

Immunomodulatory Biomaterials to Promote Tissue Repair/Regeneration of Articular Cartilage: the Impact of Dendritic Cells

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Table of Contents

Abstract……vii
Resumo..... ix
Acknowledgments..... xi
List of Figures…….....xiii
List of Tables xv
List of Abbreviations xvii
1. Structure and Composition of Articular Cartilage 21
2. Articular Cartilage Degeneration and Injury..... 25
2.1 Mechanical Injury 25
2.2 Benign and Malignant Tumors..... 26
2.3 Rheumatic Diseases 27
2.4 Osteoarthritis..... 27
2.5 Inflammation in Osteoarthritic Joints..... 29
3. Dendritic Cells 33
3.1 Dendritic Cells in Osteoarthritic Joints 34
4. Strategies for Articular Cartilage Repair..... 37
4.1 Nonsurgical Treatments 37
4.2 Treatments Based on Surgical Intervention 39
4.3 TERM Strategies..... 40
4.3.1 Recent Studies on the Development of Dendritic Cell-Based Therapy for Osteoarthritis..... 41
5. Biomaterials to Modulate Dendritic Cell Behavior..... 43
5.1 Nanomaterials to Modulate Dendritic Cell Behavior..... 45
6. Objectives..... 51
7. Materials and Methods..... 53

7.1 Carbon Nanotubes 53
7.2 Poly(lactic-co-glycolic acid) Nanoparticles 53
7.3 Human Monocyte Isolation and Differentiation into Dendritic Cells 54
7.4 Stimulation of immature Dendritic Cells 54
7.5 Lactate Dehydrogenase Viability Assay 55
7.6 Flow Cytometry Analysis 55
7.7 Cytokine Secretion by ELISA 55
8. Results 57
8.1 Carbon Nanotubes do not activate human primary Dendritic Cells 57
8.1.1 Carbon Nanotubes may be able to reduce inflammatory stimulus 58
8.2 Poly(lactic-co-glycolic acid) Nanoparticles do not activate human primary Dendritic Cells 59
8.2.1 Poly(lactic-co-glycolic acid) Nanoparticles are not able to reduce inflammatory stimulus 60
8.2.2 Increased concentrations of Poly(lactic-co-glycolic acid) Nanoparticles induce some toxicity in human primary Dendritic Cells 61
8.3 Establishing an inflammatory microenvironment for nanobiomaterial testing 62
9. Discussion 65
10. References 71
11. Supplementary Figures 85

Abstract

Articular Cartilage (AC) covers the extremities of articulating bones, allowing load transfer and gliding to occur between them. Properties of this tissue which are critical for proper joint movement include tensile strength, provided by collagen fibers, and resistance to compression, granted by proteoglycans that retain water in the extracellular matrix (ECM). AC is avascular, which results in a very limited capacity to repair, when injuries occur. If the injuries are subchondral, reaching blood vessels, a healing response is initiated, and damaged tissue is replaced by fibrotic tissue. The mechanical properties of this tissue are, however, inferior to those of the native one. Damage of AC can be caused by mechanical injury, tumors, rheumatic illnesses and degenerative illnesses, such as osteoarthritis (OA). OA degrades AC and is accompanied by chronic pain and inflammation, affecting millions of people worldwide. Dendritic cells (DCs) have been found in samples of synovial tissue and fluid from OA patients. It has also been observed that DC proliferation and inflammation are increased in early stage OA, in an animal model. DCs are highly effective antigen presenting cells (APCs) with the ability to interact directly with biomaterials and trigger an immune response. A number of studies have evaluated how biomaterial composition, structure and size can affect DC maturation. There are several reports of biomaterials promoting maturation of DCs, as well as cytokine release, cellular uptake, and migration to the lymph nodes. These findings show great promise for the development of novel biomaterials and therapeutic approaches aiming to modulate DC phenotype and activity. Such biomaterials could potentially be used not only to alleviate OA symptoms, like pain and inflammation, but also prevent further degradation of AC and promote repair of this tissue. The goal of this dissertation work was to study the potential immunotoxicity and/or immunomodulation capacity of carbon nanotubes (CNTs) and poly(lactic-co-glycolic acid) nanoparticles (PLGA NPs), both empty and loaded with ibuprofen, on primary human DCs. We found that both nanobiomaterials were not toxic towards DCs and did not activate them, at the tested concentrations of 0,45 μg/mL and 100 μg/mL, respectively. Furthermore, stimulation with these nanobiomaterials induced low secretion of anti and pro-inflammatory cytokines into culture supernatants. Increased concentrations of PLGA NPs were found to induce some cell toxicity. Optimization of a culture model that better resembles the inflammatory microenvironment of OA is still required, to better assess the ability of CNTs and PLGA NPs to reduce inflammation.

Resumo

A cartilagem articular reveste a superfície dos ossos em articulações, permitindo que estes deslizem um sobre o outro, e que ocorra a transferência de cargas entre eles. Propriedades deste tecido que são críticas para o funcionamento normal de uma articulação incluem resistência à tensão e à compressão, características conferidas por fibras de colagénio e proteoglicanos que retêm água na matriz extracelular, respetivamente. A cartilagem articular é avascular, o que resulta numa capacidade limitada de reparação, em contexto de lesão. Se os danos se localizarem no osso sob a cartilagem, local onde se encontram vasos sanguíneos, uma resposta de reparação é iniciada, e o tecido danificado é substituído por tecido fibrótico. As propriedades mecânicas deste tecido são, no entanto, inferiores às do tecido nativo. Os danos em cartilagem articular podem ser provocados por lesões mecânicas, tumores, doenças reumáticas e degenerativas, como a osteoartrite. Esta doença degrada a cartilagem articular, e é acompanhada de dores e inflamação crónicas, afetando milhões de pessoas, globalmente. É possível encontrar células dendríticas em amostras de líquido e tecido sinovial recolhidas de pacientes com osteoartrite. Um aumento na proliferação de células dendríticas e de inflamação foi observado em estados iniciais de osteoartrite, num modelo animal. As células dendríticas são células apresentadoras de antigénios altamente eficientes, com a capacidade de interagir diretamente com os biomateriais, e desencadear uma resposta imune. Diversos estudos avaliaram como a composição, estrutura e tamanho de biomateriais pode afetar a maturação de células dendríticas. Existem várias indicações de biomateriais que foram capazes de promover a maturação de células dendríticas, assim como a secreção de citoquinas, internalização celular, e migração para os nódulos linfáticos. Estas descobertas são muito promissoras para o desenvolvimento de novos biomateriais e abordagens terapêuticas, com o objetivo de modular o fenótipo e a atividade de células dendríticas. Tais biomateriais poderiam ser utilizados não só para aliviar sintomas de osteoartrite, tais como dor e inflamação, como também para prevenir degradação de cartilagem articular, e promover reparação deste tecido. O objetivo do trabalho desta dissertação foi estudar a potencial imunotoxicidade e/ou capacidade imunomodulatória de nanotubos de carbono (CNTs) e nanopartículas de poli(ácido lático-co-ácido glicólico) (PLGA NPs), vazias e com ibuprofeno encapsulado, em células dendríticas primárias humanas. Os nossos resultados demonstraram que ambos os nanobiomateriais não induziram toxicidade nas células e não as ativaram, nas concentrações testadas de 0,45 μg/mL e 100 μg/mL, respetivamente. Em adição, estimulação com estes nanobiomateriais induziu secreção reduzida de citoquinas anti e pró-inflamatórias nos sobrenadantes de cultura celular. Concentrações acrescidas de PLGA NPs induziram alguma toxicidade nas células. É

necessário ainda otimizar o modelo de cultura celular que se assemelhe ao microambiente inflamatório característico da osteoartrite, a fim de poder aferir a capacidade de CNTs e PLGA NPs de reduzir inflamação.

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List of Figures

Figure 1.1 - Scheme depicting the different regions of AC, and their cell distribution……..21

Figure 1.2 - Depiction of the chemical structure of three common GAGs…….22

Figure 1.3 - Scheme depicting the process of chondrogenesis……24

Figure 2.1 - Lesions in AC, with varying depths…….26

Figure 2.4 - Scheme representing the structural differences between a healthy joint (left) and a joint affected by OA (right)……..29

Figure 2.5 - Structural differences between a healthy and inflamed synovial membrane……...30

Figure 3.1 - Scheme representing the different dendritic cell subtypes……..31

Figure 8.1 - DC response to carbon nanotubes (CNTs)……..58

Figure 8.1.1 - Impact of carbon nanotubes (CNTs) on stimulated DC…….....59

Figure 8.2 - DC response to Poly(lactic-co-glycolic acid) Nanoparticles (PLGA NPs)…….60

Figure 8.2.1 - Impact of Poly(lactic-co-glycolic acid) Nanoparticles (PLGA NPs) and ibuprofen on stimulated DC……..61

Figure 8.2.2 - DC response to increased concentrations of Poly(lactic-co-glycolic acid) Nanoparticles (PLGA NPs) and ibuprofen……..62

Figure 8.3 - DC response to pro-inflammatory stimuli……..63

Supplementary Figure 1…….....85

List of Tables

Table 1 - Biomaterial microparticles and films of varying composition, and their effect on dendritic cells……...43
Table 2 - Nanobiomaterials of varying composition, shape and size, and their effect on dendritic cells……...46

Table 3 - Nanobiomaterials and molecules tested on immature dendritic cells……..55

List of Abbreviations

AC Articular Cartilage

ACI Autologous Chondrocyte Transplantation

ADAMTs A Desintegrin and Metalloproteinase with Thrombospondin Motifs

$Al(OH)_3$ Aluminum hydroxide

APC Antigen Presenting Cells

Au Gold

BC Buffy Coats

BM Bone Marrow

BMDCs Mouse Bone Marrow-derived Dendritic Cells

BMMNCs Bone Marrow Mononuclear Cells

CCL2 C-C Motif Chemokine Ligand 2

CCN4 Cellular Communication Network Factor 4

CCR7 C-C Motif Chemokine Receptor Type 7

CD Cluster of Differentiation

cDC1 Conventional type I Dendritic Cells

cDC2 Conventional type II Dendritic Cells

CDP Common Dendritic Cell Progenitor

Ch Chitosan

Ch/PGA Chitosan/Polyglycolic Acid

Ch/γ-PGA Chitosan/Poly(γ-glutamic acid)

CHUSJ Centro Hospitalar Universitário de São João

CLP Common Lymphoid Progenitor

CNTs Carbon Nanotubes

CO_2 Carbon Dioxide

-COOH Carboxyl

COPD Chronic Obstructive Pulmonary Disease

COX-2 Cyclooxygenase-2

CS Chondrosarcoma

CS-PLGA Poly (lactic-co-glycolic acid) Nanoparticles coated with Chitosan

Da Dalton

DAMPs Damage-Associated Molecular Patterns

DCs Dendritic Cells

Dex Dexamethasone

DWCNTs Double-Walled Carbon Nanotubes

ECM Extracellular Matrix

ELISA Enzyme-linked Immunosorbent Assay

FBS Fetal Bovine Serum

FDA United States Food and Drug Administration

FLC Fibroblast Like Cells

FLT3L FMS-like tyrosine kinase 3 ligand

g(Ch/PGA) Chitosan/Polyglycolic Acid cross-linked with genipin

GAGs Glycosaminoglycans

GM-CSF Granulocyte-macrophage Colony-Stimulating Factor

HA Hyaluronic Acid

HLA Human Leukocyte Antigen

HMGB1 High Mobility Group Box Protein 1

HPBM Human peripheral blood monocyte

HSC Hematopoietic Stem Cells

IA Intra-Articular

ICAM-1 Intercellular Adhesion Molecule 1

iDCs Immature Dendritic Cells

IL Interleukin

JIA Juvenile Idiopathic Arthritis

LDH Lactate Dehydrogenase

LDH Layered Double Hydroxide

LPNs Lipid-polymer Hybrid Nanoparticles

LPS Lipopolysaccharide

MACI Matrix-Associated Autologous Chondrocyte Transplantation

M-CSF Macrophage-Colony Stimulating Factor

mDCs Mature Dendritic Cells

MDP Macrophage-Dendritic cell Progenitor

MFI Mean Fluorescence Intensity

$Mg(OH)_2$ Magnesium Hydroxide

MHC Major Histocompatibility Complex

MMPs Matrix Metalloproteases

MNCs Mononuclear Cells

moDCs Monocyte derived Dendritic Cells

MPs Microparticles

MRI Magnetic Resonance Imaging

mRNA Messenger Ribonucleic Acid

MSCs Mesenchymal Stem Cells

MW Molecular Weight

MWCNTs Multi-walled Carbon Nanotubes

NCs Nanoclusters

NF-κB Nuclear Factor Kappa B

NK Natural Killer

NLC Nanostructured lipid carriers

NP[OVA] Poly (lactic-co-glycolic acid) Nanoparticles with Ovalbumin

NP[OVA+Dex] Poly (lactic-co-glycolic acid) Nanoparticles with Ovalbumin and Dexamethasone

NPs Nanoparticles

NSAIDs Non-Steroidal Anti-Inflammatory Drugs

OA Osteoarthritis

OVA Ovalbumin

PAMPs Pathogen-Associated Molecular Patterns

PBMC Peripheral Blood Mononuclear Cells

PBS Phosphate Buffered Saline

pDCs Plasmocytoid Dendritic Cells

PFA Paraformaldehyde

PGE2 Prostaglandin E2

PLGA Poly (lactic-co-glycolic acid)

PRG4 Proteoglycan 4

RA Rheumatoid Arthritis

RT Room Temperature

ROS Reactive Oxygen Species

SCF Stem Cell Factor

S-DCs Oligodeoxynucleotide-induced Dendritic Cells

SLE Systemic Lupus Erythematosus

SpA Spondyloarthropathies

SWCNTs Single-Walled Carbon Nanotubes

TCP Tissue Culture Polystyrene

TERM Tissue Engineering/Regenerative Medicine

TGF-β Transforming Growth Factor β

T_H T Helper

TiO_2 Titanium Dioxide

TLRs Toll-Like Receptors

TNF-α Tumor Necrosis Factor α

tolDCs Tolerogenic Dendritic Cells

T_{regs} Regulatory T cells

VCAM-1 Vascular Cell Adhesion molecule-1

VEGF Vascular Endothelial Growth Factor

vitD3 1,25-dihydroxyvitamin D3

WNVE West Nile Virus Envelope

ZnO Zinc Oxide

γ-PGA–Phe γ-PGA-graft-l-phenylalanine ethylester

1. Structure and Composition of Articular Cartilage

Articular cartilage (AC) is an aneural and avascular connective tissue that covers the articulating surface of bones, allowing for load transfer to occur between them without friction [1]. Cartilage is not vascularized, so it exchanges nutrients and waste products through diffusion [2]. It is mainly constituted by a dense extracellular matrix (ECM), which accounts for 95 to 99% of its composition, and cells, which account for the remaining 1 to 5%. The ECM is highly hydrated, with 70 to 80% water content [3]. AC differs in structural organization and composition throughout its depth, making it possible to identify a mineral and a non-mineral layer. Three different zones can be identified in the non-mineral layer: surface, middle and deep zone (Fig. 1.1) [1].

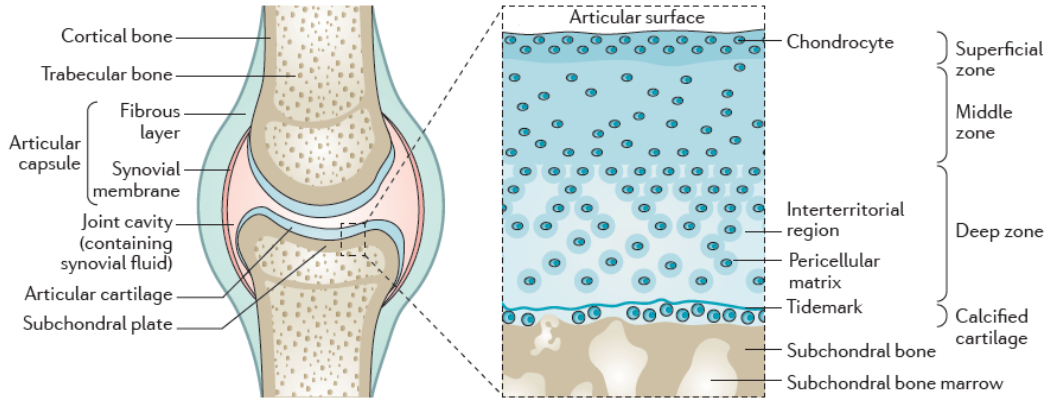


Figure 1.1 - Scheme depicting the different regions of AC, and their cell distribution. Throughout the tissue depth, chondrocyte morphology and distribution varies. Collagen fibrils orientation and density also varies in the different layers [4].

The surface zone corresponds to the articulating surface, and it constitutes close to 10% of AC height. It contains progenitor cells and chondrocytes with an elliptical/flattened shape, entrapped in the ECM. Cells produce and release proteins which aid in the lubrication of the joint, namely proteoglycan 4 (PRG4), a protein also known as lubricin [5]. This zone is highly hydrated and short collagen fibrils (between 4 to 12 nm) run parallel to its surface [1]. The middle zone amounts to 60% of the AC, and has high proteoglycan content. Chondrocytes

shape becomes spherical, and collagen fibrils are longer (between 9 to 60 nm) and thicker, constituting a disorganized network [2]. The remaining 30% of the non-mineralized layer correspond to the deep zone, where chondrocytes retain their shape and can be found in stacks, perpendicular to the surface of the joint [2]. The ECM, while still hydrated, has slightly lower water content than the superficial and middle zones. Glycosaminoglycan (GAG) content is high, and long collagen fibrils (between 60 to 140 nm) are arranged radially. A calcified layer serves as a transition between cartilage (non-mineralized) and bone (mineralized) tissue [6, 7]. The mineralized layer, which consists of calcified cartilage, can be found between the deep zone and the subchondral bone [1]. Chondrocytes are hypertrophic and trapped in the mineral matrix, which is rich in proteoglycans and interwoven collagen type II fibers that extend from the deep zone into the mineralized layer, and anchor the non-mineralized cartilage to the bone. Collagen type IX and X fibers are also present. Collagen fibrils are also responsible for diffusing and distributing compressive loads, transmitting them from cartilage to the subchondral bone, as well as reducing stress at the joint interface. Despite its proximity to the subchondral bone, the mineralized layer is not vascularized [1]. The ECM of AC comprises a network of collagen fibers that retains other molecules, such as GAGs, glycoproteins and proteoglycans [3]. Proteoglycans are macromolecules with a protein core connected to GAGs, through covalent bonds. Aggrecan is the most prevalent proteoglycan in AC ECM. GAGs include keratan sulfate, dermatan sulfate and hyaluronan [3]. GAGs can attract fluid and retain it in the collagen network of the ECM [4]. The negative charge of GAGs allows them to attract and retain water, conferring AC shock absorbent properties. When AC suffers compression, water moves from the ECM to the synovial fluid. Once it is decompressed, water re-enters the matrix, filling the interstices between GAGs. These molecules contain either sulphuric or carboxylic groups, which, due to electrostatic interactions, repel each other, creating interstitial space amongst GAGs (Fig. 1.2) [2].

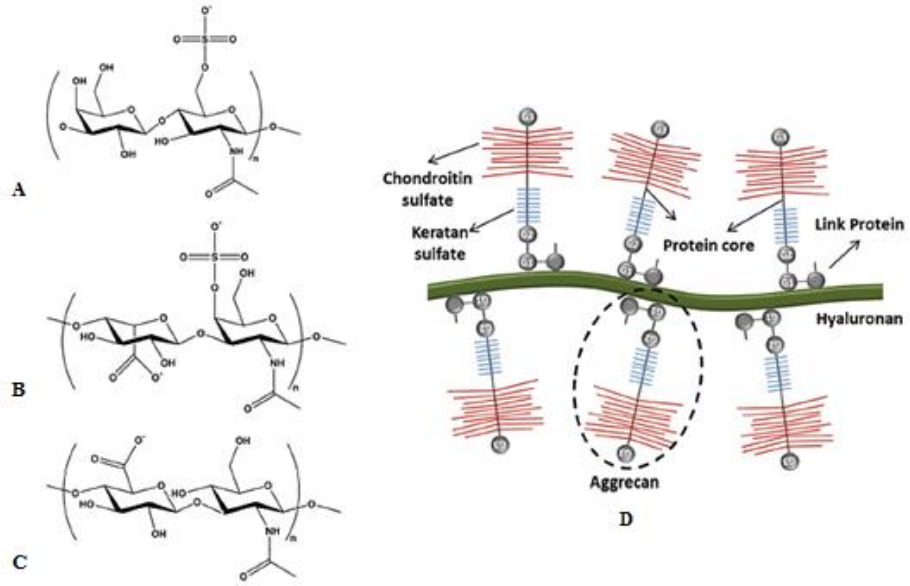


Figure 1.2 - Depiction of the chemical structure of three common GAGs. Keratan sulfate (A), dermatan sulfate (B) and hyaluronan (C), and aggrecan bonded to hyaluronan (D). In (D), keratan sulfate and chondroitin sulfate, another GAG, are bound to a core protein, forming aggrecan. The link protein enables interaction between hyaluronan and aggrecan. The negative charge between GAGs causes them to repel each other, in a "brush like" behavior [3].

The most abundant type of collagen in AC ECM is type II, but other collagens are also present, namely collagen "III, VI, IX, X and XI" [8]. Collagen confers tensile and shear strength. AC is a rather heterogeneous tissue, due to the presence of solid and liquid components, differences in ECM collagen fiber orientation and proteoglycan distribution, and due to the strains that the tissue is submitted to [2]. However, this heterogeneity grants AC its unique properties, namely its ability to withstand compression, and resistance to shear stress, which result from the mechanical interactions between liquid and solid components [8]. Another important characteristic of AC is that it allows relatively frictionless motion to occur at the synovial joint, further aided by the lubrication provided by synovial fluid [1, 8]. AC is naturally lubricated with interstitial fluid that confers it stiffness, which is further reinforced by collagen fibers' tensile strength. The fluids' viscosity increases with hyaluronic acid (HA) content [9]. PRG4 is considered, however, to be the main lubricant, since it was observed that HA digestion had no effect in the lubrication provided by synovial fluid [10, 11]. Proteolytic digestion, on the other hand, decreased lubrication in the joint surface, where PRG4 is usually deposited [10, 11]. It has been shown that increased levels of shear stress up-regulate PRG4 production [12]. Despite this, HA is still considered by many to provide efficient lubrication [13]. At the synovial joint, AC and subchondral bone allow stress and load distribution to adjacent bones [9]. The modulus of tension in the AC of the human knee joint is highest in the superficial zone, where collagen fibers are abundant and organized. AC exhibits anisotropic behavior, where increasing levels of strain result in increasing tissue stiffness. Strain and flux of interstitial fluid are highest in the superficial zone, whereas hydrostatic pressure is very low. The opposite can be verified in the deep zone, where no flux of interstitial fluid occurs and strain values are reduced, while hydrostatic pressure is at its highest. The tissues viscoelastic nature is due its organization and composition [14].

Chondroblasts that differentiate from mesenchymal stem cells (MSCs) are the precursor cells of chondrocytes, the cellular component of AC (Fig. 1.3). Chondrocytes have the ability to divide, and are responsible for producing and renovating AC ECM components [2, 8], but turnover of matrix components is very slow [8]. They are large cells, enclosed in collagen type VI, and encapsulated by pericellular matrix [2]. Chondrocytes present different morphology, organization and density, depending on their depth in the tissue [1, 8]. As chondrocytes produce matrix components, they are driven apart from each other and become trapped in cavities named *lacunae*. Chondrocytes in the deep zone become hypertrophic and produce collagen X, which contributes to cartilage mineralization in this zone. Cartilage is described as hypertrophic when its chondrocytes are enlarged and unable to divide, accumulating molecules such as alkaline phosphatase, lipids, and glycogen [2].

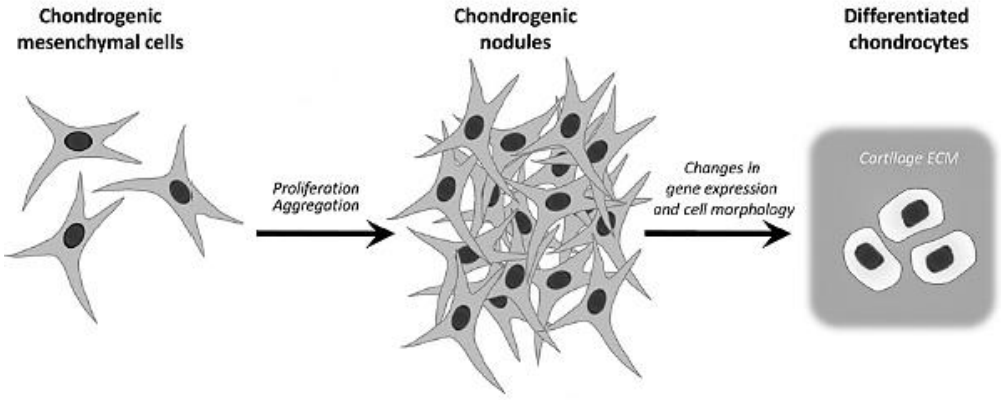


Figure 1.3 - Scheme depicting the process of chondrogenesis. Initially, chondrogenic MSCs are scattered and present an elongated morphology. In the condensation period, MSCs proliferate and cluster together, forming nodules. This process occurs in the presence of cell adhesion molecules, soluble factors, and intracellular signaling molecules. Cells then undergo differentiation, assuming a round morphology, and acquiring a genetic expression pattern that is essential for synthesis and maintenance of ECM cartilage components [15].

Cartilage may also be divided in different regions, according to the organization of ECM components that are produced by chondrocytes: pericellular, territorial and inter-territorial region [3]. The pericellular region consists of a protective layer that surrounds chondrocytes, keeping them from interacting with ECM components of the inter-territorial region. The pericellular matrix maintains chondrocytes in a differentiated state, where they perform slow turnover of ECM components [16, 17]. It contains proteoglycans and collagen VI fibers, and functions as a transducer of both chemical and mechanical signals to the chondrocytes [3]. The pericellular region is surrounded by the territorial region. Proteoglycans which contain chondroitin sulfate are abundant in the territorial region, where there is an increase in collagen fiber thickness. Proteoglycans from the inter-territorial region, which surrounds the territorial region, are rich in keratin-sulphate [3].

2. Articular Cartilage Degeneration and Injury

The unique composition and properties of AC make it capable of withstanding compression and tension [3]. However, this tissue may be damaged in every-day accidents [18], and degenerative illnesses of inflammatory nature [4, 19], and even tumors may form in AC [20]. Since AC is avascular and turnover of ECM is very slow, the repair response in this tissue is very limited and, when injuries/illnesses are not addressed, the damage that occurs may be irreversible [3].

2.1 Mechanical Injury

AC traumatic injuries occur mainly during practice of sports and due to accidents. The resulting tissue defects can be separated in two categories: chondral and full thickness (Fig. 2.1) [18]. Chondral defects affect only superficial chondral tissue, whereas full AC defects reach the subchondral bone. Since chondral defects are limited to AC alone, repair does not occur as it does in vascularized tissues. Once the superficial layer of AC is damaged, it can reduce the mechanical properties of the joint and affect load and stress distribution. Further tissue degeneration can take place, as time advances, if these lesions are not addressed [18]. Lesions that reach the subchondral bone, which is vascularized, allow blood clots to form, and so a reparative response can take place. As AC does not regenerate, native tissue is replaced by fibrocartilage, providing a short term fix for the defect. The properties of fibrocartilaginous tissue are between those of hyaline cartilage and dense connective tissue (such as ligaments and tendons) [21]. The fibrocartilage ECM is packed with dense collagen fibers, making it very resistant to compressive forces. Unlike hyaline cartilage, fibrocartilage ECM is rich in type I collagen, while GAG content is reduced. Type II collagen fibers are also present, and chondrocytes are few and in alignment with collagen fibers [2]. Although this tissue offers resistance against compression, it lacks the tensile and shear strength of native AC [2, 3]. Since fibrocartilage possesses mechanical properties which are inferior to those of native tissue, the joint will have reduced mechanical properties and further damage may occur, over time [18]. AC that has suffered chronic or acute lesions may also be inflamed. Inflammation results in the release of endogenous damage-associated molecular patterns (DAMPs) which activate cells at the injury site through their cell surface receptors, namely toll-like receptors (TLRs) [18]. Once activated, cells release cytokines and chemokines, further attracting circulating inflammatory cells to the damaged tissue. This positive feedback mechanism continues until the amount of

DAMPs is diminished. Resident macrophages present in the tissue, as well as infiltrating macrophages, which arise from circulation and adjacent tissues, are responsible for clearing debris and can often be associated with tissue repair in the injured area [18]. However, continuous infiltration of macrophages and other immune cells leads to chronic inflammation, a hallmark of several rheumatic diseases (section 2.3) [19]. Lastly, the age of the injured patient also influences AC function and structure. Changes related with age include a decrease in cell number, cartilage thickness and matrix integrity [1]. Cell density in the superficial zone is reported to decrease roughly 50% between the ages 20 and 90 years old [22]. With fewer chondrocytes present, the tissues' potential to repair is decreased even further, and tissue degradation and fibrillation are more likely to occur at the joint, as well as degenerative illnesses such as osteoarthritis (OA), which will be discussed further ahead (section 2.4) [1].

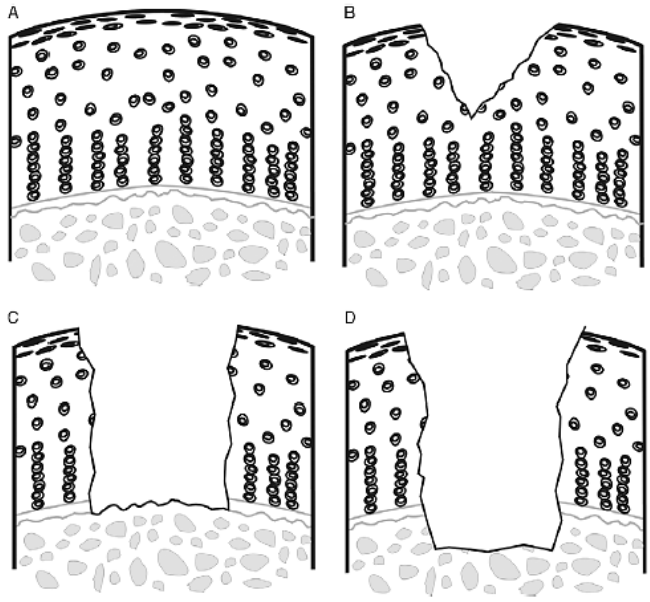


Figure 2.1 - Lesions in AC, with varying depths. Healthy and intact AC is depicted in A. B and C showcase chondral defects, with severity increasing with the depth of the injury. These lesions can be more superficial (as in B), or even reach subchondral bone (as in C) without, however, extending to the bone marrow. In D, a full thickness defect is depicted. This reaches the subchondral bone and marrow, triggering a reparative response [23].

2.2 Benign and Malignant Tumors

Another cause for AC damage can be the formation of tumors, either benign or malignant. Chondrosarcoma (CS) are heterogeneous bone tumors, mainly malignant, that are characterized by abnormal formation of hyaline cartilage [20]. These tumors are generally resistant to radiation and chemotherapy, and mainly affect adults, with close to 3 new cases per million people, each year [20, 24]. However, only 5 to 10% of cases of conventional CS present high potential to metastasize [25]. CS can be classified as primary or secondary. Primary CS occurs in patients that do not have any previous lesions, whereas secondary CS occurs in patients that have a benign cartilaginous tumor, such as an osteochondroma or an endochondroma [20]. Osteochondroma and endochondroma represent 34% and 30% of benign tumors found in cartilage, making them the most prevalent forms of benign cartilaginous tumors [26]. Many patients with benign tumors do not experience symptoms and, as a result, this problem may

remain undiagnosed. In symptomatic cases, these cartilaginous tumors may cause swelling and pain, lesions and even fractures, due to weakening of bone [27]. Benign cartilage tumors are usually excised or removed with a curette and, if necessary, bone grafts are used to reconstruct the area [19]. CS is also characterized according to the region where it first appears, as central or superficial. Central CS arises from the medullary cavity, while peripheral CS arises from the surface of newly formed bone outgrowths. Cases of primary CS are mostly central, while cases of secondary CS can be either central or peripheral [20]. If benign tumors develop into malign forms, which rarely occur, both the tumor and adjacent bone must be removed, to ensure that no tumor cells remain [19, 28, 29]. This intervention requires complex tissue reconstruction, and is often associated with morbidity, which can reduce the patient's life quality, and even result in disabilities, depending on the original location of the tumor [30, 31]. Strategies such as *cutterage* aided by cryosurgery have been developed to help preserve bone tissue and reduce morbidity, while still allowing for efficient removal of tumors [32, 33].

2.3 Rheumatic Diseases

AC can be affected by a number of different rheumatic diseases that can lead to arthritis, and whose inflammatory nature can worsen joint damage. Such diseases include juvenile idiopathic arthritis (JIA), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), gout, and seronegative spondyloarthropathies (SpA) [19]. JIA includes forms of arthritis that affect people below the age of 16, and whose cause is unknown [34]. RA is autoimmune, and the most prevalent inflammatory articular disease, currently affecting up to 1% of the worldwide population [35, 36]. In RA, arthritis can affect several synovial joints, and patients also suffer from chronic systemic inflammation. This illness can aggravate degradation of cartilaginous tissue and synovial joints, if not addressed [36]. SLE is also an autoimmune illness. Anti-nuclear antibodies found in circulation are a key aspect of this disease. SLE may have detrimental effects in several organs other than joints, such as the lungs, brain, skin, heart and kidneys [37]. SLE can occur in children and adults. However, prognosis for SLE development from childhood is worse than for SLE development during adulthood [38]. Out of all forms of inflammatory arthritis, gout is the most common. This illness occurs when crystals of uric acid deposit in joints or other tissues. Crystal deposition may result in acute gout attacks that cause severe pain, damage of the joint structure, formation of kidney stones and even renal failure [39]. SpA includes several rheumatologic diseases, whose development is believed to be correlated with the HLA-B27 gene. Clinical symptoms include peripheral arthritis, back pain of inflammatory nature, and inflammation of the eyes and sites were ligaments and tendons are inserted [40, 41].

2.4 Osteoarthritis

AC damage may also happen due to degenerative illnesses, such as OA, which affects synovial joints, impairing their mobility and causing chronic pain. It can occur in any synovial joint, but incidence is most common in the hips, knees, spine and hands [4]. OA affects the entire joint and surrounding tissue, such as the synovial membrane and subchondral bone [18]. It is characterized by the destruction of cartilaginous tissue and remodeling of the bone that lies beneath it, as well as by inflammation of the synovium [4]. Just in 2017, 303 million people were reported to suffer with OA [42]. OA can be categorized as either primary or secondary,

depending on its development. Primary OA occurs in spite of the absence of structural abnormalities of the joint, whereas secondary OA can be developed due to the presence of abnormalities in the joint or cartilaginous tissue, and also due to traumatic injuries [4]. OA development is related with age, gender and high body mass index, and prior injuries are also considered risk factors [43-45]. Other likely contributing aspects include mechanical stress and cytokines which promote inflammation. OA affects all the components of the joint, not just AC (Fig. 2.4). Alterations caused by OA include remodeling of bone and cartilage matrix, bone outgrowths (osteophytes) in joint margins, and cell proliferation [46]. Other alterations include increased expression of pro-inflammatory cytokines, a desintegrin and metalloproteinase with thrombospondin motifs (ADAMTs), and matrix metalloproteases (MMPs), which display catabolic activity, degrading AC ECM [4, 46]. Due to ECM degradation, both in terms of structure and composition, chondrocyte phenotype is altered, causing these cells to become hypertrophic and terminally differentiated [46].

As age progresses, whenever there is an unbalance that favors catabolic processes in detriment of anabolic ones, illnesses such as OA are more likely to occur. In addition, wear at the joints is greater in older patients, increasing the likelihood of OA development even further. The specific mechanisms that trigger joint degeneration related with age are yet unknown. However, it has been observed that areas of cartilage which are not exposed to compression are the first to suffer degeneration. For instance, Bullough proposed that areas of cartilage which are not subjected to loading forces suffer from degeneration and, with joint remodeling, become exposed to excessive levels of load which overtax the joint and degenerate cartilage even further, possibly leading to OA [47, 48]. OA could also arise due to damage located in the subchondral bone, which may lead to its collapse and cause lesions to appear in formerly healthy AC [49].

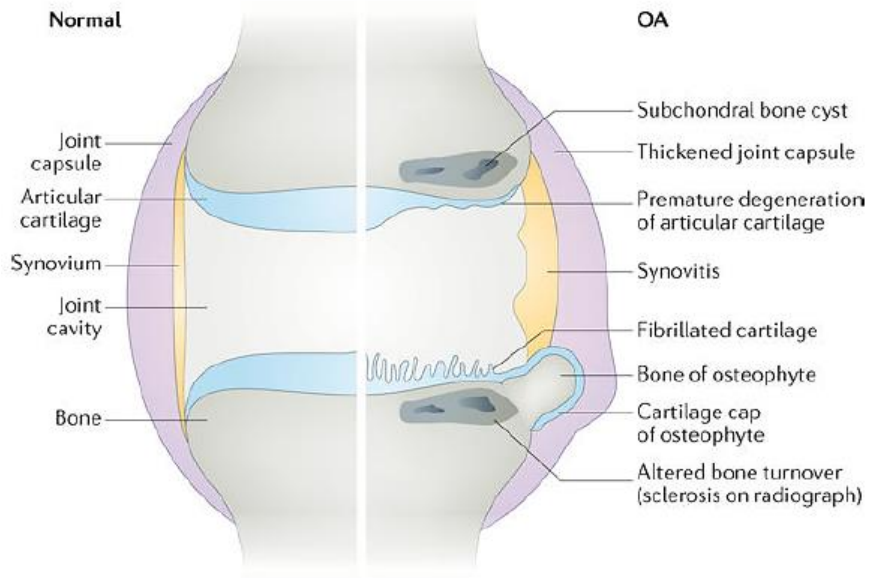


Figure 2.4 - Scheme representing the structural differences between a healthy joint (left) and a joint affected by OA (right). As depicted, the structural changes are not limited to AC, extending to several components of the joint, such as the synovial membrane and the subchondral bone. Therefore, OA is viewed as a disease which affects the whole joint [50].

A focal aspect of OA is inflammation of the synovium, which is related with pain and degradation of AC. The synovium is a membrane that surrounds synovial joints. It is very important, since it can remove byproducts of cellular metabolism and provide nutrients to the chondrocytes [51]. Synovitis is a condition which develops when the synovial membrane becomes inflamed, and it is believed to correlate with the advancement of OA throughout the joint structure [51]. Inflammation of the synovium is also related with hypertrophy and hyperplasia of this membrane. Synovitis is believed to further cartilage degradation, through the production of pro-inflammatory mediators [50]. Traumatic lesions, such as meniscal tears, are thought to cause synovial inflammation [52]. Synovial fluid collected from joints affected with OA has been found to contain mediators which are pro-inflammatory, stimulating cartilage degradation and impairing ECM synthesis [53]. Additionally, thickening of the synovium has been observed in people suffering from mild and early stage OA, through magnetic resonance imaging (MRI) [54]. Inflammation of the synovium can also take place second to AC degeneration, since products which result from this tissue deterioration activate synovial and immune cells, triggering inflammation [51]. By co-culturing synovial tissue alongside cartilage explants, it was possible to study the influence that active synoviocytes, which are cells that constitute the synovial lining, have in cartilage degradation linked to OA [55]. During the culture period, high levels of interleukin (IL) 6, IL-8 and osteoprotegerin were secreted by the synovial tissue. In cartilage explants, GAG content decreased over the 21 day period, in which it was exposed to synovial tissue. It was then possible to observe that mediators produced by the inflamed synovial membrane could further the inflammation and induce catabolic activity in cartilage, therefore being capable of steering the progression of OA [55].

2.5 Inflammation in Osteoarthritic Joints

Inflammation of the synovial membrane in osteoarthritic joints is linked to pain and destruction of cartilaginous tissue and it is critical to pathogenesis and development of OA, due to secretion of inflammatory mediators [51]. The lining of a healthy synovial membrane contains macrophage and fibroblast like synoviocytes, also known as macrophage-like type A synoviocytes, and fibroblast-like type B synoviocytes. As this region is vascularized, circulating immune cells can also be found, but in small amounts. When OA installs in a joint, inflammation leads to a number of changes in the synovium, namely hyperplasia, due to the arrival and infiltration of immune cells, such as macrophages, mast cells, T and B lymphocytes, dendritic cells (DCs), and Natural Killer (NK) cells [51, 56]. Hypertrophy of the membrane also occurs, due to proliferation of synovial macrophages and fibroblasts, which thickens the synovial lining (Fig. 2.5) [51]. Recently, a study by Kriegova *et al.* determined which cell types could be found in the synovial fluid of patients suffering with knee OA. Results showed that 90% of the content of the synovial fluid consisted of hematopoietic cells, and lymphocytes were the predominant cell population (44,8%), with cluster of differentiation (CD)4^+T lymphocytes outnumbering $CD8^+$T cells. Cell populations from the "monocyte/macrophage lineage" [57] were also prevalent, and included monocytes (33,8%), macrophages (14,8%) and monocyte derived DCs (16,4%). The remaining cell populations consisted of neutrophils (8%), NK cells (3,8%), regulatory T cells (T_{regs}) (1,2%), plasmocytoid DCs (1,1%) and, finally, mast cells (0,3%). This study also reported differences between the composition of synovial fluid of the female and male OA patients. Comparing with male patients, synovial fluid collected from female patients had a lower percentage of $CD8^+$T cells

and monocytes, while the $CD4^+$T cell and macrophage populations were more abundant. These results may suggest that OA treatment may need to be adjusted for patients, according to cell phenotypes which are more predominant in the affected joint [57]. Furthermore, determining the contribution of immune cells towards synovial inflammation is critical to understand how OA develops [51].

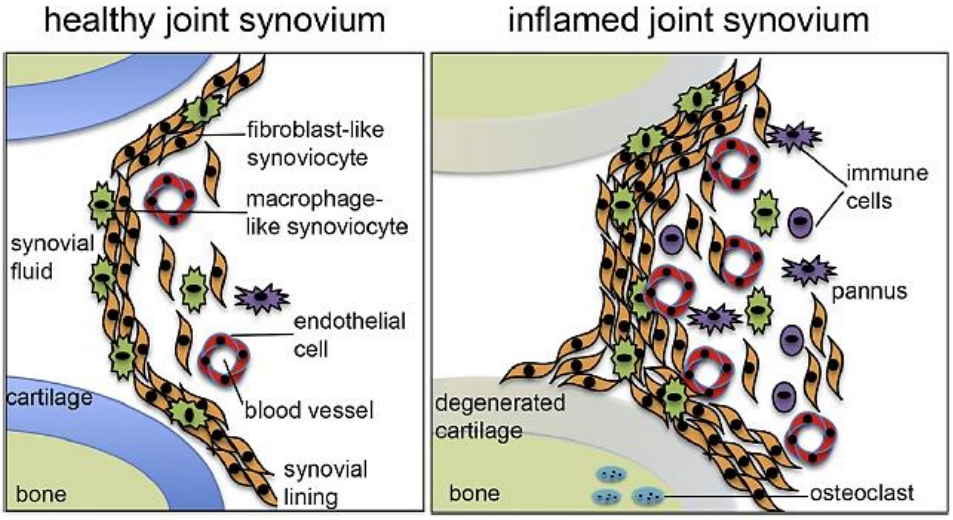


Figure 2.5 - Structural differences between a healthy and inflamed synovial membrane. In the absence of inflammation, the synovial membrane consists of a thin layer of macrophage and fibroblast-like synoviocytes. Blood vessels adjacent to the sub-intimal layer of the synovium allow exchange of electrolytes and nutrients with the synovial fluid. In a joint affected by inflammation, the synovial membrane becomes engorged, forming a pannus, due to accumulation of macrophage and fibroblast-like synoviocytes. The latter degrade AC and bone. Bone is further eroded due to osteoclastic activity. Vascularization in the sub-intimal space increases, and inflammatory immune cells infiltrate the synovium [58].

Synoviocyte-like macrophages, or simply, synovial macrophages, can be found in the lining of the synovial membrane, as well as the sublayers beneath it. They are known as "primary cellular mediators" [51] of inflammation of the synovium in OA joints [51]. These macrophages are antigen presenting cells, with the ability to phagocyte damaged cells and debris, and produce inflammatory cytokines that contribute to joint degradation in OA, such as IL-1β, IL-6, tumor necrosis factor α (TNF-α) and oncostatin-M [4, 59]. TNF-α and IL-1β induce catabolic activity in chondrocytes, which downregulate proteoglycans and collagen II, major components of AC ECM, while upregulating MMP-9 and the enzyme cyclooxygenase-2 (COX-2) [51, 60]. Matrix metalloproteinases such as MMP-1, MMP-3 and MMP-13 are involved in AC ECM destruction during OA [4, 46]. IL-1β can also increase COX-2 and prostaglandin E2 (PGE2) expression in human chondrocytes, which may contribute to the calcification of cartilaginous matrix [61]. Prostaglandins also contribute towards inflammation and pain [62]. An *in vitro* study showed how M1 phenotype macrophages can inhibit MSC differentiation into cartilage in the joint synovium, whereas the M2 phenotype presented an opposite effect [63]. This difference is likely due to secretion of pro-inflammatory cytokines by M1 macrophages, such as IL-6, IL-12, and TNF-α. Since M2 macrophages release anti-inflammatory cytokines (like IL-10), they may favor cartilage repair [64]. The combination of IL-10 and IL-4 has been shown to prevent blood-induced cartilage degradation, *in vitro* [65]. Products that are released from cells or from the ECM during OA can also contribute to joint

degradation. These include molecules such as S100 (an alarmin) and high mobility group box protein 1 (HMGB1), or fragments of components of AC ECM, like collagen or proteoglycans [4]. HMGB1, which is secreted by synovial macrophages, also functions as an inflammation mediator, once macrophages are stimulated with lipopolysaccharide (LPS), IL-1β or TNF-α. Signals that are pro-inflammatory can also lead to HMGB1 secretion, which will then display activity similar to that of pro-inflammatory cytokines, furthering inflammation [66]. HMGB1 was found to be expressed by synoviocytes from healthy joints, as well as joints affected by OA. It acts in synergy with IL-1β, augmenting the expression of mediators that favor inflammation [67]. HMGB1's synergistic activity also extends to molecules produced by synovial fibroblasts in cases of OA, namely IL-6, IL-8, TNF-α and MMP-3 [68]. Furthermore, when released by chondrocytes, HMGB1 has been shown to attract osteocytes and osteoblasts, therefore influencing the formation of bone [68]. HMGB1 release may be linked to formation of osteophytes, a known occurrence in OA [69, 70]. Studies in OA models established in murines have also shown that synovial macrophages can contribute to osteophyte formation. Particularly, they are capable of promoting transforming growth factor β (TGF-β) mediated osteophyte formation [71, 72].

The inflammatory cytokines that are released by synovial macrophages may induce neighboring cells of the synovium to also release pro-inflammatory mediators, further accentuating the inflammatory response. Synoviocyte-like fibroblasts, or synovial fibroblasts, are also part of the lining of the synovial membrane [51]. When stimulated with IL-1β, synovial fibroblasts from joints affected by OA were reported to produce vascular endothelial growth factor (VEGF), IL-6 and IL-8 [73]. Laminin-111, a protein found in the pericellular matrix of AC, can bind to fibroblasts from the synovium when TGF-β is present, activating them and inducing secretion of pro-inflammatory mediators IL-6 and IL-8. This mechanism poses a contribution of synovial fibroblasts to OA pathology, when acute inflammation is not occurring [74]. Vascular cell adhesion molecule-1 (VCAM-1) expression is upregulated by synovial fibroblasts, in response to C-C motif chemokine ligand 2 (CCL2) and cellular communication network factor 4 (CCN4), and both these molecules have been detected in synovial fluid removed from patients suffering from OA. When VCAM-1 expression is increased, mononuclear cells (MNCs) may adhere more easily to sites where inflammation is occurring [75, 76]. All of these observations show a potential of synovial fibroblasts to potentiate inflammatory responses, either due to activation of inflammatory mediators, or due to facilitation of infiltrating immune cells' adhesion to sites of inflammation [51]. However, synovial fibroblasts, like chondrocytes, can also express IL-10, which can augment the expression of proteoglycans and collagen II. IL-10 can also stimulate heme oxygenase-1 activity, an enzyme that provides protection to cartilage [77]. Additionally, MMP-3 and MMP-13 activity is inhibited by the presence of IL-10, which also reduces chondrocyte apoptosis induced by TNF-α and IL-1β [78, 79]. Overall, though it is feasible to expect a contribution from synovial fibroblasts towards inflammation of the synovium, resident macrophages and infiltrating immune cells are believed to be the main cells responsible for OA progression [51].

Mast cells are immune cells of hematopoietic origin that are associated with inflammation and allergic reactions. They possess granules, where they store molecules that mediate inflammation, such as histamine and heparin, and proteases, such as tryptase [80]. Inflammation in OA is believed to be linked to the presence of mast cells. In fact, high levels of mast cells

have been detected in synovial fluid from osteoarthritic patients, along with high levels of tryptase, histamine and nitrate [81, 82]. The increase of mast cells has been attributed to expansion of tryptase containing population, as studies have reported an increase of this population in synovial tissue of patients with OA, when compared to controls [83]. Tryptase can induce epithelial cells to express intercellular adhesion molecule 1 (ICAM-1) and release IL-8, as well as upregulate gene expression of both IL-1β and IL-8 in endothelial cells. These findings suggest that tryptase may influence inflammation through immune cell recruitment [84, 85]. Lastly, TNF-α, which can be released by synovial macrophages in joints affected by OA, has also been shown to induce chemotaxis of mast cells [86].

T lymphocytes, or T cells, reside in lymphoid organs, such as the thymus, and are essential to initiate adaptive immunity [87]. T cells have been detected in the synovium of patients with early and late stage OA [51, 88]. $CD4^+$ effector T cells are divided in subsets, depending on the functions they perform and cytokines they express. T helper 1 (T_H1) cells produce IL-2 and interferon γ (IFN-γ). They can initiate cell mediated inflammation, e.g., through cytotoxic T-cell and macrophage activation, which will enable intracellular removal of pathogens [89]. T helper 2 (T_H2) cells produce IL-4, IL-5 and IL-10, leading to superior production of antibodies, and removal of infections of parasitic origin [89]. $CD8^+$ cytotoxic T cells and $CD4^+$ effector T cells have been identified in the innermost layer of the synovial membrane, in OA patients [90]. In addition, comparing with IL-4, increased Il-12 and IFN-γ expression was detected, indicating that T_H1 cells were predominant in the synovium [90]. Other works have reported that infiltration of macrophages and $CD4^+$ effector T cell in the synovial tissue was connected to increased expression of VEGF and ICAM-1 [91]. Macrophages can express VEGF, which has been linked to OA pathogenesis in mice [92]. Inflammatory stimuli can upregulate ICAM-1 production in OA synoviocytes, which enables T cell migration through the endothelium, and may further facilitate infiltration of lymphocytes in the synovial membrane [93, 94].

Although DCs are found in the synovial membrane and synovial fluid of OA patients [51, 56, 57], how they contribute towards synovial inflammation and OA development has not been described as thoroughly as for other immune cells found in osteoarthritic joints. The role of DCs and their relevance in OA are described in section 3.

3. Dendritic Cells

DCs are the most efficient antigen presenting cells (APC), bridging the innate and adaptive immune systems, by processing and presenting antigens to naïve T cells [95]. This ability is considered to be critical for successful integration and function of the implanted biomaterial [96]. Different DC populations can be found in the body, including plasmocytoid DCs (pDCs), conventional type I DCs (cDC1s), conventional type II DCs (cDC2s) and monocyte derived DCs (moDCs), and their abundance changes depending on homeostasis and inflammatory conditions (reviewed in [97]) (Fig 3.1). Particularly, moDCs result from monocyte differentiation in peripheral tissues, in the context of inflammation. They have been mainly studied in *in vitro* settings, and they are known to stimulate $CD4^+$T cell differentiation into T_H1, T_H2, or T_H17 phenotypes, depending on the molecular cues and inflammatory microenvironment [97, 98].

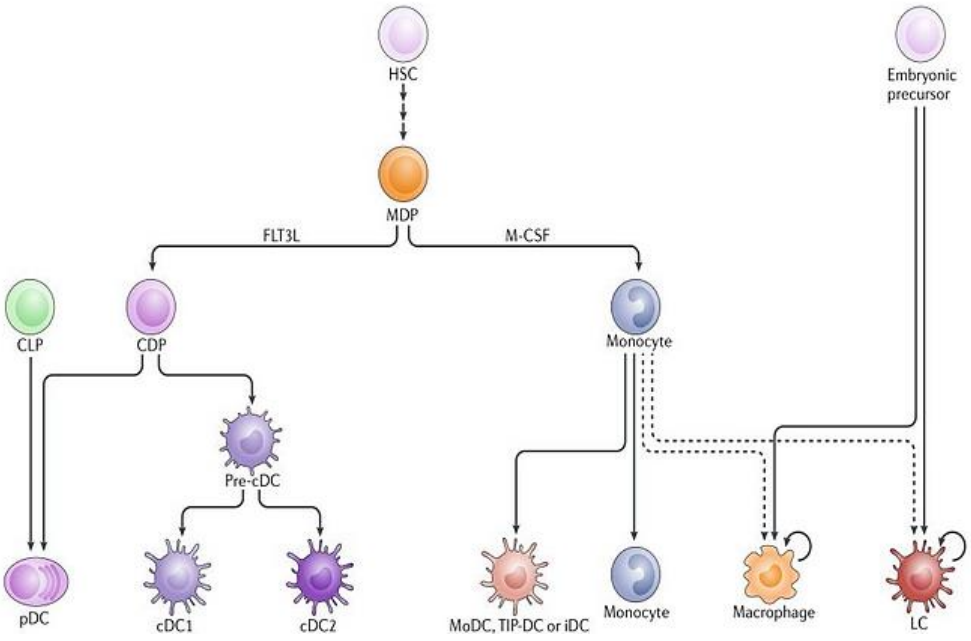


Figure 3.1 - Scheme representing the different dendritic cell subtypes. Hematopoietic stem cells (HSC) give rise to macrophage–DC progenitors (MDP) which, depending on stimulus, differentiate into common DC precursor (CDP) or monocytes. Conventional DCs (cDCs) and plasmocytoid DCs (pDCs) development and expression occurs in the presence of FMS-like tyrosine kinase 3 ligand (FLT3L), which is a transcription factor. Common lymphoid progenitor (CLP) cells may also give rise to pDCs. In the presence of macrophage colony stimulating factor (M-CSF), MDP give rise to monocytes, where monocyte derived DCs (moDCs) originate from [99].

Under homeostatic conditions, immature DCs (iDCs) can be found in peripheral tissues, where they recognize DAMPs and pathogen-associated molecular patterns (PAMPs) through their PRRs, being then able to internalize and process antigens. In this state, they are characterized by a low expression of major histocompatibility complex (MHC) I and II and costimulatory molecules. As they mature, DCs migrate towards secondary lymphoid organs, where they can activate T-cell and B-cell responses, kick starting the adaptive immune response [87]. Mature DCs (mDCs) present higher expression levels of MHC-I, MHC-II, and costimulatory molecules at their surface, such as CD40, CD83 and CD86. They are able to synthesize and secrete chemokines and inflammatory cytokines (e.g. IL-6, IL-8, IL-12 and TNF-α). They suffer a switch in chemokine receptor expression, for instance, with the upregulation of C-C motif chemokine receptor type 7 (CCR7), responsible for their migration to the lymph nodes. Importantly, DCs can also contribute towards immune tolerance, removing thymocytes that are self-reactive, promoting T cell anergy, or inducing T_{regs} polarization [87].

As described above, DCs play a critical role in starting adaptive immunity, but they can also take part in the healing response and tissue homeostasis [100]. Differentiation of tolerogenic DCs has also been considered as a strategy to address refractory chronic inflammatory illnesses [101, 102], and recent studies have started to explore the potential of DCs in OA therapy (Section 4.3.1).

3.1 Dendritic Cells in Osteoarthritic Joints

Alarmins secreted in OA joints, such as HMGB1 and S100 proteins, have been shown to recruit DCs and other immune cells to the inflamed synovium, thereby inducing adaptive immunity and perpetuating the illness [103]. Infiltration of differentiated DCs in synovial tissue is common in several rheumatic diseases, but particularly in RA. Migration of MNCs such as DCs towards sites of inflammation appeared, however, to be nonspecific and independent of the disease/cause of inflammation [104]. In an early study by Pettit *et al.*, researchers collected synovial biopsies from patients suffering with RA, SpA, OA and gout, in order to assess the specificity of differentiated DC infiltration in synovial tissue, in diseases of varying inflammatory nature. Results showed that DCs were found in aggregates of MNCs surrounding blood vessels (perivascular region), for all diseases previously mentioned, and that DC infiltration was closely associated with lymphocyte infiltration. However, infiltration of DCs in synovial tissue was lower for OA patients, comparing with RA and SpA patients. The study concluded that perivascular infiltration of MNCs, including DCs, was related with inflammation in a nonspecific manner, and that signals from RA and SpA synovial tissues resulted in increased infiltration and accumulation of immune cells than in OA synovial tissues [104]. Also, Takakubo *et al.* compared DC distribution in synovial tissue and fluid from RA and OA patients, observing that, in RA samples, myeloid DCs (or cDCs) were prevalent in lymphoid aggregations, and pDCs were dispersed near areas of perivenular infiltration (around veins) and lymphoid aggregations [105]. There was a significant increase in the ratio of myeloid DCs to pDCs, and grading was superior in RA synovial fluid tissues, comparing with

OA synovial tissues. Myeloid DCs are known to play a key role in T cell priming, which may explain their abundance in lymphoid aggregation, as well as increased inflammation verified in RA samples [105, 106]. Results point to a key role of myeloid DCs towards inflammation in RA, but not in OA [105]. In 2012, Xiaoqiang *et al.* studied the role of DCs in OA, as well as DC distribution in the synovium of rabbits with knee OA, established through surgery [107]. Several DC molecular markers were detected in lymphoid aggregations and perivenular infiltrates from the synovial membrane of OA rabbits. In early post-operative stage, a large amount of DCs was present in the synovial membrane, and the number of DCs was found to increase with synovial inflammation. Expression levels of TNF-α and IL-1β were higher during this stage, and decreased over time with synovial inflammation [107]. In another study, the differentiation of pDCs from bone marrow mononuclear cells (BMMNCs) from RA and OA patients was evaluated. Synovial tissues affected by RA are often sites of pDCs accumulation [108]. Flow cytometry analysis showed that percentages of $BDCA2^+$ and $CD34^+$ populations within BMMNCs did not differ significantly between RA and OA samples. BMMNC from RA samples did contain, however, a superior percentage of $BDCA2^+CD34^+$ cells (corresponding to pre-pDCs) than BMMNCs from OA samples. In agreement with this result, the percentage of $BDCA2^+CD34^+$ cells in bone marrow (BM) $CD34^+$ population was superior for RA, comparing with OA, and was also correlated with the extent of synovial proliferation. Altogether, results show that in RA pre-pDC generation from BM $CD34^+$ cells is superior to OA, and the amount of pDCs arising from BM can be linked with synovial proliferation [108]. These different studies point to a possible role of DCs in pathogenesis of OA, by contributing towards an inflammatory response, which is more evident in early stage OA [107].

DCs are known to cross-talk with different cell populations and adaptive immunity. Zhang *et al.* [109] showed that NK cells could promote differentiation of monocytes into DCs in some arthritic diseases. NK cells accumulate in tissues suffering with chronic inflammation, and in lymph nodes. In this study, peripheral blood mononuclear cell (PBMC)-derived monocytes differentiated into T_H1 promoting DCs, *in vitro*, in the presence of NK cells [109]. Synovial fluid from psoriatic arthritis and RA patients induced differentiation of monocytes into DCs, in the presence of NK cells, but the same did not occur when monocytes were in the presence of synovial fluid from OA patients. NK cells could contribute towards maintenance of the inflammatory phenotype (mediated by T_H1) in diseases such as RA, providing a microenvironment that enables monocyte differentiation into DCs. However, NK do not appear to promote monocyte differentiation into DCs in OA [109]. T cells are among the immune cells found in the inflamed synovium of OA joints [51]. In a 2012 study, distribution of T_{regs} in the synovial membrane of patients with RA and OA was assessed, as well as their interactions with DCs [110]. It was possible to observe a great number of T_{regs} in lymphoid aggregates and in regions of perivenular infiltration in the synovial membrane of RA samples. DC molecular markers were also present in areas of perivenular infiltration and lymphoid aggregates in RA samples. T_{regs} and DCs were found together mainly in regions of lymphoid aggregates. For RA tissue samples where inflammation was more severe, there was a greater increase of DCs than T_{regs} [110]. Expression levels of messenger ribonucleic acid (mRNA) for DC molecular markers and for T_{regs} cellular markers were higher in RA synovium, comparing with OA synovium. Even though there was a simultaneous upregulation of DCs and T_{regs}, there was a greater increase of DCs, pointing to their dominant role in activation and regulation of the immune response

in RA. These results also suggest that T_{regs} did not inhibit DC maturation in synovial membrane of RA patients [110].

Also, the capacity of BM derived pDCs to differentiate into fibroblast like cells (FLC), or type B synoviocyte-like cells was investigated. pDCs and $CD34^+$ cells were purified from BM derived MNC, and cultured in the presence and absence of TNF-α, GM-CSF and stem cell factor (SCF) [111]. It was possible to verify differentiation of OA BM pDCs and RA BM pDCs into FLC, particularly in the presence of TNF-α. Results suggest that type B synoviocytes can arise from pDCs in the presence of inflammatory stimuli. Furthermore, BM pDCs could be contributors in the pathogenesis of OA and RA [111].

Overall, previous work shows that immune cell infiltration and proliferation (including DCs) is higher in cases of RA, and so is inflammation at the joint, comparing with cases of OA. Furthermore, the extent of cell infiltration in RA joints has been linked with the degree of inflammation. In OA joints, the release of alarmins by the damaged tissue was shown to lead to DC recruitment, as well as other immune cells, and inflammation and DC infiltration were shown to be increased in cases of early stage OA. It has also been shown that BM derived pDCs, from both RA and OA samples can differentiate into type B synoviocytes in the presence of pro-inflammatory stimuli, suggesting that BM pDCs could play a key role in pathogenesis of OA, since accumulation of type B synoviocytes (or fibroblast-like B synoviocytes) is a hallmark of synovial inflammation.

4. Strategies for Articular Cartilage Repair

To address injuries and defects in AC, clinically available treatments can be based on nonsurgical strategies [23], or surgical interventions [1, 15]. Nonsurgical strategies target symptoms, like pain and discomfort caused by lesions and degeneration of the joint [23], while surgical interventions attempt to repair or replace the damaged tissue [1, 15]. Currently, in the fields of tissue engineering and regenerative medicine, a variety of materials (of natural and synthetic origin), fabrication methods and molecular factors are being explored in order to develop novel strategies to address defects and lesions in AC, and to tailor treatments and therapies to the patients' needs [3].

4.1 Nonsurgical Treatments

Nonsurgical strategies are used to diminish symptoms such as pain and inflammation in the joints, as well as delay the progression of pathologies responsible for degeneration of AC. These include non-pharmacological treatments, which don't rely on drugs, or pharmacological treatments, in which therapy is drug-based [23].

Non-pharmacological treatments view to control joint pain caused by OA, and improve the patients' mobility and life quality [23, 112]. They include physical therapy and other forms of exercise, such as aerobics. Moderate exercise is important to improve mechanical function at the joint, as well as reduce risk of other conditions, such as diabetes or cardiovascular illnesses [112]. The use of orthoses, such as unloading braces, is also common, as these devices are a relatively accessible, simple and cost effective way to alleviate pain at the joint and improve patient mobility [113]. Overall education of the patient and adjustment of daily activities and eating habits is important to minimize discomfort and prevent worsening of tissue damage [23].

Pharmacologic treatments include analgesics and supplements that can be obtained over the counter, medication that targets inflammation, local injection of corticosteroids, and viscosupplementation [23]. Inhibitors of cyclooxygenase, such as non-steroidal anti-inflammatory drugs (NSAIDs) and acetaminophen have commonly been used to address symptoms in mild to severe cases of OA, being that NSAIDs have proved to be superior to acetaminophen [114]. However, these medications may cause side effects in the hematological, renal, gastrointestinal and cardiac systems, for which prolonged use is not recommended [114]. NSAIDs can also be applied topically, and have proved to be only slightly less effective than systemic NSAIDs. They have also been shown to only provide short

term benefits, usually for up to a week of daily application [115]. Intra-articular (IA) delivery of substances through injections has also been explored. Corticosteroids, HA and platelet rich plasma are examples of substances that can be delivered to OA joints through IA injection [116]. This technique allows OA symptoms to be addressed locally in the joint, and reduce undesirable systemic effects [117]. IA injection has been reported to outperform oral NSAIDs and other medications. However, benefits of IA injection have also been associated with placebo effect [117].

Corticosteroids have immunosuppressive and anti-inflammatory action [116]. They act on nuclear receptors for steroids, interrupting the "inflammatory immune cascade" [118, 119], reducing expression of genes that are pro-inflammatory [120]. Corticosteroids can decrease the permeability of blood vessels, hinder accumulation of inflammatory cells and phagocytosis. Furthermore, they inhibit production of metalloprotease and metalloprotease activator, and avert production and secretion of inflammatory mediators like leukotrienes and prostaglandin [118, 119]. This ability is thought to contribute towards alleviation of pain, and increased mobility of the joint, in knee OA [116, 121]. Corticosteroids approved by the United States Food and Drug Administration (FDA) include: dexamethasone, methylprednisolone acetate, betamethasone acetate, betamethasone sodium phosphate, triamcinolone acetate and triamcinolone hexacetonide [116]. Dexamethasone, e.g, is frequently used to help manage inflammation in OA patients. This glucocorticosteroid acts by increasing gene transcription, a process known as transactivation, and transrepression, which results from its binding to nuclear factor kappa B (NF-κB), which then becomes inactive [122, 123]. Dexamethasone is also used *in vitro*, in order to induce chondrogenesis in BM derived MSCs, although its role in differentiation is yet to be fully understood [122, 123]. Application of dexamethasone in the joint could increase chondrogenic differentiation and reduce inflammation and its' detrimental effects on chondrocyte differentiation. Long term exposure to this compound can improve deposition of matrix, perhaps due to inhibition of signals which favor inflammation. Long term exposure *in vivo*, however, leads to less collagen production and increased structural damage [122, 123]. HA is a natural component of the ECM of AC, and can also be found in synovial fluid [3]. Besides its lubricant function and shock absorbing capacity, HA is also thought to have anti-oxidant and anti-inflammatory functions [116, 121]. In knee affected by OA, HA content decreases, and so does its molecular weight [116, 121]. Viscosupplementation with HA is meant to address this issue, however, there is still no consensus as to whether this treatment has benefits or not [116, 121, 124].

Non-pharmacological treatments cannot help repair defects or lesions in AC. However, changes such as introducing moderate exercise and healthier eating habits in a patients' routine are beneficial for their overall health. The use of devices such as unloading braces can also improve their mobility and reduce discomfort. Non-pharmacological strategies are also relatively inexpensive and easy to implement [23].

Pharmacological treatments, while still unable to repair damage in AC, can provide relief and reduce symptoms such as pain and inflammation. These substances can be administered orally, topically, or as IA injection, in the affected joint [23]. However, this relief is not long-lasting, and may even be attributed to placebo effect, and does not address any defects that might be present in AC, which will worsen overtime [23]. Furthermore, continuous use of drugs may have deleterious side effects for the overall health of patients [114].

4.2 Treatments Based on Surgical Intervention

Clinically available surgical procedures that aim at repairing damaged cartilaginous tissue include abrasion arthroplasty, subchondral drilling, microfracture, and osteochondral transplantation [1, 15].

Abrasion arthroplasty, subchondral drilling and microfracture are known as marrow stimulating techniques, as they all rely on accessing the marrow of subchondral bone, which is vascularized, in order to elicit a repair response [1, 15]. Abrasion arthroplasty starts with debridement of boundaries of the injured AC, countering the edges of the defect evenly, to expose "fresh collagen" [125]. Next, the bone lying beneath the defect is breached, so blood can fill it and a fibrin clot forms, which is sustained by the exposed collagen. Aftermath of this procedure tends to vary, and good outcomes are not consistent [125]. In subchondral drilling, debriding of the defect site is first conducted, in order to create a contained concave shape. High speed drilling is then performed, reaching trabecular bone, were blood vessels exist. Blood fills the defect, forming a clot and enabling a repair response to take place. Thermal osteonecrosis may occur, due to heat caused during the drilling process [125, 126]. However, solutions such as use of drills with a built in cooling system can help address this issue [127]. In microfracture, AC of the damaged area is filled with small holes, equally spaced, and with depth of four millimeters. This allows MSCs from the subchondral bone to reach the injured tissue and help it undergo repair, which starts with formation of a blood clot [128]. The small holes form an irregular surface that facilitates clot formation. Microfracture has the advantage of removing the use of drills, and therefore thermal osteonecrosis does not occur, as in subchondral drilling [125]. In marrow stimulation techniques, repair tissue consists of a mix of fibrocartilage and hyaline cartilage [125]. Although these surgical techniques are readily available, enable tissue repair and reduce discomfort at the joint, these benefits are not long-lasting [3]. Newly formed tissue has inferior mechanical properties than native one, and pain relief is usually temporary [3]. Furthermore, integration between neo and native tissue is not optimal [1, 15] and post-operative recovery time is somewhat prolonged [3].

Osteochondral transplantation, or mosaicplasty, occurs when tissue from the patient (autograft) or a cadaveric donor (allograft) is transplanted into the affected area [1][3]. The transplanted tissue consists of cartilage and underlying subchondral bone [3]. For this purpose, the lesioned area is debrided, and unstable tissue is removed. An osteochondral plug, of cylindrical shape, is removed from a donor site and transplanted into the defect [125]. In the case of autografts, the donor site must not bear weight. Autografts are commonly employed to address smaller defects, with less than 2 cm in diameter, while allografts are suitable for any size of defect [3]. The use of allografts from cadaveric donors raises concerns, as they may elicit a host immune response or transmit diseases [3]. Weight bearing capacity is limited for a few weeks after surgery, but good results are reported for autografting [129] and allografting [130]. However, this procedure is more invasive than marrow stimulating techniques, which may also increase chances of infection [131]. Currently available grafting techniques focus on regeneration of both cartilage and bone. However, cartilage graft integration with native AC and subchondral bone remains challenging. The integration between grafted and native cartilage is essential for stable repair to occur, as otherwise movement between the tissues will lead to further cartilage degeneration [1]. Bone-cartilage integration, which involves the regeneration of the osteochondral interface, is also a key factor for repair to take place, since this mineralized layer confers stability and helps maintain homeostasis and mechanical

function of the joint [1].

More recently developed approaches for repair of injuries include autologous chondrocyte transplantation (ACI) and matrix-associated autologous chondrocyte implantation (MACI), in which cells of the patients are used [3]. In ACI, a biopsy of cartilage is performed in the patient, in an area which is not load-bearing [3]. The collected tissue goes through a process of enzymatic digestion, and chondrocytes are then propagated *in vitro*, to reach a cell density of at least 10^7, and later injected into the injured area [3, 64]. A periosteal patch is employed to avoid swelling and to seal the drilled region, containing the recently injected cell suspension [3]. Currently, synthetic collagen is used instead, as patients would sometimes require another surgical intervention to address hypertrophy of the previously placed patch [132]. MACI is a technique derived from ACI, in which cells are delivered through a scaffold. This structure allows cell distribution and proliferation, and may also release ECM components [3]. Biomaterials such as collagen fibers, gels, and synthetic polymers mixed with HA are commonly used [133-135]. The scaffolds can be inserted into defects with a cylindrical shape, or even fixed utilizing fibrin glue [3]. ACI and MACI are appealing techniques, as they avoid donor site morbidity and disease transmission, which can occur in cases of graft transplantation [1, 3]. There are, however, some drawbacks to these techniques, namely their invasive nature, limited number of chondrocytes that can be harvested, and the fact they are not a suitable approach for the repair of larger lesions or defects [64]. Furthermore, cell transplantation is performed between 6 weeks to 18 months after the biopsy is obtained, which means that it is not a suitable approach for situations where repair of the defect is urgent. Post-operative recovery can also be long, as the joint has to slowly adjust to normal weight bearing functions [3].

To address severe injuries, the most common surgical intervention method still consists in replacing part of the joint, or the joint as a whole, with an artificial implant [51]. In fact, for end stage joint diseases of degenerative nature like OA, joint replacement remains the most common and cost-effective procedure [136]. However, as much as 15 to 30% of patients that have had knee replacement surgery do not report a significant improvement. A smaller percentage of patients that have had hip replacement surgery report the same. This is attributed to pain, restricted mobility, and inferior quality of life. Outcome of this procedure is greatly influenced by patients' age, gender, weight and existing health issues. The period following joint replacement and process of rehabilitation are also critical for a good outcome [136].

4.3 TERM Strategies

TERM (tissue engineering/regenerative medicine) strategies are also being researched and developed, but so far are mostly not available in clinics. These include the use of cells, biomaterials and molecular factors, alone or in different combinations, to promote repair or regeneration of AC [51]. MSCs are considered the most attractive cell type, for therapeutic purposes, due to their ability to differentiate into chondrocytes and secrete molecules with biological activity, that can modulate the immune response and promote tissue repair [51]. Autologous MSCs have been successfully injected in AC of patients suffering from OA, showcasing anti-inflammatory action and reducing pain in the joint. Strategies that hope to repair AC damaged by OA must address the inflammation that is promoted by several mediators and that could potentially further OA progression [51].

Several biomaterials, particularly hydrogels, have been produced and studied for their

possible use in cartilage repair and regeneration. In a recent study, agarose, alginate, collagen and Matrigel hydrogels were evaluated on their potential to function as substrates for expansion of donor chondrocytes, for use in ACI. The hydrogels' influence in chondrocyte proliferation and phenotype maintenance was observed. Collagen hydrogel was shown to prevent chondrocyte dedifferentiation, facilitate cell expansion and proliferation, and augment the expression of genes specific to cartilage. Expression of COL I and X was also decreased for this hydrogel, another promising result, as these markers are indicators of dedifferentiation and chondrocyte hypertrophy, respectively. Overall, results showed that the collagen hydrogel was the most suitable to support cell growth and maintain their phenotype, making it a viable substrate for expansion of donor chondrocytes, for use in ACI [137].

Hydrogels of chitosan-hyaluronic acid dialdehyde were also studied in the context of cartilage regeneration, *in vivo*. The hydrogels were tested alone and combined with chondrocytes. A rabbit model was used, and osteochondral defects of critical size were done in the knee joints of the animals. The hydrogels were then aseptically implanted in the site of injury. The sham group did not receive any type of hydrogel. Rabbit chondrocytes of allogenic origin were incorporated in the hydrogel + cells material. Twelve weeks after implantation, there was repair tissue present in the sham, hydrogel, and hydrogel + cell rabbit groups. Tissue formed in the hydrogel group had the most similar texture to the native cartilage, and the encapsulation of chondrocytes in the hydrogel did not seem to enhance the quality of the newly formed cartilage [138].

Iseki *et al.* used fibrin hydrogels, both cell free and seeded with BM-MSCs, and studied the influence of mechanical loading on a model of microfracture repair, *in vitro*. Cartilage explants from bovines were used. Cores were made in each explant and filled with a fibrin gel, with or without encapsulated BM-MSCs, or with the native cartilage than had been previously extracted to form the cores. The explants were then submitted to mechanical loading. For the fibrin+BM-MSCs group, compressive loads promoted chondrogenesis and maturation of the newly formed tissue. Cartilaginous tissue similar to hyaline cartilage formed, and favored integration between new tissue and native tissue. It was concluded that weight bearing soon after microfracture is performed can promote repair, as hyaline-like cartilage forms. In the absence of weight bearing, repair may be less effective, and even hindered [139]. Several biomaterial constructs and materials for TE and regeneration of damaged cartilage are reviewed in [140].

TERM strategies combine biomaterials and cells for specific patient needs, which may enhance their therapeutic properties. However, many of these biomaterials have yet to undergo pre-clinical and/or clinical testing, and may be difficult to mass produce, limiting their availability and widespread use.

4.3.1 Recent Studies on the Development of Dendritic Cell-Based Therapy for Osteoarthritis

Recent studies have explored the therapeutic potential of DCs in OA. A study by Guo *et al.* assessed how suppressive oligodeoxynucleotide-induced DCs (S-DCs) could potentially benefit OA therapy [141]. Overall, treatment with S-DCs diminished inflammation, even upon pro-inflammatory stimulation, and expression of surface markers and pro-inflammatory cytokines was hindered. The transfer of S-DCs into the knee of OA mice improved weight bearing capacity at the joint and controlled OA progression, as joint inflammation was

restricted by S-DCs. Results showed that S-DCs could potentially be used in OA therapies, to help control disease development [141].

TLRs expressed on DCs can mediate inflammation, and previous studies have highlighted their possible relevance in OA [142], as well on DC activation [143-145], albeit their contribution towards OA progression is yet to be established. In a work by Nie *et al.*, the role of both TLRs and DCs in the development of OA was studied, in a mouse OA model [146]. DCs in synovial fluid from OA mice had increased expression levels of "TLR family members" [146], when compared with sham mice. *In vitro*, DCs derived from OA mice produced more inflammatory cytokines after treatment with LPS and R848 (TLR agonists), and DC maturation was not affected by TLRs challenges. Furthermore, *in vivo* treatment of DCs with FP7, a TLR antagonist, decreased LPS induced inflammation. This study elucidated how TLRs expressed by DCs contribute towards inflammation, indicating that they may be a possible target in development of successful OA therapies [146].

Finally, recent studies regarding vitamin D have highlighted its capability to perform immunomodulation. In a work by Chen *et al*., LPS induced inflammation in DCs was inhibited, when the cells were treated with 1,25-dihydroxyvitamin D3 (vitD3) [147]. Tolerogenic DCs (tolDCs) are known to express low levels of MHC-II and co-stimulatory molecules, produce reduced amounts of pro-inflammatory cytokines and inducing "peripheral T cell tolerance" [148], helping to regulate immune responses [148, 149]. Wong *et al.* isolated DCs from PBMCs of OA patients, and treated them with a combination of dexamethasone and vitD3 (Dex/vitD3) [150]. This treatment lowered secretion of inflammatory cytokines, while it increased secretion of TGF-β and IL-10. Treatment of DCs with LPS led to a higher percentage of IFN-γ and IL-17 producing T cells, while treatment of DCs with vitD3/Dex led to a higher percentage of IL-10 and FoxP3 producing T cells. For DCs treated with vitD3/Dex followed by LPS, levels of the previously mentioned cytokines decreased. This study also found that release of IL-10 by stimulated T cells caused autologous T cell proliferation to be suppressed, through soluble IL-10 and direct cell contact. In addition, T_{regs} and tolDCs were able to suppress chondrocyte secretion of MMP-1 and MMP-3. These results show that treatment of DCs with Dex/vitD3 successfully induced a tolerogenic phenotype, and these cells were able to exert tolerogenic functions [150], as in previous studies [151, 152]. Novel therapeutic strategies for OA could benefit from incorporating tolDCs [150].

5. Biomaterials to Modulate Dendritic Cell Behavior

The performance of a given biomaterial after implantation greatly depends on its interactions with the host immune system. Macrophage interactions with biomaterials have been widely studied, as these cells, along with neutrophils, constitute one of the first cell types to arrive at the implant site [153, 154]. DCs, although less studied in this context, have been gathering increasing attention since they can also perform direct interactions with the implanted biomaterials and trigger immune responses, in particular adaptive immunity [95]. A number of studies have reported how biomaterials properties, such as size, composition and structure, can effect DC maturation, cytokine release and expression of co-stimulatory molecules. These are summarized in Table 1.

Table 1 - Biomaterial microparticles and films of varying composition, and their effect on dendritic cells.

Biomaterial	DC origin	Effects on DCs	Source
Ch films	HPBM derived DCs	Promoted DC maturation; Increased secretion of pro-inflammatory cytokines; Increased expression of HLA-DQ, CD44, CD80, CD83 and CD86; Increased levels of allostimulatory capacity; Reduced endocytic capacity.	[155]
PLGA films (75:25;100000 Da)			
Alginate films		DC maturation was affected similarly to stimulation with Ch and PLGA films	
HA films		Suppression of DC phenotype; Decreased secretion of pro-inflammatory cytokines; Decreased expression of HLA-DR, CD40, CD80 and CD86; Decreased allostimulatory capacity.	
Agarose films		Promoted DCs to retain an immature phenotype.	
PLGA MPs (50:50; 65000 Da)	BMDCs	MPs had no effect on DC maturation.	[156, 157]
PLGA MPs PLGA Films (75:25)	BMDCs	Biomaterial shape influenced DC maturation; Both biomaterials promoted maturation of DCs; Both biomaterials increased expression of CD80 and CD86; Both biomaterials increased release of TNF-α and IL-6.	[158]

PLGA MPs PLGA Films (75:25)	HPBM derived DCs	Biomaterial shape influenced DC maturation, Maturation of DCs by expression of co-stimulatory molecules and MHC-II; MPs promoted proliferation of allogenic T cells, which did not occur with films.	[159]
Ch films	HPBM derived DCs	Cell polarization towards a pro-inflammatory phenotype; Increased expression of CD86, but not CD83 and HLA-DR; Increased secretion of TNF-α and IL-1β, but not IL-12, IL-23; Decreased secretion of IL-10; Activity of MMP-2 and MMP-9 was not affected.	[160]

Footnote: Chitosan (Ch), poly (lactic-co-glycolic acid) (PLGA), Hyaluronic Acid (HA), Microparticles (MPs), Human peripheral blood monocyte (HPBM), Mouse Bone Marrow-derived Dendritic Cells (BMDCs), Nanoparticles (NPs), Chitosan/Poly(γ-glutamic acid).

In a study performed by Park and Babeensi, the effect of material composition on DC maturation was analyzed [155]. Human peripheral blood monocyte (HPBM) derived DCs were exposed to films of chitosan (Ch), agarose, alginate, HA and poly (lactic-co-glycolic acid) (PLGA). The latter was formulated with a molar ratio of 75:25 and had a molecular weight (MW) of 100 000 Dalton (Da) [155]. DCs cultured on Ch and PLGA films presented morphology of mDCs, secretion of pro-inflammatory cytokines, human leukocyte antigen (HLA)-DQ and expression of costimulatory molecules, namely CD44, CD80, CD83 and CD86, as well as increased levels of allostimulatory capacity. Compared with iDCs, these mDCs displayed reduced ability to perform endocytosis. Stimulation of DCs with alginate films affected cell maturation similarly to stimulation with Ch and PLGA films [155]. Comparing with iDCs, DCs cultured on HA films resulted in phenotype suppression, decreased secretion of pro-inflammatory cytokines and reduced expression of HLA-DR, CD40, CD80 and CD86, as well a reduction in levels of allostimulatory capacity [155]. Stimulation of DCs with agarose films promoted cells to retain an immature phenotype [155]. Two studies with PLGA microparticles formulated with a molar ratio of 50:50 and a MW of 65 000 Da, reported that these had no effect on DC phenotypic maturation [156, 157]. The different conclusions of these studies could be attributed to differing biomaterial structure, formulation and MW. Also, DC immunophenotyping was performed at different time points, which could potentially have contributed to the divergent observations [155-157].

Previous work from our group showed that DCs derived from blood monocytes were polarized toward a pro-inflammatory phenotype, when cultured on Ch films. Ch was able to upregulate the expression of CD86 and stimulated the release of TNF-α and IL-1β, both pro-inflammatory cytokines, and this secretion was higher, when compared to that of DCs in tissue culture polystyrene (TCPs). IL-10 secretion was decreased by DCs cultured in Ch films. Furthermore, Ch films did not impact the activity of MMP-2 and MMP-9, secreted by DCs [160].

Studies have shown that PLGA microparticles and films differently affect DC phenotype [158, 159]. It has been proposed that phagocytosis and subsequent internalization of PLGA microparticles increased DC maturation, comparing with PLGA films. PLGA microparticles have been found inside phagosomal vesicles [161]. Lactic and glycolic acid, which are released due to PLGA degradation, could lead DCs to produce IL-10 and induce

immunological tolerance [162], which can justify the differences in phenotype induction by PLGA throughout different time points. Interestingly, a study by Allen *et al*. showed that internalization of PLGA microparticles by BMDCs and subsequent hydrolysis and lactic acid release led to phenotype suppression and resistance to LPS-induced maturation [163]. Results obtained reporting a contrary effect of PLGA microparticles in DC function were attributed to differences in MW of the particles and duration of co-culture [163].

5.1 Nanomaterials to Modulate Dendritic Cell Behavior

Nanotechnology allows manipulation of compounds and materials at the atomic level, rearranging their molecular organization and enhancing their biological, physical and chemical properties [164]. Nanomedicine emerged as a field from this technology, focusing on production of NPs, among other components and devices of nanometric scale [165]. Materials in the nanometric scale are particularly unique, as they showcase different behavior and properties (physical and chemical) than those that can be observed at larger scales, in bulk form [140]. In medicine, nanoparticles are used as vectors for gene delivery, as drug carriers and in cancer immunotherapies [165].

Regarding biomedical application of these biomaterials of nanometric scale, their integration in tissue engineered constructs, such as scaffolds, can help mimic the microenvironment of cartilage ECM, and improve interactions between the cells and the construct [140]. Such materials may also be incorporated or combined with other biomaterials, drugs, or active molecules, to enhance their therapeutic effects [140]. However, NP cytotoxicity and immunotoxicity in living organisms are major concerns, hindering their widespread clinical use. When designing NPs, some key aspects should be considered, namely their possible interactions with the biomolecules from the organism they will enter [165]. Their physical and chemical properties should be optimized by tailoring NP characteristics such as surface charge, size, shape and "protein corona effects" [165]. The latter consists of a layer of plasmatic proteins which envelop NPs, once they are exposed to systemic circulation, i.e, to physiological fluids, primarily blood [166]. Adsorption of proteins to NPs is critical, since they can alter the biomaterials' physical and chemical identity, their cellular targeting and uptake, the period of time they remain in circulation in the blood stream, as well as the body's response to the material and its toxicity [165]. Due to these interactions, NPs morphology may become heterogeneous, which, in turn, influences their biocompatibility and safety in the organism. NPs can enter cells directly, or through a series of different mechanisms, namely phagocytosis, pinocytosis and micropinocytosis. Afterwards, they combine with molecules which are present in the extracellular fluid, which enables their internalization by cells [165]. Over the past two decades, several NPs, with varying shape, composition and physicochemical properties have been produced, and their effects on DCs studied (Table 2).

Table 2 - Nanobiomaterials of varying composition, shape and size, and their effect on dendritic cells.

Biomaterial	DC origin	Effects on DCs	Source
Ch/γ-PGA NPs	HPBM derived DCs	Increased expression of CD40, CD86 and HLA-DR; Release of IL-12/IL-23(p40), TNF-α and IL-6.	[167]
NLC loaded with resveratrol	HPBM derived DCs	Decreased expression of CD83 and HLA-DR (after TNF-α stimulation); Decreased secretion of IL-12/IL-23;	[168]
LDH NPs ($Mg(OH)_2$ and $Al(OH)_3$)	BMDCs	For NPs with equal ratio of $Mg(OH)_2$ and $Al(OH)_3$: • Increased expression of CD40, CD86 and CCR7; • Increased secretion of TNF-α and IL-12; • Increased DC migration capacity to CCL21.	[169]
$[Gd@C_{82}(OH)_{22}]_n$ NPs	HPBM derived DCs	Promoted DC maturation; Promoted IL-12p70 release; Upregulated expression of CD80, CD83, CD86 and MHC molecules.	[170]
ZnO NPs (sheet or spherical shape) (30 cm^2/g)	BMDCs	Exposure to NPs at 30 μg/mL concentration: • Upregulated CD80 and CD86 expression; • Increased IL-6 and TNF-α release; Similar NP surface area resulted in similar effects, in spite of shape; DC phenotype was not affected for NP concentration of 10 μg/mL.	[171, 172]
Au NCs (3 nm and 12 nm)	HPBM derived DCs	Au NCs with 3 nm: • Increased IL-12 and IFN-γ synthesis; • Promoted pro-inflammatory activity of T cells. Au NCs with 12 nm: • Increased IL-4 synthesis; • Promoted a wound healing response from T cells.	[173]
TiO_2 nanostructures (diameter varying from 7 to 20 nm)	*In vitro* human construct	Induced shape dependent DC maturation; Induced ROS production; Induced pro-inflammatory cytokine secretion; Primed naïve $CD4^+$ T cell activation and proliferation.	[174]
WNVE coated Au NPs (spherical, cubical and rod shaped)	BMDCs	DC uptake was superior for rod shaped NPs; Treatment with rod shaped NPs led to IL-1β and IL-18 secretion; Treatment with spherical and cubical NPs led to IL-6, IL-12, TNF-α and GM-CSF secretion.	[175]
Au NPs (10 nm and 50 nm)	HPBM derived DCs	Hindered LPS-induced maturation; Hindered allostimulatory ability; 10 nm NPs further inhibited LPS induced expression of IL-12p70, CD83 and CD86.	[176]
Polyanhydride nanospheres and films	BMDCs	Treatment with nanospheres: • Increased expression of CD40, CD86, CD209 and MHC II; • Increased IL-12p40 production.	[177]
γ-PGA–Phe NPs (23 to 128 nm)	BMDCs	Smaller NPs resulted in lower uptake by DCs, but had a more prominent effect on their activation.	[178]
γ-PGA-Phe NPs	Murine	*In vitro*, 40 nm NPs resulted in higher DC activation;	[179]

(40, 100 and 200 nm)	raw264 cell line	*In vivo*, a greater number of DCs were able to uptake the 40 nm NPs; Each cell was able to uptake greater amounts of the larger NPs; Migration of the 40 nm NPs towards lymph nodes was quicker, and they efficiently promoted DC maturation.	

Footnote: Human Peripheral Blood Monocyte (HPBM), Nanoparticles (NPs), Chitosan/Poly(γ-glutamic acid) (Ch/γ-PGA), Nanostructured lipid carriers (NLC), Layered Double Hydroxide (LDH), Mouse Bone Marrow-derived Dendritic Cells (BMDCs), Magnesium Hydroxide ($Mg(OH)_2$), Aluminum hydroxide ($Al(OH)_3$), Zinc Oxide (ZnO), Gold (Au), Nanoclusters (NCs), Titanium Dioxide (TiO_2), West Nile Virus Envelope (WNVE), γ-PGA-graft-l-phenylalanine ethylester (γ-PGA–Phe).

Aiming to increase DC activation as an anti-cancer therapy, Castro *et al*. explored the impact of pro-inflammatory Chitosan/Poly(γ-glutamic acid) (Ch/γ-PGA) NPs on DC maturation and activation. The expression of CD40, CD86 and HLA-DR was found to be increased upon Ch/γ-PGA NPs treatment, to levels similar to those of DCs stimulated with LPS. Moreover, DCs' secretion of pro-inflammatory cytokines IL-12/IL-23(p40), TNF-α and IL-6 was stimulated by this biomaterial, when compared to the unstimulated condition and similarly to the positive LPS control [167]. Conversely, to reduce DC inflammatory stimulation, Barbosa *et al*. studied the capacity of nanostructured lipid carriers (NLC) loaded with resveratrol, an anti-inflammatory drug, to reduce DC activation in response to TNF-α, which is increased in chronic inflammatory illnesses, such as RA. These NPs were found to be able to decrease DC activation by TNF-α, reducing cell surface expression of CD83, HLA-DR, and reducing IL-12/IL-23. This reduction in DC activation in response to TNF-α was more efficient when resveratrol was encapsulated in NPs that when it was free, reducing the necessary dose of resveratrol [168].

Li *et al.* studied the ability of layered double hydroxide (LDH) NPs, considered as potential vectors for drug delivery to tissues and cells, to activate DCs. The LDH particles were formulated with different ratios of magnesium hydroxide ($Mg(OH)_2$) and aluminum hydroxide ($Al(OH)_3$). The NPs were named R1, R2 and R3, corresponding to ratios of 1:1, 2:1 and 3:1, respectively. BMDCs were exposed to these NPs. R1 NPs increased TNF-α, IL-12, CD40 and CD86 expression, in a dose dependent manner. The same effect was not verified for R2 and R3 NPs. R1 NPs also increased DC migration capacity to CCL21, and up-regulated CCR7 expression [169]. Yang *et al.*, reported that $[Gd@C_{82}(OH)_{22}]_n$ was capable of inducing maturation in DCs. $[Gd@C_{82}(OH)_{22}]_n$ is derived from C_{82} fullerene. This material stimulated DCs to produce IL-12p70, and upregulated expression of CD80, CD83, CD86 and MHC molecules [170].

The effects of metallic nanobiomaterials on DCs have also been explored. In a study by Heng *et al.*, zinc oxide (ZnO) NPs effects on BMDCs were analyzed. The NPs were sheet shaped, or spherical, with a surface area close to 30 cm^2/g. Exposure of DCs to the ZnO NPs at a concentration of 30 μg/mL resulted in upregulation of CD80 and CD86 surface makers. The NPs also promoted release of IL-6 and TNF-α, which may point to an inflammation inducing capability. These effects did not differ between NPs shape, as they were manufactured with similar surface area [171]. Andersson-Willman *et al.* also studied ZnO NPs, and found that exposure of moDCs to these NPs at a concentration of 10 μg/mL did not result in the same effects as those verified for the previous study [171] [172]. This concentration did not cause

significant alterations to DC phenotype [172].

Gold-based nanomaterials have been used to examine the impact of size and shape on DC response. Fernández *et al.* studied the effect of gold nanoclusters (Au NCs) in HPBM DCs and found that size was determinant for the outcome of these materials. Au NCs with approximately 3 nm in size promoted an increase in IL-12 and IFN-γ synthesis, in addition to pro-inflammatory stimulation of T cells, in co-culture, while Au NCs with 12 nm increased IL-4 secretion, and promoted a wound healing response from T cells [173]. In a work by Niikura *et al.*, gold nanoparticles (Au NPs) coated with West Nile Virus envelope (WNVE) protein shapes were tested with spherical, cubical, and rod shapes, with dimensions of 20 and 40 nm in diameter, 40x40x40 nm, and 40x10 nm, respectively. The coated NPs were inoculated into mice, spherical 40 nm NPs led to the highest level of anti-WNVE antibodies. NP uptake by BMDCs was superior for rod shaped NPs, leading to the conclusion that antibody production may not be dependent on the efficiency of DC NP uptake. Finally, cytokine production by DCs treated with the coated Au NPs was also assessed. DCs treated with rod shaped NPs secreted IL-1β and IL-18, while DCs treated with spherical and cubical NPs secreted pro-inflammatory cytokines, namely IL-6, IL-12, TNF-α and GM-CSF [175]. Tomić *et al.* also tested gold NPs of 10 nm (GNP10) and 50 nm (GNP50) in DCs of human origin. Results showed that the NPs were internalized and hindered DC maturation induced by LPS, as well as their allostimulatory ability. These effects were more prominent for GNP10, which further inhibited LPS induced expression of IL-12p70, CD83 and CD86 [176].

NP structure was also investigated for titanium dioxide (TiO_2) particles in an *in vitro* human immune construct, consisting of endothelial cells from veins. The particles had three common manufacturing structures: anatase, rutile and nanotube. Particle diameter varied between 7 to 10 nm, 15 to 20 nm, and 10 to 15 nm, respectively. These nanostructures induced shape dependent DC maturation, reactive oxygen species (ROS) production, and pro-inflammatory cytokine secretion. Comparing with DCs that received treatment with TiO_2 microparticles, treatment with TiO_2 nanostructures "primed activation and proliferation of naïve $CD4^+$ T cells" [174].

Petersen *et al*. tested the adjuvant effects of polyanhydride nanospheres and films in murine DCs. Comparing to DCs exposed to films, DCs in the presence of nanospheres showed increased expression of cell surface activation markers, namely CD40, CD86, CD209 and MHC II. They also produced more IL-12p40. These results were also shown to be dependent on the surface chemistry of the materials. The results indicate that polyanhydrides' properties, such as shape and surface chemistry, can be adjusted to induce activation of DCs [177]. In a study by Kim *et al.*, γ-PGA-graft-l-phenylalanine ethylester (γ-PGA–Phe) NPs of varying size (23 to 128 nm) were shown to have the same ability to encapsulate ovalbumin (OVA), as seen by the amount of OVA per mg of NPs. Smaller NPs resulted in lower uptake by DCs, but did have a more prominent effect on their activation. It was then possible to conclude that particle size influenced cellular uptake by DCs, as well as their maturation process [178]. Shima *et al.* also tested the impact of size, using 40, 100 and 200 nm γ-PGA-Phe NPs. Out of all the sizes, the 40 nm NPs resulted in higher DC activation, *in vitro*. Uptake of NPs by the cells was also affected by their size. *In vivo*, a greater number of DCs were able to uptake the 40 nm NPs, and these particles were also able to migrate more quickly towards the lymph nodes, were they efficiently promoted DC maturation. However, DCs were able to uptake greater amounts of the 100 and 200 nm NPs. For the purpose of antigen delivery, the larger sized particles would then be able to deliver larger amounts of antigens than the 40 nm NPs. These results reinforce how

the size of γ-PGA-Phe NPs can be used to regulate DC uptake, migration and maturation [179].

Overall, these works show that for biomaterials of nanometric scale, chemical composition, size and morphology can impact DC activation and phenotype, and should all be considered when designing DC-targeted therapies. Such parameters will also be likely to influence immunotoxicity of different nanobiomaterials. Furthermore, uptake of these nanobiomaterials by DCs also varies with their properties, influencing DC migration towards lymphoid organs and, therefore, the immune response that is triggered.

6. Objectives

The work of this dissertation was developed within the scope of the RESTORE Project, a collaborative European project between medical professionals and scientific investigators, aiming to develop nanoenabled materials capable of controlling inflammation and promote cartilage repair, in OA. The main goal of this work was to study the potential immunotoxicity/immunomodulation of selected nanobiomaterials towards human primary DCs. Three specific objectives were established:

(1) Assessment of DC viability upon exposure to nanobiomaterials;

(2) Assessment of nanobiomaterial capacity to modulate DC phenotype and reduce inflammatory stimulation;

(3) Assessment of DC cytokine secretion in response to the nanobiomaterials.

7. Materials and Methods

7.1 Carbon Nanotubes

CNTs are very light and thin hollow tubes, and possess high strength and conductivity [180]. They consist of one or more sheets of rolled graphene, and have a fibrous texture. Each graphene sheet is as thick as an atom. Single-walled (SW) CNTs consist of a single sheet of rolled graphene, with diameter between 0,7 to 150 nm, and length between 0,5 to 50 μm. Double-walled (DW) CNTs have two rolled sheets, with diameter between 1,3 to 5 nm, and length between and 0,5 to 40 μm [180]. Multi-walled (MW) CNTs have more than two sheets of graphene rolled and stacked, with diameter between 4 to 150 nm, and length between 0,5 to 150 μm [180]. CNTs can enter the human body through ingestion, inhalation and injection [181]. Their properties are thought to be appealing for applications such as drug delivery, imaging techniques and gene therapy. However, mechanisms of CNT transport and uptake in the human organism still need to be studied, and, more importantly, possible detrimental effects to patients' health need to be assessed [182]. CNT distribution and potential side effects in the organism have been the target of several studies in animal models [183, 184]. These particles have also been incorporated in strategies for cartilage repair/regeneration [185-187]. We tested MWCNTs functionalized with carboxyl (-COOH), with a 35 nm diameter, measuring a few microns in length. They are referred to simply as CNTs.

7.2 Poly(lactic-co-glycolic acid) Nanoparticles

PLGA is a non-toxic, biocompatible and biodegradable polymer, which is also able to perform sustained release of molecules, making it a popular biomaterial to use in the field of tissue engineering, and a suitable vehicle for drug delivery [188, 189]. In an aqueous environment, PLGA is degraded through hydrolysis of ester bonds of its polymeric chains. Scission of the polymeric chains reduces the MW of PLGA, which in turn increases hydrophilicity and further facilitates degradation of the biomaterial [190, 191]. PLGA can also degrade through a process of auto-catalysis, where byproducts of acidic nature (such as lactic and glycolic acid) remain trapped in the polymer, becoming the catalysts of PLGA degradation. Upon release of these byproducts, an acidic microenvironment is locally established, which can irritate neighboring tissue, and be detrimental to the stability of the biomaterial. In case PLGA is loaded with drugs, their stability will also be affected [192]. The physicochemical properties of PLGA NPs, such as surface area, MW and lactic acid to glycolic acid ratio influence the

materials' behavior and degradation in the body [188]. NPs with larger surface area lead to increased rate of degradation, since a larger surface is available for enzymatic breakdown [193, 194]. High MW decreases the rate of degradation, as more time is required to break down longer polymeric chains [195]. Increased glycolic acid content has also been shown to accelerate PLGA degradation, due to glycolic acid's high hydrophilicity [196]. PLGA microspheres and scaffolds applied *in vivo*, empty or loaded with drugs, have been shown to improve the regeneration of cartilage [140, 197].

7.3 Human Monocyte Isolation and Differentiation into Dendritic Cells

Primary human peripheral blood mononuclear cells were isolated from buffy coats (BC), from healthy volunteer blood donors (kindly provided by the Immunotherapy Department of Centro Hospitalar Universitário de São João (CHUSJ) from Porto, Portugal) and monocytes were isolated by negative selection, as routinely performed in the lab [160]. The collection and processing of the blood samples was conducted in accordance with the principles of the Declaration of Helsinki, and under an ethically approved protocol (ref.90/19). Blood donors provided informed written consent for byproducts of their donations to be used. All samples were analyzed anonymously.

Briefly, fresh BC were transferred to 50 mL tubes and centrifuged at room temperature (RT) for 30 min, at 1200 g, without break. The resulting layer of PBMCs was collected and incubated for 20 min at RT with *RosetteSep* human monocyte enrichment isolation kit (StemCell Technologies), according to the manufacturer's instructions. Following incubation period, the mixture was diluted at a 1:1 ratio, with phosphate buffered saline (PBS) supplemented with 2% FBS (heat-inactivated fetal bovine serum, Biowest). The diluted mixture was then layered over Histopaque-1077 (Sigma-Aldrich), and centrifuged at RT for 20 min, 1200 g, without break. The enriched monocyte layer was collected and washed with PBS to remove platelets. The washes were performed at 1300 rpm, for 6 minutes. The resulting pellet was then resuspended in RPMI 1640 medium (Corning), supplemented with 10% FBS, 100 μg/mL of streptomycin and 100 U/mL of penicillin (both from Invitrogen). To differentiate monocytes into iDCs, IL-4 and GM-CSF (ImmunoTools) were added at 50 ng/mL. The cells were cultured in TCPs, at 37ºC and 5% CO_2, for a period of 7 days [160].

7.4 Stimulation of immature Dendritic Cells

At day 4 of culture, iDCs were stimulated with different nanobiomaterials and molecules, for 72 h (Table 3). Cells treated with 50 ng/mL of LPS (Sigma-Aldrich) were used as positive controls for activated mDCs, while unstimulated cells were used as negative controls for iDCs. At day 7, conditioned media were collected, and cells were harvested for downstream analysis.

Table 3 - Nanobiomaterials and molecules tested on immature dendritic cells.

Nanobiomaterial	Tested Concentration
CNTs	0,45 μg/mL
PLGA NPs (empty)	100, 150, 200 μg/mL
PLGA NPs (loaded with ibuprofen)	100, 150, 200 μg/mL
Nanobiomaterial + Pro-inflammatory stimulus	**Tested Concentration**
CNTs + TNF-α	0,45 μg/mL; 100 ng/mL
PLGA NPs (empty) + TNF-α	100 μg/mL; 100 ng/mL
PLGA NPs (loaded with ibuprofen) + TNF-α	100 μg/mL; 100 ng/mL
Molecule	**Tested Concentration**
Ibuprofen	15, 20, 25, 30 μg/mL
Ibuprofen + TNF-α	15 μg/mL; 100 ng/mL
TNF-α	100 ng/mL
IL-1β	10, 50, 100 ng/mL
TNF-α + IL-1β	10, 50, 100 ng/mL

7.5 Lactate Dehydrogenase Viability Assay

Conditioned media from DC culture were used to assess cell viability, by measuring the presence of lactate dehydrogenase (LDH) (CytoTox 96® NonRadioactive Cytotoxicity Assay, Promega). The experimental protocol was conducted according to the manufacturer's instructions. Absorbance at 490 nm was read in a Synergy™ Mxplate reader (BioTek). Levels of cell death (relative LDH release) were expressed relative to the unstimulated DC condition.

7.6 Flow Cytometry Analysis

Cells harvested at day 7 were centrifuged at 300 g for 5 min. Each pellet was washed with 1 mL of PBS, and centrifuged as before. The supernatant was then removed, and the pellets were resuspended in 100 μL of FACS buffer (PBS with 2% FBS and 0,01% Sodium Azide) and transferred to a 96 well plate. The plate was then centrifuged and the supernatant discarded. The cells were incubated on ice for 30 min, with the following antibodies: anti-human CD1a-PE (ImmunoTools), anti-human CD86-APC (ImmunoTools) and CD83-FITC (Biorad). A volume of 2 μL of each antibody and 44 μL of FACS buffer were added, in a final volume of 50 μL. The respective isotype fluorochrome matched control antibodies were used as controls to define background labeling. After antibody incubation, cells were washed twice with FACS buffer and then fixed with 100 μL of 1% paraformaldehyde (PFA), and stored at 4ºC, protected from light. Cells were filtered before data acquisition in FACS CANTO II equipment. Results were analyzed in FlowJo software and GraphPad Prism, and presented as mean fluorescence intensity (MFI), after subtracting the respective isotype controls.

7.7 Cytokine Secretion by ELISA

Levels of pro-inflammatory cytokines TNF-α and IL-6, and anti-inflammatory cytokine IL-10 were evaluated in previously collected conditioned media of DCs, through ELISA (Legend

Max enzyme-linked immunosorbent assay kits from Biolegend), according to manufacturer's instructions.

8. Results

8.1 Carbon Nanotubes do not activate human primary Dendritic Cells

To evaluate the impact of CNTs on DC survival and stimulation, DCs were stimulated for 72 h, before cells were harvested. Levels of LDH release were measured in culture supernatants, in order to assess cell viability. Results are presented as the LDH release relative to control unstimulated cells (Fig. 8.1 (A)). LDH release by DCs treated with CNTs was very close to control DCs, and even lower than LPS stimulation, indicating that CNTs at a concentration of 0,45 μg/mL were not toxic towards DCs. Next, the harvested cells were stained for flow cytometry analysis, with anti-human CD83 and anti-human CD86 antibodies, in order to assess whether CNTs promoted DC maturation. Membrane CD83 is highly expressed by mDCs [198], and so is the co-stimulatory molecule CD86 [199]. MFI are presented in Figure 8.1 (B). Cells treated with LPS, which is able to induce DC maturation [167], expressed high levels of these surface markers, as expected. Cells treated with CNTs were not activated, displaying surface marker expression levels similar to those of unstimulated cells (negative control), which were lower than those of LPS-treated DCs. The secretion levels of TNF-α, IL-6 and IL-10 were quantified in conditioned media, through ELISA. TNF-α and IL-6 are known to have a pro-inflammatory effect, while IL-10 is anti-inflammatory [64]. Results are presented in Figure 8.1 (C) and show that all three cytokines were up-regulated in response to LPS stimulation, particularly IL-6, as expected, but their secretion was very low for DCs stimulated with CNTs, and similar to unstimulated control DCs. Results show that cells treated with CNTs were not activated, showing cell surface markers and cytokine secretion levels close to control unstimulated DCs.

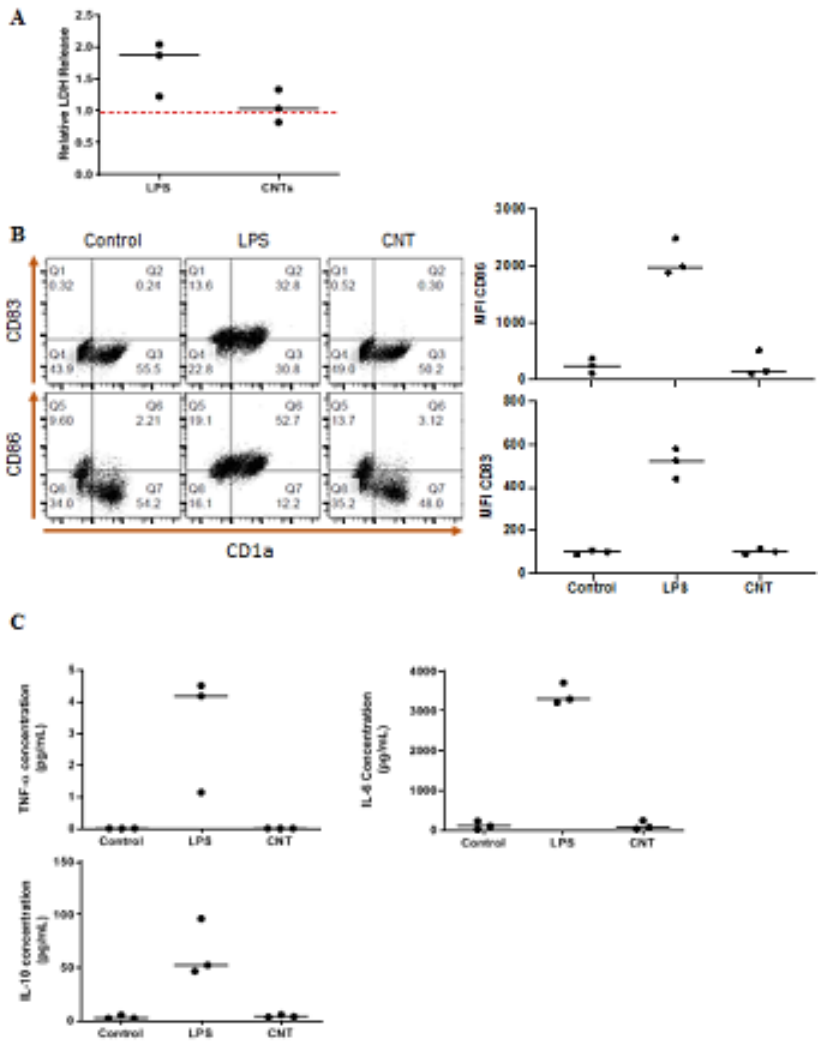


Figure 8.1 - DC response to carbon nanotubes (CNTs). DCs were differentiated for 4 days before being left either unstimulated (negative control), or stimulated with LPS (positive control) or CNTs for further 72 h. A) The presence of LDH in DC culture supernatant, after stimulation with LPS or CNTs, expressed relative to the unstimulated condition (dashed line). B) Illustrative flow cytometry dot plots of the expression of cell surface markers CD83 (upper left) and CD86 of DCs exposed to different stimuli (bottom left). Mean fluorescence intensity (MFI) levels for cell surface CD86 (upper right) and CD83 (bottom right) markers. C) Concentration of TNF-α (left), IL-6 (right), and IL-10 (bottom) released by DCs into culture supernatants, as detected by ELISA (n=3). Statistical significance was assessed by Friedman Test, followed by uncorrected Dunn's multiple comparison test.

8.1.1 Carbon Nanotubes may be able to reduce inflammatory stimulus

Next, we wanted to ascertain if CNTs could impact DC activation under inflammatory conditions, similar to what exists in OA joints. Levels of LDH release were measured in culture supernatant, in order to assess cell viability. According to the results presented in Figure 8.1.1 (A), the different stimuli led to LDH release similar to negative control cells, indicating that neither CNTs nor TNF-α were toxic to DCs. However, LDH release was slightly higher for DCs treated with a combination of CNTs and TNF-α. Following the viability assay, expression of the activation markers CD83 and CD86 on the harvested cells was evaluated by flow cytometry (Fig 8.1.1 (B)). Cells that received treatment with LPS expressed higher levels of these surface markers, indicating DC maturation, while those stimulated with TNF-α showed only a slight increase in cell surface markers expression, indicating that stimulation with TNF-α does not

activate DCs as efficiently as LPS stimulation. Cells treated with CNTs displayed surface marker expression levels similar to those of unstimulated controls cells, and inferior to LPS and TNF-α stimulated DCs. Surface marker expression levels for cells that were stimulated with CNTs and TNF-α were close to those of CNTs treated DCs, indicating that CNTs could have had an effect on reversing the inflammatory stimulus.

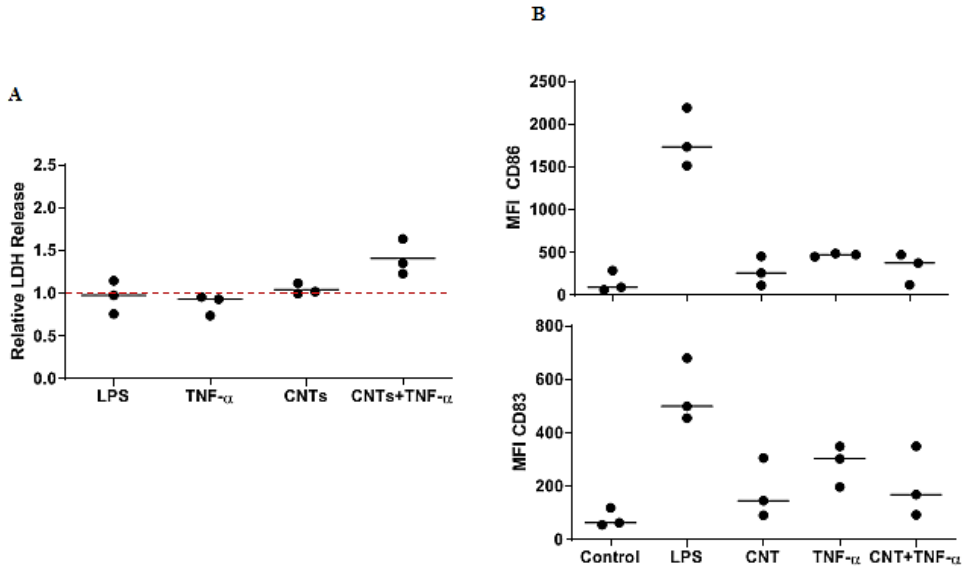


Figure 8.1.1 - Impact of carbon nanotubes (CNTs) on stimulated DC. DCs were differentiated for 4 days before being left unstimulated (negative control) or stimulated with LPS (positive control), TNF-α, CNTs alone or with TNF-α, for 72h. A) Concentration of LDH released by DCs into culture supernatants, as detected by LDH cytotoxicity assay. Data is expressed relative to the unstimulated condition (dashed line); B) Mean fluorescence intensity (MFI) levels for cell surface CD83 (top) and CD86 (bottom) markers, measured in DCs from three independent donors (n=3).

8.2 Poly(lactic-co-glycolic acid) Nanoparticles do not activate human primary Dendritic Cells

Next, we tested polymeric PLGA NPs, which can be loaded with different drugs, for their ability to stimulate DCs. Cell viability assay showed that LDH release concentration was slightly higher for DCs treated with LPS or PLGA NPs, when compared to unstimulated cells, indicating that PLGA NPs induced levels of toxicity more similar to LPS stimulation (Fig. 8.2 (A)). Next, flow cytometry was performed to evaluate expression levels of the cell surface markers CD83 and CD86 (Fig. 8.2 (B)). Cells that received treatment with LPS expressed higher levels of these surface markers, indicating DC maturation. Cells treated with PLGA NPs were not activated, displaying surface marker expression levels similar to those of unstimulated control cells, which were lower than those of LPS-treated DCs. The levels of TNF-α, IL-6 and IL-10 secretion were quantified in conditioned media, through ELISA (Fig. 8.2 (C)). TNF-α, IL-6 and IL-10 secretion was low to undetectable for DCs stimulated with PLGA NPs, and similar to those of the unstimulated control cells. Cytokine secretion was higher for LPS treated DCs, comparing with control DCs. Results show that DCs treated with PLGA NPs were not activated to a pro-inflammatory profile, showing cell surface markers and cytokine secretion levels close to control unstimulated DCs.

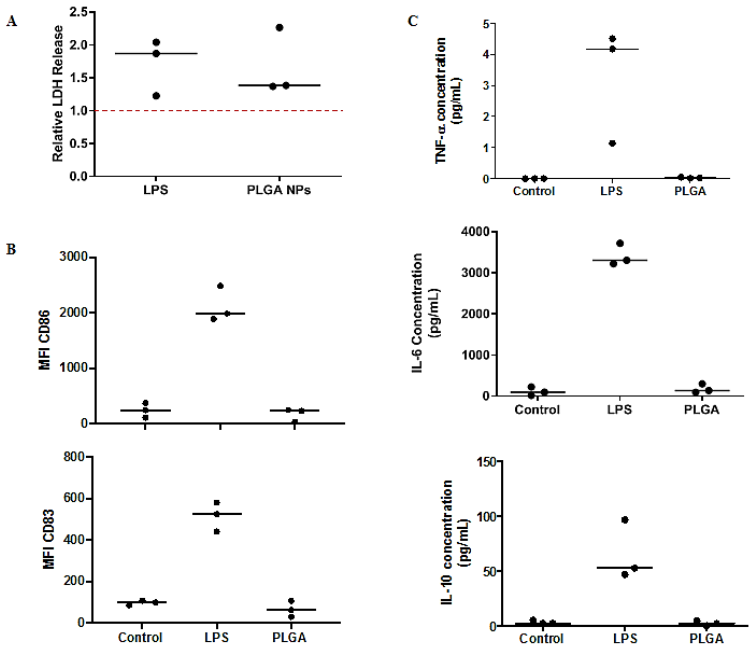


Figure 8.2 - DC response to Poly(lactic-co-glycolic acid) Nanoparticles (PLGA NPs). DCs were differentiated for 4 days before being left either unstimulated (negative control), or stimulated with LPS (positive control) or PLGA NPs, for further 72 h. A) Presence of LDH in DC culture supernatant, after stimulation with LPS or PLGA NPs, expressed relative to the unstimulated condition (dashed line). B) Mean fluorescence intensity (MFI) levels for cell surface CD86 (top) and CD83 (bottom) markers. C) Concentration of TNF-α (top), IL-6 (middle), and IL-10 (bottom) released by DCs into culture supernatants, as detected by ELISA (n=3). Statistical significance was assessed by Friedman Test, followed by uncorrected Dunn's multiple comparison test.

8.2.1 Poly(lactic-co-glycolic acid) Nanoparticles are not able to reduce inflammatory stimulus

Next we wanted to ascertain if PLGA NPs, empty or loaded with the anti-inflammatory drug ibuprofen, were able to reduce DC pro-inflammatory stimulation. The NPs and ibuprofen were tested at a concentration of 100 μg/mL and 15 μg/mL, respectively. The results of the viability assay that are presented below (Fig. 8.2.1 (A)), show high variability in the concentration levels of LDH released by cells in the different conditions. This variability is due to increased LDH release by one particular donor. For the remaining donors, LDH release was similar to unstimulated control DCs. Furthermore, LDH release for DCs treated with a combination of the pro-inflammatory stimulus and ibuprofen (in free form and incorporated in NPs) was slightly lower than LDH release by unstimulated control DCs. It is then possible to conclude that the NPs and stimulatory conditions were not toxic to cells, at the tested concentrations, at least for two of the three donors. Next, the expression levels of cell surface CD83 and CD86 markers was evaluated through flow cytometry (Fig. 8.2.1 (B)), in order to determine whether the NPs and ibuprofen could affect DC phenotype, and also to evaluate if the addition of an anti-inflammatory drug (ibuprofen) could reduce the pro-inflammatory stimulus of TNF-α. Cells that received treatment with LPS expressed higher levels of the surface markers, indicating DC maturation. Cells treated with empty PLGA NPs, loaded PLGA NPs and ibuprofen were not activated, displaying surface marker expression levels similar to those of unstimulated control DCs, which were lower than LPS-treated DCs. However, for one of the donors, DCs treated with empty PLGA NPs displayed increased expression levels of CD83 and CD86. TNF-α

treated DCs displayed increased surface marker expression, comparing with unstimulated control DCs, but not as high as DCs that received LPs stimulus. DCs treated with ibuprofen and PLGA NPs (empty and loaded) with the additional pro-inflammatory stimulus showcased surface marker expression levels similar to those of TNF-α treated DCs, indicating that ibuprofen (in free form and incorporated in the NPs) was not able to reduce the pro-inflammatory stimulus. However, the addition of TNF-α appears to grant a reduced stimulus to cells, as their levels of surface marker expression did not differ much from those of control DCs.

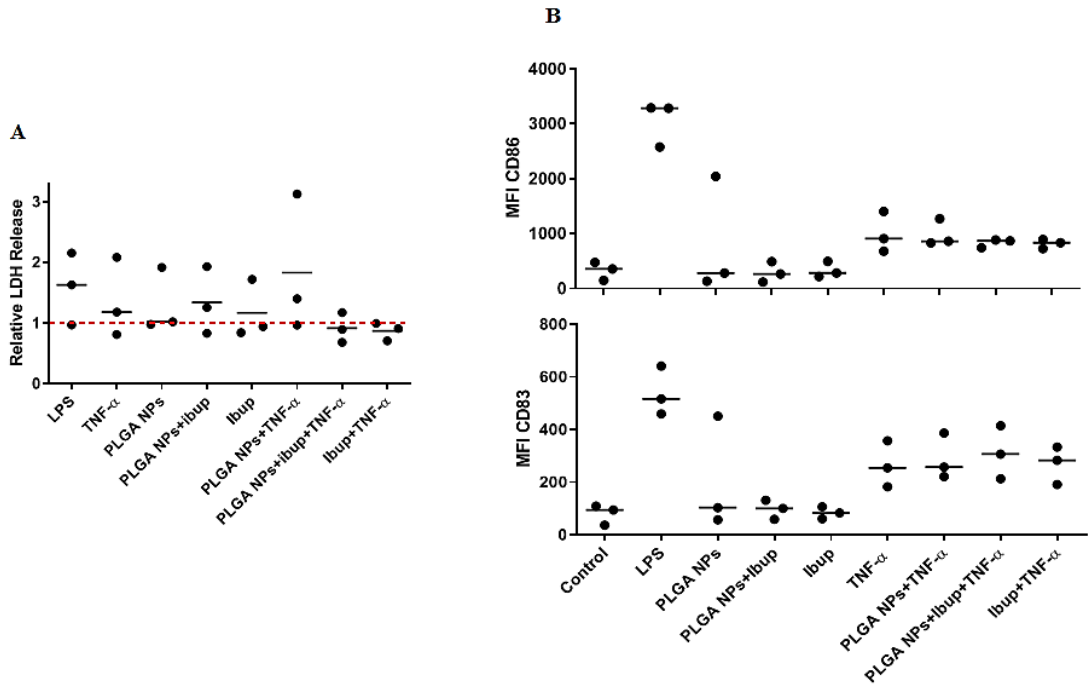


Figure 8.2.1 - Impact of Poly(lactic-co-glycolic acid) Nanoparticles (PLGA NPs) and ibuprofen on stimulated DC. DCs were differentiated for 4 days before being left unstimulated (negative control) or stimulated with LPS (positive control), TNF-α, PLGA NPs or ibuprofen, alone or with an additional TNF-α stimulus, for 72h. A) Concentration of LDH released by DCs into culture supernatants, as detected by LDH cytotoxicity assay. Data is expressed relative to the unstimulated condition (dashed line); B) Mean fluorescence intensity (MFI) levels for cell surface CD86 (top) and CD83 (bottom) markers, measured in DCs from three independent donors (n=3).

8.2.2 Increased concentrations of Poly(lactic-co-glycolic acid) Nanoparticles induce some toxicity in human primary Dendritic Cells

As PLGA NPs (empty and loaded) and ibuprofen did not appear to be toxic or activate DCs in the previously tested concentrations, a new concentration range was tested on DCs. NPs were tested at 100, 150 and 200 μg/mL, while free ibuprofen was tested at 20, 25 and 30 μg/mL. Viability assay results show some variability, and again increased LDH release levels for one particular donor (Fig. 8.2.2). Overall, levels of LDH are higher than for negative control DCs (dashed line), indicating that the tested concentrations of NPs and ibuprofen may induce some cell toxicity, but again similar to LPS stimulation and, for some conditions, even higher than that.

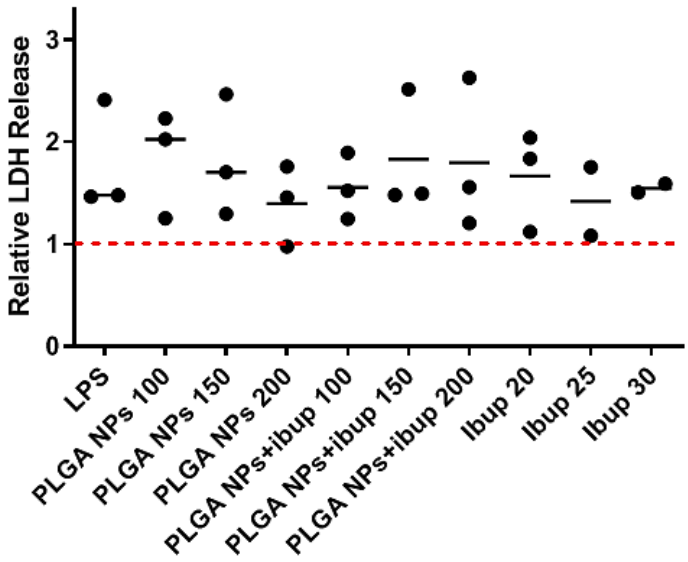


Figure 8.2.2 - DC response to increased concentrations of Poly(lactic-co-glycolic acid) Nanoparticles (PLGA NPs) and ibuprofen. DCs were differentiated for 4 days before being left either unstimulated (negative control), or stimulated with LPS(positive control), PLGA NPs or ibuprofen, for further 72 h. The presence of LDH in DC culture supernatant, after stimulation, is expressed relative to the unstimulated condition (dashed line) (n=3).

8.3 Establishing an inflammatory microenvironment for nanobiomaterial testing

As TNF-α stimulation conditions used in the first experiments, and based in previous work of our group, yield low DC activation levels we next decided to establish a culture model for biomaterial testing that could resemble the inflammatory microenvironment present in osteoarthritic joints. For this purpose, DCs were treated with pro-inflammatory cytokines at varying concentrations: TNF-α (100 ng/mL), IL-1β (10, 50 and 100 ng/mL) and a combination of these cytokines (10, 50 and 100 ng/mL). Results from the viability assay showed that LDH release by cells was very close to those of unstimulated control DCs, with the exception of one donor, where treatment with IL-1β and TNF-α (50 ng/mL) resulted in higher LDH release, and another donor, where treatment with IL-1β at 50 and 100 ng/mL also led to increased LDH release, indicating some toxicity (Fig. 8.3.1 (A)). Apart from these two cases, the remaining stimuli appear to be non-toxic to DCs. Harvested cells were evaluated for their expression levels of cell surface CD83 and CD86 markers, through flow cytometry (Fig. 8.3.1 (B)). Cells that received LPS treatment expressed higher levels of CD83, as expected. However, CD86 expression levels were similar to expression levels of unstimulated control cells. This is an unusual result, as it does not indicate DC maturation, which is induced by LPS stimulation. In fact, expression levels of CD86 for all conditions were not far from those obtained for control cells, although cells stimulated with IL-1β displayed increased expression levels, comparing with the remaining conditions. This could be attributed to malfunction of the antibody, or lack of biological activity from the selected cytokines. Regarding CD83 expression levels, these were highest for LPS-treated DCs, indicating DC maturation, as expected. Expression levels of CD83 for DCs that received TNF-α stimulation were between those of unstimulated control cells and LPS-treated DCs. Expression levels for DCs treated with IL-1β, for concentrations of 10 and 50 ng/mL, were low and closer to those of negative control DCs, while DCs treated with

both cytokines, for concentrations of 50 and 100 ng/mL, were closer to those of TNF-α treated DCs. Expression levels for DCs treated with IL-1β at 100 ng/mL and cytokine combination at 10 ng/mL were close, and between the previously mentioned conditions. Treatment with these cytokines at the chosen concentration range did not, however, lead to DC activation to the same level as LPS stimulation.

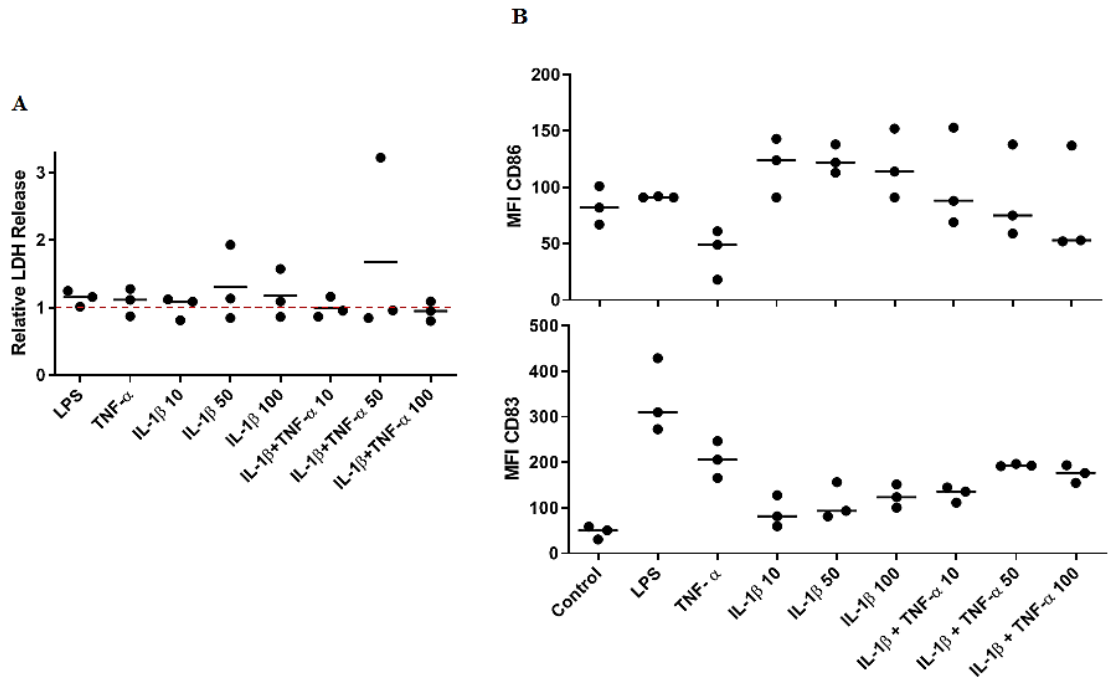


Figure 8.3 - DC response to pro-inflammatory stimuli. DCs were differentiated for 4 days before being left either unstimulated (negative control), or stimulated with LPS (positive control), TNF-α, IL-1β, or a combination of these cytokines, for further 72 h. A) Concentration of LDH released by DCs into culture supernatants, as detected by LDH cytotoxicity assay. Data is expressed relative to the unstimulated condition (dashed line); B) Mean fluorescence intensity (MFI) levels for cell surface CD86 (top) and CD83 (bottom) markers, measured in DCs from three independent donors (n=3).

9. Discussion

CNTs have been increasingly explored due to appealing qualities such as their reduced size and weight, coupled with their resistance and conductivity [180]. Although this material has begun to be incorporated in strategies that view repair and regeneration of cartilaginous tissue [185-187], its interactions with the human organism and its potential side effects are not yet known [182].

We tested MWCNTs (referred to as CNTs), functionalized with -COOH, with a 35 nm diameter, measuring a few microns in length. Our results showed that stimulation with 0,45 μg/mL of CNTs for a 72 h period was not toxic to primary human DCs, while some toxicity was verified for the combined stimulus of CNT and TNF-α (100 ng/mL), comparing with unstimulated control cells. Inoue *et al.* performed *in vitro* testing of MWCNTs on BMDCs, with concentrations ranging from 0,1 to 1 μg/mL, but did not report on MWCNTs cytotoxicity towards these cells [200]. Palomäki *et al.* stimulated BMDCs with SWCNTs and MWCNTs, at 3, 10 and 30 μg/mL, for 24 h, and reported that both nanomaterials were cytotoxic towards BMDCs, in a dose-dependent manner [201]. Laverny *et al.* found that primary human PBMC derived DCs remained viable upon stimulation with MWCNTs at 15, 30, 60 or 120 μg/mL. However, a lower number of cells were able to differentiate into moDCs, when these cells were stimulated with MWCNTs concentrations greater than 30 μg/mL [202]. Cytotoxicity assay results from the aforementioned studies support that a stimulus of 0,45 μg/mL of CNTs is safe, for DCs derived from humans and mice.

Flow cytometry analysis showed that our CNTs did not activate DCs, which retained an immature phenotype, as they expressed levels of CD83 and CD86 surface markers close to those of unstimulated control cells. Surface marker expression levels for DCs treated with CNTs and TNF-α were very similar to those of DCs treated with CNTs, which could indicate that CNTs were able to reduce the inflammatory stimulus of TNF-α. However, DCs treated with TNF-α expressed similar levels of surface markers to cells treated with CNTs and TNF-α, which may indicate that the chosen concentration of 100 ng/mL was too low, or that the cytokine itself presented decreased activity. Inoue *et al.* found that stimulation of BMDCs with MWCNTs at 0,1 and 1 μg,/mL increased the percentage of $CD86^+$ cells and $MHC\text{-}II^+$ cells. Furthermore, stimulation with 0,1 μg/mL of MWCNTs increased the percentage of double positive $MHC\text{-}II^+CD86^+$ cells [200]. Conversely, Palomäki *et al.* reported that BMDCs exposed to 3, 10 and 30 μg/mL of SWCNTs and MWCNTs did not present noticeable changes in expression of cell surface markers CD40 and CD86, comparing with LPS-treated BMDCs, which stimulated DC co-stimulatory activity [201]. Tkach *et al.* stimulated BMDCs with 6,25 and 25 μg/mL of SWCNTs, for 48 h, and found that expression levels of CD40, CD80, CD86 and MHC-II were

close to those of unstimulated cells. Furthermore, cells treated with SWCNTs and LPS displayed expression levels similar to those of LPS-treated DCs, indicating that SWCNTs did not interfere with LPS-induced maturation [203]. Laverny *et al.* reported that the addition of MWCNTs to moDCs at 15 μg/mL decreased expression levels of CD1a, CD209 and MHC-II, comparing with control moDCs, indicating that exposure to the material could have influenced cell phenotype. In addition, moDCs that were differentiated in the presence or absence of MWCNTs were stimulated with LPS, forming MWCNT mDCs and control mDCs (positive control), respectively. Comparing with control mDCs, MWCNT mDCs expressed lower levels of CD83, CD86 and MHC-II, indicating that the presence of the material affected cell maturation and may have reduced cell activation [202]. In a 2016 *in vivo* study, 50, 100 and 200 μg/kg of SWCNTs were administered to mice, in a single intratracheal instillation. The effects of instillation were observed throughout a 13 week period. Flow cytometry analysis showed that SWCNTs increased expression of CD80, CD86 and MHC-II on APCs in the lungs of mice, including DCs, in a dose dependent manner. However, as time progressed, the accumulation of SWCNTs in the lungs hindered DC maturation and activation [204]. Recently, Beyeler *et al.* also studied intrapulmonary effects caused by MWCNTs inhalation, using a mouse model of chronic obstructive pulmonary disease (COPD) [205]. Mice were administered a total of 40 μg of MWCNTs, through intratracheal instillation, representing an approximate lung deposition of 0,08 μg/cm^2. Instillation of MWCNTs resulted in increased frequency of pulmonary DCs, and lead to expansion of the CD11b$^+$CD103$^-$ population [205]. CD103$^-$ DCs are able to secrete high amounts of pro-inflammatory cytokines, while CD103$^+$ DCs are efficient in "cross-presenting antigens to T cells" [205]. Results suggest that inhalation of MWCNTs altered the frequency, activation and phenotype of pulmonary DCs and other pulmonary APCs, and were able to affect the immune system of the pulmonary mucosa [205]. Overall, studies show that, when inhaled, SWCNTs and MWCNTs are more likely to activate DC and other inflammatory immune cells in the lungs. Accumulation of SWCNTs in lung tissue over a longer period of time was also shown to inhibit DC maturation. However, the remaining studies showed that treatment of BMDCs and human moDCs with SWCNTs and/or MWCNTs, in varying concentration, did not activate these cells, as was the case for our results, as they did not increase the expression of maturation/activation surface marker molecules.

Lastly, stimulation of human primary DCs with 0,45 μg/mL of CNTs led to the secretion of low amounts of TNF-α, IL-6 and IL-10 into culture supernatant, similar to unstimulated control cells. Palomäki *et al.* reported that BMDCs treated with 3, 10, and 30 μg/mL of MWCNTs, comparing with unstimulated cells, did not experience significant increase of chemokine and cytokine mRNA expression, among them IL-10, IL-12p40 and IL-23p19 [201]. IL-12p40 is produced when inflammatory immune cells, like DCs and macrophages, are activated [206]. IL-23p19 has been implicated in inflammatory diseases such as RA, and has been recognized as a pro-inflammatory endothelial peptide [207]. In line with these results, Laverny *et al.* found that human moDCs stimulated with LPS and 15 μg/mL of MWCNTs presented lower secretion of IL-10, IL-12p40 and IL-23p19 into culture supernatant, comparing with LPS-treated moDCs [202]. These studies support that expression/release of anti and pro-inflammatory cytokines and/or chemokines is not stimulated by exposure of DCs to MWCNTs.

PLGA is widely used for tissue engineering strategies and drug delivery, due to its several desirable properties, namely biocompatibility, biodegradability and non-toxicity, as well as its ability to perform sustained release of molecules [188, 189]. Several PLGA biomaterials

intended to treat cartilage defects and lesions have been developed and studied, and some have been shown to improve regeneration of cartilage [140, 197].

We tested PLGA NPs (empty and loaded with ibuprofen) and ibuprofen, at 100 μg/mL and 15 μg/mL, respectively, in the presence and absence of pro-inflammatory stimulus (TNF-α at 100 ng/mL). Our results showed that stimulation of human primary DCs with 100 μg/mL of PLGA NPs, resulted in LDH release levels close to those of unstimulated control cells, indicating that the NPs were not toxic to cells. Treatment with loaded PLGA NPs at 100 μg/mL and ibuprofen at 15 μg/mL lead to variable LDH release by DCs from one donor, but the remaining two experienced release levels similar to those of unstimulated control cells, indicating that, for these two donors, the chosen concentrations were not toxic to cells. For cells treated with empty PLGA NPs and TNF-α, LDH release was slightly increased, comparing to levels of unstimulated control cells, indicating that this combination induced some toxicity in DCs. For cells treated with loaded PLGA NPs and ibuprofen, with the additional TNF-α stimulus, levels of LDH release were actually slightly inferior to release levels of unstimulated control cells, indicating that these combinations were not toxic to DCs. As results showed that the aforementioned concentrations of PLGA NPs and ibuprofen were not toxic to DCs, a new concentration range was selected. PLGA NPs were tested at 100, 150 and 200 μg/mL, while ibuprofen was tested at 20, 25, and 30 μg/mL. Cell viability assay showed variability, with cells from one donor one in particular showing increased LDH release levels, comparing with unstimulated control cells. Overall, levels of LDH released were higher than release levels of unstimulated control cells, indicating that the tested concentrations induced some toxicity, whereas previously tested concentrations were shown to be safe for DCs. In a study by Barillet *et al*., BMDCs and human moDCs were exposed to PLGA NPs for 24 h, at concentrations ranging from 1 to 500 μg/mL. Results showed 5 to 10% cell death, relative to control unstimulated DCs, for both types of DCs after exposure to the nanobiomaterial, indicating that cytotoxic effects of these NPs on cells was low, even for a concentration as high as 500 μg/mL [208]. In a study by Zhu *et al.*, BMDCs were stimulated with PLGA NPs, and samples were collected after 24, 48 and 72h, to be analyzed in terms of cytotoxicity. For PLGA NPs concentrations up to 800 μg/mL, cell viability was superior to 75%, comparing to unstimulated control cells [209]. Yasar *et al.* prepared core–shell structured lipid–polymer hybrid nanoparticles (LPNs) with PLGA, and PLGA NPs coated with chitosan (CS-PLGA NPs), to study "time-dependent internalization behavior of mRNA" [210] in DCs. These particles were prepared in concentrations of 10, 20, 40, 60, 100 and 160 μg/mL and incubated with $DC_{2.4}$ cells, from a murine BM-derived DC line. Viability and cytotoxicity assay showed that empty LPNs were not toxic to cells, except for the concentration of 160 μg/mL. Empty CS-PLGA NPs did not display toxic effects throughout the entire concentration range [210]. Conversely to these studies, our results showed that PLGA NPs induced some toxicity for concentrations higher than 100 μg/mL. This difference could be due to duration of exposure to the PLGA NPs, as our cells were exposed to stimuli for 72 h, while BMDCs and human moDCs in the study by Barillet *et al.* were only exposed for 24 h. However, Zhu *et al.* showed that BMDCs' viability was quite high after a 72 h treatment with PLGA NPs in concentrations as high as 800 μg/mL.

Flow cytometry analysis results showed that cells treated with PLGA NPs (empty and loaded) at 100 μg/mL and ibuprofen and 15 μg/mL did not activate DCs, since expression levels of CD83 and CD86 surface markers were close to those of unstimulated control cells. Furthermore, expression levels of surface markers for DCs treated with PLGA NPs (empty and loaded) and ibuprofen, in the presence of TNF-α, were similar to those of DCs treated with

TNF-α, showing that NPs were not able to reduce the inflammatory stimulus, even with the incorporation of ibuprofen. Barillet *et al.* exposed BMDCs and human moDCs to LPS (positive control), distilled water (negative control), or 200 μg/mL of PLGA NPs, for 24 h. Phenotypic analysis showed that PLGA NPs caused a subtle, yet significant increase in expression levels of CD83, CD86, CD40 and CD54 molecules, in moDCs. Results were similar for BMDCs, with a significant increase in expression of CD40, CD86 and CCR7 molecules. Exposure to PLGA NPs was, therefore, able to activate DCs, with more noticeable effects for human moDCs than BMDCs [208]. Kim *et al*. used PLGA to encapsulate model antigen OVA and Dex, and deliver them to phagocytic cells [211]. BMDCs were cultured for 48 h with NPs containing OVA and Dex (NP[OVA+Dex]), OVA (NP[OVA]), IFN-γ+TNF-α (mDCs), or left untreated (iDCs). The NPs had an average OVA content of 10 μg/mL. Results showed that DC stimulation with NP[OVA+Dex] decreased expression of CD80, CD86 and MHC-II molecules, comparing with mDCs and NP[OVA]-treated DCs. NP[OVA+Dex] DCs showed decreased expression of the co-inhibitory molecule PD-L1, but had the highest percentage of $CD86^{lo}PD\text{-}L1^{hi}$ cell population, out of all conditions [211]. In our results, stimulation of human primary DCs with PLGA NPs (empty and loaded) at 100 μg/mL did not activate cells, contrarily to what was observed in the study by Barillet *et al.*, for a concentration of 200 μg/mL [208]. We tested PLGA NPs at the same concentration, but did not conduct flow cytometry analysis to assess phenotypic changes. Kim *et al*. showed that particles containing Dex, which is often used to address inflammation, managed to decrease expression of activation markers, comparing with other conditions [211]. In our results, incorporation of ibuprofen in PLGA NPs was not able to reduce activation induced by the stimulation with TNF-α.

Our final results showed that treatment of DCs with 100 μg/mL of PLGA NPs led to negligible release of TNF-α, IL-6 and IL-10 into culture supernatant. Barillet *et al*. analyzed cytokine content in moDCs and BMDCs culture supernatants, after 24 h exposure to 200 μg/mL of PLGA NPs. Results showed that moDCs exposure to PLGA NPs induced secretion of pro-inflammatory cytokines (TNF-α, Il-1β, IL-6 and IL-8), while the secretory activity of BMDCs was not affected, indicating that PLGA NPs only enhanced cytokine secretion in moDCs [208]. Kim *et al.* reported that treatment of BMDCs with NP[OVA] increased secretion of TNF-α, IL-1β, Il-6 and IL-12, comparing with mDCs. Treatment with NP[OVA+Dex] suppressed secretion of the aforementioned cytokines, but significantly increased IL-10 secretion, comparing with mDCs and NP[OVA] DCs [211]. Conversely to our results, Barillet *et al.* reported that PLGA NPs enhanced secretion of pro-inflammatory cytokines [208], which could be attributed to the increased concentration of NPs. Kim *et al.* showed that PLGA NPs with Dex decreased secretion of pro-inflammatory cytokines, while it increased secretion of IL-10 [211]. In our work, we did not assess the potential effect of ibuprofen encapsulation on cytokine secretion by primary human DCs.

Lastly, we conducted testing pro-inflammatory cytokines TNF-α and IL-1β, in order to establish a culture model for biomaterial testing that could resemble the inflammatory microenvironment present in OA joints, as TNF-α stimulation alone yielded low DC activation levels. As previously mentioned, TNF-α and IL-1β are key contributors to inflammation during OA [4, 50]. For this experiment, TNF-α was tested in the usual concentration of 100 ng/mL, IL-1β was tested at 10, 50 and 100 ng/mL, and a combination of these cytokines was tested at 10, 50 and 100 ng/mL. Overall, cell viability assay results showed that the chosen concentrations were not toxic to DCs, which had LDH release levels similar to those of unstimulated control cells. Results from flow cytometry analysis where anomalous for CD86 surface marker, as LPS

and TNF-α stimuli presented CD86 expression levels similar to unstimulated control cells, which should not occur, as these stimuli induce cell maturation, and negative control DCs retain an immature phenotype, due to lack of stimulation. Expression levels of CD86 for the remaining conditions were not far from those obtained for unstimulated control cells either. These results could be attributed to a potential malfunction of the antibody or lack of biological activity from the selected cytokines. Expression levels of CD83 were highest for LPS-treated DCs, indicating DC maturation, as expected. Expression levels for DCs treated TNF-α were between expression levels of control DCs and LPS-treated DCs. CD83 expression levels for the remaining stimuli were close to those of TNF-α-treated DCs, with the exception of DCs treated with IL-1β at 10 and 50 ng/mL, which were closer to those of unstimulated control DCs. Overall, treatment with these cytokines at the chosen concentration range was not sufficient to induce cell maturation, similar to what is observed after DC treatment with LPS. Higher concentrations could be tested; however, these may induce cell toxicity. Testing cytokines with increased biological activity could potentially provide better results.

For future work, the current culture model for biomaterial testing resembling the inflammatory microenvironment would need to be improved, either by testing different concentrations of cytokines, or testing cytokines with increased biological activity. After optimization, experiments with CNTs, PLGA NPs (empty and loaded) and ibuprofen would need to be repeated in the optimized testing conditions, in order to truly assess the effect of these particles and molecule on DCs, in the presence of inflammatory stimuli. A safe concentration for testing of chitosan/polyglycolic acid (Ch/PGA) NPs, both plain and crosslinked with genipin (g(Ch/PGA)), also has to be determined, in order to perform experiments under inflammatory conditions, as previously done for the other nanobiomaterials, and assess their effects on DC phenotype, and whether they can reduce inflammatory stimuli, and possibly be used as a nanobiomaterial to promote cartilage repair.

Overall, the research and experimental work developed during this dissertation allowed for a better understanding of DC behavior upon exposure to nanobiomaterials, and their potential contributions towards OA pathogenesis. Furthermore, this work reinforces that nanobiomaterials parameters such as chemical composition, size and morphology can be explored in order to induce favorable immune responses from DCs, and potentially develop novel biomaterials and therapies for cartilage repair in OA.

10. References

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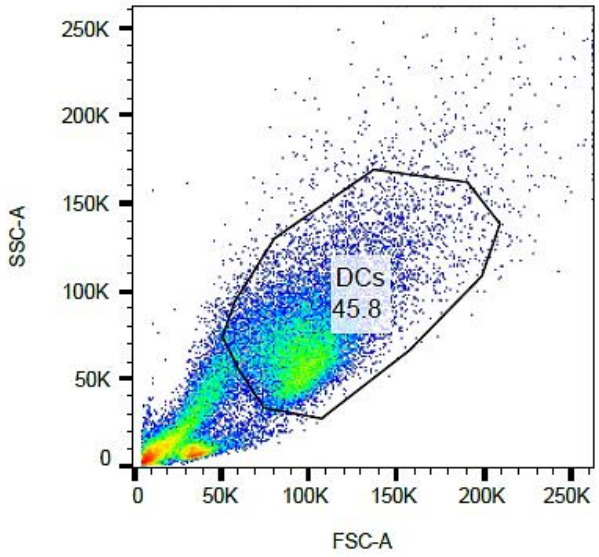
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11. Supplementary Figures



Supplementary Figure 1 – Dendritic Cell gating strategy in FACS.