# ImmunoTools *special* Award 2021

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## Dissecting the effect of inflammation in human Annulus Fibrosus matrix production and organization

Low back pain (LBP) is the leader disorder in number of years lived with disability and although established as a multifactorial condition, intervertebral disc (IVD) disorders are considered one of the main pathophysiological conditions contributing to LBP [1]. In particular, lumbar disc herniation (5-15% of LBP cases) is considered the main cause of spine surgery, so a major financial burden to the healthcare systems worldwide [2, 3]. When herniation occurs, the inner tissue, the nucleus pulposus (NP) pushes out of its normal space, pressing against the outer tissue, the annulus fibrosus (AF), pressuring the spinal canal or nerve roots, releasing inflammatory mediators that can irritate the surrounding nerves, causing inflammation and pain. Ultimately, the AF fails allowing NP extrusion. Inflammation has been strongly linked with IVD degeneration and associated pain, but there is a lack of knowledge on the pathomechanism behind AF failure and IVD herniation. Besides both NP and AF cells secrete pro-inflammatory markers, the presence of macrophages in IVD herniated tissue has been extensively documented, linking these cells to increased pain and IVD degenerative status [4].

The major goal of this project is to understand the role of the inflammatory microenvironment in the AF extracellular matrix (ECM) remodelling, turning AF more susceptible to rupture. Our hypothesis is that a pro-inflammatory environment deregulates AF native ECM production and organization and stimulates macrophages towards a pro-inflammatory profile, contributing to AF ECM disorganization.

To test this hypothesis, AF cells will be isolated from human (h)IVD biopsies from LBP patients from Centro Hospitalar Universitário S.João and Hospital CUF/Porto under informed consent and ethical agreement. The AF will be separated from NP by macroscopic observation and the AF cells will be collected by enzymatic digestion as described [5]. AF cell phenotype (mesoderm-derived) will be characterized by flow cytometry to exclude NP contamination (endoderm-derived). For that AF cells will be characterized for expression of CD73, CD90, CD105, CD146, CD44 and lack of expression of CD45, CD14, CD3 and CD24 (hematopoietic and notochordal markers) by flow cytometry [6]. AF cells will then be cultured in vitro in 3D collagen-based hydrogels with oriented fibers and stimulated with Interleukin-1β (IL-1β) and tumour necrosis factor-alpha (TNF-α) (10ng/ml), the two pro-inflammatory cytokines more frequently expressed in IVD degeneration [7]. In parallel, human primary monocytes will be isolated from buffy coats of healthy blood donors, as routinely performed in our group [8]. Monocytes will then be seeded in transwells in the top of AF 3D constructs and allowed to differentiate into macrophages. After 10 days, the construct will be fixed and processed for histological staining, while the macrophages will be collected for characterization of surface markers by flow cytometry. The culture supernatant will be collected and frozen until further use. AF cell viability will be evaluated by LIVE/DEAD assay, metabolic activity will be analysed by rezasurin conversion, phenotype stability will be evaluated by flow cytometry and ECM production (namely Collagen type1,

Aggrecan and Elastin) will be evaluated by histology/immunohistochemistry. Collagen fibers orientation will be evaluated by Transmission Electron Microscopy and reflectance confocal microscopy. In parallel, macrophage phenotype will be evaluated by flow cytometry for M1 (CCR7, CD86, HLA-DR) and M2 (CD163, CD206) markers. The inflammatory secretion profile of AF cells and macrophages will be evaluated by Enzyme-Linked Immunosorbent Assay (ELISA) (for inflammatory target proteins as TNF-α, IL-1β, IL-6, IL-8, IL-12). In the end, the AF ECM composition and organization in the presence of a pro-inflammatory environment with and without macrophages will be compared with those in basal (healthy) environment.

The selected ImmunoTools collection of recombinant cytokines and ELISA-sets selected will be of crucial importance for an extensive evaluation of alterations in human annulus fibrosus cells induced by macrophage-mediated pro-inflammatory response. The selected cytokines will allow us to establish the pro-inflammatory conditions to human AF cells. Moreover, our team will be able to characterize cytokine production in our *in vitro* model upon inflammatory stimuli. With the support of ImmunoTools our work will take a step closer to understand the pathological mechanisms behind IVD degeneration, more specifically in AF integrity during herniation, bringing our field closer to unravel new insights for the development of new technologies and treatment for LBP.

## References


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**ImmunoTools** *special* AWARD for **Ana Luísa Castro** includes 10 reagents

recombinant human rh IL-1beta, rh TNFα

human ELISA-set (for one 96 plate): IL-6, IL-8

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