

General introduction, thesis aim and outline

JOINT INJURY AND OSTEOARTHRITIS

Joints are important structures in the body, as they are anatomical locations for the union between two or more bones. The cartilage tissue that lines the long end of the bones provide sliding areas and consists of chondrocytes embedded within an extracellular matrix that contains distinct macromolecules such as proteoglycans and collagens. In contrast to various other tissues, cartilage has a very limited regeneration capacity due to the lack of vascularization of the tissue¹. Synovial joints are enclosed by a synovial membrane, also known as the synovium, which produces synovial fluid that acts as lubricant to facilitate bone movement. Inflammation of the synovium is a major feature of osteoarthritis (OA) and its progression²⁻⁴. The synovium is comprised of connective tissue in which synoviocytes/fibroblasts and low percentages of T-cells, B-cell, and mast cells reside. Another essential cell type in the synovium, is the macrophage^{5,6}. In the knee joint, the menisci and crucial ligaments are also important structures in addition to the cartilage. Studies have shown that patients who have sustained injury to the anterior cruciate ligament or menisci have an increased risk of developing OA^{7,8}. OA is a degenerative joint disease that affects a large proportion of the population. Classically, it has been considered as a 'wear-and-tear' disorder resulting in the erosion of articular cartilage and subsequently exposure of the bone, though it is now known that many other processes such as inflammation are hallmarks of OA (Figure 1.1). Worldwide, 4.8% of women and 2.8% of men suffer from OA⁹ and in the Netherlands alone, the prevalence of knee OA in people over 55 years of age is 30.5% in women and 15.6% in men¹⁰. Radiological OA appears even in 80% of the people over 70 years old¹¹. As a result, the global economic burden exceeds 975 million euro per year for surgical costs and pain suppressing drugs and indirect costs are estimated to be over 3 billion euro¹². In the last twenty-five years, it has become evident that OA should be identified as a degenerative disease that affects the whole joint and its surrounding tissues and also includes an inflammatory component¹³. In many cases, the disease leads to extensive pain and loss of function^{14,15}. Due to ageing of the population, it is expected that in the upcoming 20 years the number of OA patients will have increased with 52%¹⁶.

Current clinical treatments for OA are primarily aimed to reduce and control symptoms. Physical therapy is a conservative treatment to strengthen the muscles around the joint; however, success is not always achieved and it is not considered a long-term solution. Pain can be temporarily treated with pharmaceuticals such as paracetamol or non-steroidal anti-inflammatory drugs (NSAIDs). Intra-articular injections with corticosteroids can also relieve pain by reducing synovitis, though an important unwanted side-effect is chondrotoxicity¹⁷. Disease-modifying osteoarthritis drugs (DMOADs) are a group of mainly experimental, biological agents that are used with the intention to modify the course of the disease. Such agents include, but

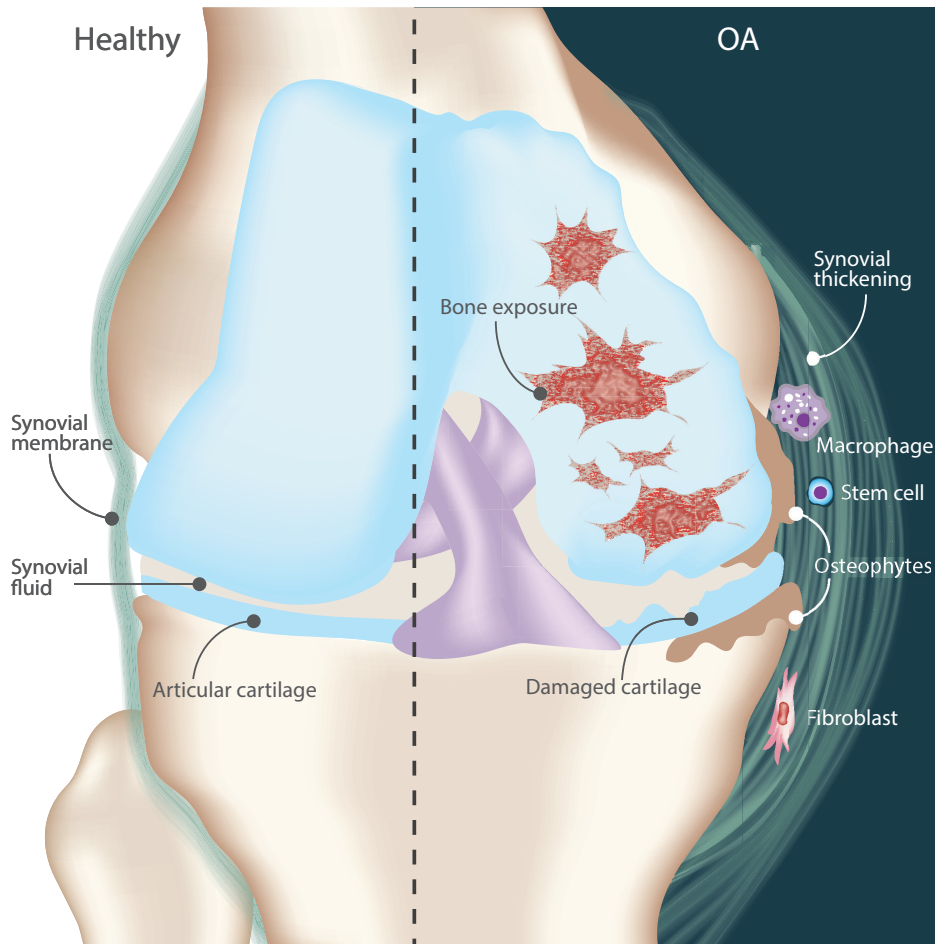


Figure 1.1: The contrast between the healthy and osteoarthritic knee joint.

are not limited to: matrix-metalloproteinases (MMPs) inhibitors, cytokine blockers, bisphosphonates, calcitonin, inducible nitric oxide synthase (iNOS) inhibitors^{18,19}, and more recently, Wnt pathway inhibitors²⁰. Another approach is not to inhibit pro-inflammatory factors, but enhancing anti-inflammatory factors. A promising study that touches upon this aspect is a clinical trial where patients received intra-articular administration of recombinant interleukin (IL)-1RA after sustaining severe knee injury²¹. The IL-1RA therapy resulted in reduced knee pain and improved knee function, though the long-term effect on OA development was not assessed. This was further investigated in a small animal study²² using the closed articular fracture model for OA in C57BL/6 mice. Intra-articular injection of IL-1RA resulted in less cartilage degeneration as evaluated by the histological Mankin score. Injection of IL-1RA also resulted in less synovitis than when the joints were injected with saline or a tumor necrosis factor alpha

(TNF α) inhibitor. Another study showed that IL-1RA knockout mice had worsened progression after induction of collagen-induced arthritis (CIA)²³. If conservative treatments fail to alleviate pain, total replacement of the joint could be a last resort. Young patients who develop post-traumatic OA, for instance due to sports injuries, are less likely to be eligible for joint replacement surgeries. Although a prosthetic joint is an excellent solution to relieve pain and solve instability problems, prosthetics have a relatively short life span. On average, knee and hip prosthetics last approximately 15 to 20 years before the need of replacement. Therefore, it is undesirable to subject these young patients to a total joint replacement surgery.

MONOCYTES AND MACROPHAGES

Macrophages in immunity and disease

Macrophages are immune cells that are virtually present in all tissues of the body. They can derive from blood monocytes and may polarize into activated macrophages when receiving cues from their microenvironment²⁴, a concept that will be discussed in the next paragraph. Depending on the polarization state, macrophages express a repertoire of surface markers and produce a profile of cytokines that in turn contribute to the degree of inflammation of the tissue. Together with dendritic cells, macrophages aid to the innate immune response by producing cytokines that promote clearance of pathogens. By producing chemokines, macrophages contribute in some cases to the regulation of extravasation of additional leukocytes from the blood that augment the repression of the infection while an adaptive immune response is developing²⁴. Unfortunately, the homeostatic function of macrophages can become compromised leading to their implication in many diseases²⁵. For instance, Kupffer cells, which are the macrophages found on the luminal surface of hepatic sinusoids, and pulmonary macrophages are both macrophage types that are associated with tissue fibrosis. In the vasculature, circulating monocytes that are recruited to a lesion site contribute to the formation of atherosclerotic plaques. Macrophages are also implicated in obesity²⁶ and inflammatory diseases such as inflammatory bowel disease, asthma, and rheumatoid arthritis²⁷⁻³³. The involvement of macrophages in numerous disorders demonstrates that it is an interesting cell type that could be deployed as a therapeutic option for the treatment of inflammatory degenerative joint diseases.

Macrophage polarization: fifty shades of grey?

Macrophage polarization is the concept in which monocytes are activated into macrophages and obtain a certain phenotype and function³⁴. The M1/M2 nomenclature, proposed in 2000 by Mills *et al.*³⁵, describes the phenotype of macrophages that can be

generated *in vitro*. This nomenclature was mainly based on the Th1 and Th2 classification of T-lymphocytes that was proposed earlier by Mosmann *et al.* in 1986³⁶. The M1/M2 classification³⁷⁻⁴¹ represents the very extremes of the macrophage phenotype spectrum: pro-inflammatory M1 or classically activated macrophages develop *in vitro* when monocytes are stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon- γ (IFN γ), lipopolysaccharide (LPS), TNF α , or a combination of these factors. These pro-inflammatory macrophages have an increased microbicidal activity and express and secrete considerable amounts of pro-inflammatory factors, such as TNF α , IL-1 β , IL-6, and chemokine (C-X-C motif) ligand (CXCL)10⁴². Anti-inflammatory M2 or alternatively activated macrophages can be further divided into the subtypes M2a, M2b and M2c. M2a-like macrophages can develop when monocytes are exposed to IL-4 and/or IL-13. These macrophages express and secrete for instance IL-10, IL-1RA, cluster of differentiation (CD)206, and C-C motif ligand (CCL)18⁴³ and have an additional role in wound healing, tissue repair, and tissue remodeling^{40,44}. The M2b-like subtype develops when exposed to immune complexes and LPS and have an immune regulatory function. This group is challenging to characterize because they produce both pro-inflammatory and anti-inflammatory cytokines, for instance low levels of IL-12 and high levels of IL-10^{39,40}. The M2c-like subtype develops when monocytes are stimulated with IL-10 and glucocorticoids, and these macrophages express and secrete for instance IL-10, CD163^{42,45,46}, and Transforming growth factor beta (TGF- β). They are characterized by their ability to down regulate the production of pro-inflammatory cytokines^{37,46} (Figure 1.2). The extremes of the macrophage spectrum of phenotypes can be acquired *in vitro*. Since the behavior of polarized macrophages can vary depending on the polarization method, Murray *et al.*⁴⁷ advocated to specifically describe the activation state of macrophages by their stimuli. For example, pro-inflammatory M1 macrophages that have been polarized *in vitro* by IFN γ and TNF α should be described as M(IFN γ +TNF α), a terminology that will be applied throughout this thesis. Defining macrophage phenotypes remains a challenging task as the plasticity of the cells cause a spectrum of phenotypes that is currently only partially explored. The plasticity of macrophages can also be seen as an opportunity. Modulating, switching or reprogramming of the phenotype of a macrophage population in either direction to influence inflammation, is nowadays of high interest in multiple research areas, also in the field of OA research.

Macrophages in osteoarthritis

Besides their importance during innate and adaptive immunity, macrophages are pivotal in joint homeostasis and inflammation⁴. Early indications that they can affect joint tissue were seen in *in vitro* studies where it was reported that bone-marrow derived macrophages⁴⁸ and peritoneal macrophages⁴⁹ can secrete enzymes that

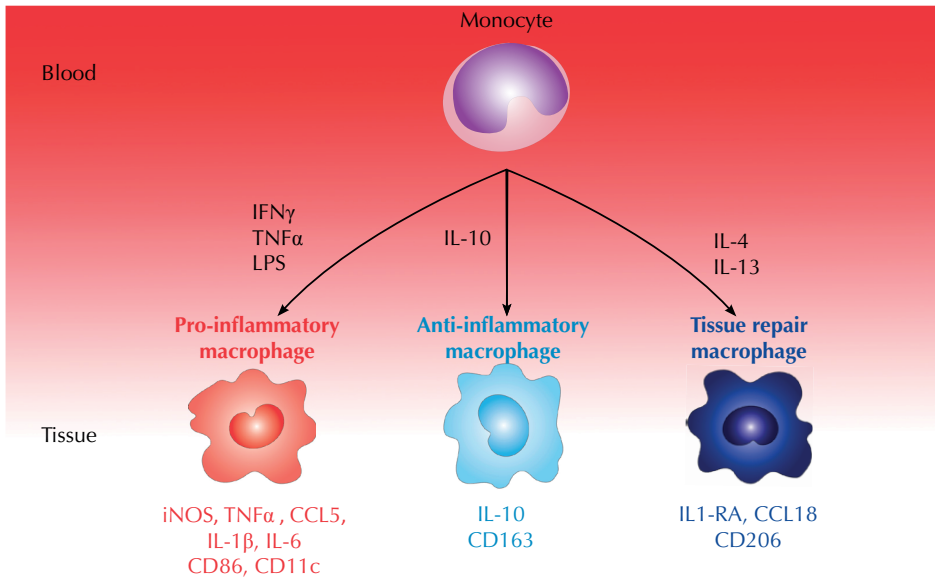


Figure 1.2: Spectrum of macrophage phenotypes. A spectrum of macrophage phenotypes can develop after monocytes receive cues from their environment.

exacerbate degeneration of cartilage tissue. In respect to OA pathophysiology, increased percentage of macrophages in the synovium are observed⁶ and synovial macrophages were shown to be pivotal in the development of the disease⁵⁰. This was demonstrated by *in vivo* studies where depletion of synovial macrophages diminished the formation of osteophyte⁵¹ and reduced cartilage destruction⁵² in OA mouse models. Recently, the association between macrophages and obesity-related osteoarthritis has also been reported^{53,54}. It was shown that induced obesity in rats resulted in infiltration of pro-inflammatory macrophages in the synovium and contributed to OA processes⁵⁰. Another study reported that temporary systemic depletion of macrophages, did not protect obese mice from actually developing OA. Additionally, the depletion caused enhanced inflammation⁵⁴. In respect to joint tissue regeneration, it was shown that chondrogenesis is negatively affected when chondrogenically-stimulated mesenchymal stem cell (MSCs) pellets were cultured with the conditioned medium of human OA synovium⁵, infrapatellar fat pad⁵⁵, and macrophages isolated from both tissues. These reports combined demonstrate that macrophages are important players during joint tissue degeneration and OA onset and progression. Therefore, in-depth research regarding the involvement of macrophages in OA has become of increasing interest in the last decade, mainly to understand the underlying pathophysiological mechanisms that could open up therapeutic possibilities.

BIOMATERIALS IN REGENERATIVE MEDICINE

Biomaterials are materials of synthetic or natural origin that are used in contact with a biological environment⁵⁶. These materials are frequently applied in the field of regenerative medicine to either support or replace damaged tissue in the body. For example, scaffolds or prosthetics used for joint reconstruction approaches or meshes that are applied during surgical procedures. Sutures are indispensable in modern medicine and though one may find it less obvious, they can be considered as biomaterials. An important aspect that all biomaterials have in common is the fact that upon implantation, the immune system of the host will interact with the material and will induce an immune reaction involving macrophages. Following the initiation by blood-material contact, an acute inflammatory phase will begin during which monocytes and neutrophils are recruited and extravasate into the tissue. After the monocytes encounter the surface of the biomaterial, they will become activated and polarize into macrophages. Depending on their phenotype they may contribute to chronic inflammation, granulated tissue development, and a foreign body reaction will occur that is often followed by fibrosis (Figure 1.3)⁵⁷. The cascade of the so-called foreign-body-reaction of the host upon biomaterial implantation, may determine the long-term integration of the material with the tissue. When this cascade is compromised, adverse effects may arise such as material-induced inflammation, excessive fibrosis, excessive coagulation, and infection⁵⁸. Influencing macrophage polarization during this stage can be an approach to improve biomaterial integration for regenerative purposes.

AIM AND THESIS OUTLINE

Macrophages are plastic cells and depending on their phenotype can contribute to the inflammatory state of tissues. The main objective of this thesis is *to explore the involvement of macrophages, in particular the role of their phenotypes, during processes of joint degeneration*. The answers of the following questions contribute to the main objective:

- How are macrophages of different phenotypes involved in inflammatory tissue degeneration and degenerative joint disease?
- How can modulation of macrophage phenotypes be applied to control either inflammation or their response to biomaterials?

Synovial inflammation, and specifically increased number of macrophages, are observed during OA². As macrophages can derive from monocytes, understanding mechanisms that are responsible for monocyte migration into tissue may be an approach

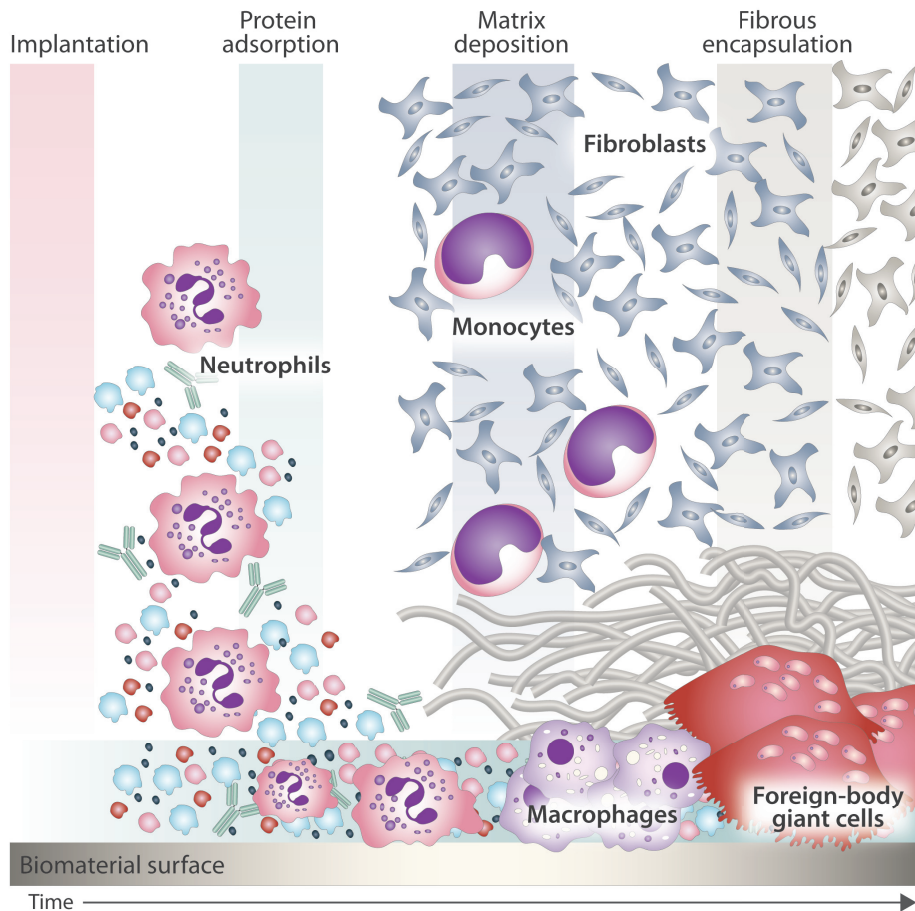


Figure 1.3: Macrophage activation by a biomaterial. Monocytes are recruited to the implantation site and become activated macrophages.

to develop new strategies to dampen tissue inflammation. Chemokines at the local site of inflammation facilitate and can regulate monocyte migration into the tissue. Therefore, we first investigate in chapter 2 the expressions of chemokines that are related to monocyte migration in synovial tissues of OA patients. We also assess associations between the chemokine expression profiles and the macrophage phenotypes present in the synovium and correlate these with the degenerative state of the articular cartilage. This knowledge may contribute to the development of management strategies focusing on monocyte/macrophage recruitment to control the progression of OA.

Macrophages of different phenotypes each have a signature comprised of a protein secretion profile, gene expression profile, and function. The effects of the secreted products of different phenotypes of macrophages on cartilage is unclear. Hence, we

evaluated in chapter 3 the *in vitro* effects of three phenotypes on human articular cartilage explants by using conditioned medium of polarized macrophages.

In order to be able to know *how* to manage the phenotypes of macrophages, it should be determined *which* phenotypes are in fact involved and present during a certain stage of the disease. Therefore, in chapter 4 we determine profiles of the presence of three different macrophage phenotypes during the course of OA in two mouse models. Furthermore, associations between the presence of these macrophage phenotypes and different features of OA are assessed. Determining the macrophage phenotype profiles over time and investigating how different macrophage phenotypes affect joint tissues, provides an indication on the optimal time to intervene and which phenotypes to target.

Managing the inflammatory state of a tissue as a therapeutic approach can be accomplished by specific modulation of a macrophage population. The possibility to modulate the macrophage phenotype in synovial tissue *in situ* and how this concept may guide synovial inflammation, is explored in chapter 5. For this study, we evaluate the modulatory capacity of common used medications on synovial tissue and primary polarized macrophages.

As biomaterials are frequently used in reconstructive joint surgeries with the goal to prevent OA progression and are also used as in other fields of regenerative medicine, we explore in chapter 6 the polarization behavior of monocytes into macrophages in an *in vitro* culture model. Since obesity is a major risk factor for OA development, the association between specific subsets of monocytes and their inflammatory response is also assessed. The behavior of macrophages after activation by a biomaterial is determined by the type of material. In chapter 7, we evaluate *in vitro* the ability to modulate the acquired phenotype of macrophages with pharmaceuticals after they have been activated by biomaterials. The materials represent classes of frequently used materials in regenerative medicine.

Finally, chapter 8 provides a summary and general overview based on the presented work in this thesis, and aims to combine the knowledge of the studies to discuss the contribution of macrophage phenotypes during degenerative joint disease. It also provides potential directions on the use of monocyte and macrophage phenotype modulation as a tool to inhibit tissue degeneration.