. The aim of this work was to evaluate, *in vivo*, the effect of previously developed chitosan/poly-γ-glutamic acid nanocomplexes (NCs) for delivery of Df.

In this study, soluble Df and Df-NCs were injected into a rat IVD lesion model with hernia formation, 24 hours after injury. NCs and Df-NCs were prepared by coacervation method, and all solutions were injected at pH 5.0. NCs and Df-NCs were obtained with sizes of 328±6 and 310±16 nm and poly dispersion indexes of 0.33±0.04 and 0.30±0.01, respectively. At 2 weeks after injection treatment, animals were sacrificed. NCs vehicle, NCs, Df and Df-NCs did not promote an increase in the percentage of disc height index nor of NP-like matrix production, namely proteoglycans, when compared to the Injury group. NCs injection alone up-regulated *IL-1β*, *IL-6* and *COX-2* by NP cells, compared to Injury. This up-regulation was statistically significant for *COX-2* ($p<0.01$). It was observed great loss of healthy NP structure and composition, namely in NC and Df-NCs conditions. In all injured animals, NP extrusion and hernia formation occurred. Df intradiscal injection seemed to slightly contribute to the decrease of hernia volume from about 0.12±0.05 to 0.04±0.03 mm^3 ($p=0.08$). Nonetheless, there were no significant changes regarding the percentage of $CD68^+$ cells macrophages infiltration into the hernias formed in the animal undergoing different treatments.

Overall, this study focused on the modulation of local inflammatory response to promote IVD regeneration by native IVD cells. A Df intradiscal-delivery approach might promote hernia regression and contribute, in the future, to pain reduction, as well as reduction of the number of patients undergoing hernia removal surgery.

1. Introduction

The degenerative disc disease is perceived as the primary cause of chronic low back pain (LBP).[1, 2] In the clinic, the progression of disc dehydration and loss of the disc height is mainly diagnosed by imaging modalities, namely magnetic resonance imaging (MRI).[3, 4] The decrease of the water signal inside the IVD is considered as an indirect sign of alterations in the composition and structure of the disc structure and tissue, and therefore of degeneration.[4]

Although not always linked with IVD rupture, LBP is very frequently caused by NP extrusion and herniation-induced pressure on over-sensitized nerve roots, that are stimulated by mechanical cues and by molecules arising from the inflammatory cascade.[5-7] Structural damage of the outer annulus provides then an opportunity for blood vessels and nerves to invade the disc.[8] Non-surgical interventions may vary between active physical therapy, education/counseling with home exercise and pain medication.[5, 9] Up to date, most treatments are transient in time, leading to neurological alterations, affecting patients' mobility, and potentially altering spine biomechanics leading to degeneration of adjacent discs.[10, 11] The regenerative therapies proposed overtime have been described to act in early stages of disease's development, and to look for less invasive, long term effective and safe approaches.[12-14] More integrated strategies that could act on different targets of the discogenic disease, such as the inflammatory process, would probably promote native tissue generation and decrease of LBP.

The local delivery of bioactive molecules, such as non-steroidal anti-inflammatory drugs (NSADs), is an interesting approach to reduce the drugs dosage and increase targeting, potentially overcoming the risk of side effects, namely in the gastrointestinal tract.[15] Chitosan (Ch)/poly-(γ-glutamic acid) (γ-PGA) nanocarriers (NCs) demonstrated a potential use as effective anti-inflammatory drug delivery system, *in vitro*.[15] Promising results were also observed with Df-NCs intradiscal administration in bovine tail IVD punches, cultured under pro-inflammatory/degenerative conditions.[16]

Several *in vivo* models of IVD degeneration (and inflammation) are described in the literature, being commonly used murine tail models of mechanical injury, namely performed by needle puncture, as reviewed by others.[17-19]

The aim of this work was to evaluate the intradiscal injection of the NCs-based anti-inflammatory drug delivery system *in vivo*, with the final goal to locally control the inflammatory response in degenerated IVD. In this context, the anti-inflammatory NCs were tested in an IVD herniation and degeneration model by rat caudal needle puncture, using a 21G needle, previously developed and validated in the group.[20, 21] This model leads to an increase in cell death in the IVD, hernia formation and its infiltration by $CD68^+$ macrophages.[21] The effect of

the injected Df-NCs in the control of inflammation and in IVD matrix remodeling was here addressed 2 weeks post-injury.

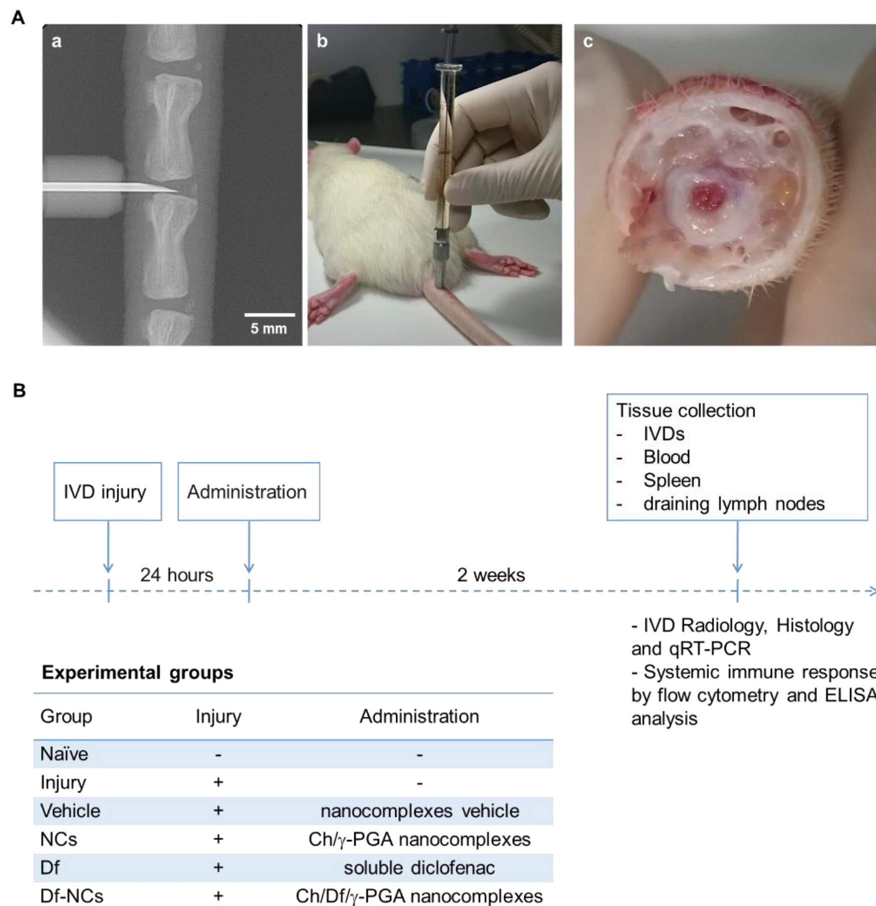
2. Materials and Methods

2.1. Nanocomplexes preparation, incorporation of diclofenac and characterization

γ-PGA with molecular weight (Mw) of 10-50 kDa and purity level of 99.5% was produced by *Bacillus subtilis* as described by Pereira et al.[22] Purified Ch (France-Chitine) with degree of acetylation (DA) of approximately 10.4% and Mw of 324±27 kDa, as determined by Antunes et al., [23] was used. NCs and Df-NCs were assembled by co-acervation as previously described.[15] A solution of 0.05 M Tris-HCl buffer containing 0.15 M NaCl was used as vehicle to prepare the NCs solution. Briefly, γ-PGA solution (0.2 mg/mL) was added dropwise to Ch solution (0.2 mg/mL) at a molar ratio of 1:1.5 (mol Ch:mol γ-PGA). Df sodium salt (Sigma-Aldrich) solution (10 mg/mL in distilled water) was incorporated in Ch/γ-PGA nanocomplexes at a molar ratio of 2:0.35:1.5 (mol Ch:mol Df:mol γ-PGA).[15] Before injection in the IVD, NCs and Df-NCs solutions were centrifuged (15000 rpm, RT) for 30 min.[24] The pellets were concentrated 50 times in the vehicle. A soluble Df solution of 2.975 mg/mL was also prepared. All solutions' pH was adjusted to 5.0. Concentrated NCs and Df-NCs were characterized concerning their size, polydispersion index (PdI) and surface electrical charge (ζ potential), determined using a Zetasizer Nano ZS (Malvern Instruments), as described elsewhere.[24] The calculation used as dispersants the original solutions of γ-PGA (γ-PGA at 0.2 mg/mL in 0.05 M Tris-HCl buffer with 0.15 M NaCl) and Ch (0.2 mg/mL in 0.2 M AcOH).

2.2. Animal experimentation

Male Wistar Han (Crl:WI/Han) rats (36 rats, n=6 per experimental group) with 2 months of age were used. Experiments were carried out at *Instituto de Investigação e Inovação em Saúde* (i3S) animal house, in accordance with European Legislation on Animal Experimentation through the Directive 2010/63/UE and approved by the Institute's Animal Ethics Committee and *Direcção Geral de Alimentação e Veterinária* through the license no. 3773/2015-02-09. The IVD lesion was performed by caudal needle puncture, as previously described by Cunha et al.[21] The animals were anaesthetized by isoflurane inhalation and placed in prone position and the tail skin was disinfected with ethanol prior to every procedure. To induce the lesion, a percutaneous puncture using a 21G needle was done in the coccygeal IVDs Co5/6, Co6/7 and Co7/8 (Fig. 1A, image a). Radiography was performed for IVDs identification.



Group	Injury	Administration
Naïve	-	-
Injury	+	-
Vehicle	+	nanocomplexes vehicle
NCs	+	Ch/γ-PGA nanocomplexes
Df	+	soluble diclofenac
Df-NCs	+	Ch/Df/γ-PGA nanocomplexes

Fig. 1. Rat model of IVD herniation for intradiscal therapy. A) Experimental procedure, needle puncture into a rat coccygeal intervertebral disc, as imaged by X-ray radiography (scale bar, 5 mm) (a), nanocomplexes injection into the IVD with Hamilton syringe (b) and rat tail cross section, showing representative lesioned IVD and adjacent tissue (c). B) Experimental timeline.

After 24 hours, lesioned animals were treated by intradiscal administration of 10 µL of vehicle, NCs alone, soluble Df and Df-NCs, using a 33G needle coupled to a microsyringe (Hamilton, image b). Naïve (healthy and non-injured) and only lesioned animals were kept as controls. Two weeks later, the animals were sacrificed for tissue collection (image c depicts a transversal cut of the rat tail, exposing the IVD and surrounding tissues). The experimental scheme and the different outputs analyzed are represented in Fig. 1B.

A pilot study with 3 animals was performed to determine the timepoint of the acute phase of the inflammatory response post-injury for the treatments' administration and to observe the Df-NCs distribution in the tissue after injection. IVD lesion was induced as previously described and the animals were followed over 50 hours (Supplementary Data).

2.3. Determination of the disc height index

Digital radiographs were acquired by the Owandy-RX radiology system equipped with an Opteo digital sensor (Owandy Radiology) and processed with QuickVision software. The

percentage of the disc height index (% DHI) was calculated by the DHI ratio between post-injury and pre-injury (% DHI = $DHI_{post\text{-}injury}$ / $DHI_{pre\text{-}injury}$ x 100), using ImageJ 1.43u software (Wayne Rasband) for radiograph measurements, as previously described.[21]

2.4. IVD RNA isolation and quantitative real-time reverse transcription polymerase chain reaction

Total RNA was isolated from the NP using TRIzol reagent (Ambion) and quantified by Nanodrop spectrophotometry (ND-1000, Thermo Fisher). RNA quality was assessed by means of RNA ratio. Samples were treated with DNase (Turbo DNA-free Kit, Thermo Fisher). Complementary DNA (cDNA) was obtained through the high-capacity cDNA reverse transcription kit, per the manufacturer's instructions (Applied Biosystems).
Gene expression levels were determined by qRT-PCR conducted on iQ5 Real-Time PCR Detection System (Bio-Rad), using TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays (Applied Biosystems) for interleukin (IL)-1β (Rn00580432_m1), IL-6 (Rn00561420_m1), cyclooxygenase (COX)-2 (Rn01483828_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Rn99999916_s1), as a reference gene. Experiments were performed in duplicate and a quantification cycle (Cq) 35 cutoff was used. Relative expression levels were calculated using the Cq method ($\Delta Ct = Ct(_{gene\ of\ interest}) - Ct(_{GAPDH})$), according to published guidelines.[25]

2.5. IVD collection and histological analysis

Target IVDs with adjacent vertebrae were collected 2 weeks' post-injury, fixed in 10% neutral buffered formalin (VWR) for 1 week at room temperature. Tissue was decalcified in EDTA-glycerol solution, processed for paraffin embedding and sequential transversal 5 µm sections of the IVD were collected. Sections were deparaffinized in xylene solution and rehydrated through a graded series of ethanol. Alcian blue/Picrosirius red and Safranin-O/Fast green stainings were performed throughout the IVD length to identify proteoglycans and collagen tissue distribution.

2.5.1. Alcian Blue/Picrosirius Red staining

A Picrosirius red solution was prepared by dilution of 3g of Sirius red (Sigma) in 500 mL of picric acid (Sigma) saturated aqueous solution. After rehydration, the sections were then incubated in Weigert's Iron Hematoxylin for 8 minutes and washed in tap water. Afterwards, the sections were incubated in Picrosirius red solution for 1 hour and then washed twice in 1% acetic acid. Afterwards, the samples were air-dried, dehydrated and mounted with DPX (VWR) and analyzed in a Zeiss Axiovert200 inverted microscope (Zeiss). Alcian blue stains the

proteoglycans and Sirius red stains collagen type I and III. The hernia area was determined, in each slide, by delimitating the proteoglycans region extruded through the AF. The hernia volume was calculated by the sum of the areas of each individual section throughout the IVD, as previously described.[21]

2.5.2. Safranin-O/Fast Green staining

For Safranin O/Fast Green staining, dehydrated sections were incubated in Gill's Hematoxylin (Sigma-Aldrich) for 5 minutes and washed in distilled water. Afterwards, the samples were immersed in 0.4% Fast Green (Sigma) solution during 5 minutes as a counterstain, and washed twice in 1% acetic acid. Sections were then immersed for 30 minutes in 1.5% Safranin O (Sigma-Aldrich) solution, which stains orange the proteoglycans. After hydration, slides were mounted and imaged as described in 2.5.1.

2.6. Detection of CD68$^+$ cells

CD68$^+$ cells distribution in the IVD was analyzed by immunohistochemistry (IHC), using the Novolink™ Polymer Detection Kit (Leica Biosystems) and following the manufacturer's instructions. Antigen retrieval was performed in paraffin sections through incubation in near-boiling point 10 mmol/L sodium citrate buffer, pH 6.0, for 1 minute, followed by incubation with 20 µg/mL proteinase K (Sigma-Aldrich) solution for 15 minutes at 37ºC. Sections were incubated with anti-CD68 (clone ED1, 1:100 dilution, Bio-Rad Laboratories) primary antibody, overnight at 4ºC.

2.7. Statistical analysis

Results are shown in dot plots, and discussed as average ± standard deviation. Normality was assessed by D'Agostino-Pearson omnibus normality test, after which statistical analysis was performed with non-parametric Kruskal-Wallis test with GraphPad v6.01 for Windows. Statistical significance was set at $^*p<0.05$.

3. Results

3.1. Characterization of the nanocomplexes used for intradiscal injection

Df was incorporated in Ch/γ-PGA NCs as previously reported by Gonçalves et al.,[15] at a molar ratio of 2.0:0.35:1.5 (Ch:Df:γ-PGA) and pH 5.0. The molar ratio, polymer concentration, pH of interaction, Df concentration and components order of addition were previously optimized.[15] About 75% of the initial amount of Df (0.06 mg/mL) was incorporated in Ch/Df/γ-PGA NCs, 1 hour after preparation.[15] To concentrate Df-NCs, in order to inject the maximum amount of NCs

and Df in the small animal model, NCs were concentrated 50 times by centrifugation. The features of concentrated NCs were then analyzed by comparison with the ones previously used in *ex vivo* experiments with bovine tail NP punches (NCs dil. and Df-NCs dil.).[16] The particle size (nm) and polydispersion index (PdI) observed for NCs, NCs dil., Df-NCs and Df-NCs dil. are summarized in Table 1. The ζ potential (mV) of NCs and Df-NCs was also analyzed. The particle size distribution plots obtained in DLS analysis are shown in supplementary Figure S1.

Table 1. Characterization of NCs and Df-NCs.

	Particle size (nm)	**Polydispersion index (PdI)**	**ζ potential (mV)**
NCs dil.	194±5	0.27±0.02	-
NCs	328±6	0.33±0.04	15.30±1.15
Df-NCs dil.	203±4	0.26±0.02	-
Df-NCs	310±16	0.30±0.01	19.2±1.39

With the concentration by centrifugation, particle size seemed to be increased about 70% and 53% for NCs and Df-NCs, respectively. This was observed also for the PdI, which seemed to increase about 22% for NCs and approximately 15% for Df-NCs, when compared to diluted solutions. The concentrated particles remained positively charged, 15.30±1.15 mV (NCs) and 19.2±1.39 mV (Df-NCs), similarly to the values previously observed for diluted NCs (20.8±1.6 mV) and Df-NCs (20.5±1.9 mV).[16]

3.2. Disc height index and local profile of pro-inflammatory markers after injury and intradiscal treatment

The intradiscal injection of this anti-inflammatory nanotechnology-based therapy was then tested in a rat caudal herniation and degeneration IVD model, previously developed by our team.[21] As previously mentioned, the IVD injury was induced by needle puncture into the coccygeal discs 5/6, 6/7 and 7/8 and, after 24 hours, the intradiscal treatments were administered. The effect of intradiscal injection of NCs alone, soluble Df and Df-NCs was directly compared, 2 weeks after injury. Naïve, injury alone and NCs vehicle alone were analyzed in parallel as control groups. The timepoint of NCs administration was determined in a pilot experiment, where 2 out of 3 animals showed high levels of systemic IL-1β and PGE_2 (see Supplementary Fig. S2), suggesting this was a peak in the acute inflammatory response upon injury. At 2 weeks' post-injury, tissue samples were analyzed. This time point was previously selected to analyze IVD herniation, since the hernia formed is reduced from 2 to 6 weeks' post-injury.[21]

IVDs were radiographed before and 2 weeks after injury. The percentage of DHI was calculated as a value inversely proportional to the degree of disc degeneration (Fig. 2A). It was observed a slight decrease of percentage of DHI in the Injury group, compared to Naïve animals (from 101±4% to 77±8%, p=0.07). Moreover, when compared to Naïve, a statistically significant decrease of percentage of DHI was observed in all the groups: NCs (68±12%, p<0.001), Df (75±4%, p<0.05) and Df-NCs (72±6%, p<0.01). When comparing Injury alone with the treated groups, no differences were observed between them.

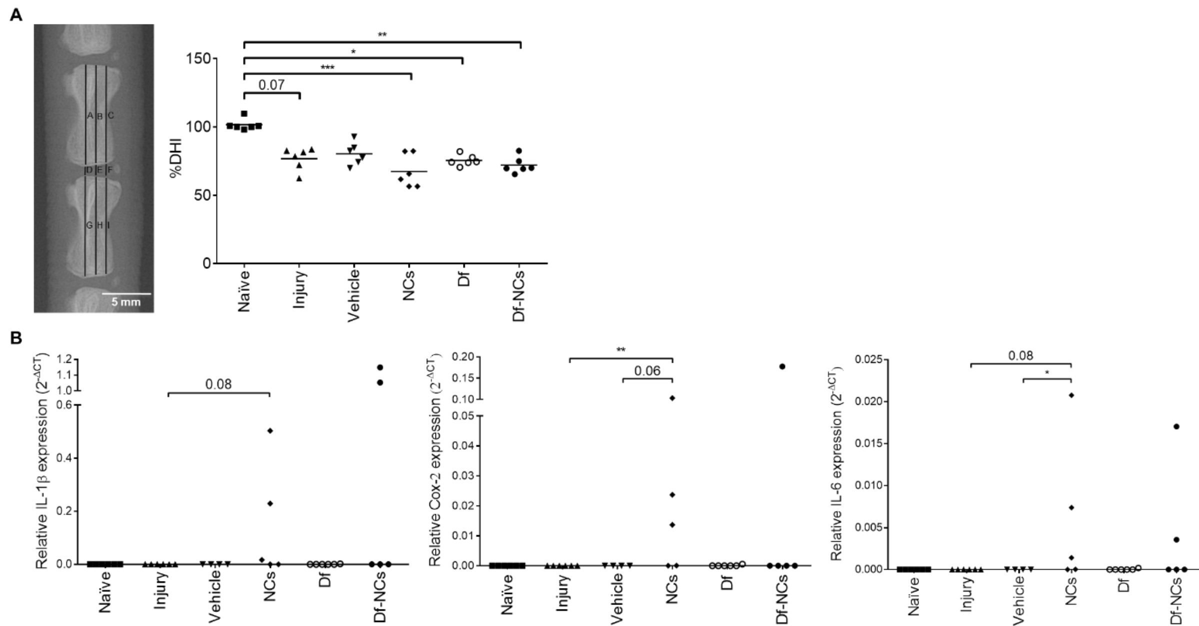


Fig. 2. Local effect of different injectable treatments in the inflammatory response and ECM remodeling of degenerated/herniated IVD, 2 weeks after injury. A) Representative digital radiograph and percentage of disc height index (% DHI). DHI was calculated by DHI=2x(D+E+F)/(A+B+C+G+H+I). $\%DHI=DHI_{post\text{-}injury}/DHI_{pre\text{-}injury}x100$. B) Relative mRNA expression of *IL-1β*, *COX-2* and *IL-6* of IVD cells. mRNA levels were normalized to *GAPDH* control gene. %DHI and mRNA expression results are presented as dot plots (n=4-6). *p<0.05; **p<0.01; ***p<0.001

The local inflammatory response in IVD samples was evaluated by gene expression analysis of pro-inflammatory markers, namely *IL-1β*, *COX-2* and *IL-6*, 2 weeks after injury (Fig. 2B). No expression of these pro-inflammatory markers was observed for the Naïve, Injury, Vehicle and Df groups. Whereas, an up-regulation of *IL-1β* (p=0.08), *COX-2* (p<0.01) and *IL-6* (p=0.08) mRNA expression was observed for the NCs group, when compared to the Injury group. The up-regulation of *COX-2* (p=0.06) and *IL-6* (p<0.05) in NCs group was also observed when compared to the vehicle group. In addition, one to two animals of Df-NCs-treated group also expressed higher *IL-1β*, *COX-2* and *IL-6* gene expression comparatively to others of the same group. The results suggest that both NCs and Df-NCs caused an increase in the local inflammatory response in some of the injected animals (max. 3 out of 6 animals). Nevertheless, the relative expression ($2^{-\Delta Ct}$) of the pro-inflammatory markers is low: under 1.2 (for *IL-1β* in Df-NCs), 0.20 (for *COX-2* in Df-NCs) and 0.021 (for *IL-6* in NCs group).

3.3. IVD ECM composition analysis

Histological analysis of the IVD ECM composition was also performed. In Fig. 3 it is depicted the NP, based on the blue staining of proteoglycans in the center of the IVD, for 3 representative animals from all the conditions tested. When analyzing qualitatively the proteoglycans content (stained in blue), there seemed to have occurred alterations in the tissue morphology and a decrease of the proteoglycans content in the NP, for all the stimulated conditions, in contrast with the Naïve animals, with exception of two animals in the Vehicle group (Fig. 3, the NP section of one of the animals is shown in image i). Of notice, in the Injury group one of the animals completely lost NP integrity and proteoglycans content, and in both NCs and Df-NCs conditions, four and five animals, respectively, also were absent of a healthy NP structure with proteoglycans, when compared to NP sections of Naïve animals.

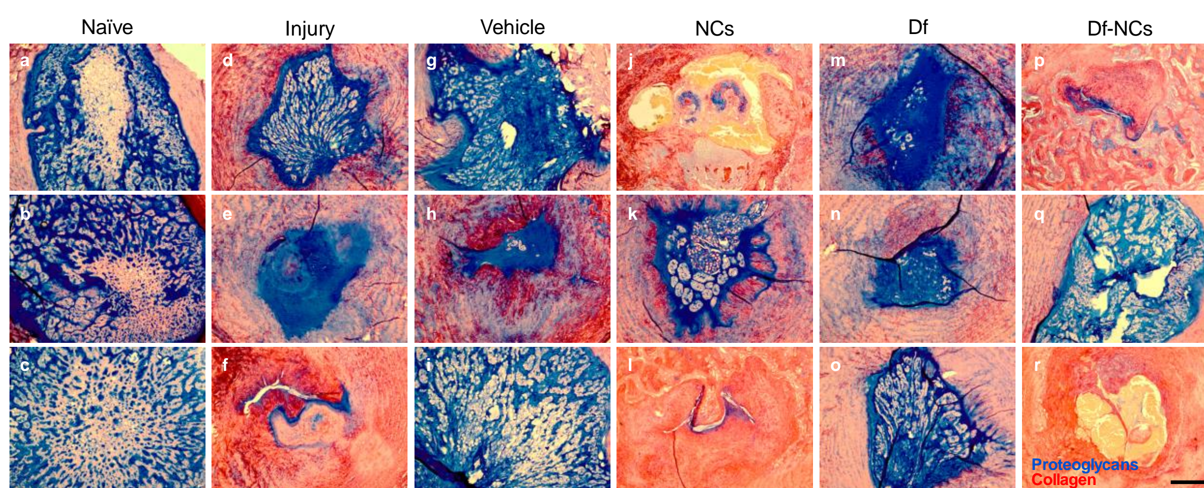


Fig. 3. Analysis performed at the IVD NP, 2 weeks after lesion. A) Alcian blue and picrosirius red staining of one IVD from three different animals, for each condition tested (n=6). Alcian blue stains proteoglycans in blue and sirius red stains collagen type I and III in red (scale bar, 500 µm).

3.4. Hernia size and immune cell infiltration

The *in vivo* model used in this study consists in a needle puncture that leads to hernia formation.[21] In this study the effect of intradiscal anti-inflammatory treatments on formation of herniated tissue was specifically addressed. The preliminary microscopic evaluation of consecutive stained sections for Alcian blue/Picrosirius red (Fig. 4A: a-e) and Safranin O/Fast green (Fig. 4A: f-j) allowed an overview of tissue morphological changes, namely NP leakage and hernia formation (delimited in white dashed lines). A pronounced hernia was formed in injured animals, with proteoglycan-rich tissue extruded, in blue in the Alcian Blue/Pricosirius Red staining, and orange in the Safranin O/Fast Green staining. In most cases, the tissue was extruded to the region between dorsal segmental muscles.

Quantification of the hernia volume (mm^3) indicated a higher tissue herniation in injured animals that seems to be slightly reduced upon Df intradiscal injection (p=0.08, Fig. 4A).

Furthermore, a detailed assessment of macrophages infiltration in the hernia was performed and it is shown in Fig. 4B for a representative lesioned animal (a; border area magnification: a´). After delimitation of the hernia area, using ImageJ software, the percentage of CD68⁺ cells was calculated (as described in Supplementary Materials and Methods). Macrophages were present in the hernias of all injured groups. The % of CD68⁺ cells present in the hernia seem to follow the same trend as in the determination of the hernia volume, for all the IVD samples analyzed (i.e. bigger hernias may have a higher % of CD68⁺ cells within). Nonetheless, no statically significant differences were found, when comparing the treated groups with the Injury alone.

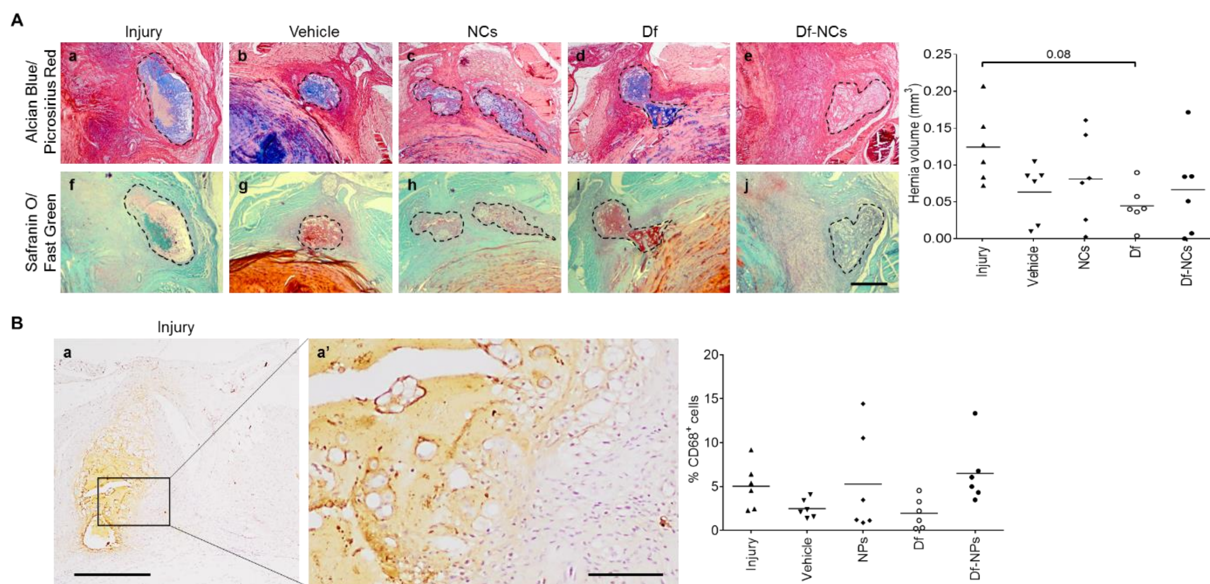


Fig. 4. Hernia formation analysis, 2 weeks after lesion. A) Herniation in the lesioned discs, showing Alcian Blue/Picrosirius Red and Safranin O/Fast Green stainings (hernia delimitated by dashed line; scale bar, 500 μm) and hernia volume (mm^3) quantification. B) Macrophages identification within the hernia by CD68 immunohistochemistry (positive cells are shown in brown). Respective image of the hernia extruded out of the IVD of an animal from the Injury group (a; scale bar, 500 μm) and magnification (a'; scale bar, 100 μm). Quantification of % of CD68⁺ cells within the hernia. Results are presented as dot plots (n=6).

4. Discussion

An intradiscal injection treatment with Df was previously evaluated in an *ex vivo* culture of bovine tail IVD punches, under pro-inflammatory/degenerative conditions.[16, 26] Df administration induced, 2 days after intradiscal injection in the degenerative/pro-inflammatory organ culture model, a significant decrease in PGE_2 production, and down-regulation of *IL-6*, *IL-8* and *MMP1* expression by IVD cells, while *ACAN* expression was up-regulated;[26] however, without changes at protein level, after 8 days of Df treatment.[16] The Df-NCs demonstrated a potential use as effective anti-inflammatory drug delivery system, *in vitro*.[15] In the work of Gonçalves et al., the Df was incorporated at a maximum concentration of 0.05 mg/mL in the

NCs. After adding Df-NCs to macrophages' culture medium at a concentration of 10% v/v, the particles were shown to be nontoxic to the cells. Moreover, the Df released could inhibit or revert PGE_2 production by lipopolysaccharide activated macrophages.[15] Promising results were also observed with Df-NCs intradiscal administration in the *ex vivo* culture of bovine tail IVD punches, under pro-inflammatory/degenerative conditions.[16] Df-NCs down-regulated the expression of pro-inflammatory markers by IVD cells, 2 days after treatment.[16] The analysis at protein level revealed that 8 days after administration, only Df-NCs significantly promoted COL2 and ACAN production by the native IVD cells, when compared to pro-inflammatory/degenerative conditions alone.[16]

Given the promising results, this therapeutic approach was here investigated *in vivo*, in the IVD needle puncture injury model.[20, 21] In previous experiments using this model, 24 hours after injury was the timepoint selected for systemic delivery of mesenchymal stem/stromal cells (MSCs), and IVD tissue analysis was also performed 2 weeks after intradiscal injection.[20] In this model, it was formerly seen spontaneous hernia regression 2 to 6 weeks' post injury.[21] Moreover, 2 weeks after intravenous administration of MSCs, it was observed in the transplanted local down-regulation of glucose transporter (GLUT)-1, a target of the hypoxia-inducible transcription factor (HIF)-1α, and significantly less NP tissue herniation, with higher number of $Pax5^+$ B lymphocytes.[20]

For this work, give the differences regarding the NP and IVD volumes in the rat tail (about 3 to 8 mm^3) and bovine tail (1 to 4 cm^3) (O'Connell et al. 2007), the volume to be injected was adapted. Therefore, the particles were concentrated 50 times and re-suspended in 10 µL of injection vehicle. Also, Df soluble was injected at a concentration 50 times higher (2.98 mg/mL) than the one used in the *ex vivo* model. NCs features were monitored and, although NCs and Df-NCs remained stable at pH 5.0, their size and PdI increased after centrifugation and concentration, suggesting some particle aggregation, as shown in the tissue, after injection (Supplementary Figure S2). In a previous work from our group, Antunes et al. also concentrated Ch/γ-PGA NCs about 10 times,[24] obtaining particles with size and PdI about 40 and 25% higher, respectively, than the diluted NCs used in the previous work from our team.[16] Although the dimensions of the NCs used in the present work were higher (about 70%, compared to NCs dil.), the PdI was similar (approximately 22% higher than the one from NCs dil.). The 10 times concentrated NCs, once injected at acidic pH in a nucleotomized IVD model from bovine origin, significantly reduced cell metabolic activity and DNA content of the NP.[24] In this study, due to increased size and PdI of the NCs, we hypothesize that lower internalization of NCs and Df-NCs by the rat IVD cells might have occurred, contrasting with the previous *ex vivo* results, in which internalization was about 92%.[16] Although Ch/γ-PGA NCs internalization may mainly occur via non-specific charge-mediated interactions,[27] these NCs are able to infiltrate cell-cell junctions.[28, 29] Additionally, Ch/DNA/γ-PGA were shown to be

internalized via macropinocytosis and caveolae-mediated pathway,[30] and γ-PGA-coated Ch/DNA complexes via a specific γ-glutamyltransferase (GGT)-mediated pathway.[31]

The use of Ch/γ-PGA NCs as drug delivery systems rely on pH changes, that allow the disruption of electrostatic interaction between both polymers and the molecule/drug incorporated, in order to release it. The pH of a healthy IVD is reported to be about 7.1,[32] and commonly drops to 6.8 up to 6.5, from mild to severe degeneration.[33] Given this, we are currently performing experiments to better understand if the pH of the rat IVD after injury also drops to the values described in the literature. Moreover, we are also performing experiments to determine the Df release kinetics from the NCs in solutions at different pH values, ranging namely from 6.5 to 7.1. We hypothesize that the much higher ratio NCs volume/IVD volume in the rat (10 µL/3 mm^3 of NP, representing over a 2 times higher injection volume than the NP volume) than in the bovine model (500 µL/1 cm^3 of NP, representing half of the theoretical bovine NP volume), may cause an increase in the pH of the IVD, hindering Df release.

In this model, 2 weeks after MSCs systemic transplantation, DHI and histological grading score seemed to indicate less degeneration; however, without alterations at the ECM level.[20] In the present work, it was seen a decrease in DHI, 2 weeks after needle puncture injury, as well as greater loss of proteoglycans after injury, and of integrity of NP-AF border, with no recovery after the application of the different treatments. Qualitative analysis might indicate formation of fibrocartilaginous matrix, as pointed out by others.[34] Matta el al. observed 6 weeks after a single intradiscal injection of recombinant transforming growth factor (TGF)-β1 and connective tissue growth factor (CTGF) proteins in a rat-tail IVD model of needle puncture, restored notochordal cell content in NP, increase expression of ACAN, COL2, Brachyury and octamer-binding transcription factor 4, compared to injured discs, or injected with PBS.[34] In this last model, the active form of IL-1β was not observed until between 8 to 10 weeks, timepoints at which was also observed an increased expression of the inflammatory mediator, COX-2 and the ECM degrading enzymes, metalloproteinases (MMPs)-3 and -13.[34]

In the present work, the inflammatory markers (*IL-1β*, *COX2* and *IL-6*) mRNA expression was very low, possibly indicating that the resolution of inflammation might be occurring. In a rat IVD degeneration model induced by prolonged upright posture, *COX2* and *IL-1β* were up-regulated about 30- and over 90-fold, respectively, in degenerated IVDs, compare to naïve animals, which were significantly decreased after a 30-days treatment with intraperitoneal injections of a NSAD, meloxicam.[35] On the other hand, in a rat tail torsion loading study, it was observed an overall down-regulation of *IL-1β*, and no effect on *IL-6* or *TNF-α* expression, for the different conditions tested, compared with sham group, 24 hours following the applied loading.[36] In future experiments, it would be interesting to analyze the acute inflammatory response at an earlier timepoint (for instance, up to 72 hours). Cuellar and colleagues evaluated up to 24 hours the protein levels of pro- and anti-inflammatory cytokines in the epidural space of a rat model

of non-compressive disc herniation-induced inflammation.[37] They detected the highest production of IL-6 at 3 hours after inflammation induction, of IL-1β at 6 to 24 hours and of TNF-α at 24 hours.[37] Moreover, a study by MacLean et al. focusing the changes in gene expression due to dynamic compression in caudal motion segments *in vivo* demonstrated that mRNA levels of most catabolic and anabolic genes reached maximum levels 24 hours following mechanical stimulation (but, some had maximum levels 8 and 72 hours following loading).[38] Nonetheless, to evaluate ECM production at protein level, the animal experiments should be kept for longer time periods of, for instance, 4 to 10 weeks, as suggested by other works *in vivo*, focusing on intradiscal injection of factors to promote IVD ECM production.[34, 39, 40]

In this study, intradiscal Df injection seemed to decrease the hernia volume. Nonetheless, it is important to highlight that a single intradiscal injection of a drug with short biological half-life, as Df, due to a very rapid metabolism,[41] may not be enough to promote ECM components production by native IVD cells. Zhang et al. evaluated the pharmacokinetic-pharmacodynamic modeling of Df in normal and Freund's complete adjuvant-induced arthritic male Sprague-Dawley rats.[42] In their model, Df was administered to arthritic rats intravenously (10 mg/kg), in the tail vein, and their results showed a decrease in plasma levels of PGE_2, in both normal and arthritic rats, up to 360 min after dosing.[42] Moreover, the inhibitory effect on PGE_2 levels was proportional to the Df concentration in plasma.[42]

Furthermore, the inflammatory process in degenerated/herniated IVD is complex and is frequently reported that is linked to the presence of macrophages.[43-46] An antigen-specific immune response is widely considered in regression of herniated disc where lymphocytes exist.[47] We consider that an initial M1 pro-inflammatory macrophage response might be important to promote hernia tissue phagocytosis in earlier time points, but a polarization of M1 to M2 macrophages might be key to promote IVD regeneration to avoid chronic inflammation.[47, 48] Therefore, it would be also of interest to prepare NCs using Ch with a 5% DA, which showed to induce a benign M2 anti-inflammatory macrophage response, compared to 15% DA Ch,[49] or incorporate pro-resolution mediators, such as inflammatory resolution lipoxin A4 (LxA4) and resolvin D1 (RvD1), to modulate the inflammatory response to chitosan, as suggested in the work by Vasconcelos et al.[50]

Rat models of mechanical injury, namely coccygeal IVD needle puncture (with 18 to 21G needle, to cause significant tissue damage), are frequently used.[21, 51-53] These present a cost-effectiveness, when compared to large animals[19] and have to follow less complex requirements for experimental approval, when compared to human trials.[18, 54] Nonetheless, the model used might present limitations regarding the small dimensions of the coccygeal IVDs, with IVD and NP volumes of approximately 8 and 3.1 mm^3, respectively.[55] Therefore, it presented also limitations in the scaling of specific parameters,[18] such as the injection of relevant volumes,[56] and the translation of the NCs and Df concentrations with success in the

ex vivo organ culture. Therefore, it is important to perform more experiments to better understand the activity of the 50 times concentrated NCs and the Df release kinetics, for the interpretation of the present experimental findings.

Overall, this study focused on the modulation of local inflammatory response to promote IVD regeneration by native cells. Df intradiscal injection seemed to contribute to the decrease of hernia volume. However, due to the need of a needle puncture, the drug administration cannot be a repeated process. So, if combined with an optimized, biocompatible drug delivery system, to better promote a controlled delivery of Df overtime, this might contribute, in the future, to hernia retraction, reducing the number of patients undergoing discectomy surgery.

Disclosure

The authors declare no competing financial interests.

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Supplementary Data

Materials and Methods

1.1. Preparation of fluorescent Ch and fluorescent Ch/γ-PGA nanoparticles with and without Df

Fluorescent NCs (ftNCs) and Df-ftNCs were prepared as described in Materials and Methods. Ch was labeled with fluorescein isothiocyanate (FITC, 5% modification of amine groups with FITC) according to Gonçalves et al.[1]

1.2. Analysis of ftCh/Df/γ-PGA nanoparticles distribution in the IVD using confocal microscopy

Df-ftNCs distribution in the rat IVD tissue was analyzed 2 hours after intradiscal administration, by confocal laser scanning microscopy (CLSM, Leica TCS-SP5, Leica microsystems). The Df-ftNCs were injected in IVDs as described in Materials and Methods. Animals were sacrificed, IVDs were collected and fixed in 10% formalin. Cells cytoskeleton was stained with Alexa Fluor 594-conjugated Phalloidin (Invitrogen), while cell nuclei were stained with DAPI. The tissue was imaged by CLSM and serial optical sections were analyzed using ImageJ 1.43u software (Wayne Rasband).

1.3. Calculation of the percentage of CD68$^+$ cells in the hernia region

Sections stained for CD68 were imaged with light microscopy, using the same settings to allow comparison. Diaminobenzidine tetrahydrochloride (DAB) staining intensity, corresponding to CD68$^+$ cells, was quantified using an ImageJ H-DAB plugin, based on a color deconvolution technique, which calculates the contributions of DAB and hematoxylin, based on stain-specific red-green-blue (RGB) absorption.[2] By applying this method, DAB and hematoxylin color channels were digitally separated, allowing quantification of color intensity only in the DAB channel. The measurement parameter was optical density (OD), obtained by log(max intensity/mean intensity), where max intensity corresponds to 255, for 8-bit images.[2] The area of CD68$^+$ cells within the previously selected region of interest (ROI) was then determined and normalized to the ROI, for each sample.

Results

3.1. Characterization of Ch/γ-PGA and Ch/Df/γ-PGA NCs

NCs and Df-NCs, obtained by coacervation method, were concentrated 50 times by centrifugation and resuspension in vehicle solution, previous to injection in rat IVDs. After concentration, these particles were compared with diluted NCs (NCs dil.) and Df-NCs (Df-NCs dil.). In Fig. S1 it is shown the size distribution of the different solutions analyzed.

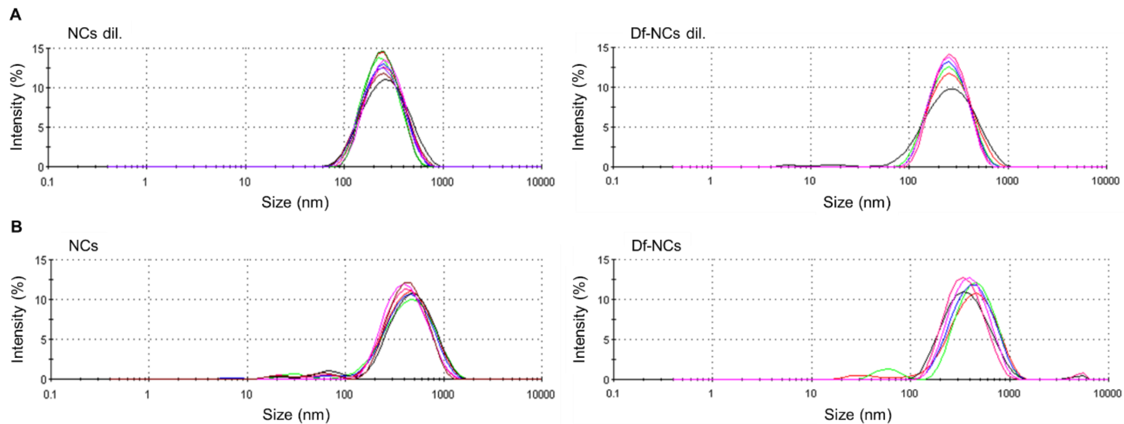

Fig. S1. Characterization of the size dispersion of different nanocomplexes solutions, analyzed by dynamic and electrophoretic light scattering (DLS). A) Diluted NCs and Df-NCs (NCs dil. and Df-NCs dil., respectively). B) NCs and DF-NCs, which were 50 times concentrated after preparation, for injection into the rat IVDs. Particle size distribution plots obtained by intensity of the scattered light (n=6-9).

3.1. Systemic inflammatory profile after IVD lesion

A pilot study was performed to determine the timepoint of administration of the intradiscal treatments after causing the needle-puncture injury into 3 consecutive coccygeal rat IVDs (Co5/6, Co6/7, Co7/8). Blood was collected from the caudal vein at 0, 1, 3, 24, 48 and 50 hours' post-injury, and analyzed for quantification of IL-1β and PGE_2 in plasma (Fig. S2A). Df-ftNCs were injected into the IVD 48 hours after inducing the injury. The animals were sacrificed 2 hours later and the IVD tissue injected with the Df-ftNCs was imaged by CLSM (Fig. S2B).

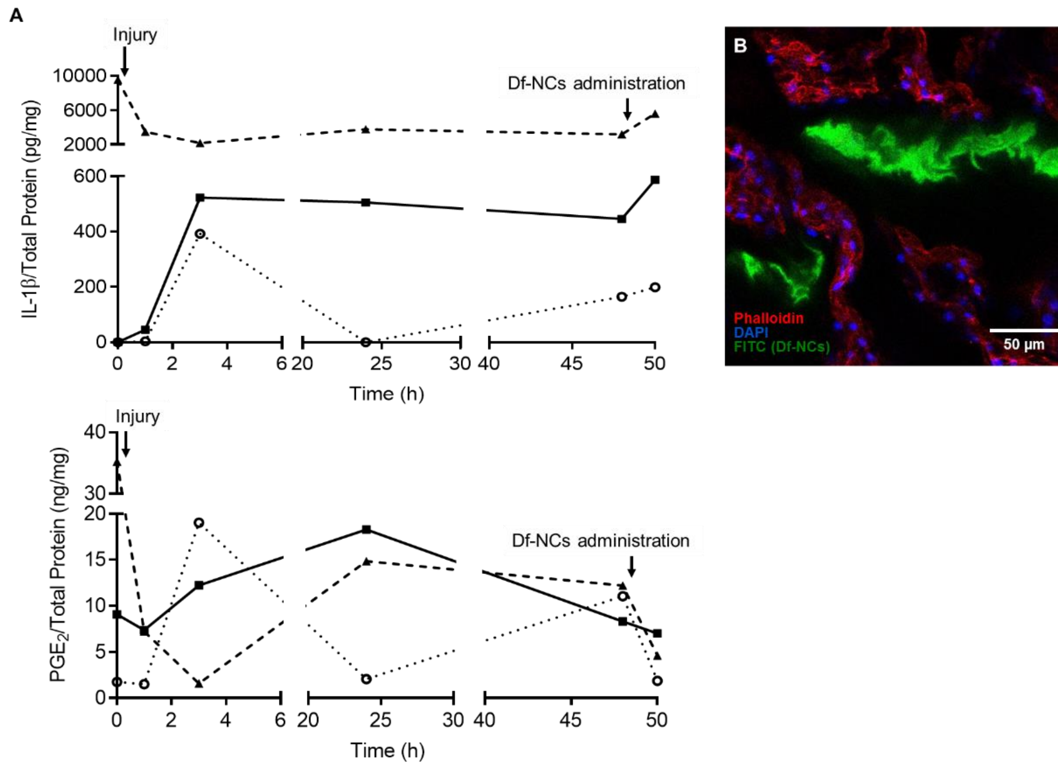

Fig. S2. Systemic inflammatory profile of animals with punctured IVD over a 50 hours' period. Blood plasma samples were collected from the lateral tail vein before injury and 1, 3, 24, 48 and 50 hours' post-lesion to trace a systemic inflammatory profile upon injury. A) Kinetics of Interleukin-1β (IL-1β) (pg/mg protein) and Prostaglandin E_2 (PGE_2) (ng/mg protein) levels in the plasma (n=3). B) Representative CLSM images (maximum intensity projection of serial optical sections) of IVD tissue with Df-ftNCs (FITC stains Ch from the Df-ftNCs in green, Alexa®594-Phalloidin stains F-actin in red and DAPI stains cell nuclei in blue; scale bar, 50 µm), acquired from time point 2 hours' post-administration (n=3).

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CHAPTER VII

Immunomodulation of human mesenchymal stem/stromal cells in intervertebral disc degeneration: insights from a pro-inflammatory/degenerative *ex vivo* model

Submitted

In this manuscript, we have investigated how the pro-inflammatory/degenerative IVD microenvironment can affect the regenerative and immunomodulatory behavior of human bone marrow-derived MSCs. As recently highlighted, for instance, by Sakai and Andersson (2015), the number of trials proposing MSCs-based therapies to treat low back pain and IVD degeneration are increasing. Moreover, although MSCs intradiscal injection (Crevensten et al. 2004) and recruitment (Illien-Junger et al. 2012, Pereira et al. 2014) have been attempted by other authors, the studies published so far do not (or poorly) address the inflammatory environment characteristic of IVD degeneration, and how this milieu can influence the MSCs response. IVD's microenvironment has been recognized to be harsh for MSCs, potentially impairing their survival and function (Rinkler et al. 2010). Furthermore, it was suggested that MSCs immunomodulatory response contributes, initially, to counteract inflammation instead of stimulating matrix formation in short-term *ex vivo* culture of osteoarthritic synovium and cartilage (van Buul et al. 2012). To increase the knowledge on this topic, human bone marrow-derived MSCs isolated by us were co-cultured with pro-inflammatory/degenerative IVD organ cultures in a model of bovine origin previously established and validated (Teixeira et al. 2015). The results obtained so far show an immunomodulatory paracrine effect of MSCs in degenerated IVD, without an apparent effect in ECM remodeling, and suggest that the mechanisms of action of MSCs are based on a cytokine feedback loop.
Importantly, we consider of great relevance to highlight that the results from this study raise the importance of investigating MSCs behavior in degenerated IVD before their widespread use for LPB treatment.

Immunomodulation of human mesenchymal stem/stromal cells in intervertebral disc degeneration: insights from a pro-inflammatory/degenerative *ex vivo* model

Graciosa Q. Teixeira[a-d], Catarina Leite Pereira[a,b,d], Joana R. Ferreira[a,b,d], André F. Maia[a,e], Maria Gomez-Lazaro[a,b], Paulo Aguiar[a,b], Mário A. Barbosa[a,b,d], Cornelia Neidlinger-Wilke[c], Raquel M. Goncalves[a,b,d]

[a]Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Rua Alfredo Allen 208, 4200-135 Porto, Portugal

[b]INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Rua Alfredo Allen 208, 4200-135 Porto, Portugal

[c]Institute of Orthopaedic Research and Biomechanics, University of Ulm, Helmholtzstrasse 14, 89081 Ulm, Germany

[d]ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal

[e]IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, Rua Alfredo Allen 208, 4200-135 Porto, Portugal

Abstract

Objective: Low back pain (LBP) is one of the causes of disability worldwide, frequently associated with intervertebral disc (IVD) degeneration and inflammation. Mesenchymal stem/stromal cells (MSCs)-based therapies to LBP have been advocated but the involvement of inflammation in the remodeling mechanism of IVD has not been explored. Here we investigated how the pro-inflammatory/degenerative IVD microenvironment affects the regenerative and immunomodulatory behavior of human bone marrow-derived MSCs, using a bovine ex vivo model.

Design: IVD punches were cultured in basal or pro-inflammatory/degenerative conditions (needle-punctured and IL-1β supplemented). MSCs were posteriorly co-cultured on top of transwells, above IVD punches, for up to 2 weeks. Cell viability and MSCs migration were analyzed. Extracellular matrix (ECM) remodeling of IVD organ cultures, MSCs response to the pro-inflammatory/degenerative environment and gene expression profile of IVD cells after co-culture with MSCs were also assessed.

Results: The pro-inflammatory/degenerative IVD conditions did not affect MSCs viability, but promoted migration, despite very few MSCs being found in IVD tissue. This has led us to investigate the possibility of MSCs acting via a paracrine mechanism. The pro-inflammatory/degenerative IVD conditions promoted IL-6, IL-8, MCP-1 and PGE_2 production by MSCs, while reducing TGF-β1. Furthermore, the presence of MSCs did not stimulate ECM production in neither basal nor inflammatory conditions, but down-regulated bovine pro-inflammatory gene expression levels (*IL-6*, *IL-8*, *TNF-α*) in IL-1β-stimulated IVDs.

Conclusions: This study provides evidence for a mechanism dependent on a cytokine feedback loop, through which MSCs are capable of immunomodulating the IVD microenvironment.

1. Introduction

Discogenic low back pain (LBP) accounts for almost 40% of chronic LBP, the leading disorder in number of years lived with disability.[1] In patients with discogenic LBP, degeneration of the intervertebral disc (IVD) without apparent nerve compression occurs, associated with increased inflammation and pain.[2] IVD degeneration is characterized by a decrease in water content, a reduction of cell numbers and the turnover of extracellular matrix (ECM) components.[3] A shift from collagen type II (COL2) to type I (COL1) production by nucleus pulposus (NP) cells, and a decrease in aggrecan (ACAN) synthesis occur.[3] Furthermore, an up-regulation of specific metalloproteinases (MMPs), MMPs with thrombospondin motifs (ADAMTS),[4,5] and a wide number of inflammatory mediators[6,7] have been described as responsible for IVD degeneration, nerve ingrowth and pain.

Cell-based therapies to stimulate IVD regeneration are being increasingly investigated, particularly using mesenchymal stem cells (MSCs).[8,9-11] However, the behavior of MSCs in IVD-associated inflammation scenarios has been neglected. Moreover, though a high number of degenerated/injured IVD animal models has been developed, there is no ideal model that both mimics progressive human disc degeneration and allows a standardized control.[12] IVD organ cultures have been arising to address specific questions, although, of course, with limitations, such as lack of the immune host response and pain assessment.[13]

In multiple *in vitro* and animal studies, MSCs have been proposed to have a beneficial effect in IVD regeneration due to their differentiation capacity into an NP-like phenotype.[14-16] MSCs are known to contribute to the regenerative process by interacting with the surrounding environment through the secretion of numerous molecules, such as growth factors, cytokines and chemokines.[17] Nonetheless, the IVD hypoxic environment and mechanical load, the high osmolarity and low pH may impair MSCs survival and function.[18] In humans, MSCs are currently being tested in several LBP clinical trials.[11,19,20] However, the results obtained so far remain controversial since the patients referred pain reduction, but no increase of disc height was observed.[11,19] In a clinical trial, in which patients received autologous bone marrow concentrate, discogenic pain reduction was reported after 12 months of follow-up.[20] Ongoing clinical trials are addressing the use of allogenic[21] or autologous MSCs transplantation[22,23] and implantation of cell-seeded scaffolds in degenerated IVD.[24,25]

Besides their multi-differentiation potential, MSCs are accepted as immunomodulatory cells, by interacting with the different immune cells.[26] Thus, MSCs might modulate the inflammatory milieu associated with IVD degeneration. This aspect has been neglected in several studies, although MSCs have already been shown to contribute to maintain IVD immune privilege by the expression of fas ligand (*FasL*).[27]

This study explores for the first time the synergic interplay between MSCs and IVD cells in the presence of pro-inflammatory/degenerative IVD conditions, namely in what refers to their pro-regenerative and immunomodulatory contributions through a paracrine mechanism. In addition, we expect to increase the knowledge of how IVD degenerative and pro-inflammatory environment can affect and change MSCs immunomodulatory profile. The knowledge generated will impact to increase their successful use in the discogenic pain treatment.

2. Materials and Methods

2.1. Culture of human MSCs

Human MSCs harvested from bone marrow were obtained from different donors who underwent bone marrow donation, hip replacement or knee joint surgery, with informed consent and following the rules of the ethical commission of the University of Ulm (Ulm, Germany) and the Portuguese authorities (*Direcção-Geral da Saúde*, Porto, Portugal). MSCs phenotypic profile was previously accessed either by immunohistochemistry for CD9, CD90, CD105, CD44, and Stro-1 staining,[28] or by flow cytometry for CD19, CD14, CD73, CD34, CD90, CD105, CD45 and HLA-DR.[29] Multi-lineage differentiation potential was also previously assessed.[28,29] Cells were expanded as reported in Almeida et al.[29] Experiments were performed with MSCs from 7 donors, in passages 3-7 (detailed information in Supplementary Table S1).

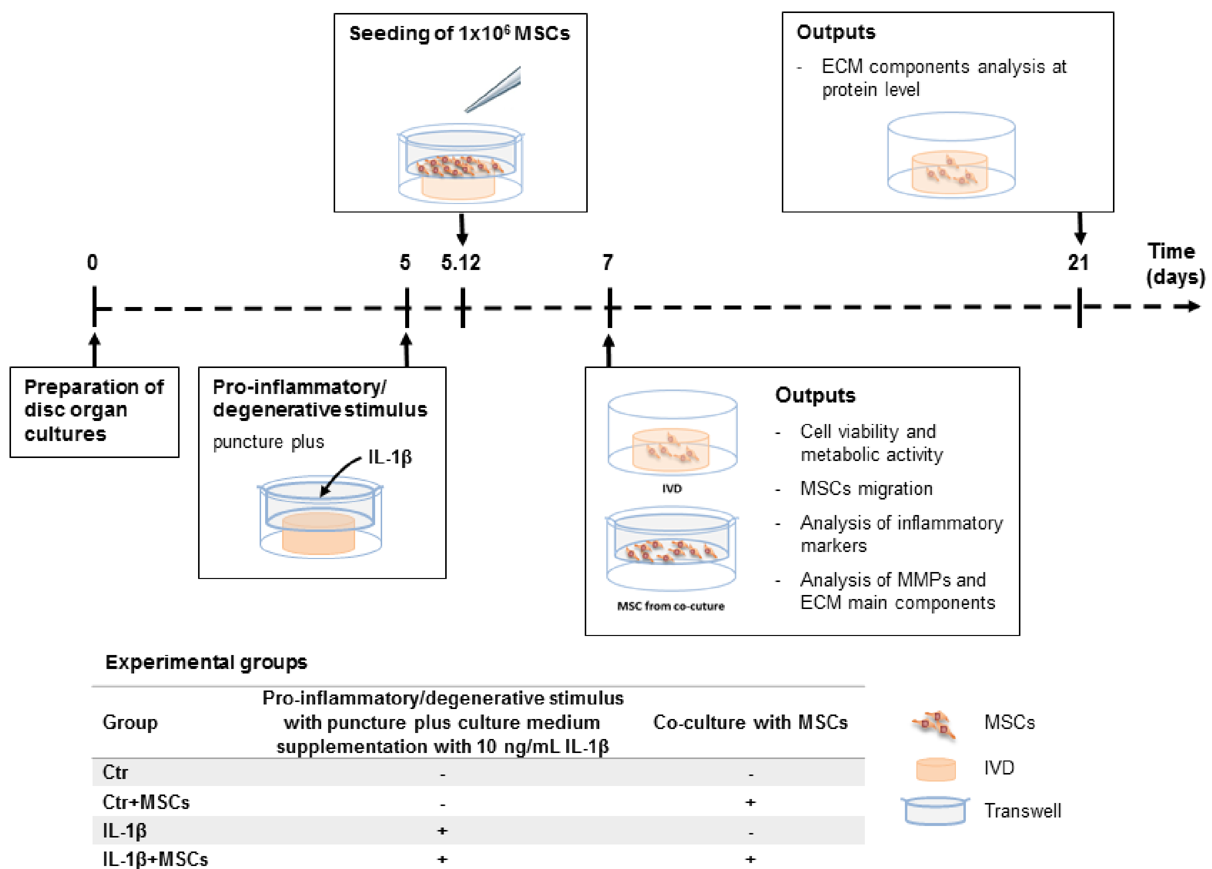
2.2. Pro-inflammatory IVD organ culture model

Bovine IVDs were isolated from young adult animals' tails (age<48 months) within 3 hours' post-slaughter, with the ethical approval of the Portuguese National Authority for Animal Health. Caudal discs were isolated and cultured in basal conditions according to Teixeira et al.[30] Pro-inflammatory/degenerative stimulation was induced as described by Teixeira et al.[30] Briefly, after 6 days of culture in basal conditions, organ cultures were injured by needle-puncture with a 21G needle and stimulated with pro-inflammatory factor IL-1β (10 ng/mL, PeproTech, UK).

2.3. MSCs co-culture with IVD

Three hours after pro-inflammatory stimulus, 1×10^6 MSCs per IVD punch were seeded on top of the transwell. MSCs were stained, prior to seeding, with CellTracker™ Blue CMAC Dye (CTB, Molecular Probes), For confocal microscopy analysis, or with CellTracker™ CM-Dil Dye (Thermo Fisher Scientific), for identification by flow cytometry analysis (Supplementary

Methods). This time point was selected based on previous work from our team.[30] Non-manipulated samples kept in BM were used as controls. Two days later it was checked if cells were still alive and if they had migrated to the disc by LIVE/DEAD and migration assays. IVD and MSCs apoptosis and viability were accessed by flow cytometry analysis, gene expression and protein production by both cell types were analyzed 2 days after stimulation and co-culture (day 7). Tissue sulphated glycosaminoglycan (sGAG) content was analyzed at days 7 and 21 of culture. ECM components were analyzed at protein level at day 21 of culture. The experimental scheme and groups are depicted in Fig. 1.



Group	Pro-inflammatory/degenerative stimulus with puncture plus culture medium supplementation with 10 ng/mL IL-1β	Co-culture with MSCs
Ctr	-	-
Ctr+MSCs	-	+
IL-1β	+	-
IL-1β+MSCs	+	+

Fig. 1. Experimental timeline and culture groups.

2.4. Human MSCs migration assessment, samples preparation, image acquisition and analysis

To evaluate MSCs migration, transwells with a polyethylene terephthalate membrane with 8 μm pore size and 4.5 cm^2 membrane surface area (Millipore) were used. After 2 days of MSCs co-culture with IVD punches, cells were fixed with 4% paraformaldehyde and rinsed with PBS. Inserts were carefully washed with PBS, and cells remaining on the upper face of the filters were removed with a cotton wool swab[31]. The filters were cut out and in two halves with a scalpel, stained with DAPI and mounted onto glass slides, with the down part facing

upwards. The number of cells that had migrated was determined by counting each half filter (spanning 172-383 microscope fields), avoiding areas with air bubbles. Images with $6.4x10^{-3}$ cm^2 were collected with a Nikon 20x/0.45 NA Plan Fluor objective in a high-throughput automated fluorescence widefield microscope (IN Cell Analyzer 2000, GE Healthcare). 2.5D Acquisition & Deconvolution mode was used integrating the signal over 2.0 µm Z section, generating a pseudo 3D projection.

Quantification of the number of cells that migrated through the transwell was performed with Developer Toolbox 1.9.2 (GE Healthcare). Briefly, a nuclear segmentation algorithm was used to identify and quantify the number of migrated cells. Data are expressed as number of migrated cells per cm^2. The corrected number of migrated MSCs per cm^2 was obtained by the sum of the results for both halves of the insert, and subtraction of the number of cells per cm^2 counted in the respective control conditions without MSCs (corrected $(cells/cm^2)_{IL\text{-}1\beta+MSCs} = (cells/cm^2)_{IL\text{-}1\beta+MSCs} - (cells/cm^2)_{IL\text{-}1\beta}$), due to the transwell direct contract with the IVD tissue, from which IVD cells can attach to the polymeric surface. The results include independent experiments with 6 different bovine IVD donors, and 3 different human MSCs donors in passages 4 to 7.

2.5. Mitochondrial metabolic activity of IVD cells in the organ culture model

Cell mitochondrial metabolic activity was accessed by resazurin assay. Resazurin solution (0.1 mg/mL) was added to IVD culture medium at a final concentration of 10% v/v. Samples were incubated for 3 hours at 37ºC. Fluorescence intensity was measured in a spectrophotometer microplate reader (BioTek Synergy HT), with 530 nm excitation filters and 590 nm emission filters. A calibration curve was previously designed to exclude saturated values.

2.6. MSCs identification and LIVE/DEAD assay

MSCs were stained before seeding with CellTracker™ Blue CMAC Dye (CTB, Molecular Probes) for further identification after culture. Cell viability was qualitatively assessed through fluorescence-based LIVE/DEAD Cell Viability/Cytotoxicity kit (Invitrogen), by confocal laser scanning microscopy (CLSM, Leica TCS-SP5, Leica microsystems). Briefly, IVD tissue samples collected from the center of the disc punch by sagittal cut were incubated with Calcein Acetoxymethyl Ester (Calcein AM; 1 mM) and Ethidium Homodimer-1 (EthD-1; 2.5 mM) for 45 min at 37ºC. Calcein AM (Ex 485 nm/Em 530 nm) stains live cells green, indicating intracellular esterase activity, while EthD-1 (Ex 530 nm/Em 645 nm) stains dead cells red, indicating loss of plasma membrane integrity. Images were analyzed, using ImageJ 1.43u software (Wayne Rasband).

2.7. DNA quantification

DNA content of IVD punches was quantified using Quant-iT PicoGreen double standard DNA kit (Invitrogen), and normalized to the wet weight of the digested tissue. Tissue digests were obtained by incubation of IVD minced samples with proteinase K solution (0.5 mg/mL) overnight at 56ºC.

2.8. Quantitative real-time reverse transcription polymerase chain reaction

Gene expression levels were determined by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) on cDNA derived from disc samples. Specific primer pairs for bovine were designed using published gene sequences (PubMed, NCBI Entrez Nucleotide Database) and Primer 3 software48. The bovine primer sequences for *TNF-α* and *MMP13* are in Table 1, while *IL-6*, *IL-8*, *MMP1*, *MMP3*, collagen type II (*COL2*), *ACAN*, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) are published in Teixeira et al.[30] The analysis was carried out using SYBR Green method. Briefly, IVD punches were digested enzymatically as described above, cell pellets were recovered and total RNA was extracted with ReliaPrep RNA Cell Miniprep System (Promega), as per the manufacturer's instructions. Total RNA was quantified by a NanoDrop spectrophotometer (ND-1000, Thermo) and RNA quality was assessed by means of RNA ratio. Total RNA was reverse transcribed into cDNA using SuperScript® III Reverse Transcriptase kit (Invitrogen). Gene expression levels were determined by qRT-PCR conducted on iQ5 Real-Time PCR Detection System (Bio-Rad), and using iQ™ SYBR® Green Supermix (Bio-Rad). Statistical analysis was performed on ΔC_t values, as described by MacLean et al.[32] Fold changes in gene expression were presented as $2^{-(average\Delta\Delta Ct)}$. The average Ct value of each triplicate measurement of each sample was normalized to the house-keeping gene *GAPDH* in each sample ($\Delta Ct = Ct_{(gene\ of\ interest)} - Ct_{(GAPDH)}$). The ΔCt of each stimulated sample was related to the respective ΔCt of each control sample. Normalized values of samples collected at the end of the experiments were compared with the control and between the different experimental groups.

Table 1. Bovine oligonucleotide primers.

Gene	Forward and reverse primer, 5'-3'	Product length (bp)	NCBI reference sequence
TNF-α	CCATCAACAGCCCTCTGGTT GAGGGCATTGGCATACGAGT	134	AF011926
MMP13	CATGAGTTTGGCCATTCCTT GGCGTTTTGGGATGTTTAGA	179	NM_174389

2.9. Detection of relative protein expression

A commercially available array of 40 human inflammatory factors (Human Inflammation Array C3, AAH-INF-3, RayBiotech) was used to evaluate the relative levels of cytokine production in the IVD punches' culture supernatants. A pool of 8 culture supernatants of each experimental group was prepared for this determination, and 1 mL of the prepared pool was used. Data shown are from 25.5 sec exposure in Chemidoc XRSþ (BioRad). Quantification of the results was generated by quantifying the mean spot pixel density from the array using image software analyses (ImageLab 4.1; BioRad). Briefly, the pixel intensities gathered from the array spots were obtained using the volume tools option of the software. It was defined an area of interest of the reference spots by surrounding it with a circle, and then equal circles were used for all spots of the array. Afterwards, the circles were analyzed and the densities of signals were normalized by the background.

2.10. Protein quantification in culture supernatants

Culture medium collected at day 8 was centrifuged (3000 rpm, 5 minutes) and kept at -20ºC for posterior analysis. PGE_2 (Arbor Assays), free active TGF-β1 (BioLegend), and human IL-6, IL-8, monocyte chemoattractant protein (MCP)-1, IL-1β and regulated on activation, normal T-cell expressed and secreted RANTES, also called chemokine ligand 5 (CCL5) (PeproTech) were quantified by ELISA, as per the manufacturers' instructions.

2.11. Sulphated glycosaminoglycans quantification

Sulphated glycosaminoglycan (sGAG) content of IVD punches was assessed at day 8 by reaction with 1,9-dimethyl-methylene blue zinc chloride double salt (DMMB, Sigma-Aldrich) dye reagent solution, containing 40 mM sodium chloride (NaCl, Roth), 40 mM Glycine (Roth) and 46 µM DMMB, previously adjusted to pH 3.0. Chondroitin sulphate A sodium salt from bovine trachea (Sigma) was used as standard. Results were normalized by DNA content.

2.12. Detection of type II collagen and aggrecan in the IVD

COL2 distribution was analyzed by immunofluorescence (IF) staining. ACAN production and distribution was analyzed by immunohistochemistry (IHC). For IHC, Novolink™ Polymer Detection Kit (Leica Biosystems) was used, following the manufacturer's instructions. For both, antigen retrieval was performed in paraffin sections through incubation with 20 µg/mL proteinase K solution for 15 minutes at 37ºC. For COL2 staining, after a blocking step, sections were incubated for 2 hours at 37ºC with anti-collagen II-II6B3 (Developmental Studies Hybridoma Bank) at a 1:50 dilution. Alexa Fluor 594-labeled goat anti-mouse

(Invitrogen-Molecular Probes, 1:1000) was used as secondary antibody. For ACAN, sections were incubated overnight with primary antibody (H-300) sc-25674 (Santa Cruz Biotechnology) to a 1:50 dilution. All samples were stained at the same time for comparison purposes.

Sections stained for COL2 were mounted in Fluorshield with DAPI (Sigma). Control sections for each labeling excluded primary antibody staining. In COL2 staining, representative images of the slides (covering all section) were taken using an inverted fluorescence microscope (Axiovert 200 M, Zeiss), and the same exposure time for all samples. COL2 intensity was quantified using a custom-made MATLAB (The MathWorks Inc., Natick MA, USA) script, the IntensityStatisticsMask Software.[33]

Sections stained for ACAN were imaged with light microscopy, using the same settings to allow comparison. Diaminobenzidine tetrahydrochloride (DAB) staining intensity, corresponding to ACAN deposition in the tissue, was quantified using a custom ImageJ H-DAB plugin, based on a color deconvolution technique, that calculated the contribution of DAB and hematoxylin, based on stain-specific red-green-blue (RGB) absorption.[34] By applying the macro, DAB and hematoxylin color channels were digitally separated, allowing quantification of color intensity only in the DAB channel. The measurement parameter was optical density (OD), obtained by log(max intensity/mean intensity), where max intensity corresponds to 255, for 8-bit images.[34] ACAN negative ($ACAN^-$) and positive ($ACAN^+$) cells were quantified using another custom-made MATLAB script, the ImmunoCellCounter Software, as previously described.[35]

2.13. Statistical analysis

Results are presented as Median±Interquartile Range (IQR) in box and whiskers plots. Data normality was first analyzed by D'Agostino and Pearson Normality Test after which statistical analysis was performed with either non-parametric Mann-Whitney or Kruskal-Wallis test and Dunns multiple comparison as post hoc test. MSCs migration was compared with Wilcoxon test for paired analysis. Graph Pad v6.02 for Windows. Tests were two-sided, and a confidence level of at least 95% (*, $p<0.05$) was used.

3. Results

3.1. *Metabolic activity, viability and apoptosis of MSCs and IVD in healthy vs pro-inflammatory/degenerated IVD environment*

To simulate the pro-inflammatory environment associated with IVD degeneration an *ex vivo* model of bovine IVD organ culture stimulated with IL-1β and puncture, previously established and validated by us was used.[30] Briefly, bovine disc punches were isolated and cultured

under static loading, and the degenerative/pro-inflammatory environment was induced by stimulation with needle puncture (21G) and medium supplementation with 10 ng/mL IL-1β.[30] Three hours after stimulus, $1x10^6$ human BM-derived MSCs were cultured on the top of the transwells, above the IVD punches. After 48 hours, IVD punches and MSCs on the top of the transwells were analyzed separately for metabolic activity and cell apoptosis/death, as schematically represented in Fig. 2(A). The mitochondrial metabolic activity of IVD punches was maintained in the presence of IL-1β and slightly decreased in the presence of MSCs for both control and IL-1β-stimulated IVDs [Fig. 2(B)]. Mitochondrial metabolic activity of MSCs remaining in the transwells was similar in the presence of IL-1β (IL-1β+MSCs) and non-stimulated group [Fig. 2(B)]. Cell apoptosis/death were analyzed by Annexin V (AnxV) and propidium iodide (PI) staining by flow cytometry, and these levels were overall low (under 20%) [Fig. 2(C)]. Results are presented as Median±IQR fold change. No differences were observed in cell apoptosis but an increase in the number of dead IVD cells ($AnxV^+PI^+$ cells) in the presence of MSCs was observed (from 6±2% to 14±7% in Ctr+MSCs and from 9±3% to

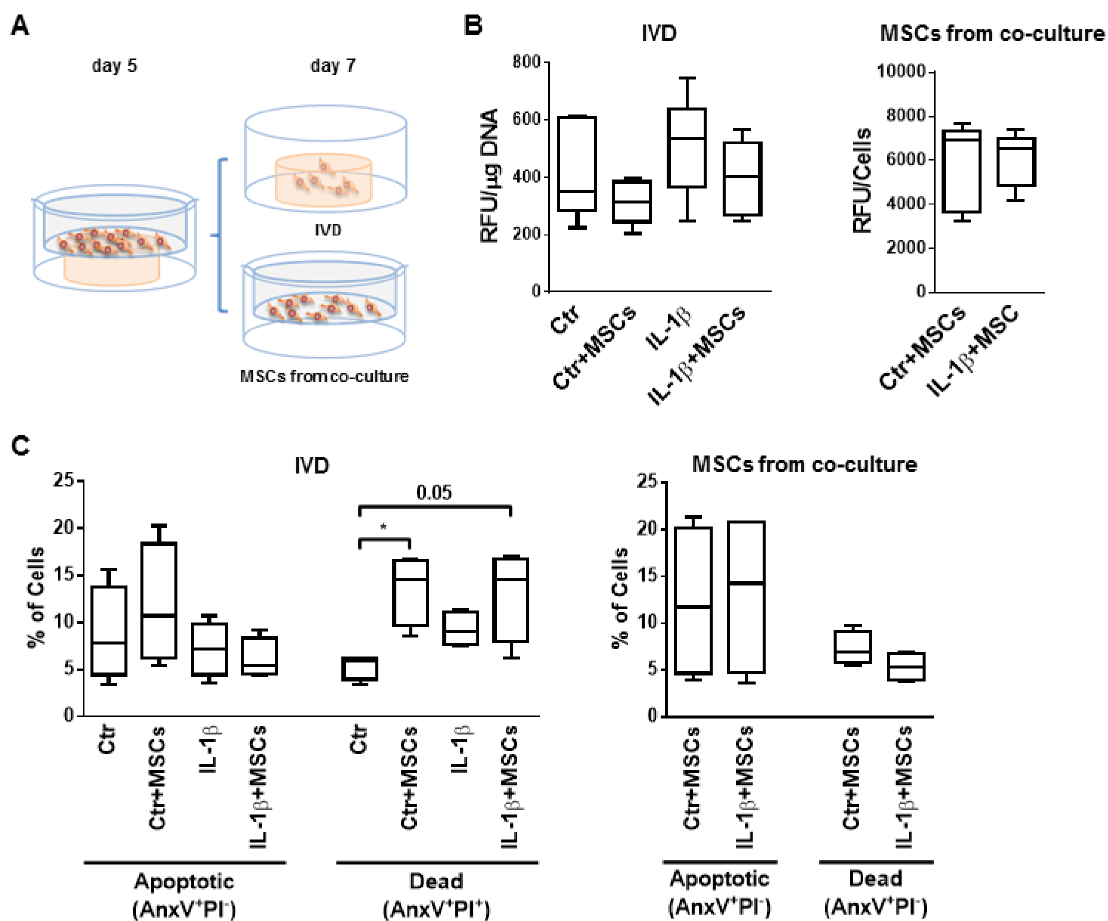


Fig. 2. Viability of the IVD organ culture model, 2 days after proinflammatory stimulus (puncture+10 ng/mL IL-1β) and co-culture with MSCs. (**A**) Experimental scheme of the samples used for the analysis performed (IVD tissue and MSCs from co-culture). (**B**) Mitochondrial metabolic activity of IVD tissue and MSCs in the transwells. (**C**) Early and late apoptosis/death of cells isolated from IVD tissue and MSCs in the transwells, by Annexin V/PI double staining. Results are shown as box and whiskers plots (n=4-5). *$p<0.05$

15±9% in IL-1β+MSCs), being this increase statistically significant in the Ctr groups ($p<0.05$). Nevertheless, apoptosis/death of MSCs collected from transwells was maintained in Ctr and IL-1β-stimulated conditions.

In parallel, MSCs labeled with CTB dye were seeded in the transwells above IVD punches. After 48 h MSCs collected from transwells remained blue [Fig. 3(A): a] and a microscopic evaluation of cell viability by LIVE/DEAD assay of a sagittal tissue section showed presence

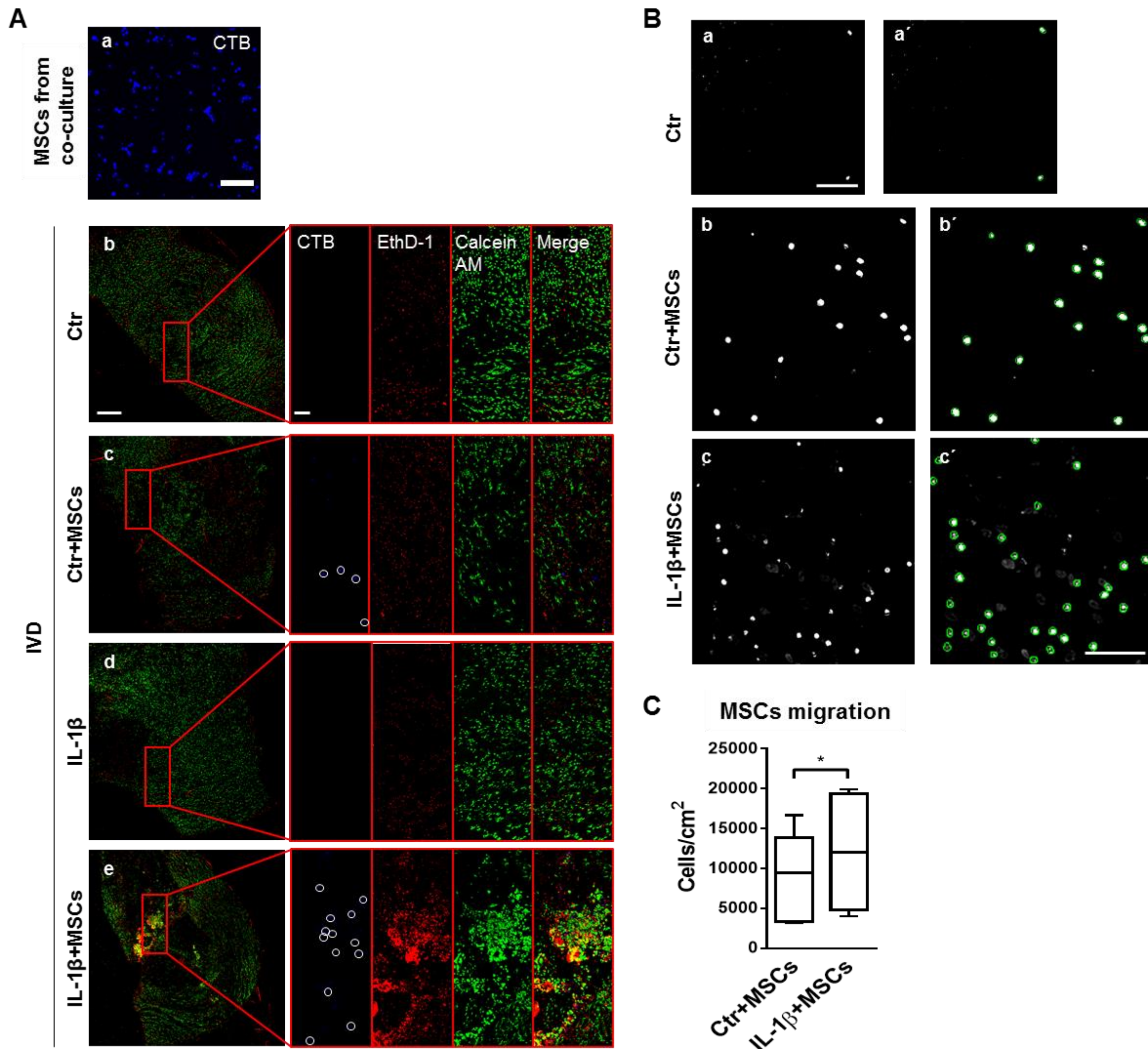


Fig. 3. Cell viability and MSCs identification, after 2 days in co-culture with IVD tissue, under control or proinflammatory/degenerative conditions. (A) Representative CLSM images (maximum intensity projection of serial optical sections) of MSCs from co-culture stained with CellTracker Blue (CTB, a; scale bar, 200 μm), and LIVE/DEAD cytotoxicity/viability assay (b-e; scale bar, 1mm), with higher magnification of splitted and merged channels, for all the conditions tested (CTB stains MSCs, ethidium homodimer-1 (EthD-1) stains dead cells and calcein AM stains live cells in green; MSCs, white arrow; scale bar, 200 μm). (B) Migration of human MSCs trough transwells with 8-μm pore size, when in co-culture with IVD punches alone (Ctr+MSCs), or in presence of 10 ng/mL IL-1β in the culture medium (IL-1β+MSCs). It is shown representative micrographs for groups Ctr (a), Ctr+MSCs (b) and IL-1β+MSCs (c), and their respective segmentation masks (a´, b´ and c´) for cell counting (counted cells in green; scale bar, 100 μm). (C) MSCs migration results normalized by the imaged area (cm^2) and respective controls. Results are shown as box and whiskers plots (n=6). *$p<0.05$

of dead cells in the IVD, with no apparent differences between Ctr and IL-1β-supplemented cultures [Fig. 3(A): b and d]. However, an increase of cell death in MSCs and IL-1β+MSCs groups was apparently observed [Fig. 3(A): c and e], in accordance with results from AnxV/PI staining. In basal conditions, the higher number of dead cells appears to be found in the lower half of the disk, being MSCs found closer to the upper IVD tissue border. On the other hand, in pro-inflammatory conditions, cell clusters formation was observed with higher number of MSCs. In addition, MSCs migration trough transwells on the top of the IVDs was analyzed by DAPI staining of the lower part of the membrane [Fig. 3(B)]. Representative images of Ctr, Ctr+MSCs and IL-1β+MSCs conditions are presented [Fig. 3(B): a, b and c, respectively]. MSCs migration was significantly increased in the presence of IL-1β ($p<0.05$). Of notice, flow cytometry analysis of IVD cells upon tissue digestion did not reveal the presence of labeled MSCs, suggesting that their frequency in the IVD was below to 0.01%, the equipment's detection limit (data not shown).

3.2. Screening of inflammatory factors produced by MSCs under pro-inflammatory/ degenerative culture conditions

To evaluate whether pro-inflammatory/degenerative IVD conditions could influence MSCs cytokine profile the protein content in the culture supernatants was evaluated by a human inflammatory cytokines array (40 proteins). A pool of samples from 8 independent experiments was used. The human factors detected only in the presence of MSCs are shown in Fig. 4(A), for Ctr+MSCs and IL-1β+MSCs groups. The results show that the IVD punches induced the production of IL-6, IL-8 and MCP-1 by MSCs, with higher intensity in pro-inflammatory/degenerative conditions (IL-1β+MSCs group). Also, tissue inhibitor of metalloproteinase (TIMP)-2 and IL-4 production seemed to increase in IL-1β+MSCs. To validate the array, IL-6, IL-8 and MCP-1 protein content in the supernatants was quantified by ELISA [Fig. 4(B)]. Results showed a statistically significant increase of IL-6, IL-8 and MCP-1 by MSCs in presence of IL-1β of about 6-fold for IL-6 ($p<0.0001$), 41-fold for IL-8 ($p<0.0001$) and 2-fold for MCP-1 ($p<0.05$). In the groups without MSCs (Ctr and IL-1β) IL-6, IL-8 and MCP-1 were glucose), osmolarity (400 mOsm), hypoxia (6% O_2 and 8.5% CO_2) and pro-inflammatory stimulus (10 ng/mL IL-1β) expressed higher *IL-6* and *IL-10*, produced higher PGE_2, while in presence of IL-1β [Supplementary Fig. S1]. Furthermore, other immune regulatory cytokines such as, TNF-α, IL-10, indoleamine-2,3-dioxygenase (IDO) and TNF-α stimulated gene/protein 6 (TSG-6) were also analyzed by ELISA, but if present, their values were below the detection limit of the technique.

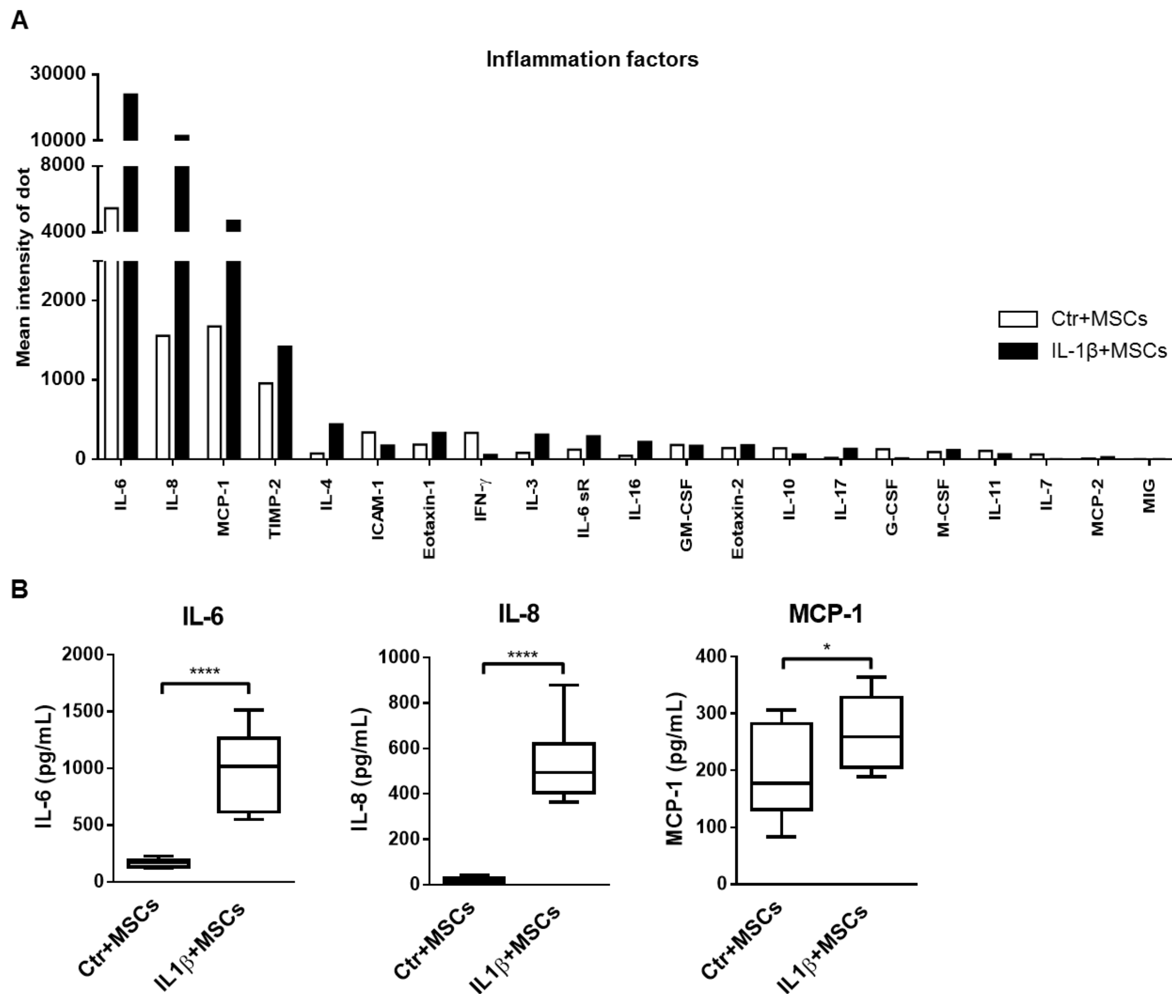


Fig. 4. MSCs production of inflammation factors, after 2 days in co-culture with IVD tissue, under control or proinflammatory/degenerative conditions. (A) Inflammation factors detected by human membrane cytokine array, for Ctr+MSCs and IL-1β+MSCs groups. Values are expressed as average of two dots. (B) Concentration of IL-6 (pg/mL), IL-8 (pg/mL) and MCP-1 (pg/mL) in co-culture supernatants. Results are presented as box and whiskers plots (n=8-13). *p<0.05; ****p<0.0001

3.3. Influence of MSCs in the profile of MMPs and ECM components

In the IVD organ culture model previously established, *MMP1* and *MMP3* were shown to be up-regulated, while ECM components *COL2* and *ACAN* were down-regulated in the IVD tissue under degenerative/pro-inflammatory conditions.[30] Here, MMPs and the main ECM components of IVD were analyzed 2 days after IL-1β stimulation and co-culture with MSCs [Fig. 5(A)]. The presence of MSCs did not induce by itself an up-regulation of *MMP1* and *MMP3* by IVD cells. On the other hand, it was observed up-regulation in IL-1β+MSCs, when compared to Ctr+MSCs co-culture, of *MMP1* of approximately 3-fold (p=0.05) and of *MMP3* of about 7-fold (p<0.05). *MMP13* expression was down-regulated in Ctr+MSCs, relatively to Ctr (0.3±0.6, p<0.05). Furthermore, MSCs were not able to down-regulate the increased

levels of MMPs in presence of IL-1β. Concerning ECM proteins, *COL2* and *ACAN* were significantly down-regulated in all the conditions tested in comparison to the control. Furthermore, it was also seen a down-regulation of *COL2* after IL-1β-stimulated discs' co-culture with MSCs to 0.09±0.06, compared both with Ctr+MSCs (0.2±0.2) and IL-1β (0.2±0.2) groups (p<0.01). *ACAN* expression did not seem to be altered after IL-1β+MSCs stimulation, when compared either with Ctr+MSCs or IL-1β alone groups.

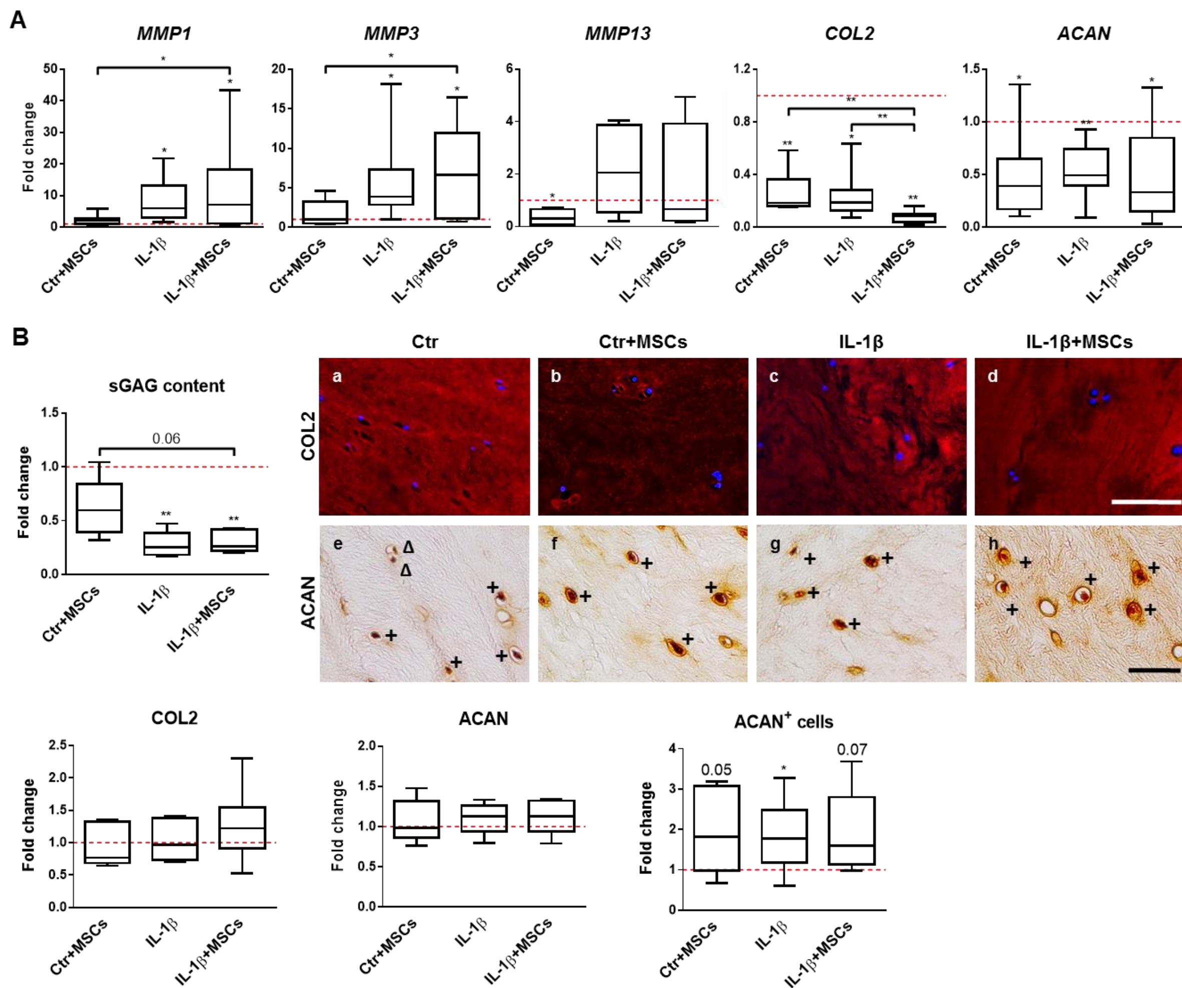


Fig. 5. Effect on the IVD ECM composition of proinflammatory/degenerative stimulus and co-culture with MSCs, after 2 and 14 days. (A) mRNA expression of bovine *MMP1*, *MMP3*, *MMP13*, *COL2A1* and *ACAN* of IVD cells, after 2 days of co-culture. mRNA levels were normalized to *GAPDH* control gene and to the unstimulated discs (control level=1; dashed line). (B) Analysis at protein level of IVD ECM components, 14 days after co-culture. Biochemical analysis of sGAG content of IVD punches, normalized to control. Representative sagittal sections of disc punches stained for COL2A1 (a-d; scale bar, 100 μm) and ACAN (e-h; scale bar, 50 μm), displaying ACAN negative ($ACAN^-$, Δ) and positive ($ACAN^+$, +) cells. COL2A1 fluorescence intensity normalized to control. ACAN intensity in the tissue, determined by measuring the optic density (OD) of the DAB staining, normalized to control. Percentage of $ACAN^+$ cells normalized to the imaged area (mm^2) and to control group. Results are shown as box and whiskers plots (n=5-20). **P* < 0.05; ***P* < 0.01

3.4. Evaluation of ECM remodeling in longer-term pro-inflammatory MSCs/IVD co-culture

To evaluate the effects of MSCs co-culture on IVD tissue ECM at protein level, the organ cultures were maintained for 21 days, after which sGAG content was quantified, and COL2 and ACAN deposition were analyzed by IHC [Fig. 5(B)]. A statistically significant decrease of sGAG content of about 0.3 in both IL-1β and IL-1β+MSCs groups was observed, compared to Ctr ($p<0.01$). Also in the Ctr+MSCs group sGAG content seemed to be lower in comparison with Ctr (0.6±0.4). COL2 staining [Fig. 5(B): a-d] fluorescence intensity was quantified using the IntensityStatisticsMask software and is presented as fold change to Ctr group. Results indicated similar COL2 for Ctr+MSCs and IL-1β groups, compared to Ctr. Yet, IL-1β+MSCs samples seemed to have slightly higher COL2 content, when compared to Ctr (1.2±0.6-fold) and to Ctr+MSCs (about 2-fold). ACAN deposition (brown) [Fig. 5(B): e-h], as well as cells negative ($ACAN^-$, Δ) and positive ($ACAN^+$, +) for ACAN deposition were quantified for the different conditions and normalized to Ctr. ACAN deposition in tissue was determined by color intensity measurements in DAB channel and presented as OD fold change to unstimulated IVD punches, for each donor. As mentioned in Supplementary Materials and Methods, all slides were stained at the same time to allow comparison between them. Overall, results indicated similar ACAN content in all the different conditions tested. Nonetheless, the numbers of $ACAN^+$ and $ACAN^-$ cells were quantified using the ImmunoCellCounter software, and presented as % of $ACAN^+$ cells, normalized to the imaged area (mm^2) and in fold change to Ctr. Results showed that IVD stimulation with MSCs alone (Ctr+MSCs), IL-1β or IL-1β+MSCs appeared to increase the % of $ACAN^+$ cells/mm^2, compared to Ctr discs. These results represented a fold change of approximately 2-fold for all Ctr+MSCs, IL-1β and IL-1β+MSCs. Overall, no significant effect of MSCs in ECM production was observed, when compared to either control or IL-1β cultures.

3.5. Inflammatory gene expression in cells isolated from the organ culture, 2 days after culture in pro-inflammatory conditions

IVD cells pro-inflammatory gene expression profile was assessed by the expression of *IL-6*, *IL-8* and *TNF-α*, 2 days after IL-1β stimulation and co-culture with MSCs [Fig. 6(A)]. In the IVD organ culture model an up-regulation of inflammatory markers *IL-6* and *IL-8* (14±29-fold and 8±8-fold, respectively; $p<0.01$) were here observed in the presence of IL-1β, in accordance with previous results[30]. *TNF-α* gene expression was similar between the IL-1β group and the control. Interestingly, IVD cells in co-culture with MSCs in basal conditions expressed similar *IL-6* and *IL-8* levels and *TNF-α*, when compared to Ctr.

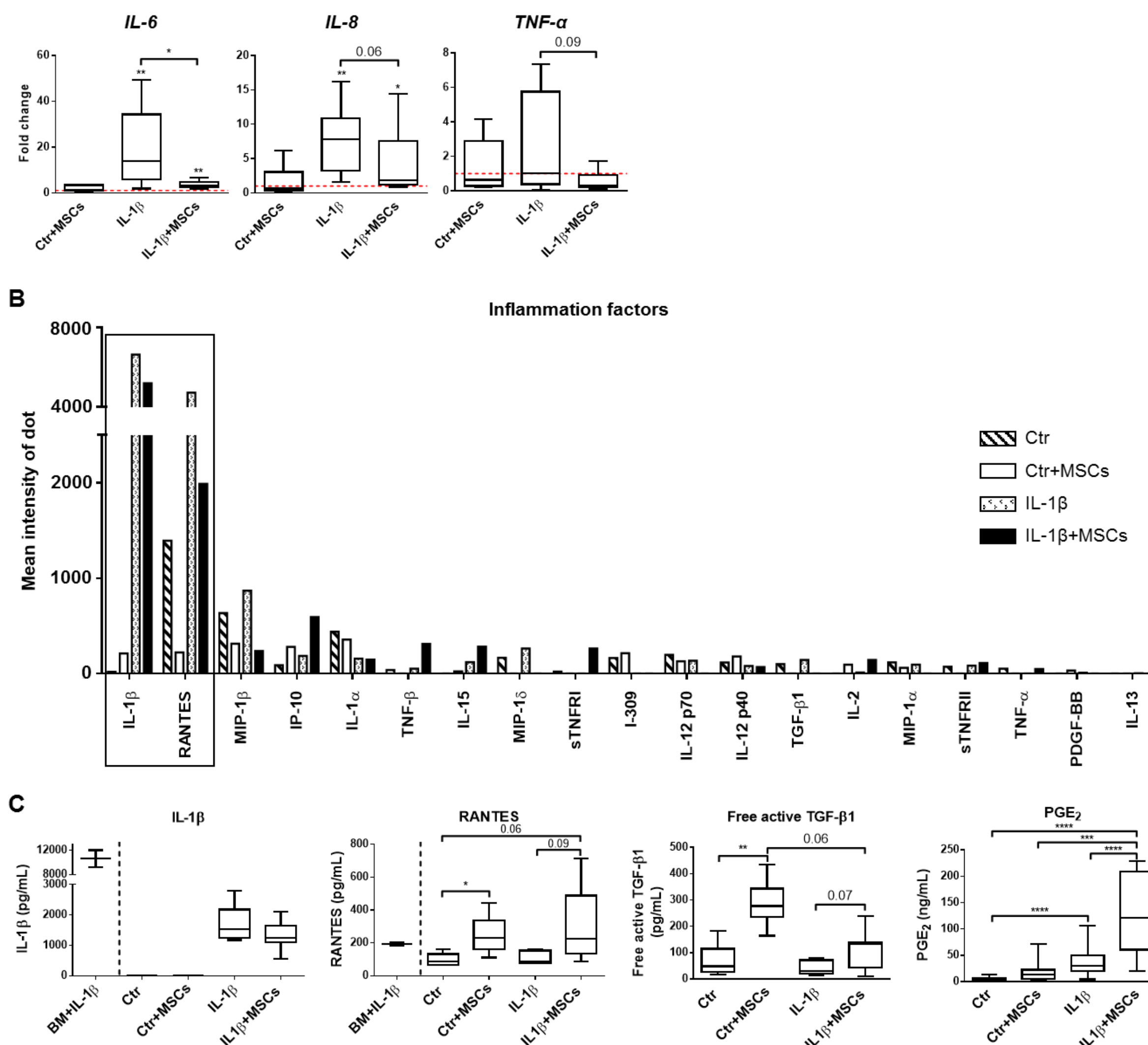


Fig. 6. Inflammatory profile of IVD cells and MSCs, after 2 days in co-culture under control or proinflammatory/ degenerative conditions. (A) mRNA expression of bovine proinflammatory markers *IL-6*, *IL-8*, *TNF-α* by IVD cells. mRNA levels were normalized to *GAPDH* control gene and to the unstimulated discs (control level=1; dashed line). (B) Quantification of the detected cytokines of array membranes obtained for all conditions tested, values are expressed as average of two dots. (C) Concentration of IL-1β (pg/mL), RANTES (pg/mL), TGF-β1 (pg/mL) and PGE_2 (ng/mL) in co-culture supernatants. Results are presented as box and whiskers plots (n=6-34). **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001

In presence of IL-1β, MSCs significantly down-regulated *IL-6* (from 14±28- to 3±2-fold; p<0.05). In addition, MSCs also seemed to down-regulated bovine *IL-8* levels (from 8±8- to 2±6-fold, p=0.06) and *TNF-α* (from 1±5-fold to 0.3±0.7-fold; p=0.09). Although gene expression of these pro-inflammatory players remarkably decrease in the presence of MSCs, their expression levels were still significantly up-regulated compared to control IVDs.

In addition, the inflammatory cytokine array performed on IVDs culture supernatant allowed us to screen the factors involved in the immunoregulatory crosstalk between MSCs and IVD cells. The factors identified either in MSCs/IVD co-cultures and IVD cultures alone are

depicted in Fig. 6(B). IL-1β, RANTES and macrophage inflammatory protein (MIP)-1β concentrations were lower in IL-1β+MSCs than in IL-1β-stimulated samples alone. On the other hand, it seemed that IP-10 concentration was higher in IL-1β+MSCs in comparison to IL-1β. To validate the array's qualitative results, IL-1β and RANTES were quantified by ELISA, in supernatants from experiments other than the ones used in the array analysis, as well as free-active TGF-β1 and PGE_2 [Fig. 6(C)]. In basal conditions, IL-1β was not detected (Ctr and Ctr+MSCs), but a consumption of IL-1β in both groups in pro-inflammatory conditions (IL-1β and IL-1β+MSCs) was observed from 10.6±2.8 ng/mL to approximately 1.5±0.9 and 1.2±0.5 ng/mL, respectively. RANTES was detected in the basal medium, as well as in all the experimental conditions tested, and was apparently consumed in the Ctr and IL-1β conditions, showing a reduction about 0.4. RANTES was produced in the presence of MSCs, with increase of approximately 1.2-fold, in both Ctr+MSCs and IL-1β+MSCs groups. RANTES concentration in presence of MSCs was significantly higher in basal conditions (about 2.7-fold to Ctr, $p<0.05$) and slightly higher in pro-inflammatory conditions (circa 2.6-fold to IL-1β). In addition, although total TGF-β1 was not identified in the inflammation array, free active form of TGF-β1 was detected in all experimental conditions. MSCs in Ctr IVDs contributed to an increase of TGF-β1 from 49±89- to 277±107-fold ($p<0.01$), while in the IL-1β groups, an increase from 30±51- to 132±97-fold was observed in the presence of MSCs ($p=0.07$). A significantly higher production of PGE_2 was observed in IL-1β-stimulated samples, compared to Ctr ($p<0.0001$), similarly to what was previous described by us.[30] PGE_2 production slightly increased from 4±3- to 13±18-fold when MSCs were co-cultured with IVD punches in basal conditions. But under pro-inflammatory conditions, MSCs remarkably increased PGE_2 from 30±29- to 121±148-fold ($p<0.0001$).

4. Discussion

This study investigated the regenerative and immunomodulatory role of MSCs in a pro-inflammatory/degenerative IVD *ex vivo* model. This organotypic culture consists in bovine IVD explant cultures stimulated with needle puncture, IL-1β supplementation (10 ng/mL) and 0.46 MPa static loading and was previously validated by us.[30] An up-regulation of pro-inflammatory markers (*IL-6*, *IL-8*, *MMP1* and *MMP3*), as well as a down-regulation of ECM components (*COL2* and *ACAN*) were observed in this model,[30] in accordance with human disc degeneration,[7,36] in which IL-1 was proposed as a key regulator of matrix-degrading enzymes.[37-39] In this study, $1x10^6$ MSCs/IVD were selected as the optimal cell concentration based in previous works with other models.[40] MSCs were added to IVD culture 3 hours after pro-inflammatory stimulus. Maidhof et al. studies in a rat disc stab injury model suggest that cell administration at an early stage of injury/disease progression might decrease matrix loss,

through a potentially higher MSCs activity, due to the inflammatory microenvironment associated with injury.[41] Nonetheless, higher metabolic activity might also be connected to cell senescence.[42,43] Cell apoptosis and death remained low in our study and MSCs did not present an anti-apoptotic effect, in contrast with findings from Yang and colleagues.[44] Of notice, there was formation of cell cluster inside the NP and higher MSCs migration in presence of IL-1β-stimulated IVDs, which could be due to an increased production of chemotactic recruitment mediators such as, for example, RANTES,[45] TNF-α and/or IL-1β.[46]

In this pro-inflammatory/degenerative IVD model, MSCs exhibited overall a pro-inflammatory profile, producing higher amounts of IL-6, IL-8, MCP-1, TIMP-2 and IL-4, and contributing to an increase of PGE_2 production, while seemed to have decreased free active TGF-β1 production. MSCs pro-inflammatory profile was also previously observed when MSCs were injected into the IVD.[30] In the work of van Buul et al., MSCs stimulated with TNF-α and interferon (IFN)-γ showed higher production inflammation markers (IL-6, IDO) and anti-catabolic TIMP-2, whereas TGF-β1 decreased.[47]

It has been proposed that MSCs can create negative feedback loops as mode of action.[48] For instance, while TNF-α and other pro-inflammatory cytokines from resident macrophages have shown to activate MSCs to secrete TSG-6 on injured cornea,[49] pro-inflammatory cytokines, nitric oxide, and other damage-associated molecules from injured tissues have also been shown to activate MSCs to secrete PGE_2, which bound to macrophages and polarized them to an M2 phenotype that secreted IL-10.[50] IVD cultures in degenerative and conditions stimulated MSCs to significant increase the expression of chemokine ligand CCL5/RANTES and chemokine receptors CCR1 and CCR4,[45] as well as to produce factors as MCP-1 and MIP-1α, described to have a variety of pro-inflammatory activities, including chemotaxis.[51]

TGF-β is known to enhance proteoglycans and COL2 in NP 3D cultures,[52] and it is conventionally used to induce MSCs differentiation into a NP-like phenotype.[8] This cytokine has a potent regulatory and inflammatory activity and, among others, regulates MSCs immune responses.[53] In a human MSCs/IVD fragments co-culture model it was observed, over time in culture, up-regulation of *TGF-β1* by MSCs, and a decrease of *TNF-α*, stabilization of *IL-1α* and up-regulation of *IL-1β* expression by IVD cells.[38] This is in opposition with our observations, in which a decrease of TGF-β1 production was related with a decrease of inflammatory markers *IL-6*, *IL-8* and *TNF-α* of bovine IVDs. We hypothesize that it may be due to the differences between study models (human *vs* bovine) and to the culture stimulation with IL-1β in the bovine and not in the human model that may modify MSCs mode of action.

PGE_2 is known to be produced by both IVD cells[54] and MSCs,[55] in response to pro-inflammatory cytokine signaling, particularly IL-1β, as it was observed in this co-culture

model. Activated by environmental signals, PGE_2 from MSCs exert regulatory influence on the activation status, proliferation, differentiation and function of immune cells from adaptive and innate immunity.[53] Additionally, it has been shown that cyclooxygenase (COX)-2/PGE_2 pathway may be one of the modulators of MSCs anti-inflammatory mechanism of action in osteoarthritic chondrocytes.[26]

MSCs stimulation of proteoglycans and COL2 production in IVD were already reported in different literature models, in which the inflammatory environment was not addressed.[35,56,57] Furthermore, *in vivo* observations showed increased ECM components only after 12[58] to 48 weeks.[56] In the present inflammatory conditions, *MMP1* and *MMP3* expression by IVD cells was up-regulated 2 days after co-culture with MSCs and no stimulatory effect of MSCs was observed at ECM level after 14 days, which we hypothesized that could be due to be an early time point. In agreement with our results, van Buul et al. observe significant down-regulation of COL2 gene expression in human osteoarthritic cartilage explants cultured with TNF-α and IFN)-γ stimulated MSC-conditioned medium.[47] The literature suggests that MSCs have an immunomodulatory response when in an inflammatory environment,[26] and that they are mainly triggered to first counteract inflammation instead of stimulating matrix formation.[47]

Overall, MSCs co-culture with IVDs under degenerative/pro-inflammatory conditions contribute to a less pro-inflammatory profile of native IVD cells. This immunomodulatory action was already described in osteoarthritic chondrocytes cultures with adipose-derived MSCs by a reduction of IL-6, IL-8, IL-1β, MCP-1, MIP-1α and RANTES[26] and in rat NP cells, co-cultured with human synovial MSCs, where it was observed down-regulated gene expression of, for example, nuclear factor, interleukin 3 regulated, *IL-15,* IL-6 signal transducer, IL-11 receptor, alpha chain 1, *TSG-6* and TNF receptor superfamily, member 6.[59] However, MSCs influence in IVD inflammatory response, degeneration and regeneration has not yet been extensively characterized.[10]

Here, MSCs seemed to possess anti-inflammatory, but not anti-catabolic properties in the pro-inflammatory/degenerative IVD. This mode of action seems to occur via a negative feedback loop, with increasing production of pro-inflammatory factors by MSCs. Overall, this study calls the attention to the need of more thorough studies before the widespread use of MSCs-based approaches for LBP. Moreover, differences in people's genetic predisposition may impact on the response to MSCs immunomodulation and thus affect LBP and IVD function. In the future, it would be interesting to explore the effect of MSCs in more complex models of IVD degeneration/inflammation. It is crucial to better understand the interactions between MSCs, IVD cells and immune cells in the context of the degenerated intervertebral disc and associated inflammation and pain.

Contributions

GQT, CN-W, MAB and RMG substantially contributed to the study conception and design. GQT and RMG contributed to the acquisition, analysis and interpretation of data, and to drafting the article. CLP and JRF contributed to data acquisition. AFM and MG-L contributed to image acquisition and analysis. MAB and RMG provided the funding for this study. All authors have critically revised the manuscript for important intellectual content and approved its final version.

Competing interests

The authors declare no competing financial interests.

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Supplementary Data

Materials and Methods

Culture of human MSCs

The data regarding age, gender, bone marrow origin and passaged used for the experiments conducted for the present work are shown in Table S1.

Table S1. Human MSCs Donors.

Donor	Age	Gender	Bone marrow origin	Passage used
1	18	male	Healthy donor for bone marrow for transplantation	3
2	21	male	Aspirate from knee joint surgery	5, 6, 7
3	22	male	Aspirate from knee joint surgery	5
4	25	female	Aspirate from knee joint surgery	5
5	34	male	Aspirate from knee joint surgery	3
6	45	male	Hip replacement surgery	4
7	56	female	Hip replacement surgery	3

Human MSCs 2D culture and quantitative real-time reverse transcription polymerase chain reaction

In parallel with IVD punches co-culture with MSCs in the transwells, a 2D culture was performed. MSCs ($1x10^6$) were seeded in 6-well plates and stimulated by IVD culture medium supplemented with 10 ng/mL IL-1β (IL-1β 2D). MSCs cultured in IVD basal medium were used as control (Ctr 2D).

MSCs gene expression was analyzed 2 days later by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Specific primer pairs were designed using published gene sequences (PubMed, NCBI Entrez Nucleotide Database) and Primer 3 software for human *GAPDH*, *TNF-α*, *IL-6*, *IL-8*, *IL-10*, *MMP1*, *MMP3*, *MMP13*, *COL2* and *ACAN* (Table S2), and synthesized by Thermo Fisher Scientific.

Table S2. Human oligonucleotide primers

Gene	Forward and reverse primer, 5'-3'	Product length (bp)	NCBI reference sequence
GAPDH	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTTC	224	NM_002046
TNF-α	AACCTCCTCTCTGCCATCAA GGAAGACCCCTCCCAGATAG	100	HQ201306
IL-6	AGGAGACTTGCCTGGTGAAA CAGGGGTGGTTATTGCATCT	180	NM_000600
IL-10	CTGGGTTGCCAAGCCTTGTCTGA ATCGATGACAGCGCCGTAGCC	154	NM_000572.2
MMP1	ATGCTGAAACCCTGAAGGTG CTGCTTGACCCTCAGAGACC	234	NM_002421

MMP3	GGAGATGCCCACTTTGATGAT CATCTTGAGACAGGCGGAAC	187	NM_002422
MMP13	TTGAGCTGGACTCATTGTCG GGAGCCTCTCAGTCATGGAG	172	NM_002427
COL2	CGCACCTGCAGAGACCTGAA TCTTCTTGGGAACGTTTGCTGG	162	XM_056481
ACAN	TCTGTAACCCAGGCTCCAAC CTGGCAAAATCCCCACTAAA	199	XM_007701

Results

Evaluation of MSCs anti-inflammatory potential in the proinflammatory conditions 2 days after 2D culture

After 2 days of culture, MSCs gene expression analysis showed a significant up-regulation of proinflammatory interleukin *IL-6*, anti-inflammatory *IL-10*, matrix degrading enzymes *MMP1* and *MMP13*, and ECM component *ACAN*, compared to Ctr 2D ($p<0.05$) [Fig. S1(A)]. *TNF-α* expression was similar between IL-1β 2D and Ctr 2D groups. Furthermore, *MMP3* and *COL2* expression was not detected in MSCs 2D cultures, although they were previously shown to be expressed by human by nucleus pulposus cells.[1]

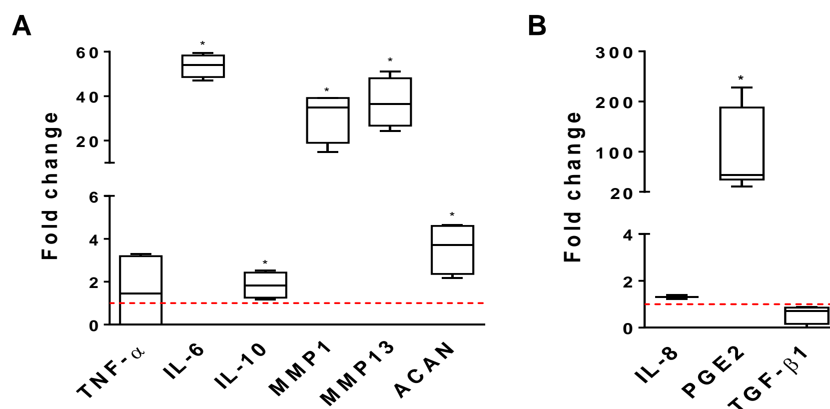

Fig. S1. Effect on MSCs in 2D culture of proinflammatory stimulus with culture medium supplementation of 10 ng/mL IL-1β, for 2 days. (A) mRNA expression of proinflammation markers (*TNF-α*, *IL-6*, *IL-10*), matrix degrading enzymes (*MMP1, MMP13*), and an ECM component (*ACAN*) in MSCs 2D culture. Levels of mRNA were normalized to *GAPDH*. The ratio of stimulation to control (Crt 2D) indicates the fold change of induction after stimulation (control level = 1; dashed line). (B) Fold change of IL-8, PGE_2 and free active TGF-β1 concentrations normalized to control (Crt 2D) (n=3-7). *$p<0.05$

IL-8, PGE_2 and free active TGF-β1 productions were quantified in culture supernatants [Fig. S1(B)]. After 2 days of stimulation with IL-1β, PGE_2 production by MSCs was significantly increased ($P = 0.02$), while TGF-β1 production seemed to have decreased about 0.7±0.7, in comparison to control.

These results indicate that MSCs cultured in 2D are sensitive to the presence of proinflammatory conditions, namely IL-1β, presenting a more proinflammatory profile.

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CHAPTER VIII

General discussion and future perspectives

The work on this thesis was developed in view of enhancing knowledge concerning the inflammatory response of degenerated IVD, as well as to propose immunomodulatory therapies for this disorder.
As proposed by other authors, the modulation of inflammation is key for promoting matrix synthesis by the cells in the IVD microenvironment, both by native or transplanted cells (Wuertz and Haglund 2013, Risbud and Shapiro 2014, Gorth et al. 2015). For instance, herniated tissues, mostly extruded but also with intact AF structure (Lee et al. 2009, Phillips et al. 2015), are often described to contain abundant macrophage infiltration (Peng et al. 2006, Kokubo et al. 2008, Shamji et al. 2010, Wuertz and Haglund 2013). AF tear and consequent NP leakage is recognizable to the immune system as a foreign body, activating immune cell migration (lymphocytes, such as natural killer, T and B cells, and monocytes/macrophages) and infiltration in the extruded tissue, which together with the produced cytokines, amplify the inflammatory response, leading to increased innervation and associated pain (Sun et al. 2013b, Risbud and Shapiro 2014). The control of the inflammatory response for successful tissue repair/regeneration has been explored in other tissues, such as bone (Rozen et al. 2007, Santos et al. 2013, Lin et al. 2017), skin (Cardoso et al. 2011), tendon (Shen et al. 2016), cartilage (Pers et al. 2015, Kim et al. 2016, Sakata and Reddi 2016), peripheral nerve (Bombeiro et al. 2016), spinal cord (Watanabe et al. 2015), kidney (Semedo et al. 2009) or cardiac tissue (Han et al. 2015, Reina-Couto et al. 2016, Zlatanova et al. 2016), among others. In this work, the modulation of inflammation in degenerated IVD was attempted to promote tissue repair. However, it is important to highlight that the healthy human IVD has specific characteristic that may render difficult the reestablishment of homeostasis upon degeneration. The IVD is the largest avascular and aneural tissue in the body (Urban and Roberts 2003, Raj 2008, Huang et al. 2014), populated by a small number of resident cells in a slightly acidic and hypoxic environment (Huang et al. 2014). Moreover, it has access to low nutrient supply and is subjected to high mechanical and osmotic pressures (Rinkler et al. 2010).

The first contribution of this thesis to the field was the establishment of a study model of IVD degeneration and inflammation *ex vivo* (Chapter IV) that was shown to be adequate to test different therapeutics for IVD degeneration and associated inflammation (Teixeira et al. 2015, Teixeira et al. 2016). Due to the low accessibility of healthy human IVD tissue, this revealed to be a simple and inexpensive model (Alini et al. 2008), with high availability, enabling the design of more complex experiments requiring higher number of replicates. Nonetheless, due to the IVD's great swelling potential (Urban et al. 1979), and variability of IVD sizes within the same tail, it is difficult to establish a reproducible model of IVD tissue without endplate. To overcome these issues, standardized punches were performed to collect similar tissue samples from the different discs along the tail (with few surrounding AF) and these were cultured with membrane

filter inserts on top and under 0.46 MPa static loading, to prevent swelling (Teixeira et al. 2015). This pro-inflammatory/degenerative model was developed with needle puncture and IL-1β stimulation, a more physiological approach than most of the models available at the time, that simulated tissue degeneration by: 1) tissue removal (Pereira et al. 2014), 2) injury (Korecki et al. 2008) and/or 3) loading (Korecki et al. 2007, Illien-Junger et al. 2012, Pattappa et al. 2014), or also by tissue digestion with 4) chemical compounds, such as papain (Bucher et al. 2013, Chan et al. 2013, Malonzo et al. 2015) or trypsin (Jim et al. 2011, Gawri et al. 2014a, Mwale et al. 2014, AlGarni et al. 2016). Other *ex vivo* models in the presence of TNF-α were established for bovine (Purmessur et al. 2013b) and rat IVD (Walter et al. 2015, Walter et al. 2016). Ponnappan and colleagues (2011) had developed a rat lumbar IVD pro-inflammatory/degenerative model with IL-1β and TNF-α stimulation. This system is an atraumatic *in vitro* model of early IVD degeneration, and therefore, based on changes in the microenvironmental cues that promote wide changes in the expression of several genes linked to the IVD degenerative process (Ponnappan et al. 2011, Markova et al. 2013). The culture of the IVD with endplates allows for a better preservation of tissue structure, avoiding excessive swelling. Nonetheless, given the differences regarding the NP and IVD volumes from the rat lumbar (about 5 to 19 mm^3), bovine tail (1 to 4 cm^3), and human lumbar (5 to 20 cm^3) regions (O'Connell et al. 2007), bovine IVDs promote a similar environment to human, namely for translation of intradiscal injection treatment volumes. Also, bovine discs suffer similar loss of notochordal cell content as humans, while rat IVDs preserve them in great number throughout adulthood, which confers a higher regenerative capacity to this model, that is far away from the human IVD potential (Alini et al. 2008). Moreover, bovine tails are cost-efficient and easily available for our studies. Recently, Krupkova et al. (2016) proposed an *ex vivo* bovine NP tissue culture in presence of IL-1β and TNF-α, to simulate the pro-inflammatory environment. In their work, the NP tissue is cultured inside a hypertonic polyethylene glycol structure, used as an artificial annulus system that was previously shown to prevent tissue swelling and proteoglycans loss, while maintaining cell viability for 42 days (van Dijk et al. 2013). Nonetheless, our NP punches culture with few surrounding AF, membrane filter inserts on top and under 0.46 MPa static loading also prevent excessive swelling (Teixeira et al. 2015).

Though our model was shown to be suitable to test the direct effect of intradiscal therapies on IVD cells, which can be analyzed without the complex *in vivo* cell crosstalk, we consider that this model can be improved. The *ex vivo* model was developed without simulation of the normal physiological loadings to which discs are exposed *in vivo* (Haschtmann et al. 2006, Junger et al. 2009, Gawri et al. 2014a), which might influence the outcome. Moreover, using disc punches are a reliable simplification step, but not as physiological when compared to more complex organ culture approaches which use complete discs with the adjacent endplates and additional dynamic loading application (Illien-Junger et al. 2012, Pattappa et al. 2014, Walter

et al. 2015, Walter et al. 2016). Another limitation is the absence of vascularization and innervation, and therefore, of immune cells in the disc and surroundings. To improve this, it would be interesting to complex the *ex vivo* model to focus on inflammation studies and correlation with pain mediators, since not all patients diagnosed with degenerative disc disease report pain, which suggests that only some specific features of disc degeneration are associated with LBP (Cheung et al. 2009, de Schepper et al. 2010, Adams et al. 2014). The establishment of a co-culture of the IVD punches with, for instance, macrophages (currently being performed in our team) will allow a better understanding of the local interactions between these cells and the IVD, in presence of different treatments. Previous *ex vivo* works showed that macrophage-IVD interactions promote the secretion of matrix degrading enzymes linked to a positive effect in spontaneous hernia regression (Haro et al. 2000, Doita et al. 2001), but on the other hand they play a major role in sciatica and in the production of TNF-α, IL-6, IL-8 and PGE_2 (Takada et al. 2004, Takada et al. 2012). Therefore, such model would be of great interest to further test the intradiscal therapies proposed in this thesis.

Nanotechnology-based therapies present several advantages for drug delivery. Chitosan (Ch) and poly(γ-glutamic acid) (γ-PGA) nanoparticles/nanocomplexes (NCs) have been previously used in our group to deliver an anti-inflammatory drug, diclofenac (Df). These Df-NCs inhibited and reverted macrophage activation *in vitro* (Gonçalves et al. 2015). In this thesis, Df-NCs intradiscal injection was attempted using the pro-inflammatory organ culture previously established. IVD cells were able to internalize the particles, which promoted down-regulation of *IL-6*, *IL-8*, *MMP1* and *MMP3* expression and decreased PGE_2 production, while NCs by themselves only significantly decreased *MMP3*. Moreover, Df-NCs promoted an increase in matrix proteins production by native cells, namely COL2 and ACAN, while NPs alone increased ACAN production only. γ-PGA is one of the most appealing natural polymers, mainly due to its biodegradability into glutamate residues. Since γ-PGA is anionic (pKa 2.19) it can be easily combined by electrostatic interaction with cationic polymers as Ch (Antunes et al. 2011), forming polyelectrolyte complexes with great potential as delivery systems. γ-PGA has been pointed out by prior works from our team to promote earlier chondrogenic differentiation of MSCs in pellet culture (Antunes et al. 2015) and to enhance COL2 production in a nucleotomized IVD *ex vivo* model (Antunes et al. 2017).

Despite the promising results ex vivo, we failed to determine the most adequate concentration of Df-NCs for intradiscal injection in the rat caudal injury model (Cunha et al. 2015), as discussed in Chapter VI. The injection of 10 µL of Df and Df-NCs 50 times concentrated represented a too high NCs/NP volume ratio (over 2 times higher injection volume than the NP volume). Moreover, given that these solutions were injected at pH 5.0, a high acidification of

the IVD environment may have occurred, impeding the release of Df from Df-NCs. We look forward to improve the delivery system and to better understand if the pH of the rat IVD after injury drops under healthy physiological values of around 7.1 (Ichimura et al. 1991). Moreover, we are also performing experiments to determine the Df release kinetics from the NCs in solutions at different pH values, ranging namely from 6.5, pointed out the be characteristic of a severely degenerated environment (Kitano et al. 1993), to 7.1. On the other hand, further complexation of this system is required to promote prolonged drug release. Nanocapsules production in a layer-by-layer methodology could facilitate a single intradiscal injection with prolonged release periods.

Nonetheless, although the degeneration/herniation rat caudal model is of great interest to study molecular mechanisms in a complex environment, such as cell recruitment (Cunha et al. 2016), it presented limitations in the translation from *ex vivo* bovine organ cultures (as seen in Chapter VI), namely related with the determination of the most adequate volume and dosage for intradiscal injection of therapeutics or implantation of engineered tissue constructs, as reviewed by Zhang et al. (2011a). Large animal models, reviewed in Chapter I, as sheep or goat present several advantages regarding similar loss of notochordal cells and IVD size as humans, and even if quadruped, they suffer similar mechanical loadings applied on the lumbar spine region (O'Connell et al. 2007, Alini et al. 2008, Daly et al. 2016), facilitating the translation of volumes and concentrations, compared to rat (Zhang et al. 2011a).

Mao et al. studied the effect of injection volume on disc degeneration in a rat tail model, and observed significantly higher histologic score in IVDs, 1 week after injection of 3 µL of saline solution, and more severe degeneration, particularly during week 4, when compared to animals injected with 2.5 µL of saline solution or less (Mao et al. 2011). Rat degenerate discs treated with 2 µL simvastatin in a hydrogel carrier demonstrated, after 6 weeks, radiographic and histologic features resembling non-injured IVDs (Than et al. 2014). Nonetheless, other studies of rat intradiscal injection of about 8 µL of growth factors solutions have shown to promote matrix synthesis (Walsh et al. 2004, Matta et al. 2017). The needle diameter used is also of great importance to minimize the risk associated with further IVD degeneration. Elliott et al. (2008) review of several animal IVD models of needle puncture or sham injection denoted that needle injection in models where needle diameter/disc height ratio was smaller than 25%, no significant disc changes seemed to be observed regarding degenerative features.

In addition, the degree of acetylation (DA) of the Ch used to produce the NCs is about 11%. Nonetheless, Vasconcelos et al. (2013) showed that Ch scaffolds with 5% DA induced the adhesion of lower numbers of inflammatory cells after implantation in a murine air-pouch model, having the adherent macrophages predominantly an anti-inflammatory phenotypic profile (M2), compared to scaffold produced with Ch with 15% DA, predominantly with pro-inflammatory M1 macrophages both adherent to the scaffold and in the exudates. Therefore,

it would be of interest to produce NCs with different DA, namely 5%, and analyze *in vivo* if differences would be observed regarding the percentage of macrophages migration to the hernia, as well as their inflammatory profile. Additionally, the incorporation of immunomodulatory molecules, such as TGF-β1 (Yang et al. 2015), or resolvins (Vasconcelos et al. 2015a, Vasconcelos et al. 2015b) on the NCs could trigger a shift in the macrophage response towards an M2 phenotype, to promote resolution of inflammation and tissue repair *in vivo*.

IVD's ability to regenerate benefits from the presence of cells capable of proliferating and differentiating into NP-like cells. Although IVD progenitor cells have been found in the human IVD, after birth, the number of notochordal cells decreases very rapidly (Blanco et al. 2010, Feng et al. 2010, Liu et al. 2011). An exhaustion of these cells with ageing and degeneration limits IVD's potential to counteract degeneration (Sakai et al. 2012, Sakai and Andersson 2015). Therefore, cell-based therapies to LBP, with the purpose to stimulate regeneration of the IVD, are being increasingly used (Sakai and Andersson 2015). Autologous or allogeneic MSCs transplantation is suggested as an adequate cell source (Yoshikawa et al. 2010, Sakai and Andersson 2015). In this work, the influence of the pro-inflammatory/degenerative environment of IVD in MSCs behavior was evaluated, namely in their immunomodulatory role. MSCs help minimize organ damage caused by the inflammation and cells activated by the immune system (Zachar et al. 2016). Several mechanisms of action have been proposed for MSC immunomodulation, including the secretion of soluble factors, among others, as reviewed (Caplan and Dennis 2006, English 2013). Initial striking clinical trials showed that in patients suffering from lumbar IVD degeneration with associated LBP, autologous bone marrow MSCs grafted percutaneously to degenerated IVDs (Yoshikawa et al. 2010) or injections into the NP (Orozco et al. 2011) did not seem to promote an increase in disc height, but increased MRI signal intensity and improved pain symptoms, at 1 and 2 years after surgery. Moreover, autologous bone marrow concentrate disc injections also seemed to have reduced patients' discogenic pain after 12 months (Pettine et al. 2015). In rabbits, MSCs injection in nucleotomized discs promoted COL2 synthesis by native cells, inhibited the expression of degrading enzymes and inflammatory cytokines, indicating a possible immunomodulatory effect (Miyamoto et al. 2010). Nonetheless, as suggested by others and by our results (Chapter VII), in a pro-inflammatory environment MSCs are firstly triggered to modulate inflammation instead of stimulating matrix production (van Buul et al. 2012, Manferdini et al. 2013). MSCs have been shown to differentiate to an NP-like phenotype *in vitro* (Risbud et al. 2004, Richardson et al. 2008b, Strassburg et al. 2010). *In vivo*, MSCs transplantation showed to increase COL2 expression, while decreasing cell apoptosis in the disc (Yang et al. 2010). MSCs can be recruited by chemoattractants to IVD (Pereira et al. 2014), but their role in the

pro-inflammatory/degenerative conditions of degenerated IVD seems to be somehow committed (Chapter VII).

Currently, bone marrow is the primary used source of adult MSCs, in which one of 10^5 nucleated cells is an MSC. This low cell number leads to the need of in vitro cell expansion to obtain sufficient cell numbers for clinical application (Hoogendoorn et al. 2008, Kregar Velikonja et al. 2014). In alternative, adipose-tissue is an abundant, expendable and easily accessible source of MSCs. For instance, Serigano et al. (2010) suggested 10^6 MSCs/disc as optimum cell number for transplantation into a dog disc degeneration model. The use of adipose-derived stem cells (ASCs) could reduce the need for *in vitro* expansion and subsequently one-step regenerative treatment strategies could be developed (Hoogendoorn et al. 2008, Kregar Velikonja et al. 2014). Few works have addressed the potential of ASCs in the IVD microenvironment, especially at long-term. However, it has been demonstrated that co-culture with degenerative NP tissue (Li et al. 2005) and cells (Choi et al. 2011) increase ASCs expression of COL2 and ACAN. Moreover, ASCs have also shown to promote matrix synthesis and cell proliferation of degenerated NP cells (Song et al. 2015). ASCs implanted in a rabbit model of traumatic degeneration of lumbar discs, showed proliferation 10 weeks after cell injection, ECM secretion and less ossification of damaged NP, compared with degenerative control discs (Chun et al. 2012). Recently, ASCs were shown to modulate inflammation in autoimmune arthritis (Lopez-Santalla et al. 2016).

Additionally, MSCs secrete numerous soluble factors in response to the microenvironmental cues, tuning several mechanisms in neighbor tissues via paracrine signaling (Caplan and Dennis 2006, Brisby et al. 2013). Thus, several studies focused on, for example, MSCs secretome for cardiac tissue repair (Dai et al. 2007) and recovery of hepatic (Parekkadan et al. 2007) and kidney (van Koppen et al. 2012) functions. MSCs secretome was suggested to stimulate IVD progenitor cells activity within degenerated human IVD tissue samples toward the repair process (Brisby et al. 2013). Hence, our group is currently comparing the therapeutic potential of not only MSCs, but also their secretome in the established degenerative/pro-inflammatory organ culture model.

Ultimately, the establishment of an experimental setup of IVD/macrophages co-culture, under pro-inflammatory/degenerative conditions could be used to evaluate the MSCs immunomodulatory effect, either in co-culture with the macrophages colonized IVD tissue or of their secretome. Moreover, given the results from the work in Chapter VII, which point out that in the degenerative environment, MSCs have a pro-inflammatory profile, while contributing to a less pro-inflammatory profile of native IVD cells, it would be of interest to treat the system with the anti-inflammatory Df-NCs. Taking this, if addition of the Df-NCs would decrease production of pro-inflammatory molecules by all cells, MSC could possibly differentiate and produce IVD-like ECM components.

This thesis focused mainly in the NP tissue. As future work, it would also be interesting to investigate the effect of different anti-inflammatory and immunomodulatory therapies in mechanical and biochemical properties of AF for repair stimulation. Aside the hostile environment of the NP, also the risk of AF tear has been challenging alternative solutions for IVD degeneration/herniation. As stated by Long et al. (Long et al. 2016), although discectomy is the most effective surgical procedure to treat hernia-associated LBP (Asch et al. 2002, Gray et al. 2006, Weinstein et al. 2008), this requires an incision in the AF, which may contribute, together with pre-existing annular injury, to worsening the IVD biomechanical stability (Masuda et al. 2005, Elliott et al. 2008, Michalek and Iatridis 2012). Failure of the AF is often associated with disc degeneration, whereas this structure gets too weak to restrain the hydrated NP material (Adams and Roughley 2006, Stefanakis et al. 2012, Stefanakis et al. 2014). As described before, the AF consists in concentric lamellae of regularly arranged collagen fibers, interconnected by a network of elastin and fibrillin, the so called translamellar bridging network (TLBN), which increase tensile strength of the annular wall (Yu et al. 2007, Schollum et al. 2009, Yu et al. 2015). But the pathomechanism leading to mechanical weakness (and ultimately rupture) of AF and consequent disc herniation is not yet fully understood.
The studies regarding the inflammation in the pathomechanism of disc degeneration and the inflammatory targets proposed for therapeutic strategies in degenerated IVD, previously discussed in Chapter II, are mostly focused in the NP tissue, often disregarding the influence of pro-inflammatory conditions on the TLBN of AF. Therefore, the influence of pro-inflammatory conditions on the mechanical properties of the AF tissue should be further addressed, along with the impact of anti-inflammatory treatment strategies. For this, an IVD *ex vivo* AF model, under physiological and degenerative biomechanical loading conditions (Neidlinger-Wilke et al. 2014), is being established to contribute with further knowledge in the field of therapeutic approaches for IVD regeneration. This model results from a new collaborative project between Portugal and Germany, that will enhance the knowledge on immunomodulatory strategies for IVD, that hopefully will increase the success of LBP therapeutic approaches.

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APPENDIX

Image licenses

Chapter I

Figure 1. “Schematic representation of healthy intervertebral disc.”
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Chapter II

Figure 1. “Role of the cytokines involved in different phases of intervertebral disc degeneration and herniation, leading to back and radicular pain.”
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