European Cells and Materials Vol. 39 2020 (pages 227-248) DOI: 10.22203/eCM.v039a15 ISSN 1473-2262

eCELLS & MATERIALS OPEN ACCESS

INTERVERTEBRAL DISC AND ENDPLATE CELLS RESPONSE TO IL-1β INFLAMMATORY CELL PRIMING AND IDENTIFICATION OF MOLECULAR TARGETS OF TISSUE DEGENERATION

P. De Luca[1], L. de Girolamo[1], D. Kouroupis[2], M. Castagnetta[3], C. Perucca Orfei[1], D. Coviello[4], S. Coco[5], D. Correa[2,6], M. Brayda-Bruno[7] and A. Colombini[1,*]

[1] IRCCS Istituto Ortopedico Galeazzi, Orthopaedic Biotechnology Lab, Milan, Italy
[2] Department of Orthopaedics, UHealth Sports Medicine Institute, University of Miami, Miller School of Medicine, Miami, FL, USA
[3] Ospedale Galliera, Laboratorio di Istocompatibilità/IBMDR, Genoa, Italy
[4] IRCCS Istituto Giannina Gaslini, Laboratorio di Genetica Umana, Genoa, Italy
[5] IRCCS Ospedale Policlinico San Martino, Lung Cancer Unit, Genoa, Italy
[6] Diabetes Research Institute and Cell Transplant Centre, University of Miami, Miller School of Medicine, Miami, FL, USA
[7] IRCCS Istituto Ortopedico Galeazzi, Scoliosis Unit, Department of Orthopaedics and Traumatology-Spine Surgery III, Milan, Italy

Abstract

Inflammation represents an important factor leading to metabolic imbalance within the intervertebral disc (IVD), conducive to degenerative changes. Therefore, a thorough knowledge of the IVD and endplate (EP) cell behaviour in such pathological environments is essential when designing regenerative therapeutic strategies. The present study aimed at assessing the molecular response of the IVD constitutive nucleus pulposus (NPCs)-, annulus fibrosus (AFCs)- and endplate (EPCs)-derived cells to interleukin (IL)-1β treatment, through large-scale, high-throughput microarray and protein analysis, identifying the differentially expressed genes and released proteins. Overall, the inflammatory stimulus downregulated stemness genes while upregulating pro-inflammatory, pro-angiogenic and catabolic genes, including matrix metalloproteases, which were not balanced by a concomitant upregulation of their inhibitors. Upregulation of anti-inflammatory and anabolic tumour necrosis factor inducible gene 6 protein (*TNFAIP6*), of IL-1 receptor antagonist (IL-1Ra) (at gene and protein levels) and of trophic insulin-like growth factor 1 (*IGF1*) was also observed in all cell types; *IGF1* particularly in AFCs. An overall inhibitory effect of tumour necrosis factor alpha (TNFα) signal was observed in all cell types; however, EPCs showed the strongest anti-inflammatory behaviour. AFCs and EPCs shared the ability to limit the activation of the signalling mediated by specific chemokines. AFCs showed a slightly senescent attitude, with a downregulation of genes related to DNA repair or pro-mitosis.

Results allowed for the identification of specific molecular targets in IVD and EP cells that respond to an inflammatory environment. Such targets can be either silenced (when pathological targets) or stimulated to counteract the inflammation.

Keywords: Intervertebral disc cells, endplate cells, interleukin 1 beta, gene array, protein array, inflammation markers.

***Address for correspondence**: Alessandra Colombini, Istituto Ortopedico Galeazzi, Orthopaedic Biotechnology Lab, Via R. Galeazzi 4, 20161, Milan, Italy.
Telephone number: +39 0266214067 Email: alessandra.colombini@grupposandonato.it

Copyright policy: This article is distributed in accordance with Creative Commons Attribution Licence (http://creativecommons.org/licenses/by-sa/4.0/).

List of Abbreviations

ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
ADGRB1	adhesion G protein-coupled receptor B1
ADH1B	alcohol dehydrogenase 1B
ADIPOQ	adiponectin, C1Q and collagen domain containing
AF	annulus fibrosus
AFCs	AF cells
ANGPT	angiopoietin

ARPP21	cAMP regulated phosphoprotein 21
BCA	bicinchoninic acid
BCL2	BCL2 apoptosis regulator
BCL2A1	BCL2-related protein A1
BLM	BLM RecQ-like helicase
C3AR1	complement component 3a receptor 1
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor
CD40LG	CD40 ligand
CDH5	cadherin 5
CEBPB	CCAAT enhancer binding protein beta
CHEK1	checkpoint kinase 1
CHGA	chromogranin A
CHI3L1	chitinase-3-like
CILP2	cartilage intermediate layer protein 2
CLCA2	chloride channel accessory 2
CLIC6	chloride intracellular channel 6
CMPK2	cytidine/uridine monophosphate kinase 2
CNMD	chondromodulin
COL4A3	collagen type IV alpha 3 chain
CSF	colony stimulating factor
CSF1R	CSF 1 receptor
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
DCLRE1B	DNA cross-link repair 1B
ECM	extracellular matrix
EGF	epidermal growth factor
EHF	ETS homologous factor
ELISA	enzyme-linked immunosorbent assay
EME1	essential meiotic structure-specific endonuclease 1
EP	endplate
EPCs	EP cells
EREG	epiregulin
F3	coagulation factor III, tissue factor
FASLG	Fas ligand
FBS	foetal bovine serum
Fc	fold change
FGF	fibroblast growth factor
FGFBP1	fibroblast growth factor binding protein 1
FGFR3	fibroblast growth factor receptor 3
FST	follistatin
GDF	growth differentiation factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GRP	gastrin releasing peptide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HG-DMEM	high-glucose Dulbecco's modified Eagle's medium
ICAM	intercellular adhesion molecule 1
IDO	indoleamine 2,3-dioxygenase
IFI	interferon alpha inducible protein
IFN	interferon
IGF	insulin-like growth factor
IGFBP	IGF-binding protein
IL	interleukin
IL1R2	interleukin 1 receptor type 2
IL-1Ra/*IL1RN*	IL-1 receptor antagonist
IL-6sR	IL-6 soluble receptor
INHBA	inhibin subunit beta A
iNOS	inducible nitric oxide synthase
IP-10	interferon-gamma-induced protein 10
IVD	intervertebral disc
KRAS	KRAS proto-oncogene, GTPase
KYNU	kynureninase
LAMP5	lysosomal associated membrane protein family member 5
LCN2	lipocalin 2
LDB2	LIM domain binding 2
LEFTY2	left-right determination factor 2
LEP	leptin
LIF	LIF interleukin 6 family cytokine
LRRN3	leucine rich repeat neuronal 3
LSP1	lymphocyte specific protein 1
LTA	lymphotoxin alpha
LTB	lymphotoxin beta
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1
MAP1LC3C	microtubule associated protein 1 light chain 3 gamma
MCP-1	monocyte chemoattractant protein 1
MIP	macrophage inflammatory protein
MMPs	metalloproteinases
MSC	mesenchymal stem/stromal cell
MSTN	myostatin
MYBPH	myosin binding protein H
MYL1	myosin light chain 1
NAMPT	nicotinamide phosphoribosyltransferase
NEFM	neurofilament medium
NEURL3	neuralised E3 ubiquitin protein ligase 3
NO	nitric oxide
NOD2	nucleotide binding oligomerisation domain containing 2
NOS	NO synthase
NOTCH4	notch receptor 4
NOX1	NADPH oxidase 1
NP	nucleus pulposus
NPCs	NP cells
NR4A3	nuclear receptor subfamily 4 group A member 3
NRG1	neuregulin 1
NRP	natriuretic peptide receptor
NTF4	neurotrophin 4
OLFML	olfactomedin-like
OSM	oncostatin M
P3	passage 3
PBMCs	peripheral blood mononuclear cells
PDE5A	phosphodiesterase 5A
PDGF	platelet-derived growth factor
PLG	plasminogen

PLK1	polo-like kinase 1
PPARG	peroxisome proliferator activated receptor gamma
PPP2R1B	protein phosphatase 2 scaffold subunit A beta
PRKCB	protein kinase C beta
PROK	prokineticin
PTGS1	prostaglandin-endoperoxide synthase 1
RASD1	Ras-related dexamethasone induced 1
RIMS1	regulating synaptic membrane exocytosis 1
RIN	RNA integrity number
RIPK2	receptor interacting serine/threonine kinase 2
RNH1	ribonuclease/angiogenin inhibitor 1
RSAD2	radical S-adenosyl methionine domain containing 2
RSPO3	R-spondin 3
S100A8	S100 calcium binding protein A8
SCRG1	stimulator of chondrogenesis 1
SELE	selectin E
SIRT2	sirtuin 2
SLC	solute carrier
SLPI	secretory leukocyte peptidase inhibitor
SMOC2	SPARC-related modular calcium binding 2
SOSTDC1	sclerostin domain containing 1
SPINK5	serine peptidase inhibitor Kazal type 5
SPP1	secreted phosphoprotein 1
SD	standard deviation
sTNF-RI	soluble tumour necrosis factor receptor I
TBP	TATA-box binding protein
TDO2	tryptophan 2,3-dioxygenase
TGF	transforming growth factor
TGFBR1	transforming growth factor beta receptor 1
Th	T helper
TIMP	tissue inhibitor of MMP
TLR	toll-like receptor
TNFα	tumour necrosis factor alpha
TNFAIP6	TNF inducible gene 6 protein
TNFRSF1B	TNF receptor superfamily member 1B
TNFSF	TNF superfamily member
TPH1	tryptophan hydroxylase 1
TWIST2	twist family bHLH transcription factor 2
TYMP	thymidine phosphorylase
TSG-6	TNF-stimulated gene 6
VCAM1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor

Introduction

Around 540 million people worldwide suffer from low-back pain (Web ref. 1), mainly due to spine disorders strictly associated with age-related degenerative processes of the IVD (Rodrigues-Pinto *et al.*, 2014). These disorders have high socio-economic costs related both to their long-lasting but transiently beneficial multidisciplinary management (*i.e.* conservative, analgesic or surgical) and to the progression of the IVD degeneration (Raj, 2008). For these reasons, the development of biological strategies to counteract IVD degeneration (Hughes *et al.*, 2012) would be a valuable alternative to the current clinical management. In this context, a clear understanding of anatomical and pathophysiological features of the IVD is crucial (Colombini *et al.*, 2008).

The IVD is a heterogeneous structure composed of a central NP, a surrounding AF and a bony-cartilaginous EP delimiting the disc. The functional properties of the disc (*e.g.* mechanical) largely depend on the integrity of the physiological ECM, produced, maintained and remodelled by few resident cells. Alterations to the cellular metabolism adversely affect the tissue composition and performance, providing a starting point for degenerative changes (Colombini *et al.*, 2008).

Maintenance of the metabolic balance in the IVD is a delicate equilibrium, because of a predominant chronic catabolic environment under pathological conditions. This condition is driven and sustained by the presence of higher levels of pro-inflammatory cytokines (Risbud and Shapiro, 2014), mainly TNFα and IL-1β (Burke *et al.*, 2002; Hamamoto *et al.*, 2012; Le Maitre *et al.*, 2005; Le Maitre *et al.*, 2003; Risbud and Shapiro, 2014; Solovieva *et al.*, 2004), and ECM-degrading enzymes, such as matrix MMPs and ADAMTS (Kepler *et al.*, 2013; Le Maitre *et al.*, 2004; Wang *et al.*, 2011), associated with low levels of their inhibitors (TIMPs) (Liao *et al.*, 2019; Vergroesen *et al.*, 2015). Subpopulations of human NPCs, AFCs and EPCs showing phenotypic plasticity and a stem-like immunophenotype are present in degenerated IVDs (Brisby *et al.*, 2013; Liu *et al.*, 2011; Wang *et al.*, 2016) and ideally would represent tissue specific cells to be used in therapies aiming at counteracting the degenerative microenvironment.

De Luca *et al.* (2020) have recently reported that NP, AF and EP cell populations isolated from pathological IVDs and EPs express specific phenotypic and stemness markers, as well as clonogenic, adipogenic and osteogenic potential, similar to MSC cells. Considerable similarities in pro-inflammatory and ECM-degrading factors involved in the degenerative processes have been observed in articular cartilage and IVD (Rustenburg *et al.*, 2018). In fact, as for IVD and EP, chondrogenic progenitor cells have been found in human cartilage obtained from osteoarthritic patients and, together with chondrocytes, they show an increased secretion of cytokines and expression of MMPs, without a corresponding production of TIMPs after IL-1β stimulation (De Luca *et al.*, 2019).

Given the significant impact of the local environment on cell behaviour, the analysis of potential IVD cellular alterations in response to inflammatory conditions becomes essential.

The *in vitro* exposure of human IVD cells to IL-1β and TNF-α, the two main inflammatory mediators involved in IVD degeneration, causes the upregulation of inflammatory molecules such as iNOS, NO, IL-1β, IL-6, IL-8, IL-20, prostaglandin 2 and TNF-α (Kang *et al.*, 1997; Klawitter *et al.*, 2012a; Klawitter *et al.*, 2012b; Millward-Sadler *et al.*, 2009; Sinclair *et al.*, 2011; Wuertz *et al.*, 2011; Wuertz *et al.*, 2013). Furthermore, both mediators also contribute to disc disease through the induction of the catabolic enzymes ADAMTS-4, -5 and MMP-1, -2, -3, -4, -13, -14 and the concomitant decreased expression of connective tissue growth factor, aggrecan and type II collagen, leading to a progressive ECM degradation (Bachmeier *et al.*, 2009; Jimbo *et al.*, 2005; Johnson *et al.*, 2015; Le Maitre *et al.*, 2005; Séguin *et al.*, 2005; Wang *et al.*, 2011; Wang *et al.*, 2014; Tran *et al.*, 2010; Tran *et al.*, 2014). Nevertheless, although it is not definitively established whether either IL-1β, TNF-α – or both – drive ECM degradation, IL-1β is the key cytokine involved in this process (Hoyland *et al.*, 2008; Le Maitre *et al.*, 2007).

Based on this evidence, there are resulting issues that are of particular interest. To the best of the authors' knowledge, the molecular responses of NPCs, AFCs and EPCs to inflammatory and catabolic environments have not yet been fully investigated. Consequently, the aim of the present study was to investigate the response of NPCs, AFCs and EPCs to IL-1β, a master regulator of IVD catabolic processes (Phillips *et al.*, 2015), using large-scale high-throughput microarray and protein array analyses to identify differentially expressed genes and released proteins. These findings would provide new insights into the molecular targets of tissue degeneration, defining the usefulness of this specific inflammatory cell priming, and help identify the best resident and highly specialised cell agent to be used/stimulated in harsh condition for the treatment of disc degenerative processes.

Materials and Methods

Cell isolation and expansion

The study was approved by the Institutional Review Board (Protocol GenVDisc Version 1, 20 November 2015) and specimens were collected after patient informed consent was given. NP, AF from lumbar IVDs and EP of 3 male and 1 female patients (average age 50.5 years) affected by discopathy were harvested during discectomy through a careful macroscopic dissection performed by an experienced surgeon, discriminating the lamellar AF and the jelly-like NP and avoiding the transitional zone of the inner AF. NPCs, AFCs and EPCs were isolated by enzymatic digestion as previously described (Colombini *et al.*, 2015; Lopa *et al.*, 2016). The cell populations were cultured in control medium consisting of 4.5 mg/mL HG-DMEM, supplemented with 10 % FBS (Lonza), 0.29 mg/mL L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM HEPES, 1 mM sodium pyruvate (all from Life Technologies). Cells were seeded at 5 × 10^3 cells/cm^2 and expanded up to P3.

In vitro model of inflammation

Cells at P3 were stimulated by adding 1 ng/mL IL-1β to the culture medium for 48 h according to a previously validated *in vitro* model of IVD and EP cell inflammation (De Luca *et al.*, 2018; Kim *et al.*, 2013). Then, supernatants and cells were collected for further analyses.

RNA isolation and quality assessment for microarray analysis

RNA was isolated from a pool of cells obtained from 4 donors by RNeasy Plus Mini Kit (Qiagen). Residual genomic DNA digestion was performed using RNase-Free DNase Set (Qiagen). RNA quantification and quality control were performed spectrophotometrically (Nanodrop, Thermo Scientific). RIN (value range from 1, totally degraded, to 10, intact) was evaluated by Agilent RNA ScreenTape System (Agilent Technologies). All RNA samples were intact and showed a RIN value of 10.

Gene expression microarray

The expression profiling was performed using a custom-made gene expression microarray, allowing for the analyses of a maximum of 3000 genes considering a minimum of 5 replicates for each gene. The custom-made array was constructed based on an Agilent Technologies algorithm (Web ref. 2). Gene symbols and NM of the selected genes divided into housekeeping (14), chondrogenic/IVD/growth factors- (332), stemness- (1279), inflammation- (235), senescence- (140) and angiogenic-related (79) genes are reported in Supplementary Table 1. The choice of the genes of interest was performed based on the ones previously analysed (Takahashi *et al.*, 2007; Xu *et al.*, 2008; Yoo *et al.*, 2011) or present in commercially available microarray and, then, implemented with genes analysed in studies focusing on the IVD (Liu *et al.*, 2015; Minogue *et al.*, 2010; Power *et al.*, 2011; Rutges *et al.*, 2010; Tang *et al.*, 2012).

cRNA was obtained from 100 ng of total RNA that was labelled and amplified using a Low Input Quick Amp Labeling Kit, one-color. A spike mix was also added to each RNA sample (One-Color RNA Spike-In Kit) to obtain the correct annealing between 10 optimised positive control transcripts and the complementary probes on the chip. Then, auto- and cross-hybridisation was evaluated. Next, cRNA was purified using the RNeasy® Plus Mini Kit (Qiagen) and employed for the slide hybridisation using the Gene Expression Hybridization Kit. To obtain a high-resolution image of the fluorescence values for each probe, the chip was washed and processed using the SureScan Microarray Scanner and Feature Extraction 12.0 software. Data analysis was performed by

Genespring GX software. Reagents, instruments and software were purchased by Agilent Technologies, unless differently specified.

The ontology-based pathway analyses were performed using Panther, NCBI, QuickGO and GeneCards databases. Only values with an Fc ≤ − 2 or ≥ + 2 were considered. Heat maps were generated using the online package ClustVis (Metsalu *et al.*, 2015; Web ref. 3).

Gene expression analysis through real-time PCR

The expression of the most common inhibitors of MMPs, *TIMP1* (Hs00171558, Life Technologies) and *TIMP3* (Hs00165949, Life Technologies), was evaluated also by real-time PCR. For consistency, also the expression of *MMP1* (Hs00899658, Life Technologies), *MMP3* (Hs00968305, Life Technologies) and *MMP13* (Hs00233992, Life Technologies) was assessed through the same method. The evaluation was performed at P3 and after IL-1β treatment in a total of 8 donors: the same 4 donors as used for the pool of gene and protein arrays and 4 new donors (mean age 51.9 ± 6.9 years; 4 males and 4 females) to strengthen and validate the results obtained from the other assays.

RNA was isolated from all cells using the RNeasy® Plus Mini Kit (Qiagen), subsequently quantified spectrophotometrically (NanoDrop, Thermo Scientific) and reverse-transcribed with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Gene expression was evaluated by real-time PCR (StepOne Plus, Life Technologies) using TaqMan® Gene Expression Master Mix and TaqMan® Gene Expression Assays (Life Technologies).

Since *TBP* (Hs00427620_m1, Life Technologies) represents the most stable gene observed in the array, according to previously published data (Lopa *et al.*, 2016), it was chosen as the housekeeping gene. Data are expressed as Fc according to the ΔCt method.

Protein array

ELISA-based protein arrays (RayBio® C-Series, RayBiotech, Peachtree Corners, GA, USA) were used to evaluate the levels of inflammatory mediators in culture media obtained from disc cells, stimulated or not with IL-1β. For each cell population, conditioned media were obtained from 4 donors, pooling 3 technical replicates for each of them. Data were normalised by the total protein content through BCA assay. The arrays showed a detection sensitivity up to pg/mL of protein and were performed following the manufacturer's instructions. Data represent 40 s exposures in a FluorChem E chemiluminescence imaging system (ProteinSimple, San Jose, CA, USA). Results were generated by quantifying the mean spot pixel density using the protein array analyser of ImageJ (NIH website). The signal intensities were normalised to the background, whereas separate signal intensity results represented the average pixel density of two spots per inflammatory mediator. The relative concentration of the antigen in the sample was proportional to the signal intensity for each spot.

Determination of IL-1Ra

The levels of soluble IL-1Ra in cell culture medium after 48 h of treatment with IL-1β were determined for 8 total donors, as aforementioned, by a commercially available ELISA assay, according to the manufacturers' instructions (PeproTech). The detection range was 23-1500 pg/mL.

Statistical analysis

Data are expressed as mean ± SD. Normal distribution of values was assessed by the Kolmogorov-Smirnov normality test. Statistical analysis was performed using paired and unpaired Student's *t*-test for normally distributed data and Wilcoxon (for paired data) or Mann-Whitney (for unpaired data) test in the presence of a non-normal distribution (GraphPad Prism v5.00; GraphPad Software). Level of significance was set at $p < 0.05$.

Results

Gene expression analysis

Stemness-related genes

After IL-1β treatment, 320 genes out of 1279 were modulated. Among these, 47 were upregulated, 271 downregulated and 2 either upregulated or downregulated in different cell populations (Supplementary Table 2). The three cell types shared 16 upregulated and 32 downregulated genes. Concerning the downregulated genes, 86 were found in NPCs, 123 in AFCs and 139 in EPCs. Heat maps and clustering showed that NPCs and EPCs shared similar behaviours in term of entity of stemness genes upregulation (Fig. 1**a**) or downregulation (Fig. 1**b**) after IL-1β treatment. In Supplementary Table 2 Fc ≥ + 2 or ≤ − 2 are reported.

Inflammation-related genes

After IL-1β stimulation, NPCs, AFCs and EPCs showed an upregulation of 77 out of 235 genes related to inflammation (either upregulated or downregulated in the different cell populations), 53 of which shared by the three cell types (Fig. 2). Among the shared genes, there were those coding for *SPP1* and *MMP2*, *MMP3*, *MMP7*, *MMP10*, *MMP12* and *MMP13*, involved in catabolic pathways. The upregulation of *MMP3* and *MMP13* after IL-1β treatment was confirmed by real-time PCR, which revealed also an upregulation of *MMP1*, without a corresponding upregulation of their inhibitors *TIMP1* and *TIMP3*. The latter was in fact downregulated in all cell types (Fig. 3). *IDO1*, *KYNU*, *NAMPT*, *SAAS* and *TNFAIP6*, also belonging to metabolic pathways, were upregulated in all three cell populations. The reactive free radical *NOS2*, *IL6*, *CXCL8*, *IL1α*, *IL1β*, *IL17C*, *SELE*, the chemokine ligands *CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, *CXCL6*, *CXCL8*, *CXCL10*, *CCL2*, *CCL3*, *CCL5*, *CCL7*, *CCL20*, their receptors *CXCR3*, *CXCR4* and *CCR7* and the complement component *C3*, all involved in pro-inflammatory pathways, were upregulated. An upregulation of *CSF2*, *CSF3*, *IFI27*, *IL11*, *IL23A*, *LCN2*, *LIF*, *NOD2*, *OSM*, *RSAD2* and of the anti-inflammatory *IL1RN* was also observed. The anti-apoptotic *BCL2A1* and *INHBA*, related to TGF signalling cascade, and *EHF*, *RSPO3*, *LRRN3*, *RASD1* and *SLC7A2* were also upregulated in all three cell populations. NPCs and AFCs had in common the upregulation of *CMPK2*, *RIPK2* and *IFI44L*, AFCs and EPCs shared the upregulation of *NEFM*, *NR4A3* and of the pro-inflammatory *CCL4*, *CCL11* and *CCL13*. Finally, NPCs and EPCs shared the upregulation

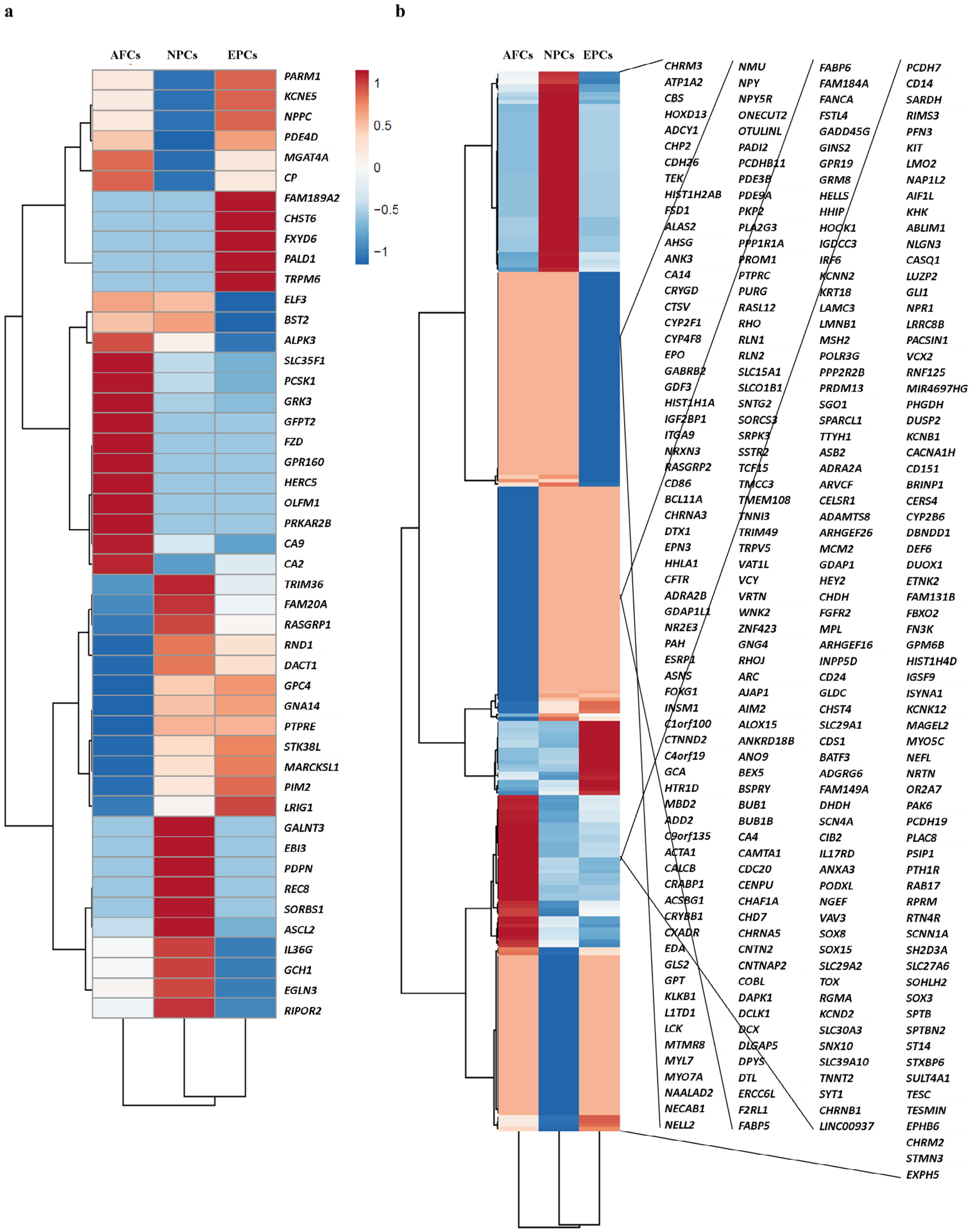


Fig. 1. Heat maps and clustering representing the stemness-related genes modulated by IL-1β treatment. (**a**) Upregulated (Fc ≥ + 2) and (**b**) downregulated (Fc ≤ − 2) genes (n = 4).

of the pro-inflammatory *IL32*, *SERPINB2* and the anti-apoptotic *TNFRSF1B*. *TNF* and *VCAM1* were upregulated only in NPCs; the catabolic *ADAMTS4* and *ADAMTS9* as well as *CCL26*, *CXCL9*, *CLIC6*, *NEURL3* and *TWIST2* only in AFCs. The anti-inflammatory *IL1R2*, the pro-inflammatory *IL20* and *CEBPB*, *CHI3L2* and *SLPI* were instead exclusively upregulated in EPCs.

IL-1β treatment caused the downregulation of 79 out of 235 genes related to inflammation, of which 18 in common among the three cell populations (Fig. 2**b**): the catabolic *ADAMTS15*; the pro-inflammatory *CCL22*, *CCR1* and *IL17A*; the metabolic *ADH1B*, *PDE5A*, *SCRG1* and *SLC40A*. Similarly, *GDF5* and *RIMS1* were downregulated as well as *LAMP5*, *LDB2*, *LSP1*, *MAP1LC3C*, *MYBPH*, *OLFML2A*, *OLFML2B* and *SMOC2*. Interestingly, AFCs and EPCs shared a downregulation of 42 genes belonging to different pathways: the pro-inflammatory *C3AR1*, *CCL17*, *CCL21*, *CCL24*, *CCR2*, *CCR4*, *CCR5*, *CCR6*, *CXCR2* and *IFNG*; the anti-inflammatory *CXCL4*, *IL4*, *IL5*, *IL13*; genes involved in cytokine pathways such as *IFNA1*, *IFNA2*, *IFNA4*, *IFNA5*, *IL12B*, *IL2*, *IL9*, *IL21*, *IL22*, *IL23R*, *LTB*, *TNFSF8* and *CD40LG*. Furthermore,

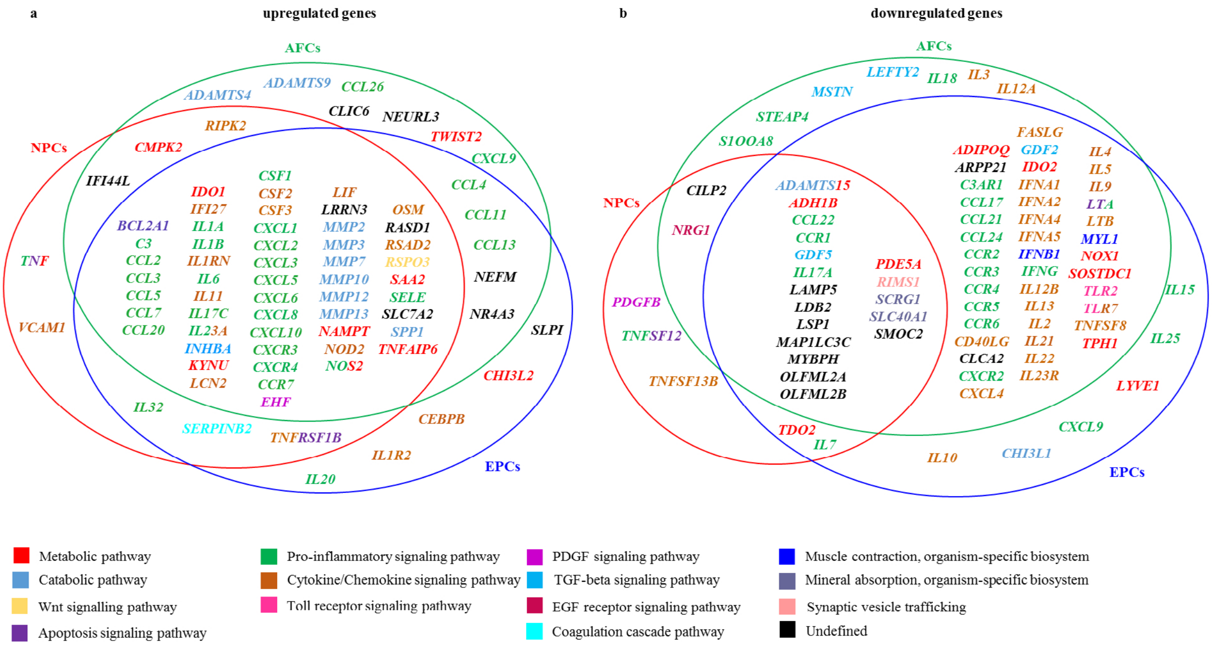


Fig. 2. Venn diagram showing all the genes belonging to the inflammation panel modulated by IL-1β treatment. (**a**) Upregulated (Fc ≥ + 2) and (**b**) downregulated (Fc ≤ − 2) genes and the respective pathway, single or in common with the other cell populations (*n* = 4).

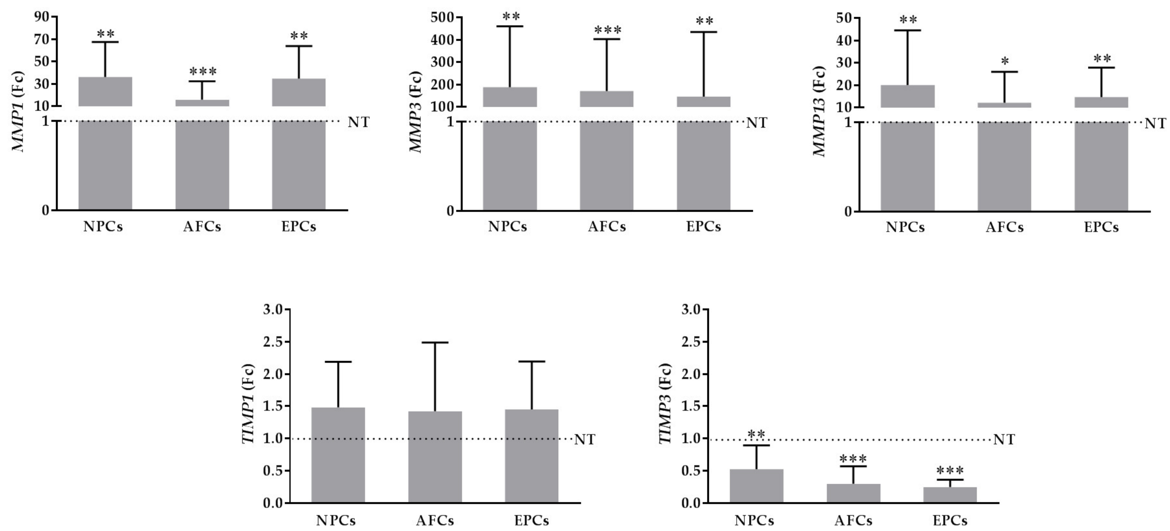


Fig. 3. Gene expression analysis (real-time PCR) represented as Fc of MMPs (*MMP1*, *MMP3*, *MMP13*) and their inhibitors (*TIMP1*, *TIMP3*) with respect to each non-treated sample (NT). * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ indicate IL-1β treated *vs.* NT, in each cell populations (*n* = 8). Data are expressed as mean ± SD.

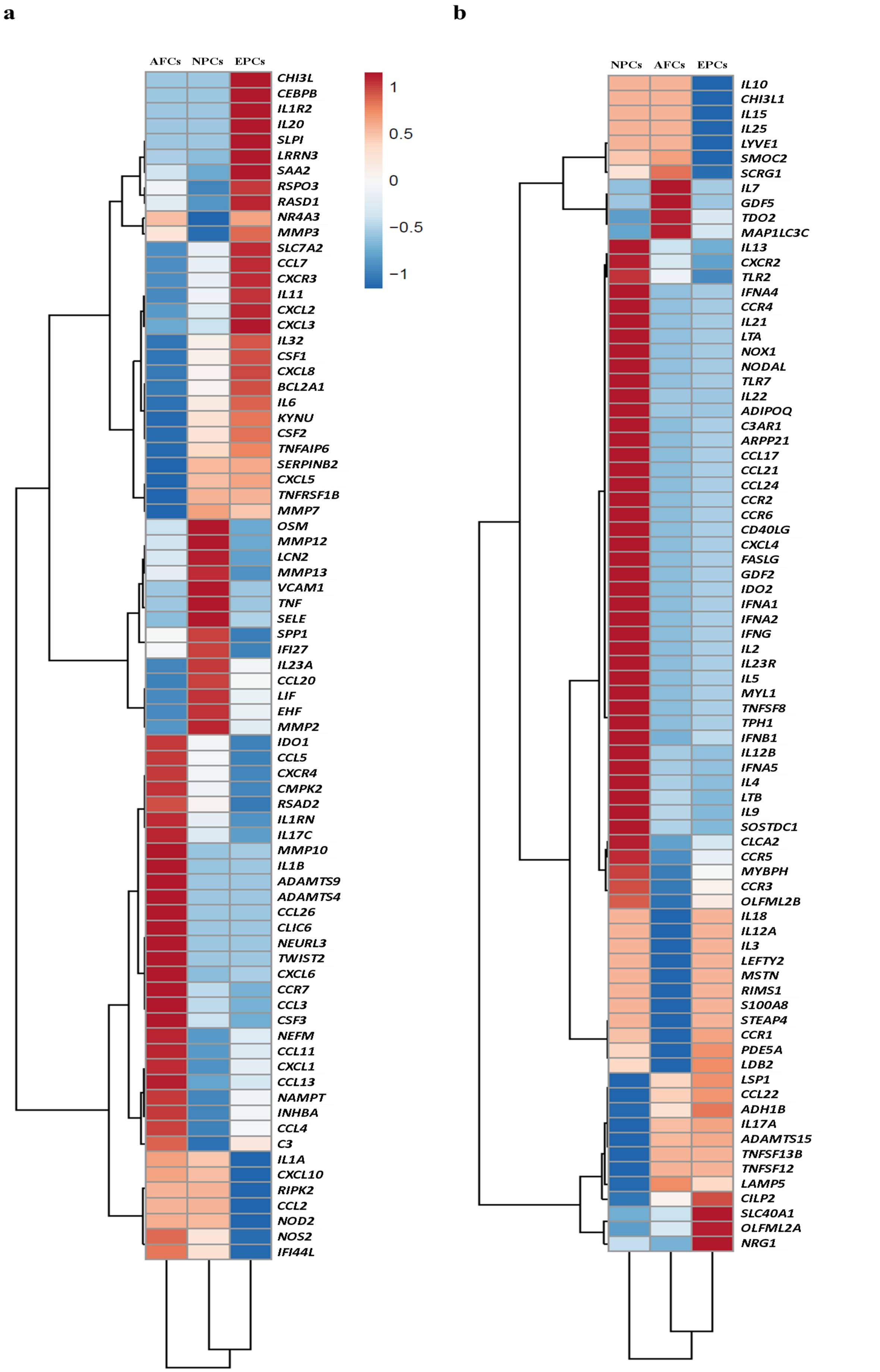


Fig. 4. Heat maps and clustering representing the inflammation-related genes modulated by IL-1β treatment. (**a**) Upregulated (Fc ≥ + 2) and (**b**) downregulated (Fc ≤ − 2) genes (*n* = 4).

Table 1a. Upregulated or downregulated inflammation-related genes after IL-1β treatment with Fc ≥ + 2 or ≤ − 2 in the different cell populations; (-) no differences.

Gene	Fc NPCs	Fc AFCs	Fc EPCs
ADAMTS15	− 12.8	− 7.1	− 6.8
ADAMTS4	-	2.6	-
ADAMTS9	-	2.3	-
ADH1B	− 28.2	− 13.5	− 8.0
ADIPOQ	-	− 2.3	− 2.3
ARPP21	-	− 2.4	− 2.2
BCL2A1	3.2	2.6	3.7
C3	4.3	6.9	6.0
C3AR1	-	− 2.4	− 2.2
CCL11	-	10.4	3.8
CCL13	-	21.8	5.8
CCL17	-	− 2.4	− 2.2
CCL2	9.5	9.5	7.7
CCL20	1098.9	390.3	734.1
CCL21	-	− 2.4	− 2.2
CCL22	− 4.2	− 2.5	− 2.2
CCL24	-	− 2.4	− 2.2
CCL26	-	4.0	-
CCL3	13.4	16.5	12.9
CCL4	-	3.5	2.2
CCL5	93.2	148.0	45.2
CCL7	5.7	5.2	6.5
CCR1	− 4.0	− 13.7	− 3.2
CCR2	-	− 2.4	− 2.2
CCR3	-	− 6.6	− 2.4
CCR4	-	− 2.4	− 2.3
CCR5	-	− 4.2	− 2.2
CCR6	-	− 2.4	− 2.2
CCR7	5.5	11.9	4.5
CD40LG	-	− 2.4	− 2.2
CEBPB	-	-	2
CHI3L1	-	-	− 2.2
CHI3L2	-	-	2.2
CILP2	− 6.4	− 2.2	-
CLCA2	-	− 3.1	− 2.1
CLIC6	-	3.0	-
CMPK2	2.2	3.8	-
CSF1	3.2	2.4	3.8
CSF2	61.6	12.1	82.7
CSF3	25.7	77.0	15.5
CXCL1	102.5	162.4	124.1
CXCL10	12.2	12.9	2.7
CXCL2	33.8	22.2	59.7
CXCL3	59.5	47.3	114.0
CXCL4	-	− 2.4	− 2.2
CXCL5	17.3	9.4	17.9
CXCL6	442.9	1702.1	512.0
CXCL8	501.9	438.6	555.9
CXCL9	-	5.0	− 2.2
CXCR2	-	− 2.3	− 3.4
CXCR3	3.2	2.5	4.3
CXCR4	17.2	33.2	4.2
EHF	44.4	8.5	23.3
FASLG	-	− 2.4	− 2.2
GDF2	-	− 2.4	− 2.2

Table 1b. Upregulated or downregulated inflammation-related genes after IL-1β treatment with Fc ≥ + 2 or ≤ − 2 in the different cell populations; (-) no differences.

Gene	Fc NPCs	Fc AFCs	Fc EPCs
GDF5	− 3.0	− 2.5	− 3.0
IDO1	17.7	32.6	4.8
IDO2	-	− 2.4	− 2.2
IFI27	14.2	10.5	7.1
IFI44L	9.2	12.3	-
IFNA1	-	− 2.4	− 2.2
IFNA2	-	− 2.4	− 2.2
IFNA4	-	− 2.4	− 2.3
IFNA5	-	− 2.3	− 2.4
IFNB1	-	− 2.8	− 2.3
IFNG	-	− 2.4	− 2.2
IL10	-	-	− 2.8
IL11	30.4	26.2	36.3
IL12A	-	− 2.4	-
IL12B	-	− 2.4	− 2.5
IL13	-	− 2.2	− 2.9
IL15	-	-	− 2.1
IL17A	− 5.4	− 2.4	− 2.2
IL17C	4.5	6.0	3.9
IL18	-	− 3.8	-
IL1A	14.4	15.5	5.0
IL1B	5.3	10.9	5.4
IL1R2	-	-	2.8
IL1RN	8.4	15.1	4.6
IL2	-	− 2.4	− 2.2
IL20	-	-	2.2
IL21	-	− 2.4	− 2.3
IL22	-	− 2.4	− 2.4
IL23A	368.2	153.1	251.6
IL23R	-	− 2.4	− 2.2
IL25	-	-	− 3.1
IL3	-	− 2.4	-
IL32	2.7	-	3.9
IL4	-	− 2.4	− 2.6
IL5	-	− 2.4	− 2.2
IL6	66.5	46.3	78.0
IL7	− 3.1	-	− 2.9
IL9	-	− 2.4	− 2.8
INHBA	3.1	4.2	3.6
KYNU	16.6	13.8	17.7
LAMP5	− 9.1	− 5.7	− 6.4
LCN2	433.5	228.0	158.7
LDB2	− 6.0	− 11.1	− 4.7
LEFTY2	-	− 2.6	-
LIF	4.2	3.7	3.9
LRRN3	4	4.2	7.6
LSP1	− 5.3	− 3.9	− 3.6
LTA	-	− 2.4	− 2.3
LTB	-	− 2.4	− 2.8
LYVE1	-	-	− 2.4
MAP1LC3C	− 21.5	− 17.2	− 20.5
MMP10	11.5	19.2	11.7
MMP12	142.4	95.3	83.4
MMP13	24.8	13.3	7.2
MMP2	3	2.4	2.6
MMP3	65.1	84.5	93.6

Table 1c. Upregulated or downregulated inflammation-related genes after IL-1β treatment with Fc ≥ + 2 or ≤ − 2 in the different cell populations; (-) no differences.

Gene	Fc NPCs	Fc AFCs	Fc EPCs
MMP7	5.7	2.8	5.4
MSTN	-	− 2.6	-
MYBPH	− 2.7	− 2.9	− 2.8
MYL1	-	− 2.4	− 2.2
NAMPT	4.6	5.5	5.0
NEFM	-	6.9	2.8
NEURL3	-	2.6	-
NOD2	20	20.2	12.4
NODAL	-	− 2.5	− 2.4
NOS2	40.4	49.9	20.0
NOX1	-	− 2.4	− 2.3
NR4A3	-	2.3	2.4
NRG1	− 2.5	− 3.1	-
OLFML2A	− 6.8	− 6.3	− 4.9
OLFML2B	− 2.1	− 3.9	− 2.8
OSM	7.7	3.7	2.8
PDE5A	− 5.6	− 7.6	− 5.2
RASD1	2.6	2.7	2.9
RIMS1	− 2.3	− 2.4	− 2.3
RIPK2	2.1	2.1	-
RSAD2	4.3	6.1	2.1
RSPO3	5.1	6.2	7.6
S100A8	-	− 2.4	-
SAA2	19.5	26.2	53.3
SCRG1	− 3.1	− 2.8	− 3.8
SELE	32.8	8.0	9.9
SERPINB2	3.1	-	3.2
SLC40A1	− 6.0	− 5.9	− 5.4
SLC7A2	51.3	49.8	53.7
SLPI	-	-	2.1
SMOC2	− 3.0	− 2.8	− 4.8
SOSTDC1	-	− 2.4	− 2.7
SPP1	2.9	2.6	2.3
STEAP4	-	− 5.9	-
TDO2	− 4.9	-	− 3.3
TLR2	-	− 2.4	− 4.8
TLR7	-	− 2.3	− 2.2
TNF	3.4	-	-
TNFAIP6	68.4	21.5	81.7
TNFRSF1B	3.4	-	3.4
TNFSF12	− 2.2	-	-
TNFSF13B	− 2.2	-	-
TNFSF8	-	− 2.4	− 2.2
TPH1	-	− 2.4	− 2.2
TWIST2	-	3.0	-
VCAM1	2.5	-	-

the pro-inflammatory and pro-apoptotic *LTA*, the pro-apoptotic *FASLG*, the toll receptor signalling *TLR2* and *TLR7*, the metabolic *ADIPOQ*, *IDO2*, *NOX1*, *SOSTDC1* and *TPH1*, the TGF-β signalling *GDF2* were also downregulated after inflammation. Moreover, downregulation of *IFNB1* and *MYL1*, belonging to the muscle contraction pathway, and of *ARPP21* and *CLCA2* was observed. NPCs and AFCs presented a common downregulation of *NRG1*, involved in the EGF receptor pathway, and of *CILP2*; NPCs and EPCs shared the downregulation of *TDO2* and the pro-inflammatory *IL7*. An exclusive downregulation was observed in NPCs of the pro-inflammatory and pro-apoptotic *TNFSF12* and *TNFSF13B* and of *PDGFB*; in AFCs of the pro-inflammatory *IL18*, *S100A8* and *STEAP4* and of *IL3*, *IL12A*, *MSTN* and *LEFTY2*. EPCs showed a selective downregulation of the pro-inflammatory *CXCL9*, *IL15*, *IL25*, of the

Table 2. Upregulated or downregulated angiogenesis-related genes after IL-1β treatment with Fc ≥ + 2 or ≤ − 2 in the different cell populations; (-) no differences.

Gene	Fc NPCs	Fc AFCs	Fc EPCs
ADGRB1	− 3.1	− 2.7	− 2.4
ANGPT1	3.1	4.1	5.0
ANGPT2	-	− 2.3	− 2.2
ANGPTL1	-	2.6	-
CD55	7.9	5.9	8.8
CDH5	-	− 2.9	− 2.2
CHGA	-	− 2.4	− 2.3
CNMD	− 3.0	− 3.1	− 2.2
COL4A3	-	− 2.4	− 2.2
CXCL11	-	− 2.3	− 2.2
CXCL12	− 3.8	− 2.9	− 2.8
CXCL13	-	− 2.4	− 2.3
EREG	14.0	8.4	12.0
F3	2.5	2.4	2.9
FGF2	-	2.1	-
FGFBP1	9.3	16.2	7.5
FGFR3	− 2.2	-	− 2.2
FST	2.3	2.2	2.4
GRP	-	− 2.4	-
IL17F	-	− 2.4	− 2.2
KITLG	2.3	-	2.3
LEP	− 2.1	-	-
NOS3	− 2.9	-	− 2.8
NOTCH4	-	2.7	-
NRP1	-	− 2.2	-
NRP2	2.9	3.4	2.7
PECAM1	-	-	-11.8
PLG	-	− 2.5	− 2.4
PPBP	-	− 2.4	− 2.2
PROK1	− 3.8	-	− 3.5
PROK2	-	− 2.4	− 2.2
PTGS1	4.3	3.7	4.6
RNH1	− 2.5	− 2.8	− 3.1
SERPINC1	-	− 2.4	-
SPINK5	− 2.9	− 2.3	− 2.5
TGFB2	− 2.2	-	− 2.9
TGFBR1	3.0	2.6	3.0
TYMP	2.2	2.6	2.3
VEGFB	-	− 2.4	-
VEGFD	-	− 2.4	− 2.3

anti-inflammatory *IL10*, of the catabolic *CHI3L1* and of the metabolic *LYVE1*.

Heat maps and clustering showed that NPCs and EPCs shared a similar entity of upregulated (Fig. 4**a**) whereas AFCs and EPCs of downregulated (Fig. 4**b**) inflammation-related genes after IL-1β treatment. Table 1**a**, **b**, **c** report Fc ≥ + 2 or ≤ − 2.

Angiogenesis-related genes

After IL-1β treatment, 40 genes were modulated (14 upregulated and 26 downregulated), 9 of which involved in inhibition of angiogenesis, 27 in promotion of angiogenesis and 4 with an unclear function in angiogenesis. All 9 genes with an inhibitory role were downregulated by the treatment with IL-1β: *ADGRB1*, *CNMD*, *RNH1* and *SPINK5* in all three cell populations; *SERPINC1* only in AFCs; *ANGPT2*, *CHGA*, *COL4A3* and *IL17F* in AFCs and EPCs. Among the pro-angiogenic, *ANGPT1*, *CD55*, *EREG*, *FGFBP1* and *PTGS1* were highly upregulated (3.1 ≤ Fc ≤ 16.2) as well as *F3*, *FST*, *NRP2*, *TGFBR1* and *TYMP*, even if to a lesser extent (2.2 ≤ Fc ≤ 3.4), in all the cell populations. Moreover, *ANGPT1*, *FGF2* and *NOTCH4* were upregulated only in AFCs. After

IL-1β treatment, some pro-angiogenic genes such as *CXCL12* were downregulated in all analysed cell populations. *CDH5*, *CXCL11*, *PLG*, *PROK2* and *VEGFD* showed the same trend in both AFCs and EPCs, whereas *FGFR3*, *LEP*, *NOS3* and *PROK1* in both NPCs and EPCs. *GRP*, *NRP1* and *VEGFB* were downregulated only in AFCs and *PECAM1* only in EPCs, with the highest downregulation (Fc = − 11.8). In Table 2, all modulated genes with their respective Fc are reported.

Growth-factor-related and trophic genes

After IL-1β treatment, 11 growth factors and trophic genes were modulated, of which 7 were upregulated (*FGF7*, *IGF1*, *IGFBP4* and *IGFBP5* were shared by all cell populations), 3 were downregulated and one (*AREG*) was both upregulated (EPCs) and downregulated (AFCs). Among the upregulated genes, *IGFBP2* was shared by NPCs and AFCs, *TGFB3* was shared by NPCs and EPCs, *IGF2* was only upregulated in NPCs and *AREG* in EPCs. Concerning the downregulated genes, *AREG* and *CSF1R* were downregulated only in AFCs and *FGF6* and *NTF4* were shared by AFCs and EPCs. In Table 3, all modulated genes with their respective Fc are reported.

Senescence-related genes

After IL-1β treatment, 12 senescence-related genes were modulated. All, except for *PPP2R1B* that was upregulated, were downregulated. In particular, *BCL2* and *PPARG* were downregulated in all cell populations; *EGF*, *KRAS* and *PRKCB* in AFCs and EPCs; *SIRT2* in NPCs and EPCs; *CHEK1* only in NPCs; *BLM*, *DCLRE1B*, *EME1* and *PLK1* only in AFCs. In Table 4, all modulated genes with their respective Fc are reported.

Protein profiling

In basal condition, the three cell populations showed no differences in the production of inflammatory mediators, whereas this was affected by the presence of IL-1β (Fig. 5**a**). After IL-1β treatment, all cells released more pro-inflammatory ICAM-1 and IL-1β and anti-inflammatory IL-11 and GM-CSF. In all cell populations, the anti-inflammatory IL-1Ra levels were higher after the inflammatory stimulus in comparison with basal levels ($p < 0.01$) (Fig. 5**b**). NPCs showed an increase in the pro-inflammatory IL-8 and IL-6sR, accompanied by a decrease in the anti-inflammatory IL-10. Moreover, AFCs showed increased levels of the pro-inflammatory IL-8, MIP-1-α and MIP-1-β. EPCs were the most responsive cells to IL-1β, showing metabolic inhibition characterised by decreased levels of the pro-inflammatory eotaxin-2, IL-3, IL-15, IL-16, IL-17, MCP-1 and PDGF-BB and of the anti-inflammatory IL-10, IL-13, sTNF-RI and TIMP-2 proteins. The anti-inflammatory IP-10 was increased in both AFCs and EPCs. The protein analysis confirmed the gene expression results for GM-CSF, IL-1β, IL-11, IL-15, MCP-2 and MIP-1-δ in all cell populations, for IL-8 in NPCs and AFCs, for IP-10 in AFCs and EPCs, for MIP-1-α and MIP-1-β in AFCs, for IL-10, IL-13, IL-17 and eotaxin-2 in EPCs. Despite the upregulation or downregulation of the gene expression, no significant changes were observed in the release of most of the analysed proteins. No changes in *ICAM1* expression, but an increase in protein release in all cell populations was observed. Finally, no changes were observed in the expression of *IL16*, *sTNF-RI* and *PDGF-BB* in EPCs and *IL10* in NPCs despite a decreased release of these proteins. The comparison between gene expression and protein release is reported in Table 5.

Table 3. Upregulated or downregulated growth-factor-related and trophic genes after IL-1β treatment with Fc ≥ + 2 or ≤ − 2 in the different cell populations; (-) no differences.

Gene	Fc NPCs	Fc AFCs	Fc EPCs
AREG	-	− 2.3	3.5
CSF1R	-	− 2.4	-
FGF6	-	− 2.4	− 2.3
FGF7	2.8	2.6	3.2
IGF1	6.5	20.9	5.5
IGF2	2.4	-	-
IGFBP2	2.5	6.9	-
IGFBP4	2.3	3.3	2.3
IGFBP5	2.4	2.5	4
NTF4	-	− 2.4	− 2.5
TGFB3	2.5	-	2.3

Discussion

The findings of the present study showed that AFCs and EPCs were more molecularly responsive to IL-1β treatment than NPCs. In particular, AFCs showed the largest release of pro-inflammatory-related proteins, whereas EPCs showed the greatest anti-inflammatory ability.

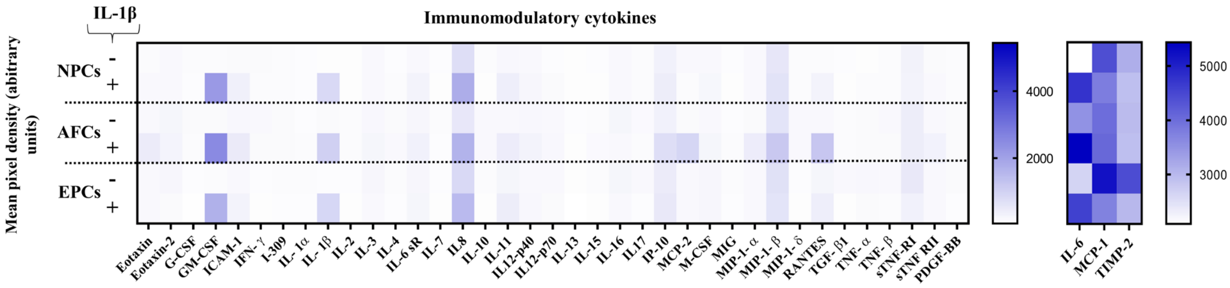
As far as it can be ascertained, the present is the first study comparing side-by-side the molecular profile of NPCs, AFCs and EPCs in response to *in vitro* inflammation. The responses include the release of stemness, pro/anti-inflammatory, angiogenic and trophic factors and the expression of genes participating in multiple signalling cascades and biological processes. The inflammatory stimulus induced a marked downregulation (5.7 times more than the upregulated genes) of the stemness genes. All cell types showed comparable numbers of upregulated stemness genes, whereas AFCs and EPCs showed a slightly larger number of downregulated genes in comparison with NPCs, sharing with EPCs, on the other hand, a similar entity of modulation.

Recently, the expression of a very large panel of genes in IVD- and EP-expanded cells derived from

Table 4. Upregulated or downregulated senescence-related genes after IL-1β treatment with Fc ≥ + 2 or ≤ − 2 in the different cell populations; (-) no differences.

Gene	Fc NPCs	Fc AFCs	Fc EPCs
BCL2	− 2.6	− 2.5	− 3.1
BLM	-	− 2.3	-
CHEK1	− 2.1	-	-
DCLRE1B	-	− 2.3	-
EGF	-	− 2.3	− 2.3
EME1	-	− 2.3	-
KRAS	-	− 2.4	− 2.2
PLK1	-	− 2.1	-
PPARG	− 4.2	− 4.0	− 3.8
PPP2R1B	-	-	2.8
PRKCB	-	− 2.4	− 2.2
SIRT2	− 2.1	-	− 2.1

a



Cell type	Significantly ($p < 0.05$) upregulated post-IL-1β stimulation	Significantly ($p < 0.05$) downregulated post-IL-1β stimulation
NPCs	GM-CSF, ICAM-1, IL-1β, IL-6sR, IL-8, IL-11	IL-10
AFCs	GM-CSF, ICAM-1, IL-1β, IL-8, IL-11, IP-10, MIP-1-α, MIP-1-β	-
EPCs	GM-CSF, ICAM-1, IL-1β, IL-11, IP-10	Eotaxin-2, IL-3, IL-10, IL-13, IL-15, IL-16, IL-17, MCP-1, sTNF-RI, PDGF-BB, TIMP-2

b

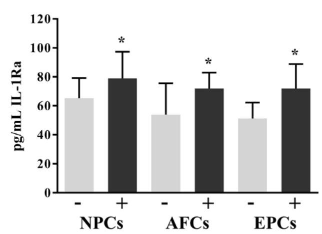


Fig. 5. Protein secretion in conditioned media obtained from NPCs, AFCs and EPCs at basal (-) and post-stimulation with IL-1β (+). (**a**) Secretome multiplex analysis of immunomodulatory cytokines presented as overall heat maps of mean pixel intensity. Table shows significantly upregulated and downregulated molecules after IL-1β stimulation (n = 4). (**b**) ELISA assay of IL-1Ra release. * $p \leq 0.05$ indicates IL-1β treated *vs.* basal, in each cell populations (n = 8).

the same donors has been evaluated. AFCs present the largest number of selectively highly expressed stemness and chondrogenic/tissue specific genes (De Luca *et al.*, 2020), therefore representing the most promising IVD cell population for the treatment of IVD degeneration. Differently from what observed in basal conditions, the results of the present study did not provide a strong indication about the identification of AFCs as having the greatest stemness potential in the presence of an inflammatory stimulus.

Interestingly, the trophic *IGF1*, marker of notochordal cells (Peck *et al.*, 2017), appeared upregulated in the presence of IL-1β, especially in AFCs (Fc = 20.9). Previous *in vitro* and *in vivo* studies have demonstrated that IGF-1 exerts anabolic, proliferative and anti-apoptotic effects on disc cells (Day *et al.*, 2005; Masuda and An, 2006; Masuda *et al.*, 2004; Osada *et al.*, 1996; Pratsinis and Kletsas, 2007; Sakai, 2008). These data suggest a tissue protective response to inflammation. In addition, in the present study, an upregulation of *IGF2*, a competitor of IGF-1 for the binding with IGF-R1 (Travascio *et al.*, 2014; Zhang *et al.*, 2013a), was observed only in NPCs. A concomitant upregulation of *IGFBP2* in NPCs and AFCs and of *IGFBP4* and *IGFBP5* in all the analysed cells was observed. This suggests an attempt of these

Table 5. Protein array and expression of their encoding genes; increase (+), decrease (–) or no variation (=).

Gene	Protein	NPCs		AFCs		EPCs	
		Gene	Protein	Gene	Protein	Gene	Protein
CCL2	MCP-1	+	=	+	=	+	–
CCL3	MIP-1-α	+	=	+	+	+	=
CCL4	MIP-1-β	=	=	+	+	+	=
CCL5	RANTES	+	=	+	=	+	=
CCL8	MCP-2	=	=	=	=	=	=
CCL11	Eotaxin	=	=	+	=	+	=
CCL15	MIP-1-δ	=	=	=	=	=	=
CSF1	M-CSF	+	=	+	=	+	=
CXCL8	IL-8	+	+	+	+	+	=
CXCL9	MIG	=	=	–	=	–	=
CXCL10	IP-10	+	=	+	+	+	+
ICAM1	ICAM-1	=	+	=	+	=	+
IFNG	IFNγ	=	=	–	=	–	=
IL1A	IL-1α	+	=	+	=	+	=
IL1B	IL-1β	+	+	+	+	+	+
IL2	IL-2	=	=	–	=	–	=
IL3	IL-3	=	=	–	=	=	–
IL6	IL-6	+	=	+	=	+	=
IL7	IL-7	–	=	=	=	–	=
IL11	IL-11	+	+	+	+	+	+
IL15	IL-15	=	=	=	=	–	–
IL16	IL-16	=	=	=	=	=	–
IL17A	IL-17	–	=	–	=	–	–
TNF	TNF-α	+	=	=	=	=	=
LTA	TNF-β	=	=	–	=	–	=
GSF3	G-CSF	+	=	+	=	+	=
IL4	IL-4	=	=	–	=	–	=
IL10	IL-10	=	–	=	=	–	–
IL13	IL-13	=	=	–	=	–	–
TNFRSF1A	sTNFRI	=	=	=	=	=	–
TNFRSF1B	sTNFRII	+	=	=	=	+	=
PDGFB	PDGF-BB	–	=	=	=	=	–
IL12B	IL12p40/IL12p70	=	=	–	=	–	=
CCL24	Eotaxin2	=	=	–	=	–	–
CSF2	GM-CSF	+	+	+	+	+	+

cells to promote the increase of IGF-1 and IGF-2 half-life, mediated by the binding with IGFBPs (Asfour *et al.*, 2015; Elmasry *et al.*, 2016; Zhang *et al.*, 2013a).

ECM disruption is a major hallmark of IVD degeneration and many studies have demonstrated that degradation enzymes such as MMPs and ADAMTS are upregulated in pathological discs (Wang *et al.*, 2015). After IL-1β treatment, all cell populations showed an upregulation of genes coding for several MMPs involved in gelatine, collagens, proteoglycans, laminin, fibronectin and elastin degradation. In particular, despite the physiological role of MMPs in repair and remodelling and their low expression in normal tissue, the catabolic effect of inflammation was demonstrated through the upregulation of *MMP1*, *MMP2*, *MMP3*, *MMP10* and *MMP12*, confirming what observed in IVD pathological tissues (Bachmeier *et al.*, 2009; Canbay *et al.*, 2013; Gruber *et al.*, 2014b; Richardson *et al.*, 2009; Tang *et al.*, 2014; Xu *et al.*, 2014a; Xu *et al.*, 2014b).

Along with MMPs, ADAMTS contribute to the physiological disc ECM turnover. In the present study, IL-1β induced an upregulation of *ADAMTS4*, a highly active aggrecanase-1 (Gendron *et al.*, 2007), and *ADAMTS9* in AFCs. ADAMTS4 is significantly increased in human degenerated IVD tissue in comparison with normal tissue (Pockert *et al.*, 2009; Zhang *et al.*, 2012) and IL-1β promotes its upregulation in NPCs (Wang *et al.*, 2011). In contrast, a downregulation of *ADAMTS15*, generally increased in human degenerated IVD tissue (Pockert *et al.*, 2009; Zhang *et al.*, 2012), was observed in the analysed cell populations. The general catabolic switch induced by IL-1β was not balanced by a concomitant upregulation of *TIMP1*, *TIMP2* and *TIMP3*. Nevertheless, a strong upregulation of *TNFAIP6* (21.5 < Fc < 81.7) was observed in all cell types. The TSG-6 protein encoded by this gene is not normally present in healthy adult tissues, but it is induced by pro-inflammatory cytokines such as IL-1β (Milner and Day, 2003) and plays a protective role against cartilage matrix degradation and inflammation (Glant *et al.*, 2002; Wisniewski and Vilcek, 1997). Moreover, a co-localisation of IL-1β and TSG-6 is observed in normal and degenerated IVD tissues (Roberts *et al.*, 2005), suggesting this interplay also in the disc. The study results showed an anti-inflammatory and anabolic response of all the analysed disc cells mediated by TSG-6 in inflamed conditions. TSG-6 is also able to inhibit neutrophil migration (Getting *et al.*, 2002) and likely it counteracted the upregulation of chemokine ligands, particularly *CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, and even more *CXCL6* and *CXCL8* after IL-1β stimulation in the three cell populations. The expression of *CXCR2*, receptor of these ligands, was slightly downregulated in AFCs and EPCs, indicating an attempt to inhibit the effect of these chemokines on these cells. In general, considering the modulation of the inflammatory genes by IL-1β, there was a balance between the number of upregulated or downregulated genes, mainly encoding for cytokines, chemokines and their receptor or antagonist, with a very similar behaviour shared by AFCs and EPCs. Moreover, a similar entity of upregulation was shown by NPCs and EPCs, whereas AFCs and EPCs shared a similar modulation of downregulated genes.

CCL2, a chemoattractant for monocytes and basophils, together with its receptors CCR2 or CCR4, plays a role in the induction of the inflammatory process in herniated discs, as demonstrated in a mouse model of IVD degeneration induced by TNF-α (Nakawaki *et al.*, 2019), and it is expressed by NPCs as a protein related to the histological degenerative tissue changes (Phillips *et al.*, 2013). After IL-1β treatment, an overexpression of *CCL2* ($7.7 < Fc < 9.5$) in all cell populations was observed in agreement with an increased expression of this cytokine, as previously shown in human AFCs (Gruber *et al.*, 2015). The level of the released MCP-1, encoded by *CCL2*, decreased in EPCs after IL-1β treatment, probably due to a negative feedback control promoted by these cells, as recently demonstrated for murine IVD cells exposed to TNFα (Nakawaki *et al.*, 2019). Monocytes recruitment/mobilisation is also obtained from the binding of CCL7 with its receptors CCR2 or CCR3 (Sokol and Luster, 2015). NPCs are a source of CCL7, whose release increases along with IVD degeneration grade (Phillips *et al.*, 2013). In the present study, an upregulation of *CCL7* was observed in all cell populations, suggesting a possible monocyte recruitment in inflamed discs. A concomitant downregulation of *CCR2*, *CCR3* and *CCR4* could be ascribed again to the attempt to limit the effect of CCL2 and CCL7 in AFCs and EPCs.

Another chemoattractant is CCL3; through its receptors CCR1 and CCR5, this chemokine induces white blood cell recruitment and promotes IVD inflammation and degeneration (Liu *et al.*, 2015; Wang *et al.*, 2013). After the pro-inflammatory stimulation, a higher expression of *CCL3* ($13.4 < Fc < 16.5$) was observed in all IVD cells as well as a downregulation of *CCR1* and *CCR5* in AFCs and EPCs, probably to counteract the effect of *CCL3* on disc cells. Other two chemoattractant of white blood cells were upregulated by the inflammatory stimulus, *CCL4* in AFCs and EPCs and *CCL5* in all the cell populations ($45.2 < Fc < 148$). CCL5 is significantly high in disc cells derived from patients with severe disc degeneration (Gruber *et al.*, 2014a; Weber *et al.*, 2015) and its expression levels correlate with the IVD degenerative grade (Gruber *et al.*, 2014a). *CCR1*, *CCR3* and *CCR5*, receptors of CCL5 (Gruber *et al.*, 2015; Sokol and Luster, 2015) and CCL4, were all downregulated; in particular, *CCR1* in all cell populations, *CCR3* and *CCR5* in AFCs and EPCs, highlighting that AFCs and EPCs share a similar behaviour to counteract this signalling. In contrast, IL-1β treatment induced upregulation of *CXCL10*, a chemokine promoting a Th1-orienting (pro-inflammatory) attitude (Romagnani *et al.*, 2005), in all the analysed cell populations and downregulation of *CXCL4* (Romagnani *et al.*, 2005) and *CCL17* in AFCs and EPCs and of *CCL22* in all cell populations (Sokol and Luster, 2015). All these chemokines are involved in the Th2 (anti-inflammatory) response. The concomitant upregulation in AFCs and EPCs of *CXCR3*, receptor of CXCL10, suggests a pro-inflammatory behaviour and probably is responsible for the downregulation of IL-4, IL-5 and IL-13 observed in the same populations (Romagnani *et al.*, 2005). Taken together, these results suggest a promotion, by all analysed cells, of white blood cells recruitment and of a pro-inflammatory switch induction after IL-1β stimulation.

CCL20 ($390.3 < Fc < 1,098.9$), involved in Th17 response (Tesmer *et al.*, 2008), was found among the highest upregulated genes in all cell populations, along with a downregulation of its receptor *CCR6* and of the pro-inflammatory *IL17A* in AFCs and EPCs, likely an attempt of these cells to limit self-detrimental effects of CCL20-mediated signalling. CCL20 production is observed in degenerated and cultured NPCs and its release further increases after IL-17A or TNF-α stimulation (Zhang *et al.*, 2013b). Interestingly, the same authors reported the expression of *CCR6* in PBMCs derived from patients with IVD degeneration and of IL-17A in pathologic IVD tissues (Shamji *et al.*, 2010; Zhang *et al.*, 2013b).

Inflammatory cytokines such as TNFα, IL-6 and in particular IL-1β affect matrix metabolism and apoptosis of IVD cells, causing disc degeneration (Kalb *et al.*, 2012; Wuertz and Haglund, 2013). In the presence of IL-1β, an upregulation of *TNF* in NPCs and of IL-1β (both at gene and protein level) and *IL6* ($46.3 < Fc < 78.0$) in all cell types was observed, probably depending on a positive feedback loop created by IL-1β treatment (Jimbo *et al.*, 2005). In this regard, all three cell populations showed a significant upregulation of IL-1Ra at the gene and protein level aimed to counteract IL-1β-mediated inflammation. Interestingly, in EPCs there was also a slight upregulation of the expression of the decoy receptor *IL1R2*, strengthening the anti-inflammatory attitude of these cells towards IL-1β. In addition, only in NPCs, the release of IL-6sR increased after IL-1β treatment. Based on what previously reported, although the IL-6/IL-6sR binding induces a pro-inflammatory response, it also induces, even if to a lesser extent, the upregulation of *TIMP1*, indicating a protective role in cartilage metabolism (Silacci *et al.*, 1998).

Intriguingly, after IL-1β treatment, in NPCs and EPCs, together with the over-expression of *TNF*, an upregulation of one of its receptor was observed, *TNFRSF1B*, also known as *TNFR2*, that antagonise TNF effects. Moreover, a downregulation of TNF ligands such as *TNFSF12* and *TNFSF13B* (in NPCs) and *TNFSF8* (in AFCs and EPCs) was observed, showing an inhibitory overall effect of TNF signalling.

Another well-known marker involved in pathological (Kang *et al.*, 1996) and/or inflamed IVD (Asahara *et al.*, 1996) cells is NO. In agreement with previously reported upregulation of iNOS and NO in NPCs after IL-1β treatment (Bai *et al.*, 2019), after the same inflammatory stimulation, *NOS2* was upregulated in all the analysed disc and EP cells. In addition, it was observed in cerebrospinal fluid of patients affected by degenerative lumbar disease (Asahara *et al.*, 1996).

Concerning the angiogenic-related genes evaluated, all the cell populations showed a slight pro-angiogenic behaviour after inflammation, with no inter-population difference. This is in line with what previously reported for human degenerated or inflamed IVDs (Binch *et al.*, 2014).

Between all the analysed cells, AFCs had a slightly senescent attitude, showing a downregulation of *DCLRE1B*, *EME1* and *MSH2*, related to DNA repair process, of the pro-mitogenic *EGF* and of *PLK1*, related to cell cycle control.

One limitation of the present study was the use of only one *in vitro* model of inflammation. However, IL-1β is the strongest inflammatory stimulus for IVD degeneration (Khan *et al.*, 2017; Johnson *et al.*, 2015; Molinos *et al.*, 2015; Le Maitre *et al.*, 2005; Wuertz and Haglund, 2013). Another limitation was the use of a pool of cells obtained from 4 donors for the gene array. Nevertheless, the expression of the panel of genes was confirmed by protein array (same 4 donors not pooled), ELISA and real-time PCR (same 4 donors and further new 4 donors, not pooled). Finally, functional assays would have been necessary to confirm the role of inflammatory genes in order to ascribe a clear function on the different IVD cells.

Conclusion

IVD and EP cells were responsive to IL-1β, as demonstrated by the massive downregulation of stemness genes and upregulation of pro-inflammatory and catabolic genes. In the presence of this inflammatory stimulus, all the analysed cell populations attempted to molecularly counteract the degradative process of the matrix. In particular, EPCs showed the most anti-inflammatory response, while AFCs secreted the largest number of pro-inflammatory mediators. AFCs and EPCs, on the other hand, exhibited a common protective response by repressing the receptors involved in the activation of the signalling mediated by specific chemokines inducing white blood cell recruitment. Molecular targets specific for one or more IVD and EP cell populations in the presence of IL-1β were identified. In particular, in the presence of an inflammatory environment, the anti-inflammatory and anabolic properties of IL-1Ra, IGFs and TSG6 can be exploited to suppress the identified pathological targets upregulated in these cells.

Acknowledgement

The study was funded by the Italian Ministry of Health, “Ricerca Corrente”. Diego Correa and Dimitrios Kouroupis thank the Soffer Family Foundation and the Diabetes Research Institute Foundation (DRIF) for their generous funding support. These funding sources were not involved in any step of the study design, collection, analysis, interpretation of the data or writing of the manuscript.

Diego Correa is a paid consultant of Lipogems USA, LLC. The other authors declare no conflict of interest.

References

Asahara H, Yokoi I, Tamada T, Kabuto H, Ogawab N, Mori A, Inoue H (1996) Increased cerebrospinal fluid nitrite and nitrate levels in patients with lumbar spondylosis. Res Commun Mol Pathol Pharmacol **91**: 77-83.

Asfour S, Travascio F, Elmasry S, de Rivero Vaccari JP (2015) A computational analysis on the implications of age-related changes in the expression of cellular signals on the role of IGF-1 in intervertebral disc homeostasis. J Biomech **48**: 332-339.

Bachmeier BE, Nerlich A, Mittermaier N, Weiler C, Lumenta C, Wuertz K, Boos N (2009) Matrix metalloproteinase expression levels suggest distinct enzyme roles during lumbar disc herniation and degeneration. Eur Spine J **18**: 1573-1586.

Bai X, Ding W, Yang S, Guo X (2019) Higenamine inhibits IL-1β-induced inflammation in human nucleus pulposus cells. Biosci Rep **39**. pii: BSR20190857. DOI: 10.1042/BSR20190857.

Binch AL, Cole AA, Breakwell LM, Michael AL, Chiverton N, Cross AK, Le Maitre CL (2014) Expression and regulation of neurotrophic and angiogenic factors during human intervertebral disc degeneration. Arthritis Res Ther **16**: 416. DOI: 10.1186/s13075-014-0416-1.

Brisby H, Papadimitriou N, Brantsing C, Bergh P, Lindahl A, Barreto Henriksson H (2013) The presence of local mesenchymal progenitor cells in human degenerated intervertebral discs and possibilities to influence these *in vitro*: a descriptive study in humans. Stem Cells Dev **22**: 804-814.

Burke JG, Watson RW, McCormack D, Dowling FE, Walsh MG, Fitzpatrick JM (2002) Spontaneous production of monocyte chemoattractant protein-1 and interleukin-8 by the human lumbar intervertebral disc. Spine (Phila Pa 1976) **27**: 1402-1407.

Canbay S, Turhan N, Bozkurt M, Arda K, Caglar S (2013) Correlation of matrix metalloproteinase-3 expression with patient age, magnetic resonance imaging and histopathological grade in lumbar disc degeneration. Turk Neurosurg **23**: 427-433.

Colombini A, Lombardi G, Corsi MM, Banfi G (2008) Pathophysiology of the human intervertebral disc. Int J Biochem Cell Biol **40**: 837-842.

Colombini A, Lopa S, Ceriani C, Lovati AB, Croiset SJ, Di Giancamillo A, Lombardi G, Banfi G, Moretti M (2015) *In vitro* characterization and *in vivo* behavior of human nucleus pulposus and annulus fibrosus cells in clinical-grade fibrin and collagen-enriched fibrin gels. Tissue Eng Part A **21**: 793-802.

Day TF, Guo X, Garrett-Beal L, Yang Y (2005) Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell **8**: 739-750.

De Luca P, Castagnetta M, de Girolamo L, Coco S, Malacarne M, Ragni E, Vigano M, Lugano G, Brayda-Bruno M, Coviello D, Colombini A (2020) Intervertebral disc and endplate cell characterisation highlights annulus fibrosus cells as the most promising for tissue-specific disc degeneration therapy. Eur Cell Mater **39**: 156-170.

De Luca P, Kouroupis D, Vigano M, Perucca-Orfei C, Kaplan L, Zagra L, de Girolamo L, Correa D, Colombini A (2019) Human diseased articular cartilage contains a mesenchymal stem cell-like population of chondroprogenitors with strong immunomodulatory responses. J Clin Med **8**. pii: E423. DOI: 10.3390/jcm8040423.

De Luca P, de Girolamo L, Perucca Orfei C, Viganò M, Cecchinato R, Brayda-Bruno M, Colombini A (2018) Vitamin D's effect on the proliferation and inflammation of human intervertebral disc cells in relation to the functional vitamin d receptor gene foki polymorphism. Int J Mol Sci **19**: 2002. pii: E2002. DOI: 10.3390/ijms19072002.

Elmasry S, Asfour S, de Rivero Vaccari JP, Travascio F (2016) A computational model for investigating the effects of changes in bioavailability of insulin-like growth factor-1 on the homeostasis of the intervertebral disc. Comput Biol Med **78**: 126-137.

Gendron C, Kashiwagi M, Lim NH, Enghild JJ, Thogersen IB, Hughes C, Caterson B, Nagase H (2007) Proteolytic activities of human ADAMTS-5: comparative studies with ADAMTS-4. J Biol Chem **282**: 18294-18306.

Getting SJ, Mahoney DJ, Cao T, Rugg MS, Fries E, Milner CM, Perretti M, Day AJ (2002) The link module from human TSG-6 inhibits neutrophil migration in a hyaluronan- and inter-alpha -inhibitor-independent manner. J Biol Chem **277**: 51068-51076.

Glant TT, Kamath RV, Bardos T, Gal I, Szanto S, Murad YM, Sandy JD, Mort JS, Roughley PJ, Mikecz K (2002) Cartilage-specific constitutive expression of TSG-6 protein (product of tumor necrosis factor alpha-stimulated gene 6) provides a chondroprotective, but not antiinflammatory, effect in antigen-induced arthritis. Arthritis Rheum **46**: 2207-2218.

Gruber HE, Hoelscher GL, Ingram JA, Bethea S, Cox M, Hanley EN Jr (2015) Proinflammatory cytokines modulate the chemokine CCL2 (MCP-1) in human annulus cells *in vitro*: CCL2 expression and production. Exp Mol Pathol **98**: 102-105.

Gruber HE, Hoelscher GL, Ingram JA, Bethea S, Norton HJ, Hanley EN Jr (2014a) Production and expression of RANTES (CCL5) by human disc cells and modulation by IL-1-β and TNF-α in 3D culture. Exp Mol Pathol **96**: 133-138.

Gruber HE, Ingram JA, Cox MD, Hanley EN Jr (2014b) Matrix metalloproteinase-12 immunolocalization in the degenerating human intervertebral disc and sand rat spine: biologic implications. Exp Mol Pathol **97**: 1-5.

Hamamoto H, Miyamoto H, Doita M, Takada T, Nishida K, Kurosaka M (2012) Capability of nondegenerated and degenerated discs in producing inflammatory agents with or without macrophage interaction. Spine (Phila Pa 1976) **37**: 161-167.

Hughes SP, Freemont AJ, Hukins DW, McGregor AH, Roberts S (2012) The pathogenesis of degeneration of the intervertebral disc and emerging therapies in the management of back pain. J Bone Joint Surg Br **94**: 1298-1304.

Jimbo K, Park JS, Yokosuka K, Sato K, Nagata K (2005) Positive feedback loop of interleukin-1beta upregulating production of inflammatory mediators in human intervertebral disc cells *in vitro*. J Neurosurg Spine **2**: 589-595.

Johnson ZI, Schoepflin ZR, Choi H, Shapiro IM, Risbud MV (2015) Disc in flames: roles of TNF-α and IL-1β in intervertebral disc degeneration. Eur Cell Mater **30**: 104-117.

Kalb S, Martirosyan NL, Kalani MY, Broc GG, Theodore N (2012) Genetics of the degenerated intervertebral disc. World Neurosurg **77**: 491-501.

Kang JD, Georgescu HI, McIntyre-Larkin L, Stefanovic-Racic M, Donaldson WF 3rd, Evans CH (1996) Herniated lumbar intervertebral discs spontaneously produce matrix metalloproteinases, nitric oxide, interleukin-6, and prostaglandin E2. Spine (Phila Pa 1976) **21**: 271-277.

Kang JD, Stefanovic-Racic M, McIntyre LA, Georgescu HI, Evans CH (1997) Toward a biochemical understanding of human intervertebral disc degeneration and herniation. Contributions of nitric oxide, interleukins, prostaglandin E2, and matrix metalloproteinases. Spine (Phila Pa 1976) **22**: 1065-1073.

Kepler CK, Markova DZ, Dibra F, Yadla S, Vaccaro AR, Risbud MV, Albert TJ, Anderson DG (2013) Expression and relationship of proinflammatory chemokine RANTES/CCL5 and cytokine IL-1β in painful human intervertebral discs. Spine (Phila Pa 1976) **38**: 873-880.

Khan AN, Jacobsen HE, Khan J, Filippi CG, Levine M, Lehman RA Jr, Riew KD, Lenke LG, Chahine NO (2017) Inflammatory biomarkers of low back pain and disc degeneration: a review. Ann N Y Acad Sci **1410**: 68-84.

Kim JH, Choi H, Suh MJ, Shin JH, Hwang MH, Lee HM (2013) Effect of biphasic electrical current stimulation on IL-1β-stimulated annulus fibrosus cells using *in vitro* microcurrent generating chamber system. Spine **38**: E1368-E1376.

Klawitter M, Quero L, Klasen J, Liebscher T, Nerlich A, Boos N, Wuertz K (2012a) Triptolide exhibits anti-inflammatory, anti-catabolic as well as anabolic effects and suppresses TLR expression and MAPK activity in IL-1β treated human intervertebral disc cells. Eur Spine J **21** Suppl 6: S850-S859.

Klawitter M, Quero L, Klasen J, Gloess AN, Klopprogge B, Hausmann O, Boos N, Wuertz K (2012b) Curcuma DMSO extracts and curcumin exhibit an anti-inflammatory and anti-catabolic effect on human intervertebral disc cells, possibly by influencing TLR2 expression and JNK activity. J Inflamm (Lond) **9**: 29. DOI: 10.1186/1476-9255-9-29.

Le Maitre CL, Freemont AJ, Hoyland JA (2004) Localization of degradative enzymes and their inhibitors in the degenerate human intervertebral disc. J Pathol **204**: 47-54.

Le Maitre CL, Freemont AJ, Hoyland JA (2005) The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration. Arthritis Res Ther **7**: R732-745.

Le Maitre CL, Hoyland JA, Freemont AJ (2007) Catabolic cytokine expression in degenerate and herniated human intervertebral discs: IL-1beta and TNFalpha expression profile. Arthritis Res Ther **9**: R77. DOI: 10.1186/ar2275.

Le Maitre CL, Richardson SMA, Baird P, Williamson B, Ross R, Freemont AJ, Hoyland JA (2003) IL-1; Role in degeneration of the intervertebral disc and its inhibition using IL-1Ra gene transfer. Molecular Therapy **7**: S405-S405.

Liao ZW, Wu XH, Song Y, Luo RJ, Yin HP, Zhan SF, Li S, Wang K, Zhang YK, Yang C (2019) Angiopoietin-like protein 8 expression and association with extracellular matrix metabolism and inflammation during intervertebral disc degeneration. J Cell Mol Med **23**: 5737-5750.

Liu C, Fei HD, Sun ZY, Tian JW (2015) Bioinformatic analysis of the microarray gene expression profile in degenerative intervertebral disc cells exposed to TNF-α. Eur Rev Med Pharmacol Sci **19**: 3332-3339.

Liu LT, Huang B, Li CQ, Zhuang Y, Wang J, Zhou Y (2011) Characteristics of stem cells derived from the degenerated human intervertebral disc cartilage endplate. PLoS One **6**: e26285. DOI: 10.1371/journal.pone.0026285.

Lopa S, Ceriani C, Cecchinato R, Zagra L, Moretti M, Colombini A (2016) Stability of housekeeping genes in human intervertebral disc, endplate and articular cartilage cells in multiple conditions for reliable transcriptional analysis. Eur Cell Mater **31**: 395-406.

Masuda K, An HS (2006) Prevention of disc degeneration with growth factors. Eur Spine J **15** Suppl 3: S422-4432.

Masuda K, Oegema TR Jr, An HS (2004) Growth factors and treatment of intervertebral disc degeneration. Spine (Phila Pa 1976) **29**: 2757-2769.

Metsalu T, Vilo J (2015) ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. Nucleic Acids Res **43**:W566-570.

Millward-Sadler SJ, Costello P W, Freemont A J, Hoyland J A (2009) Regulation of catabolic gene expression in normal and degenerate human intervertebral disc cells: implications for the pathogenesis of intervertebral disc degeneration. Arthritis Res Ther **11**: R65. DOI: 10.1186/ar2693.

Milner CM, Day AJ (2003) TSG-6: a multifunctional protein associated with inflammation. J Cell Sci **116**: 1863-1873.

Minogue BM, Richardson SM, Zeef LA, Freemont AJ, Hoyland JA (2010) Characterization of the human nucleus pulposus cell phenotype and evaluation of novel marker gene expression to define adult stem cell differentiation. Arthritis Rheum **62**: 3695-3705.

Molinos M, Almeida CR, Caldeira J, Cunha C, Goncalves RM, Barbosa MA (2015) Inflammation in intervertebral disc degeneration and regeneration. J R Soc Interface **12**: 20150429. DOI: 10.1098/rsif.2015.0429.

Nakawaki M, Uchida K, Miyagi M, Inoue G, Kawakubo A, Kuroda A, Satoh M, Takaso M (2019) Sequential CCL2 expression profile after disc injury in mice. J Orthop Res **38**: 895-901.

Osada R, Ohshima H, Ishihara H, Yudoh K, Sakai K, Matsui H, Tsuji H (1996) Autocrine/paracrine mechanism of insulin-like growth factor-1 secretion, and the effect of insulin-like growth factor-1 on proteoglycan synthesis in bovine intervertebral discs. J Orthop Res **14**: 690-699.

Peck SH, McKee KK, Tobias JW, Malhotra NR, Harfe BD, Smith LJ (2017) Whole transcriptome analysis of notochord-derived cells during embryonic formation of the nucleus pulposus. Sci Rep **7**: 10504. DOI: 10.1038/s41598-017-10692-5.

Phillips KL, Chiverton N, Michael AL, Cole AA, Breakwell LM, Haddock G, Bunning RA, Cross AK, Le Maitre CL (2013) The cytokine and chemokine expression profile of nucleus pulposus cells: implications for degeneration and regeneration of the intervertebral disc. Arthritis Res Ther **15**: R213. DOI: 10.1186/ar4408.

Phillips KL, Cullen K, Chiverton N, Michael AL, Cole AA, Breakwell LM, Haddock G, Bunning RA, Cross AK, Le Maitre CL (2015) Potential roles of cytokines and chemokines in human intervertebral disc degeneration: interleukin-1 is a master regulator of catabolic processes. Osteoarthritis Cartilage **23**: 1165-1177.

Pockert AJ, Richardson SM, Le Maitre CL, Lyon M, Deakin JA, Buttle DJ, Freemont AJ, Hoyland JA (2009) Modified expression of the ADAMTS enzymes and tissue inhibitor of metalloproteinases 3 during human intervertebral disc degeneration. Arthritis Rheum **60**: 482-491.

Power KA, Grad S, Rutges JP, Creemers LB, van Rijen MH, O'Gaora P, Wall JG, Alini M, Pandit A, Gallagher WM (2011) Identification of cell surface-specific markers to target human nucleus pulposus cells: expression of carbonic anhydrase XII varies with age and degeneration. Arthritis Rheum **63**: 3876-386.

Pratsinis H, Kletsas D (2007) PDGF, bFGF and IGF-I stimulate the proliferation of intervertebral disc cells *in vitro via* the activation of the ERK and Akt signaling pathways. Eur Spine J **16**: 1858-1866.

Raj PP (2008) Intervertebral disc: anatomy-physiology-pathophysiology-treatment. Pain Pract **8**: 18-44.

Richardson SM, Doyle P, Minogue BM, Gnanalingham K, Hoyland JA (2009) Increased expression of matrix metalloproteinase-10, nerve growth factor and substance P in the painful degenerate intervertebral disc. Arthritis Res Ther **11**: R126. DOI: 10.1186/ar2793.

Risbud MV, Shapiro IM (2014) Role of cytokines in intervertebral disc degeneration: pain and disc content. Nat Rev Rheumatol **10**: 44-56.

Roberts S, Evans H, Menage J, Urban JP, Bayliss MT, Eisenstein SM, Rugg MS, Milner CM, Griffin S, Day AJ (2005) TNFalpha-stimulated gene product (TSG-6) and its binding protein, IalphaI, in the human intervertebral disc: new molecules for the disc. Eur Spine J **14**: 36-42.

Rodrigues-Pinto R, Richardson SM, Hoyland JA (2014) An understanding of intervertebral disc development, maturation and cell phenotype provides clues to direct cell-based tissue regeneration therapies for disc degeneration. Eur Spine J **23**: 1803-1814.

Romagnani P, Maggi L, Mazzinghi B, Cosmi L, Lasagni L, Liotta F, Lazzeri E, Angeli R, Rotondi M, Fili L, Parronchi P, Serio M, Maggi E, Romagnani S, Annunziato F (2005) CXCR3-mediated opposite effects of CXCL10 and CXCL4 on TH1 or TH2 cytokine production. J Allergy Clin Immunol **116**: 1372-1379.

Rustenburg CME, Emanuel KS, Peeters M, Lems WF, Vergroesen PA, Smit TH (2018) Osteoarthritis and intervertebral disc degeneration: quite different, quite similar. JOR Spine **1**: e1033. DOI: 10.1002/jsp2.1033.

Rutges J, Creemers LB, Dhert W, Milz S, Sakai D, Mochida J, Alini M, Grad S (2010) Variations in gene and protein expression in human nucleus pulposus in comparison to annulus fibrosus and cartilage cells: potential associations with aging and degeneration. Osteoarthritis Cartilage **18**: 416-423.

Sakai D (2008) Future perspectives of cell-based therapy for intervertebral disc disease. Eur Spine J **17** Suppl 4: 452-458.

Séguin CA, Pilliar RM, Madri JA, Kandel RA (2008) TNF-alpha induces MMP2 gelatinase activity and MT1-MMP expression in an *in vitro* model of nucleus pulposus tissue degeneration. Spine (Phila Pa 1976) **33**: 356-365.

Shamji MF, Setton LA, Jarvis W, So S, Chen J, Jing L, Bullock R, Isaacs RE, Brown C, Richardson WJ (2010) Proinflammatory cytokine expression profile in degenerated and herniated human intervertebral disc tissues. Arthritis Rheum **62**: 1974-1982.

Silacci P, Dayer JM, Desgeorges A, Peter R, Manueddu C, Guerne PA (1998) Interleukin (IL)-6 and its soluble receptor induce TIMP-1 expression in synoviocytes and chondrocytes, and block IL-1-induced collagenolytic activity. J Biol Chem **273**: 13625-13629.

Sinclair SM, Shamji MF, Chen J, Jing L, Richardson WJ, Brown CR, Fitch RD, Setton LA (2011) Attenuation of inflammatory events in human intervertebral disc cells with a tumor necrosis factor antagonist. Spine (Phila Pa 1976) **36**: 1190-1196.

Sokol CL, Luster AD (2015) The chemokine system in innate immunity. Cold Spring Harb Perspect Biol **7**. pii: a016303. DOI: 10.1101/cshperspect.a016303.

Solovieva S, Kouhia S, Leino-Arjas P, Ala-Kokko L, Luoma K, Raininko R, Saarela J, Riihimaki H (2004) Interleukin 1 polymorphisms and intervertebral disc degeneration. Epidemiology **15**: 626-633.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell **131**: 861-872.

Tang Y, Wang S, Liu Y, Wang X (2014) Microarray analysis of genes and gene functions in disc degeneration. Exp Ther Med **7**: 343-348.

Tang X, Jing L, Chen J (2012) Changes in the molecular phenotype of nucleus pulposus cells with intervertebral disc aging. PLoS One **7**: e52020. DOI: 10.1371/journal.pone.0052020.

Tesmer LA, Lundy SK, Sarkar S, Fox DA (2008) Th17 cells in human disease. Immunol Rev **223**: 87-113.

Tran CM, Markova D, Smith HE, Susarla B, Ponnappan RK, Anderson DG, Symes A, Shapiro IM, Risbud MV (2010) Regulation of CCN2/connective tissue growth factor expression in the nucleus pulposus of the intervertebral disc: role of Smad and activator protein 1 signaling. Arthritis Rheum **62**: 1983-1992.

Tran CM, Schoepflin ZR, Markova DZ, Kepler CK, Anderson DG, Shapiro IM, Risbud MV (2014) CCN2 suppresses catabolic effects of interleukin-1β through α5β1 and αVβ3 Integrins in nucleus pulposus cells: implications in intervertebral disc degeneration. J Biol Chem **289**: 7374-7387.

Travascio F, Eltoukhy M, Cami S, Asfour S (2014) Altered mechano-chemical environment in hip articular cartilage: effect of obesity. Biomech Model Mechanobiol **13**: 945-959.

Vergroesen PPA, Kingma I, Emanuel KS, Hoogendoorn RJW, Welting TJ, van Royen BJ, van Dieen JH, Smit TH (2015) Mechanics and biology in intervertebral disc degeneration: a vicious circle. Osteoarthritis Cartilage **23**: 1057-1070.

Wang H, Zhou Y, Chu TW, Li CQ, Wang J, Zhang ZF, Huang B (2016) Distinguishing characteristics of stem cells derived from different anatomical regions of human degenerated intervertebral discs. Eur Spine J **25**: 2691-2704.

Wang X, Wang H, Yang H, Li J, Cai Q, Shapiro IM, Risbud MV (2014) Tumor necrosis factor-α- and interleukin-1β-dependent matrix metalloproteinase-3 expression in nucleus pulposus cells requires cooperative signaling *via* syndecan 4 and mitogen-activated protein kinase-NF-κB axis: implications in inflammatory disc disease. Am J Pathol **184**:2560-2572.

Wang J, Tian Y, Phillips KL, Chiverton N, Haddock G, Bunning RA, Cross AK, Shapiro IM, Le Maitre CL, Risbud MV (2013) Tumor necrosis factor α- and interleukin-1 β-dependent induction of CCL3 expression by nucleus pulposus cells promotes macrophage migration through CCR1. Arthritis Rheum **65**: 832-842.

Wang JR, Markova D, Anderson DG, Zheng ZM, Shapiro IM, Risbud MV (2011) TNF-α and IL-1β promote a disintegrin-like and metalloprotease with thrombospondin type I motif-5-mediated aggrecan degradation through syndecan-4 in intervertebral disc. J Biol Chem **286**: 39738-39749.

Wang WJ, Yu XH, Wang C, Yang W, He WS, Zhang SJ, Yan YG, Zhang J (2015) MMPs and ADAMTSs in intervertebral disc degeneration. Clin Chim Acta **448**: 238-246.

Weber KT, Jacobsen TD, Maidhof R, Virojanapa J, Overby C, Bloom O, Quraishi S, Levine M, Chahine NO (2015) Developments in intervertebral disc disease research: pathophysiology, mechanobiology, and therapeutics. Curr Rev Musculoskelet Med **8**: 18-31.

Wisniewski HG, Vilcek J (1997) TSG-6: an IL-1/TNF-inducible protein with anti-inflammatory activity. Cytokine Growth Factor Rev **8**: 143-156.

Wuertz K, Quero L,Sekiguchi M, Klawitter M, Nerlich A, Konno S, Kikuchi S, Boos N (2011) The red wine polyphenol resveratrol shows promising potential for the treatment of nucleus pulposus-mediated pain *in vitro* and *in vivo*. Spine (Phila Pa 1976) **36**: E1373-E1384.

Wuertz K, Haglund L (2013) Inflammatory mediators in intervertebral disk degeneration and discogenic pain. Global Spine J **3**: 175-184.

Xu H, Mei Q, He J, Liu G, Zhao J, Xu B (2014a) Correlation of matrix metalloproteinases-1 and tissue inhibitor of metalloproteinases-1 with patient age and grade of lumbar disk herniation. Cell Biochem Biophys **69**: 439-444.

Xu HD, Mei Q, Xu B, Liu G, Zhao JN (2014b) Expression of matrix metalloproteinases is positively related to the severity of disc degeneration and growing age in the east asian lumbar disc herniation patients. Cell Biochem Biophys **70**: 1219-1225.

Xu J, Wang W, Ludeman M, Cheng K, Hayami T, Lotz JC, Kapila S (2008) Chondrogenic differentiation of human mesenchymal stem cells in three-dimensional alginate gels. Tissue Eng Part A **14**:667-680.

Yoo HJ, Yoon SS, Park SY, Lee EY, Lee EB, Kim JH, Song YW (2011) Gene expression profile during chondrogenesis in human bone marrow derived mesenchymal stem cells using a cDNA microarray. J Korean Med Sci **26**: 851-858.

Zhang L, Smith DW, Gardiner BS, Grodzinsky AJ (2013a) Modeling the insulin-like growth factor system in articular cartilage. PLoS One **8**: e66870. DOI: 10.1371/journal.pone.0066870.

Zhang Q, Huang M, Wang X, Xu X, Ni M, Wang Y (2012) Negative effects of ADAMTS-7 and ADAMTS-12 on endplate cartilage differentiation. J Orthop Res **30**: 1238-1243.

Zhang W, Nie L, Wang Y, Wang XP, Zhao H, Dongol S, Maharjan S, Cheng L (2013b) CCL20 secretion from the nucleus pulposus improves the recruitment of CCR6-expressing Th17 cells to degenerated IVD Tissues. PLoS One **8**: e66286. DOI: 10.1371/journal.pone.0066286.

Web References

1. https://www.oulu.fi/sites/default/files/news/TheLancet_LowBackPain.pdf [06.05.2020]

2. https://earray.chem.agilent.com/earray/ [06.05.2020]

3. https://biit.cs.ut.ee/clustvis/ [06.05.2020]

Discussion with Reviewers

Reviewer 1: Please discuss the potential limitations when using passaged cells for this type of analysis, especially in relation to the fact that the number of adhering cells was not controlled for.

Authors: In general, although cell stability in culture has been demonstrated up to several passages, a potential limitation of using expanded cells consists in the partially loss of their tissue-specific phenotype. However, expanded cells had to be used because of the limited number of collected cells immediately after isolation, not sufficient to perform all the experiments. To indirectly control the number of adhering cells, cell counts have been performed after isolation and after each passage, by maintaining the same seeding density and assessing their doubling time throughout expansion. On the other hand, an advantage of using passaged cells is represented by the fact that future cell therapy will be based on cell expansion in order to obtain a suitable number of homogeneous cells. Therefore, the characterisation of the response to IL-1β of expanded cells would represent a more realistic clinical scenario.

Marianna Peroglio: In the last sentence of the abstract it is mentioned that the study allowed for the identification of specific molecular targets that can be either silenced (when pathological targets) or stimulated to counteract the inflammation What would be the key molecular targets for each strategy? Which strategy could have a better chance of success in humans?
Authors: Among the three kind of analysed cells, EPCs showed the most anti-inflammatory response to IL-1β treatment, along with a protective attitude to repress the inflammation-activated pathways involved in white blood cell recruitment. For this reason, in an inflammatory context, they appear as the more promising tool for cell therapy. On the other hand, if considering the identified molecular target, a huge plethora of catabolic and inflammatory mediators were upregulated in presence of IL-1β, and IL-1Ra, IGFs and TSG6, in particular, appeared to be suitable candidates to be stimulated in IVD and EP cells for counteracting degenerative processes. These results suggested exploiting EPCs and stimulating the release of the identified anti-inflammatory and anabolic mediators as a potentially successful strategy to control inflammation.

Marianna Peroglio: In a previous study, the authors have investigated the response of osteoarthritic cartilage to IL-1β stimulation. What similarities and differences can be drawn between osteoarthritic chondrocytes' response to IL-1 β stimulation and IVD and EP cells response to this same cytokine?
Authors: Maintaining the same experimental conditions, some similarities were observed in terms of catabolic and secretory markers produced by IVD and EP cells after IL-1β treatment and osteoarthritic chondrocytes. In particular, in all the analysed cell populations, the inflammatory stimulus promoted the upregulation of specific MMPs (1, 3 and 13), without a corresponding production of their TIMPs (1 and 3) to balance the catabolic induction. Rather, IVD and EP cells showed a downregulation of TIMP3. In both spine cells and osteoarthritic chondrocytes, IL-1β treatment also increased the production of a plethora of secretory molecules, such as the anti-inflammatory IL-1Ra, GM-CSF and IL-11 as well as the pro-inflammatory ICAM-1 and IL-1β. In general, EPCs and osteoarthritic chondrocytes were the most responsive cells to IL-1β. The former showing metabolic inhibition, whereas the latter showing an increased secretion of the pro-inflammatory/modulatory molecules. Moreover, IL-1β promoted a more pro-inflammatory behaviour of NPCs, AFCs and osteoarthritic chondrocytes, resulting from an increased secretion of the pro-inflammatory IL-6sR, MIP-1-α, MIP-1-β and IL-8.

Marianna Peroglio: Could the authors comment on the inter-donor variability in terms of response to IL-1β and how this could potentially impact the envisioned therapy?
Authors: The inter-donor variability is a fundamental aspect to consider when using primary cells. In the present study, despite the inter-donor variability, markers of specific biological processes were identified as being modulated by IL-1β treatment. However, further evaluations should be performed to confirm the results obtained in a pool of donors in single donors too. Confirming of these results and performing specific functional tests would add further value to the therapeutic potential of the identified molecular targets.

Editor's note: The Scientific Editor responsible for this paper was Mauro Alini.