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TOWARDS THE USE OF MESENCHYMAL STEM CELLS SECRETOME TO TREAT INTERVERTEBRAL DISC DEGENERATION

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Abstract

Intervertebral disc (IVD) degeneration, generally considered the major cause of spine disorders, is normally associated with Low Back Pain (LBP), one of the main contributors to crippling disability worldwide. Current therapies focus only on symptoms management, disregarding the true cause of the pathology and even accelerating the degenerative process in adjacent tissues.

Alternative therapies have been proposed for LBP and IVD degeneration, namely cell-based therapies. Among these, Mesenchymal Stem/Stromal Cells (MSCs) appear to be the most appealing. MSCs regenerative potential has been mostly linked to their differentiation potential, but their paracrine mechanism of action is being increasingly appointed as a main therapeutic factor, with many studies achieving equally promising results with MSCs secretome. Nevertheless, MSCs therapeutical properties are strongly influenced by cells' microenvironment but how this affects MSCs secretome is not yet well described.

This work aims to evaluate the immunomodulatory and regenerative potential of MSCs secretome in IVD degeneration and to study the influence of oxygen and pro-inflammatory stimuli on MSCs secretome. For that, human bone marrow-derived MSCs were pre-conditioned for 48h with IL-1β (10 ng/mL), TNF-α (10 ng/mL) or both (IL-1β+TNF-α), in either normoxia (21% O_2) or hypoxia (6%O_2). MSCs viability, metabolic activity and phenotype were maintained within different preconditioning conditions. Different MSCs secretomes (secMSCs) were collected and used to culture bovine IVD punches in pro-inflammatory/degenerative culture conditions (puncture+IL-1β stimulus), as previously established in the group. The treated IVDs remained viable and secMSCs down-regulated inflammatory markers of the pro-inflammatory/degenerative IVDs cultures (IL-6, IL-8, TNF-α, PGE_2, TGF-β) after 48h in general, which was not observed in the presence of MSCs. After 14 days, the ECM production of treated IVDs was promoted only in the presence of MSCs and secMSCs of three conditions (control, IL-1β+normoxia, IL-1β+TNF-α+hypoxia), which interestingly, present higher levels of key immune regulatory molecules (TNF-α, IL-6, IDO). Overall, the results demonstrate the potential of

secMSCs to control inflammation and the possible link between inflammatory response and ECM remodeling in degenerated IVD, opening new perspectives for LBP treatment.

Resumo

A degeneração do Disco Intervertebral, geralmente considerada a principal causa de doenças da coluna, é normalmente associada à Dor Lombar, um dos principais fatores que contribuem para a incapacidade física mundial. As terapias atuais focam-se apenas na gestão dos sintomas, ignorando a verdadeira causa da patologia e contribuindo ainda para uma aceleração do processo degenerativo em tecidos adjacentes.

Têm sido propostas terapias alternativas para a Dor Lombar e para a degeneração do disco Intervertebral. De entre estas, as Células Estaminais/Estromais Mesenquimais (MSCs) apresentam-se como a mais atrativa. O potencial regenerativo das MSCs tem sido maioritariamente ligado ao seu potencial de diferenciação, embora o seu mecanismo de ação parácrino venha a ser cada vez mais indicado como o principal fator terapêutico, com diversos estudos demonstrando resultados igualmente promissores com o seu secretoma. As propriedades terapêuticas das MSCs são fortemente influenciadas pelo microambiente em que se encontram, mas ainda não se encontra bem descrita a forma como isto afeta o seu secretoma.

Este trabalho tem como objetivo a avaliação dos potenciais regenerativo e imunomodulatório do secretoma das MSCs na degeneração do disco Intervertebral assim como da influência do oxigénio e do estímulo pró-inflamatório nas suas características. Nesse sentido, MSCs humanas da medula óssea foram pré-estimuladas durante 48h com IL-1β (10ng/mL), TNF-α (10ng/mL) ou ambas (IL-1β+TNF-α) em condições de normóxia (21% O_2) ou hipoxia (6% O_2).

A viabilidade, a atividade metabólica e o fenótipo das MSCs foram mantidos nas diferentes condições de pré-condicionamento. Diferentes secretomas de MSCs (secMSCs) foram recolhidos e usados para cultura de porções de disco em condições pró-inflamatórias/degenerativas (estímulo com punção + Il-1β), como descrito anteriormente pelo grupo. Os discos tratados mantiveram-se viáveis e o uso de secMSCs reduziu a produção de marcadores inflamatórios da cultura de discos em condições pró-inflamatórias/degenerativas (IL6, IL-8, TNF-α, PGE2, TGF-β) após 48h, o que não foi observado na presença de MSCs. Após 14 dias, a produção de ECM dos discos tratados foi promovida

apenas na presença de MSCs e secMSCs de três condições (controlo, IL-1β+normoxia, IL-1β+TNF-α+hipoxia), que, curiosamente, apresentam níveis mais elevados de moléculas com um papel chave na regulação imune (TNF-α, IL-6, IDO).

Em resumo, os resultados demonstram o potencial do secretoma das MSCs para controlar a inflamação e a possível ligação entre a resposta inflamatória e a remodelação da matriz extracelular do disco intervertebral degenerado, introduzindo novas perspetivas no tratamento da dor lombar.

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Contents

Acknowledgements 7
Contents 9
List of Figures 11
List of Abbreviations 12
A. Introduction 15
1. Low Back Pain and Degenerative Disc Disease 15
2. Mesenchymal Stem/Stromal Cells (MSCs) as a promising cell source 17
3. Evidences of MSCs beneficial effects in DDD 18
4. MSCs Secretome as a powerful therapy 22
4.1.MSCs secretome in Neurologic disorders 22
4.2 MSCs secretome in Cardiovascular diseases 23
4.3 MSCs secretome in Bone and Cartilage Repair/Regeneration 23
4.4 MSCs secretome in Liver Disorders 24
4.5 MSCs secretome in Kidney Disorder 24
4.6 MSCs secretome in DDD 24
5. Influence of preconditioning on MSCs secretome 25
B. Materials and Methods 29
1. Culture and pre-conditioning of human bone marrow MSCs 29
1.1 Isolation of primary human bone marrow MSCs 29
1.2 Routine culture of human MSCs 29
1.3 MSCs preconditioning 30
2. Bovine intervertebral discs isolation and culture 30
2.1 IVD pro-inflammatory stimulation 31
3. Analysis of the effect of MSCs preconditioning on cell metabolic activity, viability, phenotype and inflammatory profile 32
3.1 Mitochondrial Metabolic Activity Analysis 32
3.2. Trypan Blue Exclusion Test of cell viability 32
3.3 Flow Cytometry Analysis of MSCs phenotypic markers 32
3.4. Gene Expression Analysis of MSCs 33
3.5. Growth factors/cytokine analysis in MSCs secretome by ELISA 34
4. Analysis of the effect of MSCs secretome on IVD metabolic activity, viability and inflammatory profile 34

4.1 Mitochondrial Metabolic Activity Analysis 34
4.2. IVD apoptosis/viability analysis by flow cytometry 34
4.3 Gene Expression Analysis of IVD cells 34
4.4. Analysis of IVDs supernatants 35
5. Analysis of MSCs secretome long-term effect on IVD matrix content 35
5.1. Sulphated glucosaminoglycans quantification 35
5.2. Proteoglycans detection by safranin O/light green staining 35
5.3. Collagen II and Aggrecan detection 36
6. Statistical analysis 36
C. Results 37
1. The influence of preconditioning on MSCs 37
1.1. Effect of MSCs preconditioning on cell viability and phenotype 37
2. Effect of MSCs secretomes (secMSCs) on the degenerated IVD 38
2.1. Influence of different secMSCs on the pro-inflammatory/degenerated IVDs apoptosis/viability 39
2.2. Effect of secMSCs on the IVDs inflammatory state 40
2.3. Effect of secMSCs on initial IVDs matrix degradation 42
2.4. Effect of secMSCs on IVDs matrix degradation at protein level 43
3. Influence of preconditioning on MSC immunomodulatory profile 46
D. Discussion 48
E. Conclusion 53
F. References 54
G. Supplementary Materials and Methods 67
1. Flow Cytometry analysis of phenotypic markers 67
2. MSCs differentiation assays 67
2.1 Osteogenic differentiation 67
2.2 Adipogenic differentiation 67
2.3 Chondrogenic differentiation 68
3. Primers used for Gene Expression Analysis of MSCs/IVDs 68
H. Supplementary Results 69
1. Phenotypic and Functional Characterization of isolated MSCs 70
2. Overview on secMSCs effect on the degenerated IVD 71

List of Figures

Figure 1| The IVD niche and its degeneration 15
Figure 2| Preconditioning conditions design 29
Figure 3| Experimental Design 30
Figure 4| Analysis of MSCs after pre-conditioning with IL-1β (10ng/ml), TNF-α (10ng/ml) or IL-1β + TNF-α (10ng/ml) in either normoxic (6% O2) or hypoxic (21% O2) culture conditions 36
Figure 5| Analysis of IVD condition after pro-inflammatory stimulus with IL-1β (10ng/mL) + puncture and posterior treatment with MSCs or MSCs secretomes 38
Figure 6| Effect of different secMSCs on IVDs inflammatory response, 2 days after pro-inflammatory stimulus and treatment. Quantitative analysis of pro-inflammatory markers. 39
Figure 7| Effect of different secMSCs on IVDs matrix maintenance and content, 2 days after pro-inflammatory stimulus and treatment. 41
Figure 8| Effect of different secMSCs on inflammatory/degenerated IVD model matrix content, at the protein level, 14 days after pro-inflammatory stimulus and treatment. 43
Figure 9| Effect of different secMSCs on inflammatory/degenerated IVD model matrix content, at the protein level, 14 days after pro-inflammatory stimulus and treatment. 44
Figure 10| Analysis of MSCs inflammatory-related gene expression and molecular secretion profile , after pre-conditioning with IL-1β (10ng/ml), TNF-α (10ng/ml) or IL-1β + TNF-α (10ng/ml) in either normoxic (6% O2) or hypoxic (21% O2) culture conditions. 46
Figure S1| Analysis of newly isolated MSCs differentiation capacity and phenotype 68
Figure S2| Overall results overview represented as heat map, generated using software Gene E, considering median calculated from replicas of each condition for every factor analyzed. 69

List of Abbreviations

AF	Annulus Fibrosus
Agg	Aggrecan
ALP	Alkaline Phosphatase
ASC	Adipose Tissue-derived MSC
AT	Adipose Tissue
BDNF	Brain-Derived Neurotrophic Factor
BM	Bone Marrow
BMP	Bone Morphogenic Protein
CM	Conditioned Medium
Coll II	Type II Collagen
DDD	Degenerative Disc Disease
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldehydes 3-phosphate dehydrogenase
GDNF	Glial Cell line-Derived Neurotrophic Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HGF	Hepatocyte Growth Factor
HIF-1/2α	Hypoxia-Inducible-Factor-1/2α
IBMX	Isobutyl-1-methylxanthine
ICAM	Intercellular Adhesion Molecule
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN-γ	Interferon γ
IGF	Insulin-Like Growth Factor
IHC	Immunohistochemistry
IL-	Interleukin
ITS	Insulin-Transferring-Selenium
IVD	Intervertebral Disc
KRT	Keratin
LBP	Low Back Pain

LIF	Leukemia Inhibitory Factor
LPS	Lipopolysaccharides
MCP	Monocyte Chemoattractant Protein
MGP	Matrix Gla Protein
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem/Stromal Cell
NGF	Nerve Growth Factor
NO	Nitric Oxide
NP	Nucleus Pulposus
P/S	Penicillin/Streptomycin
PBS	Phosphate Buffer Saline
PDGF	Platelet-Derived Growth Factor
PFA	Paraformaldehyde
PGE_2	Prostaglandin E2
qRT-PCR	quantitative Real-Time Reverse Transcription Polymerase Chain Reaction
SC	Hepatic Stellate Cell
SCM	Standard Culture Medium
SDF-1	Stromal Cell-Derived Factor 1
sGAG	sulphated Glucosaminoglycans
Shh	Sonic Hedgehog
STC-1	Stanniocalcin-1
TGF-β1	Transforming Growth Factor β1
TIMP	Tissue Inhibitor of Metalloproteinase
TNF-α	Tumor Necrosis Factor α
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
TSG-6	Tumor Necrosis Factor-Inducible Gene 6
UCB	Umbilical Cord Blood
VEGF	Vascular Endothelial Growth Factor

A. Introduction

1. Low Back Pain and Degenerative Disc Disease

Low back pain (LPB) is on the top of the list of the major causes of disability in the world, being expected that 2/3 of the world population will suffer from it at least once in their lives [1]. LBP contributes with more than 10% to the global number of years lived with disability, or 83 million years lived with disability [2], and has a relevant economic burden associated [2-4]. Degeneration of the intervertebral disc (IVD) is considered the major cause of spine disorders [5] and is commonly linked to degenerative disc disease (DDD) and LBP [6, 7], although this last one is known as a multi-factorial disorder [8-10].

The IVDs are key structures to sustain the weight applied to the spine and provide spine flexibility, multi-axial motion and load transmission throughout the spine [11, 12]. They are composed by an outer ring of fibrous cartilage (annulus fibrosus-AF) surrounding a gelatinous nucleus (nucleus pulposus-NP) that is delimited, above and below, by cartilage endplates [11]. The NP is the largest avascular tissue in the body and forms a hypoxic and hyperosmotic environment [13], which is possible only due to the cells' constitutively expression of hypoxia-inducible-factor-1/2α (HIF-1/2α) [14]. As its main function is to resist compressive loads, the NP extracellular matrix (ECM) is mostly composed of aggrecan (Agg) and type II collagen (Col II) in a characteristic ratio of >20 [15]. NP cells can also be characterized based on a set of specific markers such as sonic hedgehog (shh), brachyury and CD24, even though most known NP markers are species dependent and still controversial [16]. NP contains mainly two different cell populations (chondrocyte-like and notochordal cells) but Molinos et al has recently published the discovery of three distinguishable sub-populations in the NP by flow cytometry [17]: P1, presenting a seemingly more progenitor-like phenotype with high expression of CD146 and CD44 in most cells and only a few expressing CD45 and CD34; P2 and P3, CD29 expressing populations, distinguishable mainly by P2-restrict expression of CD45 and CD146.

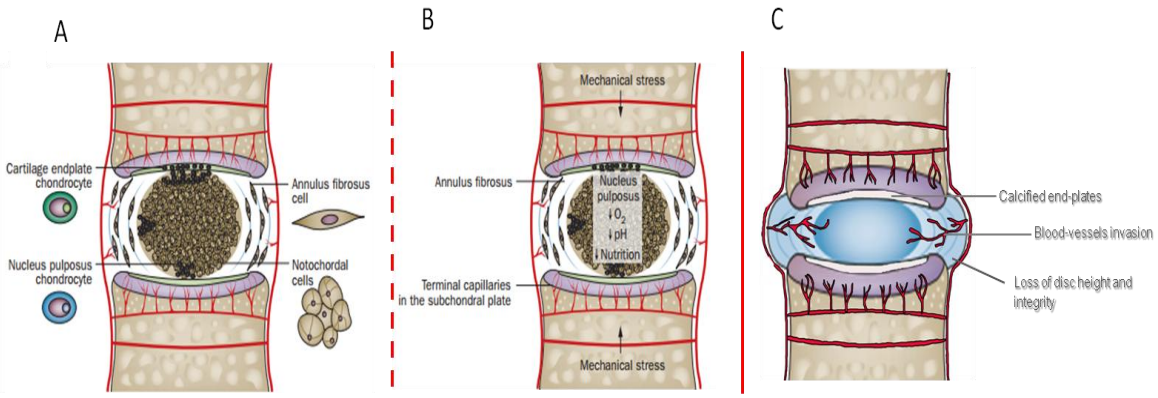


Figure 1| The IVD niche and its degeneration

A. The IVD presents an heterogeneous group of different cell populations, namely: notochordal cells (large and vacuolated progenitor cells), mature nucleus pulposus cells (chondrocyte-like cells), annulus fibrosus cells (fibroblast-like cells) and cells of the endplate (also chondrocyte-like cells). **B.** IVD niche is very specific and harsh environment, presenting low pH and oxygen levels and a poor nutrients supply due to its avascular nature, alongside an intense mechanical load. **C**. In the process of disc degeneration, there is loss of disc height and integrity, with possible disc extrusion. The calcification of the endplates is also a natural outcome of this process, further inhibiting nutrients supply. There's also invasion by nerves and blood vessels. Adapted from: Sakai, D. and G.B. Andersson, Stem cell therapy for intervertebral disc regeneration: obstacles and solutions. Nat Rev Rheumatol, 2015. 11(4): p. 243-56

IVD degeneration is believed to originate in the NP (Fig. 1A), the central region of the IVD that ensure ECM maintenance [12]. Age related changes, mostly progressive cell senescence [21, 22] seem to indicate this connection.

In this characteristic environment, degeneration begins with increased production of matrix metalloproteinases (MMPs) that results in ECM increased degradation [23] with loss of disc height and integrity [24, 25]. Nutrition becomes impaired with calcification of the end plates, normally responsible for the diffusion of gases and nutrients to the disc [26, 27]. Disc invasion by nerve and blood vessels was observed, being appointed as a possible cause of the pain induced [28, 29] (Figure 1). Up-regulation of a wide number of inflammatory cytokines (Tumor Necrosis Factor α (TNF-α), IL-1β, IL-6, IL-8, Nitric Oxide (NO) and Prostaglandin E2 (PGE_2) among others) has also been observed, indicating involvement of an inflammatory process [30-32]. Although this inflammatory reaction remains still scarcely understood in the context of IVD degeneration, ECM breakdown products generated during tissue damage or as a consequence of degenerative metabolic dysfunction seem to elicit a local inflammatory response. Indeed, Quero et al. observed that in human IVD cells, hyaluronan fragments provoked increase in expression of inflammatory genes such as *IL-1β, IL-6, IL-8* and matrix-degrading genes *MMP1* and *MMP13*

[33]. Accordingly, IVDs seem to endogenously present inflammatory-like cells able to phagocyte apoptotic bodies [34] that present immune-specific markers [35] and produce inflammatory cytokines, even in non-degenerated conditions [36]. Additionally, infiltrated leukocytes were found in IVDs with intact NP still isolated from vascular supply, suggesting an endogenous source for the inflammatory reaction [36]. In more advanced degenerative states, when the NP becomes exposed to the immune system, the invasion of immune cells attracted to the local inflammatory stimulus, further aggravate the reaction [37]. The presence of high levels of some of these inflammatory cytokines in the IVD, may mediate catabolic effects, such as loss of ECM proteoglycan content and stimulation of MMP expression [31, 38].

2. Mesenchymal Stem/Stromal Cells (MSCs) as a promising cell source

Currently, DDD is mainly either conservatively treated with pain management through medication or physical therapy, or surgically treated with removal or substitution of complete/partial disc tissue or in addition, vertebral fusion [19, 39]. These approaches are not only not treating the disorder cause, seeking merely symptomatic relief [40], as they are also not always effective, impairing normal motion when fusion methods are used, with possible acceleration of degeneration in adjoining segments and maintenance of LBP [41, 42]. Alternative approaches have been explored, recurring to growth factors injection [43], gene therapy [44, 45] and tissue engineering [19, 44]. However, with known limited success. In addition, cell-based therapies have been purposed [46, 47], with promising results, either with IVD autologous cells [23], NP progenitor cells [48, 49], immortalized NP-cell line [50], fetal spinal cells [51] and stem-cells [52-55], namely MSCs [56, 57].

MSCs named by Caplan [58] and first isolated by Friedenstein and colleagues [59] are defined as plastic-adherent fibroblast-like cells with multipotent differentiation capacity in vitro [60] and an expression pattern of $CD90^{+}CD105^{+}CD73^{+}CD14^{-}CD34^{-}CD45^{-}$ [61]. MSCs are also $MHCI^{+}MHCII^{-}$ $CD40^{-}CD80^{-}CD86^{-}$, an immune phenotype that inhibits T-cell activation by lack of secondary stimuli (provided by MHCII, CD80 and CD86, for example), even when there is recognition (by interaction between T-cells and MHCI molecules), making them non-immunogenic [62, 63]. The production of specific soluble

factors such as members of the TGF-β family, IL-6 and IL-10, MMPs and nitric oxide interferes with a variety of pathways necessary for the immune response [64]. Besides suppressing T-cell proliferation [65], MSCs induce T-regulatory cells activity [66] and inhibit the function and maturation of dendritic cells [67]. Both immunological suppressive [65-68] and stimulating capacities [69, 70] have been described, supporting the current definition of immunomodulatory cells.

Although these defining characteristics, there is still not an unique set of markers for MSCs and many other markers may be analyzed [60]. Traditionally, adult MSCs are isolated from bone marrow (BM) [59]. Nonetheless, MSCs have also been isolated from adipose tissue (AT) [71], umbilical blood cord (UCB) [72], placenta [73], peripheral blood [74] and other multiple sources [75-79]. When expanded in vitro, they have been shown primarily to have osteogenic potencial, and later, to be able to differentiate in other skeletal tissues such as chondrogenic and adipogenic lineages, all from mesodermal origin [80, 81]. More recently, MSCs were shown to have regenerative potential in ectodermal and endodermal lineages' tissues [82-88], even though the question whether they do so by differentiating or by molecular signaling remains controversial. Although MSCs physiological role is yet to be elucidated, it seems to be established their involvement in hematopoietic microenvironment formation and maintenance [89], modulation of immune response [90, 91] and regenerative processes throughout the human body [92], as exemplified in table 1.

3. Evidences of MSCs beneficial effects in DDD

The majority of the current cell therapies aim to restore native IVD matrix, focusing particularly in increasing NP proteoglycan content [19, 93]. Several studies focus on the possibility of tissue repopulation with IVD native cells, succeeding in delaying degeneration and maintaining disc height with ECM production. However, studies have shown that the isolation of cells from this tissue could create or accelerate pre-existing degeneration, suggesting a downside to this possible future therapy [94]. Additionally, the recruitment of cells to increase the supply of viable cells, recurring to cytokine and chemokine provision, seems to be difficult. Although IVD progenitor cells have been

Table 1| MSCs regenerative potential - Several examples

Study	MSCs Source	Study Model	Major Conclusions
Bone & Cartilage			
Garimella et al. 2015[50]	AT	Mouse rheumatoid arthritis model	Reduction of clinical symptoms and joint pathology.
Zscharnack et al. 2010[172]	BM	Chronic osteochondral defect in ovine stifle joints	Significant improvement of morphological characteristics of hyaline cartilage.
Koh et al. 2012[76]	AT	Patients with knee osteoarthritis	Improvement of clinical symptoms.
Nervous System			
Zhao et al. 2007[169]	BM	Mouse amyotrophic lateral sclerosis model	Delay in disease onset and progress. Increase in mice lifespan
Bouchez et al. 2008[20]	BM	Rat Parkinson's disease model	Reduction of behavioral symptoms. Partial restoration of dopaminergic markers
Zhang et al. 2008[168]	BM	Patients with traumatic brain injury	Significant improvement of neurologic function
Bang et al. 2005[11]	BM	Patients suffering with post-stroke severe neurologic deficit	Consistent improvement of functional scores
Heart			
Hamdi et al. 2011[56]	AT	Rat myocardial infarction model	Increase in survival rate and cell engraftment. Preservation of ventricle geometry
Amado et al. 2006[6]	BM	Pig cardiac infarction model	Active contractile tissue recovery with increase in myocardium thickness. Scar tissue reduction
Viswanathan et al. 2010[156]	BM	Myocardial infarction patients	Perfusion improvement in the infarcted area and ejection fraction
Immune System			
Le Blanc et al. 2008[79]	BM	Patient with severe graft-versus-host disease	No adverse effects directly related. Higher survival rate.
Sun et al. 2008[149]	BM	Systemic lupus erythematosus mice model	Reconstruction of BM osteblastic niche with reversion of multiorgan dysfunction.
Ciccocioppo et al. 2015[35]	BM	Patients suffering from Crohn's disease	Disease remission achieved after 1 and 5 years.
Cancer			
Yulyana et al. 2015[166]	Fetal bone marrow	Hepatocellular carcinoma mouse model	Inhibition of cancer cell proliferation. Enhancement of chemotherapeutic drugs effect.
Skin			
Santos et al. 2015[135]	UCB	Rat wound splinting model	Faster and better wound resolution with complete tissue and mature vascular system regeneration.
Pancreas			
Holmes 2014[62]	BM	Type 1 diabetes mellitus patients	Preservation of c-peptide response after 1 year.
Bladder			
Snow-Lisy et al. 2015[145]	BM	Rat bladder augmentation model	Increase in blood vessel numbers and muscle content. Peripheral nerve regeneration
Kidney			
Gatti et al. 2011[51]	BM	Nephrectomy and renal artery and vein occlusion in rats	Inhibition of cell apoptosis. Reduced renal function impairment.
Liver			
Zhang et al. 2012[167]	UCB	Patients suffering from decompensated liver cirrhosis	Significant improvement in liver function

discovered in NP [95], AF [96] and end-plates [97] and shown to have multipotency in vitro and in vivo [98, 99], in 2012, Sakai and colleagues have demonstrated that there is an exhaustion of these cells with ageing and degeneration [22] and so, attempts to stimulate them for matrix repair could be impossible due to a lack of sufficient cells [25].

Transplantation of cells from different sources, such as chondrocytes [100, 101] and MSCs, has been contemplated, with mainly positive results. MSCs transplantation in the degenerated disc has been demonstrated initially in the works of Sakai et al. In this work, it was shown that cells seeded in a collagen scaffold, after implantation in a rabbit degenerated disc model, induced ECM production while disc height was maintained [102, 103]. Following the trend, hydrogel [104, 105], hyaluronan [106], fibrin [107, 108] and collagen [109]-based scaffolds seemed to work synergistically with cell therapy leading to matrix recovery, enhancing its effects. Additionally, scaffolds were shown to prevent possible osteophyte formation in near tissues due to cell leakage [109, 110]. In 2010, Yang and colleagues introduced a TGF-β1(Transforming Growth Factor β1) loaded fibrin scaffold, demonstrating higher regenerative effect influenced by the cell-growth factor interaction to increase NP and Col II content [108], supporting earlier evidence that pointed towards the importance of this [111, 112] and other factors [105, 113-115], such as hypoxia [113] and load [111, 114] in induction of MSC differentiation in NP-like cells for disc regeneration. Nonetheless, the clinical application of biomaterials as scaffolds for cell therapy may need more research to become feasible. On the one hand, there's the difficulty in tuning biomechanical properties adequate for use in load-bearing tissue such as the IVD, in order to avoid problems such as material extrusion [116]. On the other hand, the surgical techniques commonly used to introduce the material in the tissue have been shown to accelerate IVD degeneration [117].

Cell transplantation proved to be successful even without the use of a scaffold in small [118-120] and large animal models [121, 122]. In a rabbit degenerated disc model, MSC injection not only promoted Col II synthesis, but lead to an alteration of NP cells genetic profile as well, with suppression of degradative enzymes and inflammatory cytokines' gene expression, indicating a possible immunomodulatory effect [118]. Another work in a dog degenerated disc model

established 10^6 MSCs/disc as an optimum cell number for transplantation, to maintain a higher survival rate and to promote the maintenance of structural microenvironment and ECM content [122], effects previously observed elsewhere [121].

Two human clinical trials have recently been done, with more underway. In 2010, two Japanese elderly female patients received an autologous BM-derived MSC seeded collagen scaffold transplant to treat lumbar spinal canal stenosis with vacuum phenomenon related instability. Scaffold pieces grafted percutaneously to degenerated IVD lead to improvements in vacuum phenomenon as well as reduction in associated instability and maintenance of high moisture content, indicating maintenance of ECM content [123]. In 2011, 10 patients suffering from lumbar disc degeneration with associated LBP received autologous BM-MSC injections to the NP, resulting in a rapid pain and disability improvement, reaching 71% of optimal efficacy. Water content has remained elevated even though disc height was not recovered [54]. A follow-up phase I-II clinical trial is currently underway to further study this effect, recurring to MSV, an expanded BM-MSC [124]. Six other clinical trials, currently recruiting, will study the effect of adipose tissue-derived MSCs in lumbar disc degeneration, either recurring to direct cell injection [125, 126] or to the use of a supporting scaffold [127-130].

Despite the increasing evidence towards a beneficial effect of MSCs injection on the regeneration on degenerated IVD, some questions have arisen regarding practical and safety issues. As aforementioned, osteophyte formation due to cell leakage can occur. Vadalà et al observed the migration of injected MSCs to surrounding tissues with undesirable bone formation while no marked MSCs seemed to be found inside the IVD [110], which suggests not only a safety concern but also the possibility of total inefficiency of the procedure. Furthermore, even when injected cells engraft into the IVD tissue, some authors question whether they can survive and actively function in that hostile environment long enough to generate an effect in the progressive degeneration [25].

At the same time, the recruitment of cells from the surrounding environment is not only an alternative approach, but also a natural response of IVD cells to degeneration. In 2012, Illien-Junger et al demonstrated that a degenerative IVD

environment induces the production of factors that promote MSCs recruitment [131]. In 2014, Pattappa et al. showed that RANTES (CCL5) and CXCL5, key chemoattractant molecules, are produced in response to induced tissue degeneration [132], confirming the observation from Phillips and colleagues [36]. Meanwhile, Pereira et al. developed a chemoattractant-delivery system composed of a thermoreversible hydrogel enriched with stromal cell-derived factor 1 (SDF-1) that displayed great potential in MSCs recruitment to degenerated IVD, unfolding new possibilities of regenerative approaches [133]. Nevertheless, the avascular nature of this tissue represents a major obstacle to exogenous cell recruitment as few cells actually migrate from other regions towards the IVD [25, 134].

4. MSCs Secretome as a powerful therapy

As stated by Richardson and Hoyland [94], the most important stem cell asset is commonly assumed to be the ability to differentiate into the desired phenotype, as it can lead to tissue regeneration through cell repopulation. This seems to be supported by a large number of publications that have observed MSC differentiation as a major contribution for the positive results achieved. However, MSCs mode of action goes beyond their multi-differentiation potential. These cells secrete a large variability of bioactive molecules in response to local environment that act in near tissues through a paracrine effect [135]. An increasing number of publications consider this paracrine action to be a primary function of MSCs [92, 135, 136] and there has been much investigation to ascertain if some of the effects of the MSCs observed until now are due to trophic effects, as in many cases the number of differentiated cells isn't enough to explain the observed response [135]. MSCs secretome's therapeutic potential has been and is being increasingly explored in most tissues and in a large variety of pathologic conditions.

4.1.MSCs secretome in Neurologic disorders

MSCs injection in the brain tissue of a stroke model rat was shown to improve coordinated function with inhibition of scar tissue formation and cell apoptosis, and stimulation of angiogenesis and endogenous progenitors' activity [137]. Authors, however, reported absence of MSCs' neural differentiation. Other

study used concentrated conditioned medium (CM) from human MSCs culture in a rat model of traumatic brain injury. Results showed that the bioactive factors produced by the MSCs (namely Hepatocyte Growth Factor (HGF) and Vascular Endothelial Growth Factor (VEGF)) stimulated neurogenesis and improved motor and cognitive function [138]. Hypoxic preconditioning seemed to enhance these effects. Additionally, MSCs exosomes were shown to mediate the transfer of miRNA 133b to neuronal cells, contributing to neurite outgrowth and functional recovery after stroke [139], suggesting a relevant part in the neuronal protection capacity exhibited by MSCs. One group has directly established that MSCs secretome can promote neuronal survival and differentiation both in vivo and in vitro [140] and has reviewed the presence of factors such as BDNF (Brain-Derived Neurotrophic Factor), GDNF (Glial Cell line-Derived Neurotrophic Factor) , NGF (Nerve Growth Factor) and IGF (Insulin-Like Growth Factor) in the secretome, with different roles in neuronal protection in various related disorders (such as Parkinson's disease and spinal cord injury) [141, 142]. This work demonstrates the vast existent evidence indicating the importance of these soluble factors in the MSCs action.

4.2 MSCs secretome in Cardiovascular diseases

In 2007, Dai et al. observed that using MSCs-CM in myocardial infarction rat model when compared to cells transplantation induced an effect only slightly less intense, indicating that part of the effect could be attributed to soluble factors [143]. Other study has achieved cardiac function restoration only 3 days after MSCs injection in infarcted heart tissue, leading to the conclusion that the speed of the response couldn't be explained by a massive differentiation phenomenon [144] as MSCs differentiation in cardiomyocyte-like cells can take up to 9-14 days to happen [145]. Recently, MSC-derived exosomes were shown to improve cardiac function after myocardial infarction, with reduction of infarct size and maintenance of systolic and diastolic performance, as a result of neorevascularization and modulation of the inflammatory response [146, 147].

4.3 MSCs secretome in Bone and Cartilage Repair/Regeneration

Surgically created mandible lesions in a rabbit model were regenerated through ASC (Adipose Tissue-derived MSC)-CM administration. CM was obtained from

cells incubated in serum-free medium, in hypoxic conditions for 24h. There were detected 43 angiogenic factors, from which 11 elevated molecules appeared to be involved in bone regeneration: IGF-1, TGF-β1, VEGF, angiogenin, IL-6, PDGF-BB, bFGF, EGF, RANTES, MCP-1 and MCP3 [148]. This expression pattern seemed to be in accordance with reported MSC-CM composition as other authors had found similar key molecules [149-152], including HGF [150] and BMP-1 [152]. Notably, VEGF, bFGF, HGF and IGF-1 were also significantly increased in injured hearts treated with MSCs [144]. HGF seemed to be a key factor in hepatic fibrosis aversion [153]. Knee joint MSCs' injection in a severe osteoarthritis goat model also lead to meniscus regeneration without detectable cell differentiation [154]

4.4 MSCs secretome in Liver Disorders

MSCs co-culture with hepatic stellate cells (SC), responsible for hepatic fibrosis formation, induced SC apoptosis mainly through HGF signaling while inhibiting SC proliferation and collagen synthesis due to IL-6 induced IL-10 and TNF-α production, indicating immunomodulation of activated SCs [153]. Others demonstrated the therapeutic action of MSC-derived exosomes in fibrotic liver as its administration seemed to have a protecting effect on hepatocytes, reducing hepatic inflammation and collagen deposition [155]. Parekkadan and colleagues showed that the administration of MSCs-CM to a rat fulminant hepatic failure model, not only reduced mortality rate and hepatocellular death, but also diverted immune cells from the injured liver, supporting the importance of MSCs immunomodulatory action [156].

4.5 MSCs secretome in Kidney Disorder

In a chronic kidney disease rat model, MSC-CM succeeded in partially rescuing kidney function, mainly through endothelial cells attraction, angiogenesis and wound closure stimulation [157]. Important reno-protective paracrine factors were suggested to be VEGF, HGF and IGF, recurring important factors in MSC-CM for various different organs. In 2011, Gatti and colleagues managed to protect rats from acute kidney injury with a single administration of MSC-derived microvesicles that inhibited apoptosis and stimulated tubular epithelial cell proliferation [158].

4.6 MSCs secretome in DDD

Despite the work of many investigators indicating MSCs differentiation as a necessary part of the natural IVD regenerative process [105, 111, 112, 114, 159, 160], there's strong evidence of existence of an essential paracrine crosstalk between the two types of cells [98, 161, 162]. In 2008, Yang and colleagues studied this interaction with a co-culture assay, which showed MSCs enhanced proliferation and Col II production in response to NP-like cells activity. A significant proliferation and Agg production increase in NP-like cells due to MSCs influence was also observed [161]. These results were later confirmed by Zhang et al. that further analyzed MSC-CM effect in NP-like cells gene expression, finding up-regulation of *KRT19* and downregulation of *MMP12* and MGP [162]. As *MMP12, KRT19* and *MGP* have been associated with IVD degeneration, authors suggest that an healthy NP-like phenotype could be rescued. Another work indicated that MSC-CM could stimulate IVD progenitor cells activity [98], once again confirming the existence of a paracrine effect on degenerated IVD that could be further explored for therapeutic use. One such exploratory work, intending to understand the communication mechanisms between MSCs and NP cells, observed that one main via of communication is secured by secretion and internalization of microvesicles by both cell types [163], indicating, as in other organs, that the study of microvesicles and exosomes could represent a step further in the understanding of MSCs action mechanisms and in the pursuit of a regenerative therapy.

5. Influence of preconditioning on MSCs secretome

MSCs paracrine action is influenced by the microenvironment to which MSCs are subject to. This is demonstrated by the complexity shown by these cells' response to different physiologic and pathologic situations and by the constant dual role they have of stimulation/inhibition, performing as modulatory cells in most scenarios. In this context, much investigation is being conducted in the effect of MSCs preconditioning in their characteristics, mainly exploring hypoxia, inflammatory stimuli and tridimensional culture as preconditioning factors. Some authors have also pursued pharmacological conditioning in specific pathological contexts [164, 165].

MSCs secretome, even when is produced without any prior conditioning of the cells, seems to be rich in chemokines, immunomodulatory molecules and growth factors (Table 2). Liu et al. analyzed a large array of factors, demonstrating that most abundant factors seemed to be IL-6, IL-8, TIMP-1 and TIMP-2, with high levels of MCP-1, LIF, VEGF and IGF observed [166]. Kyurkchiev et al. reviewed this subject, indicating IL-10, TGF-β, RANTES, IDO, ICAM and PGE_2 as other factors highly produced by MSCs [167]. Other authors also repeatedly observed significant levels of not only VEGF, but also FGF, IGF, HGF, EGF, PDGF and BMP [148, 150-152, 166, 168, 169], demonstrating MSCs secretome richness in growth factors that are involved in regenerative processes of many different systems, accounting for MSCs outstanding regenerative capacity. On the other hand, MSCs ability for immunomodulation seems secured by the diverse and abundant production of factors involved in inflammation and immune response in general. Especially, IL-6 was indicated as the key cytokine involved in the immunoregulatory effects by MSCs [170] and PGE_2 and IDO seem to be major effector molecules in MSCs immunoregulation [171, 172]. Effectively, this triad production is stimulated by the presence of pro-inflammatory factors, such as IL-1β, TNF-α, IFN-γ and LPS [67, 166, 172-174], that mostly elicit an immunomodulatory response from MSCs, aside from the production of a cocktail of growth factors, indicating a close relationship between inflammation and regeneration. Hypoxia, in turn, primarily activates the expression of hypoxia inducible factor (HIF-1α), leading to the stimulation of angiogenesis by production of factors such as vascular endothelial growth factor (VEGF) and Angiogenin [175, 176], an unwelcomed effect in the context of the avascular healthy IVD. Other growth factors seem to also be upregulated [177]. Secondly, it seems to induce multipotency maintenance [178, 179] and enhance cellular proliferation [180]. These effects can be explained by the fact that MSCs tend to exist in hypoxic areas of the body, contributing to the maintenance of the microenvironment there found.

MSCs have proven to have a wide therapeutic potential in many diseases and conditions, as they present multipotent differentiation capacity alongside powerful immunomodulation ability and the aptitude to strongly interact with the surrounding environment, either collecting signals and reacting accordingly or

Table 2| Overview on the factors produced by MSCs cultured under different conditions

	No stimulation	Hypoxia	Inflammatory stimuli
MCP-1[148, 149, 164]	*	+	
MCP-3[148, 149]	*	+	
RANTES [148, 149, 164]	*	+	
Oct4 [165]	*	+	
Rex1[165]	*	+	
Angiogenin [148]	*	+	
PDGF [148, 150, 151]	*	+	
EGF [148]	*	+	
FGF [148, 150, 151, 165, 166]	*	+	
VEGF [148, 150, 151, 164-167]	*	+	+
IGF [148, 150, 151, 165-167]	*	+	+
HGF [150, 165, 167]	*	+	+
IDO [164, 165, 168]	*	+	+
TGF-β [150, 164-166]	*	+	+
IL-6 [148, 149, 164, 166, 169]	*	+	+
Factor H [165]	*		+
Gal-9 [165]	*		+
BMP [150-152, 165]	*		+
PGE_2 [164, 165, 168]	*		+
TSG-6 [165]	*		
IL-24 [165]	*		
STC-1 [165]	*		
CXCR4 [165]	*		
TRAIL [165]	*		
CD82 [165]	*		
IL-10 [164]	*	-	
IL-8 [166]	*		
IL-1β [166]	*		
GM-CSF [166]	*		
IL-3 [149]	*		
MMP-1 [152]	*		
MMP-2 [152]	*		
TIMP-1 [152, 166]	*		
TIMP-2 [166]	*		
SDF-1 [150, 151]	*		
LIF [166]	*		

producing effects in adjoining tissues, via paracrine effect. As MSCs are also easy to isolate from a variety of sources and relatively easy to expand in vitro, they have become an attractive regenerative element for future therapies and have been studied under such pretense.

In degenerated IVD, the current knowledge indicates that MSC therapy is safe in short-term and effective in promoting decrease in tissue degeneration [181]. But MSCs secretome constitutes a promising alternative for degenerated IVD, as its harsh, avascular environment hampers cell survival and recruitment. More work is needed to better understand the MSC-based regenerative mechanism in this disorder, to tune MSCs secretome towards IVD regeneration.

The objective of this work is to analyze the effect of MSCs secretome in a degenerative/inflammatory IVD model [182], depending on various MSCs preconditioning environments.

B. Materials and Methods

1. Culture and pre-conditioning of human bone marrow MSCs

1.1 Isolation of primary human bone marrow MSCs

MSCs were obtained from BM aspirates collected from the tibia of young patients undergoing knee surgery who did not suffer from any known inflammatory disease. Patients gave informed written consent for tissue use for research purposes and procedures were carried out in accordance with the relevant guidelines approved by the Centro Hospitalar São João Ethics Committee. Cells were isolated by density gradient centrifugation and selection of adherent cells in tissue culture plastic, as previously established in our group [183]. After Lymphoprep gradient density centrifugation at 1100g for 30 min, at 20ºC (no break), nucleated cells were collected and cultured in MSCs standard culture medium (SCM): Dulbecco's modified Eagle's medium (DMEM) with low glucose, 10% selected inactivated FBS (Gibco) and 1% penicillin/streptomycin (Invitrogen). Cells were incubated at 37ºC, with 5% CO_2.After 72h, non-adherent cells were removed and new medium was added. The medium was changed twice a week until cells reached approximately 80% confluence, after which, cells were detached by treatment with 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA; Invitrogen) and expanded in 150 cm^2 tissue culture flasks (BD Falcon). Isolation of MSCs was confirmed by surface expression of CD105, CD73 and CD90 and absence of expression of CD45, CD34, CD14, CD19 and HLA-DR and by testing the cells capacity to differentiate towards the chondrocyte, osteoblast or adipocyte lineage.

1.2 Routine culture of human MSCs

Experiments were performed with 2 different MSCs donors, tested for their phenotype and differentiation potential (as described in Supplementary Materials and Methods). The *ex vivo* routine expansion of human MSCs was performed using standard culture medium (SCM): Dulbecco's Modified Eagle's Medium with low glucose (DMEM, Gibco), supplemented with 10% v/v fetal bovine serum (FBS, MSCs qualified, Life Technologies Gibco) and 1% v/v P/S (Gibco). Cells were expanded in monolayer culture, at 37ºC, in a humidified

atmosphere with 5% v/v CO_2 in air. Medium was changed twice a week and cells were trypsinized when 70% confluence was reached and re-seeded at a concentration of 3000 cells/cm^3. Experiments were performed with cells in passages 4-7.

1.3 MSCs preconditioning

MSCs stimulation was performed 1 week after cell seeding in T25 flasks, by medium supplementation with either recombinant human IL-1β (10 ng/mL, PeproTech), recombinant human TNF-α (10ng/mL, Immunotools) or both, in normoxic (37ºC, in a humidified atmosphere with 5% v/v CO_2 in air) or hypoxic (37ºC under reduced oxygen atmosphere with 6% O_2 and 8.5% CO_2 and saturated humidity) culture conditions, as depicted in Figure 2.

After 48h, media were collected and stored at -80ºC for further use in *ex vivo* disc culture, or at -20ºC for later content analysis.

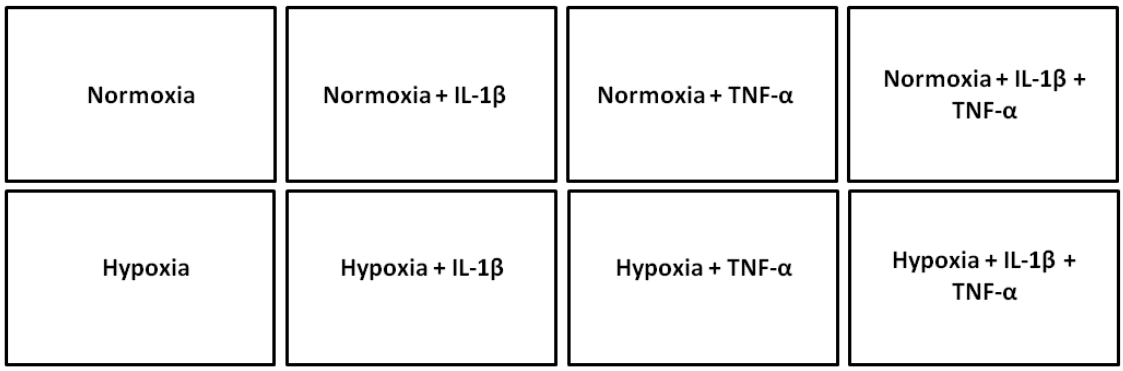


Figure 2| Preconditioning conditions design

2. Bovine intervertebral discs isolation and culture

Bovine IVDs were isolated from tails of young animals (less than 1 year), three hours after slaughter in the nearest accredited slaughterhouse. Tails were dissected under sterile conditions and the surrounding tissues (muscles, ligaments, nerves) were removed, exposing vertebrae and IVDs. Discs were then isolated from the adjacent vertebral bodies as close as possible to the endplates, punched to a standardized diameter of 9 mm, containing the NP and minimum AF lamellae in the borderline, and kept in phosphate buffer saline (PBS) with 1% v/v P/S and 0.5% v/v fungizone (Biowest).

After isolation, discs were cultured in 6-well tissue culture plates with membrane filter inserts on the top and 0.46 MPa static loading to prevent tissue swelling in

5 mL of DMEM medium supplemented with 5% v/v FBS, 1% v/v P/S, 0.5% v/v fungizone 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 (IVD medium) [182]. Discs culture was always conducted at 37ºC under reduced oxygen atmosphere with 6% O_2 and 8.5% CO_2 and saturated humidity as described [46]. Medium was changed every second day, for a period of 6 days.

2.1 IVD pro-inflammatory stimulation

At day six of culture, discs were punctured with a 21G needle that was introduced laterally up to the NP and rotated 180º three times clockwise and three times anticlockwise, and kept inserted for approximately 30sec. Additionally to this physical stimulus, culture medium was supplemented with 10 ng/mL recombinant human IL-1β. Three hours after this stimulus, medium was replaced with previously thawed MSCs conditioned media, similarly supplemented with recombinant human IL-1β (10 ng/mL, PeproTech). This condition was either maintained for 48h or 14 days, during which, two partial media exchanges were performed, renewing 1mL of conditioned media (~20% medium exchange).

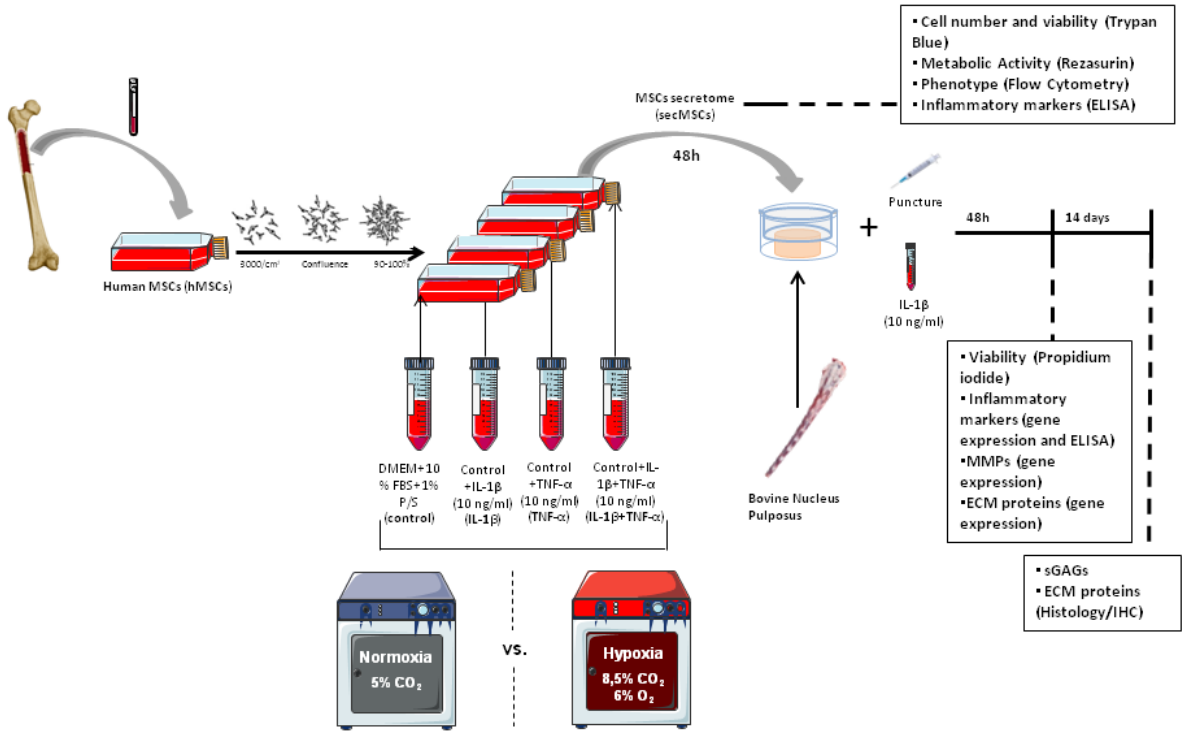


Figure 3| Experimental Design

3. Analysis of the effect of MSCs preconditioning on cell metabolic activity, viability, phenotype and inflammatory profile

3.1 Mitochondrial Metabolic Activity Analysis

Mitochondrial metabolic activity of MSCs was accessed by resazurin reduction assay. MSCs seeded on the cell culture inserts were transferred separately to new medium supplemented with 0.1 mg/mL resazurin sodium salt (Sigma), in the dark, for 3h at 37ºC. A blank well with resazurin working solution without cells was used. After 3h, resazurin reduction into resorufin fluorescence levels were read in a spectrophotometer microplate reader (BioTek Synergy HT), with a 560 nm excitation / 590 nm emission filter set. The discs were then washed three times with PBS for 10 min and frozen at -20ºC for later analysis.

3.2. Trypan Blue Exclusion Test of cell viability

MSCs expansion rate were ascertained by trypan blue dye exclusion assay. For that MSCs were trypsinized, centrifuged and resuspended in 1 mL of complete cell culture medium. Of these, 10 µL were collected and mixed with Trypan Blue dye in a 1:1 proportion of which 10 µL were transferred to a Neubauer chamber. The samples were then observed under the microscope and live/dead cells counted.

3.3 Flow Cytometry Analysis of MSCs phenotypic markers

MSCs phenotype was analyzed by flow cytometry, accordingly with the International Society for Stem Cell Research (ISSCR) criteria [184]. For flow cytometry analysis, cells were trypsinized, washed in FACS buffer (0.01% Sodium Azide/0.5% BSA in PBS) and centrifuged at 1200 rpm, for 7min. After discarding the supernatant, cells were resuspended with the following fluorochrome-conjugated antibodies: FITC-labelled CD105 (Immunotools), PE-labelled CD73 (BD) and APC-labelled and CD90 (eBioscience). As controls, IgG2a FITC, IgG1 PE and IgG1 APC were used. After incubation, cells were washed with FACS buffer and fixed in 4% paraformaldehyde (PFA). At least 10000 events were counted in a Flow Cytometer (FACSCanto, Becton Dickinson). Data was analyzed with FlowJo X software.

3.4. Gene Expression Analysis of MSCs

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed in duplicate on cDNA derived from MSC samples, using the SYBR Green method with specific published primer pairs designed using Primer 3 software to be inter-exonic and obtained from Eurofins Genomics (Ebersberg, Germany) for Interleukin 6 (IL-6), IL-10, TNF-α, IL-1β, TGF-β and β-actin [185] (in Supplementary Materials and Methods: Table S1), conducted on iQ5 Real-Time PCR Detection System (Bio-Rad) Primers sequences are presented in Table S1.

Briefly, the frozen cells were thawed on ice, total RNA extracted using ReliaPrep RNA Cell Miniprep System (Promega), according to manufacturer´s instructions, quantified using a Nanodrop spectrophotometer and transcribed into cDNA with IQTM SYBR Green Supermix (Bio-Rad). Before reverse transcription, RNA quality was assessed by means of RNA ratio of absorbance (abs 260/abs 280) provided by the Nanodrop spectrophotometer. RNA samples were diluted to the same concentration with nuclease-free water and purified by DNAse mediated digestion of contaminant DNA for 30 min at 37ºC. Inactivating Reagent (4 µL) was added and the mix centrifuged, precipitating the contaminant DNA. Of each RNA sample, 25 µL were transcribed into cDNA in two steps. First, the reaction mix was completed with deoxynucleotides (dNTPs, 2 µL) and random hexamers (2 µL) in a total volume of 29 µL, and incubated for for 5 min at 65ºC. Then, 1 µL SuperScript® III enzyme, 2 µL 0.1 DTT and 8 µL of buffer for reverse transcription (all from Invitrogen) were added to the mix. Samples were incubated for a cycle of 10 min at 25ºC, 60 min at 50ºC and 15 min at 70ºC, followed by storage at 4ºC.

Gene expression levels were determined by qRT-PCR mixes contained 1 µL cDNA, 10 µL iQTM SYBR® Green Supermix (Bio-Rad), 0.5 µL forward primer, 0.5 µL reverse primer and 8 µL RNase free water. Gene expression analysis was performed on ΔCt values according to a modified method described by MacLean et al. [47]. The average Ct value of each duplicate measurement of each sample was normalized to the house-keeping gene GAPDH (ΔCt) and the ΔCt of each stimulated sample was related to the ΔCt of each respective control (ΔΔCt). Fold changes in gene expression were then presented as $2^{-(\Delta\Delta Ct)}$, an

index that when <1 represents a down-regulation of the gene expression compared to its expression in the control sample and when >1 represents an opposite up-regulation.

3.5. Growth factors/cytokine analysis in MSCs secretome by ELISA

Culture medium collected from MSCs culture was centrifuged at 3000rpm for 5min to remove cell debris. The following growth factors/cytokines were analyzed by human ELISA kits, according to manufacturer's instructions,: PGE_2 (Arbor Assays), TGF-β (Biolegend), IL-8 (Peprotech), TNF-α (Biolegend), IL-10 (Biolegend), IL-12 (Biolegend), TSG-6 (RayBiothec) and IDO (Abbexa).

4. Analysis of the effect of MSCs secretome on IVD metabolic activity, viability and inflammatory profile

4.1 Mitochondrial Metabolic Activity Analysis

Mitochondrial metabolic activity of IVDs was accessed by resazurin reduction assay as described in section 3.1. Previous to the assay, discs were cut in half with a scalpel and one half was prepared for metabolic activity analysis. The other half was stored at -80ºC for later analysis.

4.2. IVD apoptosis/viability analysis by flow cytometry

IVDs were minced in small pieces and digested in DMEM with 1 mg/mL collagenase type I (Sigma-Aldrich). The digestion was conducted under stirring, reduced oxygen atmosphere with 6% O2 and 8.5% CO2 and saturated humidity, at 37ºC. After 2h of digestion, the mixture was passed through a cell strainer (100 µm pore size), washed with PBS and centrifuged at 400g for 10 min. Cells were labeled with FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) according to manufacturer's instructions (FITC-Annexin V and Propidium Iodide). Cells were then run on the FACSCalibur™ system (BD Biosciences). Results were analyzed with FlowJo software version 10.

4.3 Gene Expression Analysis of IVD cells

Gene expression of IVD cells was analyzed by qRT-PCR and SYBR Green method as mentioned in section 3.4. Specific primer pairs were designed using published gene sequences and Primer 3 for Interleukin 6 (IL-6), IL-8,

Metalloproteinase 1 (MMP1), MMP3 and MMP13, Collagen type II (Col II), Aggrecan (Agg) and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (in Supplementary Materials and Methods: Table S1), conducted on iQ5 Real-Time PCR Detection System (Bio-Rad) [186].

4.4. Analysis of IVDs supernatants

Culture medium was collected after IVDs pro-inflammatory stimulus and treatment with MSCs secretome (secMSCs) and centrifuged at 3000rpm for 5min to remove cell debris. The IVDs conditioned medium was analyzed by ELISA, according to manufacturer's instructions, for the following molecules: PGE_2 (Arbor Assays) and TGF-β (Biolegend).

5. Analysis of MSCs secretome long-term effect on IVD matrix content

MSCs secretome ability to impact matrix content on degenerated IVDs was assessed through quantification of sulfated glucosaminoglycans, proteoglycans, collagen II and aggrecan by histological analysis after 14 days of culture.

5.1. Sulphated glucosaminoglycans quantification

IVDs sulphated glucosaminoglycans (sGAG) content was assessed by reaction with 1,9-dimethyl-methylene blue zinc chloride double salt (DMMB, Sigma-Aldrich) dye reagent solution, containing 40mM sodium chloride (NaCl, Roth), 40mM glycine (Roth) and 46μM DMMB, previously adjusted to pH 3.0. Condroitin sulphate A sodium salt from bovine trachea (Sigma) was used as standard. Results were normalized by DNA content.

5.2. Proteoglycans detection by safranin O/light green staining

IVDs collected at day 14 of culture were fixed with formalin, processed and embedded in paraffin. Sections of 5 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 and Olympus CX31 light microscope equipped with a DP-25 camera (Imaging Software CellB, Olympus) using the 20x objective.

5.3. Collagen II and Aggrecan detection

Coll II distribution was analyzed by immunofluorescence staining and Agg production and distribution was analyzed by immunohistochemistry (IHC). For IHC, NovolinkTM 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 min at 37ºC. For Coll II staining, after the blocking step, sections were incubated for 2h at 37ºC with anti-collagen II-II6B3 (Developmental Sudies Hybridoma Bank) at a 1:50 dilution. Alexa Fluor 694-labeled goat anti-mouse (Invitrogen-Molecular Probes, 1:1000) was used as a secondary antibody. For Agg, sections were incubated overnight with Agg 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 200M, Zeiss) and the 10x objective, for Coll II staining. Coll II intensity was quantified using a custom-made MATLAB (The MathWorks Inc., Natick MA, USA) script, the IntesityStatiscticsMask Software [186, 187]. An average of three quantifications performed in three different sections of the analyzed disc was used. Agg stained sections were imaged with light microscopy with 2x objective for qualitative analysis.

6. Statistical analysis

Results are presented as scatter dot plots with represented median. Statistical analysis was performed using GraphPad Prism (vs. 6). Due to low number of replicates, non-parametric tests were used. Unpaired tests as Mann-Whitney and Kruskal-Wallis were used to compare 2 or more groups, respectively. Nevertheless, due to the low number of replicates no statistical significance (confidence level 95%) was observed.

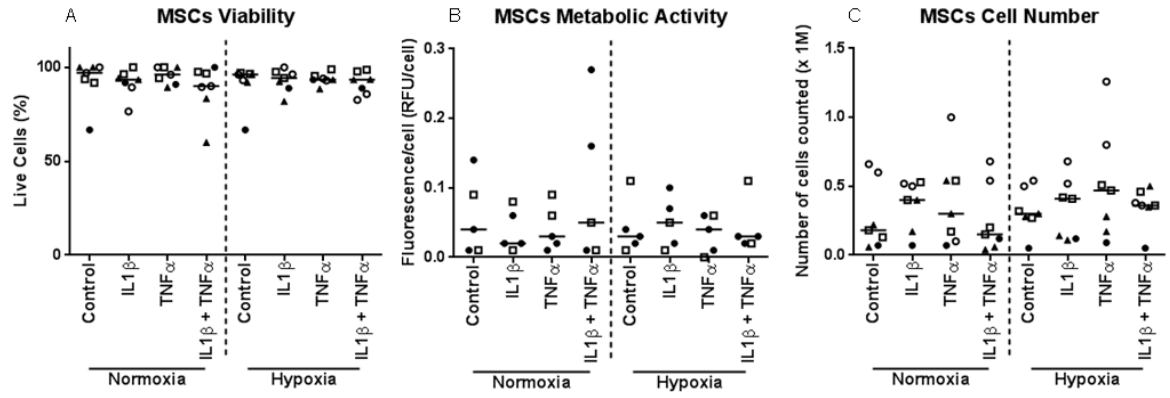
C. Results

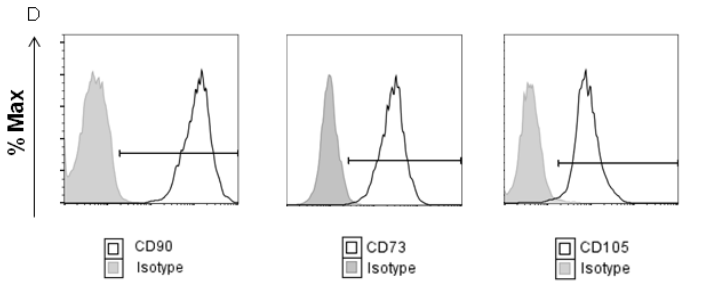
The work here described focus on the influence of pre-conditioned secMSCs on a degenerated/pro-inflammatory bovine disc ex vivo model, under the pretense that MSCs paracrine action has a decisive role on the positive results reported by previous works on degenerated IVD's immunomodulation and tissue regeneration. The experimental layout is designed in Figure 3.

1. The influence of preconditioning on MSCs

In the context of this work how MSCs preconditioning could affect cells metabolic activity, proliferation, viability and phenotype was first addressed.

1.1. Effect of MSCs preconditioning on cell viability and phenotype





%	CD105	CD90	CD73
MSCs before pre-conditioning	93,24	98,66	97,42
Should be	>95%	>95%	>95%
N	95,67	98,12	97,1
N + IL1β	89,47	97,52	93,4
N + TNFα	95,27	98,52	97,5
N + IL1β + TNFα	96,07	98,52	97,9
H	91,47	98,62	96
H + IL1β	91,77	98,62	97,6
H + TNFα	94,07	98,62	98
H + IL1β + TNFα	94,97	98,62	98,1

Figure 4| Analysis of MSCs after pre-conditioning with IL-1β (10ng/ml), TNF-α (10ng/ml) or IL-1β + TNF-α (10ng/ml) in either normoxic (6% O2) or hypoxic (21% O2) culture conditions.

A. Number of cells after 48h of pre-conditioning, by Trypan Blue Exclusion Assay . Results are presented as scatter dor plots with differentiated experiments. **B.** Percentage of live cells, by Trypan Blue Exclusion Assay. Results are presented as scatter dor plots with differentiated experiments. **C.** Analysis of MSCs metabolic activity by Resazurin Reduction Assay. Fluorescence was read at 560/590 nm (excitation/emission wavelenghts). Results are presented as scatter dor plots with differentiated experiments. **D.** Histogram profile and quantitative analysis of MSCs phenotype exhibiting expression of CD90, CD105, CD73.

The effect of MSCs pre-conditioning with inflammatory cytokines - IL-1β (10 ng/mL), TNF-α (10 ng/mL) or IL-1β+TNF-α (both 10 ng/mL) and oxygen levels - normoxia (21% O_2) or hypoxia (6% O_2) - on cell metabolic activity, viability and phenotype was addressed 48h after stimulus. The results indicate that MSCs viability and metabolic activity was maintained with the different pre-conditioning conditions after 48h (Fig 4A and 4B). Still, IL-1β- or TNF-α-preconditioning in normoxia and hypoxia by itself seemed to slightly increase cell proliferation when compared to control (normoxia without pro-inflammatory stimuli) (Fig 4C). To guarantee that MSCs phenotype was maintained with these culture conditions, the expression of MSCs positive surface markers - CD90, CD73 and CD105 – was addressed and no differences were observed when compared with surface expression of the same markers in MSCs without pre-conditioning (Fig 4D).

2. Effect of MSCs secretomes (secMSCs) on the degenerated IVD

To take advantage of MSCs immunomodulatory potential as well as their paracrine mode of action, different secMSCs were produced by MSCs pre-conditioning (as described in 1) and used to cultivate bovine IVD punches previously stimulated with IL-1β (10 ng/mL) and needle puncture, consisting in an ex-vivo model that mimics the pro-inflammatory environment of a degenerated disc. These cultures were maintained for 48h to study the effect that these secretomes could have in the inflammatory state of the disc and in the early signs of matrix unbalance, or for 14 days to observe the effects produced on the matrix at protein level. Three different controls were used: non-stimulated IVDs (Control), IVDs needle punctured and stimulated with IL-1β (10ng/mL) to simulate degenerative/pro-inflammatory conditions (IL-1β) and IL-1β-stimulated IVDs co-cultured with MSCs seeded on the culture inserts (IL1β+MSCs).

2.1. Influence of different secMSCs on the pro-inflammatory/degenerated IVDs apoptosis/viability

In order to evaluate the effect of the secMSCs on the IVD cells apoptosis/viability, IVDs were digested and IVD cells stained with PI/AnnexinV and analyzed by flow cytometry (Fig 5A). Both cell apoptosis (AnnexinV+/PI-) (Fig. 5B) and cell death (AnnexinV+/PI+) (Fig. 5C) did not seem to be significantly affected by any of the conditions tested, with percentages about 20%.

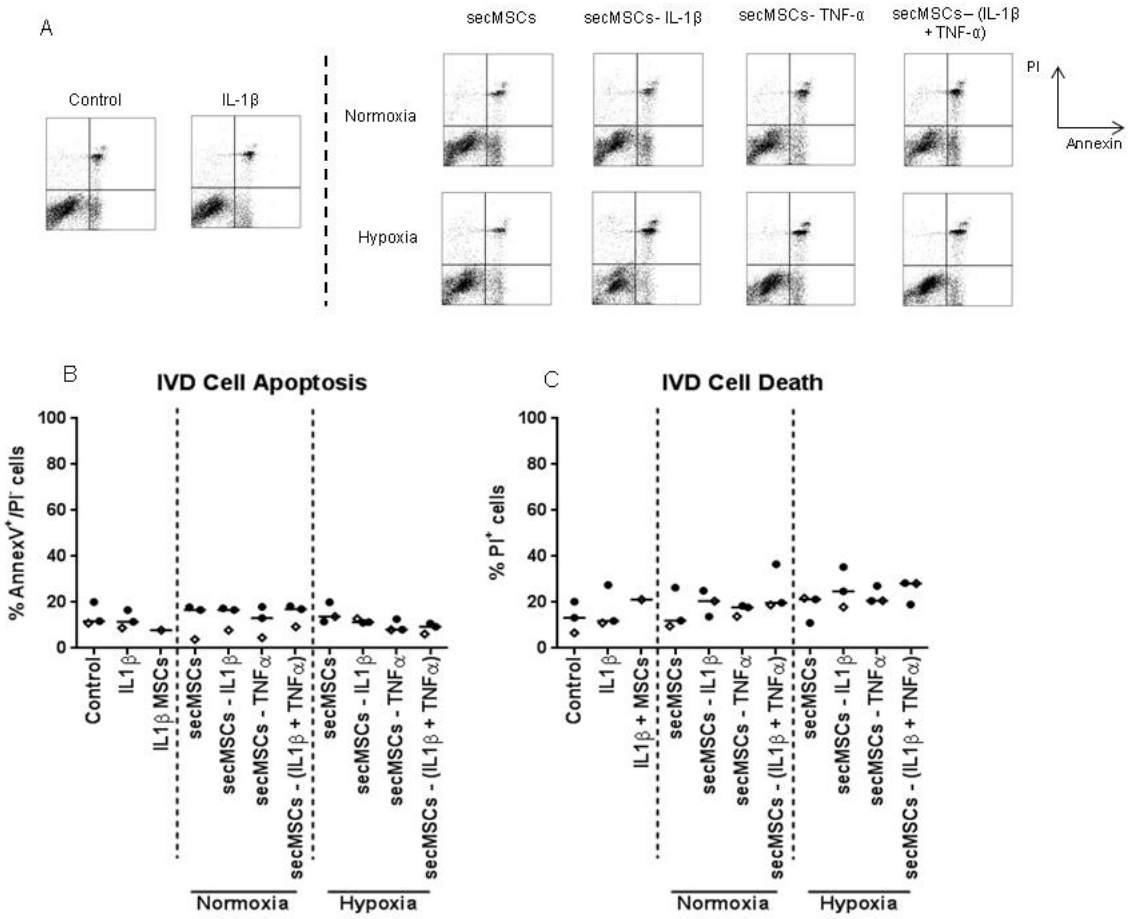


Figure 5| Analysis of IVD condition after pro-inflammatory stimulus with IL-1β (10ng/mL) + puncture and posterior treatment with MSCs or MSCs secretomes.

A. Dual parameter flow cytometry analysis of IVD cells with PI/Annexin fluorescent labeling to define percentage of necrotic and late apoptotic cells (upper quadrants), early apoptotic cells (right lower quadrant) and live cells (left lower quadrant). **B**. Quantitative analysis of flow cytometry analysis with definition of percentage of apoptotic cells. **C**. Quantitative analysis of flow cytometry analysis with definition of percentage of apoptotic cells. Results are presented as scatter dot plots with differentiated experiments.

2.2. Effect of secMSCs on the IVDs inflammatory state

Considering MSCs immunomodulatory potential, the effect of different secMSCs on the pro-inflammatory/degenerated disc model was assessed. Gene expression and protein production of some factors involved in the inflammatory state was analyzed 48h after inflammatory stimulus and treatment with secMSCs. As illustrated in Figure 6A, *IL-6*, *IL-8* and *TNF-α* expressions were upregulated in the discs stimulated with IL-1β, as expected, as these are cytokines globally associated with an inflammatory state [38, 188, 189]. Co-culture with MSCs seemed to maintain IL-8 up-regulation, increase *IL-6*

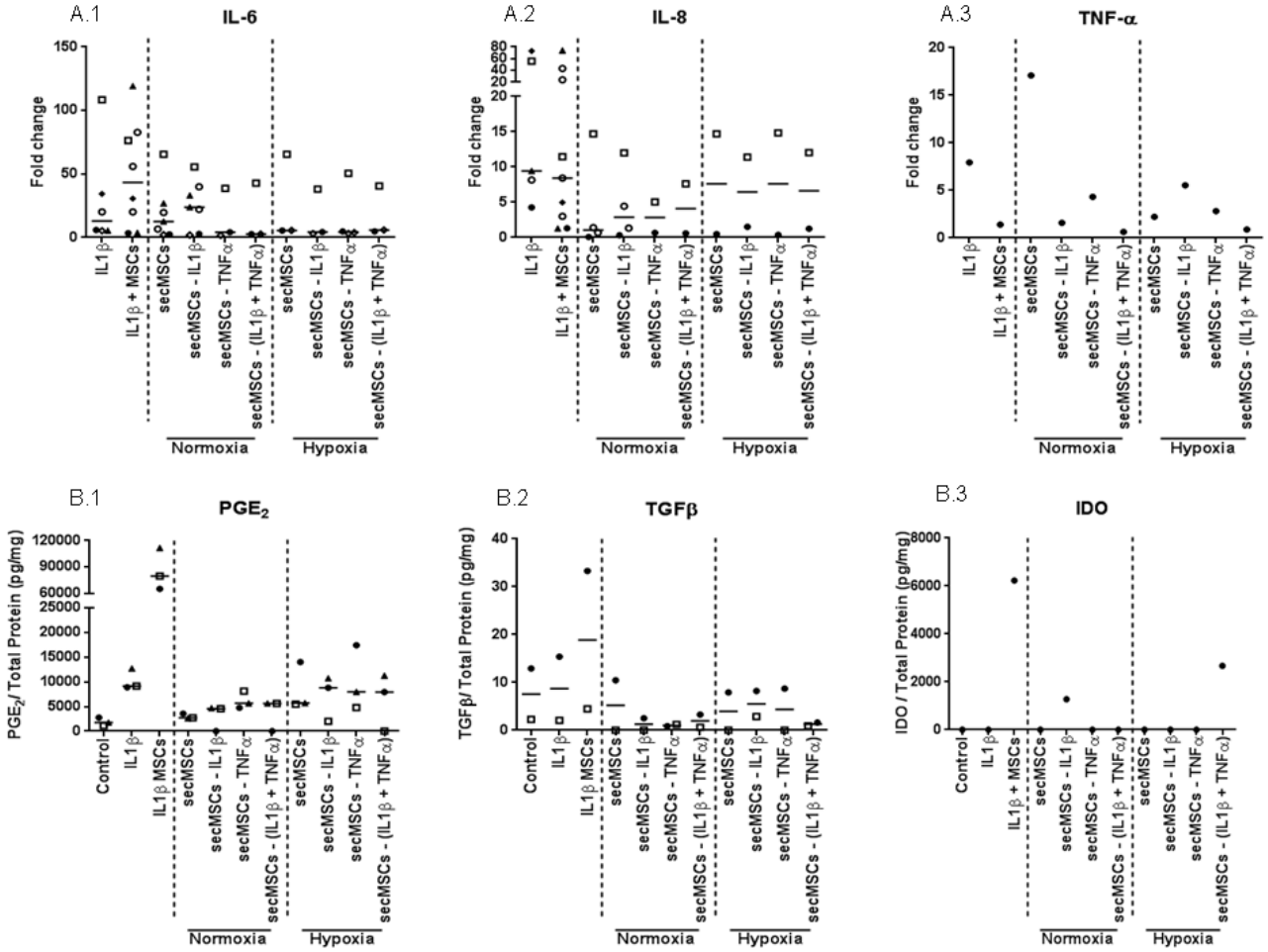


Figure 6| Effect of different secMSCs on IVDs inflammatory response, 2 days after pro-inflammatory stimulus and treatment. Quantitative analysis of pro-inflammatory markers.

A. mRNA expression of IL-6 (**A.1**), IL-8 (**A.2**) and TNF-α (**A.3**). Levels of mRNA were normalized to GAPDH. The ratio of stimulation to control condition indicates the fold change of induction after stimulation (control level=1). Results are presented as scatter dor plots with differentiated experiments. **B.** PGE2 (**B.1**), TGF-β (**B.2**) and IDO (**B.3**) concentration, normalized to total protein concentration in culture medium minus concentration found in secMSCs prior to IVDs treatment. Results are presented as scatter dor plots with differentiated experiments.

expression (about 3-fold increase) and downregulate *TNF-α* (more than 5 times decrease), in the one indicative experiment that yielded detectable results for this cytokine.

Treatment with secMSCs seemed to considerably reduce *IL-6* and IL-8 up-regulation caused by the stimulus with IL-1β and even downregulate its expression comparatively to control, in some animals. For *IL-6*, only unstimulated secMSCs or produced in normoxia with IL-1β alone maintained or presented a slight up-regulation compared with the IL-1β condition (approximately 2-fold increase). IL-8 seemed to be upregulated when discs were treated with secMSCs produced in hypoxia when compared with those produced in normoxia. The unstimulated secMSCs decreased IL-8 expression to control levels, completely countering the up-regulation elicited by the stimulation with IL-1β. In both cases, treatment with secMSCs seemed to reduce cytokine expression, especially in secretomes produced in normoxia.

For *TNF-α*, treatment with unstimulated secMSCs seemed to highly increase its expression and treatment with secMSCs preconditioned with both cytokines seemed to maintain control levels.

Regarding the analysis of protein produced to the medium (Figure 6B), the stimulus with IL-1β seemed to increase PGE_2 production (from 1754 pg/mg protein to 9156 pg/mg) and have no significant effect on TGF-β levels (about 7,59-8,73 pg/mg protein), indicating that under inflammatory stimulation, IVD cells produce more PGE_2, known as an inflammatory marker, but not TGF-β that is recognized as an anti-inflammatory player. IDO was undetectable in either control or IL-1β conditions. For all three molecules, co-culture with MSCs seemed to significantly increase protein levels in supernatant. Treatment with secMSCs seemed to decrease PGE_2 (to 5536 pg/mg protein (median)) and TGF-β (to 1,42 pg/mg protein (median)) protein levels, an effect generally pronounced in conditions where secretome was produced in normoxia conditions.

Levels of IDO were only detectable in discs treated with two different secretomes: secretome produced in normoxia with IL-1β (1276 pg/mg) and secretome produced in hypoxia with both cytokines present (2669 pg/mg). Both levels were lower than the level obtained with MSCs in co-culture (6229 pg/mg).

2.3. Effect of secMSCs on initial IVDs matrix degradation

As the main consequence of IVDs degeneration is the loss of ECM the effect of various secMSCs on expression of MMPs and matrix main components – *Col II* and *Agg* – was analyzed, 48h after pro-inflammatory stimulus and treatment with secMSCs, by qRT-PCR.

The pro-inflammatory stimulus by itself, up-regulated MMPs levels comparatively to control, particularly *MMP1* and *MMP3* (8,7 and 9,1-fold increase respectively). Co-culture with MSCs maintained *MMP3* and increased *MMP1* expression levels (to 43,4 fold-increase), when compared with discs

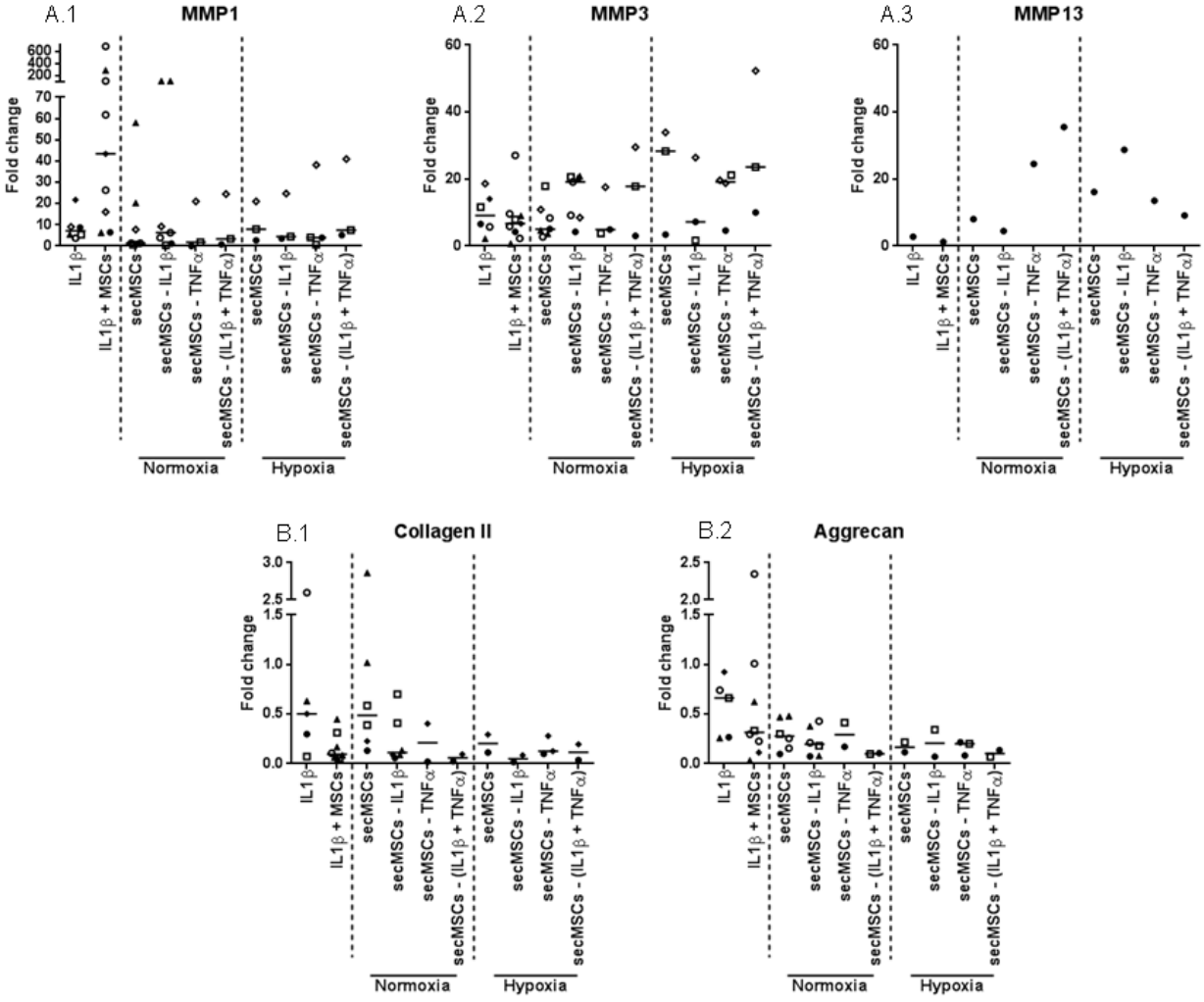


Figure 7| Effect of different secMSCs on IVDs matrix maintenance and content, 2 days after pro-inflammatory stimulus and treatment.

Quantitative analysis of MMPs (matrix metaloproteinases). **A**. mRNA expression of MMP1 (**A.1**), MMP2 (**A.2**), MMP13 (**A.3**) **B.** Matrix components Collagen type II (**B.1**) and Aggrecan (**B.2**). Levels of mRNA were normalized to GAPDH. The ratio of stimulation to control condition indicates the fold change of induction after stimulation (control level=1). Results are presented as scatter dor plots with differentiated experiments.

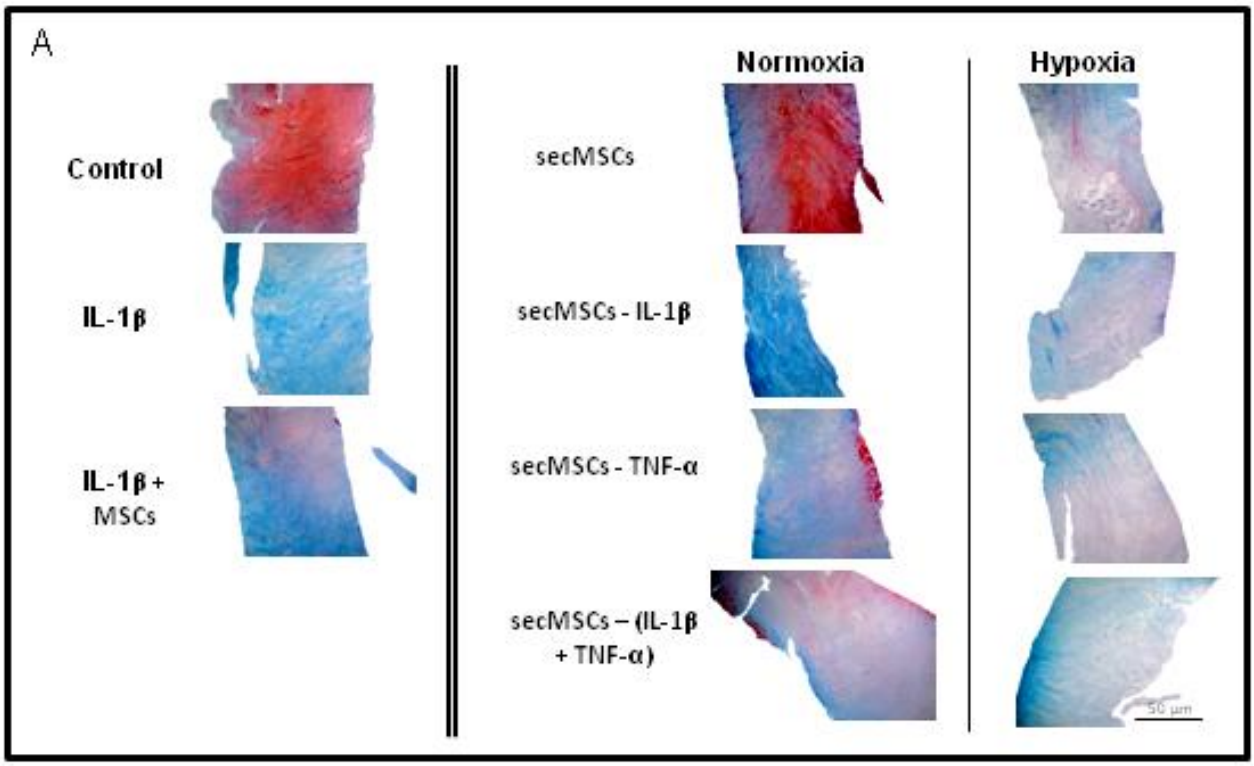
solely stimulated with IL-1β. Contrarily, MSCs seemed to down-regulate *MMP13* comparatively with the pro-inflammatory condition, in the only experiment that yielded detectable levels of expression.

Treatment with secMSCs appeared to always decrease *MMP1* expression, independently of preconditioning, while co-culture with MSCs presents a broader range of results, demonstrated in Figure 7A. On the other hand, *MMP3* expression seems to be stimulated in discs treated with secMSCs produced in hypoxia (except for the condition secMSCs-IL-1β) (20,4-fold increase) and normoxia-IL-1β (19,1-fold increase) or normoxia-(IL-1β+TNF-α) (17,8-fold). In general, secMSCs seem to contribute to an increase in the expression of this enzyme and the effect of IL-1β seems to be dependent on oxygen availability. Regarding *Col II* and *Agg* (Figure 7B), stimulation with IL-1β elicited a downregulation in expression (down to 0,5-0,7 fold-change), comparatively to control, as expected [186]. Treatment with MSCs and secMSCs seemed to further downregulate their expression (down to 0,12-0,21 fold-change), 48h after treatment.

2.4. Effect of secMSCs on IVDs matrix degradation at protein level

To fully understand how both pro-inflammatory stimulus and treatment with secMSCs could influence disc degenerative state, their effects in the degenerated disc ECM were investigated after a 14-days culture period. Discs were prepared for histological analysis of proteoglycans (Safranin staining) and Col II and Agg (IHC) content. Biochemical analysis of sGAG content was also performed.

Relatively to proteoglycans content (depicted in Figure 8A), discs stimulated with IL-1β appeared to lose proteoglycan content (stained in red), comparatively to control, confirming that an inflammatory state seems to be linked to tissue degeneration. On the other hand, co-culture with MSCs seemed to slightly increase the proteoglycan content of the disc, indicating that the presence of the MSCs may have a positive effect in the matrix regeneration. Discs treated with secMSCs without pre-conditioning and TNF-α-pre-conditioned also seemed to recover some proteoglycan content. On the other hand, secMSCs produced under hypoxic conditions didn't have an effect in proteoglycan content.



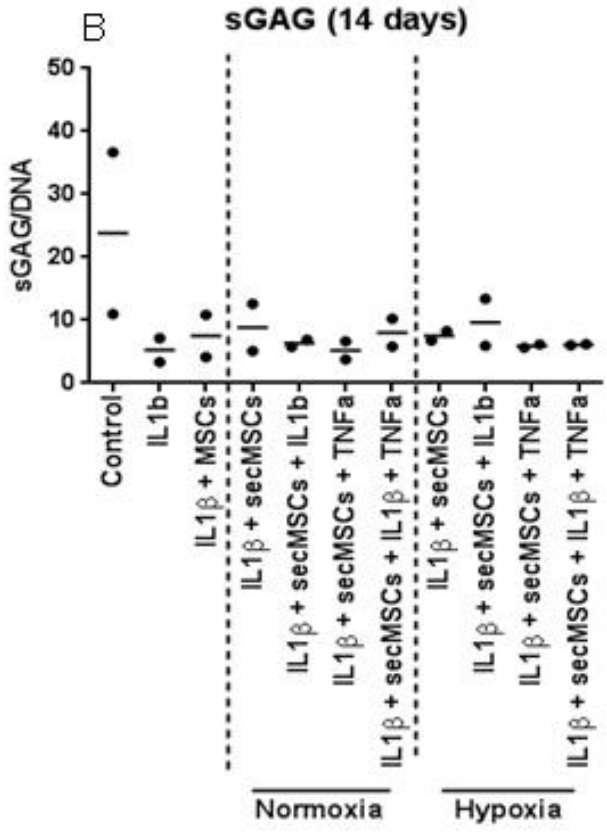
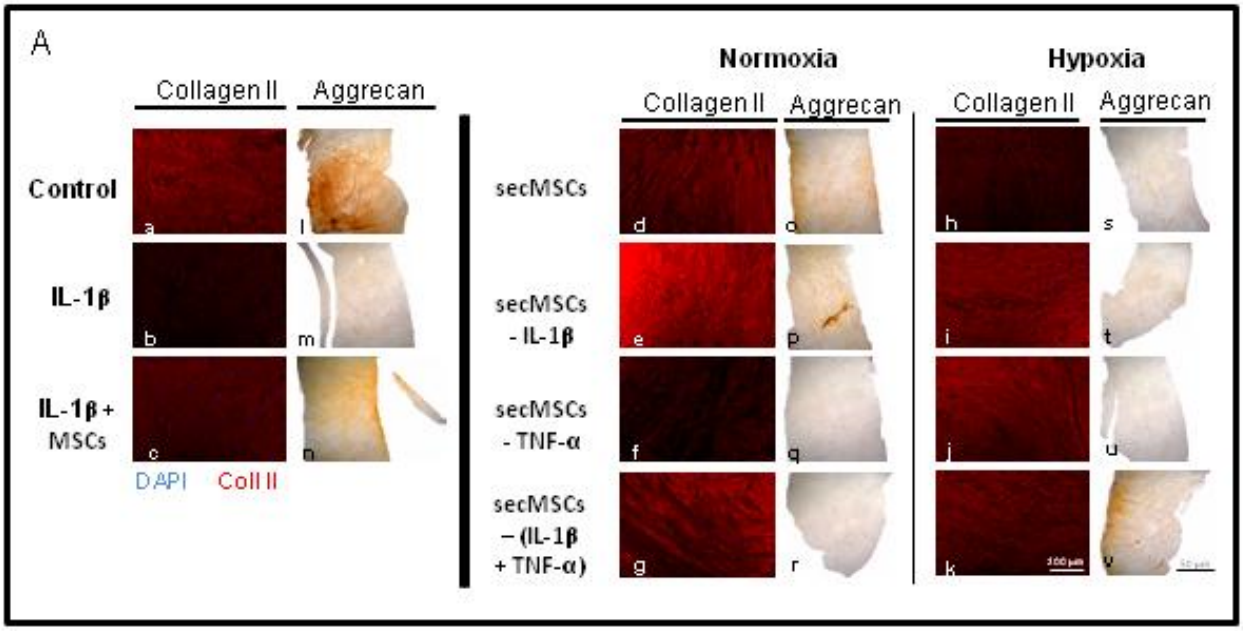


Figure 8| Effect of different secMSCs on inflammatory/degenerated IVD model matrix content, at the protein level, 14 days after pro-inflammatory stimulus and treatment.

A. Saggital sections of disc punches stained for proteoglycans (scale bar: 50 µm). **B.** Biochemical analysis of sGAG content of IVD punches.



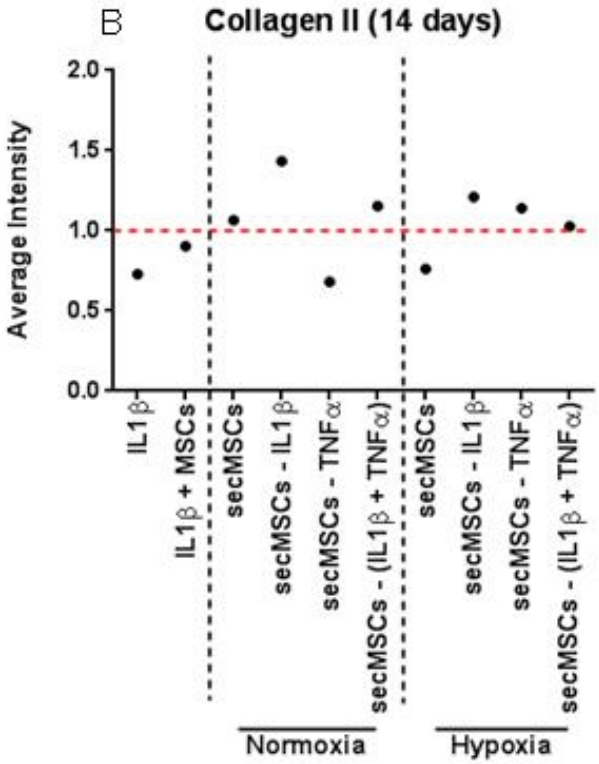


Figure 9| Effect of different secMSCs on inflammatory/degenerated IVD model matrix content, at the protein level, 14 days after pro-inflammatory stimulus and treatment.

A. Saggital sections of disc punches stained for collagen type II (a-k, scale bar: 200μm), aggrecan (l-v, scale bar: 50μm). **B.** Collagen II fluorescence intensity normalized to control (dashed line).

The pro-inflammatory stimulus seemed to reduce sGAG content (Figure 8B) (from 23,9 to 5,25 sGAG/DNA) and neither MSCs nor secMSCs were able to promote tissue recovering at this point (the median level of GAGs of IVDs treated with secMSCs was 6,16).

A qualitative analysis of Agg content was also performed upon visual evaluation, as well as a quantification of collagen fluorescence intensity, using a custom-made MATLAB script software [187]. Representative images are presented in Figure 9A. Comparatively to control, and as observed for proteoglycans and sGAG, IL-1β treatment of IVDs induced a significant loss of Agg and Col II (down to 0,72 fold-change) content that co-culture with MSCs appeared to partially recover (up to 0,9 fold-change). The secMSCs treatment in general increased Col II content, independently of oxygen availability, specially using secretome produced without pre-conditioning and IL-1β-preconditioned cells (up to 1,06 and to 1,43 fold-change, respectively). Concerning Agg production, only secMSCs obtained without pre-conditioning, IL-1β-preconditioned and (IL-1β+TNF-α)-preconditioned in hypoxia were able to increase Agg production. Overall, treatment of pro-inflammatory/degenerated IVD with secMSCs produced in normoxia and with IL-1β-preconditioning appear to promote Coll II and Agg production, while TNF-α-preconditioning appears to contribute to proteoglycans production.

3. Influence of preconditioning on MSC immunomodulatory profile

MSCs immunomodulatory capacity has been widely explored in the context of diverse immune disorders and can, perhaps, explain some of the observed modifications in degenerative/inflammatory disc response when treated with different secMSCs. Here, gene expression of immune-related factors in MSCs and inflammatory cytokines production to the culture medium was assessed 48h after preconditioning.

Gene expression analysis of *IL-6, IL-10, TNF-α* and *IL-1β* was tested, with only *IL-6* wielding results as all others were kept under detection limit; *IL-6* was upregulated in IL-1β-preconditioned MSCs (6,5-fold increase), independently of oxygen availability and the presence of TNF-α (Fig. 10A). Cytokine analysis of secMSC revealed that IL-10, IL-1β and TNF-α were undetectable also.

Similarly, IL-12, IDO and TSG-6 were not possible to detect by ELISA. IL-8 production increased by pre-conditioning MSCs with either IL-1β or TNF-α only (from 101 to 154 and to 163 pg/mg protein), but not with both cytokines (Fig.10B).

Oxygen levels didn't seem to influence IL-8 production, although only two experiences were performed. PGE_2 production was increased in the presence of IL-1β and particularly in (IL-1β+TNF-α)-treated cells (5-8-fold increase), independently of oxygen availability (Fig 10C). Free active TGF-β1 levels seems to be slightly increased in the presence of IL-1β (from 6,7 to 11,3 pg/mg protein) while decreased when both, IL-1β and TNF-α (1,3 pg/mg), were used, while hypoxia seemed to reduce TGF-β production in all conditions except in the presence of TNF-α (down to a median value of 2,1 pg/mg) (Fig 10D).

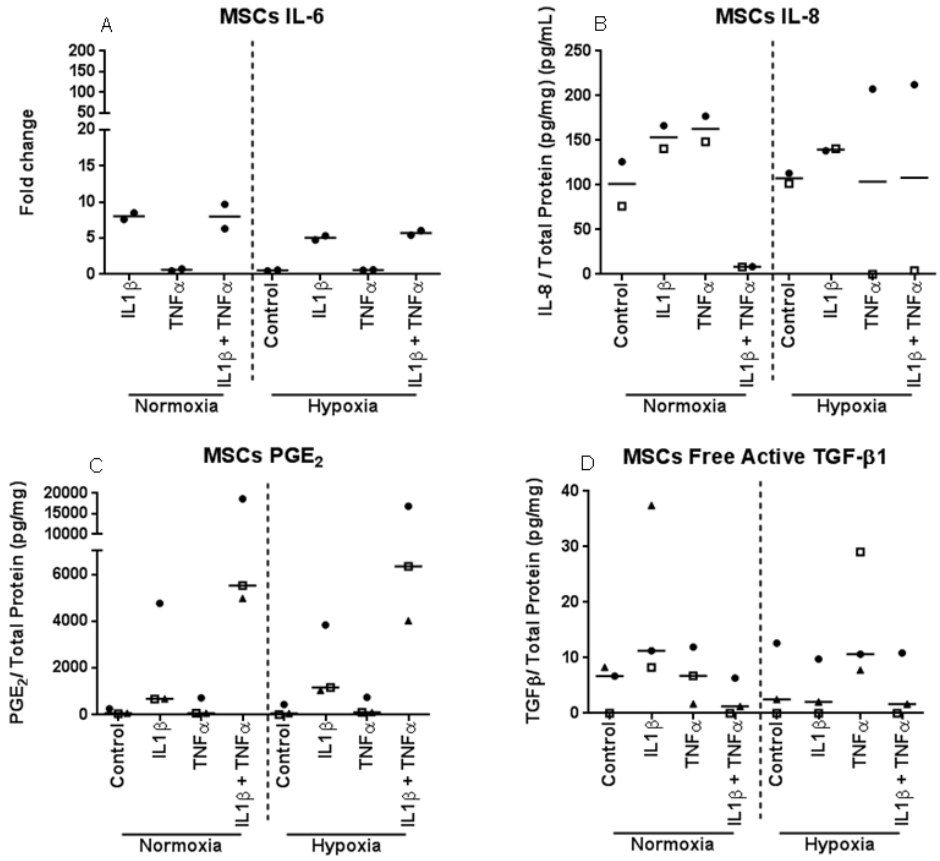


Figure 10| Analysis of MSCs inflammatory-related gene expression and molecular secretion profile , after pre-conditioning with IL-1β (10ng/ml), TNF-α (10ng/ml) or IL-1β + TNF-α (10ng/ml) in either normoxic (6% O2) or hypoxic (21% O2) culture conditions.

A. mRNA expression of IL-6 in MSCs. Levels of mRNA were normalized to β-actin. The ratio of stimulation to control condition (MSCs) indicates the fold change of induction after stimulation (control level=1). Results are presented as scatter dor plots with differentiated experiments**. B.** IL-8 concentration in culture medium. Results are presented as scatter dor plots with differentiated experiments. **C.** PGE_2 concentration, normalized to total protein concentration in culture medium. Results are presented as scatter dor plots with differentiated experiments. D. TGF-β concentration, normalized to total protein concentration in culture medium. Results are presented as scatter dot plots with differentiated experiments

D. Discussion

The main goal of this study was to evaluate the immunomodulatory capacity of secMSCs in degenerated IVD. For that, an ex vivo model consisting in an organotypic culture of bovine IVDs stimulated with needle puncture and pro-inflammatory cytokines previously established in the group was used [182]. This *ex vivo* approach is more reliable than any 2D *in vitro* culture due to the increased complexity represented and to the fact that IVD cells cultured *in vitro* lose the ability to produce native ECM [190]. Explant cultures of bovine discs seem to be suitable for the study of the IVD degenerative process as they are easily available, allow for well-controlled culture conditions [191, 192] and are similar to the human IVD, having identical physical-chemical properties [193], identical size and considering that the musculature of the bovine tail maintain a pressure in the disc that is approximately the same as the one existent in human lumbar discs in the prone position [194]. Moreover, although *in vivo* models could be more reliable, the *in vivo* models of disc injury, normally established by IVD puncture or NP enzymatic digestion, do not simulate the natural process of human IVD degeneration and do not mimic the mechanical forces caused by the biped position of the human spine [194]. The inflammatory environment was promoted using needle puncture to simulate disc degeneration, as demonstrated in other studies [182, 195, 196], in addition to medium supplementation with IL-1β. This cytokine is highly expressed during IVD degenerative process [197] and has been shown to lead to the loss of ECM, mainly sGAG [198], inducing the desired degenerative state.

MSCs have been proven to elicit a regenerative response in the degenerated IVD, either as a cell therapy [54, 123, 199, 200] or recurring to their secretome [161, 162]. In this study, it was hypothesized that MSCs preconditioning with pro-inflammatory cytokines and hypoxia, present in degenerated IVDs environment, could stimulate MSCs to produce a secretome with immunomodulatory and/or regenerative properties, with therapeutic potential in the DDD context. IL-1β and TNF-α are pro-inflammatory cytokines constitutively expressed in the IVD, particularly in degenerative states [31]. TNF-α has been associated to disc herniation and nerve in-growth [201, 202] and both cytokines have been demonstrated to induce up-regulation of matrix-degrading enzymes

gene expression [197, 203]. These inflammatory factors, as well as hypoxia, were reported to stimulate MSCs regenerative and immunomodulatory abilities on other contexts [168, 204-207].

The secMSC was obtained 48h after cells preconditioning with IL-1β and/or TNF-α under hypoxic (6% O_2) or normoxic culture conditions. MSCs metabolic activity, viability, proliferation and phenotype was maintained in all the conditions tested, in agreement with the literature [208-212], although the effect of hypoxic preconditioning on MSCs viability seems to gather contradictory results in other studies, with one group observing increase in number of MSCs [213] and another reporting decreased cell viability [214], although in these cases the hypoxia values were 1% O_2. In this study the oxygen level was selected based on NP average normoxic conditions that were previously studied and optimized in the context of the degenerative/inflammatory disc model used [215].

The secMSCs obtained upon different pre-conditioning conditions were used to culture pro-inflammatory/degenerative IVD organ cultures stimulated with a needle puncture and IL-1β (10ng/mL), as previously established [182]. As a control, discs were co-cultured with MSCs in similar number to those used to obtain secMSCs. IVD viability was maintained within all the conditions tested.

Considering the high number of factors analyzed concerning degenerated IVDs response to the treatment with secMSCs, a heat map was created with an overview of all the results observed at 48h to help visualize connections and tendencies between results (Figure S2).

When stimulated with IL-1β, the expression of pro-inflammatory markers *IL-8, TNF-α* and *IL-6,* as well as the production of PGE_2, increases, contrarily to what was observed for TGF-β1. This is in conformity with the observations of others that have linked an higher production of pro-inflammatory factors in degenerated IVD [30, 31, 37]. IL-1β stimulation of IVD also upregulated MMPs expression, particularly *MMP1* and *MMP3*, demonstrating that the inflammatory state induced stimulates matrix catabolism. This result is also expressed by the decrease in the ECM main components, *Agg* and *Col II*, in IL-1β-stimulated IVDs.

48h after treatment with secMSCs obtained in normoxia and pre-conditioning with IL-1β, the levels of immune regulatory cytokines in IVD decreased (*IL-6*, PGE_2, TGF-β and IDO), while *IL-8* was moderately maintained.

In what concerns ECM remodeling at this time point, secMSCs in general appear to stimulate *MMP3* and *MMP13* compared to IL-1b-treated IVDs and cannot revert *Agg* and *Col II* gene expression levels, previously down-regulated by IL-1b treatment. Only secMSC obtained from MSCs without pre-conditioning elicit less loss of *Col II* expression and less increase in MMPs mRNA levels.

MSCs co-culture with IVDs in pro-inflammatory conditions was performed as a control of secMSCs. In this case, MSCs induce an increase in the pro-inflammatory markers *IL-6* and PGE_2 while maintain *IL-8* expression levels, which was contrarily to the treatment with secMSCs. A decrease in TGF-β and *TNF-α* was observed, this last one previously reported by Bertolo et al. [216]. Miyamoto et al. demonstrated an overall downregulation of all inflammatory cytokines analyzed, mostly IL-cytokines (such as *IL-6, IL-11, IL-3* and *IL-15*) and of the *TNF* family and *MMPs* as well as an up-regulation of *Col II* when rat NP cells were co-cultured with human MSCs [217]. However, in a recent *in vivo* rat degenerated disc model, treatment with MSCs seemed to downregulate TNF-α, along with IL-13 and up-regulate IL-6 along with other interleukins such as IL-2, IL-4 and IL-10 [218], similarly to the results obtained so far in this study.

In addition, IVD cultures were maintained until 14 days to evaluate the effect of secMSCs on ECM components at the protein levels. Pro-inflammatory stimulation of IVDs was confirmed to cause loss of matrix components, as observed in other studies [140, 203]. MSCs co-culture seemed to slightly induce ECM recovering in accordance with the literature [101, 102, 118, 122]. secMSCs obtained without pre-conditioning generally increase proteoglycan levels and Col II production, while secMSCs obtained from preconditioning with IL-1β seemed to block that effect. Agg production appeared to be moderately recovered in discs treated with secMSCs without pre-conditioning, secMSCs-IL-1β in normoxia and secMSCs-(IL-1β+TFN-α) in hypoxia. Curiously, these were the same secretomes that demonstrated a higher reduction of the pro-inflammatory mediators in IVD, 48h after treatment, as well as a stimulation of

immune-regulatory cytokines such as TGF-β and IDO. To our knowledge, this is the first report on this interplay between IDO levels and matrix degradation. Even though this observation is based in only one experiment, it may wield interesting results if further explored.

Therefore, to dissect the mechanisms behind the immune-modulatory capacity of secMSCs in the pro-inflammatory IVD organ culture, several inflammatory mediators were analyzed by gene expression in pre-conditioned MSCs and by ELISA in secMSCs. MSCs preconditioning with IL-1β seemed to up-regulate *IL-6* expression and increase PGE_2 production, independently of oxygen availability. Although both proteins were shown to be stimulated under pro-inflammatory conditions [167, 189, 219], in this study TNF-α by itself did not have a direct effect in *IL-6* and PGE_2, contrary to the observations of Aggarwal et al. and Heo et al. [67, 220] that indicated a definite link between MSCs exposure to TNF-α and increased production of IL-6 and PGE_2 in response to the inflammatory environment. Also, Bouffi et al. demonstrated a symbiotic relationship between IL-6 and PGE_2, with IL-6 dependent PGE_2 secretion and IL-6 activated pathways through PGE_2 up-regulation [221]. Additionally, when exposed to high levels of IL-6, NP cells were shown to increase the production of PGE_2, namely through enhancement of IL-1β and TNF-α, further confirming the link between these two cytokines and demonstrating their relevance in the context of IVD degeneration [222].

In our study, hypoxia seemed to decrease TGF-β content in secMSCs, contrarily to previous studies that demonstrated that TGF-β production is stimulated in hypoxic conditions [223], through HIF-1 binding [224]. This contradictory result may be, perhaps, due to the difference in available oxygen, as cited studies maintained cells cultured under 1% O_2, while here 6% O_2 was used to culture MSCs. Interestingly, TNF-α-preconditioning appeared to block this effect. Classically, TGF-β is known for an anti-inflammatory action [167, 225], meaning that IL-1β would stimulate the production of pro-inflammatory cytokines (IL-6, PGE_2) while hypoxia would inhibit anti-inflammatory signaling.

IL-6 and $PGE_{2,}$ together with IDO, are considered to be the major molecules responsible for the immune regulatory ability of MSCs [167, 171, 226], due to their dual pro- and anti-inflammatory role, making it extremely more complex to understand the practical implications of these differences.

Although interesting results were observed, an insufficient number of replicas to obtain statistical significance was considered, so future experiments need to be conducted to ensure the results observed. Moreover, although conducted in an organ culture model previously validated, this model has limitations such as the presence of immune cells, present in human degenerated discs that are often in close contact with blood supply, as there is an increase in angiogenesis as a result of the degenerative process [12]. It would be valuable to understand the influence of discs treated with secMSCs in immune cells, as this could be a key factor in the regenerative process. Also, future *in vivo* experiments could contribute profoundly to the understanding of these processes.

E. Conclusion

This study suggests that secMSCs can modulate the inflammatory response and ECM of degenerated disc. MSCs preconditioning with pro-inflammatory mediators and low oxygen availability elicited different cell responses in degenerated IVD. secMSCs produced in normoxia (independently of the pro-inflammatory stimuli) reduced expression of pro-inflammatory markers in IVD. Unstimulated secMSCs and IL-1β-stimulated secMSCs promoted matrix regeneration in degenerated IVDs. MSCs pre-conditioning with IL-1β at normoxia stimulates MSCs to produce higher concentration of immune-regulatory factors (IL-6, IL-8, TGF-β, PGE_2) in their secretome. Preconditioning with hypoxia appears to affect secMSCs immunomodulatory profile, which consequently does not seem to promote matrix recovery. IVD matrix regeneration seems to be closely linked to initial inflammation management, as well as matrix-degrading enzymes levels. Unstimulated secMSCs and IL-1β-stimulated secMSCs should be further explored as they are offering clues to their regenerative potential in the context of IVD degeneration.

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G. Supplementary Materials and Methods

1. Flow Cytometry analysis of phenotypic markers

For flow cytometry analysis, cells were trypsinized, washed in FACS buffer (0.01% Sodium Azide/0.5% BSA in PBS) and centrifuged at 1200 rpm, for 7min. After discarding the supernatant, cells were resuspended with the following fluorochrome-conjugated antibodies: FITC-labelled CD105 (Immunotools), CD34 (Caltag Lab) and HLA-DR (Immunotools), PE-labelled CD19 (Immunotools), CD14 (Immunotools) and CD73 (BD) and APC-labelled CD45 (Immunotools) and CD90 (eBioscience). As controls, IgG1 FITC, IgG2a FITC, IgG1 PE and IgG1 APC were used. After incubation, cells were washed with FACS buffer and fixed in 4% paraformaldehyde (PFA). At least 10000 events were counted in a Flow Cytometer (FACSCanto, Becton Dickinson). Data was analyzed with FlowJo X software.

2. MSCs differentiation assays

2.1 Osteogenic differentiation

Cells were seeded in a total of 15000 cells/well in a 24-well plate and induced to differentiate by SCM supplementation with 100 nM dexamethasone, 10mM β-glycerophosphate and 50mM ascorbic acid. Medium was changed twice a week and osteogenic differentiation was evaluated after 7, 14, 21 and 28 days with alkaline phosphatase (ALP) staining. At each time point, cells were fixed with 4% PFA, for 15 min, washed with pure water and incubated for 45 min with ALP staining solution (4% Naphtol AS-MX phosphate alkaline solution in Fast Violet B solution). At days 21 and 28, alizarin and Von Kossa staining were performed. For alizarin staining, cells were incubated for 5-10 min with freshly prepared alizarin red solution. For Von Kossa staining, cells were serially incubated (and serially washed in between) with: silver nitrate 2.5% (w/t) (for 30 min under UV light), sodium thiosulfate 5% (w/t) (for 3 min) and nuclear fast red (for 5 min).

2.2 Adipogenic differentiation

Cells were seeded in a density of 6000 cells/well in a 24-well plate and left in SCM until confluence was reached. Differentiaton twards the adipogenic lineage was induced by SCM supplementation with 100μM dexamethasone, 500μM 3-

isobutyl-1-methylxanthine (IBMX), 10 μg/mL insulin and 100μM indomethacin. Medium was replaced every 2 or 3 days with either insulin medium (10 μg/mL insulin) or adipogenic medium. After a 28-days culture, cells were fixed as mentioned above. For Oil Red O staining, cells were washed with pure water and incubated with 60% isopropanol for 2-5 min, followed by incubation with Oil Red O staining solution for 5 min and with hematoxylin for 1 min to counterstain.

2.3 Chondrogenic differentiation

For chondrogenic differentiation assessment, $0{,}2x10^6$ cells were suspended in 15mL conical tubes, centrifuged at 450g for 10 min and left in DMEMhigh glucose (4,5g/L), 1% P/S, 250μM ascorbic acid, 40μg/mL L-proline, 100μg/mL sodium pyruvate and 100μg/mL insulin-transferring-selenium (ITS), supplemented with 100nM dexamethasone and 10ng/mL TGF-β, at 37ºC. Medium was changed twice a weed.After 14 and 28 days, cells were fixed as mentioned above. Cell pellets were then transferred to histology cassettes, dehydrated and embedded in paraffin blocks. Pellet sections of 3-5 μm-thick were rehydrated and stained with 1% toluidine blue/1% sodium borate solution, for 2 min.

3. Primers used for Gene Expression Analysis of MSCs/IVDs

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed in duplicate on cDNA derived from MSC/IVD samples, using the SYBR Green method as specified in sections 3.4 and 4.2. The primers used are presented in Table S1.

Table S1| Primers used for qRT-PCR analysis

Human Gene	**Forward Primer Sequence**	**Reverse Primer Sequence**	**GenBank Number**
β-Actin	5'-TACCTCATGAAGATCCTCA-3'	5'-TTCGTGGATGCCACAGGAC-3'	[NM_001101]
IL-6	5'-CAATCTGGATTCAATGAGGAGACT-3'	5'-CTGTTCTGGAGGTACTCTAGGTAT-3'	[NM_000600]
IL-10	5'-GGAGAACCTGAAGACCCTCA-3'	5'-TATAGAGTCGCCACCCTGAT-3'	[NM_000572]
IL-1β	5'-CTTCAGCCAATCTTCATT-3'	5'-CACTGTAATAAGCCATCAT-3'	[NM_000576]
TNF-α	5'-TCTCTCTAATCAGCCCTCTG-3'	5'-TGCTACAACATGGGCTACAG-3'	[NM_000594]
TGF-β1	5'-CCTGGACACCAACTATTG-3'	5'-CTTGCGGAAGTCAATGTA-3'	[NM_000660]
Bovine Gene	**Forward Primer Sequence**	**Reverse Primer Sequence**	**NCBI reference**
GAPDH	5'-ACCCAGAAGACTGTGGATGG-3'	5'-CAACAGACACGTTGGGAGTG-3'	XM_001252511
IL-6	5'-ACCCCAGGCAGACTACTTCT-3'	5'-GCATCCGTCCTTTTCCTCCA-3'	EU276071
IL-8	5'-ATTCCACACCTTTCCACCCC-3'	5'-ACAACCTTCTGCACCCACTT-3'	AF232704
MMP-1	5'-ATGCTGTTTTCCAGAAAGGTGG-3'	5'-TCAGGAAACACCTTCCACAGAC-3'	NM_174112.1
MMP-3	5'-AATCAGTTCTGGGCCATCAG-3'	5'-CTCTGATTCAACCCCTGGAA-3'	AF069642
MMP-13	5'- CATGAGTTTGGCCATTCCTT-3'	5'-GGCGTTTTGGGATGTTTAGA-3'	NM_174389
Collagen II	5'-CCTGTAGGACCTTTGGGTCA-3'	5'-ATAGCGCCGTTGTGTAGGAC-3'	X02420
Aggrecan	5'-ACAGCGCCTACCAAGACAAG-3'	5'-ACGATGCCTTTTACCACGAC-3'	NM_173981

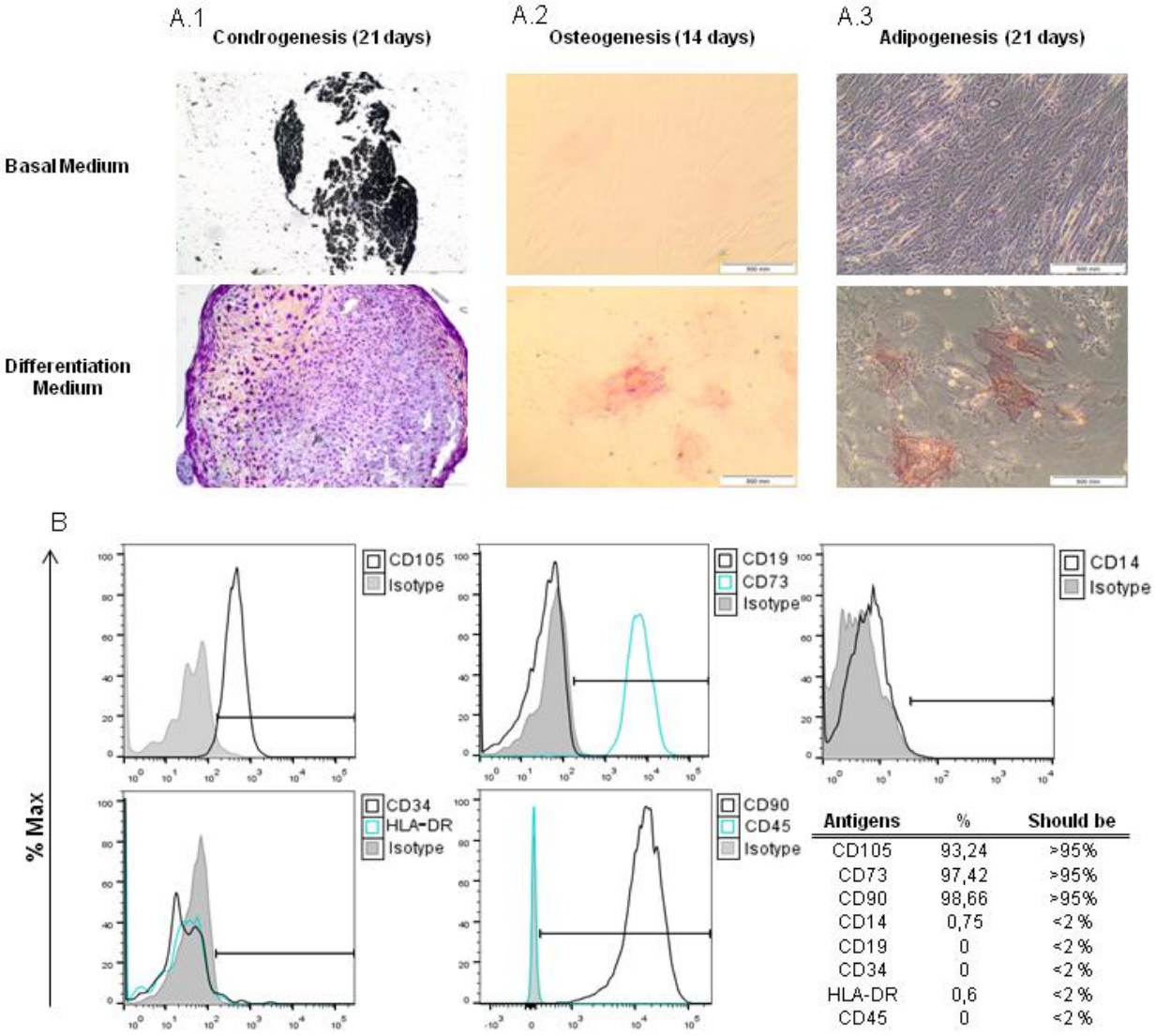
H. Supplementary Results

1. Phenotypic and Functional Characterization of isolated MSCs

Human primary MSCs were isolated and phenotypically and functionally characterized to ensure that the cells used fulfilled the defined criteria to be considered MSCs. These criteria were established in 2006 [184], stating that true MSCs are demonstrated when three standard results are achieved: cell adherence to plastic (assured by isolation protocol used); multipotent differentiation potential and expression of certain surface antigens (CD105, CD90 and CD73, specific MSCs markers) or lack of expression of other surface antigens (CD19 – B cells marker, CD14 – expressed by monocytes and macrophages, CD45 – hematopoietic marker, CD34 – expressed in early hematopoietic progenitors and endothelial cells and HLA-DR – expressed in activated MSCs). The MSCs used in this study were shown to differentiate to osteoblasts, chondroblasts and adipocytes using standard in vitro tissue culture-differentiating techniques (Fig. S1, Supplementary Data), demonstrating their capacity for trilineage mesenchymal differentiation. Furthermore, they were shown to have the desired surface expression profile (Fig. S1, Supplementary Data).

2. Overview on secMSCs effect on the degenerated IVD

A heat map, encompassing all results related to the effect provoked on degenerative/inflammatory IVD at 48h by the secMSCs, was generated using software GENE-E (vs. 3.0.206, The Broad Institute of MIT and Harvard, USA), considering median calculated from all replicas of each condition for every factor analyzed.



Antigens	%	Should be
CD105	93,24	>95%
CD73	97,42	>95%
CD90	98,66	>95%
CD14	0,75	<2 %
CD19	0	<2 %
CD34	0	<2 %
HLA-DR	0,6	<2 %
CD45	0	<2 %

Figure S1| Analysis of newly isolated MSCs differentiation capacity and phenotype.

A. MSCs differentiation in chondrogenic, osteogenic and adipogenic lineages. **A1.** representative images of toluidine blue staining. **A.2** Representative images of alkaline phosphatase (ALP) staining. **A.3** Representative image of Oil Red O staining. **B.** Histogram profile of MSCs phenotype exhibiting expression of CD90, CD105, CD73, CD14, CD19, CD34, CD45 and HLA-DR.

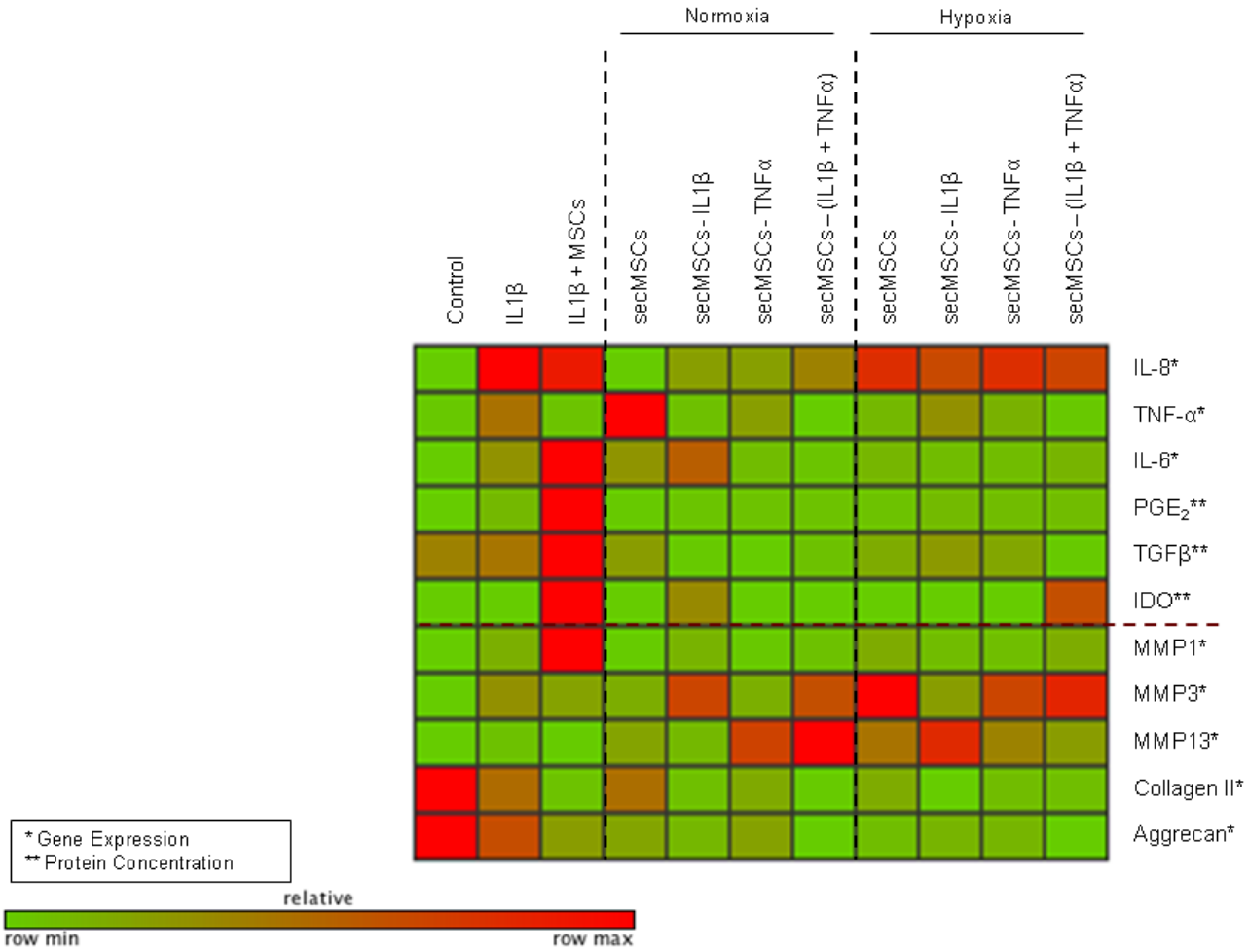


Figure S2| Overall results overview represented as heat map, generated using software Gene E, considering median calculated from replicas of each condition for every factor analyzed.

Results relative to IVDs condition after inflammatory stimulus and treatment with secMSCs, at 48h