Magnetic actuation and magnetic responsive materials to modulate inflammation and their impact in cell behavior for tendon regeneration

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Universidade do Minho
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Tese de Doutoramento em Engenharia de Tecidos, Medicina Regenerativa e Células Estaminais

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Doutora Manuela E. Gomes

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Magnetic actuation and magnetic responsive materials to modulate inflammation and their impact in cell behavior for tendon regeneration

ABSTRACT

Tendon pathologies are a significant cause for disability in an increasingly active and ageing population. There is a substantial gap on the understanding of molecular and cellular responses leading to the onset and evolution of tendinopathic conditions but growing evidence suggests that persistent inflammatory events play a key role and may contribute to either triggering tendon injuries and/or prevent regenerative processes. Therefore, inflammatory events are can be the ultimate target for assisting new therapeutic strategies. In this thesis, we investigated magnetic stimuli applying pulsed electromagnetic field (PEMF) in combination with magnetically responsive materials using complementary cell based approaches for guiding and stimulating tendon regeneration through modulation of the inflammatory response.

We firstly explored the signature features of tendon- and ligament- derived cells as endogenous living agents with predictive tenogenic behavior, and the influence of cryopreservation on their functional behavior (**Chapter IV**). Subsequently, (**Chapter V**), we selected IL-1β, a powerful pro-inflammatory mediator, to artificially induce an inflammatory profile in human tendon-derived cells (hTDCs) in culture. With these studies, we established the exogenous supplementation of IL-1β as an *in vitro* inflammation model for tendon tissue which was used in further studies to assess PEMF role in controlling the inflammatory response of hTDCs (**Chapter V**), envisioning the design of non-invasive and remote-actuated systems to modulate pathophysiological responses. Macrophages have a pivotal role in healing and in the orchestration of the inflammation process. As macrophage communication with hTDCs may provide insights in tendon repair mechanisms, co-cultures of IL-1β-treated-hTDCs and macrophages were further investigated in **Chapter VI**. Taking advantage of a potential synergistic effect of PEMF action over magnetic nanoparticles, the response of IL-1β-treated-hTDCs was further assessed in different magnetic based strategies envisioning translational approaches. These included magnetic cell sheet constructions (**Chapter VII**), which provide more complex and dynamic cell to cell and cell-matrix interactions, and magnetic responsive polymeric membranes (**Chapter VIII**) to externally trigger and guide tendon derived cell responses. The achievements from this Thesis support the emergent role of magnetic based strategies in general and of PEMF in particular, in the modulation of tendon cell responses, antagonizing the effect of inflammatory mediators and consequently, stimulating tendon regeneration.

Keywords: Inflammation, Magnetic actuation, Tissue Regeneration

Estímulo magnético e materiais magnéticamente responsivos para modular a inflamação e o seu impacto no comportamento cellular para a regeneração do tendão

RESUMO

As patologias de tendão constituem uma causa significativa de incapacidade numa população cada vez mais ativa e envelhecida. Há uma lacuna substancial na compreensão das respostas moleculares e celulares que despoletam e/ou permitem a evolução das condições tendinopáticas, embora estudos recentes sugiram que a presença de eventos inflamatórios persistentes contribua para o avanço das lesões tendinosas e, portanto, podem auxiliar em novas estratégias terapêuticas. Nesta tese, investigamos a influência de estímulos magnéticos na resposta celular, aplicando um campo eletromagnético pulsado (CEMP) ou um CEMP combinado com materiais magneticamente responsivos usando abordagens baseadas em células estaminais para orientar e estimular a reparação do tendão.

Explorámos inicialmente as características das células derivadas de tendões e ligamentos como agentes endógenos com comportamento tenogénico preditivo e a influência da criopreservação na manutenção do seu fenótipo no **Capítulo IV**. Em seguida, no Capítulo V, selecionamos um mediador pro-inflamatório, IL-1β para induzir um perfil inflamatório, *in vitro*, em células derivadas de tendão humano (hCDTs). Estabelecemos a suplementação exógena de IL-1β como um modelo de inflamação in vitro para abordagens de tendão que foi posteriormente usado para avaliar o papel do CEMP no controlo da resposta inflamatória de hCDTs (**Capítulo V**), prevendo a utilização de métodos não invasivos e sistemas remotamente ativados para modular as respostas fisiopatológicas. Os macrófagos têm um papel fundamental na cicatrização e na orquestração do processo inflamatório. A comunicação de macrófagos com hCDTs pode fornecer informações sobre os mecanismos de reparação do tendão, pelo que co-culturas de hCDTs tratadas com IL-1β e macrófagos foram investigadas no **Capítulo VI**. Tirando vantagem de um efeito combinado do CEMP sobre nanopartículas magnéticas, a resposta de hCDTs tratadas com IL-1β foi ainda avaliada baseada em diferentes estratégias magnéticas que preveem abordagens translacionais. Estes incluíram matrizes celulares magnéticas (**Capítulo VII**), que fornecem interações célula-célula e célula-matriz mais complexas, e membranas poliméricas magnéticas (**Capítulo VIII**) para guiar as repostas celulares de células derivadas de tendão. Os resultados desta tese fornecem informações sobre o papel emergente das estratégias magnéticas em geral e do CEMP em particular, na modulação das respostas celulares do tendão, antagonizando o efeito dos mediadores inflamatórios e, consequentemente, estimulando a regeneração do tendão.

Palavras-chave: Estímulo magnético, Inflamação, Regeneração de tecidos

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LIST OF ABBREVIATIONS

A

A/A – Antibiotic/antimycotic

Abs – Absorvance

ABTS – 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)

ACL – Anterior cruciate ligament

ADAMTS5 - A disintegrin and metalloproteinase with thrombospondin motif

ADSCs – Adipose derived stem cells

AKT – Protein B

ANOVA – Analysis of variance

APC – Allophycocyanin

Arg-1 – Arginase 1

Arg-Gly-Asp (RGD) – Arginylglycylaspartic acid

B

Bax – Bcl-2 associated X-protein

bFGF – basic fibroblast growth factor

BGN – Byglican

BMSC – Bone marrow-derived mesenchymal stem cells

BSA – Bovine serum albumin

C

Calcein-AM – Calcein-Acetoxymethyl

$CaCl_2$ – Calcium chloride

CCL-2 – Chemokine (C-C motif) ligand 2

CCL-17 – Chemokine (C-C motif) ligand 17

CCL-24 – Chemokine (C-C motif) ligand 24

CD80 – Cluster of differentiation 80

CD169 – Cluster of differentiation 163

CL – Cruciate ligaments

c-Myc – Cellular myelocytomatosis

CO_2 – Carbon dioxide

COL1A1 – Collagen type I, alpha 1

COL3A1 – Collagen type III, alpha 1

COX-2 – Cyclooxygenase-2

CTGF – Connective tissue growth factor

CTRL – Control

Cx – Connexins

D

DAPI – 4',6-diamidino-2-phenylindole

DAMPs – Damage-associated molecular patterns

DCN – Decorin

DMEM – Dulbecco's modified eagle medium

DMSO – Dimethylsulfoxide

DNA - Deoxyribonucleic acid

dsDNA - Double-stranded DNA

2D – 2-Dimensional

3D – 3-Dimensional

E

ECM – Extracellular matrix

EDS - Energy dispersive spectrometer

EGF – Epidermal growth factor

ELISA – Enzyme-linked immunosorbent assay

ERK1/2 – Extracellular signal-regulated kinase 1/2

EVs – Extracellular vesicles

F

FACS – Fluorescence-activated cell sorting

FAK – Focal adhesion kinase

FBS – Fetal bovine serum

FDA - Food and Drug Administration

Fe – Iron

Fe_3O_4 – Iron oxide

FGF – Fibroblast growth factor

FITC – Fluorescein isothiocyanate

G

g – g force

G' – Storage modulus

G'' – Loss modulus

GAGs – Glycosaminoglycans

GDF-5 - Growth and differentiation factor 5

GFs – Growth factors

GTP – Guanosine triphosphate

H

h – hours

hASCs – Human adipose stem cells

hAFSCs – Human amniotic fluid stem cells

H&E – Hematoxylin and Eosin

HMGB1 – Intracellular high mobility group box 1

HMSO - Hexamethyldisiloxane

Hz – Hertz

H_2O_2 – Hydrogen peroxide

I

ICP – Inductively coupled plasma

IFM – Interfascicular matrix

IFNy – Interferon gamma

IGF-1 – Insulin-like growth factor 1

IκB – Inhibitors-of-kappa B

IL-1R1 – Interleukin 1 receptor type 1

IL-1β – Interleukin 1 beta

IL-4 – Interleukin 4

IL-4Rα – Interleukin 4 receptor alpha

IL-6 – Interleukin 6

IL-8 – Interleukin 8

IL-10 – Interleukin 10

IL-12 – Interleukin 12

IL-13 – Interleukin 13

IL-16 – Interleukin 16

IL-17 – Interleukin 17

iNOS-2 – Inducible nitric oxide synthase 2

J

JAK-STAT – Janus kinases/signal transducer and activator of transcription proteins

JNK – Jun N-terminal kinase

K

kDa – kiloDalton

L

LDCs – Ligament derived cells

LPS - Lipopolysaccharide

M

MAPK – Mitogen-activated protein kinase

magCSs – Magnetic cell sheets

MCP-1 – Monocyte chemoattractant protein-1

MEM – Minimum essential médium

MF – Magnetic field

Micro-BCA™ – Micro-bicinchoninic acid™

Micro-CT – Micro-computed tomography

Min – Minute

MMPs - Matrix metalloproteinases

MMP-1 – Matrix metalloproteinase 1

MMP-2 – Matrix metalloproteinase 2

MMP-3 – Matrix metalloproteinase 3

MMP-13 – Matrix metalloproteinase 13

MNPs – Magnetic nanoparticles

MRC-1 – C-type mannose receptor 1

MRI - Magnetic resonance imaging

mRNA – Messenger RNA

MSCs – Mesenchymal stem cells

MSC-EVs – Mesenchymal stem cell-derived extracellular vesicles

mT – Intensity

MTS – Tetrazolium

MRI - Magnetic resonance imaging

MKX – Mohawk

Mϕ – Macrophages

N

N/A – Not applicable, Not available

NF-κB – Nuclear factor kappa-beta

NLRP3 – Leucine-rich repeat containing protein-3

NO – Nitric oxide

O

Oct-4 – Octamer-binding transcription factor 4

P

PAMPs – Pathogen-associated molecular patterns

PBS – Phosphate-buffered saline

PCL - Poly(ε-caprolactone)

PCL – Posterior cruciate ligament

PDL - Periodontal ligament

PDGF – Platelet-derived growth factor

PE – Phycoerythrin

PEMF – Pulsed electromagnetic field

PGE_2 –Prostaglandin E2

PGs – Proteoglycans

PI – Propidium iodide

PI3K – Phosphatidylinositol 3-kinase

PT – Patellar tendon

R

Ras – Rat Sarcoma virus

RELMα – Resistin-like molecule alpha

Rho – Ras homologous

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RP – Rapid prototyping

RPM – Rotations per minute

RT – Room temperature

S

SCX - Scleraxis

SD – Standard deviation

SEM – Scanning electron microscopy

Singlec-1 – Sialic acid-binding Ig-like lectin 1

SMF – Static magnetic fields

Sox-2 – Sex determining region Y-box 2

SPCL – Starch poly(ε-caprolactone)

SSEA4 – Stage-specific embryonic antigen 4

STAT3 – Activator of transcription 3

SVF – Stromal vascular fraction

T

TDCs – Tendon-derived cells

TDSCs – Tendon-derived stem/progenitor cells

TE – Tissue engineering

TEM – Transmission electron microscopy

TERM – Tissue engineering and regenerative medicine

TGF-β1 – Transforming growth factor-beta 1

Th1 – T helper 1

Th2 – T helper 2

Thr – Threonine

TIMP-1 – Tissue inhibitor 1 of metalloproteinases

T/L – Tendon and ligament

TLR-4 – Tool-like receptor 4

TNC – Tenascin C

TNFα –Tumor necrosis factor alpha

TNMD – Tenomodulin

TSC – Tendon stem cells

TTE – Tendon tissue engineering

Tyr – Tyrosine

U

UV – Ultraviolet

(v/v) – Percentage of volume/volume

V

VEGF – Vascular endothelial growth factor

W

(w/v) – Percentage of weight/volume
%wt. – Percentage of weight

λ – Waveleng

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SHORT *CURRICULUM VITAE*

Carla Adriana Araújo Vinhas was born on the 17th of August 1992, in Vila Nova de Famalicão, Portugal. She is a PhD candidate enrolled in the Doctoral Program in Tissue Engineering, Regenerative Medicine and Stem Cells (TERM&SC) of the 3B's Research Group, I3Bs Research Institute on Biomaterials, Biodegradables and Biomimetics of the University of Minho.

She graduated in Biotechnology at Polytechnic Institute of Viana do Castelo – Escola Superior Agrária, Ponte de Lima, Portugal with a final grade of 15/20, in 2013. She obtained a Master degree in Applied Biochemistry with specialization area of Biomedicine at School of Sciences, University of Minho, Braga, Portugal. She developed her master thesis at the Institute of Biomedical Sciences of Abel Salazar (ICBAS), Porto, Portugal with a final grade of 17/20, in 2015. In her MSc thesis, she worked with rat cardiac myofibrobalsts for understanding the interaction between transporters, enzymes and purinergic receptors, which resulted in a peer reviewed publication. In September 2016, she was awarded with a PhD grant from the Doctoral Program in Tissue Engineering, Regenerative Medicine and Stem Cells (TERM&SC) at the 3B's Research Group, and started developing her PhD work under the supervision of Prof. Dr. Manuela E. Gomes and co-supervision of Dr. Márcia T. Rodrigues. Her PhD thesis focused on tendon tissue engineering strategies and explored the role of magnetic stimulation and magnetically responsive materials in tendon inflammation for development of new insights, under the scope of ERC CoG Project MagTendon.

Adriana Vinhas has been involved in a EC projects proposal preparation for a RIA Project 'InRegenera' and she was involved in the organization of 'PhDynamics', a Program created at the 3B's Group by the PhD students for science dissemination and 'soft' skills development.

As a result of her research work, she authored and co-authored 8 full length papers published in international journals (5 published, 3 submitted), 1 book chapter, 2 reviews paper. She participated in international conferences with 4 oral and 7 poster communications. She was awarded the Biology young investigator poster presentation in EORS 26th Annual Meeting, September 2018, held in Galway, Ireland.

LIST OF PUBLICATIONS

The work performed during the PhD period resulted in the publications listed below:

Papers in international Refereed Journals (as first author):

Vinhas A., Almeida A.F., Rodrigues M.T, Gomes M.E. Prospects of magnetically based approaches addressing inflammation in tendon tissues, submitted, 2020

Vinhas A., Gonçalves A.I, Rodrigues M.T, Gomes M.E. The impact of cryopreservation in signature and immunomodulatory profile of tendon and ligament derived cells, submitted, 2020

Vinhas A., Rodrigues M.T, Gonçalves A.I, Reis R.L, Gomes M.E. Pulsed Electromagnetic Field Modulates Tendon Cells Response in IL-1β-Conditioned Environment, Journal of Orthopaedic Research, 38, doi: 10.1002/jor.24538, 2019

Vinhas A., Almeida A.F., Gonçalves A.I, Rodrigues M.T, Gomes M.E. Magnetic Stimulation Drives Macrophage Polarization in Cell to-Cell Communication with IL-1β Primed Tendon Cells, International Journal of Molecular Sciences, 21(5441), doi: 10.3390/ijms21155441, 2020

Vinhas A., Gonçalves A.I, Rodrigues M.T, Gomes M.E. Human tendon derived cell sheets created by magnetic force-based tissue engineering holds tenogenic and immunomodulatory potential, submitted, 2020

Vinhas A., Rodrigues M.T, Gonçalves A.I, Reis R.L, Gomes M.E. Magnetic responsive materials modulate the inflammatory profile of IL-1β conditioned tendon cells, Acta Biomateriallia, doi.org/10.1016/j.actabio.2020.09.028, 2020

Book chapter (as first author):

Vinhas A., Rodrigues M.T., Gomes M.E. Exploring stem cells and inflammation in tendon repair and regeneration. Advances in Experimental Medicine and Biology Series – Cell Biology and Translational Medicine, Volume 2, pp 37-46, Kursad Turksen (eds), 2018

Conference oral presentations (as first author and as speaker):

Vinhas A., Gonçalves A.I., Rodrigues M.T., Reis L.R., Gomes, M.E. Pulsed electromagnetic field modulates the inflammatory environment induced by interleukin-1β on human tendon-derived cells, 26TH Annual Meeting of the European Orthopaedic Research Society, 25-28TH September, Galway–Ireland, 2018

Vinhas A., Gonçalves A.I., Rodrigues M.T., Reis L.R., Gomes, M.E. Magnetic actuated materials modulate inflammatory cues underlying human tendinopathy. TERMIS EU, 27-31ST May, Rhodes-Greece, 2019

Vinhas A., Rodrigues M.T., Gonçalves A.I., Reis L.R., Gomes, M.E. Tendon inflammation control through magnetic actuated approaches. First Achilles Conference, 8-10TH July, Porto-Portugal, 2019

Vinhas A., Gonçalves A.I., Rodrigues M.T., Reis L.R., Gomes, M.E. Magnetic actuation on human tendon cells exposed to inflammatory cues. 1st Discoveries Forum on Regenerative and Precision Medicine, 25-27TH September, Porto-Portugal, 2019

Poster presentations (as first author):

Vinhas A., Rodrigues M.T., Gonçalves A.I., Berdecka D., Reis L.R., Gomes M.E. Influence of Pulsed Electromagnetic Field on human tendon-derived cells response using an induced model of inflammation, TermStem/Forecast, 13-17TH November, Porto–Portugal, 2017

Vinhas A., Rodrigues M.T., Gonçalves A.I., Berdecka D., Reis L.R., Gomes M.E. Evaluation of tenocyte response to magnetically actuated biomaterials in experimentally induced model of tendon inflammation, Chem2Nature Second School Conference, 5-9TH June, Porto–Portugal, 2017

Vinhas A., Gonçalves A.I., Rodrigues M.T., Reis L.R., Gomes M.E., Effect of pulsed electromagnetic actuated biomaterials in tendon cell behavior under inflammatory stress, Summer School Chem2Nature, 3-6TH June, Porto–Portugal, 2018

Vinhas A., Gonçalves A.I., Rodrigues M.T., Reis L.R., Gomes M.E. Effect of Pulsed electromagnetic actuated biomaterials in tendon cell Behavior under inflammatory stress, 26TH Annual Meeting of the European Orthopaedic Research Society, 25-28TH September, Galway–Ireland, 2018

Vinhas A., Gonçalves, A.I., Rodrigues M.T., Reis, R.L., Gomes M.E. Unveiling the role of pulsed electromagnetic field on human tendon-derived cells response in an IL-1β inflammation model, 5th TERMIS World Congress, 4-7TH September, Kyoto – Japan, 2018

Vinhas A., Rodrigues M.T., Gonçalves A.I., Reis L.R., Gomes, M.E. The interplay between macrophages and tendon cells under magnetic actuation: insights on intercellular communication envisioning tendon regenerative approaches. Second Achilles conference, 4-5TH November, Braga-Portugal, 2019

Vinhas A., Rodrigues M.T., Gonçalves, A.I., Reis, R.L., Gomes M.E. The interplay between macrophages and tendon cells in an induced model of inflammation, EORS 2019, 2-4TH October, Maastrich, Netherlands, 2019

Poster presentations (as co-author):

Almeida A.F., **Vinhas A.,** Gonçalves, A.I., Miranda M.S., Rodrigues M.T., Gomes M.E. Development of magnetically responsive cell-instructive systems to target inflammatory cues in tendinopathy. First Achilles Conference, 8-10TH July, Porto-Portugal.

Almeida A.F., **Vinhas A.,** Gonçalves, A.I., Miranda M.S., Rodrigues M.T., Gomes M.E. Tendon regeneration strategies using magnetic responsive cell-instructive systems to target inflammatory cues. 1st Discoveries Forum on Regenerative and Precision Medicine, 25-27TH September, Porto-Portugal.

Almeida A.F., Miranda M.S., **Vinhas A.,** Gonçalves, A.I., Rodrigues M.T., Gomes M.E. Pursuing inflammatory cues in tendinopathy using a magnetically responsive cell-instructive system. Second Achilles conference, 4-5TH November, Braga-Portugal.

Other collaborative works:

Papers in international scientific journals with referees (as co-author):

Almeida A.F., **Vinhas A.,** Gonçalves, A.I., Miranda M.S., Rodrigues M.T., Gomes M.E. Magnetic triggers in biomedical applications – prospects for contact free cell sensing and guidance. Journal of Materials Chemistry B, doi:10.1039/d0tb02474k, 2020

Awards

Biology Young Investigator Poster Presentation "Effect of Pulsed Electromagnetic Actuated Biomaterials in Tendon Cell Behavior Under Inflammatory Stress" as part of the EORS 26th Annual Meeting, 25-28TH September, Galway, Ireland, 2018.

Contest CHEM2BRAIN organized as part of the CHEM2NATURE Summer School, 03-06TH June, Porto, Portugal, 2018.

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"*Persistence is the shortest path to success*"

Charles Chaplin

SECTION 1

GENERAL INTRODUCTION

Chapter I

Prospects of magnetically based approaches addressing inflammation in tendon tissues

Chapter I

Prospects of magnetically based approaches addressing inflammation in tendon tissues

ABSTRACT

Tendon afflictions constitute a significant share of musculoskeletal diseases and a primary cause of incapacity worldwide. Unresolved/chronic inflammatory states have been associated to tendinopathic conditions contributing to undesirable immune-stimulation and detrimental effects on tissues. The dual contributions of inflammation and inflammatory signals for pathophysiological processes may assist important developments and innovative solutions to address impaired healing and tendon regeneration. This review overviews the impact of inflammation and inflammatory regulators in tendon niches, unveiling the importance of tendon cell populations and their signature features, and the influence of microenvironmental factors to inflamed and injured tendons. The demand for non-invasive instructive strategies to manage persistent inflammatory mediators, guide inflammatory pathways and modulate cellular responses will be also approached exploring the role of pulsed electromagnetic field (PEMF). PEMF alone or combined with more sophisticated systems triggered by magnetic fields will be pondered aiming at designing successful therapies to control inflammation in tendinopathic conditions.

This chapter is based on the following publication: ***Vinhas A.,*** *Almeida A.F., Rodrigues M.T., Gomes M.E. Prospects of magnetically based approaches addressing inflammation in tendon tissues, submitted*

I-1. THE PROBLEMATIC BEHIND TENDON HEALING

Tendon tissues have a limited intrinsic healing potential and injuries to these tissues can lead to debilitating conditions with poor clinical outcomes in the long term. Together with age, gender, lifestyle and exercise levels, changes in cellularity and in the extracellular matrix (ECM) activity may contribute to advancing tendon injuries and disorders. Clinical treatments are often lengthy, expensive and insufficient to provide functional and permanent solutions to an increasingly active ageing population. The clinical, social and economic impact to overcome tendon lesions and impaired regeneration inspires the understanding of tendon biology and advanced approaches to stimulate the formation of new functional tendon, enabling to regain tissue biomechanics to a pre-injury state.

Tendinopathies refer to an umbrella term to characterize a multifactorial spectrum of tendon disorders usually associated with swelling, prolonged pain, overuse activities and impaired performance. Tendinopathies are common, accounting for up to 30% of general practice musculoskeletal consultations [1], and can ultimately lead to the loss of tissue integrity with full or partial rupture of the tendon [2]. The pathological features of tendinopathy include collagen degeneration, fibers thinning and disorganization compared with normal tendon, abnormal amount of collagen type III towards collagen type I, high content of glycosaminoglycans (GAGs) and proteoglycans (PGs) [3] and hypercellularity with more prominent and numerous rounded nuclei instead of spindle shaped tenocytes [4]. The failure of matrix adaptation results in a weaker tendon prone to future injuries.

Advances in gene and protein analysis have shown that the changes in the tendinopathic matrix composition are in part mediated by inflammatory cues [5]. Increasing evidence reports the process of inflammation as a common link between proper healing and pathological states yet the basis for this connection is not fully understood. Inflammatory processes have been observed both in early stages as well as in established tendinopathies being widely accepted that “molecular inflammation” plays a critical role in the pathogenesis of tendinopathy [1].

Since age-related diseases have been linked to low chronic inflammation states and impaired healing, therapeutic approaches targeting inflammation microenvironments could prevent and manage tendon disorders with substantial benefits for the elderly population.

I-2. TENDON NICHE

Tendon tissues are characterized by a dense ECM with a unique hierarchical organization essential for the tendon biomechanical performance.

Tendon ECM is mainly composed of tightly bundled collagen fibers, predominantly collagen type I. Collagen molecules are hierarchically arranged into fibrils, fibers, fiber bundles and fascicles that confer the biomechanical strength of tendon [6]. The collagen type III is also present in the matrix and is essential for normal collagen fibrillogenesis, being responsible for regulating the size of collagen type I fibrils. Collagen type III together with collagen type I provides mechanical strength to the newly synthesized matrix [7] while the collagen V provides a template for fibrillogenesis. In addition to collagens, PGs and GAGs such as fibromodulin and chondroitin sulfate, respectively, are present in this matrix and provide viscoelastic properties [8], which are essential for physiological biomechanics. The tendon unit and tendon sub-units are surrounded with loose connective sheaths, the epitenon and the endotenon, respectively. Moreover, tendons which bend sharply around joints (e.g. Achilles) have a sheath called the paratenon that reduces friction allowing free tendon movement against surrounding tissues [2], and sustains the vascular network that penetrates the endotenon and epitenon. Taken together, the sheaths ensure vascular, lymphatic and nerve supply.

Tendon ECM is synthesized and maintained by a sparse population of resident cells; the tenoblast/tenocyte and provides a physical microenvironment with an intricate network of biochemical and biomechanical cues [9, 10]. As cell-ECM communication is dynamic, the activities of local cells are also regulated by the ECM constituents [11]. The interactions between resident cells and their biological context define cell fate and function, and are of critical importance for assuring tendon functional competence and structural integrity.

Cell niches constitute specialized microenvironments that vary according to their anatomic location and regulate cell biology within tissues and organs. Tendons are relatively hypocellular tissues composed by a mixed population of tenocytes, and tendon-derived stem/progenitor cells [12], with contributions of other cell types as immune, vascular, synovial and non-resident mesenchymal stem cells [13].

Tenoblasts/tenocytes are organized in longitudinal rows between collagen fibers, and as mentioned before, are responsible for the formation and turnover of the ECM. Despite their role in establishing a complex network of cytoplasmic processes that link adjacent cells via gap-junctions, they have a limited

mitotic activity in adult and aged individuals, which may compromise the healing process after injury, especially in later stages of life.

Different tenocyte populations were identified with distinct biological responses. Tenocytes located within fascicles and in the interfascicular matrix (IFM), which surrounds endotenon, are more active [2, 12], and the populations of IFM proliferate more quickly than tenocytes located in fascicles, and have a higher turnover of ECM [14]. Tenocytes commonly express tendon markers, such as scleraxis (SCX), a basic helix-loop-helix transcription factor, tenomodulin (TNMD), a type II transmembrane protein, and collagen type I. It has been demonstrated that SCX is expressed in mature tendons of the limb and trunk, and in their progenitor cell population [15]. SCX is required for the proper embryonic development of tendons, and is the earliest detectable marker for differentiated tendon cells [16].

A population of tendon-derived stem/progenitor cells (TDSCs) share the microenvironment with tenocytes with an important role in the tissue renewal and repair [17]. TDSCs exhibit a classical adult mesenchymal stem cell profile with self-renewal capability, clonogenicity and differentiation into osteoblasts, chondrocytes, tenocytes, myocytes or adipocytes [2, 18, 19]. Interestingly, no specific marker has been identified to the date that enables to distinguish TDSCs from mature tenocytes or from other stem/progenitor cells. TDSC populations share with tenocytes high amounts of SCX [20] as well as Mohawk (MKX) and Tenascin C (TNC) during tendon maturation phase [21]. TDSCs also express TNMD [12], which is positively regulated by SCX, and often reported as a specific marker of mature tenocytes and ligamentocytes [22]. Other tendon associated molecules can be found in both TDSCs and tenocytes as collagen type I or collagen type III.

Recently, studies demonstrated that Nestin, a type IV intermediate filament protein, was highly expressed in TDSCs isolated from human Achilles in comparison to mature tenocytes, suggesting Nestin as a promising candidate marker for TDSCs [23]. Also, TDSCs were positive for nuclear proteins, such as octamer-binding transcription factor 4 (Oct-4), Nanog, nucleostemin, stage-specific embryonic antigen 4 (SSEA-4), cellular myelocytomatosis (c-myc) and sex determination region Y-box 2 (Sox-2), that are known pluripotency-inducing factors [24, 25].

Different TDSC populations were identified in tendon proper and tendon sheath [26]. Published works reported that stem cell populations from tendon proper hold increased potential for the regeneration of tendon structure, nevertheless stem cells from peritendon hold higher expression of progenitor cell markers [27] and can reinforce the expression of tendon markers in tendon proper stem

cells and tenocytes [28]. Endothelial-, perivascular-, smooth muscle-, synovial, neuronal, fat and immune cells (e.g. macrophages) [8, 29, 30] may also be present in paratenon, and are likely to participate in tendon extrinsic healing (Figure I-1). Despite the multiple (stem) cell subsets identified in tendon tissues and their plasticity, the involvement of these populations in tendon (neo)formation, homeostasis, adaptation to loading, and healing is largely undefined, posing barriers to the development of novel regenerative solutions.

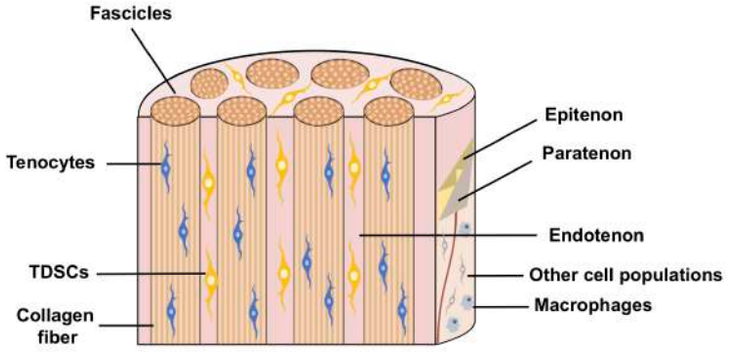

Figure I-1 Schematic representation of tendon tissues and localization of the different cell populations influencing tendon healing. Tenocytes align in between collagen fibers which are organized into structural units of increased complexity (fascicles). Endotenon and epitenon constitute non-tenogenic components of tendon tissues responsible for the vascular, neural and lymphatic supply. Paratenon and epitenon also constitute sources of stem cells and other cell populations involved in extrinsic healing of tendons, which relies in the migration of cells from the surrounding tissues.

I-3. INFLAMMATION IN TENDON REPAIR

Tendon regenerative approaches have been challenged by the still limited understanding of the multiple factors governing cellular, mechanical, and biochemical networks shared by tendon homeostasis, repair and pathologic conditions.

Inflammation is a complex process described in normal physiology and in disease pathology suggesting a tightly controlled regulatory mechanism in healthy tissues [1].

In healthy tendons, inflammatory mediators are detected after sudden exposure to mechanical stresses or overload and are necessary to preserve the tissue integrity. Studies on human tendon fibroblasts isolated from healthy human patellar tendons were shown to produce transforming growth factor-beta (TGF-β), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) after stretching the tissue at 1 hertz (Hz), 5% amplitude for 15 min and 60 min [31]. However, a prolonged exposure to these cues may compromise tendon homeostasis and advance pathological conditions.

Due to tendon limited intrinsic ability to heal, damage on the tendon structure impacts the biomechanical environment of tendon cells, and the cell mediated mechanisms leading to ineffective repair, increasing the risk of re-injury [32]. The self-recovery of tendon involves a complex and coordinated series of events.

The tendon healing starts with monocytes migrating into tissues and subsequently differentiating into macrophages, which hold a central position in the immediate response to tissue damage. Tenocytes participate in inflammation, the first phase of healing, synthesizing tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), prostaglandin E2 (PGE_2) and interleukin 10 (IL-10) that contribute to macrophage infiltration into the damage tendon [33].

Macrophages respond to inflammatory signals with the secretion of inflammatory cytokines, production of free radicals and enhanced phagocytosis of apoptotic cells [34]. Macrophages can switch into a M1 or M2 functional phenotypes. M1 macrophages are pro-inflammatory cells with anti-microbial activities that promote T helper (Th1) cell responses. M1-phenotype is common in the early inflammatory process (1-5 days after injury) [35] contributing with the release of several pro-inflammatory cytokines and mediators such as IL-1β, IL-6, TNFα, interleukin 12 (IL-12), cyclooxygenase-2 (COX-2) and reactive nitrogen and oxygen species [13, 36]. Furthermore, M1 can promote ECM breakdown, apoptosis and phagocytosis [37].

After the early inflammatory phase subsides, macrophage population assumes predominantly a M2 phenotype, responsible for the attenuation of inflammation and the beginning of a cell proliferation and tissue deposition phases [36]. M2 macrophages are immunosuppressive cells that support T helper 2 (Th2)-associated effector functions [34] and appear in later stages (4-10 days) of healing[35, 38].

M2 macrophages secrete a variety of immunoregulatory proteins such as IL-10, TGF-β1, resistin-like molecule alpha (RELMα) and arginase 1 (Arg-1) to decrease the magnitude and duration of inflammatory responses [34, 36, 37]. Specifically, Arg-1 was previously linked to tissue regeneration [39],metabolizing arginine into ornithine which in turn is a precursor of prolines and polyamines, promoters of collagen synthesis and cell proliferation, respectively [40, 41]. Moreover, M2 macrophages produce numerous growth factors (i.e. PDGF, TGF-β1, insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF) associated to cellular proliferation and angiogenesis and release soluble mediators that recruit and stimulate fibroblasts to synthetize ECM components [42].

The critical importance of M2 macrophages in mediating the healing process has been demonstrated in many other studies. For instance, abrogation of interleukin 4 receptor alpha (IL-4Rα) signalling in macrophages impaired wound repair in an *in vitro* model of wound healing [43]. While a deficient M2 macrophage response leads to impaired wound closure, excessive M2 macrophage activation promotes fibrotic tissue formation. Interleukin 13 (IL-13) can drive to pathologic fibrosis through excessive M2 activation. In models of helminth-induced fibrosis, the IL-13 driven inflammation and fibrosis were alleviated with depletion of Th2-associated macrophages [44].

The balance between M1/M2 type polarization clearly influences the final repair outcome, thus, understanding the transition of phenotypes and the regulation of inflammatory events that hinders the endogenous response to damage may contribute to new regenerative tools to manage and treat tendon pathologies.

I-4. CONTRIBUTIONS OF MICROENVIRONMENTAL CUES TO TENDON REGENERATION

I-4.1. Soluble factors mediating pro-inflammatory cues

I-4.1.1. Cytokines

Despite the importance of inflammation for the optimal healing persistent inflammatory cues are related to poor regeneration. For example, IL-1β can induce human tenocytes to produce inflammatory mediators such as COX-2, PGE_2 and matrix metalloproteinase-1 (MMP-1) which in turn accelerate the degradation of ECM thus, negatively affecting tendon mechanical properties [45]. Similarly, TNFα can strongly stimulate tenocytes to amplify their own TNFα and produce other pro-inflammatory molecules, such as IL-1β and IL-6 which subsequently inhibit ECM formation [46].

Recent studies suggest that tenocytes influence the macrophage polarization during the early phase of inflammation via paracrine communication [47, 48]. Hetero cultures of macrophages with tenocytes pre-stimulated with the pro-inflammatory cytokines TNFα, interferon gamma (IFNy) and IL-6 increased the expression of cluster of differentiation 80 (CD80) in macrophage and enhanced the release of IL-6, interleukin 8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) [47]. The fact that co-cultured cells released higher levels of pro-inflammatory cytokines than monocultures of tenocytes suggests a molecular

communication between these cell types, and shows that tenocytes respond to inflammatory environments *in vitro* adjusting the cytokines' profile and surface markers of both cell types, influencing the macrophage polarization. Indeed, a co-culture assay of human dermal fibroblasts with alternatively activated human macrophages demonstrated that the later cells stimulated fibroblasts proliferation with consequent influence upon the production of collagen and alpha smooth muscle actin synthesis [49]. Such networking is also observed with macrophages and tendon neighbouring cell populations and may also contribute to the healing response. The interplay between TNFα-stimulated synovial fibroblasts and macrophages suppressed the macrophage production of interferon beta. Conditioning the co-cultures with TNFα modulated the expression of Janus kinase/signal transducers and activators of transcription (JAK-STAT) signaling pathway necessary for macrophage polarization [50].

I-4.1.2. Extracellular Vesicles

The biomodulation of cells and biochemical cues to regulate microenvironmental stimuli offer attractive possibilities for scar-free healing and therapeutic strategies in mature tendons.

The mesenchymal stem cell (MSC)-derived extracellular vesicles (MSC-EVs) are enriched with bioactive molecules such as nucleic acids (e.g. mi/si/mRNA)), and immunomodulatory factors that precisely contribute to immunomodulation responses and to the regulation of phenotype and function during tissue repair [51]. Chamberlain et al., demonstrated that fibrin with EVs isolated from MSC conditioned media reduced a M1/M2 ratio and increased the number of endothelial cells 14 days upon injury in an Achilles model after unilateral surgical transection. In this study, the tendons treated with fibrin containing MSC-derived EVs demonstrated alignment and compact collagen fibers and higher expression of tendon related markers, Collagen type I, SCX and TNMD in comparison to control groups. Also, MSC-derived EVs were shown to successfully educate macrophages into a M2-like phenotype, modulating local inflammatory response and, thus accelerating tendon healing [52]. An intraperitoneal injection of EVs isolated from bone marrow-derived MSCs (BMSC-EVs) was reported to improve tendon repair in a rat patellar injury. The administration of BMSC-EVs decreased the number of C-C motif chemokine receptor 7 (CCR7+) cells and the levels of inflammatory cytokines IFNy, IL-1β and IL-6. Moreover, BMSC-EVs led to an increase in the number of cluster of differentiation 163 (CD163+) macrophages and higher expression of the M2 genes, interleukin 4 (IL-4) and IL-10 [53].

The role of MSC-EVs in cellular communication has also been approached in other musculoskeletal tissues and inflammatory diseases, supporting the relevance of intercellular signaling for effective regenerative responses. MSC-derived exosomes isolated from murine bone marrow protected chondrocytes from apoptosis, and blocked macrophage activation in an osteoarthritis model, increasing the expression of collagen type II and aggrecan, and reducing the values of metalloproteinase-13 (MMP-13), of A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5), and of inducible nitric oxide synthase (iNOS) [54]. Furthermore, mice with ulcerative colitis showed an increase of IL-10 and TGF-β levels and a reduction in IFNy, IL-12, TNFα, chemokine (C-C motif) ligand 24 (CCL24), chemokine (C-C motif) ligand 17 (CCL17) levels, after MSC-EVs-treatment [55].

More recently, exosomes were isolated from tendon stem cells (TSC-Exos) and their therapeutic effects evaluated *in vitro* and *in vivo* in an Achilles tendon injury. TSC-Exos promoted the proliferation and migration of tenocytes *in vitro*, which relied on the activation of the phosphatidylinositol 3-kinase (PI3K)/protein B (AKT) and mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathways. *In vivo*, TSC-Exos were mixed in gelatin methacryloyl and placed in the Achilles defect. The mixture was converted to the gel state by irradiation for 10-20 s, with a blue light. Two and eight weeks after *in situ* administration, tendon treated with TSC-Exos demonstrated a continuous and regular arrangement of ECM in comparison with control groups, that exhibited a disorganized ECM, suggesting that TSC-Exos could assist the healing of injured tendons [56].

In sum, these studies highlight the therapeutic potential of soluble mediators, regulating the paracrine communication between cell populations which may provide important information to elucidate the mechanisms driving immunomodulatory responses and its impact in tendon regenerative approaches.

I-4.2. Cell to cell communication to inflammatory mediators

Cell-cell junctions are functional units for the correct and efficient flow of information in multiple cell processes including adhesion, communication, and differentiation of cells. These complexes are able to generate surface polarity which permits cells to sense and respond to their local environment.

In tendon tissues, cells are coupled via connexins Cx32, and Cx43. Connexins integrate gap-junctions, which are membrane channels that function as conduits between the cytoplasm of contiguous cells. They enable the direct commute of ions, nucleotides, small molecules and second messengers between neighboring cells [57] working as active social networks. The involvement of gap-junctions in the

communication of inflammatory signals and in tendon regeneration it is not fully elucidated. Experimental studies with rabbit Achilles tendon cells showed that Cx43-mediated gap junctional intercellular communication may be important in the broadcasting of inflammatory markers following intense exercise and body temperature increase [58]. In this study Cx43 was not affected by a heat treatment but the inhibition of gap-junctions with B-glycyrrhetinic acid led to an increase in the expression of MMP-1, IL-1β, and IL-6. Moreover, when Cx43 was overexpressed in heat stress, lower levels of Collagen type I, IL-1β, MMP-1 and IL-6 were observed.

The permeability of gap junctions between tenocytes is modified in response to mechanical loading. Maeda et al., showed that Achilles tenocytes mechanically stimulated with 4% strain for 1 h increased the expression of Cx43 and localization at the cell membrane. Interestingly, when a 8% strain was applied to the cells Cx43 expression diminished but did not affect Cx43 localization [59].

The time of mechanical loading also influences tenocyte communication. A 10 min loading did not affect gap-junction communication, while loading for 1 h significantly reduced intercellular gap-junction permeability, thus inhibiting cell networks.

The regulation of collagen production in tendon matrix may also rely on gap-junction mechanisms. In chick tenocytes, the inhibition of Cx43 resulted in a significant production of collagen type I mRNA while the use of non-specific inhibitors of gap-junctions (e.g. octanol) suppressed the collagen synthesis [60]. However, under mechanical loading Cx32 stimulates collagen synthesis in tenocyte monolayers [61], suggesting a strong interaction between collagen, loading and gap junctions in tendons.

I-4.3. The dynamic interplay of Cell-ECM

The properties of tendon ECM are adjusted during the stages of healing in response to the disorganization of tendon matrix upon injury, represented by discontinuous, crimped and thinned collagen fibers. Remodelling events or persistent abnormal triggers will induce changes in the signaling potential of the matrix and of the ECM components, contributing to abnormal cell responses and persistent impaired functionality (e.g. excessive tissue remodelling) until balance is re-established. An extensively damaged ECM structure has been related to autophagic and to apoptotic cell death events in degenerative rotator cuff tendons [62]. Moreover, the loss of matrix integrity is accompanied by a reduction of total collagen and increased production of PGs and GAGs, resulting in tendon stiffening [8].

Matrix stiffness plays an important role in the regulation of proliferation and differentiation processes in stem cell niches, including the ones associated to BMSCs[63] and adipose-derived stem cells (ADSCs) [64]. In TDSCs, the proliferation was shown to increase with increasing matrix stiffness, despite the fact that TDSCs differentiation into tenogenic, chondrogenic, and osteogenic lineages was inhibited through focal adhesion kinase (FAK)-ERK1/2 activation [65]. Interestingly, it is also described in the literature that tendinopathic tissues have a significantly decreased stiffness in comparison with healthy tissues [66]. On the other hand, matrix softness seems to maximize the ability of MSCs to produce paracrine factors that are implicated in monocyte production and chemotaxis upon inflammatory stimulation by TNFα [67]. In a clinical study with athletes suffering from patellar tendinopathy, reduced stiffness of the tendon correlated to better clinical outcomes. Lower stiffness implies a more extensible tendon which may facilitate the tendon's capacity to mechanically buffer the force transmitted through the tendon body [68].

ECM degradation products formed from collagen, fibronectin, hyaluronic acid and biglycans are potential damage-associated molecular patterns (DAMPs) that upregulate pro-inflammatory mediators like IL-1β, TNFα and IL-6 through NF-κB activation [69]. The ECM-derived DAMPs are responsible for pro-inflammatory features, however depending on the pathway involved, DAMPs can also exert a modulatory action over the production or activity of inflammatory mediators as TGF-β and IL-1β. Such mechanisms also enable to regulate the function of immune cells that respond to these bioactive factors.

Another important aspect of ECM degradation is the abnormal cell communication due to the misalignment of the fibers, scar tissue or to a complete interruption of cell networks in the case of the most severe injuries. In a study performed by Schoenenberger et al., it was reported that disorganized fiber surfaces can prime tendon cells toward pro-inflammatory signaling. This hypothesis was supported by the fact that MMPs activation via inflammatory cytokines was more significant in tendon fibroblasts in contact to biomaterials with a misaligned fiber topography. Such scaffold topographies mimic the architecture of the tendinopathic ECM, and it is plausible that matrix organization plays a vital role in the perpetuation of tissue inflammation [38]. Accordingly, Thankam et al., associated ECM disorganization and inflammation with intracellular high mobility group box 1 (HMGB1) upregulation and leucine-rich repeat containing protein-3 (NLRP3) inflammasome activation in rotator cuff tendon injuries [69].

Exploring cell-matrix interactions is complex as the relationships established are dynamic and cyclically linked (Figure II-2).

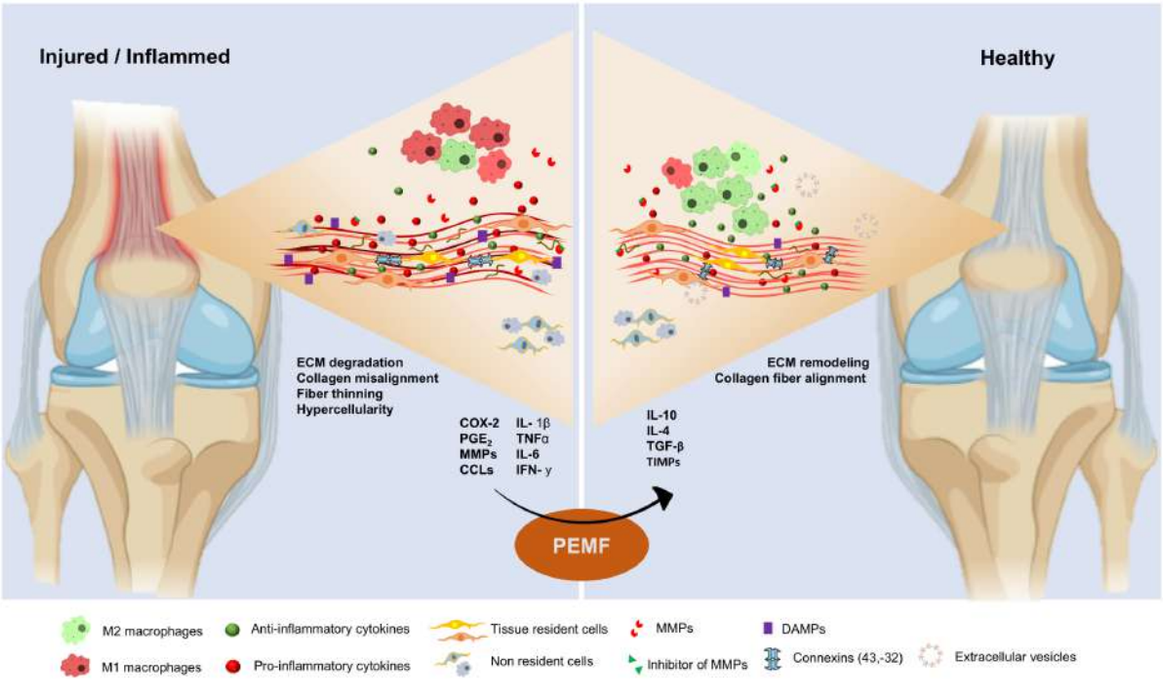

Figure I-2 Molecular and cellular interplay in injured/inflamed versus healthy tendons. The scheme represents the regulation of cell populations and microenvironmental cues through juxtacrine and paracrine communication in tendon niche. The overall balance will dictate a favorable or detrimental influence towards tendon repair mechanisms.

However, the interplay between ECM dynamics, immune cells and mechanical loading during tendon healing is only just starting to develop. It is unquestionable its importance to acknowledge the most relevant players and establish spatiotemporal stages during tissue remodelling and neotendon assemble to unveil the mechanisms behind tendon healing and to boost the development of improved regenerative tools for tendinopathy therapies.

I-5. INVESTIGATING INFLAMMATORY PATHWAYS FOR DESIGNING SUCCESSFUL THERAPIES

Inflammatory pathways have a pivotal molecular basis in the pathogenesis of many chronic diseases. The process begins with an initial stimulus (e.g. injury) that surface pattern receptors recognize and inflammatory pathways are triggered. The pathways can either regulate inflammatory mediators' levels in tissue resident cells as well as stimulate inflammatory cells recruitment.

Cytokines are among the most studied molecules for the activation of pathways. For instance, IL-1β, a key inflammatory mediator, is released in response to pathogen-associated molecular patterns (PAMPs) and DAMPs. IL-1β occurs on a continuum which may contribute for exacerbated damage during chronic

disease and acute tissue injury. Although IL-1β is mainly associated to immune cells it is also produced by tenocytes [70]. IL-1β interaction with its cognate receptor IL-1RI, can trigger important intracellular signaling pathways, including the nuclear factor kappa-B (NF-κB) and the MAPK pathways [71, 72].

In inflammatory niches, NF-κB typically responds to pro-inflammatory cytokines such as IL-1β and TNFα. In homeostasis NF-κB is bounded to inhibitors-of-kappa B (IκB) that keep the NF-κB pool mainly in the cytoplasm by inhibiting its nuclear localization and association with DNA. In case of activation (non-homeostasis), the bound between NF-κB and IκB is phosphorylated and consequently NF-κB dimers translocate to the nucleus, bind to the DNA and regulate the transcription of innumerous pro-inflammatory genes, including cytokines and chemokines genes [73, 74].

Increased NF-κB expression has been associated to early rotator cuff tendinopathy [75]. Tendon stromal cells isolated from patients with supraspinatus tendon disease revealed an increased expression of IFNy and NF-κB genes compared with tendon cells obtained from healthy controls [76]. In a recent work performed by us, the NF-κB activation by exogenous IL-1β supplementation to human tendon derived cells translated into an increase of IL-6, TNFα, IL-8 and COX-2 [77]. Also in human tenocytes, the suppression of IL-1β-induced activation of the NF-κB pathway resulted in increased collagen production and in a higher expression of TNMD and SCX, while the genetic expression of COX-2, matrix metalloproteinases (MMPs), Bax and caspase-3 were inhibited [78, 79]. The preclinical studies on a mouse rotator cuff model evidenced that NF-κB activation by IL-1β causes tendon degeneration, which is accompanied by an increase of IL-6 and chemokine (C-C motif) ligand 2 (CCL-2) [75].

The activation of NF-κB in inflammatory cells such as macrophages, induces the expression of TNFα, IL-1β and IL-6 cytokines and the c-Rel complexes. The c-Rel is important for scaling the inflammatory response based on transient/persistent tool-like receptor 4 (TLR-4) stimulation, which can prevent hyperinflammatory responses [80]. Additionally, Courtine et al., showed that mice lacking both c-Rel and p50 NF-κB proteins have impaired innate immune responses to sepsis, with deficient macrophage response for phagocytosis and bacterial killing [81].

The MAPK p38 signaling pathway is commonly activated by stressful or pro-inflammatory stimuli and plays an important role in the regulation of mechanical loading, and to the regeneration of tissue injury. The MAPK are a family of serine/threonine kinases that include the extracellular-signal-regulated kinase (ERK) 1/2, p38 MAPK and c-Jun N-terminal kinases (JNK). Activation of the MAPKs generates the phosphorylation and activation of p38 transcription factor in the cytoplasm or nucleus, initiating the

inflammatory response [82]. The MAPKs are often activated via phosphorylation or through interaction with small GTP-binding protein of the Ras/Rho family in response to external stimuli (for example, cytokines and growth factors). Kinases activation leads to the phosphorylation and activation of MAPK activity, through dual phosphorylation on threonine (Thr) and tyrosine (Tyr) residues located in the activation loop of kinase subdomain VIII. The phosphorylation of these residues is essential for enzymatic activities, originally demonstrated for ERK1/2 [83].

Inhibition of p38 MAPK in rats results in a significantly reduction of IL-6 in Achilles tendon and a modest decrease in the expression of Collagen type I and type III [84]. The inhibition of MAPK with SB203580 in a rat supraspinatus tear caused a reduction of IL-1β, IL-6 and COX-2, and an increase of IL-10 gene expression [85]. Likely, Miller et al., reported the activation of MAPK in human tendon-derived cells derived from hamstring tendon tissue subjected to hypoxic environment. When SB203580 was provided IL-6 and IL-8 expression diminished [86].

Dysregulation of NF-κB and MAPK activity has been associated with inflammatory, autoimmune and metabolic diseases. Pharmacological inhibitors of NF-κB and MAPK pathways have been used (e.g. SB203580) for the management of inflammatory diseases. The inhibitor of NF-κB repressed transcription of IL-6 and CCL-2 cytokines in human tendon fibroblasts [75]. Moreover, the inhibition of MAPK pathway in tendon cells results in a significantly reduction of pro-inflammatory molecular events in tendinopathy [86].

Therapies to assist the resolution of inflammatory events targeting key molecules associated to the activation of signaling pathways could lead to balanced inflammatory signals which may modulate the progression of tendon diseases and improve treatment outcomes.

I-6. MAGNETIC FORCE BASED STRATEGIES FOR INFLAMMATION REGULATION AND TENDON HEALING

I-6.1. Exploring magnetic fields in the modulation of pro-inflammatory microenvironments

Magneto-therapy has been mostly applied to rehabilitation treatments to reduce the pain and for the treatment of bone delayed union [87] and of osteoarthritis [88]. This therapeutic modality employs magnetic stimulation, which can be provided by static-, alternating- or pulsed electro- magnetic fields, to restore the cellular and biochemical balance of tissues. The magnetic field more frequently used for

medical treatments is the pulsed electromagnetic field (PEMF), which is generated by an alternating electric current traveling through a solenoid, and applied in pulses. Exploring magnetic stimulation under a cellular and molecular perspective would enable new non-invasive, contact-less and cost-effective approaches in comparison to current pharmacological or rehabilitation regimens. Short-term exposure to low frequency electromagnetic fields has been shown to influence cell dynamics [89-92] at the cell membrane level in the transport of proteins and on the flow of Ca^{2+}, Na^{2+}or K^{+} [93] as well as on the proliferation rate and protein synthesis [94], which could be further explored for improving ECM deposition and collagen fiber alignment during tendon healing [95, 96].

Magnetic fields are defined by several parameters including frequency and intensity. The intensity of a PEMF relies on the amount of charge to stimulate cells or tissues. The main challenge for magnetic guidance of cell processes resides in establishing the most optimal parameters for biological responsiveness; overstimulation may be detrimental resulting in apoptosis and cellular death while under stimulation may cause no-effect at all. Low-intensity (< 1 Gauss) and low frequency (<3,000 Hz) PEMF is suffice to interact with cells and tissues and to exert a beneficial effect in inflammation control. Girolamo et al., compared different dosages of PEMF (75 Hz), varying the field intensity (1.5 mT or 3 mT) and exposure time (4 h, 8 h, and 12 h) applied to healthy human tendon cells. These studies demonstrated that an exposure to a PEMF with 75Hz, 1.5 mT, and 0.1% duty cycle decreased the release of IL-1β, TNFα and IL-6 after 8 h and 12 h and increased IL-10 levels suggesting a modulatory behavior of PEMF over cytokine profiles [91, 92]. Concordant outcomes were observed in human fibroblast-like cells exposed to PEMF (50 Hz, 2.25 mT) for 15 min, evidencing a decrease in the IL-1β and TNFα after 14 and 21 days, and with IL-10 values increased on day 21 [97].

In a recent work by our group, different combinations of PEMF parameters were investigated in IL-1β conditioned tendon cells. Some combinations of PEMF parameters were more favourable for biological processes, namely 5 Hz, 4 mT and 50% duty cycle while others, 5 Hz or 17 Hz combined with 5 mT and 50% duty cycle induced lower metabolic activity and reduced cell viability rates. Supporting these works, a PEMF with 5 Hz, 4 mT and 50% duty cycle decreased IL-6, TNF, IL-8 and COX-2, and upregulated IL-10 and IL-4. Moreover, under magnetic stimulation, the gene expression of MMP-1,-2,-3 was reduced while a tissue inhibitor 1 of metalloproteinases (TIMP-1) levels increased [98]. Although it is not clear the impact of magnetic stimuli in cell responses from different tissues, studies with human annulus fibrosus cells conditioned to IL-1β evidenced an analogous modulatory effect of PEMF (3.85 Hz pulsed, 15 Hz

burst and maximum 10 T/s rate, 25% duty cycle), on the gene expression of the pro-inflammatory mediators IL-1α, IL-1β, interleukin 16 (IL-16), and interleukin 17 (IL-17) [99].

Another major challenge of magnetic stimulation relies on the standardization of magnetic parameters and of the selection of the magnetic field applied for comparable outcomes. Most of the studies are performed in custom designed systems which poses barriers to the reproducibility of the work, and consequently to advances in the understanding of magnetic effects in cellular structures and functionality. Despite these limitations, preclinical models support the modulatory effect of PEMF over inflammatory cues. In a work by Lee et al., a daily application of PEMF (17 Hz, 15 min/day, five sessions a week) led to a reduction of inflammation on Achilles tendonitis [100]. The effect of PEMF (3.846 kHz and 25% duty cycle) applied 4 hr/day was also reported on acute inflammation in a rat-tail intervertebral disc injury model. The inhibition of TNFα gene expression and of IL-6 and IL-1β occurred 7 days after PEMF stimulation [101]. These outcomes were also verified in the knee joint, where a 3 month PEMF therapy (75 Hz, 1.6 mT, 6 h/day) reduced the progression of osteoarthritis lesions [102].

Independently of the cell population and tissues investigated, it seems that PEMF stimulation holds a desirable effect over inflammatory mediators on pathological environments. Interestingly, PEMF (75 Hz, 1.3 ms pulsed for 24 h) not only modulated the cytokine production but the PEMF stimulation over adenosine receptors previously treated with IL-1β was associated to the inhibition of the NF-κB pathway in chondrocytes and hFOB 1.19 osteoblasts cells [103].

Overall, the potential for PEMF-based therapeutics is huge addressing inflammatory cues and assisting improved therapeutics of inflammation associated diseases. However, to reach translational relevance, it is imperative to establish therapeutic ranges and models to accelerate the scientific findings and provide advanced solutions with clinical impact.

I-6.2. Immunomodulatory effects of PEMF stimulation to macrophages

Magnetic fields were shown to control the production of cytokines by tissue resident cells. However, the predominant cytokine producers are the immune cells that hold plasticity and coordinated action in the articulation of multiple cell responses. Thus, insights on immune cell magnetic responsiveness, in particular on magnetic oriented strategies to macrophages, may assist the establishment of more effective

cell communication networks to prevent persistent detrimental signals and accelerate a pro-healing response.

Most of the studies report that whether macrophages are LPS stimulated or not, there is a decrease in the secretion of IL-6 and TNFα after PEMF stimulation, even when different PEMF conditions (15 Hz, 2 mT for 15 min or 5 Hz, 4 mT and 50% duty cycle) are applied [104, 105]. Moreover, particular PEMF conditions (5 Hz, 4 mT and 50% duty cycle) could increase IL-4 and IL-10 and affect macrophage polarization toward M2 by an upregulation of Arg-1, mannose receptor C-type 1 (MRC-1) and Sialic acid-binding Ig-like lectin 1 (Singlec-1) [105].

The control over cytokine production in macrophage populations may be related to a reduction in NF-κB activation induced by a continuous exposition of RAW264.7 macrophage-like cells to PEMF (75 Hz, 1.5 mT an 10% duty cycle). This study also showed that the phosphorylation of p38 was inhibited under the PEMF conditions and consequently the TNFα production by macrophages [106]. A study by Wosik et al., proposed that the morphological elongation of macrophages can be induced by a magnetic field [107], switching the upregulation of M2-specific genes as ARG-1 and decreasing the iNOS expression (M1) to undetectable levels. In a more complex approach Kang et al., developed a magnetic remotely controlled nanocage conjugated to a bioactive ligand arginylglycylaspartic acid (Arg-Gly-Asp (RGD) peptide) to regulate *in vivo* the adhesion and resultant polarization of macrophages to an implantable surface [108].

Although there are still questions to be addressed, especially considering the interactive and dynamic role of macrophages in tendon inflammation and healing, these works reinforce the potential effect of magnetic forces for promoting the resolution of inflammation and to provide precise cell oriented tools to guide tissue regeneration.

I-6.3. Magnetotherapy as non-conventional treatment and prospects of magnetic based systems

Magnetic field-based strategies are arising as a prospective, non-invasive, and safe physical therapy strategy to accelerate tissue repair. PEMF devices have been approved by the Food and Drug Administration (FDA) to treat non-union fractures and to treat post-operative pain and edema [109].

Our knowledge on magnetic fields and consequently on PEMF on biological processes is still scarce but the combination of low frequency, low intensity and suitable exposure time produce cell membrane

disturbances that lead to the activation of intracellular pathways. Interestingly, PEMF therapy demonstrated benefits to multiple tissues affected by different pathological conditions [110], suggesting the likely universal therapeutic applicability of PEMF in the human body, and demanding for a deeper understanding on the cell and tissue responsiveness to magnetic fields.

The mechanosensitive nature of tendon tissues may confer particular relevance for studying tendon cells in the presence of magnetic fields as external sources of mechano-magnetic stimulus through the activation of mechanosensitive complexes assisting tenogenic mechanisms. In this sense, PEMF approaches may be attractive to guide cell-mediated responses (e.g. bioactive molecules, ion channels, and secondary messengers) through contact-less instructive signals. More recently, PEMF has gained visibility in tissue engineering and regenerative medicine (TERM) strategies due to the increasing number of reports supporting tissue healing and regeneration as well as to the wide range of possibilities for designing sophisticated approaches combining PEMF with magnetic components, as magnetic nanoparticles. Such approaches enable the remote control over advanced magnetically responsive systems that can be designed to operate as smart delivery systems of bioactive molecules for controlling cell behavior [108, 111], to act as oriented triggers to precisely bind and activate cell receptors [112, 113] or intra-cellular molecules [114] or even to guide the maturation of the tissue engineered constructs in scaffolding strategies [115]. The combination of PEMF with magnetically responsive elements offers synergistic and complementary strategies to resolve persistent inflammatory cues and guide the healing process, holding valuable contributions for the repair of damage tissues and organs. Moreover, the promising role of PEMF on the different phases of healing in combination with magnetically responsive biomaterials can provide new prospects to exert a modulatory effect over the immune system and favour immunomodulatory responses to assist enhanced functional recovery of tissues.

I-7. CONCLUSIONS

Understanding the role of inflammation in injured tendons will support new opportunities to resolve inflammation, overcoming persistent inflammatory cues, and proceed with proper healing. While an inflammatory response is necessary for healing to occur, high levels of pro-inflammatory cytokines may result in collateral tissue damage and impaired tendon healing. These inflammatory mediators influence the composition and properties of tendon ECM, and may induce the phenotypic and functional changes of tendon cell populations. Magnetic stimulation may provide a relatively simple and contact-free solution

to modulate the inflammatory events with precise action at the cellular level influencing intracellular messengers and signalling pathways highlighting the promising future of magnetic based technologies in inflammatory associated conditions and for tendon regenerative approaches.

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Chapter II

Exploring stem cells and inflammation in tendon repair and regeneration

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ABSTRACT

Tendon injuries are frequent and are responsible for substantial morbidity both in sports and in the workplace. Despite the endogenous mechanisms of tendon repair and regeneration, tendon healing upon injury is slow and often insufficient to restore complete biomechanics functionality. Inflammation has a pivotal role in tendon healing and failed healing responses contribute to the progression of tendinopathies. However, the molecular and cellular mechanisms involved are poorly understood requiring further insights. During inflammation, bioactive molecules such as cytokines secreted locally at the injury site, influence resident stem cells that contribute as modulatory agents over the niche towards homeostasis, holding great promise as therapeutic agents for tendon pathological conditions associated to unresolved inflammation and failed healing. This review overviews the role of cytokines and resident cells, focusing on the participation of tendon stem cell population in inflammation and tendon healing upon injury and their potential action in resolution of pathological conditions.

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II-1. INTRODUCTION

II-1.1. Tendon Niche

Tendons are dense connective tissues that connect muscles to bone and transmit the mechanical forces generated during contraction to the skeleton, therefore essential for locomotion [1, 2]. Tendons are hypocellular tissues mainly composed of tenocyte, and a stem and progenitor cell population [3]. However, other cell types may be also present, for instance endothelial cells, mast cells, chondrocytes, synovial and vascular cells. Tenocytes are responsible for extracellular matrix (ECM) maintenance, which is mainly composed of collagen, in particular fibrillar collagens, namely collagen type I and III, although other types of collagen are also present, such as collagen type III, V, VI, XII, XIV and XV [4-6]. Tendon ECM is also composed of proteins and proteoglycans, such as decorin, biglycan, aggrecan and elastin [2, 5, 7-9].

Morphologically, tendons follow a hierarchical architecture of collagen molecules that gather to form collagen fibrils. These fibrils assemble into fibers to form collagen fiber bundles. Finally, the bundles organize into tendon fascicles. The presence and alignment of collagen fibers is oriented for providing resistance to tendon and increased tensile strength [7, 10-12], while reducing stress during muscle contraction [13].

In tendon milieu, tendon cells and the ECM coordinate actions in promoting damage repair and tissue regeneration. Since vascular supplies in tendon are confined to endotenon and epitenon, it is likely that stem cell recruiting through the vascular system may be restricted to the surrounding areas of these layers. Thus, the resident cell populations, stem cells and tenocytes, have a critical role in physiological homeostasis and regulation of the tendon matrix. When this delicate fine-tuned balance is disturbed the susceptibility to tendon damage increases [14].

Growing evidence supports tendon stem cells, rather than tenocytes, as the main responsible for the healing response in acute injuries. Beyond the self-renewal capacity, proliferation and multi-lineage potential, stem cells are a secretory source of cytokines and growth factors with paracrine and autocrine activities. These soluble factors support the growth and differentiation of stem and progenitor cells and have a angiogenic, chemotatic, anti-apoptotic, anti-scarring or immunomodulatory activity [15] in local environments. The secretion of a broad range of bioactive molecules with paracrine effects resulting from

the dynamic communication between stem cells and niche environment is believed to be the main mechanism by which mesenchymal stem cells achieve their therapeutic effect [15].

Other intrinsic agents such as age, genetics, nutrition, body habitus and metabolic diseases are also involved in homeostasis as well as extrinsic factors namely pharmacological influences and mechanical stresses, including loading, disuse, compression and exogenous damage [16, 17]. When tendon injuries occur, there is a local failure in physiological conditions, whose attempt to be solved is mediated by tendon healing and regeneration processes.

II-2. TENDON REPAIR MECHANISMS

The healing process is a prolonged and complex response of the host to injury and is crucial for the mechanisms of tissue regeneration. It is also a window of opportunity envisioning new therapies for improving impaired healing [18] and understanding the molecular entities and mechanisms behind regeneration. Because tendons possess a limited intrinsic regeneration capacity with low cellularity, low vascularization and poor innervation, tendon healing results in healed tissues with impaired mechanical capabilities. The potential of tendon healing also depends on the anatomical location and local environment [19].

Most tendons heal spontaneously upon injury but the load-bearing functions are frequently dominated by fibrotic scaring, which can result in adhesion formation and consequent failure to achieve proper biomechanics [20]. Thus, a major challenge in tendon healing is to control the scar tissue formed (over scar remodeling) that deeply compromises the normal function [4]. Overuse or repetitive stretching during physical activities, which are the major cause of tendon lesions leading to microdisruption of tendon fibers, are known to trigger the release of pro-inflammatory mediators [21, 22]. Increase levels of inflammatory cytokines have been associated to tendon degeneration and disease [23].

The lack of understanding on the cell mediated mechanisms disturbing the endogenous repair/regeneration process results in limited knowledge for effective treatment. The inflammation process has a pivotal role in the healing upon injury and failed healing responses contribute to the progression of tendinopathies, which represent a significant medical problem worldwide. The development of tendinopathy compromises tendon structure and function and is characterized by pain, swelling and dysfunction [24, 25], affecting athletes and general population.

The mechanisms supporting tendon healing are still a subject of debate. Two types of tendon healing were proposed: intrinsic and extrinsic. In intrinsic healing, tenocytes from the epitenon and endotenon migrate and proliferate into the site of injury, reorganizing the ECM and giving support to the internal vascular networking [6]. Conversely, extrinsic healing is achieved by the invasion of cells from the surrounding sheath and synovium. Extrinsic healing has been associated to facilitate scar formation and, consequently inferior biomechanics. Other studies suggest that both intrinsic and extrinsic pathways are fundamental to the early stages of tendon healing [26].

The tendon healing process typically includes three main phases: inflammation, proliferation and remodeling [7] influenced by a temporal and spatially controlled array of mediators and the microenvironment events [19] (Figure I-1).

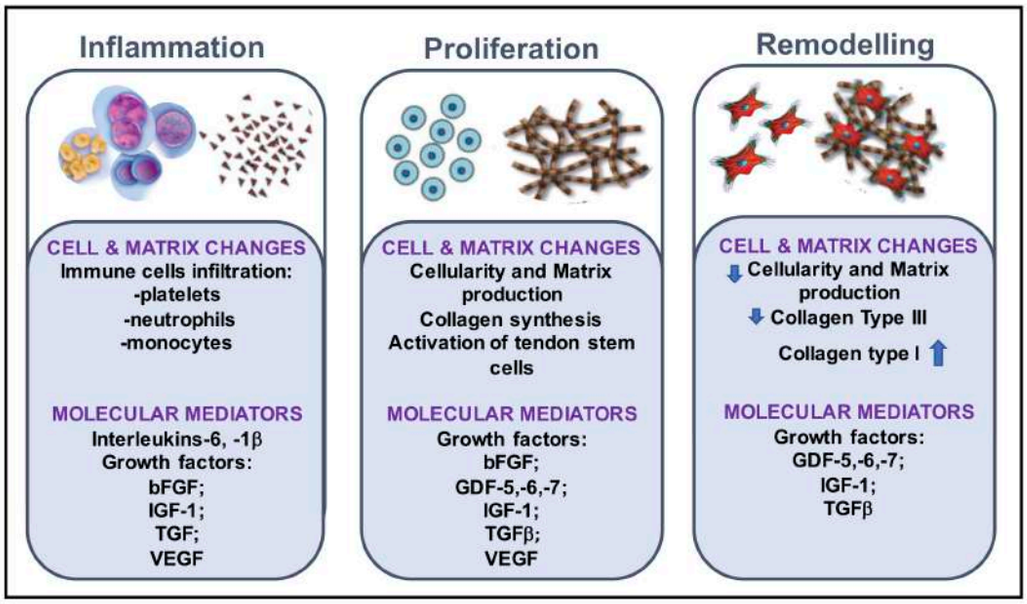

Figure II-1 Representation of the main phases of tendon repair. Inflammation, proliferation and remodeling phases and molecular, cellular and matrix changes during these phases.

The first phase is often rapid and of short duration and characterized by the infiltration of inflammatory cells like monocytes, macrophages, neutrophils and platelets. These cells release chemotactic factors that favor the migration and activation of tendon cells from nearby regions of the injury and from tendon sheaths. In the next phase, tendon cells proliferate and produce a collagen rich ECM, re-establishing the alignment of tenocytes between collagen fibrils. Finally, during the remodeling phase, ECM becomes more organized with axial arrangement of collagen fibers. In rat flexor tendons, MMP-9 and MMP-13 mediate tissue degradation, while MMP-2, MMP-3 and MMP-14 were associated to

the remodeling phase [27]. The remodeling of the ECM is a crucial process of tendon healing for gaining biomechanical competence.

During healing, the inflammatory mediators such as Il-6 and TNF-α are secreted by tendon cells [28, 29] assisting the crosstalk between cells and the ECM synthesis and arrangement contributing for the reparative versus degenerative process that drives tendon remodeling [30, 31].

Inflammation is the physiological response to injuries and is part of tendon healing process. If the injury is not resolved, the response becomes chronic and pathologic. The magnitude and duration of the inflammatory response is adjusted by regulatory mechanisms at the injury site [32, 33].

Persistent inflammation disrupts the balance between MMPs and TIMPs contributing to scared tendon healing and chronic matrix degradation [34, 35]. Scared tissue results in poor rearrangement of collagen fibrils and separation of collagen bundles. The rupture on collagen fibers may be resulted in calcifications [36]. Thus, modulation of the inflammatory response is necessary for recovery of tendon function [37].

Conservative treatments for tendon healing fight inflammation with anti-inflammatory drugs for tissue recovery in an attempt to diminish an abnormal or prolonged inflammation often associated to pathophysiology conditions. However, interrupting inflammation overruns important beneficial effects that are required for proper healing to occur.

The role of inflammation in tendinopathy is a subject of debate. Although several studies point a relation between inflammation and tendinopathy [30, 31, 38, 39], the onset and development of tendinopathy are poorly understood. Growing evidence suggests that inflammation may not be the cause of several tendinopathies but the failure to resolve inflammation will likely contribute to a complex environment of inflammatory mechanisms (stromal, immune-sensing and infiltrating compartments such as immune cells) [40] affecting tendon homeostasis and exacerbating symptoms and tissue degeneration.

Tendinopathies are associated to changes in cellularity and in the remodeling activity of tendon ECM resulting in significant structural and biomechanical alterations of the host niche [41, 42]. Histological examination of tendinopathy tissues showed collagenolytic injuries and an active healing process, focal hypervascularity and metaplasia. Moreover, the collagen fibers show unequal and irregular crimping, loss the transverse bands, separations and rupturing of the fibers with an increase of type III collagen. The type III collagen is deficient in the number of cross-links between and within the tropo-collagen units [16, 36, 38]. Inflammatory mediators including, IL-1, IL-6 and COX-2 were reported to be increased in Achilles

tendinopathy [43]. In a degenerative tendon model, the expression of IL-6, IL-11, IL-15 and TNF-α was up-regulated and accompanied by increased expression of MMP-13 and IL-1β [30, 43]. MMP-13 levels were also increased in human cuff tendon injuries. MMP-13 together with MMP-1 and MMP-8 participate in the cleavage of type I collagen present in tendons. The excessive collagen degradation during turnover results in chronic injuries [27].

II-2.1. Role of Cytokine in Tendon Healing

Cytokines are small proteins with the ability to influence and regulate biological activities [30, 44] of cells that contribute to the healing response [6, 45, 46]. Cytokines constitute the major mediators of inflammatory response with a relevant role in cell signaling and communication, holding potent immunomodulatory properties. An endogenous expression of inflammatory cytokines, namely TNF-α, IL-1β, IL-6, IL-4 and IL-10 has been demonstrated in human injured and healthy tenocytes [47, 48]. Additionally, some of these cytokines may also be involved in the (self)regulation of tenocyte processes as IL-6 stimulation was reported to increase the proliferation capacity of tenocytes and inhibition expression of tendon cell markers [19]. Cytokine expression is also affected by external stimuli as mechanical stimuli/exercise, which impacts the cytokine profile (including IL-1β and TNFα) during the tendon healing process [49].

The biochemical profile within a tissue niche during healing and other physiological events is of ultimate importance as it can be indicative of homeostatic, inflammatory or pathological conditions. Thus, the biochemical relevance of soluble factors as cytokines in tendon niches anticipates their application as potential diagnosis and therapeutic tools for repair and regeneration strategies (Table I-1).

II-3. Tendon Derived-Stem Cells (TDSCs) To Modulate Inflammation and Improve Tendon Healing

The interplay of mesenchymal stem/stromal cell (MSCs) with the tendon niche is essential for the modulation of the inflammatory response following injury [42, 57] and is strongly dependent on a balance of soluble factors, cell-cell communication and cell-matrix interactions. During inflammation, MSCs interact with resident cells to promote cell migration and proliferation, which could allow a faster re-colonization of the defect, and matrix synthesis [58].

Table II-1 Cytokines in homeostasis, inflammation and pathogenesis of tendon tissues.

Cytokine	Function in homeostasis	Function in inflammation	Pathogenesis	Refs
IL-1β	Regulation of temperature, sleep and feeding Modulation of cellular metabolism	Stimulation of MMPs production Synthesis of pro-inflammatory cytokines (e.g. TNF-α, IL-6 and IL-8)	Acute and chronic inflammatory disorders	[50]
TNFα		Favors ECM degradation (MMPs) Induction inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-10) Suppression of collagen type I - Increases the elasticity (reduction of ECM stiffness)	Participates in degeneration of tendon	[51, 52]
IL-6	Maintain metabolic homeostasis	Increases collagen synthesis Amplifies the inflammatory response Induction of VEGF and IL-10	Induces acute-phase responses	[48]
IL-4	Associated to ECM homeostasis in may disease models	Modulates and suppresses pro-inflammatory cytokines Decreases the synthesis of inflammatory cytokines (TNF-α, IL-1β, IL-6) and inhibits the secretion of MMPs Reduces tendon strength		[51, 53-55]
IL-10		Inhibition of the synthesis of inflammatory cytokines (TNF-α and IL-2) and MMPs Synthesis of collagen type II and aggrecan Induces proliferation and survival of tenocytes		[48]
IL-17A		Promote tissue destruction and degeneration Induces the production of cytokines (IL-1, IL-6, TNF-α), MMPs and NO synthase in tenocytes	Early inflammatory response in human tendinopathy; Mediates inflammation and tissue remodeling in human tenocytes	[56]

The crosstalk between inflammation cues and stem cells is important to elucidate the mechanisms of how stem cells respond to tissue damage avoiding scar formation and tuning cell-based mechanisms for regenerative approaches.

Previous studies with non-tendon mesenchymal stem/stromal cell suggested that a MSCs treatment could attenuate scar formation and compromised function by improving tissue strength after ligament and tendon injuries. This was due to a paracrine-mediated immunosuppressive effect, through which MSCs modulate macrophage phenotypes [19, 58]. It is expected that local stem cell populations within different tissues may also exert a similar effect. Thus, tendon stem/progenitor cells hold potential to contribute to the resolution of inflammation and pathophysiology of tendinopathies modulating biological responses at the injury site (Figure I-2).

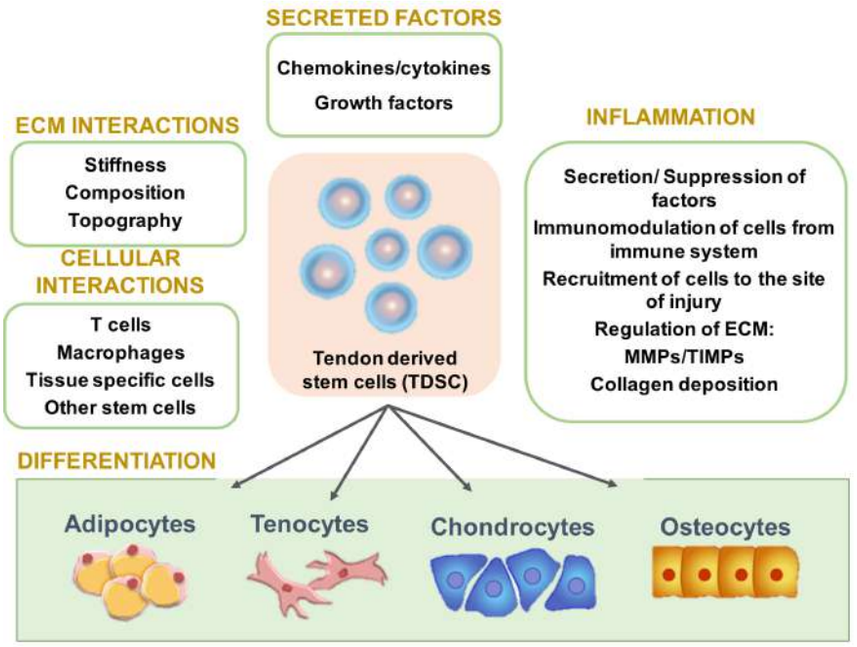

Figure II-2 Schematic representation of the TDSCs in tendon niches as modulators of tissue repair and regeneration.

A local tendon stem cell population could be beneficial over other stem cell sources due to their inherent pro-tenogenic abilities, which are likely more prone to produce tendon components under the influence of tendon environments [30, 59].

In 2007, a population of tendon stem/progenitor cells was firstly identified in tendons from mouse and humans, by Bi et al [3]. Tendon-derived stem cells (TDSCs) present universal stem cell characteristics such as the ability to self-renewal, clonogenicity and multi-lineage differentiation capacity [3]. TDSCs were reported to *in vitro* differentiate into tenocytes, chondrocytes, osteocytes and adipocytes and to originate

tendon, cartilage, bone and tendon-to-bone tissues in several animal models such as, nude mouse and rat, rabbit patellar and Achilles tendon [60, 61].

TDSCs have also shown evidence as cell source for tendon repair [34, 62, 63] (Table I-2). TDSCs cultured in fibrin glue constructs were shown to promote earlier and improved tissue repair assessed by increased collagen production and fiber alignment in a patellar tendon window defect model [64]. The TDSCs seeded in knitted silk-collagen sponge scaffolds also demonstrated ability to promoting regeneration of rotator cuff in rabbit model by inducing tenogenic differentiation and secretion of anti-inflammatory cytokines that prevented immunological rejection [65].

The resident stem cell populations present in different regions of the tendon can be subject to different biochemical stimuli and contribute in distinctive ways for the reparative response to injury, and thus play different yet interactive roles in inflammation and healing.

Table II-2 The role of tendon derived stem cell population in tendon healing and repair.

	TDSCs in tendon healing and repair	Model	Refs
TPes	Reinforces tendon differentiation genes synthesis of matrix	*In vitro*	[63]
CTGF enriched CD146$^+$ TDSCs	Induced anti-inflammatory factors: IL-10 and TIMP-3 expression Reduced pro-inflammatory M1 in the early healing phase	Full-transected patellar tendons (rat)	[34]
TDSCs in fibrin glue	Increased collagen production and fiber alignment Earlier and improved tissue repair	Patellar window defect (mouse)	[64]
Allogenic TSC in silk–collagen scaffold	Reduced number of lymphocytes	Rotator cuff (rabbit)	[65]

A study by Mienaltowski *et al.* compared the properties of proper-(TPs) and peritenon-(TPes) derived progenitor cells from embryonic Achilles tendon in an *in vitro* regenerative tendon construct model. The anatomical origin of TSCs (TPs or TPes) contributed differently for tendon-like tissue formation and the secretome of TPes bolster the expression of tenogenic differentiation markers and matrix assembly genes

in TPs and tenocytes. These findings highlight an additional potential role of TPes in tendon repair besides the synthesis of provisional matrix [63].

TDSCs also participate in the regulation of inflammation during healing of acute tendon injuries [34]. Connective tissue growth factor (CTGF) enriched CD146+ TDSCs were shown to reduce pro-inflammatory M1 cells in the early healing phase and express anti-inflammatory IL-10 and TIMP-3 vis JNK/signal transducer and activator of transcription 3 (STAT3) signaling [34].

II-4. Conclusions and Future Directions

Despite the insights from recent years on the cellular and molecular cues involved in tendon healing, the knowledge on biological mechanisms to recapitulate tendon regeneration remains at the infancy.

Findings on tendon (stem cell) biology will likely contribute for better understanding of tendon homeostasis and proper healing. Inflammation as a necessary step for healing to occur should not be blocked but modulated and controlled. New studies are required to insight on the role of the mediators involved in unresolved and chronic inflammation to unveil new homeostatic or pathological markers and assist diagnosis tools for the treatment of tendon conditions. Ultimately, the knowledge gathered would enable the control of tendon healing response to injury toward a complete restoration of functional biomechanical cues.

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SECTION 2

EXPERIMENTAL SECTION

Chapter III

Materials and Methods

Chapter III

Materials and Methods

OVERVIEW

This chapter aims to describe in detail the experimental procedures used throughout this thesis. The chapter comprises a general perspective on the techniques, methods and protocols complemented with the rationale for the selection of the materials, cells and techniques used for each experimental setup.

III-1. MATERIALS

III-1.1. Starch/poly(ε-caprolactone) polymeric blend

Polycaprolactone (PCL) is a polyester widely investigated in tissue engineering approaches of various tissues such as skin, bone, tendon/ligament, cardiac, blood vessel and nerve [1-3]. PCL it is non-toxic and biocompatible, and exhibits a low degradation rate when implanted in the body (2-3 years)[4]. Furthermore, PCL is currently approved by the Food and Drug Administration (FDA) in specific applications as drug delivery device, suture, or adhesion barrier [5].

Unlike PCL, starch is a completely biodegradable biopolymer that offers a very attractive low cost base and is relatively easy to process. Our group has developed a great amount of studies based on the use of a polymeric blend of starch and poly-E caprolactone (SPCL, 30/70 wt%), including the development of 2D and 3D structures that have shown to support the proliferation and/or differentiation of different cell types, including human adipose-derived stem cells (hASCs) [2, 3, 6] and bone marrow stromal cells [7-9] highlighting the SPCL applicability in tissue engineering and regenerative medicine.

The SPCL blend used to produce magnetic responsive membranes described in **Chapter VIII** was purchased from Novamont (Novara, Italy).

III-1.2. Magnetic nanoparticles (MNPs)

Magnetic nanoparticles (MNPs) are important nanomaterials that include metals, alloys and ferrites, widely used in biomedical applications from imaging agents to cell/biomolecule tracking/separation [10]. MNPs characterized by an iron oxide core of magnetite (Fe_3O_4) or its oxidized form maghemite (y-Fe_2O_3) hold promising value in tissue engineering and regenerative medicine due to its unique magnetic properties, biocompatibility, no immunogenicity [11, 12]. MNPs show very low cytotoxicity below 0.06 mg Fe/ML [13], and the biodegradation and clearance of MNPs from liver and spleen takes place within 2 weeks [14]. The MNPs can be externally manipulated with magnetic fields that promote MNPs motion. MNPs have been applied *in vitro* and *in vivo* assisting drug delivery, radio- and bio-therapeutics, and gene delivery [15] but in recent years there is a growing interest in MNP stimulation in driving cellular processes, including cell sorting and targeting, activation of membrane receptors or ion channels, ultimately modulating cell responses [16]. In this thesis, iron oxide MNPs and magnetic actuation approaches were explored for tendon regeneration strategies. More specifically, magnetic assisted systems were investigated incorporating MNPs in SPCL membranes creating magnetic responsive membranes (magSPCL) (**Chapter VIII**) and as scaffold-free strategies resourcing to magnetic cell sheet technology (magCSs) (**Chapter VII**). The MNPs used in this thesis, were purchased to Micromod Partikeltechnologie GmbH (Germany). We selected plain iron oxide MNPs (non-functionalized) to incorporate in magSPCL membranes to render magnetic responsiveness (**Chapter VIII**), and chitosan-coated iron oxide MNPs to enhance cell internalization (**Chapter VII**), as detailed below.

III-1.2.1. (Plain) iron oxide nanoparticles

Plain iron oxide nanoparticles were incorporated in the magSPCL membranes of **Chapter VIII**. The chemical nature of plain iron oxide particles is: iron (II/III) oxide (Fe_3O_4, magnetite) [CAS: 1317-61-9] with a 250 nm diameter (45-00-252, Micromod).

III-1.2.2. Chitosan iron oxide nanoparticles

Chitosan iron oxide composite particles (nanomag®-C) of 150 nm in size were used for the production of the magnetic cell sheets in **Chapter VII** in accordance to a previous protocol established by our group [17]. Chitosan coated MNPs are characterized by an iron oxide core [CAS: 1317-61-9] 80-85

wt% and chitosan [CAS. 9012-76-4] (04-00-152, Micromod). The positive charge of chitosan iron oxide MNPs enables the establishment of electrostatically interactions with cell membranes, which are negatively charged, resulting in a more efficiently and rapidly internalization by the cells [18].

III-1.3. Magnetic SPCL membranes production by solvent casting

Solvent casting method has been used for incorporating particles in polymer matrices. This method consists of the dissolution of a polymer in an organic solvent, in which the particles are added into the solution. When the solvent evaporates, a membrane is created with particles mixed within the polymer [19]. This method was used in **Chapter VIII** to produce magnetic responsive membranes of SPCL.

Herein, SPCL granules (30/70wt%) (Novamont, Novara, Italy) were dissolved in chloroform (20% w/v) (32211, Laborspirit). Afterwards, the polymeric solution was doped with plain MNPs (1.8% w/w, 45-00-252, Micromod), and MNPs were thoroughly dispersed in the SPCL solution by sonication and occasional orbitation before casting in glass petri dishes. The membranes formed (magSPCL) were washed with ultrapure water and dried.

Non-magnetic membranes (SPCL controls) were produced in a similar manner. After SPCL dissolution, the solution was poured into a glass petri dish and allowed to dry until fully detached. The SPCL membrane was then washed with ultrapure water and air-dried.

The magnetic formulation, magSPCL-1.8% (w/w) was selected based on a previous study in our group [3]. In this work, SPCL membranes with 1.8% (w/w) of MNPs were shown to attenuate the presence of pro-fibrotic inflammatory cells in an ectopic rat model [3].

Both SPCL and magSPCL membranes were cut into 1 cm^2 discs using a hollow punch and stored in the desiccator until performing the experimental setups.

III-2. *IN VITRO* CELL CULTURE AND CELL BASED SYSTEMS

Cell cultures enable to investigate biological responses to external cues and extracellular signals under controlled artificial environments. The cell-based culture systems provide information on cellular and molecular mechanisms through the identification and measurement of relevant biomarkers, whose expression is affected by the external stimuli. Under the scope of this Thesis, we investigated the cell

behavior in tendon tissues using cultures of cells isolated from human tendon and ligament tissues (**Chapter IV**) as well as of immune cells, THP-1 derived macrophages (**Chapter VI**), and macrophages differentiated from human peripheral blood mononuclear cells (**Chapter VIII**). To more closely study inflammatory molecules affecting pathophysiological conditions, cell responses were also investigated in pro-inflammatory cytokine enriched. Monolayered single cultures of human tendon and ligament derived cells, and macrophages were explored to address cell type responses while co-culture systems enabled the exploitation of cell interactions between human tendon derived cells and macrophages. Further, in **Chapter VII** (magCSs), cell-based systems were also investigated resourcing to magnetic cell sheet technologies to assess the contribution of cell to cell and cell-matrix enriched milieu driving human tendon cell responses.

III-2.1.1. Cell isolation and expansion

III-2.1.1.1 *Isolation of human tendon and ligament derived cells*

Tendon and ligament cell populations were selected due to their natural role in the maintenance of tendon and ligament tissues and to their epigenetic commitment to respond to tendon and ligament requirements thus, mimicking, as far as possible *in vitro*, the behavior of tissue resident cells.

Human tendon/ligament derived cells (hTDCs/hLDCs), were obtained from tissue samples obtained under a cooperation agreement with Hospital da Prelada (Porto, Portugal, P.I. N. º005/2019). Samples were provided with patient's informed consent in accordance to Helsinquia declaration, approved by the ethics committees of both institutions. Samples were collected from discarded surgical pieces of adult patients undergoing programmed reconstructive/orthopedic surgeries. hTDCs were used in all experimental Chapters of this Thesis, while hLDCs were only investigated in **Chapter IV**.

Tendon samples collected during the surgeries were immersed in a sterile solution of phosphate buffer saline (PBS, P4417, Sigma-Aldrich) with 2% antibiotic/antimicotic solution (A/A, 15240062, Alfagene) for transportation to 3B's facilities.

The isolation of hTDCs and hLDCs was based on protocols established by our group and others [20-22]. In **Chapter IV**, exploratory procedures were investigated to improve the yield of cells isolated from these tissues. These included gravitational approaches, in which, cells are expected to migrate out of the

tissue to the surface of tissue treated culture flasks, and enzymatic digestion using different concentrations of collagenase solutions, trypsin or dispase to destroy tissue matrix and release the cells.

The gravitational protocol consisted on tissue fragmentation into smaller pieces which were cultured in a 75 cm^2 culture flask (Falcon) with basic medium composed of α-MEM (12000-063, Life Technologies Limited) supplemented with 10% fetal bovine serum (FBS, A3160802, Life Technologies Limited) and 1% A/A solution. The fragments of tissue were placed on adherent culture flasks. After 4 days in culture, migrated cells were rinsed in PBS and medium exchanged. The culture flasks were maintained in an incubator at 37 °C in a 5% CO_2 atmosphere.

For the enzymatic digestion protocol, tissue portions were minced using a sterile scalpel and filtered into a 50 mL Falcon tube. After minced samples were digested with an enzymatic solution of collagenase (C6885, Sigma-Aldrich) with 2M $CaCl_2$ (1:1000, 1.02378.0500, VWR) and 1% bovine serum albumin (BSA, A2153, Sigma-Aldrich). Two concentrations of collagenase were investigated; 0.01% and 0.1% (v/v). The samples were incubated with the enzymatic solution for 1h (0.1%) or overnight (0.01%) at 37 °C under constant agitation. Afterwards, the digested samples were filtered with a cell strainer (100 µm, 352.360, Enzifarma) and centrifuged three times at 290 g for 5 min. After the centrifugation, the supernatant was discarded. Independently of the isolation protocol, hTDCs were expanded in basic culture medium, α-MEM supplemented with 10% FBS and 1% A/A at 37 °C with humidified 5% CO_2 atmosphere. Adherent hTDCs and hLDCs were expanded in monolayer culture until achieving a sufficient cell density for the experimental setups. hTDCs and hLDCs were used from passage 2 to 4.

III-2.1.1.2 *Human monocyte leukemia THP-1 cell line*

Cell lines are extensively used for assessment physiological, pathophysiological and the differentiation processes. Cell lines offer an easy, inexpensive and stable platform, which permits consistent and reproducible results. On **Chapter VI** the human monocyte cell line THP-1 was selected to investigate the crosstalk between macrophages and hTDCs using co-culture systems. THP-1 has been extensively studied in cellular functions, as well as mechanisms and signaling pathways associated to monocytes/macrophages [22, 23]. In this thesis, the THP-1 cells were cultured and expanded in RPMI-1640 medium (R7755, Sigma-Aldrich) supplemented with 1% A/A at 37 °C in a humidified incubator with 5% CO_2 atmosphere. For inducing the differentiation of THP-1 monocytes into macrophages, cells were incubated with a solution of 100nM phorbol 12-myristate-13-acetate (PMA, P8139, Sigma-Aldrich)

for 24 h, followed by 24 h incubation with PMA-free medium. Adherent THP-1 derived macrophages were washed three times with RPMI and non-attached cells removed.

III-2.1.1.3 *Human peripheral blood mononuclear cells (hPBMCs)*

PBMCs are valuable cells and a useful tool for studying various aspects of pathology and immune cell biology *in vitro*.

The most commonly used procedures for human PBMCs isolation is the density gradient centrifugation method, in which cell fractions are separated based the difference in cell density among blood cells [24].

In **Chapter VIII** of this thesis, hPBMCs were isolated from buffy coats obtained from blood units of healthy donors under an established cooperation agreement with the Portuguese Institute of Blood and Transplantation (IPST, Instituto Português do Sangue e Transplantação, Portugal).

Blood fractions were separated via centrifugation at 800 g for 30 min at room temperature (RT) using a density gradient cell separation medium, Histopaque (10771, Sigma-Aldrich). Then, the lymphocyte and monocyte layer was collected into a tube with PBS/BSA solution and centrifuged again at 350 g for 10 min. The pellet was resuspended in PBS/BSA and centrifuged in 3 cycles at 350 g for 5 min at 4 °C. Isolated hPBMCs was resuspended in PBS, and incubated with human CD14 beads for 15 min at 4 °C (microBeads, 130-118-906, Miltenyi Biotec), following manufacturer's instruction. Then, cells were rinsed in PBS/BSA and centrifuge again at 300 g for 10 min and the pellet resuspended in 500 µL of PBS/BSA. Further, CD14+ cells were immunomagnetically sorted. CD14+ cells were cultured in RPMI-1640 medium supplemented with 50 ng/mL of macrophage colony-stimulated factor (M-CSF, 300-25, Peprotech) to differentiate monocytes into M0-type macrophages for 6 days at 37 °C in a humidified incubator with 5% CO_2 atmosphere.

III-2.1.1.4 *Cryopreservation of isolated cells*

The cryopreservation process enables the preservation and storage of cells in low temperature to prevent enzymatic or biochemical activity avoiding cellular aging and deterioration. Cryopreservation minimizes genetic changes and avoids loss through cross- and microbial-contamination. Cryopreservation

procedure involves slow cooling in the presence of a cryoprotectant, as dimethyl sulphoxide (DMSO, N182-5, VWR) to avoid the damaging effects of intracellular ice formation [25, 26].

Moreover, in **Chapter IV**, cryopreservation was established as an experimental condition itself to investigate the influence of cryopreservation/thawing in the metabolic activity, phenotype and immunomodulatory properties of tendon- and ligament-derived cells. The cryopreservation/thawing process was based on a routine protocol used in our group. After expansion in culture flasks, cells were suspended at a density of $1x10^6$ cells in an ice-cooled solution of 10% (v/v) DMSO in FBS in 1.8 ml cryovails (479-6841, VWR). Cell suspension was cooled down to -20 °C for 4 h and then to -80 °C for 24 h. Afterwards, cells were mid to long-term stored in the gas nitrogen phase in a cryopreservation tank (Biosystem 24, Statebourne).

Whenever necessary, cells were thawed warming the vial to 37 °C in a water bath. Cells will be cultured with warm medium (α-MEM supplemented with 10% FBS and 1% A/A) at 37 °C in a humidified incubator with 5% CO_2 atmosphere.

All research performed with human data (anonymous or coded) and/or samples not specifically collected for research purposes (including leftover material from surgeries) was processed according to the Portuguese legislation and EC standards of data protection, quality and security, as established in the European directives 95/46/CE, 2004/23/CE, 2006/17/CE.

III-2.2. Extracellular stimuli to modulate hTDCs behavior

III-2.2.1. Exogenous supplementation with IL-1β

Tendinopathic niches are associated to pro-inflammatory cytokine rich environments which stimulate inflammation, apoptosis and extracellular matrix degradation. Interleukin-1β (IL-1β) is a potent pro-inflammatory cytokine, often up-regulated in injured tendons [27]. Reports indicate that IL-1β can artificially induce inflammatory cues *in vitro*, [28-30] and may therefore be used to study cell inflammatory responses. In addition, IL-1β can induce human tendon cells to produce inflammatory mediators such as cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE_2), interleukins and matrix metalloproteinases (MMPs), with impact in the physiological properties of tendon [31, 32].

In **Chapters V**, **VI**, **VII**, and **VIII** of this thesis, human TDCs were exogenously supplemented with IL-1β (10139-HNAE-50, Alfagene) to induce inflammatory cues in hTDCs envisioning an *in vitro* inflammatory model for tendon approaches. Firstly, in Chapter V we studied the IL-1β concentrations required to induce an inflammation profile on hTDCs. The hTDCs were treated with IL-1β supplemented media for 24 h using different concentrations of IL-1β , namely 0.01, 0.1 and 1 ng/mL and their response evaluated after 1, 2, 3 or 7 days in culture at 37 °C in a humidified incubator with 5% CO_2 atmosphere.

In other **Chapters VI**, **VII**, and **VIII** hTDCs were treated with 1 ng/mL of IL-1β, which was defined to be the most effective concentration to stimulate inflammatory signals in hTDCs. hTDCs without IL-1β treatment was established as a control condition.

III-2.2.2. Magnetic field (MF) stimuli to cells

Magnetic stimulus have been shown to improve musculoskeletal inflictions, including long-bone fractures, alleviating pain [33, 34] and influencing cytokine release kinetics [35-38]. Magnetic stimuli can be provided by static-, alternating-, and pulsed electro- magnetic fields (PEMF). While static magnetic fields (SMF) are constant fields, without intensity and direction over time, PEMF are generated by the alternating electric current that goes through a solenoid and are applied in pulses, and are preferentially used for the medical treatments.

Although the precise mechanisms are unknown, cells are able to sense the magnetic field at the cell-membrane level, influencing several types of cells [38].

The effect of externally magnetic field parameters was studied in **Chapters V to VIII**, using different yet complementary strategies, anticipating the effect of magnetic actuation in the regulation of inflammation and in the management of tendinopathic conditions.

In **Chapter V**, PEMF parameters were optimized with a magneto therapy device (Magnum XL Pro, Globus, Globus Corporation, Italy). We assessed different combinations of strength (1.5, 4 and 5 mT), frequency (5 and 17 Hz) and duty cycle (10% and 50%). These parameters were described to be within clinical therapeutic ranges of already established magneto therapy programs and to hold modulatory potential in cell based models [38-41]. These combinations were used to study hTDCs response in basal or in IL-1β supplemented media and establish the optimal PEMF conditions for controlling inflammatory cues induced by IL-1β in hTDCs.

In the following **Chapters VI**, **VII**, and **VIII**, human TDCs were IL-1β-treated for 24 h before exposure to PEMF parameters of 4mT strength, 5 Hz frequency and 50% duty cycle. This combination was selected considering the outcomes of the study developed in **Chapter V**, in which it was established as the most effective combination for the modulation of inflammatory cues of tendon cells. The PEMF stimulation was set at 1 h. Cell response was evaluated at 1, 2, 3 and 7 days after PEMF stimulation.

The combination of magnetic fields with magnetic responsive materials as MNPs can synergistically enhance the cell responses [16] and assist improved outcomes in tendon regeneration. Thus, approaches to insight on the synergistic action of magnetic stimuli and MNPs were explored in **Chapter VIII** with the production of magnetic cell sheets (magCSs) combining hTDCs and MNPs, and in **Chapter VII** using magSPCL membranes.

III-2.2.3. Single- and co-culture systems with hTDCs and macrophages

In all Chapters but **Chapter IV**, single monolayered cultures were approached to characterize cell responses to IL-1β supplementation and to PEMF stimuli. Nevertheless, anticipating the involvement of immune cells in the process of tendon inflammation and in tendinopathic niches, we explored the crosstalk between macrophages and hTDCs establishing co-culture systems in **Chapter VI**.

hTDCS were cultured on 24-well plates at a density of 10.000 cells/cm^2 for 1 h followed by an IL-1β treatment for 24 h. Direct (cell to cell contact) of hTDCs and THP-1 derived macrophages were established seeding THP-1 cells (10. 000/cm^2) on the top of hTDCs. Indirect co-cultures were established seeding THP-1 cells in a Transwell™ (pore size: 1.0 um, 662610, Corning) over the wells were hTDC were cultured. 24 h after THP-1 seeding, co-cultures were exposed to PEMF (5Hz, 4mT and 50% duty cycle) for 1 h as optimized in **Chapter V**, at 37 °C in a humidified incubator with 5% CO_2 atmosphere. One day after PEMF exposure, hTDCs and macrophage were characterized. Single cultures of hTDCs or macrophages were considered as experimental controls as well as cultures without IL-1β or without PEMF stimulation (non-PEMF control).

III-2.2.4. Production of magnetic cell sheets

The cell sheet technology has been receiving attention due to its therapeutic potential for the repair of damage tissues and organs, bypassing the need for artificial devices and their shortcomings. Although

cell sheet applications have been investigated for the regeneration of different tissues [42], magnetic cell sheets remain quite unexplored. Magnetic cell sheets enable the preservation of cell-ECM and cell-cell interactions that can offer an effective means to stimulate implantable constructs in a non-invasive manner [43].

In **Chapter VII**, magnetic cell sheets were constructed with hTDCs cells and MNPs. hTDCs were seeded onto 24 well plates at a density of 500,000 cells per well and left to adhere for 2 h at 37 °C inside the humidified incubator with 5% CO_2 atmosphere. Cells were cultured in α-MEM supplemented with 10% FBS and 1% A/A on top of a 24-well and continuously exposed permanent magnet array plate (Magnefect Nano II, N2DUO-10048) for up to 3 days. After 2 h, the chitosan coated iron oxide MNPs (400 µg/mL) were added to the hTDCs followed by overnight incubation. Then, 16 h after MNPs addition, magCSs were washed with D-PBS (21600-044, Alfagene) and the culture medium replaced by α-MEM supplemented with 10% FBS and 1% A/A and IL-1β (1 ng/mL). 24 h after IL-1β supplementation, magCSs were stimulated with PEMF using a Magnetotherapy device at 4mT strength, 5Hz frequency and 50% duty cycle for 1 h in a humidified incubator 5% CO_2 atmosphere. The magCSs were characterized after 3 days in cultures, for cell viability, proliferation, morphological analysis, collagen proteins and gene expression of tendon and inflammatory markers.

III-2.3. Characterization and analysis of cell responses

III-2.3.1. Assessment of metabolic activity, cell viability and proliferation

Assays to assess cell viability measure metabolic activity, ATP content or cell proliferation, which rely on the assessment of the redox potential, the integrity of cell membranes or the activity of cellular enzymes such as estereases to indicate the cell response after procedures that cells were subjected to [44].

III-2.3.2. MTS assay

In the all Chapters of this thesis, at each defined time in culture, metabolic activity was determined using a colorimetric assay named CellTiter96® AQueous One Solution Cell Proliferation Assay (G3581, Promega). This assay is based on the bioreduction of a tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)] into a brown formazan produced, converted by mitochondrial activity for living cells. The intensity of formazan is dependent of the viable cell number in culture [45]. Briefly, samples were washed with D-PBS and incubated in a mixture containing serum-free cell culture medium DMEM (without phenol red, D2902, Sigma-Aldrich) and MTS reagent at 5:1 ratio for 3 h at 37 °C in a humidified incubator with 5% CO_2 atmosphere, protected from light. The supernatant was then transferred to a 96-well plate and the absorbance read at 490 nm using a microplate reader (Synergy™HT, BIO-TEK Instruments). The blank (no cells) was performed.

III-2.3.3. Live/Dead assay

Live/dead staining allows determining the viability of cells using calcein acetoxymethyl (AM) which stains live cells, and ethidium homodimer-1 (PI) which is internalized by dead cells. Calcein AM is membrane permeable and is cleaved by esterases in live cells resulting in a green fluorescence molecule. The PI is membrane-impermeable and is not stained by healthy cells. However, if the cell membrane is compromised (e.g. in apoptotic and dead cells), the PI will enter the cell and stain nuclei acids(red fluorescence) [46]. The ratio of live to dead cells can be determined by a simple counting. Herein, the live/dead assay was performed on **Chapter V** and **VII**. At each defined time in culture, hTDCs were incubated for 20 min with calcein AM (2 μgmL^{-1}) (C3099, Thermo Fisher Scientific), and PI (3 μgmL^{-1}) (P1304MP, Molecular Probes), diluted in α-MEM supplemented with 10% FBS and 1% A/A. Afterwards, the cells were washed with D-PBS and visualized under a fluorescence inverted microscope (Axio Observer, ZEISS).

III-2.3.4. Proliferation/Cell content quantification

Cell proliferation is measured through an increase in cell number over time in culture. Therefore, cell proliferation assays are typically investigated through nucleoside-analog incorporation during DNA synthesis, cell cycle-associated proteins, and cytoplasmic proliferation dyes [46]. Proliferation of hTDCs, in **Chapters V**, **VI** and **VII**, was assessed by DNA quantification using PicoGreen® dsDNA assay. This assay detects double strand DNA (dsDNA) as low as 25 pg/ml. After each time point selected for cell characterization, cells were washed with D-PBS, then collected into sterile microtubes containing 1 mL of ultrapure water and stored at -80 °C until analysis.

Prior to dsDNA quantification, according to the manufacture instructions the various specimens were thawed and sonicated for 15 min. The standard curve was defined using pDNA standards prepared at concentrations ranging from 0 to 2 µg/mL. Briefly, DNA quantification was performed by adding 28.7 µL of sample or standard to a well of a 96-well white polystyrene plate, mixed with 100 µL of Tris-EDTA buffer (10 mM Tris-HCL, 1 mM EDTA, pH 7.5) and 71.3 µL of 1X Quant-It™ PicoGreen® reagent, in a total volume of 200 µL and RT incubated for 10 min. All reagents used were part of the Quant-It™ PicoGreen® dsDNA Assay Kit (P7589, Thermo Fisher Scientific. The samples and the standards were read in triplicate with a microplate reader (Synergy™HT, BIO-TEK Instruments) at an excitation of 485/20 nm and emission 528/20 nm wavelength. The DNA concentration of each sample was calculated using a standard calibration curve.

III-2.4. Assessment of multilineage potencial of hTDCs and hLDCs

III-2.4.1. Histological processing and staining with chondrogenic dyes

Histological processing is a standard procedure for tissue examination, either for research or diagnostic purposes, enabling both qualitative and quantitative measurements. In order to increase the contrast and better visualize the morpho-structure, samples are processed in four distinct steps: 1) fixation of the specimen to prevent the cell structure from degradation and contamination; 2) dehydration in a grade series of alcohol baths for water removal and clearance in xylene to enable paraffin infiltration; 3) embedding the specimen into paraffin blocks; 4) microtome sectioning to obtain a series of micro-thick sections of paraffin embedded tissue [47]. In **Chapter IV**, the chondrogenic differentiation of hTDCs and hLDCs in cell pellets was histological processed before staining with chondrogenic specific dyes. After 21 days in culture, cell pellets were fixed and embedded into an 4% agarose/PBS solution (A9539, Sigma-Aldrich) before being processed into paraffin blocks. 4 µm thick sections were obtained using a microtome (Microm, HM355S, Thermo Ficher Scientific). Sections were heated to 70°C to melt the paraffin, followed by xylene clearance and rehydration before staining with Safranin O and Toluidine Blue.

III-2.4.2. Safranin O

Safranin O (84120, Fluka) is a cationic dye with a high affinity for acidic proteoglycans in tissues, forming a reddish orange complex. In **Chapter IV**, the proteoglycans associated to chondrogenic ECM

were assessed in hTDCs and hLDCs pellets. Firstly, deparaffined sections were stained with Weigert's iron haematoxylin working solution (88028, Thermo Fisher Scientific) for staining nuclei for 7 min followed by a 5 min incubation with fast green solution (44715, Fluka) to stain collagen and reticular fibers and then by 0.1% safranin O for 5 min. The sections were washed in water between steps, and afterwards mounted for visualization in a microscope.

III-2.4.3. Toluidine Blue

Toluidine blue staining is a well-established histological procedure for the characterization of cartilaginous- and chondrogenic-differentiated tissues [48]. Toluidine blue form complexes with anionic glycoconjugates such as proteoglycans (PG) and glycosaminoglycans (GAG). In **Chapter IV**, paraffin-embedded sections of hTDCs and hLDCs pellets were stained with Toluidine Blue. A Toluidine blue solution was prepared by dissolving 1% toluidine blue (89640, Sigma-Aldrich) in distilled water containing 0.5 g sodium borate (Riedelde-Haën). Sections were dipped in for 2-3 s in Toluidine solution and then were rinsed in running tap water before stained with eosin-Y (Bio-optica) for 30 s. Then, slides were dehydrated, cleared in xylene and mounted for microscopy visualization.

III-2.4.4. Oil Red staining

Oil Red is a lipophilic dye has been used for staining fat and lipid components in biological samples. It is a lysochrome (fat-soluble dye) diazo dye used for staining of neutral lipids (triglycerides and diacylglycerols), staining them to a red hue [49], and is quite used for investigating adipogenic lineage commitment by stem cells [50]. Oil Red was used in **Chapter IV** for in hTDCs cultured in α-MEM supplemented with 10% FBS and 1% A/A. Cells were incubated with the Oil Red solution (O0625, Sigma-Aldrich) for 10 min and then washed with distilled water.

III-2.4.5. Alizarin Red staining

Alizarin red stains calcium and other divalent cations, and has been used to identify calcium deposits in ECM-associated with bone tissues. In order to characterize the osteogenic differentiation of hTDCs and hLDCs in **Chapter IV**, we qualitatively assessed Alizarin red stain in hTDCs cultures after 21 days in osteogenic medium. For that, cells were washed with PBS, fixed in a 10% neutral buffered formalin (05-

K01005Q, Bio-Optica Milano S.p.a) for 30-60 min, and kept at 4 ºC before a 2 min incubation with a, 2% of alizarin red solution (1.06278.0025, Merck). This solution was prepared in distillated water and pH adjusted to 4.1-4.3. After that, the samples were rinsed in water and microscopically visualized under a microscope (Axio Imager Z1m, Zeiss).

III-2.4.6. Sirius Red/Fast Green Staining

Sirius Red/Fast Green collagen staining is a semi-quantitative assay that provides a simple quantitative tool for determining the total amount of collagen and non-collagenous proteins in culture cell and tissue sections [51, 52]. This assay was used in **Chapters IV** and **VII** for detection and quantification of extracellular proteins produced by cultured cells. Sirius red dye binds to collagen by allowing its sulphonated acid side-chain groups to interact with the basic amino acid side groups of collagens, whereas Fast green binds to non-collagenous proteins.

The hTDCs, hLDCs and magCSs were collected for collagen staining (9046, Chondrex) were washed with PBS, fixed in a 10% neutral buffered formalin, for 30-60 min, and kept at 4 °C until further used. Following, the dye solution was added enough to completely immerse the samples and incubated for 30 min at RT. After that, the supernatant was aspirated carefully and the stained cells rinsed in distilled water repeatedly until the water runs clear. Finally, a dye extraction buffer was then mixed and the OD values read in a spectrophometer (Synergy™HT, BIO-TEK Instruments) at 540 nm and 605 nm. The collagen and non-collagenous proteins was normalized with total protein, correcting the values of OD 540 and OD 605.

III-2.5. Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked Immunosorbent Assay (ELISA) is an analytical biochemistry assay commonly used to detect and measure soluble ligands, often antibodies, antigens, proteins or peptides in biological samples [53]. The principle of this assay relies on antigen-antibody binding, in which the antigen is quantified using a solid-phase enzyme immunoassay [54]. This procedure has high specificity and sensitivity, because of an antigen-antibody reaction, high efficiency, simple procedure and generally safe and eco-friendly [53]. Under the scope of this thesis, hTDCs, hLDCs and macrophages secreted products with biological relevance were assessed using commercial ELISA kits in **all Chapters** of this thesis.

At first, the culture medium was collected and frozen at -80 °C, until analysis. The ELISA assay was performed for human pro-inflammatory cytokines: IL-6 (900-K16, Peprotech), IL-1β (900-K95, Peprotech), TNFα (EK0525, Tebu-Bio), iNOS-2 (MBS723617, Mybiosource), COX-2 (KA0323, Abnova) and PGE_2 (KHL1701, Thermo Fisher Scientific), and anti-inflammatory cytokines: IL-10 (900-K21, Peprotech), Arginase-1 (BMS2216, Invitrogen).

Afterwards, the MAPK protein phosphorylation was also determined in cytoplasmic protein extracts using the PhosphoTracer ERK1/2 (pT202/Y204) + p38 MAPK (pT180/Y182)+ JNK1/2/3 (pT183/Y185) Elisa Kit (ab119674, Abcam). The protein extracts were detailed described in the bellow.

For development kits purchased from Peprotech, the primary capture antibodies were incubated overnight at RT in a 96-well plate (Nunc-Immuno MicroWell 96-well solid phases). Then, a blocking step was performed (1% BSA in PBS) for 1 h at RT to ensure that the antigen would specifically bind to the immobilized antibody. After 1 h, the standards and the samples were incubated for 2 h at RT, according the manufacturer's instructions. In last step, 100 µL of an 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS, A3219, Sigma-Aldrich) liquid substrate was added to each well, and the color development was monitored in a microplate reader at 405 nm, with a wavelength correction set at 650 nm. The concentration and OD values were then calculated from a quadratic equation with software (Graphpad prism vs 6).

The ELISA assays of other companies Tebu-Bio, MyBiosource, ThermoFisher Scientific, Invitrogen and Abcam ELISA kits were already pre-coated with monoclonal antibody in strips. For that, standards and samples were added in strips and incubated at RT. Then, 100 µL of 3, 3', 5, 5' – Tetramethylbenzidine (TMB) liquid subtract was added to each sample to be assessed. The reaction was stopped by the addition of an equal volume of 1M acid solution and the absorbance read in a microplate reader (Synergy HT, Bio- TEK) at 450 nm.

III-2.6. Reverse transcription polymerase chain reaction (RT-PCR) technique

RT-PCR assays are a common method for the assessment of gene expression and for comparing mRNA levels in different samples. This technique allows the detection of a specific RNA transcript. After RNA isolation from cells or tissues, there is a reverse transcription of RNA into cDNA using a reverse transcriptase enzyme (RT) [55]. The RNA isolation is the first step in performing molecular techniques such as RT-PCR which is used to amplify RNA targets. Under the scope of this thesis, in **all Chapters** of

this thesis, the method used for RNA extraction and isolation was based on the Trizol reagent, which is a ready-to-use reagent for the isolation of high-quality total RNA, DNA and protein from biological samples.

At each experimental time, in all chapters of this thesis, the samples were washed with PBS, immersed in Tri reagent (15596018, TermoFisher Scientific), and kept at -80 °C for later RNA extraction. To perform the RNA extraction, samples were defrosted in ice, following the chloroform (C2432, VWR) addition for phase separation into two distinct phases: aqueous and organic. Samples were vigorously agitated (15 s) and incubated for 15 min in ice. Then, the samples were centrifuged at 12 000 g for 15 min at 4 °C, the supernatant collected for a sterile 1.5 mL microtube, and an equivalent volume of isopropanol was added. The aqueous phase contains RNA and is recovered by the precipitation with isopropanol (437423R, VWR). The RNA was let to precipitate overnight at -20 °C. Afterwards, samples were centrifuged at 12 000 g for 10 min at 4 °C, the supernatant discarded and ethanol 75 % was added in order to wash away the isopropanol. Samples were vigorously agitated (15 s) and centrifuged at 7500 g for 5 min at 4 °C. The supernatant was carefully removed, and the pellet was left to air dry under sterile conditions. The pellet was ressuspended in 15 µL of DNase free water (733-1631, Lonza Versiers SPRL). The concentration and purity of the extracted RNA were determined with a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies) and using the Mastercycler® ep realplex gradient S machine (Eppendorf) using an initial amount of total RNA of 1 µg in a total volume of 20 µL.

After that, RT-PCR was used in all experimental Chapters of this thesis for detecting gene expression of tendon (**Chapters IV, V, VII**, and **VIII**), inflammation (**Chapters IV, V, VI, VII, VIII**), ECM (**Chapters IV, V, VI, VII,** and **VIII**) related genes.

RT-PCR detection of PCR products is possible using a fluorescent molecule that reports an increase in the amount of double-stranded DNA (cDNA) (e.g. SYBR green) with a proportional increase of a fluorescent signal that reflects the amount of amplified product in each cycle. There are three major steps in each cycle in a real-time PCR reaction: 1) denaturation of double-stranded DNA into single strands by DNA polymerase; 2) annealing of complementary sequences (primers); and 3) primer extension by DNA polymerase activity, which occurs at rates of up to 100 bases per second.

In the first step, RNA was reverse transcribed into cDNA using the qScript cDNA synthesis kit (733-1175, Quanta Biosciences), according the manufacturer instructions. The reaction was performed using the MiniOpticon real-time PCR detection system (BioRad Laboratories, USA). Subsequently, the obtained cDNA was used as a template for the amplification of the target genes shown in supplementary material

of each Chapter, according to manufacturer's instructions of the PerfeCta™ SYBR® (QUNT95072-012, Quanta Biosciences). The number of amplification cycles used for every reaction was 45. Reverse transcription followed by quantitative polymerase chain reaction (qPCR) using the PerfeCTA SYBR Green FastMix kit (Quanta Biosciences) following the manufacturer's protocol, in a Real-Time Mastercycler ep realplex thermocycler (Eppendorf). The primers were designed with Primer3 software and synthesized by MWG Biotech. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was used as reference gene, and the expression of all target genes was normalized to the expression of this gene for the same sample. The gene expression quantification was performed according to the Livak method ($2^{\Delta\Delta_{Ct}}$ method), considering the negative control as calibrator [56].

III-2.7. Immunocytochemistry

Immunocytochemistry (ICC) is often used to detect specific molecules in biological samples with the benefit of observing their expression within tissues or cells. The ICC principle relies on the sample incubation with a specific primary antibody to detect the antigen of interest. Then a secondary antibody with high affinity/specificity for the primary antibody is added to specifically bind the primary antibody and enable its indirect detection via a colorimetric/fluorochrome molecule.

This technique involves preliminary steps including fixation for preserving the chemical and structural of cell components; permeabilization to ensures that antibodies enter into the cell if the antigen is intracellularly located; blocking step for reduction of fluorescence due to non-specific antibody binding.

The immunodetection of tenogenic markers: Scleraxis, Tenomodulin and Mohawk (1:100) was assessed in chapters (**Chapters IV, VII**, and **VIII**), and of ECM-related markers: Collagen I (1:500), Collagen III (1:100) and alpha smooth muscle Actin (1:200) in **Chapters IV**, and **VIII**. Inflammatory markers: using conjugated antibodies IL-1β FITC, IL-4 PE in Chapter VI, and phospho-p44/42 MAPK (1:200) and NFkB p65 (1:200) in **Chapters V** and **VIII**. In **Chapter VI** and **VIII**, we investigated the focal adhesions: Vinculin (1:300) FAK (phosphor Y397) (1:200) and gap-junctions in **Chapters V** and **VI**: connexin 43/GJA1 (1:100). The macrophage phenotype was assessed in **Chapter VIII**, using conjugated antibodies: CD14 PE, CD16 APC, CD45 FITC, CD68 APC, CD169 (Alexa fluor 647) and CD206 FITC (Supplementary Table III-1 and III-2).

After the primary antibodies overnight incubation, was followed the incubation with secondary antibodies Alexa Fluor 488 donkey anti-rabbit (2072687, Alfagene, Life Technologies limited) or Alexa

Fluor 594 donkey anti-mouse (2069656, Alfagene, Life Technologies limited, for 45min, according to the host of the primary antibody, 1 h at RT (Supplementary Table III-2).

All the samples were collected at the defined culture time, washed with PBS, fixed in a 10% neutral buffered formalin for 30-60 min, and kept at 4 °C until further used. Samples were permeabilized with 0.025% Triton-X100 (A16046.AE, Thermo Fisher Scientific) in PBS solution, and after that samples were washed in PBS for 5 min and blocked with R.T.U. Normal Horse Serum (RTU Vectastain Kit, PK-7200, Vector) for 1 h to avoid non-specific staining. Samples were further incubated with the respective primary antibodies diluted with reducing components (Dako) overnight at 4 °C and with secondary antibodies Alexa Fluor 488 or 594 for 1 h at RT, according the host of the primary antibody or for fluorochrome-conjugated antibodies for 30 min at room temperature protected from light. Between antibodies incubations, cells were washed with PBS 3 times for 10 min. After that, in all Chapters, samples were stained with DAPI (4,6-Diamidino-2-phenyindole, dilactate) and Phalloidin. DAPI is a nuclear and chromosome counterstain that emits blue fluorescence upon biding to AT regions of DNA. DAPI (5 μg/μl, D9564, Sigma) solution was used for 10 min incubation for cell nuclei staining. Phalloidin-Tetra-methylrhodamine B isothiocyanate (Phalloidin-TRITC red) stains the cytoskeleton of cells. Phalloindin-TRITC (0.1 mg/mL, P1951, Sigma) was incubated for 20 min at RT. Afterwards, samples were observed under a fluorescence microscope (**Chapter IV** and **V)** or a confocal laser scanning microscope (Chapters **VI**, **VII** and **VIII)**, as described below.

III-2.8. Western Blotting

Western blot enables to identify specific proteins extracted from cells using the antibody-antigene reaction [57]. In this method, a mixture of proteins is separated in a gel considering their molecular weight and later transferred to a membrane where proteins, glycoproteins and/or nucleic acids are immobilized. The membrane is incubated with antibodies specific to the protein of interest, which is revealed afterwards by colorimetric or fluorimetric methods. The thickness of the band corresponds to the quantity of the protein present in the samples, which can be semi-quantitatively determined [58].

This technique was approached in **Chapters V** and **VII** for the identification of MAPK protein phosphorylation and alpha smooth muscle Actin.

Cells and magCSs were collected in PBS, resuspended, and the supernatant collected. In the particular case of Chapter VII the supernatant was subjected to magnetic separation (MPC®-S, Dynal

Biotech) for MNP removal. Then, the total cell lysates were prepared with RIPA buffer (R0278, Sigma-Aldrich) supplemented with protease inhibitor cocktail (P8340, Sigma-Aldrich). Then, samples were centrifuged for 15 min at 14,000 rpm at 4 °C and the supernatants collected for the protein content assessment using a BCA protein assay kit (23225, Thermo Fisher Scientific). The BCA protein assay kit enabled to measure protein concentration within cell lysates. The BCA reaction results in a purple-colored reaction product and exhibits absorbance at 562 nm.

The protein extracts of 15 μg were resolved in 10% sodium dodecyl sulfate-polyacrylamide gels (08091, Sigma-Aldrich) followed by semi-dry transfer to AmershamTM Protran® Western blotting membranes (nitrocellulose; 10600002, GEHealthcare), using a Pierce Power Station (Thermo Fisher Scientific). The membranes were blocked with 5% BSA in Tris-buffered saline with Tween 20 (P1379, Sigma-Aldrich) and incubated with rabbit anti-phospho-p44/42 MAPK (Erk1/2) (1:1000) (9102, Cell signaling Technology) and anti-α smooth muscle actin (1:2000) (ab32575, Abcam) Cambridge, UK) antibodies for 1 h at room temperature under mild agitation. The membranes were washed three times with TBS-T and then incubated with a secondary antibody (1:2000) (Anti-Rabbit IgG Alkaline Phosphatase antibody, A9919, Sigma-Aldrich) for 1 h at RT. A colorimetric AP substrate reagent kit (1706432, BioRad) was used for protein band detection. Digital images were acquired using a scanner equipment (Epson Perfection V550 Photo).

III-2.9. Flow Cytometry

Flow cytometry (FC) is a popular laser-based technology that enables the detection of surface and intracellular biomarkers and a multi-parameter analysis in a single cell suspension [59, 60]. Flow cytometry use lasers as light sources to produce scattered and light signals at specific wavelengths that are read by optical detectors which convert light into electronic signals detected and recorded on an optical-to-electronic coupling system.

In this thesis, FC was applied for the detection of surface and intracellular phenotypic markers in single-cell suspension of hTDCs and THP-1 cells.

In **Chapter IV** the cellular phenotypic cues associated to stemness (CD73, CD90, CD105), to cell surface proteins (CD29, CD44, CD49f) and to teno-ligamento-genic markers (Scleraxis, Tenascin C, Tenomodullin, Collagen I, Collagen III) were assessed.

hTDCs and hLDCs were washed with PBS and tripsinized using a TrypLE Express (Alfagene, 12605-028). Cells were re-suspended in medium and centrifuged (400 g, 5 min) and immunostained for 1 h with i) fluorescent-conjugated antibodies CD105 (FITC, BIORAD, 1807) CD73 (PE, BD Biosciences, 9010996), CD90 (APC, BD Biosciences, 9352389) CD29 (PE, BD Pharmingen, 42615), CD44 (PE, BD Biosciences, 9199202), CD49f (FITC, 4319153, Thermo Fisher Scientific). In the case of non-conjugated antibodies, cells were incubated with Tenascin-C (Mouse anti-human, ab6393, Abcam), Collagen I (Rabbit anti-human, ab9039, Abcam), Collagen III (Rabbit Anti-human, ab7778, Abcam) , anti-human Scleraxis (Rabbit anti-human, ab58655, Abcam), and Tenomodulin (Rabbit anti-human, ab81328, Abcam) followed by incubation with secondary antibodies Alexa Fluor 488 donkey anti-rabbit (2072687, Alfagene) or Alexa Fluor 594 donkey anti-mouse (2069656, Alfagene), for 45 min, according to the host of the primary antibody, all at RT. After washing with PBS, cells were centrifuged (400 g, 5 min) and fixed with 500 µL of 1% v/v of paraformaldehyde.

To determine the intracellular expression of pro-inflammatory cytokines IL-4 (Phycoerythin; ab95717; Abcam) and IL-1β (FITC; ab16168, Abcam) studied in **Chapter VI**, cells were previously treated with Brefeldin A. Brefeldin A is a protein transport inhibitor commonly used to enhance intracellular cytokine signals by blocking transport process during cell activation. The cells retrieved from direct co-cultures of hTDCs and macrophages were treated during 4 h with 10 µg/mL Brefeldin A (ab193369, Abcam) to block cytokine secretion before being trypsinized using TrypLE Express (12605-028; Alfagene), centrifuged, and resuspended in fresh PBS. Afterwards, cells in suspension were incubated with fluorochrome-conjugated antibodies: anti-IL-1β FITC and anti-IL-4 PE for 20 min at room temperature protected from light. Cells were then rinsed in PBS and centrifuged for 5 min at 800 g. The cells were resuspended in 500 µL of acquisition buffer and data acquired in a FACSAria III sorter equipped with blue and red lasers (BD Biosciences, Erembodegem-Aalst, Belgium).

In both cases, cells were identified by forward and side scatter. A minimum of 10,000 cells were acquired in a FACSAria III sorter equipped with blue and red lasers (BD Biosciences, Erembodegem-Aalst, Belgium). Data acquired was analyzed using FACS Diva version 7 software. Unstained cells, representative of cells auto-fluorescence were considered as negative controls.

III-2.10. Microscopy techniques

To visualize the cells and sub-cellular molecules associated to their phenotype and functions we used several imaging techniques, namely fluorescence, confocal and electron microscopies.

The principle of fluorescence microscope is projecting light at a specific wavelength into the sample which is then absorbed by the fluorophore present in the sample and reflected at different wavelengths [61]. The fluorescence microscopes used were an AxioImager Z1m fluorescence microscope (Zeiss) and an Axio Observer (Zeiss) in **Chapters IV** and **V**.

The confocal microscope is an imaging tool for increasing optical resolution and contrast through a spatial pinhole in the detector system that eliminate out-of-focus (background) light [62]. Confocal images were acquired using a laser scanning confocal microscope (LSCM, TCS SP8, Leica, Germany) in **Chapters VI, VII,** and **VIII**.

The semi-quantification of mean fluorescence intensity was performed for connexin 43 and MAPK in **Chapter VI**. We used a minimum of five images per sample from independent experiments. The mean fluorescence intensity was assessed measuring the signal intensity. The signal was calculated subtracting the mean signal intensity of to the initial raw images using ImageJ software version 1.52a, National Institutes of Health, USA.

The cell distribution and morphology of cells of the magSPCL membranes developed in Chapter VIII, and magCSs in **Chapter VII** were analysed by Scanning Electron Microscopy (SEM). SEM is an imaging technique used to analyze the surface of a solid specimen [63]. SEM provides qualitative information regarding sample's surface morphology, including microstructure, porosity, pore size, topography and composition. For SEM analysis, samples have to be electrically conductive which can be achieved using a gold or metal alloys as gold/palladium thin film over the sample to create conductivity and obtain a better image [20]. In **Chapters VII** and **VIII**, the samples were fixed with 10% neutral buffered formalin for 30-60 min, and kept at 4 °C until further used. Then, the samples were washed in PBS, dehydrated in increasing alcohol concentrations followed by 5 min immersion in hexamethyldisilazane reagent (HMDS, 440191, Sigma-Aldrich) before being sputter coated and analysed by SEM microscopy (JEOL, Japan).

III-3. STATISTICAL ANALYSIS

In all Chapters of this thesis, the results are expressed as mean ± standard error of the mean (SEM). For flow cytometry results **Chapter V**, results are expressed as mean ± standard deviation (ST). The statistical analysis of data was performed using GraphPad Prism vs 6 software.

Data was obtained from three independent experiments (n=3) analyzed in triplicate, and evaluated by One-way analysis of variance (ANOVA) by Bonferroni's and Two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for multiple comparisons.

A difference between control and cell-treated groups was considered significant with a confidence interval of 95% and whenever $p < 0.05$.

III-4. SUPPLEMENTARY MATERIAL

Supplementary Table III-1 List of fluorochrome-conjugated antibodies.

	Antibodies	Fluorochrome	Reference	Company
Fluorochrome-conjugated	**CD14**	PE	B244291	Biolegend
	CD16	APC	B2128291	Biolegend
	CD45	FITC	555482	BD Biosciences
	CD68	APC	B178818	Biolegend
	CD169	AF647	565295	BD Biosciences
	CD206	FITC	5253911	BD Biosciences
	IL-1β	FITC	ab16168	Abcam
	IL-4	PE	ab95717	Abcam

Supplementary Table III-2 List of primary and secondary antibodies.

	Antibodies	Host	Reference	Company
Primary	**Scleraxis**	Rabbit	ab58655	Abcam
	Tenomodulin	Rabbit	ab81328	Abcam
	Collagen I	Rabbit	ab9039	Abcam
	Collagen III	Rabbit	ab7778	Abcam
	alpha smooth muscle Actin	Rabbit	ab32575	Abcam
	Vinculin	Mouse	V9131	Sigma-Aldrich
	connexin 43/GJA1	Rabbit	ab11370	Abcam
	Fak (phosphor Y397)	Rabbit	Ab39967	Abcam
	phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2)	Rabbit	4377	Cell Signaling Technology
	NFkB p65 (phosphor S536)	Rabbit	ab86299	Abcam
Secondary	**Alexa fluor 488**	Rabbit	2072687	Alfagene
	Alexa fluor 594	Mouse	2069656	Alfagene

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SECTION 3

EXPERIMENTAL STUDIES

Chapter IV

The impact of cryopreservation in signature markers and immunomodulatory profile of tendon and ligament derived cells

Chapter IV

The impact of cryopreservation in signature markers and immunomodulatory profile of tendon and ligament derived cells

ABSTRACT

Tendon and ligament (T/L) engineering strategies towards clinical practice have been challenged by a paucity understanding in the identification and characterization of cellular niches. By prospecting how resident cell populations behave *in vitro*, and how cryopreservation may influence T/L-promoting factors, can bring insights for novel regenerative solutions. T/L share physiological similarities and complementary functions at the joints but their individual contributions to local regeneration is still poorly described or the impact of cryopreservation/thawing in their cellular profiles.

Therefore, we propose to study human T/L-derived cells (hLDCs and hTDCs) isolated from patellar tendons and cruciate ligaments as suitable cellular models to anticipate tendon/ligament niches responses for advanced strategies with predictive tenogenic/ligamentogenic value.

Our results show that the populations studied hold a stem cell subset and share a similar behavior in terms of tenogenic/ligamentogenic commitment identified by the expression of tenogenic markers. Both T/L-derived cells successfully undergo cryopreservation/thawing maintaining the cellular molecular profile. The major differences between cryo and fresh populations were observed at the genetic expression of *MKX*, *SCX* and *TNMD* and protein levels of collagens, in which hTDCs evidence increased values in comparison to hLDCs. In addition, low temperature storage was shown to potentiate an immunomodulatory profile of cells, especially in hTDCs leading to an increase of anti-inflammatory factors, *IL-4* and *IL-10*, as well as of the secretion of IL-10. Overall, the outcomes highlight the relevance of the cryopreserved T/L-cells and their promising immunomodulatory cues as *in vitro* models for investigating cell mediated mechanisms driving tissue healing and regeneration.

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IV-1. INTRODUCTION

Despite the different functional roles in the human body, tendon and ligament share intricate and complementary relationships. There is a lack of knowledge on the biology of tendon and ligament resident cell populations and on their regenerative potential with relevant inputs to stimulate the tenogenic ability of local stem cells and their role in immunomodulation [1, 2].

Both tissues arise from the somitic mesoderm, and are originated from the somitic compartment, the syndetome that regulates the expression of transcription factors associated to tenogenic and ligamentogenic differentiation [2]. Moreover, it is not clear whether resident cell populations respond specifically to fulfill local functional requirements or if different anatomical regions have interchangeable contributions to the process of joint regeneration [3]. Hence, there is a strategic interest in the study of resident and tissue-specific cells as well as their niche interplay in the patho-physiology of T/L tissues and in the onset and progression of T/L disorders. Bi and coworkers [4] investigated tendon-derived cells from human hamstring tendon and mouse patellar tendons. A stem progenitor cell (TSPCs) population was identified in tendons of both species, that possesses clonogenicity, self-renewal and multipotency and a high expression of Scleraxis, Tenomodulin, Comp and Tenascin C [4]. However, and to the authors´ knowledge, there is no published works assessing the role of resident cell populations from tendons and ligaments considering their physiology and complementary actions at the joints. Therefore, we propose to study human T/L-derived cells (hLDCs and hTDCs) isolated from patellar tendons (PT) and cruciate ligaments (CL) and compare their metabolic activity, morphology as well as their clonogenic and tenogenic potential.

Most of the cell based studies use freshly isolated or expanded cells despite the fact that therapeutic approaches cannot fully depend on the immediate availability of cells. Cryopreservation stands as a clinically relevant procedure to enable the storage of living cells in extremely low temperatures for mid to long periods of time. One of the major challenges of cryopreservation methods is to assure that cell functions and phenotype are maintained after thawing. The effect of cryopreservation has been investigated in cell recovery after thawing and in functional responses with controversial outcomes. While cryopreservation of periodontal ligament stem cells in cell sheet constructions did not alter their viability, proliferative capacities and multi-lineage differentiation [5], other studies demonstrate that cryopreservation may compromise cell function, although the loss of potency may be recovered with time [6].

Another important aspect for healing and regeneration is the preservation of the natural immunomodulatory cues of implanted cells for a favourable interplay with the immune system. Cryopreservation revealed efficacy in the modulation of the inflammatory profile of adipose-derived stem cells [7] resulting in increased production of IL-6 and decreased levels of pro-inflammatory cytokines IL-10 and TNFα. In another study, Antebi et al., reported also a significant inhibition of IL-1β, TNFα and INFy was also observed in cryopreserved mesenchymal stem cells (MSCs) from bone-marrow [8]. Thus, human resident cells were isolated following mechanical and chemical methods and characterized in terms of morphology, stemness potential as well as for the expression of tendon-associated markers for up to 28 days in culture. Since the mid- to long-term storage influence the recapitulation *in vitro* of the characteristics of natural tissues, we also investigated the behavior of cryopreserved T/L-derived cells after expansion in monolayer cultures, and their immunomodulatory profile at the gene and protein levels.

IV-2. MATERIALS AND METHODS

IV-2.1. Isolation of human tendon and ligament (T/L) resident cells

Human tendon and ligament (T/L) resident cells (hTDCs and hLDCs, respectively) were isolated from surplus tissues of knee surgeries, obtained under previous established protocols with Hospital da Prelada (Porto, Portugal, P.I. N. º 005/2019), and with informed consent of the patients. The written informed consent and the surgical related procedures were reviewed and approved by the Hospital Ethics Committee. A minimum of three independent donors per each processed anatomical area were considered: cruciate ligaments (CL) and patellar tendons (PT). Cells were used at passage 2 or 3.

After surgical collection, tissue samples were transported in a sterile solution of phosphate buffer saline (PBS, Sigma-Aldrich) with 2% antibiotic/antimicotic solution (A/A, Alfagene). Afterwards, T/L-samples were carefully rinsed in PBS, and divided by dissection. Each tissue sample was divided into 3 portions with approximate size and cells were isolated using gravitational or enzymatic procedures.

Gravitational isolation: One of the portions was placed in a 75 cm^2 culture flask (Falcon) containing basic culture medium composed of α-MEM (A-MEM, Invitrogen) supplemented with 10% FBS (Alfagene) and 1% A/A solution. Cells were allowed to migrate from the explanted tissues into the adherent bottom of the culture flasks by gravity. After 4 days at 37°C in a 5% CO_2, cells were rinsed in PBS and medium

exchanged. Cellular detection and morphology were assessed under an inverted microscope (Axiovert 40 CFL, Zeiss).

Enzymatic isolation: The other 2 portions were minced using a sterile scalpel. PBS drops were added in a continuous basis to keep a moist environment and reduce cell damage by mechanical forces. The excess of PBS was removed using a filtration system for 50 ml tubes (Falcon). Minced samples were collected into a 50 mL Falcon tube, already containing an enzymatic solution of collagenase (C6885, Sigma-Aldrich) with 2M $CaCl_2$ (1:1000, VWR) and 1% BSA(Sigma-Aldrich). A ratio of 1:1 of minced tissue to the enzymatic solution was considered. Two concentrations of collagenase were used: i) 0.01 % collagenase solution overnight, and ii) 0.1 % collagenase solution for 1h. To stabilize the temperature inside the tube and to assure the temperature was constant at 37°C, samples were incubated in a water bath under constant agitation (1200rpm). After incubation, digested samples were filtered and centrifuged three times (1200rpm) for 5 min. Immediately after the first centrifugation, samples were vigorously agitated for 2-5 seconds and centrifuged again.

After isolation procedures hLDCs and hTDCs were cultured in basic medium composed of α-MEM (A-MEM, Invitrogen) supplemented with 10% FBS (Alfagene) and 1% A/A solution.

IV-2.2. Expansion and cryopreservation of hLDCs and hTDCs

The cells were expanded in basic medium and the influence of the cryopreservation process was investigated. Ligament- and tendon-derived cells were cryopreserved in a 1:9 ratio of DMSO (VWR, Darmstadt, Germany) in FBS and stored in a liquid nitrogen tank (390-Vessel number SC004272-4, Biosystem 24). Cryopreserved hTDCs and hLDCs were herein termed “cryo”, and the freshly isolated cells “fresh”. Cryo and fresh cells were seeded at a density of 1 x 10^5 in 6-well plates (Falcon), cultured in basic medium and weekly evaluated. Cell cultures were investigated for morphology, genetic and protein expression for up to 28 days before and after cryopreservation.

IV-2.3. Morphological analysis of isolated cells

hLDCs and hTDCs were daily monitored using a phase contrast microscope (Axiovert 40, Zeiss). Representative sections were captured by a digital camera (PowerShot G11, Canon) at 20x magnification.

IV-2.4. Multilineage potential of hLDCs and hTDCs

For the assessment of the multilineage potential, hLDCs and hTDCs were cultured in osteogenic, chondrogenic or adipogenic differentiation media and the lineage differentiation was characterized by Alizarin Red staining (osteogenic), Safranin O and Toluidine B stains (chondrogenic) and oil red (adipogenic).

Osteogenic differentiation of hLDCs and hTDCs was assessed in 24-well plates. Briefly, cells were seeded at 5000 cells/well and cultured in osteogenic medium consisting of α-MEM supplemented with 10 % FBS (and 1 % A/A, 10^{-8} M dexamethasone, 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate) for 21 days.

Chondrogenic differentiation of hLDCs and hTDCs was performed in pellet culture. Cells pellets were trypsinized and centrifuged twice at 2000 rpm for 5 min in cryotubes (250,000 cells/cryotube) to form 3D pellets and cultured in chondrogenic medium: low-glucose DMEM (D290, Sigma-Aldrich) supplemented with 10% FBS, 1% A/A, ITS+1 Liquid Media Supplement (I2521; insulin–transferrin–selenium liquid media supplement, Sigma-Aldrich), 17 mML-ascorbic acid (PHR1008-2G, Sigma-Aldrich), 0.1 M sodium pyruvate (11360070, Alfagene), 35 mML-proline (P5607,Sigma-Aldrich), 1 mM dexamethasone (D1756, Sigma-Aldrich) and 10ng/ml human transforming growth factor-β1 (TGF-β1) (100-21C, Prepotech).

Adipogenic differentiation of hLDCs and hTDCs was assessed in 24-well plates. Cells were seeded at 5000 cells/well and cultured in adipogenic medium consisting of DMEM/F-12 (31330038, Alfagene), 10% FBS (Alfagene), 1% A/A (Alfagene), 0.5 mM isobutylmethylxanthine, 33 mM biotin (B4639, Sigma-Aldrich), 17 mM pantothenate, 5mM rosiglitazone, 1mM dexamethasone (D1756, Sigma-Aldrich), 10 mM insulin (I9278) for 21 days.

IV-2.4.1. Alizarin Red staining

After 21 days in osteogenic medium, cells were fixed in 10% buffered formalin (Bio Optica) and stained with a 2% alizarin red solution (A5533, Sigma-Aldrich) (pH adjusted to 4.1–4.3).

IV-2.4.2. Safranin O and Toluidin Blue

Cell pellets were histologically processed and embedded in paraffin blocks. Sections were stained with Safranin O and Toluidine blue for proteoglycan assessment. Safranin O staining consisted of staining the sections with Weigert's iron haematoxylin for 7 min, fast green (Sigma) for 5 min and 0.1% safranin O for 5 min. The sections were washed after each staining step, left to dry in air and then mounted for visualization.

Toluidine blue staining was prepared by dissolving 1% toluidine blue in distilled water containing 0.5 g sodium borate (Riedelde-Haën), followed by filtering, and the sections were dipped in for 2–3 s. The sections were then washed in running tap water and stained in eosin-Y (Bio-optica) for 30 s. Finally, all slides were dehydrated, cleared in xylene and mounted for visualization.

IV-2.4.3. Oil Red

After fixation with 10% buffered formalin (Bio Optica), samples were incubated for 10 min with Oil Red stain and then washed with distilled water.

IV-2.5. Metabolic activity

The metabolic activity of hLDCs and hTDCs was weekly evaluated by MTS assay (Cell Titer 96® Aqueous Solution Cell Proliferation Assay, Promega) for 28 days. Cells were rinsed in PBS before a 3h incubation in a mixture of FBS-free and phenol red free medium and MTS solution (5:1 ratio) at 37°C and 5% CO_2. The absorbance was read at 490nm (Synergy HT, Bio-TeK Instruments). Triplicates were made of each sample and a blank reading was performed.

IV-2.6. Flow cytometry analysis

To investigate stemness markers, cell adhesion molecules and tendon related markers, cells were detached using TrypLE Express (12605-028, Alfagene), centrifuged and resuspended in fresh PBS. Cells were incubated with fluorochrome-conjugated antibodies for i) stemness assessment: CD105FITC (1807, BIORAD), CD73PE (9010996, BD Biosciences), and CD90APC (9352389, BD Biosciences), and for ii)

adhesion molecules detection: CD29PE (PE, 42615, BD Pharmingen), CD44PE (9199202, BD Biosciences), and CD49fFITC (4319153, Thermo Fisher Scientific) for 20min at RT protected from light.

Tenogenic markers were investigated using non-conjugated antibodies for Scleraxis (ab58655), Tenascin-C (ab6393), Tenomodulin (ab81328), Collagen I (ab292) and Collagen III (ab7778) all purchased to Abcam. Cells were incubated with antibodies for 30min before incubation with secondary antibodies Alexa Fluor 488 (Alfagene) for 30min at RT protected from light. Finally, cells were resuspended in acquisition buffer (PBS with 1% neutral buffered formalin (INOPAT/05-k01009)) and data acquired in a flow cytometer FACS Calibur (BD Biosciences). A minimum of 10,000 cells were acquired and analysis was performed using the Cell Quest software.

IV-2.7. mRNA extraction and real-time RT-PCR

Total RNA was isolated using the TRI Reagent® RNA Isolation Reagent (T9424, Sigma) following the manufacturer's instructions, and quantified spectrophotometrically (Nanodrop® ND-1000, Thermo Scientific). RNA was reverse transcribed to complementary DNA via qScript™ cDNA Synthesis Kit (Quanta Biosciences) using an Eppendorf Mastercycler® ep realplex gradient. The expression of Tenascin C (*TNC*), Collagen type I (*COL1A1*) and type III (*COL3A1*), Decorin (*DCN*), Tenomodulin *(TNMD),* Mohawk *(MKX)*, Scleraxis (*SCX*), Cycloogenase-2 *(COX-2),* Interleukin-6 *(IL-6),* Tumor necrosis factor *(TNFα),* Interleukin-4 *(IL-4)* and Interleukin-10 *(IL-10)*, was assessed by real time RT-PCR. The transcript expression of target genes was analyzed and normalized to the expression of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). The $2^{\Delta\Delta_{Ct}}$ method was used and the results presented as relative gene expression. The primer sequences were obtained from Primer 3 software, and synthesized by MWG Biotech (Supplementary Table IV-1).

IV-2.8. Immunolocation of T/L related markers

After overnight fixation in 10% neutral buffered formalin (INOPAT), "fresh" and "cryo" cells were kept in PBS at 4°C until performing the immunolocation assays.

Cells were permeabilized with 0.025% Triton-X100 (Sigma/X100)/PBS solution and then blocked with RTU Normal Horse Serum (RTU Vectastain Kit, PK-7200; Vector) to avoid unspecific reactions. Then, cells were incubated with the primary antibodies overnight at 4°C. Antibodies for Collagen I (ab9039),

Collagen III (ab7778), Tenomodulin (ab81328) and Scleraxis (ab58655) were purchased to Abcam. Then, samples were incubated with a fluorescent secondary antibody (donkey anti-rabbit Alexa Fluor 488, 1:200; Alfagene) for 1h at RT before staining with 4,6-Diamidino-2-phenyindole, dilactate (DAPI, 5μg/μl, D9564; Sigma-Aldrich) for 10 min and with Phalloidin-Tetramethylrhodamine B isothiocyanate (P1951, 1:200; Sigma-Aldrich) for 20min. Samples were observed under a microscope (Imager Z1m, Zeiss) and images acquired using a digital camera (AxioCam MRm5).

IV-2.9. Collagen and non-collagenous proteins quantification

The amount of collagen and non-collagenous proteins deposited by ligament- and tendon-derived cells, both in "fresh" and "cryo" conditions, was determined by Sirius Red/Fast Green Collagen Staining Kit (9046, Chondrex). After fixation in 10% buffered formalin (Bio Optica), cells were incubated for 30 min in the dye solution. After adding a dye extraction buffer the OD values were read (Synergy HT, Biotek Instruments) at 540 nm and 605 nm.

IV-2.10. Cytokine release

Inflammatory mediators released into the culture media were analysed using enzyme immunoassay kits for human IL-6 (900-K16, Peprotech), TNFα (EK0525, Tebu-Bio), COX-2 (KA0323, Abnova, Taiwan) and IL-10 (900-K21; Peprotech), according to manufacturers' instructions.

IV-2.11. Statistical analysis

All quantitative results are expressed as the mean ± standard error of the mean (SEM). The statistical analysis was performed using GraphPad Prism6 software. Two-Way ANOVA was used, unless specified, followed by Bonferroni post-hoc test for multiple comparison tests. Data was obtained from 3 independent experiments (n=3) analyzed in triplicate. Statistically significant differences between hLDCs and hTDCs are shown with different degrees of confidence. Differences were significant for $p<0.05$. Different degrees of confidence were assessed and represented with the symbols *, γ, $, Ω for $p < 0.05$; **, α, θ, β, η for $p < 0.01$; ***, #, δ, ∋, φ for $p < 0.001$; and ****, ****, ψ, &, ε, μ, ∞, κ, ρ, χ, €, π, τ for $p < 0.0001$, respectively.

IV-3. RESULTS AND DISCUSSION

IV-3.1. Influence of isolation protocols on hLDCs and hTDCs phenotypic profile

The isolation protocols were based on previously reported procedures [9-11], as we intended to select the method with the highest yield of cells. Based on microscopic evaluations (Figure IV-1 A), the highest yield of cells was observed after isolation with 0.1% collagenase solution, followed by gravity and then by 0.01% collagenase solution. Moreover, cells isolated from ligament tissues showed an increased cell density after isolation compared to cells obtained from PT samples (Figure IV-1 A). This is in agreement with studies reporting that the PT structure is more hypocellular than ligaments [12].

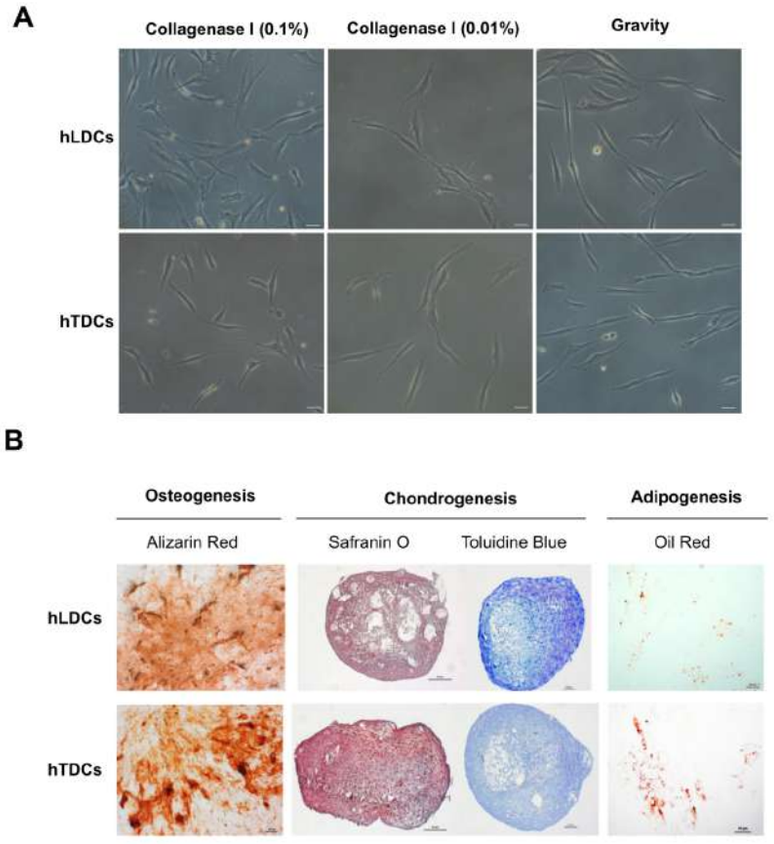

Figure IV-1 Methods for isolating hLDCs and hTDCs and assessment of cell multilineage potential. A) Morphological assessment of hLDCs and hTDCs 6 days after enzymatic digestion (collagenase solution) or tissue deposition (gravity). Magnification: 20x. D) Microscopy photographs of Alizarin Red, Safranin O, Toluidine Blue and Oil Red stains 21 days after the induction of hTDCs and hLDCs towards osteogenic, chondrogenic and adipogenic phenotype.

The multilineage potential of T/L-cell populations was confirmed with Alizarin Red, Safranin O/Toluidine blue, and Oil Red stains (Figure IV-1 B). The lineage associated stains suggest the presence of a stem cell subset within T/L-tissues, which is supported by the literature [4, 10, 13-15].

IV-3.2. Impact of cryopreservation in hLDCs and hTDCs

Cell cryopreservation enables long term storage of cells and assures the steady availability of cells for *in vitro* studies. The metabolic activity of both freshly isolated (fresh) and cryopreserved (cryo) cells, increased with time in culture (Figure IV-2 A).

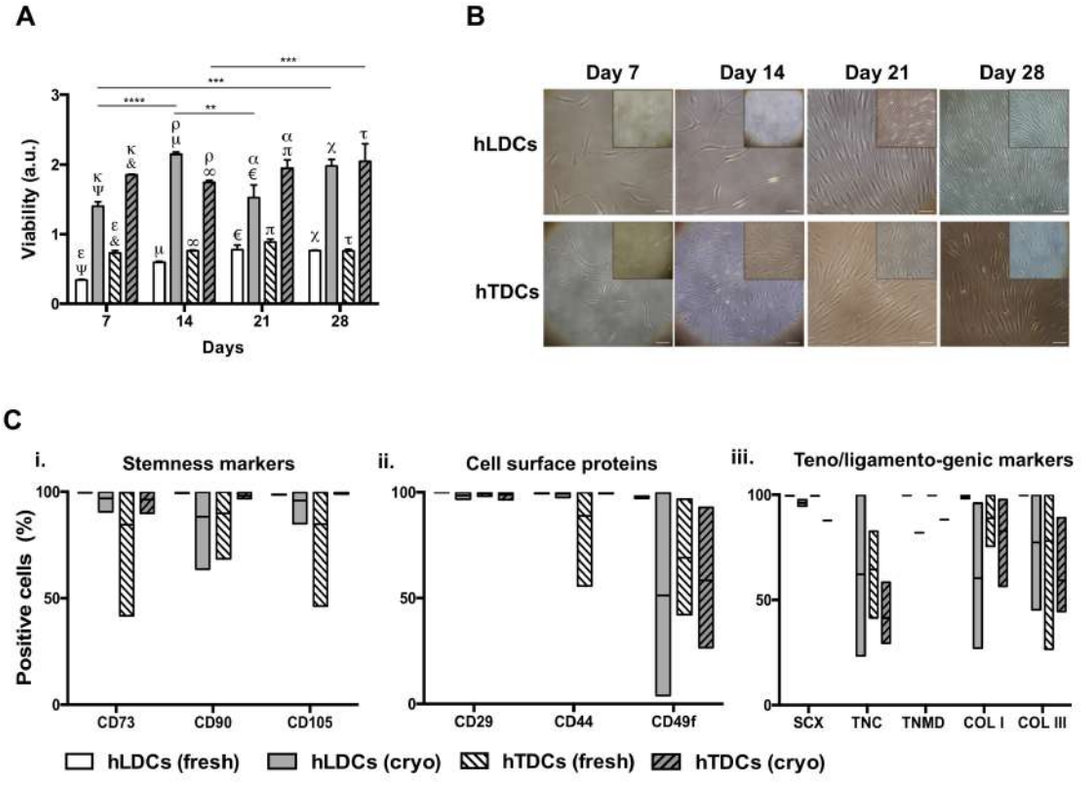

Figure IV-2 Assessment of viability, morphology and surface characterization of freshly isolated (fresh) and cryopreserved (cryo) cells up to 28 days in culture. A) Cell viability analysis by MTS assay of ligament (hLDCs) and tendon (hTDCs) cells. Statistical analysis was performed comparing cryopreserved hLDCs and hTDCs to the respective freshly isolated cells using regular two-way ANOVA followed by Dunnett's multiple test. Symbols denote statistical differences for $p<0.05$; **, α f or $p<0.01$; *** for $p<0.001$; and ****, ψ, &, ε, μ, ∞, κ, ρ, χ, €, π, τ for $p<0.0001$, respectively. B) Weekly morphological assessment of cryopreserved hLDCs and hTDCs expanded up to 28 days. Inset images represent freshly cells. Magnification: 20x; C) Percentage of positive hLDCs and hTDCs expressing different surface markers. The percentage of positive cells is represented by mean, minimum and maximum values.

At each time point, cryo cells have increased activity in comparison to freshly isolated cells ($p<0.05$), independently of their origin. Additionally, hTDCs are more metabolically active than hLDCs after cryopreservation, which is particularly evident at 7 ($p<0.0001$) and 21 days ($p<0.01$) in culture. Furthermore, hLDCs and hTDCs proliferated with the time in culture and tended to align in function of time (Figure IV-2 B). After 14 days in culture, both cell populations naturally form lines of cells, that later become parallelly distributed (at day 21 and day 28) in freshly and cryopreserved conditions. These results suggest the ability for cell machinery revival after cryopreservation, in accordance to other works [6, 8].

The stemness potential was investigated through the classical stemness markers CD73, CD90 and CD105 (Figure IV-2 Ci). was inferior when compared to cryopreserved hTDCs. The opposite was observed for hLDCs, with a higher variation in the number of cryopreserved cells expressing these molecules. Considering that local stem cells are endogenous mediators of homeostasis and healing, this result shows that cryopreserved cells highly express stem cell markers, likely due to the presence of a stem cell subset that is maintained through the cryopreservation/thawing.

Furthermore, T/L-derived cells were investigated for the adhesion molecules, CD29, CD44 and CD49f (Figure IV-2 Cii). CD29 and CD44 are involved in cell-cell and cell-matrix interactions and migration capacity [16], and are known to be present in tendon niches [17, 18]. Our results showed that nearly 100% of hLDCs and hTDCs, in cryo and fresh conditions, expressed CD29. Despite some variation in freshly isolated hTDCs, more than 90% of hLDCs and hTDCs express CD44, following previous reports [4, 19]. CD49f is an adhesion molecule available in tendon tissues [20, 21], but has also been described to enhance multipotency and stemness maintenance in MSCs [22]. With the exception of fresh hLDCs, which fully express CD49f, there is a wide variation in the expression of this marker in hLDCs cryo and hTDCs (fresh and cryo) populations. Independently of the tissue source or culture conditions, over 80% of cells express Scleraxis (SCX) and Tenomodulin (TNMD) (Figure IV-2 Ciii), which are early and late (mature) tendon associated markers, respectively, and support the T/L phenotype. Conversely, the expression of Tenascin-C (TNC), Collagen type I (COL I) and Collagen type III (COLIII) varied significantly ($p>0.05$), which may relate to the fact that most of these molecules do not accumulate in the cells and are extracellularly released to enable extracellular matrix (ECM) formation.

Overall, these results suggest that hLDCs and hTDCs populations share common properties with MSCs thus holding stemness potential, while maintaining a T/L signature. Moreover, the long term *in*

vitro expansion does not alter the cellular profile although cryopreservation/thawing may influence the expression of cell surface biomarkers as CD44 and CD49f ($p>0.05$).

The expression of tenogenic genes tended to increase after cryopreservation when compared to freshly expanded cells (Figure IV-3 A).

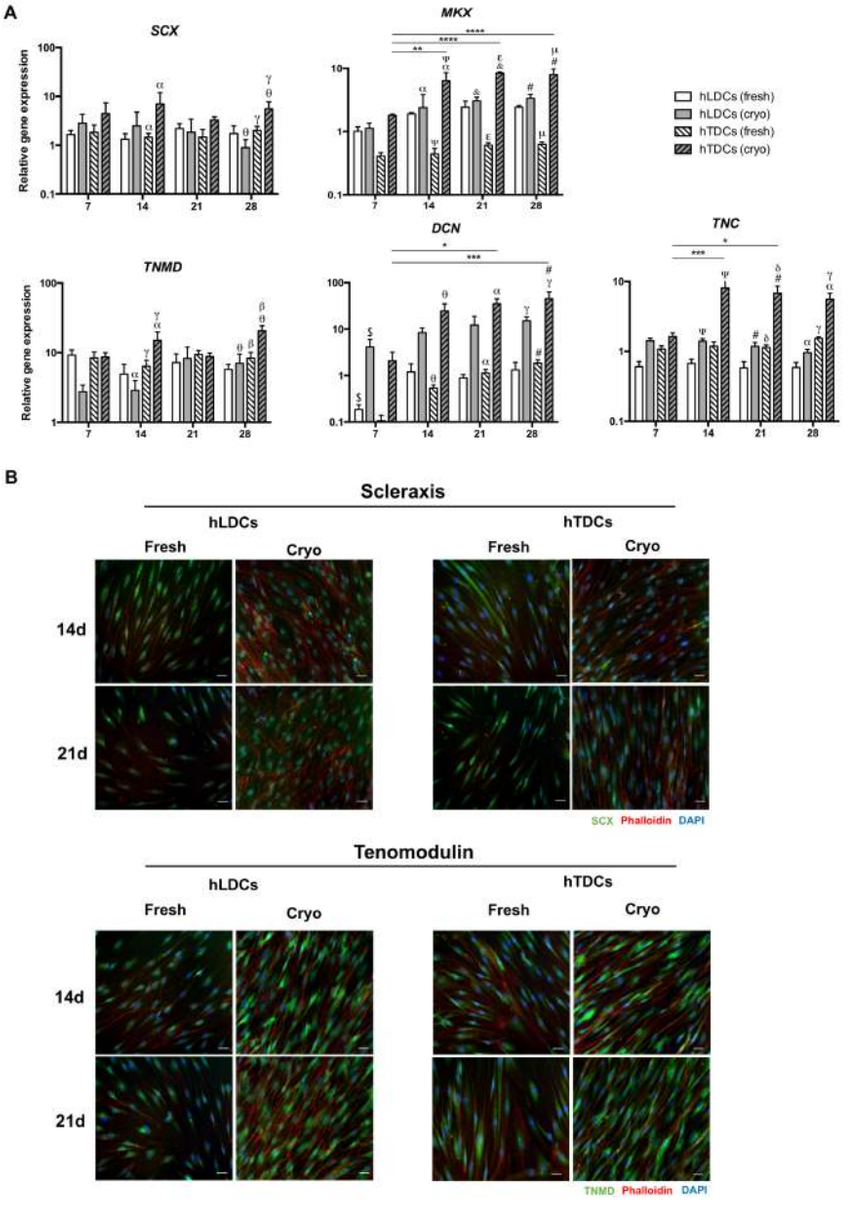

Figure IV-3 Gene expression and immunolocalization of tendon and ligament markers in freshly isolated (fresh) and cryopreserved (cryo) cells. A) Real time RT-PCR analysis of Scleraxis (*SCX*), Mohawk (*MKX*), Tenomodulin (*TNMD*), Decorin (*DCN*) and Tenascin *C* (*TNC*) for gene expression of hTDCs and hLDCs expanded up to 28 days. B) Fluorescence microscopy images for the cellular localization of the tenogenic markers (green), Scleraxis and Tenomodulin after 14 and 21 days in culture (fluorescence microscopy x20, scale bar 50 μm). Nuclei and cytoskeleton were counterstained with DAPI (blue) and phalloidin (red), respectively. Symbols *, γ, $, Ω denote statistical differences for $p<0.05$; **, α, θ for $p<0.01$; ***, #, δ for $p<0.001$; and ****, ψ, &, ε, μ for $p<0.0001$, respectively.

SCX and *TNMD* increase after cryopreservation in hTDCs, mainly at days 14 and 28 (TNMD: $p<0.05$ for 14 days and $p<0.01$ for 28 days; SCX: $p<0.01$ for 14 days and $p<0.05$ for 28 days) (Figure IV-3 A). The similar tendency of *SCX* and *TNMD* may be explained since *TNMD* expression is regulated by *SCX* [23-25]. The deposition of SCX and TNMD was also analysed by immunofluorescence (Figure IV-3 B). A consistent production of SCX and TNMD was observed over time in both fresh and cryo cells.

The relative expression of *MKX*, *DCN* and *TNC* was enhanced in hTDCs cryo when compared to hLDCs ($p<0.05$) (Figure IV-3 A). In particular *MKX* and *DCN* levels were increased from 7 to 28 days (*MKX*, $p<0.0001$; *DCN,* $p<0.001$), while *TNC* was increased from 7 to 21 days ($p<0.05$).

Overall, the genetic expression was enhanced in both types of cells after cryopreservation, however, with higher values in hTDCs ($p<0.05$ in comparison to hLDCs cryo). This is an interesting outcome since a previous study of Dai Guangchun et al., in rat PT, showed no significant differences in the expression of *SCX*, *COL I* and *TNMD* between control groups and cryo groups [26].

The ECM of T/L-tissues is mainly composed of collagen type I, interspersed with a predominantly non-collagenous matrix [27]. The expression of *COL1A1* and *COL3A1* increases in hTDCs ($p<0.05$) after cryopreservation with the exception of *COL3A1* after 14 days (Figure IV-4 Ai and Aiii).

This is consistent with the higher expression of collagen production in tendon tissues [12, 28, 29]. The synthesis of a collagen-rich matrix was confirmed by immunodetection (Figure IV-4 Aii and Aiv). Despite the significant increment in *COL1A1* of hTDCs cryo ($p<0.05$) in comparison to hLDCs cryo, the immune-detection of collagen I and collagen III was similar during the time in culture. The proper assembly of collagen molecules into a functional matrix requires non-collagenous proteins.

Thus, to infer on the quality of ECM synthetized by the cells, collagen and non-collagenous proteins were quantified (Figure IV-4 B). The concentration of collagen and non-collagenous proteins increased with time of culture, in hTDCs cryo ($p<0.01$). Moreover, cryopreserved hTDCs synthetized higher quantities of both classes of proteins ($p<0.05$ in comparison to fresh hTDCs and to hLDCs).

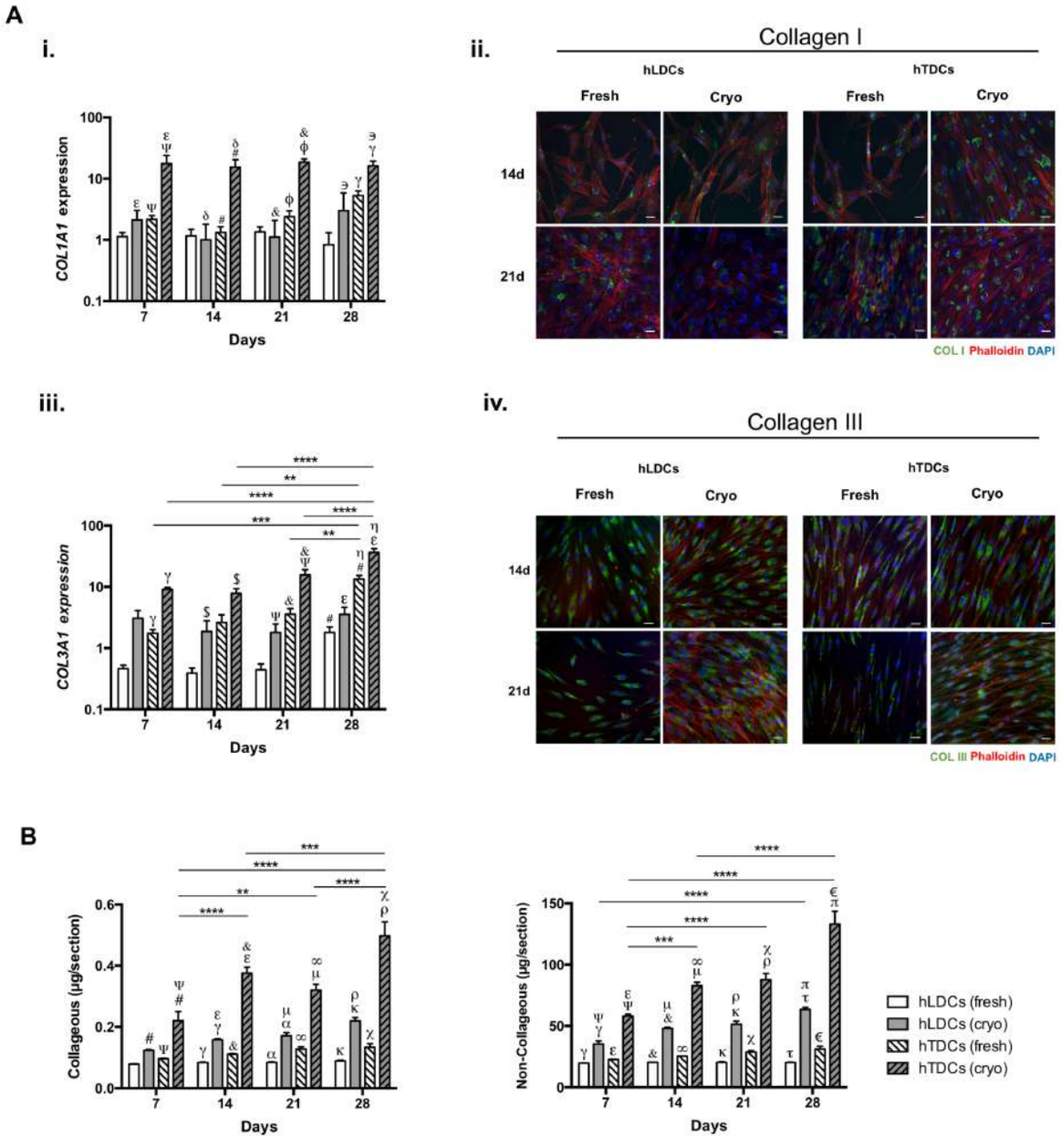

Figure IV-4 Evaluation of tendon extracellular matrix production of freshly isolated (fresh) and cryopreserved (cryo) cells. Ai, Aiii) Real time RT-PCR analysis of Collagen type I (C*OL1A1*) and Collagen type III (*COL3A1*) for gene expression of hLDCs and hTDCs expanded up to 28 days. Aii, Aiv) Immunolocation of Collagen type I and III (green) after 14 and 21 days in culture (fluorescence microscopy x20, scale bar 50 µm). Nuclei and cytoskeleton were counterstained with DAPI (blue) and phalloidin (red), respectively. B) Quantification of collagen and non-collagenous proteins secreted by hLDCs and hTDCs using a Sirius Red/Fast Green Collagen staining kit. Symbols γ, $, denote statistical differences for $p<0.05$; **, α, η for $p<0.01$; ***, #, δ, ∋, ϕ for $p<0.001$; and ****, ψ, &, ε, µ, ∞, κ, ρ, χ, €, π, τ for $p<0.0001$, respectively.

IV-3.3. Immunomodulatory capacity of cells after cryopreservation/thawing

The interplay between implanted cells and host tissues influence the successful outcomes of cell-based strategies to stimulate repair and regeneration at the implantation site. After confirming the post cryopreservation recovery of relevant functional cues, we investigated the immunomodulatory potential of hLDCs and hTDCs assessing genes and secreted forms of inflammatory mediators (Figure IV-5 A and B).

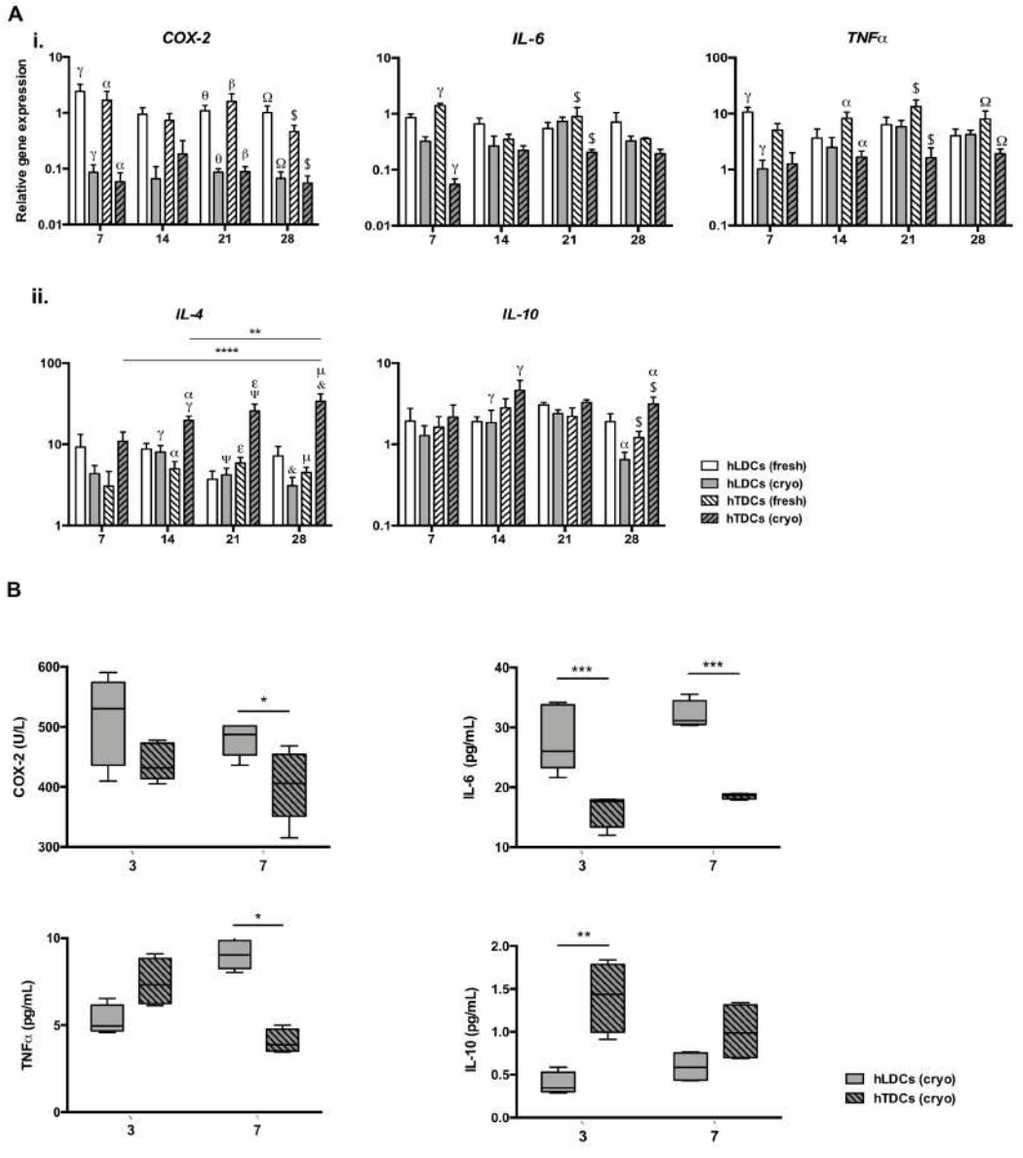

Figure IV-5 Immunomodulatory profile of freshly isolated (fresh) and cryopreserved (cryo) hLDCs and hTDCs. Real time RT-PCR analysis of (Ai) pro-inflammatory factors, *COX-2*, *IL-6*, *TNFα* and (Aii) anti-inflammatory factors, *IL-4*, *IL-10* for the gene expression of hLDCs and hTDCs expanded up to 28 days. (B) Quantification of COX-2, IL-6, TNFα and IL-10, released to the culture medium after 3 and 7 days in culture. Symbols *, γ, $, Ω denote statistical differences for $p<0.05$; **, α, θ, β for $p<0.01$; *** for $p<0.001$; and ****, ψ, ε, &, μ for $p<0.0001$, respectively.

With few exceptions at intermediary time points, the expression of *COX-2, IL-6* and *TNFα* was diminished to levels below the baseline in hTDCs cryo (*IL6: p*<0.01 for 7 and 21 days; *TNFα*: p<0.01 for 14 days and p<0.05 for 21 and 28 days, both in comparison to hTDCs fresh) (Figure IV-5 Ai). In hLDCs cryo the decrease in the expression of inflammatory genes was only significant for *COX-2 (*p<0.01 for 7 and 21 days; p< 0.05 for 28 days in comparison to hLDCs fresh) (Figure IV-5 Ai). The decrease in the gene levels is accompanied by an upregulation of the anti-inflammatory cytokine *IL-4*, especially in hTDCs, from 7 to 28 days (p<0.0001) and by a consistent expression of *IL-10* over time, with significant increased levels in comparison to hLDCs (p<0.05 for 14 and p<0.01 for 28 days) (Figure IV-5 Aii).

We further investigated the translation of inflammatory genes into secreted forms with paracrine action to nearby cells. The release of COX-2, IL-6, TNFα and IL-10 was assessed 3 and 7 days after thawing, considering the timeline of acute inflammatory responses (Figure IV-5 B). Three days after cryopreservation, hTDCs show a decrease in IL-6 cytokine (p<0.001) and an increase in IL-10 (p<0.01) in comparison to hLDCs.

The release of COX-2, IL-6 and TNFα after 7 days was significantly reduced in hTDCs cryo (p<0.05). Other works demonstrated that cryopreservation could contribute to the inhibition of TNFα and IL-1β and to an increase in IL-10 in human MSCs 24h after thawing [8]. Taken together, these results suggest that cryopreservation/thawing may influence on desirable immunomodulatory communication by hLDCs and hTDCs, offering new possibilities to explore *in vitro* the intercellular networks between resident cell populations and immune cells driving healing events.

IV-4. General Discussion

The outcomes from this work confirm the viability and tissue specific functionalities of hLDCs and hTDCs after cryopreservation/thawing, including the preservation of stemness and tenogenic cues. Despite the complementary role of tendon and ligament, the panel of specific markers assessed identified few divergences between tendon and ligament cell responses. It is likely that these results may be restrained by the limited knowledge of signature markers for tendon and for ligament. This lack of signature markers justifies the need to expand our knowledge on tendon biology to advancing new strategies for improved tendon healing.

The exploitation of cryopreservation effect on cellular phenotype is pertinent in light of the global challenge of bench-to-bedside translational therapies. Cryopreserved cells can act as off the shelf living agents to assist therapeutic approaches, including immunotherapies. Furthermore, it is also important to assure the maintenance of signature markers that preserve cellular identity and their epigenetic memory driving tissue healing. Our results support that cryopreservation/thawing maintain the phenotype of these cells, and that hTDCs demonstrate the capacity to recover more rapidly than hLDCs.

We also investigated the role of cryopreservation/thawing process to the expression and synthesis of inflammatory mediators increasing the list of benefits for using tendon and ligament cryopreserved cells to study the resident cells' contributions for tissue healing. Cryopreserved tendon cells evidenced a more promising response regarding immunomodulatory cues in relation to hLDCs with higher expression and release of anti-inflammatory factors.

In sum, this study provides relevant information on the predictive behavior of hLDCs and hTDCs as suitable *in vitro* models with immunomodulatory competences to successfully engineer optimal therapeutic solutions towards T/L functional regeneration.

IV-5. SUPPLEMENTARY MATERIAL

Supplementary Table IV-1 Primers used for real time quantitative RT-PCR analysis.

	Primer Sequence	Accession number
Human Glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)*	F – TGTACCACCAACTGCTTAGC R - GGCATGGACTGTGGTCATGAG	NM_002046.4
Tenascin C *(TNC)*	F – ACTGCCAAGTTCACAACAGACC R – CCCACAATGACTTCCTTGACTG	NM_002160.3
Collagen, Type I, alpha 1 *(COL1A1)*	F – CGAAGACATCCCACCAATCAC R – GTCACAGATCACGTCATCGC	NM_000088.3
Collagen, Type III, alpha 1 *(COL3A1)*	F – GCTGGCTACTTCTCGCTCTG R – TTGGCATGGTTCTGGCTTCC	NM_000090.3
Decorin *(DCN)*	F – CAGCATTCCTCAAGGTCTTCCT R – GAGAGCCATTGTCAACAGCA	NM_001920.3
Collagen, Type I, alpha 1 *(COL1A1)*	F – CGAAGACATCCCACCAATCAC R – GTCACAGATCACGTCATCGC	NM_000088.3
Collagen, Type III, alpha 1 *(COL3A1)*	F – GCTGGCTACTTCTCGCTCTG R – TTGGCATGGTTCTGGCTTCC	NM_000090.3
Tenomodulin *(TNMD)*	F – CCGCGTCTGTGAACCTTTAC R – CACCCACCAGTTACAAGGCA	NM_022144.2

Mohawk ***(MKX)***	F – TGTTAAGGCCATAGCTGCGT R – TCGCACAGACACCTGGAAAA	NM_173576.5
Scleraxis ***(SCXA)***	F – CGAGAACACCCAGCCCAAAC R – CTCCGAATCGCAGTCTTTCTGTC	XM_001717912
Human cyclooxygenase-2 ***(COX-2)***	F – ATGGGGTGATGAGCAGTTGT R – GAAAGGTGTCAGGCAGAAGG	NM_000963.3
Human interleukin 6 ***(IL-6)***	F – AGGAGACTTGCCTGGTGAAA R – GCATTTGTGGTTGGGTCAG	NM_000600.4
Human tumor necrosis factor ***(TNFα)***	F – ATGTTGTAGCAAACCCTCAAGC R – TGATGGCAGAGAGGAGGTTG	NM_000594.3
Human interleukin 4 ***(IL-4)***	F – GCACCGAGTTGACCGTAACA R – AGGAATTCAAGCCCGCCAG	NM_000589.3
Human interleukin 10 ***(IL-10)***	F – AAGACCCAGACATCAAGGCG R – AATCGATGACAGCGCCGTAG	NM_000572.2

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Chapter V

Pulsed Electromagnetic Field Modulates Tendon Cells Response in IL-1β-Conditioned Environment

Chapter V

Pulsed Electromagnetic Field Modulates Tendon Cells Response in IL-1β- Conditioned Environment

ABSTRACT

Strategies aiming at controlling and modulating inflammatory cues may offer therapeutic solutions for improving tendon regeneration. This study aims to investigate the modulatory effect of pulsed electromagnetic field (PEMF) on the inflammatory profile of human tendon-derived cells (hTDCs) after supplementation with interleukin-1β (IL-1β). IL-1β was used to artificially induce inflammatory cues associated with injured tendon environments.

The PEMF effect was investigated varying the frequency (5 or 17 Hz), intensity (1.5, 4 or 5mT) and duty cycle (10 or 50%) parameters to which IL-1β-treated hTDCs were exposed to. A PEMF actuation with 4mT, 5Hz and a 50% duty cycle decreased the production of IL-6 and tumor necrosis factor-α (TNFα), as well as the expression of *TNFα, IL-6, IL-8, COX-2*, *MMP-1, MMP-2* and *MMP-3*, while *IL-4, IL-10* and *TIMP-1* expression increased.

These results suggest that PEMF stimulation can modulate hTDCs response in an inflammatory environment holding therapeutic potential for tendon regenerative strategies.

This chapter is based on the following publication: **Vinhas A.**, Rodrigues M.T, Gonçalves A.I, Reis R.L, Gomes M.E. Pulsed Electromagnetic Field Modulates Tendon Cells Response in IL-1β-Conditioned Environment, Journal of Orthopaedic Research, 38, doi: 10.1002/jor.24538, 2019

V-1. INTRODUCTION

Tendons are musculoskeletal tissues with limited healing and regenerative capacity. The inadequate capacity to heal towards mechano-competent efficiency and the involvement of inflammation mediators in tendon injuries in general and tendinopathic conditions in particular demands for new approaches to improve healing and to encourage regeneration [1-3].

Interleukin 1 beta (IL-1β) is a crucial pro-inflammatory mediator, secreted by immune cells and cells of some connective tissues at the inflammation site [4] with a pivotal role in both homeostatic and pathological mechanisms. Increased levels of IL-1β have been detected in tendons after prolonged mechanical loading (overuse), a known risk factor for tendinopathy [4]. The exogenous supplementation of IL-1β increases the expression of IL-6, COX-2 and MMPs [5-7] in tendon cells [8] and reduces the expression of tendon-associated genes such as scleraxis and tenomodulin and collagen 1 and 3 in injured tendon-derived progenitor cells (TPCs) [9]. Blocking IL-1β has been also reported to resolve inflammation in several inflammatory diseases [10]. The influence of IL-1β in stimulating inflammatory cues that are known to be present in tendon pathologies has motivated its use as an *in vitro* model to study biological responses to inflammation-conditioned niches. The strategies for successful tendon treatments may rely in the creation of innovative approaches to control and guide inflammation toward proper healing envisioning contributions for tendon regeneration.

Magnetic platforms have emerged as a technological and versatile field of research to improve cell-based therapies and tissue engineering and regenerative medicine approaches [11-14] enabling the design of non-invasive and remote-actuated systems to trigger and modulate physiological processes, stimulating healing, and regeneration. The clinical potential of magnetic stimulation is enormous. Studies in the literature reported a positive effect of magnetic actuation to improve healing of non-unions and delayed unions for treatment of bone fractures and cervical spine fusion surgery [15, 16].

In recent years, pulsed electromagnetic field (PEMF) actuation, which works by emitting a pulsating, varying intensity, and frequency electromagnetic field, was reported to modulate cell processes. PEMF was shown to decrease pro-inflammatory cytokines such as IL-1β and tumor necrosis factor-α (TNFα) and to increase anti-inflammatory cytokines as IL-10 by tendon cells *in vitro* [17-19], inhibited the synthesis of PGE2 and promoted anabolic activity of the chondrocytes [17, 19, 20].

In addition, PEMF induced a downregulation of TNFα and factor nuclear factor-κB (NF-κB) on macrophages, evidencing the potential of PEMF-actuated approaches for modulating inflammatory responses. Despite the promising outcomes and the remote actuation over inflicted tissues, the cellular and molecular mechanisms involved are still unveiled. Thus, we hypothesized that IL-1β can stimulate inflammatory cues in human tendon-derived cells (hTDCs) and be used as a model for inflammatory-compromised tendon niches. We also hypothesize that the inflammatory response of IL-1β-conditioned hTDCs can be modulated towards a non-inflammatory/repair response exposing hTDCs to PEMF actuation.

In this study, hTDCs were stimulated with different concentrations of IL-1β and exposed to six combinations of PEMF parameters, namely magnetic field intensity (1.5, 4 or 5mT), frequency (5 or 17Hz) and duty cycle (10 or 50%), some of which referred to hold potential in tendon inflammation-related studies [17-19]. As inflammation mediators have been associated to tendinopathic conditions, recreating the inflammatory cues of pathological environments combined with the potential of PEMF to modulate the inflammatory profile in hTDCs anticipates new opportunities approaching tendon regeneration.

V-2. MATERIALS AND METHODS

V-2.1. Cells: isolation and expansion

The hTDCs were isolated from surplus tissue samples of the knee tendons and ligaments, namely patellar tendon, collected from four adult patients undergoing orthopedic reconstructive surgeries under protocols previously established with Hospital da Prelada (Porto, Portugal) and with informed consent of the patients. The healthy nature of the tissues was confirmed at the surgery room by the orthopaedic surgeon. The hTDCs were isolated by enzymatic digestion of tendon samples with a solution of collagenase I from *Clostridium histolyticum* [21-23]. In brief, harvested samples were immersed in a sterile solution of phosphate-buffered saline (PBS; Sigma-Aldrich, Saint Louis, MO) with 2% antibiotic/antimicotic solution (A/A; Alfagene, Life Technologies Limited, Paisley, UK) before minced and digested in collagenase (C6885; Sigma-Aldrich, Saint Louis, MO) with 2 M $CaCl_2$ (VWR, Darmstadt, Germany) (1:1000) and 1% of bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO) for 1 h at 37 °C under agitation. The digested samples were filtered, centrifuged three times at 1200 rpm for 5 min, and the supernatant discarded. The hTDCs were expanded in α-MEM (A-MEM; Alfagene, Life

Technologies Limited, Paisley, UK) supplemented with 10% of fetal bovine serum (FBS) (Alfagene, Life Technologies Limited, Paisley, UK) and 1% A/A solution.

The multilineage differentiation potential and the cell markers panel for stemness profile had been determined and confirmed in previous studies using the described isolation protocol [22].

V-2.2. Inducing inflammatory cues in hTDCs with IL-1β

The hTDCs, from passage 2-4, were incubated for 16 h with serum-deprived αMEM medium and plated at 10,000 cells/cm^2. Afterward, the hTDCs were treated with exogenous IL-1β (Alfagene, Life Technologies Limited, Paisley, UK) (0.01-1 ng/mL) and cultured for 1, 2, 3 or 7 days. The cells were assessed for cell viability, proliferation, and messenger RNA (mRNA) transcriptional levels of metalloproteinases. The media was also collected and screened for inflammatory cytokines.

V-2.3. Assessing the PEMF effect on hTDCs response to IL-1β stimulation

After pre-selecting the best concentration of IL-1β (1 ng/mL), hTDCs were IL-1β treated for 24 h before 1 h exposure to different PEMF conditions using a Magnetotherapy device (Magnum XL Pro, Globus Corporation, Italy) (Table V-1). Afterward, the cells were cultured for 1, 2, 3 and 7 days and assessed for cell viability, proliferation, mRNA transcriptional levels of inflammatory-associated markers and protein deposition. A non-PEMF group (static control) was kept in identical conditions but without PEMF stimulation.

Table V-1 Experimental Scheme of PEMF Parameters on IL-1β-Treated hTDCs. The cells were exposed to one of the following conditions for 1 h.

Frequency (Hz)	Intensity (mT)	Duty Cycle (%)
5 or 17	1.5	10
		50
	4	10
		50
	5	10
		50

V-2.4. hTDCs characterization

V-2.4.1. Metabolic activity and proliferation

The metabolic activity and proliferation of hTDCs were evaluated by MTS assay (CellTiter96® AQueous One Solution, Promega Corporation, Madison) and Quant-It PicoGreen dsDNA assay Kit (Thermo Fisher Scientific, Molecular Probes, Eugene, OR), respectively. For the MTS assay, the samples were washed with PBS and incubated with a mixture of serum-free medium without phenol red and MTS solution (5:1 ratio) for 3 h at 37 °C and 5% CO_2, protected from light. The supernatant was then transferred to a 96-well plate and the absorbance read at 490nm (Synergy™HT, BIO-TEK Instruments, Winooski, Vermont). Samples were prepared in triplicate and a blank reading (no cells) was performed.

For the dsDNA quantification assay, the samples were washed with PBS and then transferred to a microtube containing 1 mL of sterile ultrapure water and kept at -80 °C until analysis. The samples were then thawed, sonicated, and analyzed at an excitation/emission wavelength of 485/528 nm. The samples and standards were made in triplicate.

V-2.4.2. Live and Dead staining

The influence of PEMF on cell viability was also determined by Live/dead assay. Cells were incubated for 20 min with calcein AM (2 μgmL^{-1}), and propidium iodide (3 μgmL^{-1}), both from from Life Technologies Limited, Paisley, UK. Afterward, the cells were washed with PBS and visualized under a fluorescence inverted microscope (Axio Observer, ZEISS, Göttingen, Germany).

V-2.4.3. Cytokine analysis

The release of pro-inflammatory cytokines, IL-6 and TNFα by hTDCs was determined at day 1 and 3 after treatment with IL-1β and PEMF stimulation in culture medium and stored at -80 °C until analysis. The IL-6 (900-K16, Peprotech, Rocky Hill, NJ) and TNFα (EK0525, Tebu-Bio, Fremont, CA) concentrations were quantified using commercial enzyme human immunoassay kits, following the manufacturer's instructions.

V-2.4.4. RNA Isolation and Gene Expression Analysis

The total RNA was extracted using TRI reagent® RNA Isolation Reagent (T9424, Sigma, Saint Louis, MO) following the manufacturer's instructions. RNA was quantified using a Nanodrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) at 260 nm. The first-strand complementary DNA was synthesized from 1 μg of RNA of each sample (qScript™ cDNA Synthesis Kit, Quanta Biosciences, Gaithersburg, MD) in a 20 μL reaction using a Mastercycler® ep realplex gradient S machine (Eppendorf, Hamburg, Germany).

The quantification of the transcripts was carried out by quantitative polymerase chain reaction (qPCR) using the PerfeCTA SYBR Green FastMix kit (Quanta Biosciences, Gaithersburg, MD) following the manufacturer's protocol, in a Real-Time Mastercycler ep realplex thermocycler (Eppendorf, Hamburg, Germany). The primers were designed with Primer3 software (Supplementary Table V-1) and synthesized by MWG Biotech. The $2^{-\Delta\Delta c_t}$ method was used to evaluate the relative expression for each target gene [24]. The transcript expression of target genes was analyzed and normalized to the expression of endogenous housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and then to the samples collected at day 0 (n=3).

V-2.4.5. Immunolocation analysis

The cells were washed 3 times with warm PBS, before and after fixation with 10% (v/v) neutral buffered formalin overnight and kept in PBS at 4 °C until usage. Subsequently, cells were incubated with 0.025% Triton-X100 in PBS solution (Sigma-Aldrich, Saint Louis, MO, USA) and the blocking step was performed using RTU Normal Horse Serum (RTU Vectastain Kit, PK-7200, Vector, Burlingame, California). The hTDCs cells were incubated overnight with connexin 43/GJA1, 1:100 (ab11370, Abcam, Cambridge, UK) and phospho-p44/42 MAPK, 1:200 (Cell Signaling Technology, Danvers, MA), diluted in antibody diluent with background reducing components (Dako, Santa Clara, CA) at 4 °C, followed by 1 h incubation at room temperature with the secondary antibody (donkey anti-rabbit Alexa Fluor 488, 1:200; Alfagene, Life Technologies Limited, Paisley, UK). The samples were washed three times with PBS and stained with 4,6-Diamidino-2-phenyindole, dilactate (DAPI, 5 μg/μl, D9564, Sigma-Aldrich, Saint Louis, MO) for 10 min and with Phalloidin-Tetra-methylrhodamine B isothiocyanate (Phalloidin), prepared according to the manufacturer's instructions (P1951, Sigma, 1:200; Sigma-Aldrich, Saint Louis, MO).

All samples were observed under a microscope (Imager Z1m, Zeiss, Göttingen, Germany) and images acquired using a digital camera (AxioCam MRm5). A minimum of two wells per sample, condition and endpoint were analyzed. Three independent experiments were investigated for protein detection by immunofluorescence.

V-2.4.6. Western Blot analysis

The total cell lysates were prepared using RIPA buffer (Sigma Aldrich, Saint Louis, MO) supplemented with protease inhibitor cocktail (Sigma Aldrich, Saint Louis). Samples were centrifuged for 15 min at 14000 rpm at 4 °C and the supernatants collected. Protein content was evaluated using the BCA protein assay kit (23225, Thermo Fisher Scientific) according to the manufacturer's instructions.

The protein extracts of 15 μg were resolved in 10% sodium dodecyl sulfate-polyacrylamide gels, followed by semi-dry transfer to Amersham™ Protran® Western blotting membranes (nitrocellulose; 10600002, GEHealthcare), using a Pierce Power Station (ThermoScientific). The membranes were blocked with 5% BSA in Tris-buffered saline with Tween 20 (Sigma-Aldrich, Saint Louis, MO) (TBS-T) and incubated with rabbit anti-phospho-p44/42 MAPK (Erk1/2) (1:1000) (9102, Cell signaling Technology, Danvers, MA) and anti-α smooth muscle actin (1:2000) (ab32575; Abcam, Cambridge, UK) antibodies 1hour at room temperature under mild agitation. The membranes were washed three times with TBS-T and then incubated with a secondary antibody (1:2000) (Anti-Rabbit IgG Alkaline Phosphatase antibody, A9919, Sigma-Aldrich, Saint Louis, MO) for 1 h at RT. A Colorimetric AP substrate reagent kit (1706432, BioRad, Hercules CA) was used for colour development.

V-2.5. Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). The statistical analysis was performed using GraphPad Prism6 software. Data was obtained from three independent experiments (n=3) analyzed in triplicate, and evaluated by two-way analysis followed by multiple comparison tests. A difference was considered significant with a confidence interval of 95% for different degrees of confidence, $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$. A multifactorial analysis was also performed to investigate the impact of the PEMF, IL-1β treatment and times, as well as their potential interactions (PEMF×IL-1β,

PEMF×time, IL-1β×time) in the expression of individual genes, using SPSS software (vs23) with a degree of confidence of $p<0.05$.

V-3. RESULTS

V-3.1. Assessment of Human Tendon-Derived Cells Response to an IL-1β-Induced Environment

The metabolic activity and proliferation of hTDCs treated with different concentrations of IL-1β (0.01, 0.1 and 1 ng/mL) increased between day 1 and 7, and tended to increase with increasing concentrations of IL-1β (Figure V-1 A).

The MTS values showed an increase 3 and 7 days after treatment with 1 ng/mL of IL-1β in comparison to all other concentrations (*$p<0.05$*) and to non-treated hTDCs (control without IL-1β, *$p<0.05$*). Also, dsDNA increased beyond 3 days in hTDCs treated with 1 ng/mL of IL-1β (in comparison to non-treated hTDCs, *$p<0.001$*).

In Figure V-1 B, secreted IL-6 and TNFα increased in IL-1β treated hTDCs (1 ng/mL of IL1β, *$p<0.0001$*) in an IL-1β concentration dependent manner. Moreover, a significant increase in metabolic activity, gene expression and release of cytokines was observed upon hTDCs stimulation with 1 ng/mL of IL-1β (*$p<0.05$*), (Figure V-1 C and 1D). The expression of *MMP-1*, *MMP-2* and *MMP-3* increased for 1 ng/mL of IL-1β *$p<0.05$*, (Figure V-1 C).

Altogether, the 1ng/mL of IL-1β stimulated more effectively inflammatory cues in hTDCs, being selected as the most promising condition to be investigated in the following PEMF-actuated experiments.

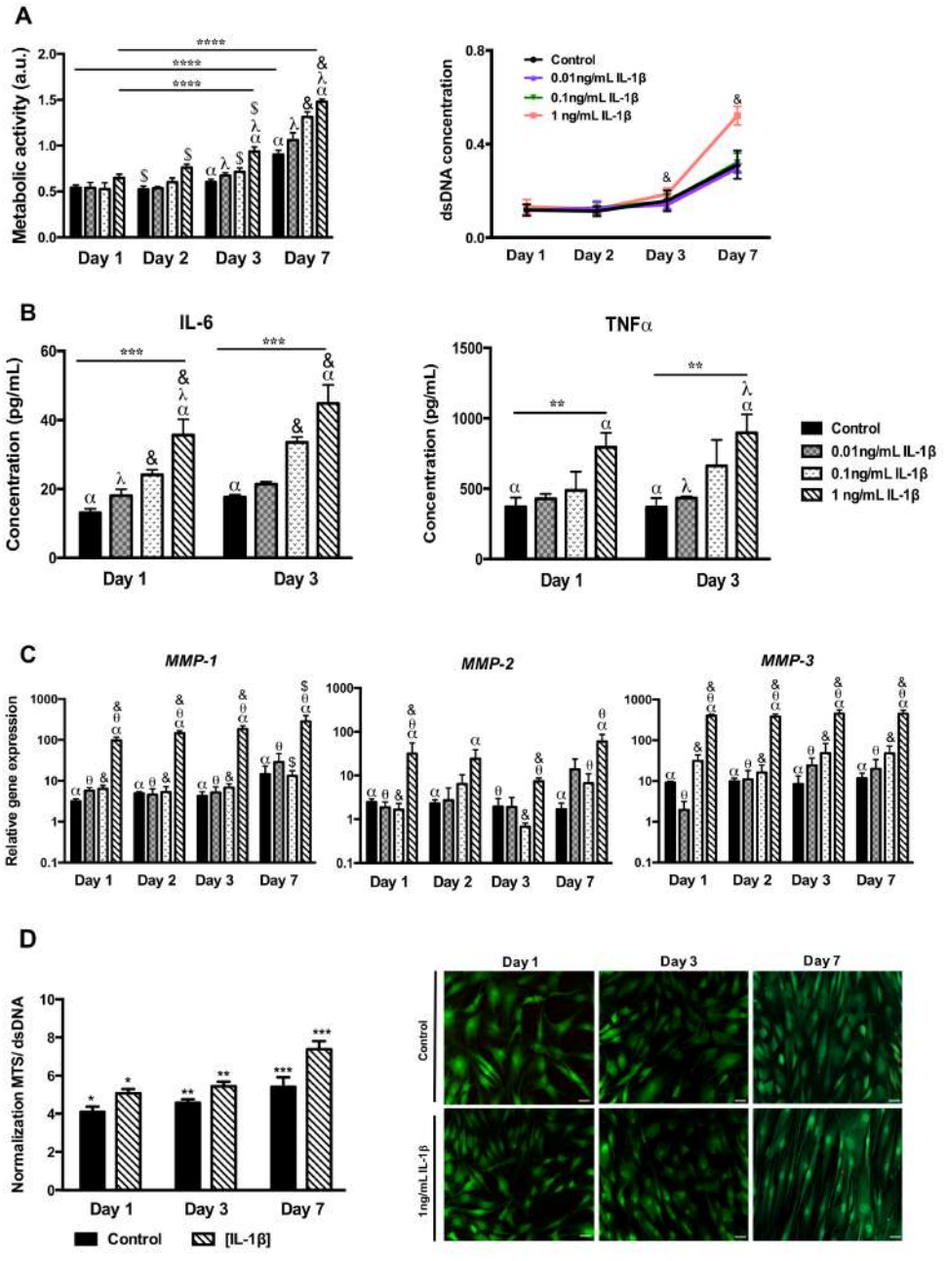

Figure V-1 Effect of interleukin-1β (IL-1β) concentration (0.01, 0.1, and 1 ng/ml) on human tendon-derived cells (hTDCs) for up to 7 days in culture. (A) The metabolic activity was determined by the MTS assay and the cell content was determined by the PicoGreen assay. (B) hTDCs relative expression of genes involved in extracellular matrix (ECM) remodeling: MMP-1, matrix metalloproteinase 1; MMP-2, MMP-3. (C) Profile of IL-6 and tumor necrosis factor-α (TNF-α) cytokines secreted by hTDCs treated with different concentrations of IL-1β after 1 and 3 days in culture. (D) Cell metabolic activity, viability, and morphology of hTDCs cultured in the presence of 1 ng/ml of IL-1β. Ratio of metabolic activity and dsDNA content of hTDCs after 1, 3, and 7 days in culture. The morphology and viability of hTDCs treated with 1 ng/ml of IL-1β was also assessed 1, 3, and 7 days after pulsed electromagnetic field (PEMF) actuation by live and dead staining. Viable and dead cells are represented in green and red, respectively (fluorescence microscopy ×20, scale bar 50 μm, merged images). Control condition refers to hTDCs without IL-1β treatment. Symbols *, $ denote statistical differences for $p < 0.05$; **, λ for $p < 0.01$; ***, &, θ for $p < 0.001$; and ****, α for $p < 0.0001$.

V-3.2. Assessment of PEMF Parameters on IL-1β-Treated hTDCs

After the establishment of the optimal concentration of IL-1β (1 ng/ml) to stimulate inflammatory cues on hTDCs, we investigated PEMF actuation to modulate the response of IL-1β treated hTDCs.

Thus, the effect of magnetic field parameters namely strength, frequency, and duty cycle were investigated on IL-1β treated hTDCs.

V-3.2.1. Effect of PEMF Actuation in Metabolic Activity and Cell Content of IL-1β Treated hTDCs

Having in consideration previous studies [17-19], different PEMF combinations of strength (1.5 mT, 4 mT, 5 mT), frequency (5 Hz, 17 Hz), and the duty cycle (10%, 50%) were assessed (Table V-1) on IL-1β treated hTDCs.

The metabolic activity of IL-1β treated hTDCs tended to increase with the time in culture for PEMF associated to both frequencies studied, 5 Hz and 17 Hz (Figure V-2 A and Figure V-3 A).

However, a decrease in the metabolic activity (Figure V-2 A and Figure V-3 A) and viability (Figure V-2 B and Figure V-3 B) of hTDCs was observed after exposure to a 5 mT PEMF with a 50% duty cycle, independently of the frequency applied.

Considering the detrimental exposure of hTDCs to 5mT strength PEMF combined with a 50% duty cycle, this condition was eliminated from the PEMF assessment of IL-1β treated hTDCs.

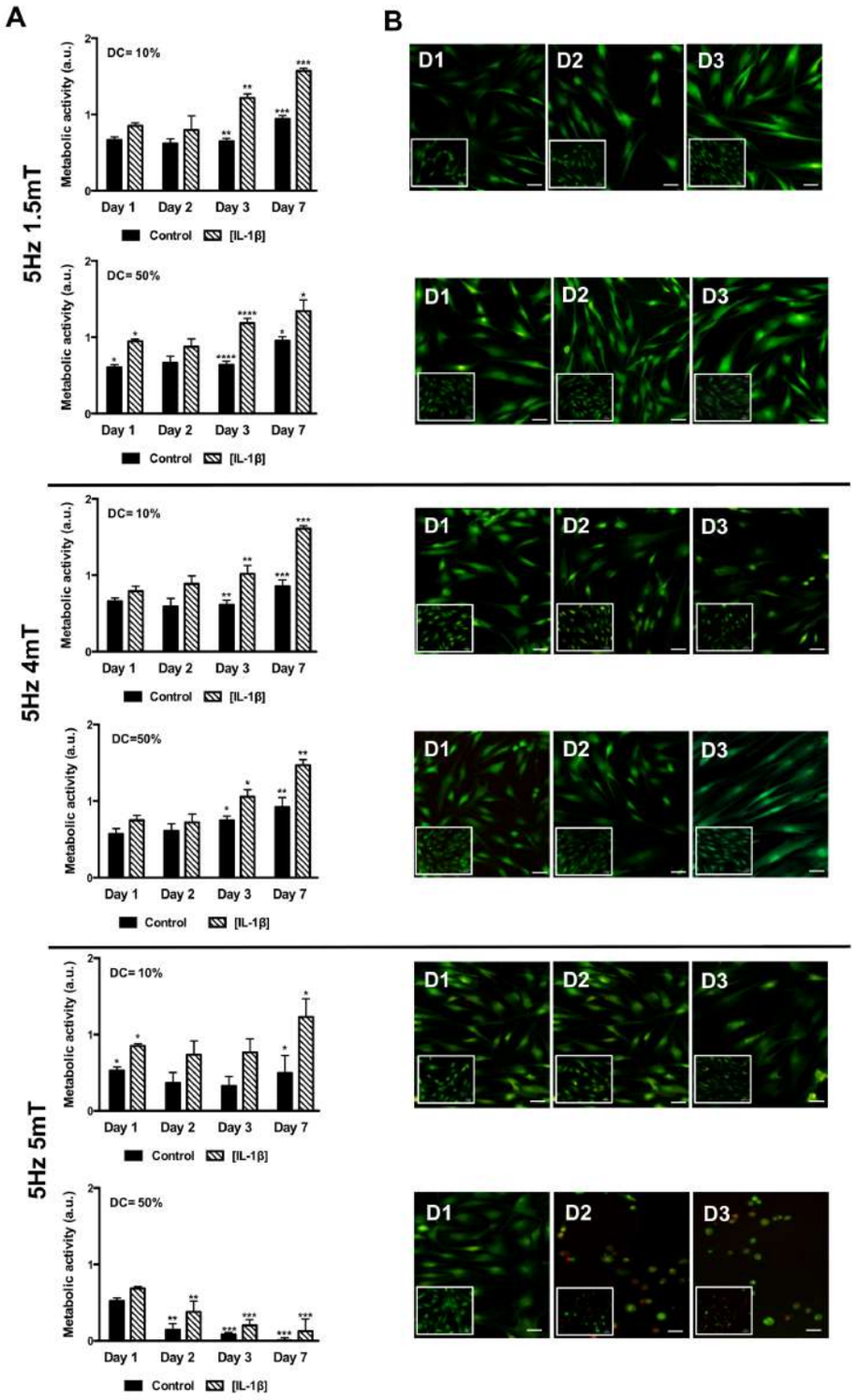

Figure V-2 Effect of 5 Hz PEMF in hTDCs treated with IL-1β up to 7 days in the culture. (A) Metabolic activity of hTDCs, with a variation of intensity (1.5–5 mT) and duty cycle (10–50%, DC, %). (B) Live and dead staining for viable (*green*) and dead cells (*red*) in hTDCs cells 1, 3, and 7 days (D1, D3, and D7), respectively, after PEMF actuation (fluorescence microscopy ×20, scale bar 50 μm, merged images). Insets are representative images of hTDCs without IL-1β treatment (control). Control condition refers to hTDCs without IL-1β treatment. Statistically, significant differences are shown with different degrees of confidence: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ in comparison with controls.

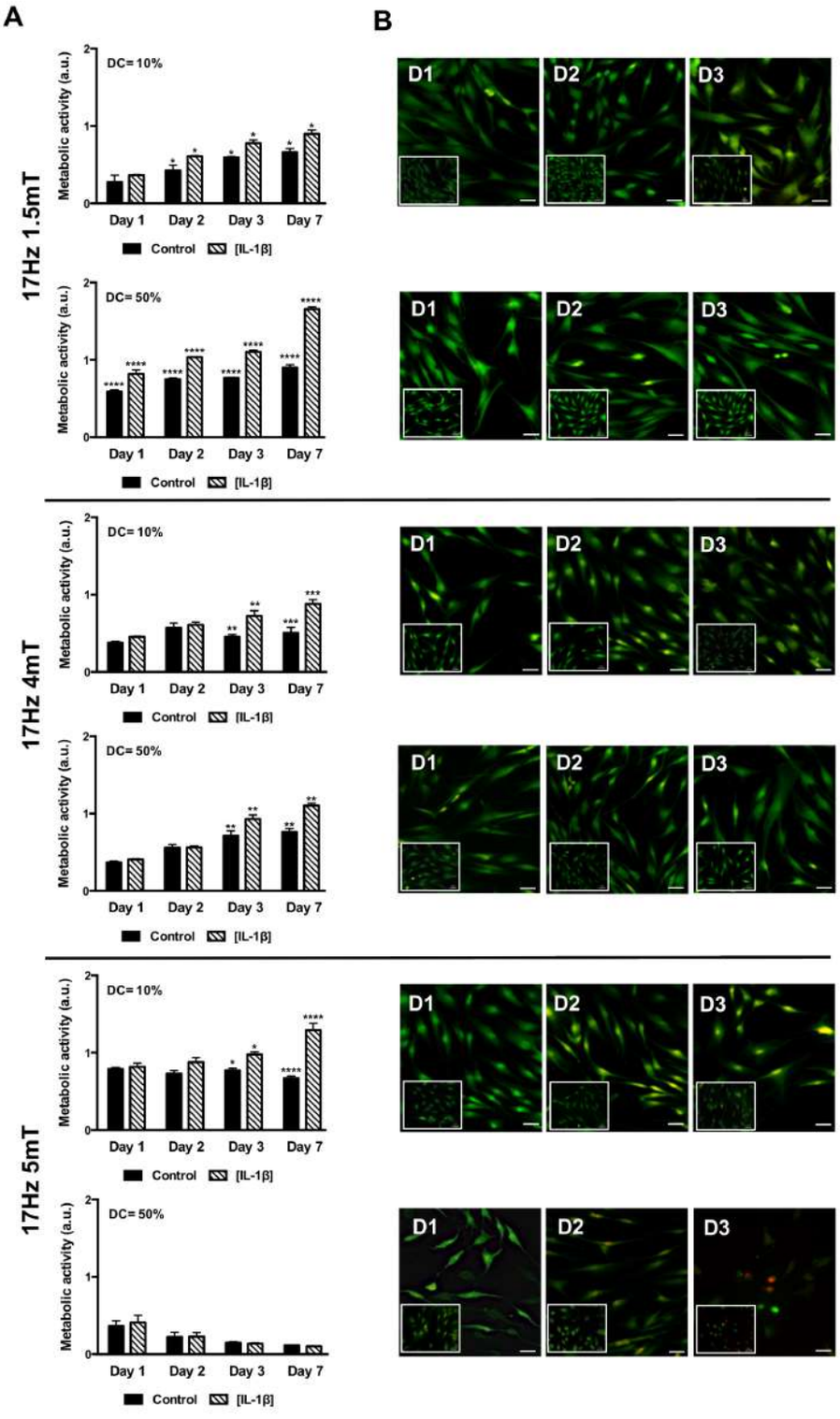

Figure V-3 Effect of 17 Hz PEMF in hTDCs treated with IL-1β up to 7 days in the culture. (A) Metabolic activity of hTDCs in function of intensity (1.5–5 mT) and duty cycle (10–50%, DC, %) of PEMF. (B) Live and dead staining for viable (*green*) and dead cells (*red*) in hTDCs cells 1, 3, and 7 days after actuation (fluorescence microscopy × 20, scale bar 50 μm, merged images). Insets are representative images of hTDCs without IL-1β treatment (control). Statistically significant differences are shown with different degrees of confidence *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$ in comparison with controls.

V-3.2.2. Influence of PEMF in the Cytokine Release Profile of IL-1β Treated hTDCs

The effect of PEMF parameters in the release of pro-inflammatory cytokines, IL-6 and TNFα, by IL-1β treated hTDCs are presented in Figure V-4 and Figure V-5, respectively.

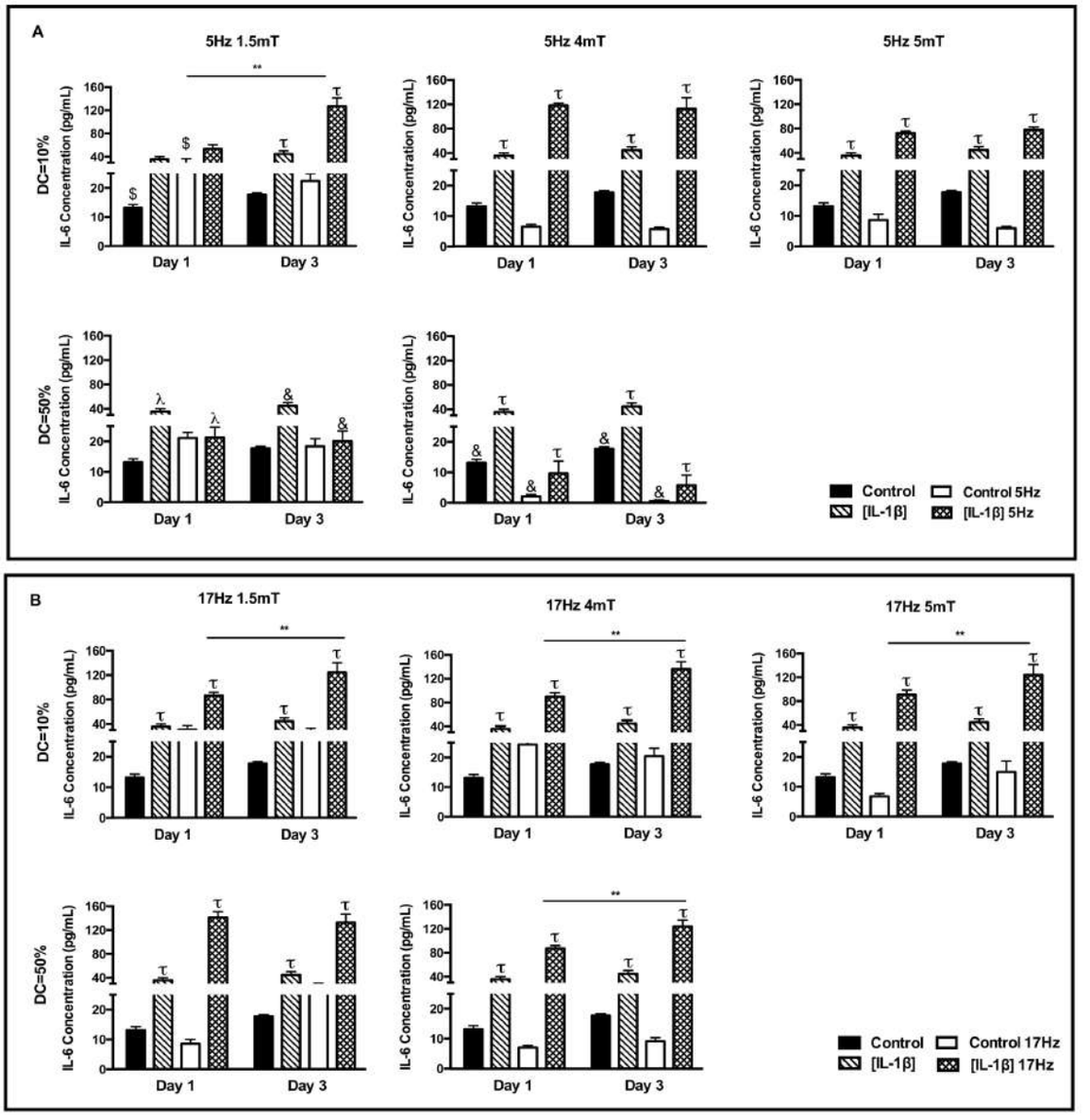

Figure V-4 Release of IL-6 after hTDCs treatment with IL-1β and PEMF stimulation. (A) The effect of 5 Hz PEMF in IL-6 levels secreted by hTDCs with a variation of intensity (1.5–5 mT) and duty cycle (10–50 DC, %); (B) The effect of 17 Hz PEMF in IL-6 levels secreted by hTDCs, with a variation of intensity (1.5–5 mT) and duty cycle (10–50 DC, %). IL-6 was quantified in the culture medium 1 and 3 days after IL-1β and PEMF stimulation. Statistically significant differences are shown with different degrees of confidence: $ for $p < 0.05$; ** and λ for $**p < 0.01$; & for $p < 0.001$, and τ for $p < 0.0001$.

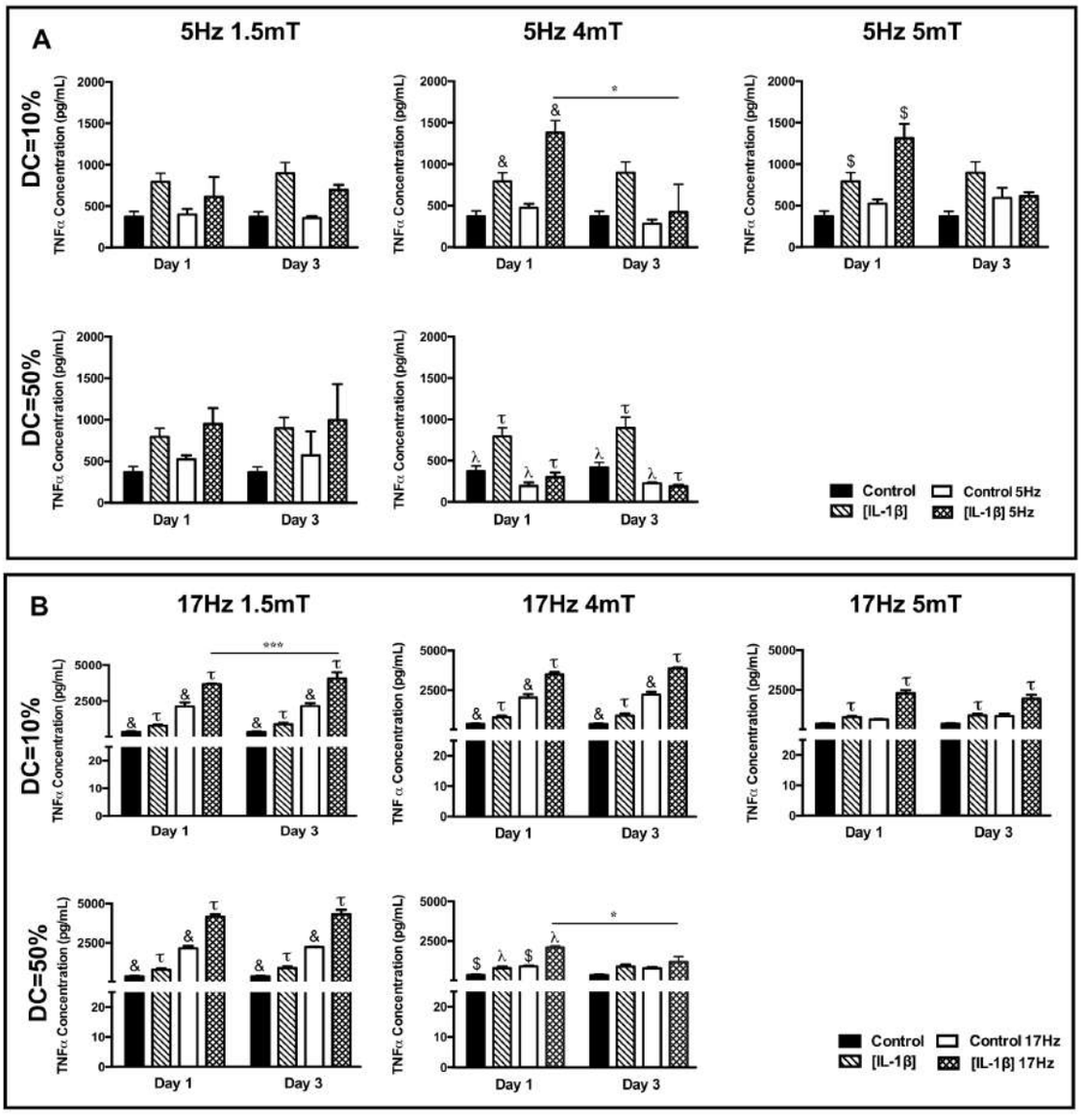

Figure V-5 TNFα secreted after hTDCs treatment with interleukin 1β (IL-1β) and PEMF stimulation. (A) The effect of 5 Hz PEMF in TNFα levels secreted by hTDCs with a variation of intensity (1.5–5 mT) and duty cycle (10–50 DC, %); (B) The effect of 17 Hz PEMF in IL-6 levels secreted by hTDCs, with a variation of intensity (1.5–5 mT) and duty cycle (10–50 DC, %), TNFα was quantified in the culture medium 1 and 3 days after IL-1β and PEMF stimulation. Statistically significant differences are shown with different degrees of confidence: *, $ for $p < 0.05$; λ for $p < 0.01$; ***, & for $p < 0.001$, and τ for $p < 0.0001$.

In non-PEMF conditions, IL-6 and TNFα concentrations tended to increase in IL-1β treated hTDCs. When a PEMF is applied, IL-6 concentration increased with 5Hz PEMF combined with a 10% duty cycle for a strength of 1.5 mT, 4 mT or 5 mT (Figure V-4 A) but not with a 5 Hz, 4 mT PEMF 50% duty cycle, in which IL-6 decreases in comparison with controls (*$p<0.001$*), (Figure V-4 A). The application of 17Hz PEMF resulted in a strongly increased IL-6 release in all conditions in comparison with IL-1β treated hTDCs without PEMF actuation (*$p<0.0001$*), (Figure V-4 B).

The release of TNFα tended to increase after PEMF exposure (Figure V-5 A and B), with the exception for the 5 Hz and 4 mT PEMF with a 50% duty cycle. As for the IL-6 release, the concentration of TNFα increased in 17 Hz PEMF conditions (Figure V-5 B).

In summary, hTDCs showed a reduced cytokine release after 1 h stimulation with a 5 Hz and 4 mT of intensity PEMF combined with 50% of duty cycle, suggesting a modulatory effect on pro-inflammatory cytokines in both physiological and inflammation-conditioned niches. Thus, this PEMF condition was further investigated in the expression of inflammation-related genes of IL-1β treated hTDCs.

V-3.2.3. PEMF Effect on the Genetic Profile of IL-1β Treated hTDCs

The gene expression of pro-inflammatory factors (*TNFα*, *IL-6*, *IL-8*, *IL-1β*, *COX-2)* was increased in IL-1β treated hTDCs in comparison with control ($p<0.01$) (Figure V-6 A). Furthermore, 5 Hz and 4 mT PEMF combined with 50% of duty cycle reduced the genetic expression of *IL-6*, *TNFα*, *COX-2* and *IL-8* in both non-treated and IL-1β treated hTDCs ($p<0.05$). In the particular case of *TNFα* and *COX-2*, the values found for IL-1β treated cells stimulated with PEMF are lower than non-PEMF stimulated hTDCs ($p<0.05$).

Conversely, PEMF stimulated hTDCs showed an increase in *IL-4* and *IL-10* in both IL-1β treated and non-treated cells ($p<0.05$) (Figure V-6 B). Overall, PEMF stimulated cells showed increased anti-inflammatory gene expression over non-stimulated cells, with IL-1β treated hTDCs and non-treated hTDCs expressing similar values.

The ECM remodelling and maturation during healing depend on the balance of catabolic MMPs and anabolic TIMPs. Thus, *MMP-1, -2,* -3 and *TIMP-1* levels were also evaluated (Figure V-6 C). *MMP-1*, *-2* and *-3* were up-regulated in IL-1β treated hTDCs in comparison with non-treated cells 1, 2 and 7 days ($p<0.05$) after IL-1β supplementation (Figure V-6 C). After PEMF actuation, MMPs expression was considerably decreased in both IL-1β treated and non-treated hTDCs ($p<0.05$). However, *TIMP-1* levels increased in non-treated hTDCs and decreased in IL-1β treated hTDCs 7 days after treatment ($p<0.01$).

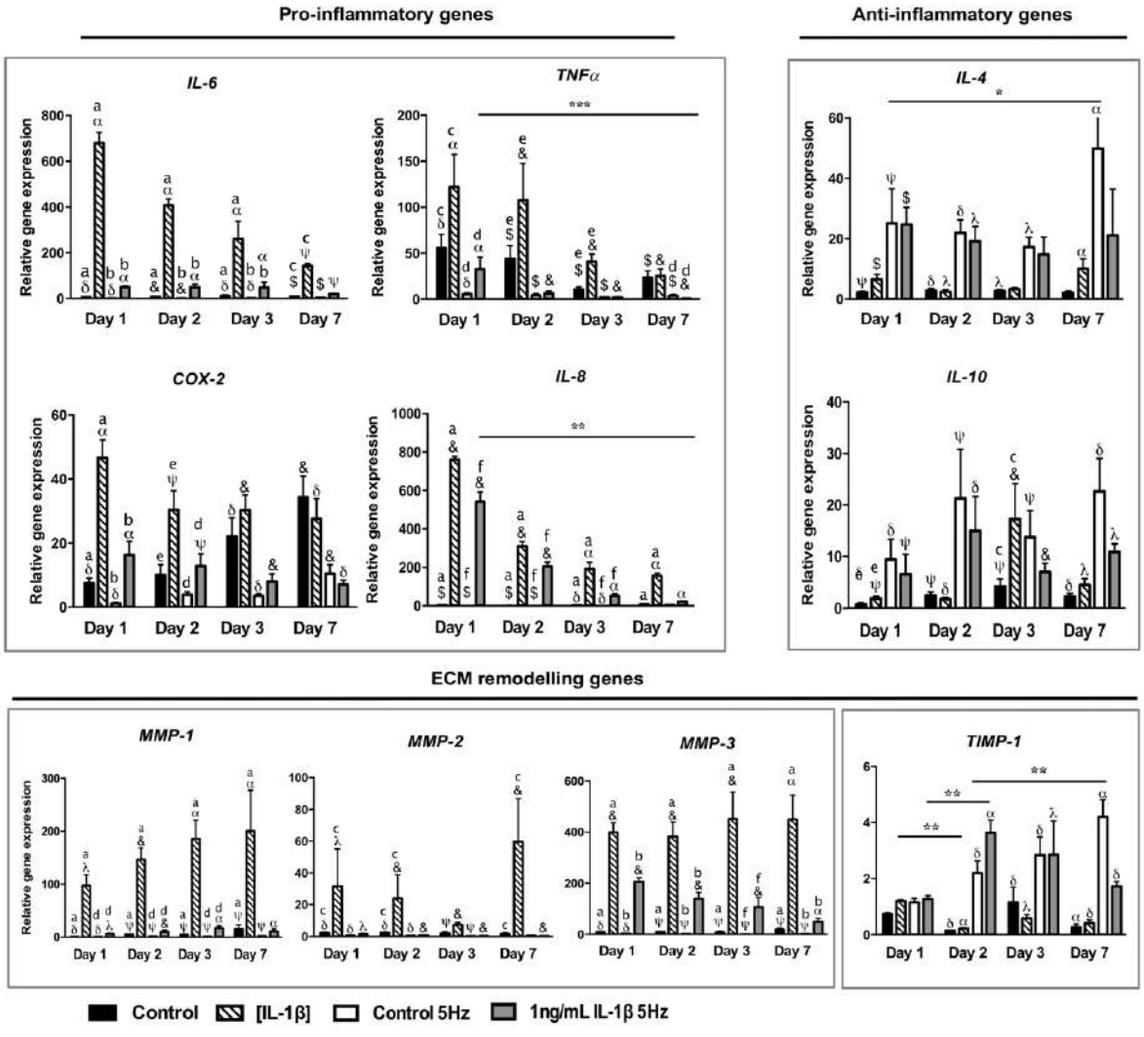

Figure V-6 Relative expression of genes related to inflammation and extracellular matrix (ECM) remodeling in human tendon-derived cells (hTDCs) treated with interleukin 1β (IL-1β) and stimulated with pulsed electromagnetic field (PEMF) by reverse-transcription polymerase chain reaction (RT-PCR) analysis. (A) Relative expression of pro-inflammatory genes: *IL-6*, *TNFα*, *IL-8*, and *COX-2*. (B) Relative expression of anti-inflammatory genes: *IL-4* and *IL-10*. (C) Relative expression of ECM-associated remodeling genes: *MMP-1*, *MMP-2*, *MMP-3*, and *TIMP-1*. Results are presented as mean ± standard error of the mean (SEM) (**n** = 3). Data were analyzed using a two-way analysis of variance (ANOVA) followed by multiple comparisons tests (GraphPad Prism). Control condition refers to hTDCs without IL-1β treatment. Statistically significant differences are shown with different degrees of confidence: *, Ψ, $, d, e for $p < 0.05$; **, λ, δ, c for $p < 0.01$; ***, &, a, b for $p < 0.001$ and α, f for $p < 0.0001$.

The multifactorial analysis performed showed that *TNFα* expression is significantly affected by all the parameters assessed: PEMF (*p=0.002*), IL-1β (*p=0.026*), and time (*p=0.042*) while *COX-2* and *TIMP-1* are influenced by the interaction of PEMF and time (*p=0.002* and *p=0.004*, respectively). *IL-4* is both influenced by time (*p=0.037*) and by the interaction of IL-1β with PEMF (*p=0.025*).ECM-related genes, namely *MMP-1*, is strongly influenced by time (*p=0.011*) and by the interaction effects of time with PEMF

(*p=0.021*) and of time with IL-1β (*p=0.041*). Interestingly, PEMF particularly influences the *MMP-2* expression (*p=0.014*) while *MMP-3* is more influenced by the PEMF and time interactions (*p=0.028*).

In summary, the cytokine gene profile was changed upon exposure of hTDCs to a PEMF with 5 Hz, 4 mT and an applied 50% duty cycle, evidencing the PEMF impact in the expression of inflammation-related genes. Moreover, the interactions between PEMF and time of culture parameters also seem to play a role in the expression of inflammatory cytokine genes and ECM-related genes.

V-3.2.4. PEMF Effect on the Cell-Cell Communication of IL-1β Treated hTDCs

Cx43 expression associated to the cell-cell dissemination of inflammatory cues is lower in IL-1β treated hTDCs after exposure to PEMF (Figure V-7 A).

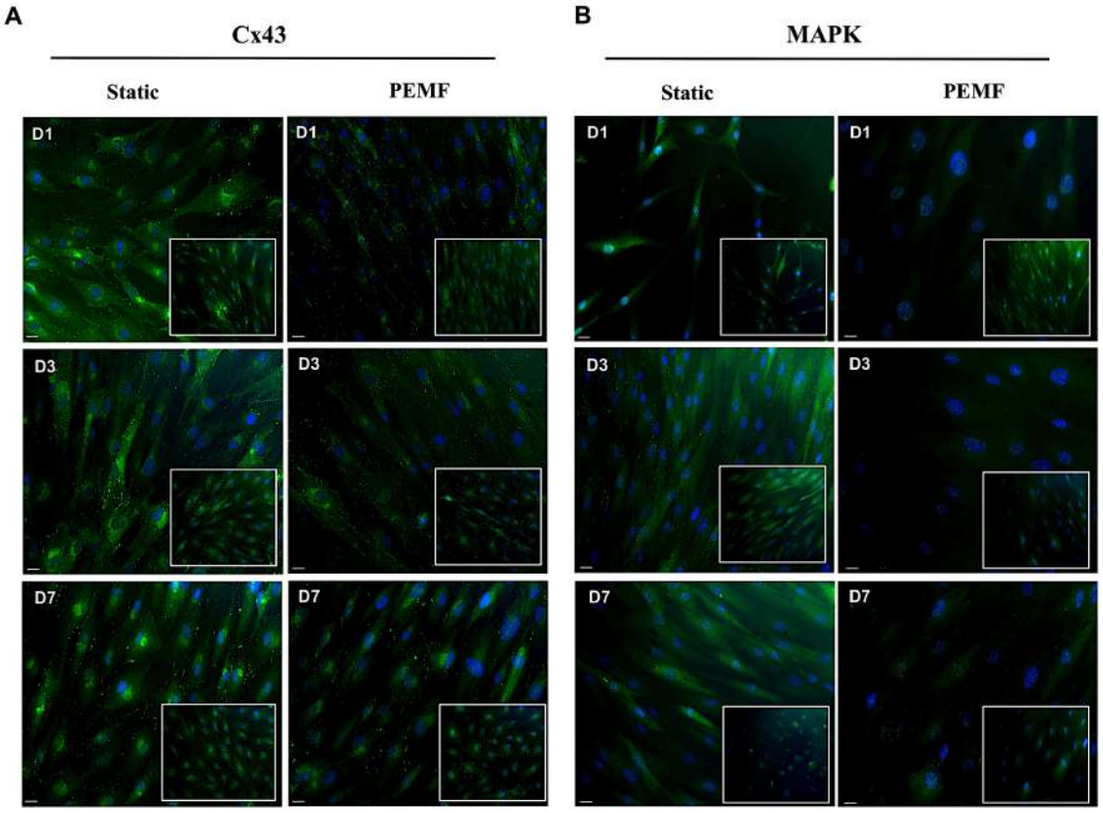

Figure V-7 Representative images of Cx43 and MAPK expression in human tendon-derived cells (hTDCs) treated with interleukin-1β (IL-1β) and stimulated with pulsed electromagnetic field (PEMF). Fluorescence images of (A) Cx43 (green), nucleus (blue) and (B) MAPK (green), nucleus (blue) immunolocation in hTDCs cells after 1, 3, and 7 days in the culture, D1, D3, and D7, respectively (fluorescence microscopy ×20, scale bar 50 μm). Insets are representative images of hTDCs without IL-1β treatment (control, bottom right) and to the negative control (top right).

The influence of IL-1β treatment and PEMF stimulation in endogenous MAPK(Erk1/2) is not as clear as for Cx43. MAPK(Erk1/2) immunolocation shows a decrease in the total protein after PEMF stimulation (Figure V-7 B).

Western blot outcomes indicate a differential detection in p42 and p44 bands of phospho-MAPK(Erk1/2). By day 1 it is increased in static control conditions (day 1) and in IL-1β treated hTDCs exposed to PEMF (day 1). With the exception of static control conditions, it tends to decrease by day 3 (Figure V-8).

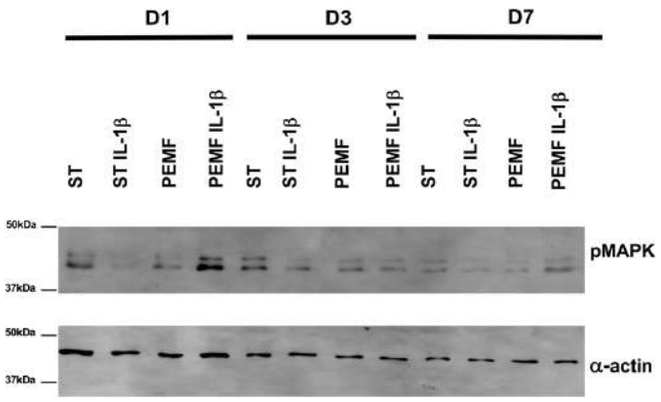

Figure V-8 Western blot assay for the detection of phosphorylated MAPK (pMAPK (Erk1/2). Whole cell protein lysates were analyzed by probing for phospho-MAPK(Erk1/2) and α-smooth muscle actin (control) in human tendon-derived cells (hTDCs) cells treated with interleukin 1β (IL-1β) for 1, 3, and 7 days in the culture (D1, D3, and D7) after pulsed electromagnetic field (PEMF) stimulation (two experimental replicates from two biological replicates).

V-4. DISCUSSION

The role of inflammation cues in healing and regeneration depends on coordinated events with the release of bioactive molecules and precise cell-to-cell communication.

Previous works have demonstrated IL-1β as an inflammation model *in vitro* [25, 26], being able to stimulate inflammatory mediators including COX-2, MMPs, TNFα and IL-6 [5].

In our study, the concentrations of IL-1β investigated increased cell proliferation beyond 3 days post-treatment ($p<0.05$), which may relate to the IL-1β influence in cell proliferation. Moreover, the significant increment in *MMPs* (-1, -2 and -3) around 100-fold suggests that hTDCs respond to IL-1β treatment. MMPs are responsible for the degradation of collagens and proteoglycans and have been linked to the degenerative changes in chronic tendinopathy [27].

The release of pro-inflammatory cytokines IL-6 and TNFα tended to increase in IL-1β concentration dependent manner, which also suggests the role of IL-1β as an inflammatory inducer for hTDCs. Although these outcomes were expected and complied with other published works [28], they were necessary to establish the optimal IL-1β concentration (1 ng/ml) to induce inflammatory cues on hTDCs without causing detrimental or cytotoxic effect to the cells and to set IL-1β model for future tendon-related studies.

In recent years, magnetic-actuated technologies propose to offer remote control and non-invasive tools to trigger and control biological processes, therefore, the promise of therapeutic value for the management and treatment of tissue pathologies. The tendon cells exposed to a 75 Hz, 1.5 mT magnetic field for 8 h showed an increment on IL-10 expression without affecting the production of pro-inflammatory cytokines [19, 29], while rat Achilles tendons showed a reduction of inflammation signals after a 17 Hz PEMF exposure for 15 min, 5 sessions a week [20]. However, studies on PEMF actuation require further insights for the identification of the main parameters or a combination of these holding a therapeutic action to potentiate proper healing at the cellular and tissue levels. Thus, we investigated sets of PEMF parameters, namely frequency, intensity, and duty cycle in modulating hTDCs cytokine profile.

An increase in cell metabolic activity, as well as an elongated hTDCs shape, was observed after PEMF exposure suggesting that the PEMF conditions investigated did not exert a cytotoxic effect. An exception was observed in hTDCs exposed to 5 mT PEMF and 50% duty cycle indicating this condition is not favorable for cellular processes. Also, these results suggest the combination of 5 mT and 50% duty cycle has more impact in cell viability than any of the frequencies applied. Overall, the PEMF parameters studied showed potential to modulate inflammatory cues of tendon cells enabling external control in non-invasive applications to assist regeneration, whose parameters can be adjusted if desirable, along the temporal healing timeline to promote the most suitable therapeutic action.

In the present study, IL-1β induces an increment in IL-6 and TNFα secretion, antagonized by PEMF actuation with 5 Hz, 4 mT and 50% of duty cycle. This effect is also verified in non-treated cells, suggesting that PEMF may also influence cytokine profile in physiological conditions. In terms of gene expression, the PEMF application shows a modulatory effect in the genetic profile of IL-1β treated hTDCs. As mRNA levels of inflammatory cytokines are remarkably upregulated in injured tendons [9], the inhibition of IL-1β via PEMF actuation could be beneficial for tendon-healing strategies. These outcomes are consistent with those of other studies applying magnetic forces combined with 5 Hz, 17 Hz or 75 Hz frequencies [17, 19, 29]. Accordingly, the strong IL-1β-induction of *MMP-1* and *MMP-3* expression has been

demonstrated in rabbit Achilles tendon cells exposed to cyclic strain and inflammatory cytokines [30]. In our work, PEMF stimulation not only decreases *MMPs* expression but favors *TIMP-1* increment, suggesting a tentative action of hTDCs towards an anabolic ECM turnover.

Connective tissues as tendons are excellent coupled cell networks that can facilitate/stop the spread of inflammatory cues [31]. The cell-cell communication established via gap junction channels as connexin 43 is of extreme importance to prevent or perpetuate inflammatory signals between cells. IL-1β was reported to stimulate an increase in Cx43 hemichannels [32] via activation of MAPK, iNOS, COX_2 and PGE_2. IL-1β was also described to increment the production of pro-inflammatory factors (IL-1, IL-17, IL-8, TGFβ and TNFα) in inflammation-associated diseases via MAPK pathway [33]. Further, the upregulation of Cx43 by synovial fibroblasts has been associated with production of pro-inflammatory factors [34], proposing a functional connection between IL-1β, Cx43 and the propagation of inflammation signals to neighboring cells.

In our study, the expression of Cx43 in IL-1β treated hTDCs was decreased after exposure to PEMF, which can limit the IL-1β-mediated signaling cascade. Besides the validation of IL-1β to induce inflammatory cues on the hTDCs, enabling studies on inflammation-conditioned niches, our outcomes disclose the potential role of PEMF as an antagonist of hTDCs response to the presence of pro-inflammatory signals. The actuation of 5 Hz 4 mT PEMF combined with 50% duty cycle led to a decrease in the expression of pro-inflammatory and MMP genes and to a diminished expression of Cx43.

MAPK signaling has been described to mediate multiple cell responses [35, 36] and could be involved in the response of IL-1β-conditioned tendon cells stimulated with PEMF. The tendon cells expressed pMAPK(Erk1/2) in all conditions studied and immunochemistry studies pointed that it was initially localized within the nucleus. Although phosphorylation of MAPK(Erk1/2) is typically associated to a translocation into the nucleus, cytoplasmic pMAPK(Erk1/2) also enables control over cell fate [37], whose expression is increased in static conditions. Moreover, in this study, p42 and p44 bands of pMAPK(Erk1/2) were detected in all conditions by western blotting. Interestingly though is the fact that thicker bands were identified in static control and IL-1β treated hTDCs exposed to PEMF. The increment in pMAPK(Erk1/2) of hTDCs exposed to PEMF treated with IL-1β did not correlate with the gene downregulation of pro-inflammatory markers TNFα and COX-2 (in comparison with PEMF or IL-1β alone). These results suggest that complex signaling networks are likely involved in triggering inflammatory cues in tendon cells and that these cues can be modulated by magnetic actuation.

V-5. CONCLUSIONS

The findings of this work show that PEMF parameters of 5 Hz, 4 mT, and 50% duty cycle can modulate the response of tendon cells to induced inflammatory stresses, influencing intracellular mechanisms at the gene and protein expression, envisioning the applicability of magnetic-actuated therapies for tendon-healing approaches.

V-6. SUPPLEMENTARY MATERIAL

Supplementary Table V-1 List of primers used for quantitative RT-PCR analysis.

	Primer Sequence	Accession number
Human Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	F - TGTACCACCAACTGCTTAGC R - GGCATGGACTGTGGTCATGAG	NM_002046.4
Human matrix metalloproteinase-1 (*MMP-1*)	F - ACCTGGAAAAATACTACAACCTGAA R – TTCAATCCTGTAGGTCAGATGTGTT	NM_002421.3
Human matrix metalloproteinase-2 (*MMP-2*)	F - GCTACGATGGAGGCGCTAAT R – TCAGGTATTGCACTGCCAACT	NM_002422.4
Human matrix metalloproteinase-3 (*MMP-3*)	F - CACTCACAGACCTGACTCGG R – AGTCAGGGGGAGGTCCATAG	NM_002422.4
Human metalloproteinase inhibitor-1 (*TIMP-1*)	F- CATCCGGTTCGTCTACACCC R- GGATAAACAGGGAAACACTGTGC	NM_003254.2
Human interleukin 4 (*IL-4*)	F- GCACCGAGTTGACCGTAACA R- AGGAATTCAAGCCCGCCAG	NM_000589.3
Human interleukin 6 (*IL-6*)	F- AGGAGACTTGCCTGGTGAAA R- GCATTTGTGGTTGGGTCAG	NM_000600.4
Human interleukin 8 (*IL-8*)	F- GGTGCAGTTTTGCCAAGGAG R- TTCCTTGGGGTCCAGACAGA	NM_001354840.1
Human interleukin 10 (*IL-10*)	F- AAGACCCAGACATCAAGGCG R- AATCGATGACAGCGCCGTAG	NM_000572.2
Human cyclooxygenase-2 (*COX-2*)	F- ATGGGGTGATGAGCAGTTGT R- GAAAGGTGTCAGGCAGAAGG	NM_000963.3
Human tumor necrosis factor (*TNFα*)	F- ATGTTGTAGCAAACCCTCAAGC R- TGATGGCAGAGAGGAGGTTG	NM_000594.3

V-7. REFERENCES

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Chapter VI

Magnetic Stimulation Drives Macrophage Polarization in Cell to–Cell Communication with IL-1β Primed Tendon Cells

Chapter VI

Magnetic Stimulation Drives Macrophage Polarization in Cell to–Cell Communication with IL-1β Primed Tendon Cells

ABSTRACT

Inflammation is part of the natural healing response, but it has been simultaneously associated with tendon disorders, as persistent inflammatory events contribute to physiological changes that compromise tendon functions. The cellular interactions within a niche are extremely important for healing. While human tendon-derived cells (hTDCs) are responsible for the maintenance of tendon matrix and turnover, macrophages regulate healing switching their functional phenotype to environmental stimuli. Thus, insights on the hTDCs and macrophages interactions can provide fundamental contributions on tendon repair mechanisms and on the inflammatory inputs in tendon disorders. We explored the crosstalk between macrophages and hTDCs using co-culture approaches in which hTDCs were previously stimulated with IL-1β. The potential modulatory effect of the pulsed electromagnetic field (PEMF) in macrophage-hTDCs communication was also investigated using the magnetic parameters identified in a previous work. The PEMF influences a macrophage pro-regenerative phenotype and favors the synthesis of anti-inflammatory mediators. These outcomes observed in cell contact co-cultures may be mediated by FAK signaling. The impact of the PEMF overcomes the effect of IL-1β-treated-hTDCs, supporting PEMF immunomodulatory actions on macrophages. This work highlights the relevance of intercellular communication in tendon healing and the beneficial role of the PEMF in guiding inflammatory responses toward regenerative strategies.

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VI-1. INTRODUCTION

Tendon pathologies are among the most debilitating orthopedic problems due to poor tissue response to currently available treatments, affecting both elderly and active populations. Tendon lesions are often multifactorial and frequently occur due to trauma, overuse activities, and aging. However, unresolved inflammation seems to be a transversal stage in all tendon disorders. Persistent inflammatory events may lead to chronic/degenerative changes in tendon structure, impairing tissue functionality and increasing the risk of re-injury [1, 2]. It is thus imperative to create new strategies to improve tendon repair and address therapeutic benefit for patients with tendon diseases [3].

In damaged tendons, tenocytes are exposed to leukocytes and inflammatory mediators. Macrophages (Mϕ), in particular, undergo distinct phenotypes and specific functional changes in response to local microenvironment signals to regulate tissue repair and regeneration [4]. In an initial response, activated macrophages (M1 subtype) are responsible for the release of pro-inflammatory cytokines (e.g., TNFα, IL-1β, IL-6, IL-12, IL-23), trophic factors as chemokines and signaling molecules into the repair site in order to promote inflammation, extracellular matrix (ECM) degradation, and debris clearance. In the later stages, pro-regenerative macrophages (M2 subtype) coordinate ECM deposition, secreting anti-inflammatory mediators (e.g., IL-4, IL-10, IL-13, TGFβ) and cell-attracting chemokines (e.g., CCL17, CCL22, and CCL24) to attenuate and assist the resolution of inflammatory events and promote tendon repair [5-7].

Although immune cells are necessary for tendon repair, their persistent activation can result in incomplete resolution of inflammation [8] and may lead to chronic injury [9]. Recent studies indicated that the adaptive and innate immune systems work with tissue resident cells to coordinate tissue repair [10], suggesting that the mechanisms to counteract inflammatory stimuli may be insufficient in natural tendon injury. Consequently, this limited intrinsic response creates new opportunities to investigate the interplay of immune cells and human tendon-derived cells (hTDCs) envisioning new molecular and cellular treatment possibilities. Thus, in this work, we approach co-culture methods of Mϕ and hTDCs to investigate intercellular communication and the interactions mediated by secreted messengers in fine-tuning cell responses after hTDCs exposure to pro-inflammatory cues (IL-1β supplemented medium), likely present in tendon injuries.

Reinforcing the physiological role of intercellular communication in the tendon, recent works demonstrated that mesenchymal stem cells (MSCs) could “educate” macrophages via paracrine

mechanisms into M2 phenotype to improve tendon healing [7] and that tenocytes isolated from ruptured tendons responded to a soluble pool of inflammatory factors, being able to influence macrophage polarization [11].

Together with complex dynamics of regulatory signals, modulation of tendon immune biology events, including the switch of macrophage functional phenotypes, may be assisted by external triggers [12]. Research has shown that the pulsed electromagnetic field (PEMF), a biophysical form of stimulation, accelerates cell differentiation, increases deposition of collagen, and modulates the activation of cell surface receptors, thereby holding relevant contributions for the re-establishment of homeostatic cell functions [13]. Thus, magnetic-based approaches have the potential to become an alternative treatment modality for regenerative therapies [14].

Low-frequency PEMF can modulate inflammatory response in MSCs and macrophages [13, 15], and specific parameters of PEMF were also shown by our group to modulate the cytokine profile of IL-1β conditioned tendon cells [16]. Therefore, magnetic stimulation may play an important role in the inflammatory process of injured tissues, resulting in enhanced functional recovery and support for tissue regeneration. Hence, in this work, we also studied the influence of the PEMF to modulate the interactions occurring between macrophages and IL-1β treated tendon cells.

VI-2. MATERIALS AND METHODS

VI-2.1. Isolation and Cell Expansion of Human Tendon-Derived Cells

hTDCs were isolated from surplus tissue samples collected from patients undergoing orthopedic reconstructive surgeries under protocols previously established with Hospital da Prelada (Porto, Portugal) and with informed consent of the patients. The content of the written informed consent and related procedures were reviewed and approved by the Hospital Ethics Committee (P.I. N.º005/2019).

Following a previous established protocol [35-37], tendon samples were minced and digested in an enzymatic solution of collagenase I (0.1 %, Sigma-Aldrich, C6885, Saint Louis, MO, USA) with 2M $CaCl_2$ (1:1000, VWR, Darmstadt, Germany) and 1 % bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO, USA) for 1 h at 37 °C under agitation. After incubation, digested samples were filtered and centrifuged three times at 1200 rpm for 5 min, and the supernatant was discarded. Isolated hTDCs were

expanded in basic culture medium composed of α-MEM (A-MEM; Invitrogen, Life Technologies Limited, Paisley, UK) supplemented with 10 % fetal bovine serum (FBS) (Alfagene, Life Technologies Limited, Paisley, UK) and 1 % antibiotic/antimicotic solution (A/A) (Alfagene, Life Technologies Limited, Paisley, UK) in a humidified 5 % CO_2 atmosphere. hTDCs from passage 2 to 4 were used to perform all the assays.

VI-2.2. Macrophage Culture and Differentiation

THP-1 cells, a human monocytic cell line that is extensively used to study monocyte/macrophage function and biology, was cultured and expanded in RPMI culture medium (Sigma-Aldrich, Saint Louis, MO, USA), supplemented with 1 % A/A in humidified 5 % CO_2 atmosphere. THP-1 derived macrophages were differentiated with 100 nM phorbol 12-myristate-13-acetate (PMA, Sigma-Aldrich, Saint Louis, MO, USA) for 24 h, followed by 24 h cultivation with PMA-free medium. Non-attached cells were removed by aspiration and the adherent THP-1 derived macrophages were washed three times with RPMI and further expanded in RPMI medium.

VI-2.3. Establishment of Co-Cultures Systems

In vitro co-cultures were established to explore potential crosstalk between macrophages and hTDCs under an inflammatory environment. hTDCs (10,000 cells/cm^2) were seeded onto 24-well plates (BD Biosciences, San Jose UK) and treated with IL-1β (1 ng/mL, Alfagene, Life Technologies Limited, Paisley, UK) for 24 h to induce inflammatory cues in hTDCs, as previously reported [16]. Co-cultures were established by culturing the macrophages (10,000 cells) either i) on top of seeded hTDCs (direct cultures) or ii) in the chamber of a Transwell™ (pore size: 1 μm; Corning, VWR, Darmstadt, Germany) placed over cultured hTDCs (indirect paracrine cultures) (Figure V-1). Upon seeding for 24 h, co-cultures were exposed to a PEMF stimulation regimen: 5 Hz, 4 mT, and 50% duty cycle for 1 h, following previous studies by our group [16] using a magnetotherapy device (Magnum XL Pro; Globus Corporation, Codogné,Italy). Co-cultures were further cultured for one day upon PEMF exposure, and then assessed for tendon and macrophage phenotypic markers (transmembranar and surface markers), focal adhesions, and for genetic and secreted cytokine profile. Outcomes were compared with single cultures of hTDCs or macrophages as appropriate.

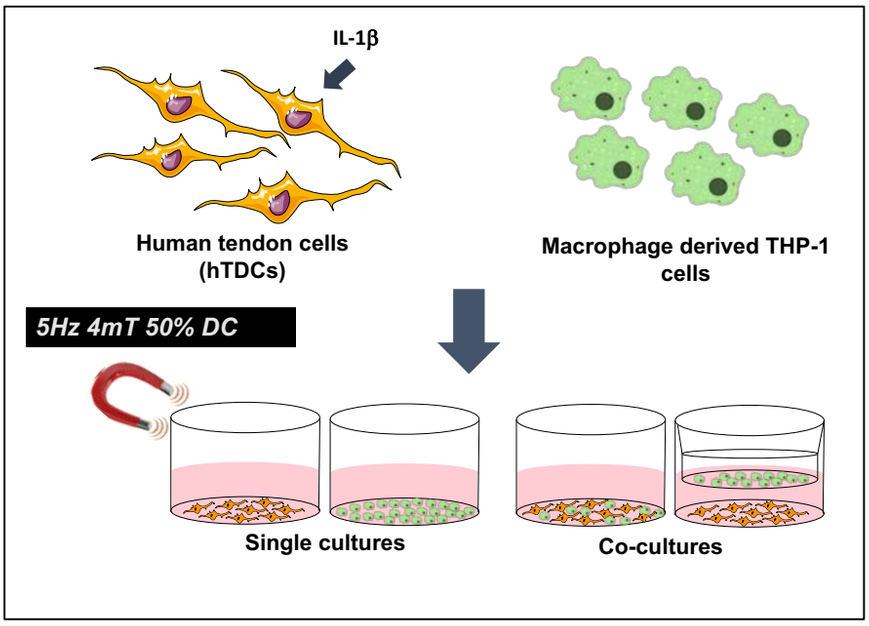


Figure VI-1 Magnetic modulation of intercellular communication between macrophage and tendon cells treated with IL-1β using co-culture systems. Single cultures were considered as experimental controls. A magnetic stimulation regimen was applied at 5 Hz, 4 mT, and 50 % duty cycle.

VI-2.4. Characterization of Tendon and Macrophage Phenotype in Co-Culture Systems

VI-2.4.1. RNA Isolation and Gene Expression Analysis

Total RNA was extracted using TRI reagent® RNA Isolation Reagent (T9424; Sigma-Aldrich, Saint Louis, MO, USA) following the manufacturer's instructions. RNA was quantified using a Nanodrop® ND-1000 spectrophotometer (Wilmington, DE, USA) at 260/280 nm. The first-strand complementary DNA was synthesized from 1 µg of RNA of each sample (qScript™ cDNA Synthesis Kit, Quanta Biosciences, Gaithersburg, MD, USA) in a 20 µL reaction using a Mastercycler® ep realplex gradient S machine (Eppendorf, Hamburg, Germany).

The quantification of the transcripts was carried out by quantitative polymerase chain reaction (qPCR) using the PerfeCTA SYBR Green FastMix kit (Quanta Biosciences, Gaithersburg, MD, USA) following the manufacturer's protocol, in a Real-Time Mastercycler ep realplex thermocycler (Eppendorf, Hamburg, Germany). The primer sequences (Supplementary Table VI-1) were designed with Primer 3 software and synthesized by MWG Biotech. The $2^{-\Delta\Delta_{Ct}}$ method was used to evaluate the relative expression level for each target gene [38].

The transcript expression of target genes (*MKX*, *SCX*, *COL1A1*, *MMP-1*, *MMP-3*, *TIMP-1*, *Arg-1*, *MRC-1*, *Singlec-1*, *NOS-2*, *IL-10*, *IL-6*, *TNFα*, *IL-4*, *IL-10*, *IL-1β*) was analyzed and normalized to the expression of endogenous housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and then to the samples collected at day 0 (n = 3).

VI-2.4.2. Quantification of Secreted Cytokines

The supernatants of single and co-cultures were assessed with respect to cytokine concentrations using commercially available enzyme immunoassay kits for Arg-1 (Arginase-1 Human ELISA Kit, BMS2216; Invitrogen, Carlsbad, California, USA), iNOS (Human Inducible nitric oxide synthase ELISA Kit, MBS723617; Mybiosource, San Diego, California, USA), IL-1β (Human IL-1β Standard ABTS ELISA Development Kit, 900-K95; Peprotech, Rocky Hill, New Jersey, USA), and IL-10 (Human IL-10 Standard ABTS ELISA Development Kit, 900-K21; Peprotech, Rocky Hill, New Jersey, USA) following the manufacturer's instructions.

VI-2.4.3. Assessment of Intracellular Cytokines in Direct Co-Cultures

The cells retrieved from direct co-cultures of hTDCs and macrophages were treated during 4 h with 10 µg/mL Brefeldin A (ab193369, Abcam, Cambridge, UK) to block cytokine secretion before being trypsinized using TrypLE Express (12605-028; Alfagene, Life Technologies Limited, Paisley, UK), centrifuged, and resuspended in fresh PBS. Afterwards, cells in suspension were incubated with fluorochrome-conjugated antibodies: anti-IL-1β (FITC; ab16168, Abcam, Cambridge, UK) and anti-IL-4 (Phycoerythin; ab95717; Abcam, Cambridge, UK) for 20 min at room temperature protected from light. Cells were then rinsed in PBS and centrifuged for 5 min at 800 *g*.

The cells were resuspended in 500 µL of acquisition buffer and data acquired in a FACSAria III sorter equipped with blue and red lasers (BD Biosciences, Erembodegem-Aalst, Belgium). Cells were identified by forward and side scatter. A minimum of 10,000 cells were acquired and analyzed using FACS Diva version 7 software. Unstained cells were considered as negative controls.

The positive population of cells expressing the markers of interest was expressed in percentage values. Data acquired and analyzed is representative of three independent experiments.

VI-2.4.4. Detection of Inflammatory Mediators in Direct Co-Cultures by Immunofluorescence

Cells were fixed with a solution of 10 % (*v/v*) neutral buffered formalin (Bio Optica, Milano, Italy) overnight, and kept in PBS at 4 °C until usage. Subsequently, cells were incubated with 0.025 % Triton-X100 (Sigma-Aldrich, Saint Louis, MO, USA) in PBS and the blocking step was performed using RTU Normal Horse Serum (RTU Vectastain Kit, PK-7200; Vector, Burlingame, California, USA). Cells were incubated for 1 h at 4 °C with anti-FAK (phosphor Y397), 1:200 (ab39967, Abcam, Cambridge, UK), or connexin 43/GJA1, 1:100 (ab11370; Abcam, Cambridge, UK), both diluted in antibody diluent with background reducing components (Dako, Santa Clara, CA, USA), followed by incubation for 1 h at room temperature with the secondary antibody (donkey anti-rabbit Alexa Fluor 488, 1:200, Alfagene, Life Technologies Limited, Paisley, UK). The direct co-cultured cells were also incubated with fluorochrome-conjugated antibodies: anti-IL-1β (FITC; ab16168, Abcam, Cambridge, UK) and anti-IL-4 (Phycoerythin; ab95717; Abcam, Cambridge, UK) for 30 min at room temperature protected from light.

Samples were washed three times with PBS before stain with 4,6-Diamidino-2-phenyindole, dilactate (DAPI, 5 μg/μl, D9564; Sigma-Aldrich) for 10 min, prepared according to the manufacturer's instructions. The outcomes of protein detection are representative of three independent experiments.

The detection of immunostained proteins, IL-4, IL-1β, FAK, and Cx43, was assessed by confocal laser scanning microscopy (CLSM, TCS SP8, Leica, Wetzlar, Germany) using 63x magnification objective and LAS X software from Leica. Images were bidirectionally scanned at 400 Hz with Argon (488) and UV (405) lasers.

The semi-quantification of mean fluorescence intensity was performed for FAK and Cx43. A minimum of five images per sample were analyzed from independent experiments. The contribution of macrophage and hTDCs to the mean fluorescence intensity was assessed measuring the signal intensity in macrophages using multiple ROI. The hTDCs signal was calculated subtracting the mean signal intensity of macrophages to the initial raw images using ImageJ software version 1.52a, National Institutes of Health, USA.

VI-2.5. Statistical Analysis

Results are expressed as mean ± standard error of the mean. In the case of flow cytometry, results are expressed as mean ± standard deviation. The statistical analysis was performed using GraphPad

Prism software version 6.0, San Diego, USA. Data was obtained from three independent experiments (*n* = 3) analyzed in triplicate and evaluated by one-way or two-way ANOVA followed by Bonferroni post-hoc test for multiple comparison tests. A difference was considered significant with a confidence interval of 95%. Different degrees of confidence, p <0.05, p <0.01, p <0.001, and p <0.0001 are represented by symbols *****, p, γ, $, λ for *p* <0.05; ******, α, β, θ, σ for p <0.01; ***, #, δ for p <0.001; and ****, ψ, &, ε for p <0.0001.

VI-3. RESULTS

VI-3.1. The hTDCs and Macrophages Phenotype Profile in Response to Co-Cultures Systems and to PEMF Stimulus

The cell-to-cell contact model was chosen for this study, as it enables both secretory and direct contact forms of communication, more closely preserving the intercellular networking and physiological behavior of populations. Since we were also exploring the effect of magnetic stimuli and IL-1β conditioned cells in the modulation of the heterotopic cell crosstalk, we investigated indirect co-cultures to confirm if cell metabolic activity and the gene transcripts of tendon and immune-associated markers were not more affected by paracrine effects rather than cellular interactions. The co-culture models were subjected to either the control basal medium (Ctrl) or medium supplemented with IL-1β cytokine (IL-1β). Both Ctrl and IL-1β treated cultures show similar metabolic activity profiles (Supplementary Figure VI-S1), independently of cell–cell contact degree or PEMF stimulus. However, the indirect co-cultures show significantly lower values in comparison to direct co-cultures (p>0.05 only for the Ctrl condition).

VI-3.1.1. Impact of Co-Cultures in the Transcript Profiles of Tendon- and Immune-Related Genes

A tendency for upregulation of tendon-related markers, namely, Mohawk (*MKX)*, Scleraxis *(SCX)*, and Collagen type 1 (*COL1A1)* was observed in both co-culture systems (Figure VI-2 A). Unlike *MKX*, *SCX*, and *COL1A1* expression in co-cultures are similar to the ones in the hTDCs group (p>0.05). Although *MKX* expression in co-cultures is lower than those in the hTDCs group, *MKX* levels are higher in direct co-cultures when compared to indirect co-cultures (direct IL-1β non-PEMF, p<0.001; indirect IL-1β non-

PEMF, $p<0.0001$). As expected, the expression of tenogenic genes in macrophage group is basal or downregulated.

The expression of *MMP-1* and *MMP-3* shows distinctive behavior in direct and indirect co-cultures. In the former, the upregulation of MMPs seems to be associated to PEMF/non-PEMF conditions, while in indirect co-cultures, the differences among the conditions studied are not so evident ($p>0.05$). In particular, *MMP-1* shows increased expression in direct co-cultures conditioned to IL-1β (non-PEMF, $p<0.001$) (Figure VI-2 B). Interestingly, the *TIMP-1* expression is upregulated in both types of co-culture, and it is not significantly affected by IL-1β (Figure VI-2 B).

The macrophage genetic profile was considered by measuring the relative expression of phenotype-switch genes *Arg-1*, *MRC-1*, *Singlec-1*, and *NOS-2* (Figure VI-2 C). In both types of co-culture, M2-like markers *Arg-1*, *MRC-1*, and *Singlec-1* show a similar pattern to the ones of macrophages group, despite the incremental expression in PEMF conditions for all these genes. The *NOS-2* expression, a M1-like marker, shows increased values in indirect co-cultures, independently of the culture medium or PEMF stimulation.

The profile of cytokine genes was also studied (Figure VI-2 D). The anti-inflammatory *IL-10* showed lower levels in indirect cultures. Nevertheless, the pro-inflammatory *IL-6* was upregulated in all conditions of indirect cultures, but not for PEMF conditions in direct co-cultures. With the exception for direct cultures treated with IL-1β, the *TNFα* expression tends to be downregulated.

Overall, the genetic expression in direct systems show values closer to hTDCs or macrophage control groups, and with distinctive patterns among the conditions studied. These outcomes suggest that the physical communication between cells enable contact-mediated signals that are not perceived via soluble factor–cell signaling.

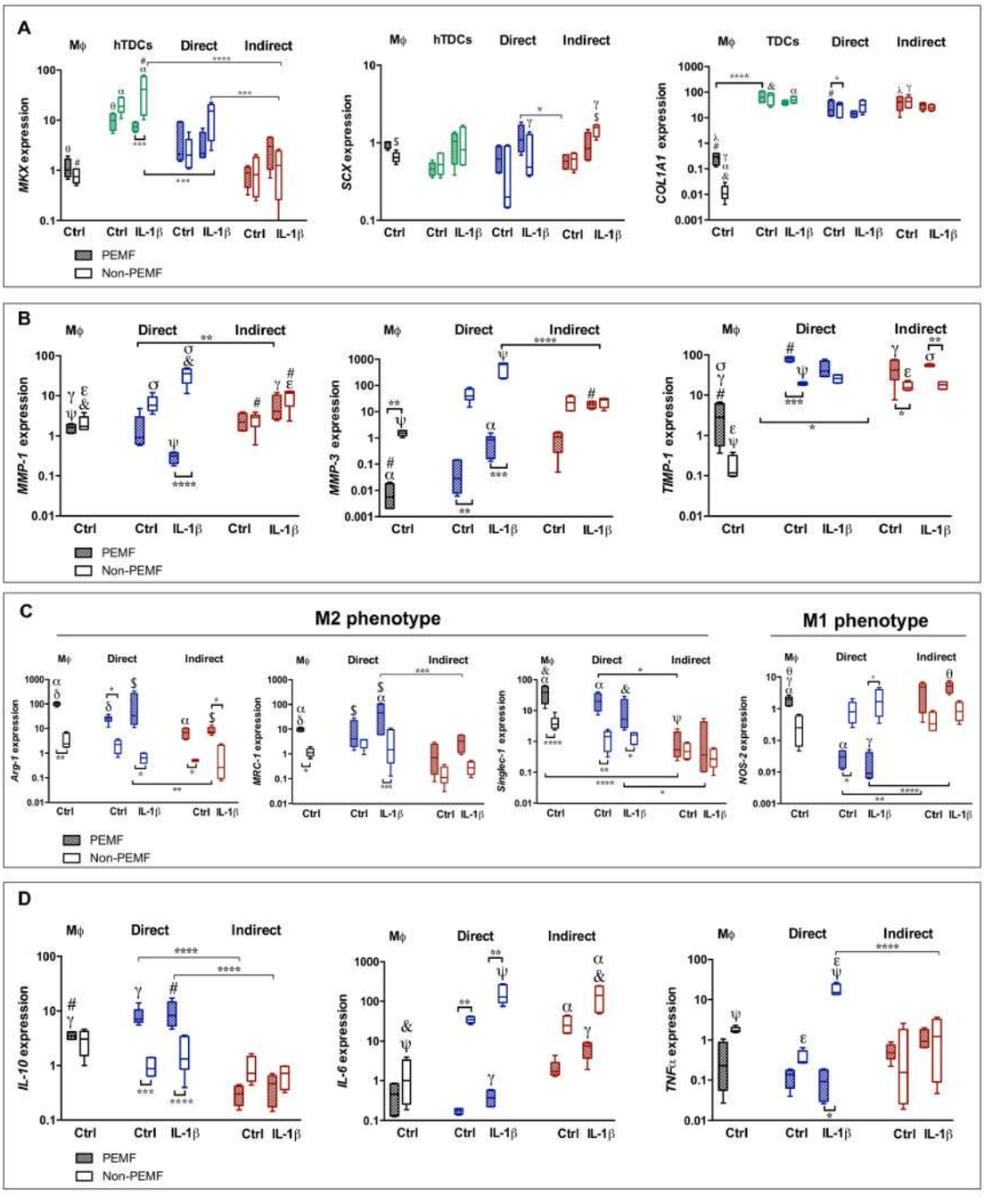


Figure VI-2 Pulsed electromagnetic field (PEMF) actuation affecting tenogenic and macrophage phenotype in co-culture models 24 h after PEMF actuation. Analysis of the gene expression of (A) tendon-related genes (*MKX*, *SCX*, and *COL1A1*), (B) extracellular matrix (ECM) remodeling genes (*MMP-1*, *MMP-3*, and *TIMP-1*), (C) markers associated to macrophage phenotype (*Arg-1*, *MRC-1*, *Singlec-1*, and *NOS-2*), and (D) cytokines (*IL-10*, *IL-6*, and *TNFα*). Control condition (Ctrl) refers to the absence of IL-1β treatment. Statistically significant differences are shown with different degrees of confidence. Symbols *, γ, $, denote statistical differences for p<0.05; **, α, θ, σ for p<0.01; ***, #, δ for p<0.001; and ****, ψ, &, ε for p<0.0001, respectively.

VI-3.1.2. Effect of the PEMF on Gene Transcription Levels in Co-Culture Systems

The effect of the PEMF was also approached in direct and indirect communication between hTDCs and macrophages.

In terms of tenogenic gene expression, *MKX*, *SCX*, and *COL1A1* were upregulated, independently of the PEMF (Figure VI-2 A). An exception is *MKX* in the IL-1β treated hTDCs group (*MKX* shows lower values under PEMF application, $p<0.01$ and *COL1A1* is enhanced in Ctrl conditions in direct co-cultures, $p<0.0001$).

In direct cultures, a dissimilar pattern of the *MMPs* and *TIMP-1* in PEMF and non-PEMF conditions is observed. PEMF actuation decreases the expression of *MMP-1* and *MMP-3* (Figure VI-2 B) and upregulates *TIMP-1*. In the case of *MMP-1*, this effect is especially noted in direct cultures treated with IL-1β ($p<0.0001$). Interestingly, PEMF causes a downregulation of *MMP-3* in direct cultures and in macrophage group, while in non-PEMF conditions, *MMP-3* is upregulated, independently of IL-1β treatment (Ctrl group, $p<0.01$ and IL-1β, $p<0.001$).

Relatively to *TIMP-1*, the gene was upregulated in all conditions, with the highest expression found in direct co-cultures actuated by PEMF and without IL-1β treatment (indirect cultures, $p<0.05$ and macrophage group under PEMF, $p<0.001$). These results comply with the upregulation of *COL1A1*, suggesting an anabolic effect of the PEMF over the synthesis of collagen type I.

Arg-1, *MRC-1*, and *Singlec-1* (M2) were upregulated in direct cultures with higher expression in the PEMF condition in both Ctrl and IL-1β treated cells ($p<0.05$) (Figure VI-2 C). In indirect co-cultures, the PEMF effect only affects *Arg-1* expression ($p<0.05$). However, expression of *NOS-2* (M1) was downregulated in direct co-cultures under the PEMF in comparison to indirect cultures (Ctrl PEMF, $p<0.01$; IL-1β PEMF, $p<0.0001$; and macrophage group, $p<0.01$).

These outcomes suggest that genes associated to macrophage polarization can be modulated by an external magnetic stimulus. Moreover, there is a combinatorial effect between the PEMF and the interplay of macrophages and tendon cells in the regulation of M1 and M2 genes in comparison to macrophage control group (Ctrl PEMF, $p<0.01$; IL-1β PEMF, $p<0.01$; *NOS-2* and IL-1β PEMF, $p<0.05$; and *MRC-1,* $p<0.01$).

Correlating with the results obtained for macrophage phenotype in Figure 1C, the transcript levels of *IL-10* were tenfold higher in PEMF-actuated cultures (in comparison with indirect co-cultures,

$p<0.0001$ and with macrophage group, $p<0.05$). Interestingly, *IL-6* and *TNFα* expression were downregulated in PEMF-exposed co-cultures (direct) and in the macrophage group, while non-PEMF cultures induced an exponential increment in *IL-6* expression, especially in IL-1β treated cells (Ctrl and IL-1β non-PEMF, $p<0.01$).

With few exceptions (enhanced *MMP-1* and *TNFα* expression in non-PEMF direct cultures), we could not find significant differences in macrophage and hTDCs cultured in Ctrl or IL-1β supplemented media. Despite the fact that IL-1β induces the synthesis of inflammatory cues [17], the interaction between hTDCs and immune cells leads to anti-inflammatory signals that decrease the impact of IL-1β in the transcript of inflammatory-associated genes.

In sum, PEMF seems to play a modulatory action on macrophage-, cytokine-, and ECM remodeling-associated genes, especially in cell-to-cell contact cultures, without a significant interference in the genetic expression of tenogenic markers.

VI-3.2. Synthesis of Proteins and Soluble Factors in hTDCs and Macrophages Co-Cultures

Cell-to-cell contact provides a more natural environment between populations. Additionally, under PEMF actuation, direct contact communication seems to be a more effective approach in the education of tendon cells and macrophages at the genetic level. At the protein level, the percentage of co-cultured cells positive for SCX and MKX were similar in all conditions studied corroborating the genetic analysis, although the number of cells producing collagen (COL1, COL3) tended to be lower in control conditions (non-PEMF and without IL-1β) (Supplementary Figure VI-2 Ai).

Furthermore, when cells are treated with IL-1β and magnetically stimulated, there is a tendency to increase the number of cells expressing CD169 (M2) in comparison to other conditions and to macrophage control group ($p>0.05$), and to decrease the number of cells positive for CD163 (M2). Interestingly, the number of cells expressing CD80 (M1) also tended to decrease in co-cultures in comparison to macrophage control group (Supplementary Figure VI-2 Aii).

Then, we focused our studies in the production and secretion of Arg-1 and iNOS. Arg-1 (Arginase 1) and iNOS (nitric oxide synthase) are two key enzymes of the arginine metabolism, related to the macrophage polarization into M2 and M1, respectively (Figure VI-3 A).

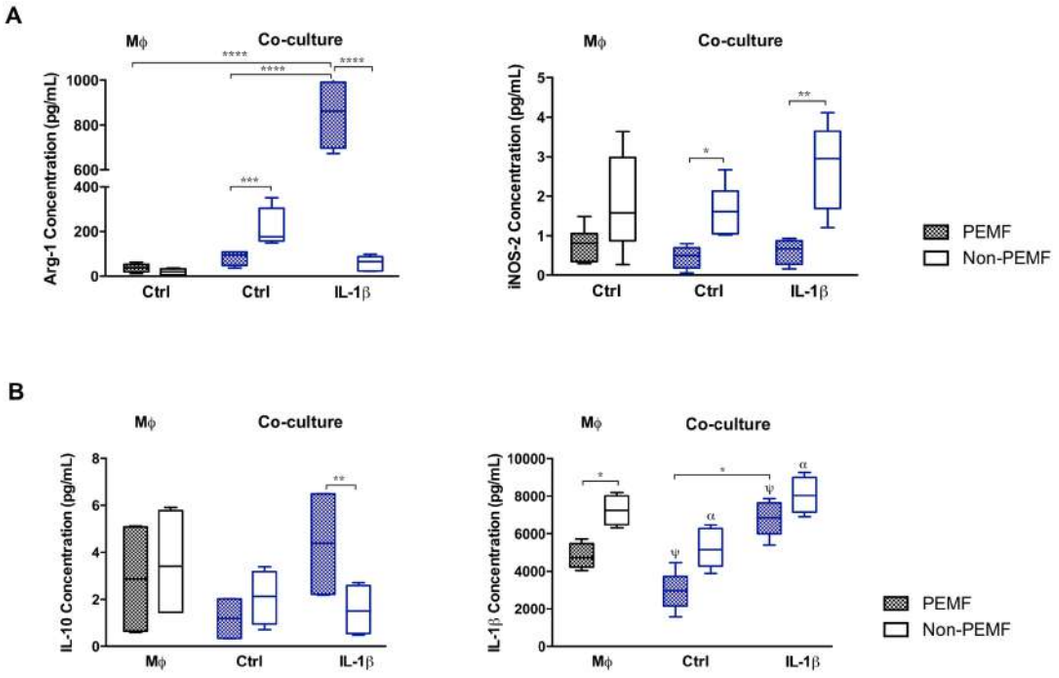


Figure VI-3 IL-1β and magnetic stimulation differently affect cell contact co-cultures. (A) Arg-1 and iNOS, and (B) IL-10 and IL-1β quantification in culture medium 24 h after PEMF and IL-1β stimulation. Data analysis was performed using two-way ANOVA followed by multiple comparisons tests (GraphPad Prism), (n = 3, three experimental replicates from three biological replicates). Statistically significant differences are shown with different degrees of confidence. Symbols: * for $p<0.05$; **, α for $p<0.01$; *** for $p<0.001$; and ****, ψ for $p<0.0001$, respectively.

The concentration of Arg-1 was higher in IL-1β PEMF condition ($p<0.0001$) in comparison to Ctrl PEMF and to macrophage group. Contrariwise, secreted iNOS was significantly decreased on PEMF-stimulated cells, with or without IL-1β treatment (Ctrl, $p<0.05$; IL-1β, $p<0.01$). Both secreted forms of Arg-1 and iNOS follow the pattern of *Arg-1* and *NOS-2* expression (Figure VI-2 C).

These results demonstrate that PEMF modulates the gene expression and the release of macrophage associated enzymes supporting a M2-like phenotype on hTDCs–macrophage cultures.

Hence, we investigated the impact of PEMF in cytokine production (Figure VI-3 B). IL-10 release was significantly increased under PEMF in IL-1β treated co-cultures when compared to non-PEMF condition ($p<0.01$).

Meanwhile, soluble IL-1β was diminished in PEMF-actuated cells (in both types of co-culture and macrophage group, $p<0.05$), proposing that PEMF modulates the production of these cytokines.

When investigating IL-4 and IL-1β produced by co-cultured cells, genetic expression demonstrates upregulation of *IL-4* but not of *IL-1β* when co-cultures were treated with IL-1β and stimulated with PEMF (in comparison to the Ctrl conditions, p<0.001) (Figure VI-4 Ai and Aii).

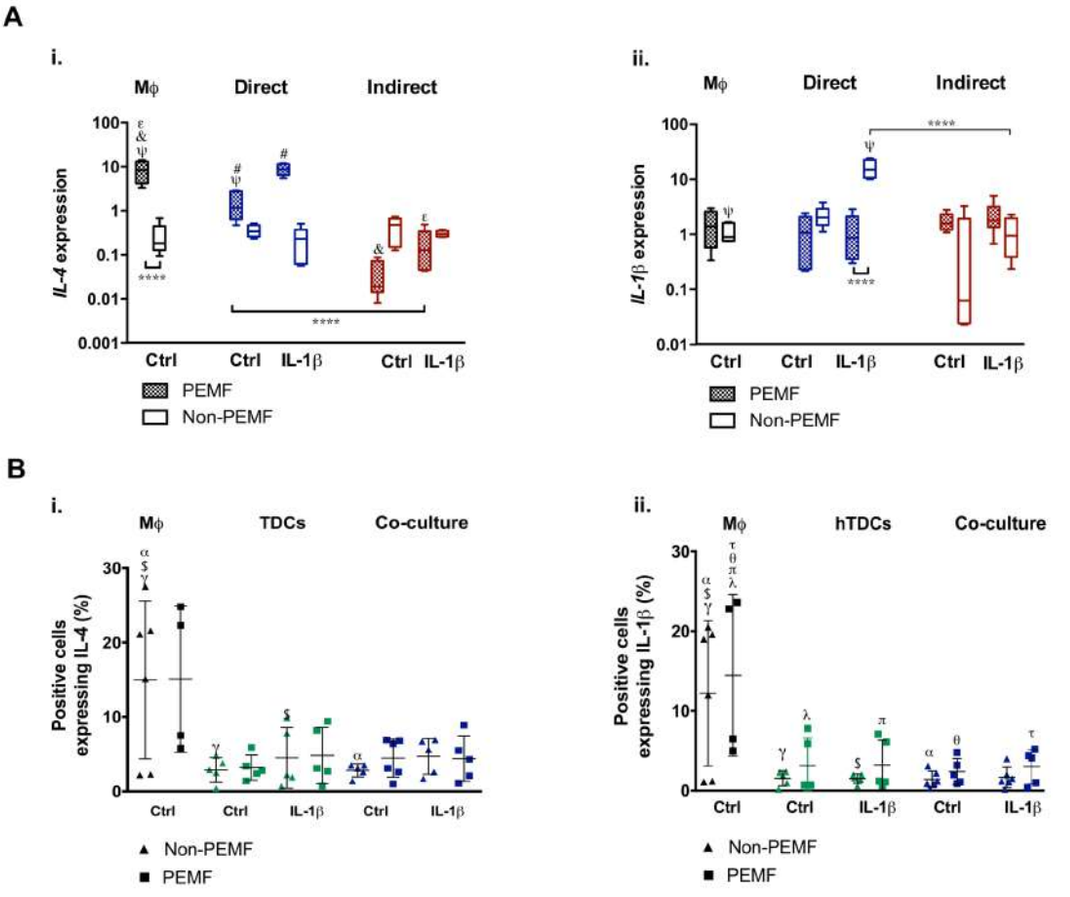


Figure VI-4 IL-1β influences pro- and anti-inflammatory markers in co-cultures under magnetic stimulation (PEMF.) (Ai, Aii) Genetic expression of *IL-4* and *IL-1β* upon PEMF and IL-1β stimulation by RT-PCR analysis. (Bi, Bii) Flow cytometry analysis of intracellular cytokines, IL-4, and IL-1β in co-cultures and in hTDCs and Mϕ (controls) upon PEMF and IL-1β stimulation. Control condition (Ctrl) refers to the absence of IL-1β. Symbols γ, $, λ, τ, π denote statistical differences for p<0.05; α, θ for p<0.01; # for p<0.001; and ****, ψ, &, ε for p<0.0001.

In addition, the percentage of cells expressing intracellular IL-4 and IL-1β was similar in all conditions studied, indicating that IL-1β treatment or PEMF stimulus do not interfere with the production of these cytokines (Figure VI-4 Bi and Bii).

To corroborate these results and assess the contributions of hTDCs and macrophages to the expression of these cytokines, immunofluorescence for IL-1β and IL-4 was assessed (Figure VI-5 B). hTDCs and macrophages can be identified in co-cultures through distinctive morphologic characteristics (Figure VI-5 Ai). More specifically, THP-1 derived macrophages have a single-cell morphology, round-

shaped with vesicle-like structures in the cytoplasm and are considerably smaller than hTDCs. On the other hand, hTDCs are fusiform elongated cells that form networks with nearby hTDCs.

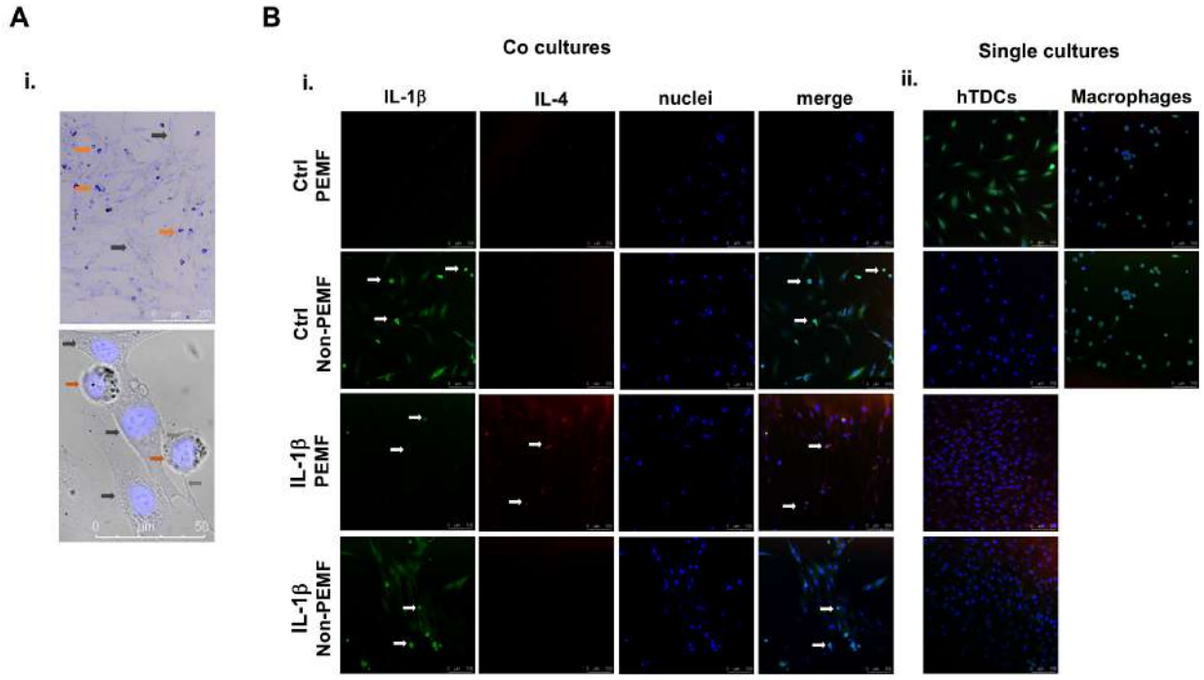


Figure VI-5 IL-1β influences pro- and anti-inflammatory markers in co-cultures under magnetic stimulation (PEMF). The white arrows identify THP-1 cells in co-cultures by their round-shaped and single-cell morphology. (Ai) Microscopic images of the dissimilar morphology of macrophages and hTDCs, enabling their identification in co-culture systems. THP-1 derived macrophages show a round and single-cell morphology (red arrows), while hTDCs are larger elongated fusiform cells (black arrows). The physical contact between THP-1 and hTDCs is evidenced by the gray arrow (bottom image). Phase contrast images counterstained with DAPI (×10; scale bar 250μm, and x63; scale bar 50 μm, respectively). (Bi, Bii) Immunofluorescence images of IL-1β (green), IL-4 (red), nuclei (DAPI, blue), and merged image 24 h after PEMF stimulation on co-cultures and on single cultures (confocal microscopy ×20, scale bar 100 μm). Control condition (Ctrl) refers to the absence of IL-1β.

Cells co-cultured under PEMF-stimulated conditions in combination with IL-1β primed hTDCs show stronger signal intensity in IL-4 (Figure VI-5 Bi). In single cell control groups, both hTDCs and macrophage contribute for IL-1β, but the signal of Il-4 is only visible in Il-1β treated hTDCs, with or without PEMF stimulation (Figure VI-5 Bii).

These outcomes suggest that hTDCs respond differently to IL-1β in the presence of macrophages, highlighting the role of communication networks in the regulation of inflammatory cues.

VI-3.3. Intercellular Communication between Tendon Cells and Macrophages on Pro-Inflammatory Environment

To more comprehensively investigate how macrophages and IL-1β conditioned hTDCs establish cell–cell contacts in the presence of magnetic cues, migration assays (Supplementary Figure VI-3) and the involvement of connexin 43 (Cx43) and focal adhesion kinase (FAK) were assessed (Figure VI-6 A and B).

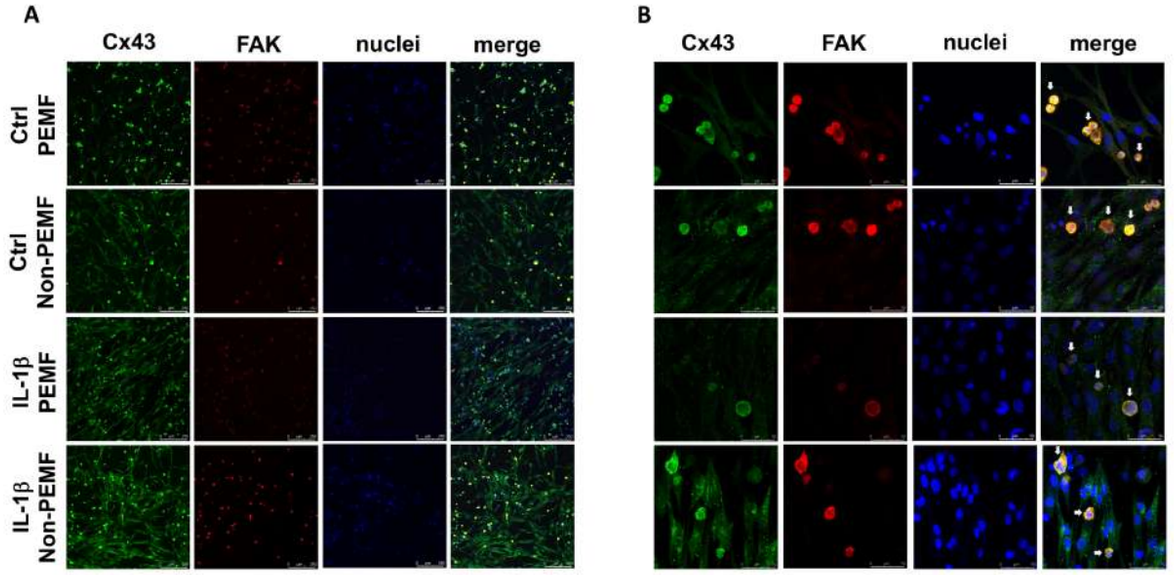


Figure VI-6 Intercellular communication in co-cultures under the PEMF. (A, B) Confocal microscopy images of Cx43 (green), FAK (red), nuclei (DAPI, blue), and merged image 24 h after PEMF stimulation and IL-1β treatment (confocal microscopy x10 and ×63, scale bars 250 μm and 50 μm, respectively). The white arrows identify THP-1 cells by their round-shaped and single-cell morphology. Control condition (Ctrl) refers to the absence of IL-1β.

Cx43 is a protein associated with cell migration and to cell–cell communication in tendon [18], while FAK integrates mechanosensory protein complexes [19] that respond to extracellular stimuli and regulate cellular responses, including cell migration and proliferation [20]. IL-1β treatment on hTDCs seems to stimulate the collective mobility of cells, with masses of both cells spreading into the void area. The migratory cells behavior seems not to be affected by PEMF.

Both Cx43 and FAK were expressed in all conditions studied (Figure VI-6 A). Although Cx43 is detected in both cell types, FAK is predominantly detected in macrophages (Figure VI-6 A and B). Overall, Cx43 signal intensity tends to increase with IL-1β treatment and PEMF stimulation ($p>0.05$), while FAK seems to be more influenced by IL-1β treatment ($p>0.05$) (Figure VI-7 A).

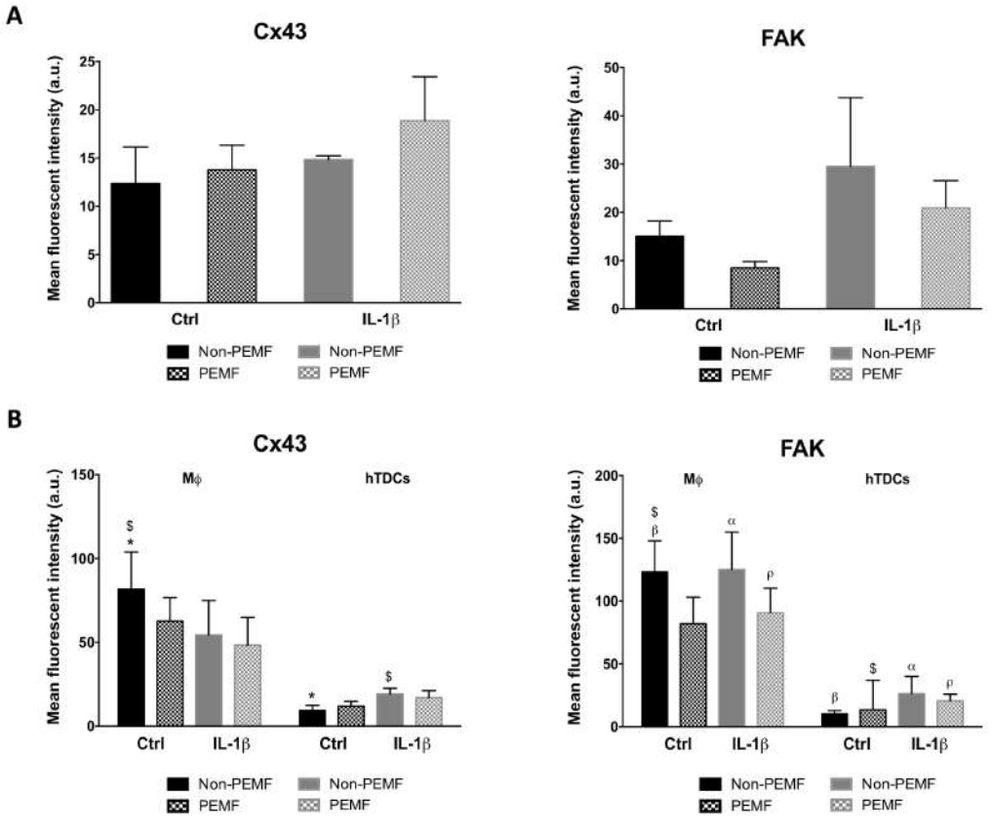


Figure VI-7 Fluorescence intensity of Cx43 and FAK. (A) Mean fluorescence intensity of Cx43 and FAK on co-cultures. (B) The contribution of macrophage and hTDCs to the mean fluorescence intensity was assessed measuring the signal intensity in macrophages and subtracting this value to the raw image to obtain the hTDCs signal. Control condition (Ctrl) refers to the absence of IL-1β. Statistically significant differences are shown as γ, $, ρ, for $p < 0.05$; β, for $p < 0.01$.

However, when Cx43 and FAK are analyzed considering each of the cell types present in the co-culture, we observed that the Cx43 signal is significantly increased in macrophages in non-PEMF conditions and without IL-1β treatment in comparison to hTDCs ($p<0.05$) (Figure VI-7 B).

In addition, the mean fluorescence intensity of FAK in macrophages is significantly increased in all conditions in comparison to hTDCs ($p<0.05$), except when cells are cultured under PEMF stimulation without IL-1β treatment ($p>0.05$) (Figure VI-7 B).

Altogether, these results suggest that macrophages are active players in the intercellular communication with hTDCs, which may be mediated by FAK signaling.

VI-4. DISCUSSION

Biological functions and homeostasis rely on complex cell responses to microenvironmental stimuli. The articulated interplay of immune cells and resident cell population is critical for timely and spatial regulation to achieve proper healing and stimulate regeneration [10]. Although inflammatory cues are necessary to trigger the repair mechanisms, if perpetuated, pro-inflammatory signals can impair regenerative response and produce a deleterious effect on tendon functionality [21].

The inflammatory response is accompanied by an increasing population of macrophages at the injury site. One of the most powerful pro-inflammatory factors released by these cells is IL-1β that helps to set an inflammatory milieu to which nearby cells respond to [22]. We have previously established an inflammatory model with IL-1β to stimulate tendon cells to exhibit a pro-inflammatory profile associated with tendon injury environments [16]. However, in this work, we aimed to assess whether "inflamed" tendon cells could communicate with immune cells and provoke a macrophage phenotype switch. For that, we studied co-cultures between tendon cells exposed to IL-1β and macrophages. Moreover, we investigated the modulatory effect of PEMF on macrophage and tendon cells interactions, using the parameters identified in our previous work [16].

The upregulation of tenogenic-related markers in both co-cultures suggests that hTDCs maintain their lineage identity, which is not affected by the direct or indirect contact with immune cells or to the exposure to PEMF.

Collagen I is the main component of connective tissues and its degradation by MMPs [23, 24] is crucial for tendon repair. As PEMF supports the decrease of MMPs and increase of *TIMP-1* expression, *COL1A1* expression is not compromised, meaning that PEMF can positively actuate on ECM turnover balancing the expression of MMPs and *TIMP-1*, displaying a regulatory action over ECM in tendon healing.

The external stimulation provided by PEMF-affected macrophage polarization toward M2, with the upregulation of *Arg-1*, *MRC-1*, and *Singlec-1*, especially in cell-to-cell contact cultures. This result is in accordance to published studies that have demonstrated the ability of magnetic forces to stimulate Arg-1 in peritoneal Mϕ [25, 26]. Conversely, the M1 markers are downregulated in direct cultures stimulated by PEMF.

Another important outcome is that PEMF seems to overpower the signals provided by IL-1β treated hTDCs, highlighting the potential immunomodulatory role of PEMF in tissue repair and regenerative

strategies. Moreover, PEMF seems to exert a dual cell action; on macrophages, switching their functional phenotype, and on IL-1β treated cells, antagonizing the pro-inflammatory signals. The impact on both cell types is likely to contribute to improve the healing response and may justify the differential response between direct and indirect cultures.

Not only are the M1/M2 associated genes influenced by the PEMF, but the profile of cytokine genes is influenced as well. Under PEMF, *IL-10* was upregulated, while *IL-6* and *TNFα* were downregulated in direct cultures exposed to PEMF. The balance toward anti-inflammatory cues also supports the immunomodulatory potential of PEMF, and correlates with outcomes reported in the literature [27].

Taken together, cell–cell signaling combined with the PEMF modulates and guides macrophages' fates toward the M2 phenotype, contributing to regulation and decreasing the influence of secreted pro-inflammatory cues. Macrophages regulate arginine metabolism via iNOS and Arg-1 with important functional responses for healing [28]. Additionally, Arg-1, inducible by IL-4, leads to the production of ornithine that assists cell proliferation and repair of damaged tissue [29]. Our results point a preferential selection for M2/Arg-1 versus M1/iNOS favoring repair signaling. Overall, PEMF positively affects the gene expression and the release of cytokines on direct cultures, supporting previous results and the immunomodulatory role of PEMF on macrophage functional responses. Due to the relevance of cell-to-cell contact in the exchange of information between macrophages and hTDCs, we investigated a possible involvement of Cx43 and FAK on cell migration and in mediating networks of inflammatory factors. Being a transmembrane protein, Cx43 participates in the flow of data between the intracellular and extracellular compartments [30]. Cx43 is expressed in both tenocytes and immune cells and can be upregulated by inflammatory influencers as TNFα and IFN-y [31]. Moreover, Cx43 is involved in cell migratory phenomena [31, 32], including endothelial cells migration during wound repair [33], and affects cell–cell contact facilitated interactions [34]. In our work, IL-1β treatment influenced the cell mobility between macrophages in comparison to hTDCs. Although an increase of Cx43 has been reported to participate in inflammation [31], we did not observe this behavior in our study. The increment in Cx43 signal in macrophages may be associated to specific functions of these cells in immunity or to the interaction with hTDCs. It is likely that the communication between tendon cells and macrophages actuated by PEMF may also privilege other communication channels. FAK was shown to be highly expressed by macrophages and tends to decrease in PEMF conditions. FAK activation in macrophages seems to be independent of IL-1β primed hTDCs, but may be associated to sensing and transmission of magnetic stimulation to immune cells.

Overall, we showed that magnetic stimulation influences the intercellular communication of tendon cells and macrophages, holding immunomodulatory action over macrophages and stimulating M2 phenotype, in which FAK signaling may be involved.

VI-5. CONCLUSIONS

This work demonstrated that direct communication established between tendon cells and THP-1 derived macrophages plays an important role in the phenotypic expression of immune cells. To modulate macrophage behavior, the impact of magnetic stimulus in cell–cell contact is more important than tendon cells conditioned to IL-1β. PEMF drives the polarization of macrophage toward a pro-regenerative phenotype, assisting the increment of soluble anti-inflammatory factors into the extracellular milieu and favoring an anabolic action over matrix turnover genes. Overall, PEMF actuation evidences an immunomodulatory role on macrophage behavior co-cultured with hTDCs.

This work provides insights on the dynamics of tendon cells and macrophage communication and supports the relevant contribution of the immunomodulatory actions of magnetic actuation for the development of new treatments and in tendon regeneration strategies.

VI-6. SUPPLEMENTARY MATERIAL

Supplementary Table VI-1 Primers used for real time quantitative RT-PCR analysis.

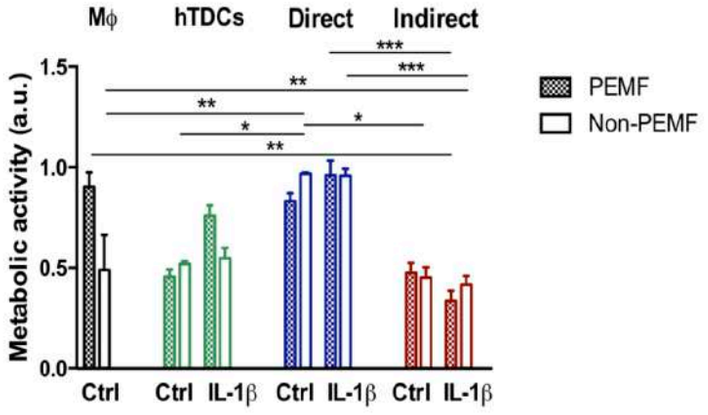
	Primer Sequence	Accession number
Human Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	F - TGTACCACCAACTGCTTAGC R – GGCATGGACTGTGGTCATGAG	NM_002046.4
Mohawk (*MKX*)	F – TGTTAAGGCCATAGCTGCGT R – TCGCACAGACACCTGGAAAA	NM_173576.5
Scleraxis (*SCXA*)	F – CGAGAACACCCAGCCCAAAC R – CTCCGAATCGCAGTCTTTCTGTC	XM_001717912
Collagen, Type I, alpha 1 (*COL1A1*)	F – CGAAGACATCCCACCAATCAC R – GTCACAGATCACGTCATCGC	NM_000088.3
Human matrix metalloproteinase-1 (*MMP-1*)	F –ACCTGGAAAAATACTACAACCTGAA R –TTCAATCCTGTAGGTCAGATGTGTT	NM_002421.3

Human matrix metalloproteinase-3 (*MMP-3*)	F – CACTCACAGACCTGACTCGG R – AGTCAGGGGGAGGTCCATAG	NM_002422.4
Human metalloproteinase inhibitor-1 (*TIMP-1*)	F – CATCCGGTTCGTCTACACCC R – GGATAAACAGGGAAACACTGTGC	NM_003254.2
Human arginase-1 (*ARG-1*)	F – GGAAAACCAAGTGGGAGCAT R – TGTGGTTGTCAGTGGAGTGT	ENSG00000118520
Human mannose receptor C-type 1 (*MRC-1*)	F – TGCTCTACAAGGGATCGGGT R – ACACGCCAAACAAGAACATGA	ENSG00000260314
Human sialic acid binding Ig like lectin 1 (*SIGLEC-1*)	F – CAACTTGCTGCGTGTGGAGA R – TGCCTGATTAGATCCTCCTCGG	ENSG00000088827
Human nitric oxide synthase 2 (*NOS2*)	F – GGACATCGCGTGGGTGAA R – TTTATCGCTCGGAGCCTGC	ENSG00000007171
Human interleukin 10 (*IL-10*)	F – AAGACCCAGACATCAAGGCG R – AATCGATGACAGCGCCGTAG	NM_000572.2
Human interleukin 6 (*IL-6*)	F – AGGAGACTTGCCTGGTGAAA R – GCATTTGTGGTTGGGTCAG	NM_000600.4
Human tumor necrosis factor (*TNFα*)	F – ATGTTGTAGCAAACCCTCAAGC R – TGATGGCAGAGAGGAGGTTG	NM_000594.3
Human interleukin 4 (*IL-4*)	F – GCACCGAGTTGACCGTAACA R – AGGAATTCAAGCCCGCCAG	NM_000589.3

VI-6.1. Assessment of metabolic activity in single and co-culture systems

The metabolic activity of hTDCs and macrophages as single or co-culture systems were evaluated by MTS assay (CellTiter 96® AQueous One Solution, Promega).

Cells were washed with PBS and incubated with a mix of serum-free culture medium without phenol red and MTS solution (5:1 ratio) for 3 h at 37 °C and 5 % CO_2 atmosphere, protected from light. After this period, the supernatant was transferred to a 96-well plate and the absorbance read at 490nm (Synergy™HT, BIO-TEK Instruments, Winooski, Vermont) (Supplementary Figure VI-1). Samples were read in triplicates and a blank sample (no cells) was assessed as control of the assay.

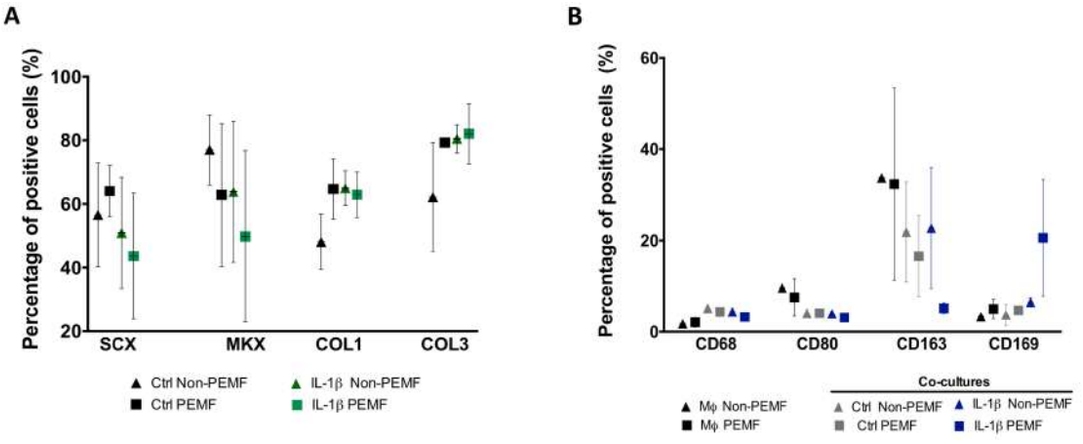


Supplementary Figure VI-1 Metabolic activity of hTDCs, macrophages (Mϕ) and co-cultures established between hTDCs and macrophage via direct or indirect (transwell) contact 24h after PEMF application (PEMF). IL-1β condition represents hTDCs previously treated with IL-1β. Symbols (*) represent significant differences to control values (Ctrl) that represent the absence of IL-1β treatment. Statistically significant differences are shown as *p<0.05; **p<0.01; ***p<0.001.

VI-6.2. Flow cytometry analysis

Cells were trypsinized using TrypLE Express (12605-028; Alfagene, Life Technologies Limited, Paisley, UK), centrifuged and resuspended in PBS. Cells were incubated with antibodies for surface specific markers: CD163 (alexa fluor 647, 8276870, BD Biosciences), CD169 (alexa fluor 647, 565295, BD Biosciences), CD206 (FITC, 5253911, BD Biosciences), CD80 (FITC, 9023585, BD Biosciences) and CD68 (E-11) (A1017, santa cruz biotechnology) or with tenogenic markers: Scleraxis (ab58655, Abcam, Cambridge, UK), Mohawk (A83377, Sigma-Aldrich), Collagen I (ab90395, Abcam, Cambridge, UK) and Collagen III (ab7778, Abcam, Cambridge, UK). Cells were incubated for 20 min at RT protected from light beforerinsed in PBS and centrifuged for 5 min at 800 g. In the case of unconjugated antibodies, secondary antibodies Alexa Fluor 488 (2072687, Alfagene, Life Technologies Limited, Paisley, UK) and 594 (2145022, Alfagene, Life Technologies Limited, Paisley, UK) were incubated with cells for 30 min at RT protected from light, according to the host species of the primary antibody.

Afterwards, the cells were ressuspended in 500 µL of acquisition buffer and data acquired in a FACSAria III sorter equipped with blue and red lasers (BD Biosciences, Erembodegem-Aalst, Belgium). Cells were identified by forward and side scatter. A minimum of 5,000 cells were acquired and analyzed using FACS Diva version 7 software. Unstained cells were considered as negative controls. The positive cell populations expressing the markers of interest were expressed in percentage values. Data acquired and analysed is representative of three independent experiments (Supplementary Figure VI-2).

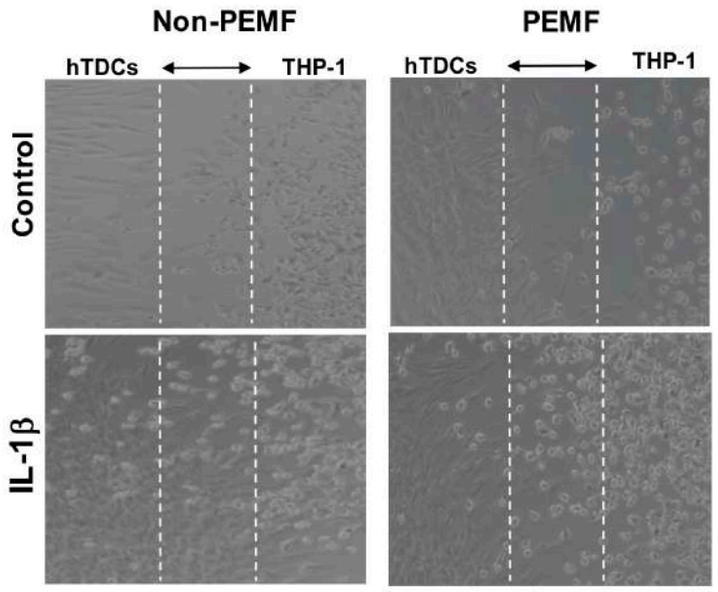


Supplementary Figure VI-2 Effect of IL-1β and magnetic stimulation on tenogenic and macrophage markers. A) Percentage of cells positive for tenogenic associated markers (SCX, MKX, COL1, COL3) and for B) macrophage phenotypic markers (CD68, CD80, CD163, CD206, CD169). The black color represents macrophage and green represents hTDCs (both single cultures). Co-cultures with or without IL-1β treatment are represented in grey and blue, respectively.

VI-6.3. Cell migration assays

For migration analysis, the hTDCs and macrophages were separately seeded in 2 well silicone insert with defined cell-free gaps (2 well in μ-dish 35 mm Ibidy, Gräfelfing, German), as previously described. The density of cells inside the well was adjusted to 5,000 hTDCs and macrophages, each. The cell laden inserts were incubated overnight at 37 °C and 5 % CO_2, allowing cells to adhere to the bottom of a 24-well well plate (BD Biosciences, UK). After 24h incubation, the debris and non-attached cells were removed rinsing the cells twice with sterile PBS. Cells cultured with RPMI medium were exposed to PEMF stimulation as described before. The mobility of cells was microscopically checked 24 h after magnetic stimulation (Supplementary Figure VI-3).

The cells were observed in a fluorescent microscope (Carl Zeiss, Germany) and images acquired using a digital camera (AxioCam MRm5) and the Axiovision version 4.8 (Carl Zeiss, Germany) software.



Supplementary Figure VI-3 IL-1β induced cell migration in cell contact co-cultures in both non-PEMF and PEMF conditions. Representative images of the cells in 2-well insert assay. Control condition (Ctrl) refers to the absence of IL-1β.

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Chapter VII

Human tendon derived cell sheets created by magnetic force-based tissue engineering holds tenogenic and immunomodulatory potential

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ABSTRACT

Cell sheet technology and magnetic based tissue engineering hold the potential to become instrumental in developing magnetically responsive living tissues analogues that can be used for both modelling and therapeutical purposes. Cell sheet constructions more closely recreate physiological niches, through the preservation of contiguous cells and cell-ECM interactions, which assist the cellular guidance in regenerative processes.

We herein propose to use magnetically assisted cell sheets (magCSs) constructed with human tendon derived cells and magnetic nanoparticles to study inflammation activity upon magCSs exposure to IL-1β, anticipating its added value for tendon disease modelling.

Our results show that IL-1β induces an inflammatory profile in magCSs, supporting its *in vitro* use to enlighten inflammation mediated events in tendon cells. Moreover, the response of magCSs to IL-1β is modulated by pulsed electromagnetic field (PEMF) stimulation, favoring the expression of anti-inflammatory genes, which seems to be associated to MAPK(ERK1/2) pathway. The anti-inflammatory response to PEMF opens new perspectives for magCSs application beyond a cell-based model towards tendon living patches holding immunomodulatory potential.

This chapter is based on the following publication: **Vinhas A.**, Gonçalves A.I, Rodrigues M.T, Gomes M.E. Human tendon derived cell sheets created by magnetic force-based tissue engineering holds tenogenic and immunomodulatory potential, *submitted*

VII-1. INTRODUCTION

Cell sheet technology introduces the possibility of providing stable cellular constructs and thus enabling the construction of tissue-like assemblies [1]. In fact, cell sheets (CSs) preserve structural elements as ion channels, growth factor receptors, and cell surface proteins as well as intercellular connections and matrix components, which assists cell sheet usefulness as either biomimetic models to address tissue functional aspects or as tissue substitutes. Previous studies reported cell sheet potential to stimulate musculoskeletal tissue regeneration [2, 3]. Furthermore, mesenchymal stem cell sheets demonstrated a satisfactory integration into native tissues accompanied by anti-inflammatory effects improving cartilage repair and treatment of osteoarthritis disease [4, 5]. With inflammation increasingly recognized as a central component both in healing and in disease progression, a model to study inflammation in tendon tissues taking advantage of CSs technology could become an important tool to advance knowledge in this field. For instances, it has been reported that adipose tissue derived stem cells (ASC) sheets improve matrix remodelling, and modulate the inflammatory phase facilitating tendon healing [6].

Magnetic cell sheets (magCSs) offer the opportunity to study inflammatory cues and cell responses through contact-free stimulation of tendon resident cells in a more tissue-like environment with intercellular signaling and structural complexity [5, 7]. Also, magnetic cell sheets technology has demonstrated promising results in vascular [8, 9], cardiac [10] and musculoskeletal [2, 11, 12] tissue engineering strategies, however, very few examples were provided in the literature of magnetic cell sheets in therapies for resolving inflammation. Our group has previously shown that magCSs exhibited a tendon-like ECM, good mechanoelastic properties and responsiveness, suggesting the applicability of these living patches in tendon therapies [2].

Moreover, magCSs could also enable to insight on pulsed electromagnetic field (PEMF) assisted immunomodulatory effects in cell-based therapies for functional tendon tissue replacement.

A controlled pro-inflammatory milieu can be provided to magnetic tendon cell sheets by the exogenous supplementation of interleukin-1β (IL-1β) to the culture medium. IL-1β is a pro-inflammatory cytokine released in inflammatory environments [13] and a well described marker of chronic inflammation [14, 15]. From previous reports by our group [16, 17] and others [18-20] exposure to magnetic stimulation was shown to modulate tendon cell response to inflammation stresses influencing the release and expression of cytokines [16, 17]. Other works also showed the effectiveness of the combination of

MNPs with magnetic field for pain control and enhanced functional recovery in the knee diseases and osteoarthritic lesions [21]. Moreover, it has been reported by Rahmi et al. [22] that bone marrow-derived mesenchymal stem cell sheets labelled with MNPs upregulated anti-inflammatory factors, TGF-β2 and IL-10, which increased the potential of magnetic cell sheets for therapeutic strategies.

In this work, we aimed at using magnetic cell sheet technologies to study tendon cell responses in inflammatory environments, induced by exposure of the cell sheets to IL-1β, and thus assess magCSs construction as a cellular model to more closely study inflammatory molecules affecting injured tendons. Considering the modulatory potential of PEMF in cell behavior favoring anti-inflammatory outcomes, we also studied the role of magCSs assisted by a remote pulsed electromagnetic field (PEMF) in IL-1β induced tendon cells responses.

VII-2. MATERIALS AND METHODS

VII-2.1. Isolation and culture of human tendon derived cells (hTDCs)

hTDCs were isolated from surplus tissue samples collected from patients undergoing orthopedic reconstructive surgeries under protocols previously established with Hospital da Prelada (Porto, Portugal) and with informed consent of the patients. The content of the written informed consent and related procedures were reviewed and approved by the Hospital Ethics Committee (P.I. N.º005/2019).

Following a previously established protocol [23-25], tendon samples were minced and digested in an enzymatic solution of collagenase (0.1%, Sigma-Aldrich, C6885, USA) with 2M CaCl2 (1:1000, VWR, Germany) and 1% bovine serum albumin (BSA) (Sigma-Aldrich, USA) for 1h at 37 °C under agitation. After incubation, digested samples were filtered and centrifuged three times at 1200 rpm for 5 min, and the supernatant discarded. Isolated hTDCs were expanded in α-MEM medium (A-MEM, Invitrogen, Life Technologies Limited, Paisley, UK) composed of α-MEM supplemented with 10% fetal bovine serum (FBS) (Alfagene, Life Technologies Limited, UK) and 1% antibiotic/antimicotic solution (A/A) (Alfagene, Life Technologies Limited, UK) in humidified 5% CO_2 atmosphere. hTDCs from passage 1 to 3 were used to perform all the experiments.

VII-2.2. Magnetic cell sheets construction, culture, and stimulation

Magnetic cell sheets (magCSs) were constructed as previously described by our group [2]. Briefly, hTDCs were seeded onto 24 well tissue culture plates (BD Biosciences, San Jose UK) at a density of 500,000 cells per well and left to adhere for 2h at 37˚C. Then, chitosan coated iron oxide MNPs (nanomag-C, 04-00-152, Micromod, Germany) were incubated with the cells at 200 pg/cell overnight, in α-MEM medium (A-MEM, Invitrogen, Life Technologies Limited, Paisley, UK) over a 24-well permanent magnet well-array plate (Magnefect nano II, Nanotherics, N2DUO-10048) for up to 3 days in humidified 5% CO_2 atmosphere.

After 16h, magCSs were washed with D-PBS (Sigma-Aldrich, Saint Louis, MO) and the culture medium replaced by either *i)* α-MEM medium (magCSs Control group) or *ii)* α-MEM medium supplemented with IL-1β (1 ng/mL, Alfagene, Life Technologies Limited, UK) (magCSs IL-1β group) to induce inflammatory cues in hTDCs, as previously established [16].

In order to assess the influence of magnetic stimulus on IL-1β primed magCSs, these were exposed to a PEMF (IL-1β PEMF group) using a magnetotherapy device (Magnum XL Pro; Globus Corporation, Italy) for 1 hour in humidified 5% CO_2 atmosphere. The PEMF parameters of 4 mT strength, 5 Hz frequency and 50 % duty cycle were previously established as optimal parameters for modulating inflammatory cues of tendon cells in monolayered cultures [16].

In sum, the following conditions were investigated: i) magCSs in α-MEM medium (Control), ii) IL-1β-treated magCSs (IL1-β), iii) IL-1β-treated magCSs exposed to PEMF (IL1-β PEMF). Magnetic cell sheets were characterized for cell viability, proliferation, morphological analysis, collagen proteins production and gene expression levels of tendon and inflammatory markers after 3 days in culture. The culture media was collected and the presence of inflammatory mediators quantified using ELISA assays.

VII-2.3. Assessment of cellular viability

Live/dead assay was performed to assess cellular viability. Briefly, the magCSs were incubated for 20 min with calcein-AM (2 μg/ml) and propidium iodide (3 μg/ml), both from Life Technologies Limited, UK. Afterwards, cells were washed with PBS and visualized under a fluorescence inverted microscope (Axio Observer, ZEISS, Germany).

The metabolic activity of magCSs was evaluated by MTS assay (Cell Titer 96 Aqueous Solution Cell Proliferation Assay, Promega). MagCSs were rinsed in PBS before a 3 h incubation in a mixture of phenol red free medium supplemented with FBS and MTS solution (5:1 ratio) at 37 ºC and 5% CO_2 atmosphere, protected from light. Then, the supernatant was transferred to a new 96-well plate and the absorbance read at 490 nm (Synergy™HT, BIO-TEK Instruments, Winooski, Vermont). Triplicates were made of each sample and a blank sample (no cells) reading was performed.

VII-2.4. Morphological characterization

The structure and morphological characterization of the magCSs was analyzed by scanning electron microscopy (Auriga Compact, ZEISS, Germany). The element composition of magCSs was also investigated by Energy dispersive x-ray spectroscopy (EDS) (JSM-6010LV, JEOL, Japan). For this purpose, after 3 days in culture, the magnetic constructs were fixed in 10% neutral buffered formalin (Bio Optica, Milano, Italy) for 1 h at room temperature and dehydrated in a series of increasing ethanol solutions (from 30% to 100% (v/v)), followed by a 5 min immersion in hexamethyldisiloxane (HMSO, Sigma-Aldrich, Saint Louis, MO, USA).

Subsequently, the samples were air-dried overnight and sputter coated with gold (30s at 20mA, Cressington, C5219, Model 108A).

VII-2.5. Gene expression evaluation by RT-PCR

The expression of tenogenic markers, namely Mohawk *(MKX)*, Scleraxis *(SCX)*, Tenomodulin *(TNMD),* Decorin *(DCN),* Metalloproteinase-1 *(MMP-1),* Metalloproteinase-2 *(MMP-2),* Metalloproteinase-3 *(MMP-3),* Inhibitor of Metalloproteinase-1 *(TIMP-1),* Collagen type I *(COL1A1),* Collagen type III *(COL3A1), and inflammation markers, namely* Interleukin-8 *(IL-8),* Interleukin-1β *(IL-1β),* Tumor necrosis factor *(TNFα),* Interleukin-6 *(IL-6),* Interleukin-10 *(IL-10),* Interleukin-4 *(IL-4),* was assessed by real time RT-PCR.

Total RNA was extracted from using TRI reagent (T9424, Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer's instructions. Briefly, TRI reagent was added to each sample and stored at -80°C. After defrosting, samples were incubated with chloroform (Sigma-Aldrich, Saint Louis, MO, USA) for 15 min and centrifuged at 12,000 g for 15 min at 4°C. The aqueous fraction was collected and isopropanol (Sigma-Aldrich, Saint Louis, MO, USA) was added. After 10 min, samples were centrifuged

at 12,000 g for 10 min at 4°C. RNA pellet was washed with ethanol (70% V/V) and subsequently centrifuged at 7,500 g for 5 min at 4°C. RNA quantity and purity were determined with a NanoDrop ND-1000 spectrophotometer (Wilmington, DE, USA). The cDNA synthesis was performed using the qScript cDNA Synthesis kit (qScript™ cDNA Synthesis Kit, Quanta Biosciences, Gaithersburg, MD, USA) in a Mastercycler Realplex (Eppendorf, Hamburg, Germany). An initial amount of total RNA of 1 μg in a total volume of 20 μL was used per each and every sample. The quantification of the transcripts was carried out by quantitative polymerase chain reaction (qPCR) using the PerfeCTA SYBR Green FastMix kit (Quanta Biosciences, Gaithersburg, MD, USA) following the manufacturer's protocol, in a Real-Time Mastercycler Realplex thermocycler (Eppendorf, Hamburg, Germany).

The primers were pre-designed with PerlPrimer v1.1.21 software (Supplementary Table VII-1) and synthesized by MWG Biotech. GAPDH was used as the housekeeping gene. The $2^{-\Delta\Delta_{Ct}}$ method was selected to evaluate the relative expression level for each target gene. All values were firstly normalized against GAPDH expression values, and then to magCSs cultured in α-MEM medium at day 3.

VII-2.6. Quantification of secreted forms of inflammatory mediators

The release of inflammatory mediators from magCSs culture was analysed using commercially available enzyme immunoassay kits for TNFα (Human TNFα ELISA Kit, EK0525, Tebu-Bio, Fremont, CA, USA), COX-2 (Human COX-2 ELISA Kit, KA0323, Abnova, Taiwan), PGE_2 (Human Prostaglandin E2 ELISA Kit, KHL1701, Thermo Fisher Scientific, Molecular Probes, Eugene, USA), IL-6 (Human IL-6 Standard ABTS ELISA Development Kit, 900-K16, Peprotech, Rocky Hill, NJ, USA), and IL-10 (Human IL-10 Standard ABTS ELISA Development Kit, 900-K21; Peprotech, Rocky Hill, NJ, USA). MAPK protein phosphorylation was also determined in cytoplasmic protein extracts using the PhosphoTracer ERK1/2 (pT202/Y204)+p38 MAPK (pT180/Y182)+JNK1/2/3 (pT183/Y185) Elisa Kit (Abcam, ab119674, Cambridge, UK). Each and every kit were performed following the manufacturers' instructions.

VII-2.7. Quantification of extracellular matrix proteins

The amount of collagen and non-collagenous proteins was determined using a semiquantitative assay, namely Sirius Red/Fast Green Collagen Staining Kit (9046, Chondrex). For this purpose, magCSs were fixed with a neutral buffered formalin solution (Bio Optica, Milano, Italy) and stored at 4 °C until

analysis. Then the dye solution from the kit was added to the magCSs followed by 40 min incubation. A dye extraction buffer was then mixed and the OD values read in a spectrophotometer (Synergy HT, Biotek Instruments) at 540 nm and 605 nm.

VII-2.8. Evaluation of tenogenic markers and MAPK phosphorylation in magCSs constructions

Deposition of Mohawk and Tenomodulin was assessed by immunocytochemistry. For this purpose, magCSs were washed three times with PBS, before and after fixation with 10% (v/v) neutral buffered formalin (Bio Optica, Milano, Italy) overnight and kept in PBS at 4 °C until usage. Subsequently, the cells were incubated with 0.025% Triton-X100 in PBS solution (Sigma-Aldrich, Saint Louis, MO) and the blocking step was performed using RTU Normal Horse Serum (RTU Vectastain Kit, PK-7200; Vector, California). The magCSs were incubated overnight with anti-MKX (Rabbit anti-human, A83377, 1:100, Sigma-Aldrich, Saint Louis, MO), anti-Tenomodulin (Rabbit anti-human, ab81328, 1:100, Abcam, Cambridge, UK), diluted in antibody diluent with background reducing components (Dako, Santa Clara, CA) at 4 °C, followed by 1 h incubation at room temperature with the secondary antibody (donkey anti-rabbit Alexa Fluor 488, 1:200; Alfagene, Life Technologies Limited, UK). The samples were washed three times with PBS and stained with 4′,6-diamidino-2-phenyindole, dilactate (DAPI, 5 μg/μl, D9564; Sigma-Aldrich, Saint Louis, MO) for 10 min. All samples were observed under a microscope (Imager Z1m; Zeiss, Germany) and images acquired using a digital camera (AxioCam MRm5). Three independent experiments were investigated for protein detection by immunofluorescence.

The immunostained samples we then analyzed by confocal laser scanning microscopy (CLSM, TCS SP8, Leica, Wetzlar, Germany). Images were bidirectionally scanned at 400 Hz with Argon (488) and UV (405) lasers and acquired using a 63x magnification objective.

The MAPK protein phosphorylation was also investigated by western blotting assay. magCSs were collected in PBS, resuspended, and the supernatant collected to a new microtube upon magnetic separation (MPC®-S, Dynal Biotech).

The total cell lysates were prepared using RIPA buffer (Sigma-Aldrich, Saint Louis, MO) with protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO). The samples were centrifuged for 15 min at 14,000 rpm at 4 °C and the supernatants collected. Afterwards, the BCA protein assay kit (Alfagene, Life Technologies Limited, UK) was used to assess the protein content of the samples according to the

manufacturer's instructions. The protein extracts were resolved in 10% sodium dodecyl sulfate-polyacrylamide gels, followed by semi-dry transfer to Amersham™ Protran® Western blotting membranes (nitrocellulose; Sigma-Aldrich, Saint Louis, MO). The transfer of the proteins to the membrane was performed in a Pierce Power Station (Thermo Fisher Scientific, Molecular Probes, Eugene, OR). The membranes were blocked with 5% BSA in Tris-buffered saline with Tween 20 (Sigma-Aldrich, Saint Louis, MO) (TBS-T) and incubated with rabbit anti-phospho-p44/42 MAPK (Erk1/2) (1:1000) (9102; Cell signaling Technology, Danvers, MA) and anti-α smooth muscle actin (1:2000) (ab32575; Abcam, UK) antibodies for 1 h at room temperature under mild agitation. The membranes were washed three times with TBS-T and then incubated with a secondary antibody (1:2000) (Anti-Rabbit IgG Alkaline Phosphatase antibody, A9919, Sigma-Aldrich, Saint Louis, MO) for 1 h at RT. A colorimetric AP substrate reagent kit (1706432; BioRad, Hercules CA) was used for color development.

VII-2.9. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The statistical analysis was performed using GraphPad Prism6 software. Data was obtained from 3-independent experiments (n=3) analyzed in triplicate, and evaluated by Two-way ANOVA followed by Bonferroni post-hoc test for multiple comparison tests. Symbols denote a different degree of confidence, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

VII-3. RESULTS

VII-3.1. Assessment of magCSs

Live/Dead analysis and metabolic activity in magCSs demonstrated high cell viability in all conditions studied (Figure VII-1 Ai and Aii). Interestingly, the cell metabolic activity in IL-1β-magCSs and IL-1β-magCSs under PEMF is higher ($p<0.05$) than in control group ($p<0.01$) (Figure VII-1 Aii).

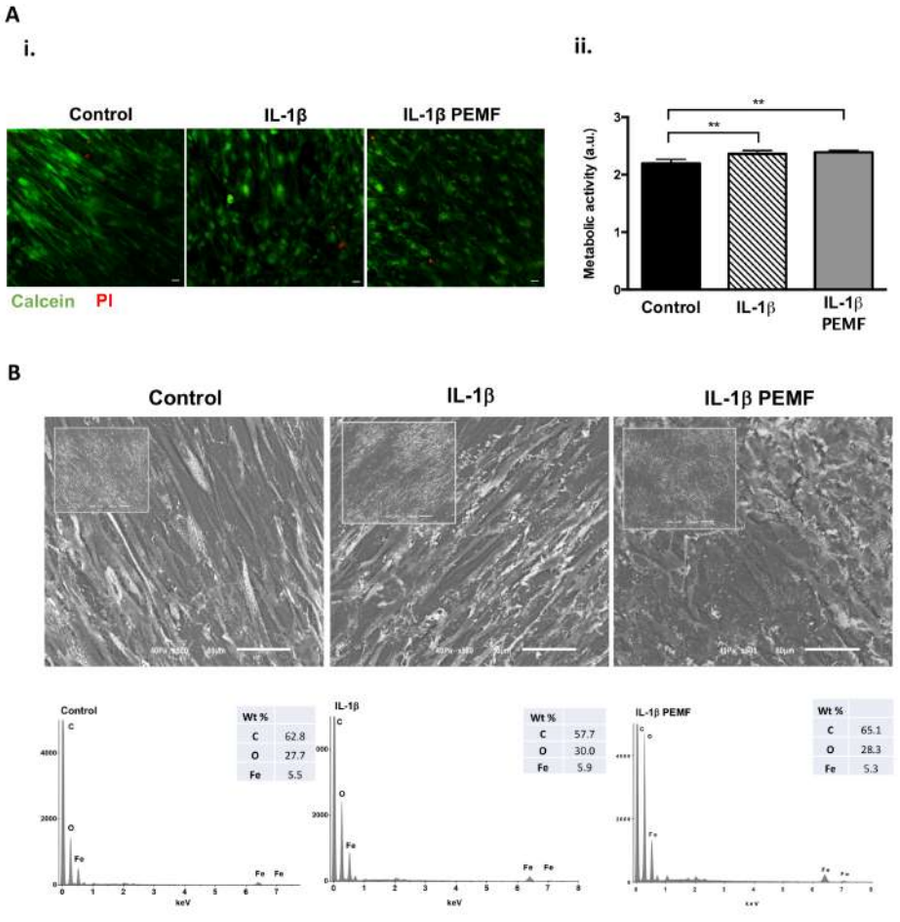

Figure VII-1 Tendon cell viability, metabolic activity and morphology of tendon cells in magnetic cell sheets (magCSs) after 3 days in culture. Ai) Live and dead staining for viable (green) and dead (red) cells in magCSs (fluorescence microscopy x20, scale bar 50μm, merged images). Aii) metabolic activity determined by MTS assay. Symbols denote statistical differences ** for $p<0.01$. B) SEM micrographs analysis (x500). Insets are representative images of magCSs in a lower amplification (x150). The graphs and the tables represent the atomic percentage (wt %) of carbon (C), oxygen (O) and iron (Fe) detected in the magCSs experimental groups. Two different samples per condition were considered. The "IL-1β" condition refers to magCSs treated with IL-1β while "IL-1β PEMF" defines magCSs treated with IL-1β exposed to PEMF. Control group refers to magCSs in the absence of IL-1β and PEMF.

According to SEM analysis (Figure VII-1 B), tendon cells showed a tendency for alignment distribution within different layers of cells suggesting the establishment of intercellular connections among cells. Moreover, the iron oxide MNPs, represented by the presence of Fe in the elemental characterization of magCSs, tended to align within cells, likely gathering along the interlinking protein filaments of cytoskeleton.

VII-3.2. Tenogenic phenotype in magCSs constructs under an IL-1β rich environment

The impact of IL-1β supplementation and PEMF exposure was assessed on the tenogenic profile of magCSs. With an exception for SCX, it is clear that the relative expression of *MKX*, *TNMD*, and *DCN* were significantly affected by the IL-1β treatment (Figure VII-2).

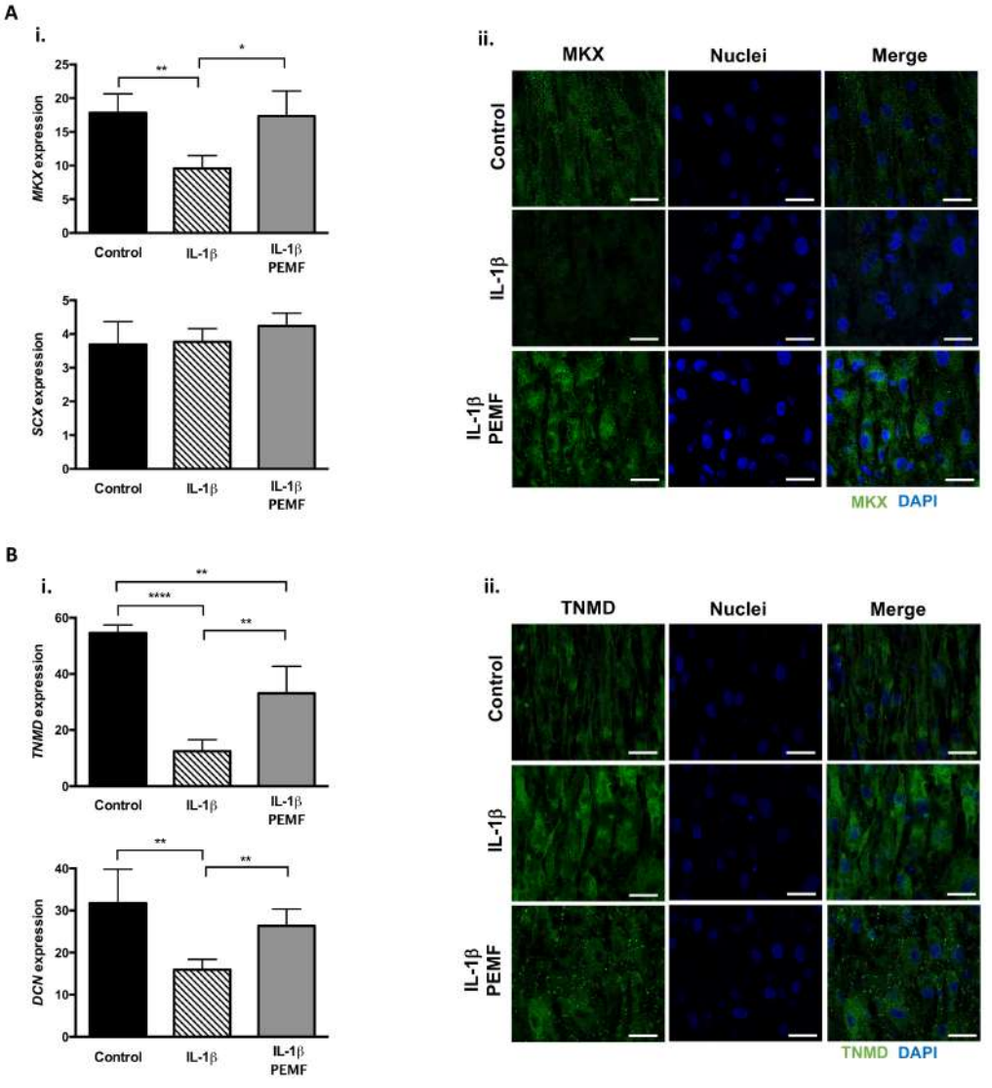

Figure VII-2 Assessement of the genetic expression and immune-location of tenogenic markers in magnetic cell sheets (magCSs) after 3 days in culture. Ai; Bi) Relative gene expression of Mohawk (*MKX*), Scleraxis (*SCX*), Tenomodulin (*TNMD*), Decorin (*DCN*) by real time RT-PCR. Expression of target genes was normalized against GAPDH housekeeping gene. Symbols denote statistical differences, * for $p<0.05$; ** for $p<0.01$ and **** for $p<0.0001$. Aii; Bii) Confocal microscopy images of the tenogenic markers (green), Mohawk and Tenomodulin were assessed at day 3 (confocal microscopy x63, scale bar 50 μm). Nuclei were counterstained with DAPI (blue). The "IL-1β" condition refers to magCSs treated with IL-1β while "IL-1β PEMF" defines magCSs treated with IL-1β exposed to PEMF. Control group refers to magCSs in the absence of IL-1β and PEMF.

MKX and *DCN* show a significant decrease in the expression values in comparison to Control and IL-1β PEMF groups ($p<0.05$) (Figure VII-2 Ai and Bi), while *TNMD* level tends to decrease with both PEMF stimulation ($p<0.01$ to Control) and IL-1β treatment ($p<0.0001$ to Control group) (Figure 2Bi). *SCX* expression seems not to be affected by IL-1β treatment or PEMF, showing similar values for all conditions studied (Figure VII-2 Ai). The maintenance of *SCX* expression levels has been also observed in our previous works [26]. Overall, IL-1β treatment combined with PEMF stimulation leads to an increment in tendon gene expression up to the levels of non-IL-1β magCSs (Control group). The presence of the tenogenic proteins MKX and TNMD were also investigated. MKX and TNMD were observed in all conditions (Figure VII-2 Aii and Bii), however the signal intensity of MKX was lower in magCSs treated with IL-1β. Overall, the combined treatment of IL-1β and PEMF seems not to particularly influence the deposition of these molecules.

VII-3.3. Composition and cohesiveness of magCSs

The normal function of tissues depends on the maintenance of the unique extracellular matrix (ECM) composition and architectural arrangements. In the particular case of tendon tissues, the matrix organization is critical to provide structure and biomechanical properties. Since we supplemented magCSs with a powerful inflammatory mediator that could contribute for architectural changes in ECM, we assessed the expression of enzymes associated to collagen degradation and to ECM remodeling phenomena.

As expected, the expression of *MMP-1*, *MMP-2* and *MMP-3* increased with IL-1β treatment in comparison to control group (*MMP-1*, $p<0.01$; *MMP-2*, *MMP-3* $p<0.0001$) and to magCSs exposed to PEMF (*MMP-1*, $p<0.05$; *MMP-2*, *MMP-3*, $p<0.0001$) (Figure VII-3 A). Interestingly MMP-1 and MMP-3 levels in IL-1β-magCSs stimulated with PEMF are higher than in control group (MMP-1, $p<0.01$; MMP-3, $P<0.0001$). The expression of TIMP-1 also seems to be upregulated in magCSs stimulated with PEMF ($p<0.0001$), while in IL-1β-magCSs was downregulated ($p<0.0001$, in comparison to non-PEMF IL-1β-magCSs). It has been reported that increased levels of MMPs and low levels of TIMP-1 are associated to the degenerative changes in chronic tendinopathy [27]. In inflammatory conditions, elevated MMPs and decreased expression of inhibitors result in the degradation of collagens, proteoglycans and elastin fibers in the ECM [28].

In sum, the IL-1β-magCSs respond to IL-1β supplementation at the gene expression level. PEMF seems to stimulate positively the expression of *MKX*, *TNMD*, *DCN* and *TIMP-1*, and inhibit *MMP-1*, *MMP-2* and *MMP-3* in IL-1β-magCSs.

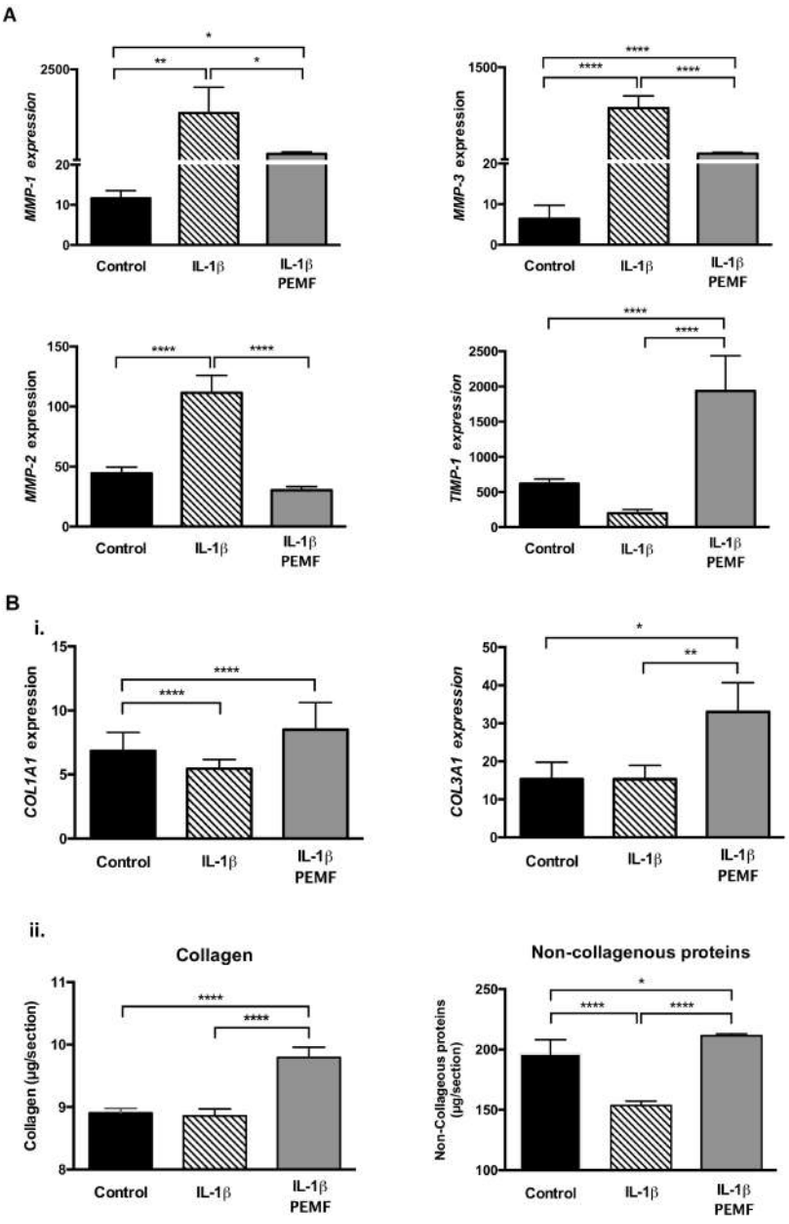

Figure VII-3 Evaluation of the extracellular matrix cohesiveness and stability in magnetic cell sheets (magCSs) after 3 days in culture. A) Relative gene expression of metalloproteinases (MMPs); metalloproteinases-1, -2, -3 (*MMP-1, MMP-2, MMP-3*) and tissue inhibitor metalloproteinase-1 (*TIMP-1*) by real time RT-PCR. Bi) Relative gene expression of collagen type I (*COL1A1)* and collagen type III (*COL3A1)* by real time RT-PCR analysis. Bii) Quantification of collagen and non-collagenous proteins by Sirius Red/Fast Green Collagen staining kit. The "IL-1β" condition refers to magCSs treated with IL-1β while "IL-1β PEMF" defines magCSs treated with IL-1β exposed to PEMF. Control group refers to magCSs in the absence of IL-1β and PEMF.

Collagen type I is the major protein in connective tissues and an increase in collagen type III has been related both to remodeling phase of healing and to fibrotic tissues [29]. We further investigated how IL-1β treatment could directly affect the degradation of collagen and other proteins in ECM produced by magCSs. The expression of collagen type I (*COL1A1)* was decreased in IL-1β-magCSs ($p<0.0001$ in comparison to control group) but collagen type III (*COL3A1)* remained unchanged ($p>0.05$) (Figure VII-3 Bi). However, in terms of collagen production, no significant differences were found between control and IL-1β-magCSs groups ($p>0.05$). Nevertheless, the production of non-collagenous proteins was deeply reduced in IL-1β-magCSs ($p<0.0001$ in comparison to Control group and IL-1β-magCSs stimulated with PEMF groups (Figure VII-3 Bii). When PEMF is applied on IL-1β-magCSs, there is an increment in the *COL3A1* expression ($p<0.001$) and in the amount of both collagenous ($p<0.0001$) and non-collagenous ($p<0.0001$) proteins. The increment is also significant in comparison to magCSs Control group ($p<0.0001$) (Figure VII-3 B), suggesting a catabolic role of PEMF in magCSs treated with IL-1β on the quality and quantity of the ECM.

Thus, PEMF seems to significantly influence the production of collagen ($p<0.05$) in IL-1β-magCSs. The fact that collagen synthesis is also increased in comparison to control group ($p<0.05$) suggests that PEMF promotes the deposition of a collagen rich-ECM matrix. Furthermore, the increased levels of *COL3A1* also supports that the synthesis and deposition of collagen type I and collagen type III is necessary for remodeling process [30].

VII-3.4. Investigation of cytokine profile in magCSs

In an inflammatory milieu, cells communicate through networks of inflammatory mediators to inform about extracellular conditions and adjust the response accordingly. The treatment of magCSs with IL-1β was envisioned to study tendon cell responses to such cues in a cellular matrix enabling juxtacrine and paracrine signaling. Pro-inflammatory genes *IL-8*, *IL-1β*, *TNFα*, and *IL-6* showed increased expression in IL-1β-magCSs, when compared with the other groups under study ($p<0.05$) (Figure VII-4 Ai). Furthermore, the transcript levels of anti-inflammatory factors *IL-10 and IL-4* were decreased in IL-1β-magCSs, in comparison to control (*IL-10*, $p<0.05$; *IL-4*, $p<0.01$) and to PEMF stimulated (*IL-10*, $p<0.0001$; *IL-4*, $p<0.0001$) conditions (Figure VII-4 Bi). These results are supported by ELISA assays, which showed higher concentrations of TNFα and IL-6 but lower amounts of IL-10 released by IL-1β-magCSs (TNFα, $p<0.05$; IL-6, $p<0.0001$ in comparison to Control group).

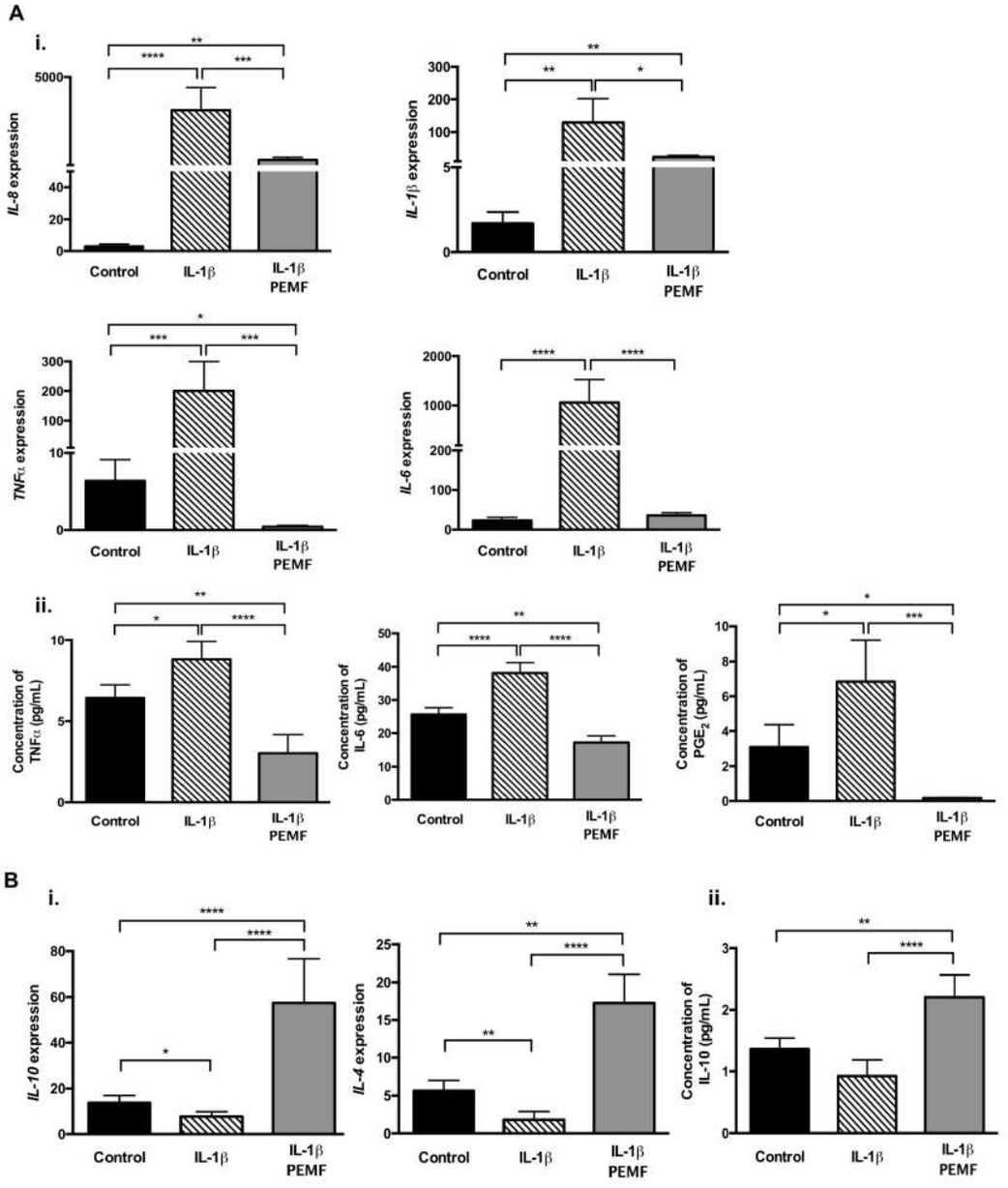

Figure VII-4 Determination of gene expression and release of pro- and anti-inflammatory factors from magnetic cell sheets (magCSs) after 3 days in culture. Ai) Gene expression of pro-inflammatory genes (*IL-8, IL-1β, TNFα*) and Bi) *IL-10* and *IL-4* by real time RT-PCR analysis. Expression of target genes was normalized against GAPDH housekeeping gene. Aii, Bii) Release of TNFα, IL-6 and PGE_2 quantified in cultured medium. Symbols denote statistical differences * for $p<0.05$; ** for $p<0.01$; *** for $p<0.001$ and **** for $p<0.0001$. The "IL-1β"condition refers to magCSs treated with IL-1β while "IL-1β PEMF" defines magCSs treated with IL-1β exposed to PEMF. Control group refers to magCSs in the absence of IL-1β and PEMF.

The effect of PEMF on the inflammatory profile of IL-1β-magCSs was also investigated (Figure VII-4 B). PEMF stimulation caused a decrease in the genetic expression of pro-inflammatory cytokines and an increase in the expression of anti-inflammatory associated genes (*IL-8*, $p<0.001$; *IL-1β,* $p<0.05$; *TNFα*, $p<0.001$; IL-6, $p<0.0001$ in comparison to IL-1β-magCSs). Interestingly, the *TNFα* showed levels of

expression even lower than the Control group ($p<0.05$), while the expression of both *IL-4 and IL-10* was approximately 3-fold higher ($p<0.05$) (Figure VII-4 Bi). The dissimilar values between PEMF and Control groups may be associated to the anti-inflammatory effect of PEMF, even in the absence of IL-1β treatment.

Correlating with the results obtained for gene expression, the release into the culture medium of TNFα and IL-6 were significantly reduced under PEMF in comparison to IL-1β-magCSs ($p<0.0001$) and to the Control group ($p<0.01$) (Figure VII-4 Aii). The concentration of PGE_2, a lipid mediator of pain and acute inflammation, also followed this trend (IL-1β-magCSs, $p<0.001$; magCSs Control, $p<0.05$) (Figure VII-4 Aii).

Additionally, the release of IL-10, was significantly increased with PEMF in comparison to the other conditions (Control group, $p<0.01$; IL-1β-magCSs, $p<0.0001$) (Figure VII-4 Bii). As expected, tendon cells in magCSs constructions respond to IL-1β treatment with pronounced pro-inflammatory cues. Moreover, the pro-inflammatory signals can be modulated by exposure to PEMF, as observed in IL-1β-magCSs, in which the expression levels of pro-inflammatory cues were lower than the ones shown for other conditions. According to these outcomes, PEMF seems to effectively reestablish the levels of anti-inflammatory factors in inflammatory conditions, necessary to the resolution of inflammation to continue healing.

VII-3.5. Involvement of MAPK signaling pathway in the regulation of inflammation cues

In the complex inflammation process, different signaling pathways may be involved and may contribute to the activation and production of multiple pro-inflammatory molecules, namely IL-1β, TNFα or IL-6 [30-32]. The p38 mitogen associated protein kinase MAPK(ER1/2) pathway is involved in many tissues responses, including tendons [33-35]. In this sense, and considering the gene and protein response of magCSs to IL-1β treatment concomitantly modulated by PEMF stimulation, we investigated the potential of MAPK/ERK1/2 on magCSs regulation (Figure VII-5). The MAPK(ER1/2) activation is a consequence of different cytokine stimuli, including IL-1β, triggering the expression of pro-inflammatory factors, such as COX-2 and PGE_2 [36].

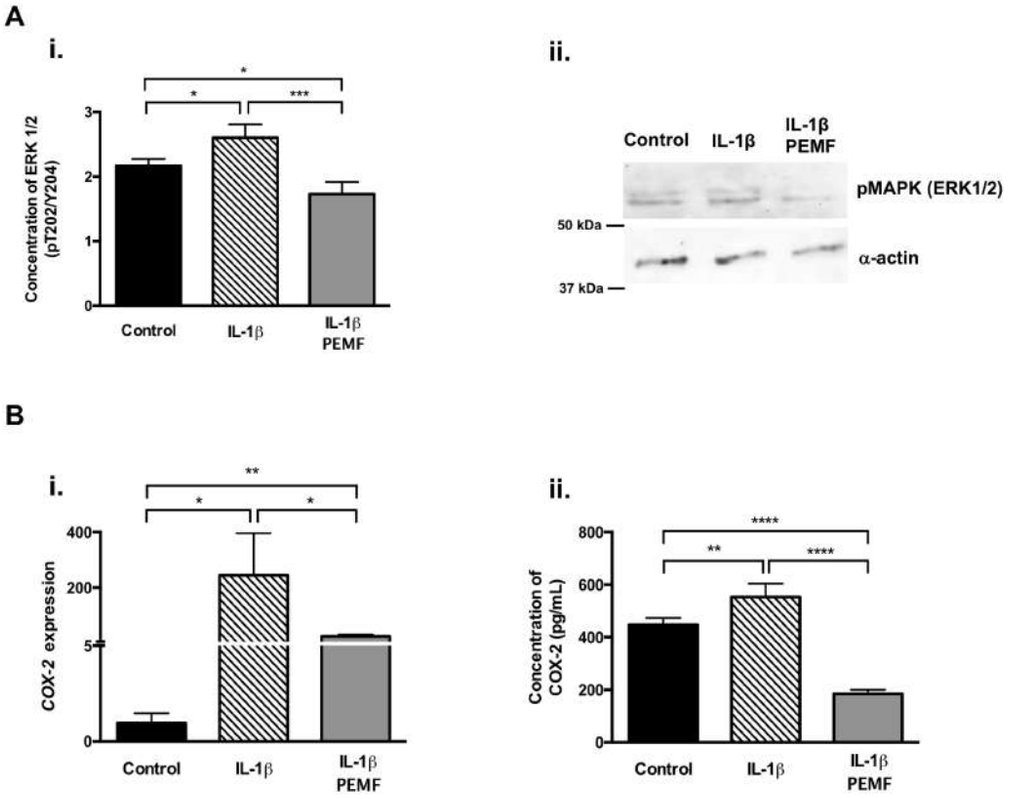

Figure VII-5 MAPK(ERK1/2) signaling pathway activation in magnetic cell sheets (magCSs). Ai) Quantification of ERK1/2 (pT202/Y204) by ELISA assay and Aii) Western blot of phosphorylated MAPK (pMAPK (Erk1/2). Protein lysates were analyzed by probing for phospho-MAPK(Erk1/2) and α-smooth muscle actin (control) in magCSs treated with IL-1β (IL-1β) and after PEMF stimulation (IL-1β PEMF) (two experimental replicates from two biological replicates). Bi) Relative expression of *COX-2* and Bii) quantification of COX-2 release in cultured medium 3 days after IL-1β and PEMF stimulation. Expression of target genes was normalized against GAPDH housekeeping gene. Symbols denote statistical differences * for $p<0.05$; ** for $p<0.01$; *** for $p<0.001$ and **** for $p<0.0001$. The "IL-1β" condition refers to magCSs treated with IL-1β while "IL-1β PEMF" defines magCSs treated with IL-1β exposed to PEMF. Control group refers to magCSs in the absence of IL-1β and PEMF.

The results of phosphorylated ERK1/2 follow the trend of the cytokine profile, with an increased concentration in IL-1β-magCSs (Control group, $p<0.05$). Again, PEMF stimulated IL-1β-magCSs show lower values in comparison to control group ($p<0.001$) (Figure VII-5 Ai). Western blot presented in Figure VII-5 Aii confirmed that PEMF reduces phospho-MAPK(ERK1/2) activation in IL-1β-magCSs, suggesting a role and a targeted action of PEMF in MAPK(ERK1/2) activation in an IL-1β induced pro-inflammatory response.

The pro-inflammatory factors, COX-2 and PGE_2, have been widely described as products resultant from MAPK pathway activation [35-37]. In this study, both *COX-2* transcripts and protein expression were increased with IL-1β treatment ($p<0.05$), and decreased with PEMF stimulation ($p<0.05$) (Figure VII-5 B).

The release of PGE_2 demonstrates a similar tendency. Interestingly, under PEMF the amounts of released PGE_2 are lower than the ones of Control group ($p<0.05$).

VII-4. DISCUSSION

Establishment of an in vitro model with magCSs

The prolonged exposure to pro-inflammatory mediators is known to contribute to impair the resolution of inflammation [38], and ultimately to tendon disorders. Exposing magnetic tendon cell sheets to an inflammatory cytokine-rich milieu, we expected to more accurately predict tendon cell behavior *in vivo* and the potential immunomodulatory contributions of magCSs for tendon therapies.

To antagonize such influence and improve cell-free strategies, we also explored the immunomodulatory effect of PEMF on magCSs conditioned to IL-1β.

The inflammatory stimulus provided by IL-1β influenced the tenogenic phenotype reducing the relative gene expression of *MKX, TNMD,* and *DCN*. Conversely, pro-inflammatory genes were increased, supported by the increment of pro-inflammatory mediators secreted by IL-1β-magCSs. These outcomes, together with the decrease in anti-inflammatory genes and soluble factors, IL-4 and IL-10, suggest that magCSs respond to IL-1β by increasing the expression and production of inflammatory factors into the environment. This response also resulted in a decrease in *COL1A1* expression and in the synthesis of ECM proteins (non-collagenous) suggesting that inflammatory stimuli may also interfere with the quality of the ECM. Afterwards, the matrix composition, organization and cell-matrix interactions are important for normal function of tendon. However, during tendinopathy conditions the normal composition of ECM suffers alterations and has been suggested to be the major factor for the development of this disorder.[30] These alterations interfere with the inflammatory cell signaling and with the cytokine release, thus affecting the important mechanisms of repair [39]. These mediators are responsible for ECM disorganization, which was recognized as a major cause for tendon injuries. In tendinopathy conditions, matrix degradation and the collagen content becomes more heterogeneous and disordered [29, 40]. The degradation of collagen is normally regulated by MMPs and by the activity of their inhibitors (TIMPs). Miller et al. [41] reported that pro-inflammatory factors as TNFα and IL-1α stimulate catabolic degradation of collagen matrix via MMP-2 in intervertebral disc cells under normal and inflammatory environments, stimulated by IL-1α.

To elucidate on the mechanisms associated to the IL-1β treatment, we investigated a possible involvement of the intracellular signaling pathway, MAPK(ERK1/2), which is activated by stressful and pro-inflammatory stimuli.

Our results indicated that IL-1β treatment leads to the activation of MAPK(ERK1/2), likely due to the increment in IL-1β expression on magCSs. In addition, results demonstrated higher expressions of COX-2 and PGE_2 by the IL-1β-magCSs. COX-2 is responsible for the high levels of prostaglandins, such as PGE_2 present in acute and chronic inflammation. MAPK (ERK1/2) signaling is involved in COX-2 expression and the inhibition of this pathway causes a reduced COX-2 expression [42]. Moreover, several studies focusing on inflammation reported that IL-1β influences the levels of COX-2 and PGE_2 through the activation of the MAPK(ERK1/2) pathway [37, 39-41, 43].

Contributions of PEMF stimulation to resolve inflammatory cues in IL-1β-magCSs

From our previous studies, PEMF has shown a great impact in the inflammatory profile of IL-1β-hTDCs influencing the release and expression of cytokines [16, 17, 37]. Despite the fact that such works were performed in monolayer cultures and in the absence of MNPs, the combination of both stimulates specific biochemical responses [44]. Herein, we also explored the role of PEMF to control the inflammatory response of tendon resident cells on magCSs constructions.

We firstly investigated a possible influence of MNPs and PEMF on cell organization and distribution within magCSs. The results showed a tendency for parallel alignment of tendon cells, within magCSs constructions. The cellular organization is an important point to obtain proper healing and disorganized architectures may favor degenerative conditions mediated by pro-inflammatory niches [40].

In relation to gene expression level, magnetic stimulation in inflamed environment provoked a pronounced increase in *MKX*, *TNMD* and *DCN*. Herein, the expression of genes encoding for matrix degrading enzymes were downregulated after the exposure to PEMF, and upregulated for the *TIMP-1* in comparison to the Control group.

Moreover, the expression of collagens increased in IL-1β-magCSs under PEMF stimulation and consequently the production of collagen proteins. During the healing process, high production of collagen fibers is essential, where collagen type III is required for the assemble of collagen type I fibers. Thus, an increase in collagen type III may suggest cellular guidance towards healing [29].

Our results demonstrate that PEMF supports the production of a tendon-like matrix, promote collagen synthesis, and a decrease in MMPs gene expression, evidencing a regulatory influence of PEMF in tendon healing process. Understanding the mechanisms associated to the expression of inflammatory mediators may provide molecular tools for improving current therapeutics [45]. The effect of PEMF in MAPK(ERK1/2) activation of IL-1β-magCSs and the consequent synthesis of COX-2 and PGE_2,was investigated in this work. Several studies have reported that PEMF can modulate the activation of intracellular signaling associated to inflammatory profiles mediated by NF-κB, mTOR or MAPK pathways [16, 46]. Additionally, the phosphorylation of p38 MAPK(ERK1/2) was shown to be inhibited by the exposure to PEMF (1.5mT; 75Hz, 10% duty cycle) in an inflammatory model of synovitis, leading to the decrease of TNFα [47].

In our study, we demonstrate not only a reduction on the phosphorylation of MAPK(ERK1/2) in IL-1β-magCSs but also reduced levels of COX-2 and PGE_2. An exposure to a PEMF (1.5 mT, 75 Hz, 1/10 duty cycle) was also shown to reduce the release of PGE_2 as well as of IL-6 and IL-8 from bovine synovial fibroblasts from osteoarthritis patients treated with IL-1β [48]. Thus, the reduction on the phosphorylation of MAPK(ERK1/2) could also indicate that PEMF may influence the expression and synthesis of pro-inflammatory cytokines and lipid mediators COX-2 and PGE_2 in magCSs through MAPK(ERK1/2). These outcomes are in accordance with published studies [46, 49] supporting the application of magnetic fields in pathologies with an inflammatory context.

Overall, these results show the applicability of magCSs as a cellular model for tendon studies. Moreover, PEMF shows a relevant influence over inflammatory mediators and in the quality and quantity of ECM in magCSs that goes beyond the expression of cytokines, and may depend on MAPK(ERK1/2) pathway.

VII-5. CONCLUSIONS

This work shows that magCSs technologies offer new possibilities as advanced cell culture models for tendon tissue engineering, including pathophysiological models of inflammation to assist the understanding of the onset and progression of tendon disorders. In accordance to previous studies, PEMF evidences a modulatory action in the inflammatory profile of IL-1β primed tendon cells in magCSs constructions, via MAPK(ERK1/2) pathway. The remote control over magCSs strengthens the role of

PEMF in tendon therapies, and highlights the promise of magCSs as a living patch to overcome sustained inflammatory events and to drive tendon repair and regeneration.

VII-6. SUPPLEMENTARY MATERIAL

Supplementary Table VII-1 Primers used for real time quantitative RT-PCR analysis.

	Primer Sequence	Accession number
Human Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	F - TGTACCACCAACTGCTTAGC R - GGCATGGACTGTGGTCATGAG	NM_002046.4
Mohawk (*MKX*)	F – TGTTAAGGCCATAGCTGCGT R – TCGCACAGACACCTGGAAAA	NM_173576.5
Scleraxis (*SCXA*)	F – CGAGAACACCCAGCCCAAAC R – CTCCGAATCGCAGTCTTTCTGTC	XM_001717912
Tenomodulin (*TNMD*)	F – CCGCGTCTGTGAACCTTTAC R – CACCCACCAGTTACAAGGCA	NM_022144.2
Decorin (*DCN*)	F – CAGCATTCCTCAAGGTCTTCCT R – GAGAGCCATTGTCAACAGCA	NM_001920.3
Human matrix metalloproteinase-1 (*MMP-1*)	F – ACCTGGAAAAATACTACAACCTGAA R – TTCAATCCTGTAGGTCAGATGTGTT	NM_002421.3
Human matrix metalloproteinase-2 (*MMP-2*)	F – GCTACGATGGAGGCGCTAAT R – TCAGGTATTGCACTGCCAACT	NM_002422.4
Human matrix metalloproteinase-3 (*MMP-3*)	F – CACTCACAGACCTGACTCGG R – AGTCAGGGGGAGGTCCATAG	NM_002422.4
Collagen, Type I, alpha 1 (*COL1A1*)	F – CGAAGACATCCCACCAATCAC R – GTCACAGATCACGTCATCGC	NM_000088.3
Collagen, Type III, alpha 1 (*COL3A1*)	F – GCTGGCTACTTCTCGCTCTG R – TTGGCATGGTTCTGGCTTCC	NM_000090.3
Human interleukin 8 (*IL-8*)	F – GGTGCAGTTTTGCCAAGGAG R – TTCCTTGGGGTCCAGACAGA	NM_001354840.1
Human interleukin 1 beta (*IL-1β*)	F – TGAGCTCGCCAGTGAAATGA R – AGGAGCACTTCATCTGTTTAGGG	NM_000576.2
Human tumor necrosis factor (*TNFα*)	F – ATGTTGTAGCAAACCCTCAAGC R – TGATGGCAGAGAGGAGGTTG	NM_000594.3
Human interleukin 6 (*IL-6*)	F – AGGAGACTTGCCTGGTGAAA R – GCATTTGTGGTTGGGTCAG	NM_000600.4
Human interleukin 10 (*IL-10*)	F – AAGACCCAGACATCAAGGCG R – AATCGATGACAGCGCCGTAG	NM_000572.2
Human interleukin 4 (*IL-4*)	F – GCACCGAGTTGACCGTAACA R – AGGAATTCAAGCCCGCCAG	NM_000589.3

VII-7. REFERENCES

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Chapter VIII

Magnetic responsive materials modulate the inflammatory profile of IL-1β conditioned tendon cells

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ABSTRACT

Tendinopathies represent half of all musculoskeletal injuries worldwide. Inflammatory events contribute to both tendon healing and to tendinopathy conditions but the cellular triggers leading to one or the other are unknown. In previous studies, we showed that magnetic field actuation modulates human tendon-derived cells (hTDCs) behavior in pro-inflammatory environments, and that magnetic responsive membranes could positively influence inflammation responses in a rat ectopic model.

Herein, we propose to investigate the potential synergistic action of the magnetic responsive membranes, made of a polymer blend of starch with polycaprolactone incorporating magnetic nanoparticles (magSPCL), and the actuation of pulsed electromagnetic field (PEMF): 5 Hz, 4 mT of intensity and 50 % of duty cycle, in IL-1β-treated-hTDCs, and in the immunomodulatory response of macrophages.

It was found that the expression of pro-inflammatory (*TNFα, IL-6, IL-8, COX-2*) and ECM remodeling (*MMP-1,-2,-3*) markers tend to decrease in cells cultured onto magSPCL membranes under PEMF, while the expression of TIMP-1 and anti-inflammatory genes (*IL-4, IL-10*) increases. Also, $CD16^{++}$ and $CD206^{+}$ macrophages were only found on magSPCL membranes with PEMF application.

Magnetic responsive membranes show a modulatory effect on the inflammatory profile of hTDCs favoring anti-inflammatory cues which is also supported by the anti-inflammatory/repair markers expressed in macrophages. These results suggest that magnetic responsive magSPCL membranes can contribute for inflammation resolution acting on both resident cell populations and inflammatory cells, and thus significantly contribute to tendon regenerative strategies.

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VIII-1. INTRODUCTION

Tendinopathy defines a multifactorial spectrum of multiple disorders often resulting from overuse activities and/or excessive mechanical loading [1, 2]. Tendinopathies are a significantly clinical problem, accounting for almost 50% of all musculoskeletal afflictions worldwide [3, 4]. Clinical diagnosis of tendinopathy relies on gradual onset of activity-related pain, decreased function and sometimes localized swelling. Current therapies target pain relief and consist of oral and locally administered anti-inflammatory drugs. Severe injury often requires surgery but in either case, the treatment is not effective, as it fails to reinstate tendon function following injury [1, 2, 5]. Inflammation is a necessary process for healing, but chronic inflammation has been related to several tendinopathies in tendons with different functions and anatomical locations [5].

Macrophages modulate inflammatory cascades switching their own phenotype to environmental stimuli. The balance between pro-inflammatory and pro-repair macrophage populations (M1 and M2, respectively) dictates inflammation regulation and resolution, being critical for improved healing [6]. Although recent studies show that macrophages are present in injured tendons, their influence on tendon resident cells and in tendon repair is unclear [7, 8]. Thus, studies aimed at finding appropriate tools for modulating inflammation and prospecting tissue regeneration, open positive perspectives for new therapeutic approaches to treat tendon disorders.

Pulsed electromagnetic field (PEMF) has been clinically applied to manage pain and delayed wound healing via remote actuation and minimally invasive approaches [9, 10]. In a previous work, we showed that a particular set of PEMF parameters modulated the pro-inflammatory profile of IL-1β-conditioned tendon cells influencing intracellular communication associated to MAPK/Erk(1/2) pathway and connexin-43 [11]. Other studies also showed that the length of exposure, the field intensity and the number of PEMF treatments differently affect tendon cell proliferation, gene expression and the kinetics released of pro- and anti-inflammatory cytokines [12].Furthermore, the impact of PEMF was reported to enhance stem cells proliferation [13] and tenogenic differentiation [14, 15], likely providing mechanical cues to the cells [16, 17], strengthening the potential of magnetic stimuli to remotely trigger and modulate cell responses.

The combination of magnetic actuation with magnetic responsive biomaterials has emerged as a platform enabling a precise action over biological processes in tissue engineering and regenerative medicine (TERM) strategies [18, 19], foreseeing sophisticated, remotely controlled systems with higher

therapeutic potential. Our group has recently demonstrated that magnetic responsive fibrous scaffolds can improve *in vitro* tenogenesis of mesenchymal stem cells through mechano-sensing mechanisms [20], and that the combination of magnetic responsive biomaterials and magnetic stimuli favors immunomodulatory responses that may contribute to tendon healing and to functional recovery [21, 22]. Additionally, strategies resourcing to magnetic responsive constructs are envisioned to potentiate more effective and real time monitoring treatments [18, 22-25].

Consequently, we hypothesized that magnetic responsive biomaterials exposed to an external magnetic field could have a synergistic action in modulating the inflammatory profile of tendon cells after IL-1β stimulation.

We and others have observed that IL-1β stimulates pro-inflammatory cues in tendon cells and could be used as an *in vitro* model in tendon inflammation studies [7, 26, 27]. Thus, tendon cells previously treated with IL-1β were cultured on magnetic responsive membranes made of a polymeric blend of poly-ε-caprolactone and starch (SPCL) incorporating iron oxide nanoparticles (magSPCL) and exposed to a magnetic field, whose intensity, frequency and duty cycle were previously optimized [11]. These membranes were previous studied, showing promising outcomes in modulating inflammation and the growth of fibrous tissue in an ectopic rodent model [22].

The phenotype and inflammatory profile of tendon cells was then investigated and compared to non-PEMF conditions. We further hypothesized that PEMF actuated magSPCL membranes could also influence macrophage populations in pro-inflammatory environments.

Thus, human macrophages were cultured on magSPCL membranes and markers associated to pro-inflammatory (M1) and repair (M2) phenotypes were investigated to provide on the immuno-modulatory effect of the PEMF and magSPCL membranes on inflammatory cells envisioning complementary and improved oriented strategies headed for tendon repair and regeneration.

VIII-2. MATERIALS AND METHODS

VIII-2.1. MagSPCL membranes preparation

Membranes made of a 30:70 blend of starch and polycaprolactone (SPCL, Novamont, Novara, Italy) were produced using the solvent casting method as reported before [22, 28]. Briefly, SPCL granules were

dissolved in chloroform 20 % (w/v) (VWR, Darmstadt, Germany). Then, the polymeric solution was doped with commercial iron oxide magnetic nanoparticles (Fe_3O_4, magnetite, MNPs) (Micromod, 45-00-252, Rostock, Germany). These MNPs form monodisperse magnetic aggregates (ϕ 250 nm), exhibit a plain surface and a polydispersity index <0.2. MNPs are stable in aqueous buffers with pH >4, and present a magnetization value of 46 emu/g iron (H= 1000 Oe), a saturation magnetization >71 emu/g iron (H>10.000 Oe) and a coercive field Hc of 0.481 Ka/m. MNPs were thoroughly dispersed in the SPCL/chloroform solution by sonication (DT100H SONOREX; VWR), which provided energy to agitate and distribute particles in the membrane, and occasional orbitation during 30 min at a temperature of 20 °C-25 °C, before casted in glass petri dishes. After detaching, membranes were washed with ultrapure water and dried. The magnetic formulation was created with SPCL/1.8 % MNPs (w/w). The casted membranes, previously shown to display an average thickness of 0.19 ± 0.02 mm [22], were cut into 1 cm^2 discs using a hollow punch and sterilized by exposure to an UV lamp for 30min before the cell culture studies.

VIII-2.2. Cells Isolation and expansion

Human tendon derived cells (hTDCs) were isolated from surplus tissue samples collected from patients undergoing orthopedic reconstructive surgeries under protocols previously established with Hospital da Prelada (Porto, Portugal) and with informed consent of the patients. The content of the written informed consent and related procedures were reviewed and approved by the Hospital Ethics Committee (P.I. N. º005/2019). The healthy nature of tendons was confirmed by the orthopedic surgeon at the surgical theater upon harvesting of tendon tissues.

Human tendon derived cells were isolated and cultured as described before [29-31]. Briefly, tissue explants were immersed in a sterile solution of phosphate buffer saline (PBS, Sigma-Aldrich, Saint Louis, MO, USA) with 2% antibiotic/antimicotic solution (A/A, Alfagene, Life Technologies Limited, Paisley, UK). Then, tissue samples were minced and digested in an enzymatic solution of 0.1 % (v/v) of collagenase (Sigma-Aldrich, C6885, Saint Louis, MO, USA) with 2M $CaCl_2$ (VWR, Darmstadt, Germany) (1:1000) and 1 % bovine serum albumin (BSA, Sigma-Aldrich, Saint Louis, MO, USA) for 1 h at 37 °C under agitation. After incubation, digested samples were filtered using a cell strainer (100µm, Enzifarma, 352360) adapted for 50 ml conical tubes (Falcon), centrifuged three times at 290 g for 5 min, and the supernatant discarded. hTDCs were expanded in basic culture medium composed of α-MEM (A-MEM, Invitrogen, Life Technologies Limited, Paisley, UK) supplemented with 10% FBS (Alfagene, Life Technologies Limited,

Paisley, UK) and 1 % A/A solution (Alfagene, Life Technologies Limited, Paisley, UK).

VIII-2.3. Human peripheral blood mononuclear cells isolation

Human peripheral blood mononuclear cells (PBMCs) were obtained from blood donations of healthy adult volunteers and collected into a heparin containing tubes to avoid coagulation. Human macrophages were obtained from buffy coat fractions separated form blood donation units of healthy volunteers under an cooperation agreement established with the Portuguese Institute of Blood and Transplantation (IPST, Instituto Português do Sangue e Transplantação, Portugal). Then, the samples were diluted in 2-4 volumes of PBS containing BSA. PBMCs were isolated from buffy coats using a density gradient cell separation medium, Histopaque (Sigma-Aldrich, Saint Louis, MO, USA), after centrifugation at 800g for 30 min at room temperature. The mononuclear cell layer, comprising lymphocytes and monocytes, was collected into a tube already containing a PBS/BSA solution and centrifuged at 350 g for 10 min. The pellet was resuspended in PBS/BSA and centrifuged in 3 cycles at 4 °C at 350 g for 5 min. Then, cells were positively immunomagnetically sorted using human CD14+ beads (microBeads, Miltenyi Biotec, Bergish Gladbach, Germany) and cultured in RPMI-1640 medium (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 50 ng/mL of macrophage colony-stimulated factor (M-CSF, Peprotech, Rocky Hill, NJ, USA) for 6 days to differentiate into M0-type macrophages.

VIII-2.4. Cell culture on magSPCL membranes (constructs) under magnetic stimulation

Human TDCs cells were seeded at a density of 1 x 10^5 cells per membrane in α-MEM medium (A-MEM, Invitrogen, Life Technologies Limited, Paisley, UK). hTDCs were treated for 24h with exogenous supplementation of IL-1β (1 ng/mL, Alfagene, Life Technologies Limited, Paisley, UK) after cell attachment and 24 h after seeding of tendon cells. Then, cells were cultured for 1, 3 and 7 days, as previously established [11]. Similarly, human macrophages were seeded at 2 x 10^5 cells per membrane and cultured in RPMI medium (Sigma-Aldrich, Saint Louis, MO, USA). Both experiments were conducted under both static (control, non-PEMF) and magnetic stimulation. A magneto therapy device (Magnum XL Pro, Globus, Globus Corporation, Italy) was used to generate a controlled magnetic field with a frequency of 5 Hz, 4 mT of intensity and 50 % of duty cycle in accordance to previous studies [11]. Cells were magnetically stimulated for a single 1 h period.

The rationale behind the selection of these PEMF parameters and the exposure time was to stimulate the cells just enough time to elicit a biological response, in this case, to modulate tendon cells inflammatory profile.

The stimulus provided was selected from a set of therapeutic programmes used in health-rehab medical treatments, pre-defined in the magnetotherapy device.

Cell laden membranes were placed and cultured between two coils (solenoids). The magnetic field was generated by the electric current that goes through a coil (solenoid) with a vertical action between the coils. Afterwards, the system was moved inside an incubator, where the temperature was maintained at 37 °C in humidified 5 % CO_2 atmosphere during the PEMF stimulation. The PEMF (5 Hz, 4 mT and 50 % of duty cycle) was applied directly over cell culture plates inside the incubator.

Two experimental controls were considered: i) hTDCs seeded on magSPCL membranes cultured in non- PEMF stimulating (static) conditions to investigate PEMF vs non-PEMF conditions, and ii) hTDCs seeded on magSPCL membranes in non-PEMF stimulating conditions without IL-1β treatment to assess tendon cell responses in non-stimulated culture conditions.

Cells-membrane constructs were further investigated for cell viability, proliferation and mRNA transcriptional levels of inflammatory and tendon-associated markers.

VIII-2.5. Morphological characterization of magnetic responsive cell laden membranes

After 1, 3 and 7 days in culture, cells laden onto magnetic responsive membranes were washed with phosphate buffer saline (PBS) and fixed in 4 % formaldehyde for 30 min at room temperature. Subsequently, samples were dehydrated using an ascending solution of ethanol (from 30 to 100%), followed by a 5min immersion in hexamethyldisiloxane (HMSO, Sigma-Aldrich, Saint Louis, MO, USA). Next, samples were air-dried overnight and sputter coated (30 s at 20 mA, Cressington, C5219, Model 108A) with gold. Images of the constructs were acquired using a scanning electron microscope (SEM, JEOL, Japan).

VIII-2.6. Human TDCs (hTDCs) characterization

VIII-2.6.1. Metabolic activity and cell proliferation

The metabolic activity and proliferation of hTDCs were evaluated by MTS assay (CellTiter 96® AQueous One Solution, Promega) and Quant-It PicoGreen dsDNA assay Kit (Thermo Fisher Scientific), respectively. For the MTS assay, cell laden membranes were washed with PBS and incubated with a mixture of serum-free culture medium without phenol red and MTS solution (5:1 ratio) for 3 h at 37 °C and 5% CO_2 atmosphere, protected from light. After this period, the supernatant was transferred to a 96-well plate and the absorbance read at 490 nm (Synergy™HT, BIO-TEK Instruments, Winooski, Vermont). Samples were read in triplicates and a blank sample (no cells) was assessed as control of the assay.

For the dsDNA quantification assay, samples were washed with PBS and then transferred to a microtube with 1ml of sterile ultrapure water and kept at -80 °C until analysis. Samples were then thawed, sonicated and analyzed according to the manufacturer's instructions, at an excitation/emission wavelength of 485/528 nm (Synergy™HT, BIO-TEK Instruments, Winooski, Vermont). Samples and standards were made in triplicate.

VIII-2.6.2. Immunofluorescence for tendon related-markers

Cell laden membranes cultured for 1, 3 and 7 days were washed three times with PBS, before and after fixation with 10 % (v/v) neutral buffered formalin (Thermo Fisher Scientific) overnight and kept in PBS at 4 °C until usage. To detect tendon ECM proteins, antibodies against collagen type I (Rabbit polyclonal Anti-Collagen I, ab9039, 1:500, Abcam, Cambridge, UK) and Tenomodulin (Rabbit anti-human, ab81328, 1:100, Abcam, Cambridge, UK) were used. The immunolocation of Scleraxis (Rabbit anti-human, ab58655, 1:100, Abcam, Cambridge, UK), NF-κB p65 (Rabbit anti-human, F0514, 1:200, Santa Cruz Biotechnology, Heidelberg, Germany), alpha smooth muscle Actin (Rabbit anti-human, ab32575, 1:200, Abcam, Cambridge, UK) and Vinculin (Mouse anti-human, V9131, 1:300, Sigma-Aldrich, Saint Louis, MO, USA) were also assessed.

The cells cultured on the magSPCL membranes were firstly permeabilized with 0.025 % (v/v) Triton X-100 (Sigma-Aldrich, Saint Louis, MO, USA) prepared in PBS for 10 min. Then, constructs were washed three times with PBS, blocked with Normal Horse Serum (RTU Vectastain Kit, PK-7200, Vector,

Burlingame, California USA) and incubated overnight with the antibodies diluted in antibody diluent with background reducing components (Dako, Santa Clara, CA, USA) at 4 °C. Afterwards, samples were rinsed in PBS, following by inactivation of endogenous peroxidase activity with hydrogen peroxide solution (0.3 % w/v, Panreac). The samples were incubated for 1 h at room temperature with fluorescent secondary antibodies Alexa fluor 488 donkey anti-rabbit (2072687, 1:1000, Alfagene, Life Technologies Limited, Paisley, UK) or Alexa fluor 488 rabbit anti-mouse (2005937, 1:100, Alfagene, Life Technologies Limited, Paisley, UK), according to the host species of the primary antibodies. Constructs were rinsed in PBS and stained with 4,6-Diamidino-2-phenyindole, dilactate (DAPI, 5 mg/ml, D9564, Sigma-Aldrich, Saint Louis, MO, USA) for 10 min. Finally, samples were incubated with a Phalloidin–Tetra- methylrhodamine B isothiocyanate (Phalloidin, P1951, 1:200, Sigma-Aldrich, Saint Louis, MO, USA) solution, which was prepared according to manufacturer's instructions.

Surface specific markers for macrophage phenotype were assessed using conjugated antibodies: CD14 PE (Anti-human, B244291, Biolegend), CD16 APC (anti-human, B2128291, Biolegend), CD45 FITC (anti-human, 555482, BD Biosciences), CD68 APC (anti-human, B178818, Biolegend), CD169 (alexa fluor 647 mouse anti-human 565295, BD Biosciences) and CD206 FITC (anti-human, 5253911). Controls were incubated in Dako diluent without the primary antibody.

VIII-2.6.3. Cytokine analysis

Cell supernatants were collected 1, 3 and 7 days after hTDCs treatment with IL-1β and PEMF stimulation and stored at -80ºC until analysis. The supernatants were tested using commercially available enzyme immunoassay kit for IL-6 (Human IL-6 Standard ABTS ELISA Development Kit, 900-K16, Peprotech, Rocky Hill, NJ, USA) and for TNFα (Human TNFα ELISA Kit, EK0525, Tebu-Bio, Fremont, CA, USA), according to the manufacturer's instructions. Data was normalized to control conditions: hTDCs without IL-1β treatment.

VIII-2.7. RNA Isolation and Gene Expression Analysis

Total RNA was extracted using TRI reagent® RNA Isolation Reagent (T9424, Sigma) following the manufacturer's instructions. RNA was quantified using a Nanodrop® ND-1000 spectrophotometer (Wilmington, DE, USA) at 260/280 nm. The first-strand complementary DNA was synthesized from 1 μg

of RNA of each sample (qScript™ cDNA Synthesis Kit, Quanta Biosciences, Gaithersburg, MD, USA) in a 20 μL reaction using a Mastercycler® ep realplex gradient S machine (Eppendorf, Hamburg, Germany).

The quantification of the transcripts was carried out by quantitative polymerase chain reaction (qPCR) using the PerfeCTA SYBR Green FastMix kit (Quanta Biosciences, Gaithersburg, MD, USA) following the manufacturer's protocol, in a Real-Time Mastercycler ep realplex thermocycler (Eppendorf, Hamburg, Germany). The primers were designed with Primer 3 software (Supplementary Table VIII-1) and synthesized by MWG Biotech. The $2^{-\Delta\Delta_{Ct}}$ method was used to evaluate the relative expression level for each target gene [32].

The transcript expression of target genes was analyzed and normalized to the expression of endogenous housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and then to the samples collected at day 0 (n=3).

VIII-2.8. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). The statistical analysis was performed using GraphPad Prism6 software. Data was obtained from 3-independent experiments (n=3) analyzed in triplicate, and evaluated by Two-way ANOVA followed by Bonferroni post-hoc test for multiple comparison tests. A difference was considered significant with a confidence interval of 95 % for different degrees of confidence, $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$.

VIII-3. RESULTS

In this work, we explored the synergism of magnetic actuation and magSPCL membranes in IL-1β treated hTDCs to be further explored as tools to promote tendon healing and regeneration.

VIII-3.1. Cytocompatibility and morphological characterization

The metabolic activity of IL-1β treated hTDCs laden on magSPCL membranes was assessed by MTS assay (Figure VIII-1 Ai). Metabolic activity increases in IL-1β primed cells stimulated with PEMF on day 1 and day 3 in comparison to control conditions (absence of IL-1β and PEMF stimulation, $p < 0.01$).

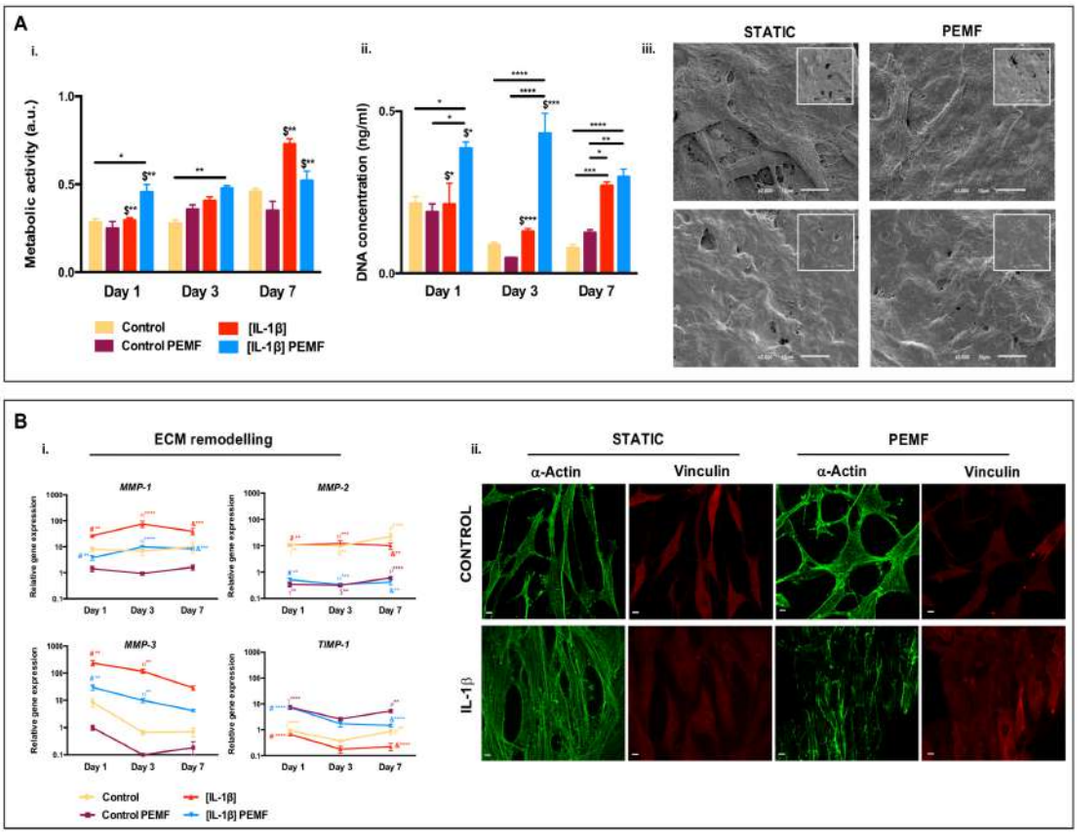

Figure VIII-1 Response of hTDCs laden on magnetic actuated membranes after IL-1β treated hTDCs and PEMF exposure. Ai) The metabolic activity was determined by the MTS assay and Aii) The cell content was determined by the PicoGreen assay. hTDCs viability and cell content was assessed on PEMF actuated membranes after 1, 3 and 7 days in culture. Aiii) Morphological analysis by SEM in hTDCs on day 1 and 7 (on upper and bottom images, respectively). Insets are representative images of hTDCs without IL-1β treatment (control). Bi) Relative gene expression of ECM remodeling genes (*MMP-1*, *MMP-2*, *MMP-3* and *TIMP-1*). Bii) Immunodetection of alpha-actin (green) and vinculin (red), respectively expressed by hTDCs after 7 days in culture (confocal microscopy x63, scale bar 20μm). Control condition (Ctrl) refers to the absence of IL-1β. significant differences are shown with different degrees of confidence. Symbols $, #, γ, α, &, p, denote statistical differences * for $p<0.05$; ** for $p<0.01$; *** for $p<0.001$; and **** for $p<0.0001$, respectively.

Furthermore, an increment in IL-1β primed cells was observed upon PEMF stimulation after 1 and 7 days in culture ($p<0.05$). In terms of DNA content (Figure VIII-1 Aii), IL-1β primed cells stimulated with PEMF showed the highest dsDNA concentration among all conditions studied ($p<0.05$) and for all time points studied. However, the increment on the cell content with time was not statistically relevant suggesting that IL-1β primed cells become metabolic more active but not more proliferative.

The morphology of hTDCs was analysed by SEM (Figure VIII-1 Aiii), showing a homogeneous distribution of hTDCs with an elongated shape on the surface of magSPCL membranes (day 1). The density of hTDCs increases with time in culture, and by day 7 several layers of hTDCs are observed,

covering the magSPCL membranes. As expected, the PEMF does not negatively affect the metabolic activity neither the cell distribution on magSPCL membranes.

VIII-3.2. Extracellular matrix and cytoskeletal organization

The extracellular matrix (ECM) and the proteins actin and vinculin were evaluated in IL-1β treated hTDCs on magSPCL membranes (Figure VIII-1 B). Independently of the time in culture, the application of PEMF causes a decrease in the *MMP-1, -2, -3* expression ($p<0.0001$, $p<0.01$, $p<0.05$) (Figure VIII-1 Bi). Nevertheless, PEMF actuation increases *TIMP-1* expression in stimulated cells ($p<0.0001$). The higher expression values of *TIMP-1* in comparison to the ones of the MMPs analysed, suggests a potential effect of PEMF actuation in the ECM remodeling process.

Staining of actin filaments (α-actin) and focal adhesions (vinculin) were also investigated (Figure VIII-1 Biii) to assess the contribution of PEMF and PEMF actuated membranes in cell mechano-sensing and cell adhesion processes. Vinculin immunodetection is observed in all conditions and in both static (non-PEMF) and PEMF conditions without significant variations. However, the expression of α-actin tends to decrease in IL-1β treated hTDCs under PEMF condition in comparison to untreated hTDCs after 7 days in culture.

VIII-3.3. Expression of tendon related markers in cell laden magSPCL membranes

Tendon related proteins were analysed on IL-1β treated hTDCs laden in magSPCL membranes, namely scleraxis (SCX), tenomodulin (TNMD), and collagen type I (COL1), (Figure VIII-2 A, B and C) to assess a possible influence of pro-inflammatory factors on the maintenance of the tenogenic phenotype of hTDCs under magnetic stimulation. The expression of SCX, an early tendon marker, was increased under PEMF stimulation compared to non-stimulated (static) cells, while for the late tendon markers, TNMD and COL1, only TNMD followed this trend. Additionally, the expression of tenogenic transcription factors; SCX and Mohawk (MKX) as well as of ECM proteins; collagen type I and type III (COL1A1, COL3A1), decorin (DCN), and tenascin C (TNC) were also investigated (Figure VIII-2C).

Overall, the genetic expression of *MKX* ($p<0.001$), *DCN* ($p<0.01$), *TNC* ($p<0.0001$) and collagens ($p<0.0001$ for *COL1A1* and $p<0.0001$ for *COL3A1*, respectively) increased under PEMF application, in comparison to non-stimulated cell laden magSPCL membranes, independently of the IL-1β treatment.

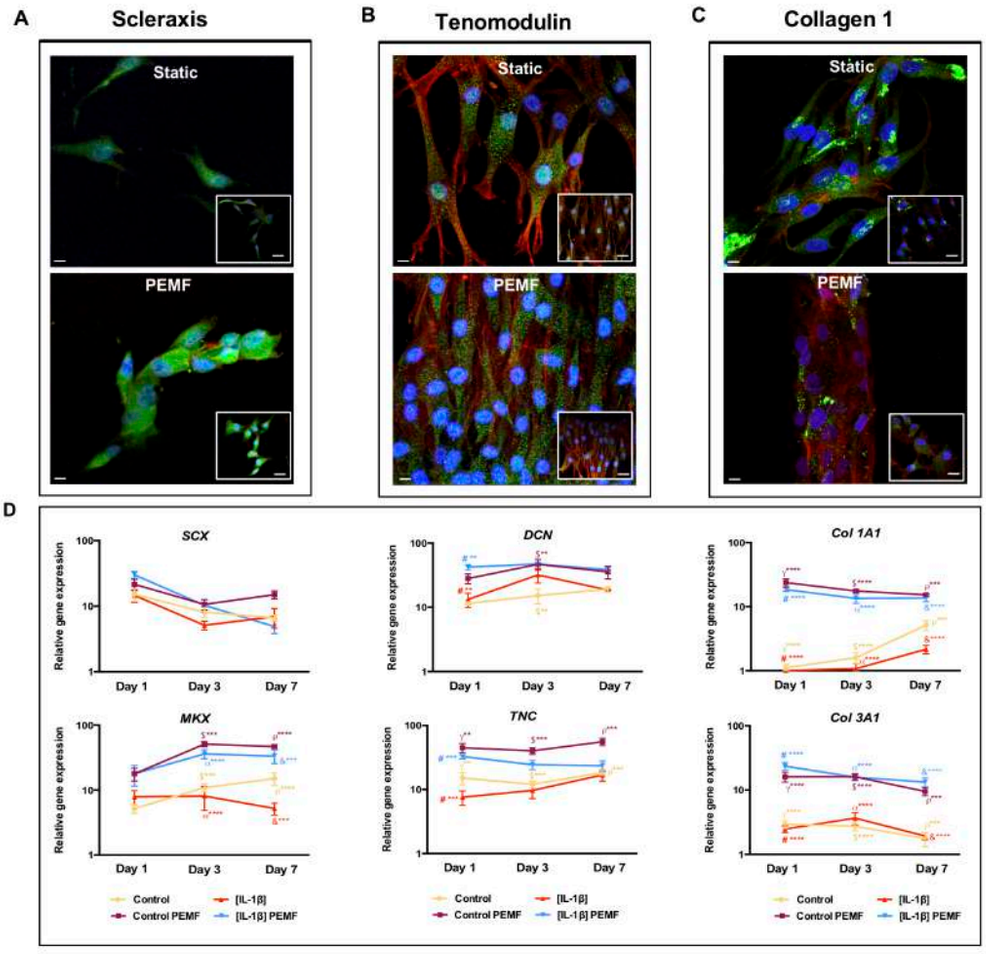

Figure VIII-2 Protein and gene expression of tenogenic markers in IL-1β treated hTDCs cultured on magnetic actuated membranes. A) Fluorescence microscopy of tenogenic-related markers (green), scleraxis (day 1), tenomodulin and collagen type I (day 3) (confocal microscopy x63, scale bar 20 μm). Insets are representative images of hTDCs without IL-1β treatment (control). Nuclei and cytoskeleton were counterstained with DAPI (blue) and phalloidin (red), respectively. B) Gene expression analysis of Scleraxis, Mohawk, Decorin, Tenascin-C, Collagen type I and III by RT-PCR. Control condition refers to the absence of IL-1β treatment. Static condition refers to the absence of PEMF stimulation. Symbols $, #, γ, α, &, p, denote statistical differences * for p<0.05; ** for p<0.01; *** for p<0.001; and **** for p<0.0001, respectively.

These results indicate that PEMF actuated membranes influence the gene and protein expression of tendon related-markers supporting the tenogenic phenotype in IL-1β treated hTDCs.

VIII-3.4. The impact of PEMF in NF-κB signaling

The potential effect of magnetic responsive magSPCL membranes on inflammatory signaling mechanisms of IL-1β was investigated by analysing the gene expression of IL-1β receptor (IL1R1) and NF-κB (Figure VIII-3A and B). In the canonical NF-κB signaling pathway IL-1β activates the IL1R1 receptor,

leading to an activation of IKKβ complex and this results in a translocation of NF-κB to the nucleus and further activation of target gene transcription of pro-inflammatory factors (TNFα, IL-1β, IL-6) (Figure VIII-3Ai). The expression of IL1R1 decreased on IL-1β-treated cells when PEMF is applied (Figure 3Aii). As expected, IL-1β treatment increased the expression of NF-κB ($p<0.01$ in comparison to non-treated hTDCs) (Figure VIII-3Bii). The effect of the IL-1β treatment seems to be antagonized by PEMF actuation which decreases the expression of NF-κB, being more evident after 3 days of culture ($p<0.0001$).

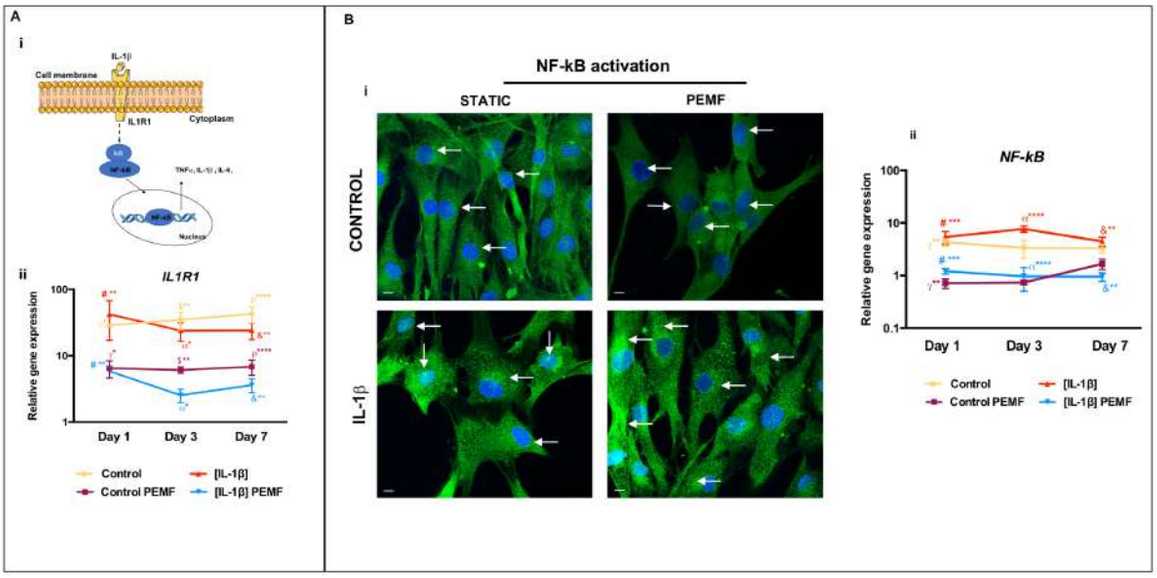

Figure VIII-3 Activation of NF-κB signaling pathway by hTDCs on PEMF actuated membranes. Ai) Schematic representation of NF-κB signaling pathway. Aii) Gene expression analysis of IL-1R1 upon hTDCs treatment with IL-1β by RT-PCR analysis. Bi) Confocal images of NF-kB (green) and nuclei (DAPI, blue) after 3 days in culture on PEMF actuated membranes (confocal microscopy x63, scale bar 20 μm). Arrows point the intracellular localization of NF-κB protein. Bii) Gene expression analysis of NF-κB upon hTDCs treatment with IL-1β by RT-PCR analysis. Control condition refers to the absence of IL-1β treatment. Static condition refers to the absence of PEMF stimulation. Symbols $, #, γ, α, &, p, denote statistical differences * for $p<0.05$; ** for $p<0.01$; *** for $p<0.001$; and **** for $p<0.0001$, respectively.

Additionally, IL-1β increased the phosphorylation of NF-κB showed by a nuclear immunodetection, especially in static (non-PEMF) conditions at day 3 (Figure VIII-3Bi). However, when IL-1β-treated-hTDCs were stimulated with PEMF (day 3), the NF-κB seems to be more concentrated in the cytoplasm (Figure 3Bii). The activation of NF-κB signaling results in the production and release of pro-inflammatory molecules such as TNFα, IL-6, IL-8 and COX-2. The gene expression of these cytokines (Figure VIII-4A) tends to decrease on PEMF stimulated constructs with or without IL-1β.

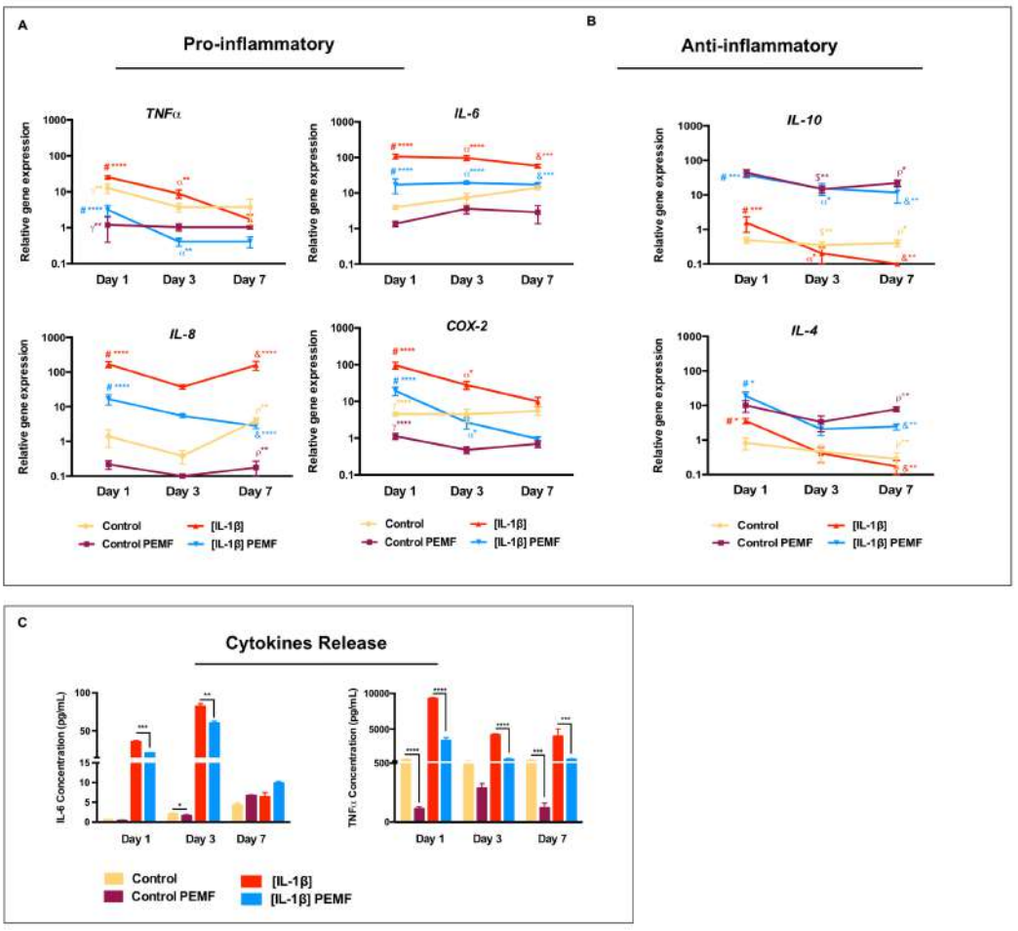

Figure VIII-4 Gene expression and release of pro and anti-inflammatory factors from hTDCs laden on PEMF actuated membranes. Relative gene expression of A) *TNFα, IL-6, IL-8, COX-2*, and B) *IL-10* and *IL-4*, on PEMF actuated membranes upon treatment with IL-1β by RT-PCR analysis. Control condition refers to the absence of IL-1β. C) Release of IL-6 and TNFα quantified in cultured medium 1, 3 and 7 days after IL-1β and PEMF stimulation. Control condition refers to the absence of IL-1β treatment. Symbols $, #, γ, α, &, p, denote statistical differences * for $p<0.05$; ** for $p<0.01$; *** for $p<0.001$; and **** for $p<0.0001$, respectively.

The release of IL-6 and TNFα was diminished following this tendency, especially after IL-1β treatment ($p<0.0001$ for both IL-6 and TNFα, in comparison to control condition) (Figure VIII-4C). Interestingly, IL-6 release was significantly diminished at day 7, in comparison to day 1 and 3 ($p<0.05$), while TNFα release was maintained from day 3 to day 7 ($p>0.05$). Conversely, the expression of anti-inflammatory cytokines (IL-4, IL-10) tends to increase in hTDCs laden on PEMF actuated magSPCL membranes ($p<0.001$ for IL-4 and $p<0.0001$ for IL-10, in comparison to control condition) (Figure VIII-4B).

Thus, PEMF seems to inhibit the expression of NF-κB in IL-1β-treated-cells, influencing the cytokine profile of hTDCs laden in magSPCL membranes.

VIII-3.5. Immunomodulatory potential of PEMF actuated magSPCL membranes

The immunomodulatory action of PEMF combined with magSPCL membranes was assessed in human macrophages (Figure VIII-5 and 6).

Macrophages under PEMF show a more elongated shape on the surface of magSPCL membranes (Figure VIII-5), which is a feature associated to a pro-healing phenotype.

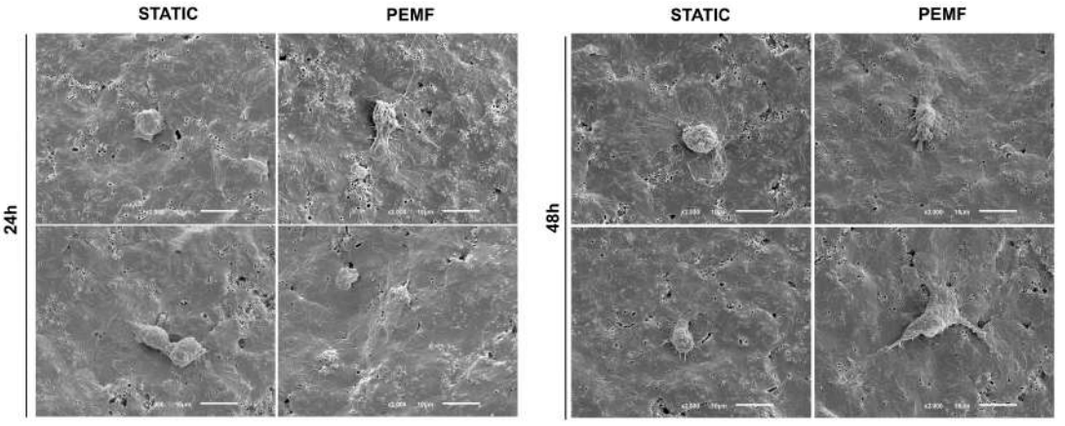

Figure VIII-5 SEM micrographs of human macrophages adhered to the PEMF actuated magSPCL membranes after 24 and 48h in culture. Scale bar, 10μm. Static condition refers to the absence of PEMF stimulation. Insets are representative images of hTDCs without IL-1β treatment (control).

Further, macrophages laden on PEMF actuated magSPCL membranes were CD45+ and CD14+. Cells also expressed CD169+, a cell adhesion molecule associated to M2-type and CD68+, a M1 marker, in static conditions. Interestingly, $CD16^{++}$ and $CD206^{+}$ (M2-type) were only detected in macrophages cultured on PEMF actuated magSPCL membranes (Figure VIII-6).

Altogether, results show that hTDCs and macrophages respond to the combination of PEMF stimulus and magSPCL membranes.

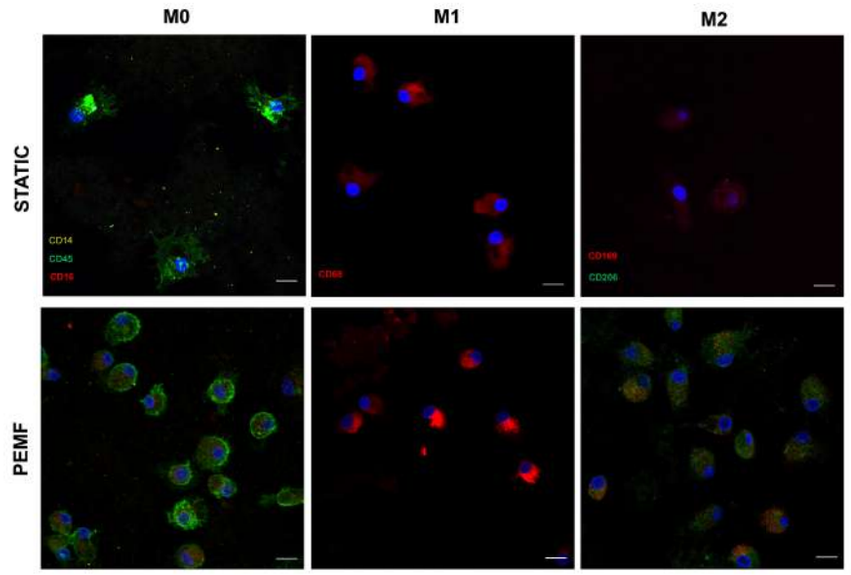

Figure VIII-6 Immunodetection of human macrophage polarization markers in response to PEMF actuated membranes. Confocal microscopy images of immunostaining against M0 phenotype, CD14 (yellow), CD45 (green) and CD16 (red); M1 phenotype, CD68 (red) and M2 phenotype, CD169 (red) and CD206 (green) after 24h in culture in macrophage laden magSPCL membranes (confocal microscopy x63, scale bar 20 μm). Static condition refers to the absence of PEMF stimulation. Insets are representative images of hTDCs without IL-1β treatment (control).

VIII-4. DISCUSSION

The increasing attention given to magnetic actuation and magnetic responsive biomaterials has gathered promising outcomes for improving repair and regenerative mechanisms, challenges to overcome in the TERM field and in current tendon treatment regimens. Having this in mind, the combination of both could accomplish more favorable biological responses and modulate symptoms associated to impair healing.

In this work, we selected a set of PEMF parameters that antagonize pro-inflammatory cues expressed by IL-1β treated tendon cells [11] to be combined with magnetic responsive magSPCL membranes to enhance the pro-regenerative response of cell populations exposed to persistent inflammatory cues. MagSPCL blend has been studied in our group using various processing techniques to fabricate different shapes and architectures for magnetic based tendon tissue engineering. magSPCL matrices have shown to be biocompatible and hold immunomodulatory properties [22] in cell-free strategies.

The results obtained here showed that IL-1β treated tendon cells were able to colonize the magSPCL membranes, increasing their metabolic activity, especially under PEMF stimulation in the days of culture.

More importantly, the gene expression of MMPs (*-1,-2* and *-3*) increased in IL-1β treated hTDCs but, when cells were exposed to PEMF the MMPs levels decreased. The expression of MMPs, enzymes implicated in cell responses to the environmental cues, including cell proliferation, migration, differentiation and in the dynamic process of ECM turnover, with relevant implications in the inflammatory and remodeling phases of healing [33], suggest that PEMF actuation can modify hTDCs behavior, decreasing the expression of MMPs associated to collagen degradation, which may contribute to inflammatory pain and tendon lesions. As cytoskeleton changes often precede MMP modulation during tissue remodelling, actin dynamics might be linked to the expression of MMP genes [34]. In our study the detection of vinculin and α-actin [35, 36] is not compromised by the PEMF stimulation. The actin expression is thus in accordance to the outcomes from MMPs, whose expression is decreased with PEMF actuation (Figure VIII-1Bi and Bii). The detection of both actin and vinculin also confirms the typical elongation of hTDCs and surface adhesion of hTDCs to magSPCL membranes, independently of IL-1β treatment or PEMF stimulation.

Overall, magSPCL membranes stimulated by PEMF maintain hTDCs viability, enable hTDCs adhesion and influence the turnover of the ECM antagonizing IL-1β effects on ECM enzymes. Afterwards, we investigated the tenogenic phenotype of hTDCs laden in PEMF responsive membranes after IL-1β treatment. The combination of PEMF and magSPCL membranes did not significantly change the expression of *DCN*, *TNC* and *SCX* during the time in culture, but stimulated an increment in *MKX* and in collagens expression. The increment of collagen type III stimulated by PEMF may relate to its role in the regulation of collagen type I fibrillogenesis and in tissue repair. Collagen III stabilizes the repair site and contributes to the ECM remodeling process highlighting the importance of collagen type III to promote a regenerative response [37]. Thus, PEMF actuation on hTDCs/magSPCL constructs contributes to the maintenance of a tenogenic phenotype at the gene and protein level. The synergistic action of PEMF and magSPCL membranes on the expression of tendon genes blurs the pro-inflammatory stimuli of exogenous IL-1β supplementation to the cells.

To elucidate the mechanisms associated to the hTDCs response to PEMF and magSPCL membranes, we searched for key mediators of the IL-1β intracellular signaling. The NF-κB pathway regulates genes involved in multiple immune processes and prolonged activation of NF-κB may lead to inflammatory diseases [38, 39]. In the canonical NF-κB signaling pathway IL-1β activates the IL-1β receptor (IL1R1) ultimately resulting in rapid and transient nuclear translocation of NF-κB, as schematically represented in Figure VIII-3Ai. The NF-κB translocation to the nucleus causes the activation

of target gene transcription of pro-inflammatory factors as TNFα, IL-1β and IL-6 [39]. Our results indicated that IL-1β treatment on hTDCs increases the expression of *IL1R1*, promotes the activation of NF-κB after 3 days in culture and induces up-regulation of *TNFα*, *IL-6*, *IL-8* and *COX-2* and downregulation of *IL-10* and *IL-4*. Interestingly, NF-κB signaling was counteracted by magSPCL membranes stimulated by PEMF. Other studies also reported significant changes in the downregulation of TNFα and NF-κB in cells continuously exposed to a PEMF at 5 Hz [40]. Altogether, these results highlight the relevance of PEMF and magnetic responsive materials to modulate hTDCs response to environments with inflammatory signatures.

Macrophages are IL-1β producing cells and major orchestrators of the inflammation process. Macrophages are present in tendon niches after tendon injury and in tendinopathy conditions, and contribute with inflammatory cues, which may prolong the inflammatory phase and influence proper healing. Magnetic stimulation seems to induce changes in macrophage morphology as these cells exhibit a more elongated shape, which has been associated to the process of macrophage polarization [41], in particular to the M2 phenotype.

Furthermore, macrophages laden in magSPCL membranes stimulated by PEMF were $CD68^{+}$, a M1 marker, but were also $CD206^{++}$ and $CD163^{++}$, which are established markers for M2 phenotype, suggesting the presence of different macrophage populations. For healing to occur, M1 macrophages should be present for the inflammatory phase and M2 macrophages are required at later stages to promote repair. Our findings suggest that PEMF responsive magSPCL membranes favour M1/M2 balance towards M2-phenotype without obliterating M1 macrophage populations. PEMF is thought to influence a number of cell-membrane processes and to activate intracellular signaling transduction. PEMF was shown to assist tenogenic phenotype [42, 43], accelerate cell differentiation [44], influence the activation of cell surface receptors [45], act on voltage-gated calcium channels [46] and in cell resting potential [46], which is also affected by inflammation. PEMF has also been related to inflammation kinetics, to participate in the control of tissue inflammation [47] and in the downregulation of TNFα and NF-κB [40], contributing for tissue repair. The specific mechanisms in immunomodulation are outshined by the complex inflammatory cascades and intricate biological reactions mediated by different signaling pathways. Some studies also suggest a PEMF action on both transcriptional and posttranscriptional regulatory mechanisms. The involvement of PEMF in epigenetic programming [41], could explain the switch in macrophage polarization, and consequently the impact on the gene expression of mediators of the inflammatory response.

Although magSPCL membranes were approached as an *in vitro* model for studying cell responses under external magnetic fields and in pro-inflammatory conditions, the immunomodulatory potential of these biomaterials could be further assessed on lesions to orient cell responses, regulate pro-inflammatory events and, ultimately improve healing outcomes. Furthermore, the investigation of cell and molecular mechanisms using commercial magnetotherapy devices used in the clinics, in which the parameters of magnetic fields are known to be safe and with therapeutic value in humans, potentially facilitates a faster translation of advanced strategies into therapeutics.

Despite the contributions of this study to the understanding of magnetic stimulation impact driving immunomodulatory cell behaviour, there are some limitations to be overcome. IL-1β model is a widely-used model of inflammation for prospective studies but quite simplistic when compared to the pathological niches and to the intricate immune networks of living organisms. Also, the involvement of NF-κB signaling requires further investigation focusing on the precise molecules, receptors and impact on immune cells to design better strategies to address persistent inflammatory environments. In sum, PEMF and magnetic responsive materials contribute for modulating and guiding biological responses in inflammatory compromised environments associated to tendon pathologies, whose mechanisms should be deeper explored in future studies. This study highlights the complementary value of magnetic stimulus on cell laden magnetic responsive materials in guiding immunomodulatory events, controlling the perpetuation of inflammatory cues and towards resolving inflammation envisioning tendon regenerative platforms.

VIII-5. Conclusions

PEMF actuated magSPCL membranes have a modulatory effect on intracellular inflammatory signals and in controlling the cytokine profile of IL-1β treated hTDCs. The influence of magnetic forces and magnetic elements may trigger IL1R1/NF-κB signaling although it requires deeper investigation. Moreover, PEMF actuated magSPCL membranes showed an immunomodulatory effect on inflammatory cells guiding macrophages towards M2 functional programs, highlighting the potential of magnetic stimulation for tendon healing strategies. This work contributes with new perspectives on cellular responses to pro-inflammatory environments and to the potential applicability of magnetic responsive systems as alternative therapeutic tools for the treatment of tendinopathy and for remotely stimulating tendon regeneration.

VIII-6. SUPPLEMENTARY INFORMATION

Supplementary Table VIII-1 Primers used for real time quantitative RT-PCR analysis.

	Primer Sequence	Accession number
Human Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	F – TGTACCACCAACTGCTTAGC R - GGCATGGACTGTGGTCATGAG	NM_002046.4
Human matrix metalloproteinase-1 (*MMP-1*)	F – ACCTGGAAAAATACTACAACCTGAA R – TTCAATCCTGTAGGTCAGATGTGTT	NM_002421.3
Human matrix metalloproteinase-2 (*MMP-2*)	F – GCTACGATGGAGGCGCTAAT R – TCAGGTATTGCACTGCCAACT	NM_002422.4
Human matrix metalloproteinase-3 (*MMP-3*)	F – CACTCACAGACCTGACTCGG R – AGTCAGGGGGAGGTCCATAG	NM_002422.4
Human metalloproteinase inhibitor-1 (*TIMP-1*)	F – CATCCGGTTCGTCTACACCC R – GGATAAACAGGGAAACACTGTGC	NM_003254.2
Scleraxis (*SCXA*)	F – CGAGAACACCCAGCCCAAAC R – CTCCGAATCGCAGTCTTTCTGTC	XM_001717912
Decorin (*DCN*)	F – CAGCATTCCTCAAGGTCTTCCT R – GAGAGCCATTGTCAACAGCA	NM_001920.3
Collagen, Type I, alpha 1 (*COL1A1*)	F – CGAAGACATCCCACCAATCAC R – GTCACAGATCACGTCATCGC	NM_000088.3
Collagen, Type III, alpha 1 (*COL3A1*)	F – GCTGGCTACTTCTCGCTCTG R – TTGGCATGGTTCTGGCTTCC	NM_000090.3
Mohawk (*MKX*)	F – TGTTAAGGCCATAGCTGCGT R – TCGCACAGACACCTGGAAAA	NM_173576.5
Tenascin C (*TNC*)	F – ACTGCCAAGTTCACAACAGACC R – CCCACAATGACTTCCTTGACTG	NM_002160.3
Human interleukin 4 (*IL-4*)	F – GCACCGAGTTGACCGTAACA R – AGGAATTCAAGCCCGCCAG	NM_000589.3
Human interleukin 6 (*IL-6*)	F – AGGAGACTTGCCTGGTGAAA R – GCATTTGTGGTTGGGTCAG	NM_000600.4
Human interleukin 8 (*IL-8*)	F – GGTGCAGTTTTGCCAAGGAG R – TTCCTTGGGGTCCAGACAGA	NM_001354840.1
Human interleukin 10 (*IL-10*)	F – AAGACCCAGACATCAAGGCG R – AATCGATGACAGCGCCGTAG	NM_000572.2
Human cyclooxygenase-2 (*COX-2*)	F – ATGGGGTGATGAGCAGTTGT R – GAAAGGTGTCAGGCAGAAGG	NM_000963.3
Human tumor necrosis factor (*TNFα*)	F – ATGTTGTAGCAAACCCTCAAGC R – TGATGGCAGAGAGGAGGTTG	NM_000594.3
Human factor nuclear kappa B (*NF-κB*)	F – GAAGCACGAATGACAGAGGC R – GCTTGGCGGATTAGCTCTTTT	NM_003998.3
Human interleukin 1 receptor (IL1R1)	F – GCATCCTACACATACTTGGGCA R – ACCTTTTAGGGCTGGAGCTATG	ENSG00000115594

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SECTION 4

GENERAL CONCLUSIONS

Chapter IX

General Conclusions and Future Perspectives

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IX-1. GENERAL CONCLUSIONS

The shortage of suitable strategies to manage and resolve tendon injuries in order to successfully restore tendon functionality to a pre-injury state motivates the development of alternative approaches that stimulate tendon regeneration. Current approaches are either conservative (e.g. immobilization), hold an unspecified action over tendons (e.g. anti-inflammatory modalities) or surgery-based in the most severe cases.

In this Thesis, we questioned the relevance and contribution of inflammation mechanisms driving tendon cell behavior. Therapeutic approaches aiming at inflammation modulation have been challenged by the fine-tuned inflammation mediated mechanisms shared by healing and by advancing pathological stages, and by the still poorly knowledge on cell and molecular responses of tendon tissues to inflammatory triggers. Conventional therapies that manage tendon pain with anti-inflammatory drugs, disrupt healing instead of properly resolving it, which may aggravate lesions in the mid to long term. The uncertain role of inflammatory events in tendon tissues as a direct cause or risk factor for disorders and injuries creates an additional challenge to study and improve our knowledge on tendon biology to properly guide tendon oriented therapies.

The magnetic actuation strategies offer great possibilities to the field of tissue engineering and regenerative medicine for assisting the guidance and stimulation of specific cells and/or tissue responses towards regeneration, in a remote/contact -free manner. Pulsed electromagnetic field (PEMF) has been applied for the treatment of musculoskeletal inflictions, including long-bone fractures and has been related to a reduction in pain. Despite the therapeutic potential to modulate inflammation and to contribute for the management of tendon injuries, the influence of magnetic stimulation over cell-mediated mechanisms in inflammatory conditioned tendon niches requires a deeper investigation. Having this mind, we have hypothesized in this Thesis that remote magnetic stimulation could modulate tendon cell responses conditioned to pro-inflammatory exploring different *in vitro* models. With these studies we provided insights on tendon cell responses to the presence of inflammatory factors, to contactless magnetic

stimulation and to magnetic stimulation combined with magnetic responsive membranes envisioning enhanced tendon regeneration. The major outcomes of this Thesis are discussed below:

i) Tendon and ligament cells as living agents of tendon niche regeneration

To understand abnormal signalling leading to pathological outcomes, it is relevant to identify signature characteristics and physiological responses of tendon cells. To acknowledge tendon cells in the presence of inflammatory factors, we firstly identified the phenotypic signature of tendon and ligament cells in **Chapter IV** to establish differences between physiological and inflammation guided behaviour. We have confirmed the constitutive expression of several tendon and ligament associated markers and the high number of cells expressing stemness markers, in conformity with previous reports. The availability of biological resources, in this case cell populations' representative of tendon and ligament tissues, is essential for studying cellular responses in controlled environments for optimal therapeutic solutions. We also reported that cryopreservation is a suitable preservation method for these cells and that cryopreservation/thawing process does not compromise tendon and ligament phenotype, retaining stem cell characteristics and increasing the tendon-related markers expression in comparison to freshly expanded cells. These outcomes are of particular interest for tendon biology advances, including studies on cell responses to specific bioactive molecules, on the identification of tendon specific markers, as well as for the assessment of microenvironmental cues or the impact of scaffolding strategies in tendon regeneration.

ii) Establishment of *in vitro* models to study and modulate the inflammatory profile of hTDCs

As dissimilar responses between healthy and pathological cell phenotypes may assist the recapitulation of the biological events associated to the onset and progression of diseases, we explored the model based on stimulation with IL-1β, previously reported for other cell types, in hTDCs. The establishment of IL-1β model to investigate cell phenotypes in the presence of pro-inflammatory stimulus resulted in an elegant and effective approach (**Chapter V**). In fact, the exposure to IL-1β induced an up-regulation of pro-inflammatory factors in hTDCs, expressed by the increment in the levels of inflammatory genes, suggesting that tendon cells respond to IL-1β adjusting their behavior accordingly.

Furthermore, not only was an exogenous IL-1β supplementation protocol optimized and established for tendon oriented strategies but its outcomes enabled its use in more complex approaches to explore

intercellular communication, and the contributions of cell-cell and cell-ECM interactions (**Chapter VI**). We demonstrated that macrophages respond to IL-1β conditioned hTDCs, highlighting the complexity of the intercellular networking during inflammatory events (**Chapter VI**), opening new perspectives on tendon cell-immune cell communication to unveil pathophysiological pathways in tendon (impaired) healing.

iii) Design of non-invasive and remote-actuated magnetic based platforms to modulate the inflammatory profile of hTDCs envisioning translational approaches.

In a complementary manner, we investigated tendon cell responses exposed to IL-1β through PEMF stimulation using clinically approved devices (Chapters V to VIII). PEMF has been applied in the clinics, which assists the translational potential of PEMF-based approaches, but its implications in cell behavior, particularly in rescuing hTDCs from inflammatory factors are largely unknown. Using the IL-1β models, we explored PEMF in tendon cells and in tendon cells/macrophage interactions as well as the PEMF contributions to cell to cell and cell-ECM relations (**Chapter V and Chapter VI**). In this Thesis, we have reported that a PEMF with 5 Hz, 4 mT and 50% duty cycle and magnetic nanoparticles was the most promising combination for tendon cell based approaches. This combination showed also to be immunomodulatory over macrophages cultured in the presence of IL-1β-treated hTDCs polarizing macrophages toward an M2 phenotype, contributing to a decrease in the pro-inflammatory cues naturally released by these cells. The repair phenotype (M2) favored by PEMF stimulation highlights the relevance of magnetic fields in orchestrating inflammatory mechanisms and in the successful integration of tissue engineered substitutes. Since FAK is also involved in this process, these results open new avenues for studying FAK activation to sensing and transmission of magnetic stimulation to immune cells.

Among the studies performed, we successfully established a magnetic tendon cell construction resourcing to magnetic-based and cell sheet- technologies. In **Chapter VII** we investigated magnetic tendon cell sheets (magCSs) taking advantage of the IL-1β model previously established, in which PEMF was shown to modulate the inflammatory profile of IL-1β-treated-magCSs, supporting the production of a tendon-like matrix, and a decrease in MMPs expression, via MAPK(ERK1/2) pathway, known to be present in acute and chronic inflammation. Not only are magnetic cell sheets a promising model for investigating inflammatory events but these constructions can be implanted as a living structural and functional substitute for tendon therapies, which could be also an asset for scaffold-free interventions. When we combined PEMF and magnetic membranes laden with IL-1β-treated-hTDCs (**Chapter VIII**), NF-κB

mediated by IL-1β/IL1R1 was counteracted, and a favourable balance towards M2 was assessed when macrophages were laden on these systems. Although the precise contribution of PEMF and magnetic membranes was not investigated in this work, this study clearly showed that magnetic membranes under a PEMF hold a modulatory action over different cell types involved in tendon healing.

The applicability of IL-1β based models to unveil the role of PEMF stimulation in hTDCs is also another important achievement of the Thesis, together with the important role of PEMF (alone or combined with magnetic responsiveness biomaterials, **Chapters V, VII and VIII**) to regulate hTDCs inflammatory profiles reversing the detrimental factors imposed by IL-1β supplementation to tendon cells. Cell modulation, especially on immune cells is quite appealing to the field of tissue engineering and regeneration medicine and may assist novel solutions for improving the integration of tendon substitutes and for meliorating the healing process after injury. Overall, the studies performed under the scope of this Thesis demonstrated that magnetic assisted strategies enable a precise and beneficial action over biological processes. PEMF assists the regulation of the inflammatory response acting as a tendon healing promoter, thus envisioning the applicability of magnetic actuated therapies for tendon regenerative approaches.

IX-2. Future Perspectives

The best comprehension of inflammatory mechanisms and its involvement in activation/inhibition of cellular cascades can contribute to the establishment of advanced cell based therapies, which can be further combined with contactless magnetically assisted strategies for improved tendon healing. In fact, we have demonstrated in different approaches the manipulation and control of cell responses through pulsed electromagnetic field alone or in combination with magnetic responsive systems. Although cell and tissue responsiveness to magnetic stimulus has been reported by others, our studies emphasize that tendon cells sense and precisely respond to a combination of magnetic parameters, conditioned or not to inflammatory inducers, enabling to control inflammation mediators. Furthermore, inflammatory priming of tendon cells has not been described in the literature and may assist insights on the role of inflammatory factors in pathological tendon niches.

Despite these contributions for a better understanding of tendon niche phenomena, it is important to question the impact of other microenvironmental factors that may be involved in tendon inflammatory mediated responses and their pathophysiological contribution to impaired tendon healing. Studies on

oxygen tension, molecular triggers (e.g. INF, chemokines), or specific matrix features (e.g. architecture, misalignment) could enlighten whether these factors share the same inflammatory pathways or if they activate non-related pathways or if different combinations of factors stimulate different cell responses in different stages of disease.

Studies resourcing to pre-clinical models should to be performed to validate the predictability of cellular responses, and consequently their potential clinical relevance. More specifically, *in vivo* studies will confirm the safety and translational potential of advanced systems, such as the magnetic cell sheets and the magnetic responsive membranes studied in **Chapters VII** and **VIII**. For instance, priming cells in implantable cell sheet constructions could enable favourable interactions with resident populations to control healing events. *In vivo* models will also confirm the magnetic based systems´ ability to extracorporeal guide cell behavior and to establish suitable regenerative programmes, as these systems may require multiple bouts for a significant and desirable modulatory effect accordingly to the type and anatomical location of the injury.

The magnetic based strategies proposed offer translational potential, and take advantage of commercially available magnetic therapy devices to provide magnetic stimuli within physiological ranges and to the accessibility of magnetic field to exert high precision control. The non-invasive nature combined with deep tissue penetration enables the expansion of these approaches to a wide range of tissues and to multiple inflammatory pathologies.

In sum, the works presented in this Thesis are pioneer in nature and represent a starting point for the exploitation of sophisticated platforms in the field of tissue engineering and regenerative medicine targeting pathophysiological inflammation processes, and prospects magnetically responsive tools for cell guidance envisioning the management of tendon injuries and to achieve tendon regeneration.