General discussion

Osteoarthritis (OA) affects a large proportion of the population worldwide and its prevalence is expected to increase due to aging and obesity[224]. For several years now, the disease is considered as an inflammatory, degenerative disease affecting the whole joint and its surrounding tissues, rather than only the erosion of the articular cartilage that lines the bones. Synovial joints are enclosed by a synovial membrane, also known as the synovium, which secretes and holds synovial fluid within the joint cavity. Current treatments for OA are mainly focused on pain management, for instance physical therapy, systemic medications, or intra-articular injections. Unfortunately, there is no definite cure available that can conserve the entire joint. Therefore, new treatment strategies to delay the progression of OA are desired. One of the approaches is focusing on inflammation of the synovial membrane, a process in which macrophages are pivotal. Macrophages are derived from monocytes and are present in virtually all tissues of the body, including the synovium. They are key during innate and adaptive immunity and essential in joint homeostasis and inflammation[4]. Macrophages are unique in terms of their plasticity as they can become activated upon stimuli from their microenvironment. This process is usually referred to as polarization and results in a spectrum of phenotypes, ranging from pro-inflammatory macrophages (M1), to anti-inflammatory macrophages or tissue repair macrophages (M2). Because their homeostatic function can become compromised, macrophages are linked to numerous pathologies, including degenerative joint diseases. The aim of this thesis was *to explore the involvement of macrophages, in particular the role of their phenotypes, during the processes of joint degeneration*.

INVOLVEMENT OF MACROPHAGE PHENOTYPES IN INFLAMMATORY TISSUE DEGENERATION AND DEGENERATIVE JOINT DISEASE.

Synovial inflammation is a major feature of OA, involving increased numbers of macrophages in the synovial tissue[2-4,6]. Early indications that macrophages in general can affect cartilage *in vitro* were reported in the '70 and '80s[48,49], where it was seen that macrophages were able to degrade cartilage explants. In the late '90s, other studies supported these early findings by use of an *in vivo* arthritis model where depletion of phagocytic synovial lining cells resulted in less influx of polymorphonuclear neutrophils. The depletion also resulted in reduced proteoglycan degradation and reduced chondrocyte death in cartilage after induction of collagen induced arthritis (CIA)[127,225]. It was further suggested that monocytes/macrophages were mainly responsible for this cartilage damage[128]. Later on, Blom *et al*. reported reduced formation of osteophytes, fibrosis, influx of inflammatory cells[51], and MMP-mediated cartilage degradation[52] when synovial macrophages were depleted from the synovial lining in

the collagenase-induced OA (CIOA) mouse model. These findings combined indicated that synovial macrophages are important regulators of joint tissue degeneration and OA development. Another study reported higher expression of *CD11c*, *TNFA*, *MMP3*, and a disintegrin and metalloproteinase with thrombospondin motifs 4 (*ADAMTS4*) in the synovium of STR/Ort mice[72], a model for spontaneous OA[226,227], than in the synovium of C57BL/6 wildtype mice. The expression levels of these genes appeared to be linked to the pro-inflammatory F4/80$^+$/CD11c$^+$ macrophage population, which was increased in STR/Ort mice compared to the C57BL/6. Moreover, the levels were significantly reduced when the macrophages were depleted from the synovium, suggesting that F4/80$^+$/CD11c$^+$ macrophages are likely regulators of *TNFA* and *MMP3* expression, factors that contribute to joint tissue degeneration.

Studies that focus on macrophages in joint diseases, mainly focus on an undefined macrophage populations or pro-inflammatory macrophages, even though the phenotype that a macrophage may acquire should be considered a spectrum, of which pro-inflammatory (M1) and anti-inflammatory (M2) are the extremes. M2 macrophages are both anti-inflammatory and involved in tissue repair. To distinguish these populations *in vitro*, M2-like macrophages are often further divided into IL-4 and/or IL-13 stimulated macrophages (M(IL4)) and IL-10 stimulated macrophages (M(IL-10)). These subsets are sometimes also referred to in literature as M2a and M2c macrophages[39,59]. As shown in **chapter 5** and in agreement with work by others, different M2 macrophage populations can be generated by using certain stimuli *in vitro*[42,43,68,73,118]. M(IL4) macrophages where shown to highly express *CD206* together with high expression and protein production of factors involved in tissue repair processes such as TGFβ, arginases, and CCL18[111,112,117,118]. M(IL-10) macrophages express high levels of *CD163* and produce high levels of IL-10 and soluble CD163, and are typically considered anti-inflammatory[45]. These phenotypes are mainly used to characterize *in vitro* polarized macrophages. The knowledge on the effects of macrophages on joint tissues needed to be further expanded by distinguishing between these phenotypes. In **chapter 3**, the effects of the secreted products of three macrophage phenotypes on articular cartilage were evaluated *in vitro*. Therefore, conditioned media of M(IFNγ+TNFα), M(IL4), and M(IL10) polarized macrophages were prepared and cultured with cartilage explants of knee OA patients. Based on changes in gene expression levels, release of nitric oxide (NO) as an indication of inflammation, and release of glycosaminoglycans (GAGs) as an indication of cartilage degradation, it was shown that the secreted products of M(IFNγ+TNFα), enhanced inflammation and degeneration of the cartilage. Expression of catabolic genes and genes encoding for inflammatory proteins were upregulated, while the expression of genes encoding for matrix proteins were suppressed. In addition, products secreted by both M(IL4) and M(IL10) macrophages did not

prevent inflammatory processes in the cartilage and were also unable to inhibit acute inflammation or degeneration of the cartilage.

As in many other pathologies, OA progresses in stages. On a microscopic level, the synovial membrane of early OA patients contains more $CD68^+$ macrophage infiltration than the synovium of late OA patients[228]. Immunohistochemical stainings of synovial tissue of end-stage hip OA patients (**chapter 2**) revealed that the majority of the macrophages in the synovium had an anti-inflammatory or tissue-repair phenotype. This was indicated by apparent staining of $CD163^+$ and $CD206^+$ cells, markers for anti-inflammatory and tissue repair macrophages, as well as increased gene expression levels identifying M(IL4+IL13) and M(IL10) macrophages. CD11c staining, a marker for pro-inflammatory macrophages, was almost completely absent. This was supported by low gene expression levels of *TNFA* and *IL1B*, markers indicative for pro-inflammatory macrophages. From these results we could speculate that the recruitment of additional monocytes into the synovial membrane by chemokines appears to result into the polarization of anti-inflammatory or tissue repair macrophage phenotypes. Together, the findings provide insights into the potential dynamics between chemokines, the phenotype of synovial macrophages, and the degenerative state of cartilage in end-stage hip OA.

In terms of knee OA, it was found that patients with bicompartmental knee OA have a higher inflammatory profile than synovial fluid of patients with unicompartmental knee OA based on multiplex analysis and the $CD14^+$ macrophages were the major cell population in both patient groups. The authors hypothesized that macrophages are relevant in the initiation stage of OA, and the stage of the disease may be predicted by the inflammatory profile of the synovial fluid[229]. Because soluble cytokines are good candidates to function as biomarkers, correlations between the presence of cytokines in the synovial fluid and OA stage have been explored extensively. The factors CCL2/MCP-1 and IL-6 are known to be produced by macrophages[68,230]. The concentrations of both CCL2 and IL-6 in synovial fluid of patients undergoing knee arthroscopy, strongly correlated with the International Cartilage Repair Society (ICRS) cartilage score[231]. More direct evidence of the involvement of macrophages in OA was demonstrated by the correlation between concentrations of soluble CD14 (sCD14) and sCD163 in synovial fluid and the presence of activated macrophages in the joint capsule, and osteophyte severity and progression. Additionally, sCD14 concentrations in the synovial fluid was correlated with joint space narrowing severity[86]. CD14 is a surface receptor that is present on all monocytes and macrophages[232,233], whereas CD163 is thought to be mainly present on anti-inflammatory macrophages[42,68,234].

The presence of these markers in the synovial fluid, indicate a dynamic presence of different macrophage phenotypes during certain stages of OA. Managing the behavior of synovial macrophages may thus be a suitable approach to prevent the degeneration

of joint tissues. However, in order to utilize macrophage phenotype modulation as a therapeutic approach for OA, it should be first known *which* phenotypes are present during the course of the disease. Because the progression rate of OA varies among patients, experimental mouse models are useful. The time profiles of the presence of three macrophage phenotypes, which represent the extremes of the phenotype spectrum, were evaluated during the course of two experimental mouse models for OA (collagenase-induced OA and destabilization of the medial meniscus). These two models differ in degree of inflammation and degeneration rate. The OA knees were histologically evaluated 1, 3, 7, 14, 28, and 56 days after OA induction. iNOS was used as an immunohistochemical marker for pro-inflammatory macrophages, CD163 for anti-inflammatory macrophages, and CD206 as a marker for tissue-repair macrophages. The presence of the three macrophage phenotypes differed during OA onset and development. Moreover, the macrophage phenotypes were also differentially associated with the development and progression of OA characteristics such as synovial thickness, presence of osteophytes, and cartilage damage, which suggests that the different phenotypes of macrophages may have a different function *in vivo* as well.

MODULATION OF MACROPHAGE PHENOTYPES TO CONTROL INFLAMMATION

An approach to manage inflammation in synovial tissue as a treatment regimen for degenerative joint diseases, may be achieved by specific modulation of a macrophage population. Chemokines at the local site of inflammation may regulate recruitment and migration of monocytes, the precursors of macrophages. In light of this concept, in **chapter 2**, expression levels of chemokines that are related to monocyte extravasation, were measured in synovial tissue of end-stage hip OA patients. *CCL2*, *CCL3*, *CCL4*, *CCL5*, and *CX3CL1* were all expressed in the synovium. *CCL2* expression levels in the synovium had an inverse correlation with the cartilage degeneration of these patients as evaluated by histological analysis. Based on these findings, it could be speculated that CCL2 is involved in the recruitment of monocytes towards synovial tissue and once migrated, the monocytes appear to polarize towards an anti-inflammatory phenotype in end-stage hip OA. A next step would be investigating the actual involvement of CCL2 on monocyte migration. Sophisticated *in vitro* systems such as microfluidic systems can be deployed to study cell extravasation[235,236]. Such information can benefit the development of strategies that influence trans-endothelial monocyte migration into joints, thereby directing synovial inflammation.

The knowledge on the profiles of the presence of these macrophage phenotypes during the course of OA (**chapter 4**), can be implemented to determine an optimal

timing to apply a treatment regimen that is focusing on modulation of macrophage phenotypes with the goal to inhibit OA progression. Therefore, in **chapter 5**, the possibility to modulate the macrophage phenotypes in synovial tissue *in situ* was assessed. To this aim, the modulatory capacity of medications or compounds that are commonly used in clinics was evaluated. The medications: dexamethasone, rapamycin, BMP-7, and pravastatin were chosen for this study and it was explored how this concept may be used to guide synovial inflammation. Each medication was able to guide the inflammatory state of the OA synovium via the modulation of specific macrophage phenotypes. More specifically, rapamycin enhanced acute inflammation of the synovial tissue, likely by specifically suppressing anti-inflammatory macrophages. On the other hand, dexamethasone reduced inflammation of the synovial explants which was likely regulated by suppression of pro-inflammatory macrophages with simultaneous enhancement of the performance of anti-inflammatory macrophages. Eventually, modulation of the inflammatory state of the synovium, can lead to an improved joint environment that may result into inhibition of cartilage degradation. This hypothesis was investigated by culturing OA cartilage explants with the conditioned medium of synovium that was modulated with the corticosteroid triamcinolone acetonide (TA) and with rapamycin (**appendix chapter 5**). The modulation of the synovium with rapamycin did not affect the degenerative state of the cartilage. Modulation of the synovium with TA however, resulted in reduced expression of the collagenases *MMP1* and *MMP13* in the cartilage, while expression of *ACAN* was enhanced. Also, hypertrophic processes appeared to be suppressed as indicated by lower expression of the hypertrophic cartilage marker *COL10A1*. Moreover, the capacity of the compounds to modulate macrophage phenotypes within the synovium, depended on the degree of inflammation in the tissue. These findings emphasize the importance of determining the inflammatory state of the synovium prior to any treatment that is focusing on macrophage modulation.

Several animal studies demonstrated that corticosteroids have to some extend the capacity to protect OA development by suppressing inflammation. In these studies however, the intervention is usually immediately applied after induction of OA[144,147,237]. In fact, in patient studies, conflicting effects on intra-articular corticosteroid injections are reported. These contradictions may likely be due to intervention timing and limitations in drug delivery. Non-post traumatic OA patients are seen in late stages of the disease by their health care provider. For instance, it has been reported that intra-articular injections of triamcinolone every 3 months over a period of 2 years, actually increased cartilage loss and did not provide beneficial effects on pain for patients with Kellgren and Lawrence (KL) scores of 2 to 3 and the Western Ontario and McMaster Universities osteoarthritis index (WOMAC) scores of $\geq$2 and $\leq$8)[238]. Additionally, prolonged exposure to high concentrations of corticosteroids have actually been proven to be toxic for chondrocytes[17,239]. The possibility to modulate synovial inflammation

was challenged by simulation of acute inflammation in the synovial explants and the modulatory capacity of the medications varied based on the inflammatory state of the synovium (**chapter 5**). The profiles of macrophage phenotypes in time after OA onset, implicate that the susceptibility to modulate may be due to the different macrophage phenotypes present during a certain stage of the disease. As it was found in chapter 4 that pro-inflammatory macrophages and tissue repair macrophages are mainly present during mid\late OA, whereas anti-inflammatory macrophages appear to be more pronounced during early OA in the mouse models, it is suggested that timing appears to be an important aspect when it comes to treatment of diseases with an inflammatory component in which macrophages have shown to be pivotal. To overcome delivery limitations, methods to encapsulate drugs (e.g., in lipid microspheres) with the goal to extend the retention of the drug, are of high interests. FX006/Zilretta (Flexion Therapeutics), is a novel formulation of TA enclosed in polylactic-co-glycolic acid (PLGA) microspheres that can remain for a prolonged period in the synovial fluid after intra-articular injection. FX006 showed promising results during Phase 2b[240] and Phase 3[241] clinical trials. Compared to injections with non-encapsulated TA and placebo, FX006 demonstrated to have beneficial effects on pain, function, and stiffness of knee OA patients[240-242]. Additionally, the formulation ensues less systemic exposure than when not encapsulated[243]. To increase the efficacy even more, methods to specifically target specific cells, for example targeting synovial macrophages, can lead to the need of low drug concentrations and avoiding toxic effects of the drug. Currently, corticosteroid amounts in the order of magnitude of milligrams are injected intra-articularily[240,244]. From the work described in **chapter 5**, we know that with lower concentrations, 200 to 2000 times lower depending on the type of corticosteroid, it is already possible to modulate specific macrophage phenotypes. These findings indicate that inhibiting OA symptoms and progression via the modulation of the phenotype of synovial macrophages, may be efficient by improving delivery systems to direct the drug towards a specific macrophage phenotype.

Biomaterials are frequently used for joint reconstructions as well as in other fields of regenerative medicine. For instance, scaffolds constructed from copolymer composites can be used for cartilage tissue engineering[245-248] with the ultimate goal to regenerate cartilage defects. To prevent further OA development due to meniscal injury or anterior crucial ligament injnury[7], repair of the damaged tissue can be achieved with fixation[249] using suture materials (e.g., monofilament polyprolylene). Upon implantation of any biomaterial, the host will interact with the material and may exert a foreign body response involving macrophages. In **chapter 6**, the polarization behavior of monocytes into macrophages was evaluated using an *in vitro* culture model. To this extend, biomaterials were chosen that represent a class of materials often used in various clinical applications or cartilage tissue engineering: polypropylene, polyethylene terephthalate,

and polylactic acid. Interestingly, the inflammatory response of the macrophages could be predicted by the presence of subsets of monocytes in the peripheral blood of the patient and was revealed to be directed by obesity, a major risk factor of OA[250,251]. Based on the findings of **chapter 6**, the capacity to modulate the phenotype of macrophages when activated by a biomaterial was investigated in **chapter 7**. The medications rapamycin, dexamethasone, celecoxib, and pravastatin were all capable to modulate the phenotype of the macrophages that were activated by polypropylene, polyethylene terephthalate, or polylactic acid, though the modulatory capacity and susceptibility depended on the type of material. Improving the environment to allow joint tissue regeneration with the aid of biomaterials, may be accomplished by macrophage phenotype modulation. For such an approach, inspiration can be taken from the normal wound healing cascade which involves an inflammatory phase, proliferation phase, and tissue remodeling phase[252]. Thus, stimulating inflammation in a controlled way, followed by controlled tissue repair processes may benefit tissue regeneration. It was reported that cartilage engineered constructs may support polarization of activated macrophages into an anti-inflammatory state, resulting in favorable cartilage generation[253]. Another study showed that producing a scaffold that is capable of sequentially releasing cytokines known to modulate macrophage phenotypes to mimic the phases of wound healing, improves bone tissue engineering[254]. The combination of such a tool and the findings of **chapters 6** and **7**, may provide insights for applying macrophage phenotype modulation for improving biomaterial-based joint tissue regeneration strategies.

FUTURE PERSPECTIVES

Functional role of macrophage phenotypes and their potential role in OA development

Besides modulation of macrophage phenotypes with the aim to suppress OA development, modulating the angiogenesis-stimulating behavior of macrophages would also be interesting. An important characteristic of OA, though less addressed in this thesis, is the alteration of the osteochondral junction, the transitional area in which cartilage meets bone. These are accompanied by the vascular invasion of the calcified cartilage, followed by the penetration of the calcified cartilage into the articular cartilage[255,256]. These events can lead to cartilage calcification and, most likely, to pain and loss of function. One study reported that vascularization of articular cartilage and synovial angiogenesis is present in early OA and is associated with cartilage damage, osteophyte formation, and synovitis[256]. Macrophages are also known to be involved in angiogenesis and vascularization, though insights in the mode of action and mechanisms during OA onset and progression are limited. Reports are somewhat contradictive, as

these cells tend to regulate vessel network growth both directly and indirectly[257]. One study reported that M(IL-4) and M(IL-10) M2 macrophages derived from murine bone marrow, promote angiogenesis in *vitro* and *in vivo* when encapsulated in Matrigel after subcutaneous implantation[258]. On the other hand, an *in vitro* study revealed that pro-inflammatory (M1) human monocyte-derived macrophages are initiators of angiogenesis and vascularization in 3D Matrigel scaffolds[257]. This initiation is directed by the macrophages via secretion of pro-angiogenic factors, such as VEGFA, FGF, and MMP9. The presence of macrophages in the synovial membrane during the course of OA was associated with the presence of osteophytes, while in the control knees no associations were found (**chapter 4**). These data combined suggest that macrophages may have a role in promoting bone formation and that this capacity may depend on the ability of macrophages to regulate angiogenesis and vascularization. A next step could be investigating the functional effects of different macrophage phenotypes during OA *in vivo*. For instance, by developing an animal model in which only one phenotype is present. This may be achieved by first depleting the macrophages, followed by reconstitution with a single phenotype of macrophages that has been generated *in vitro*[259]. After this model has been validated for this purpose, it can then be used to investigate the functional role of phenotypes in the pathogenesis of OA, and potentially elucidate further mechanisms such as calcification of articular cartilage and the identification of mechanisms promoting angiogenesis and vascularization in the joint. These findings can subsequently contribute to the identification of new potential drug targets to inhibit the invasion of subchondral bone into articular cartilage.

In vivo imaging of macrophage phenotypes in OA

Ideally, a minimally invasive tool to either predict OA progression or to assess the stage of the disease would be desirable, so that a macrophage modulation-based therapy can be applied efficiently. One study reported an association between synovial inflammation detected by ultrasound and the development of bone erosions in hand OA[260]. Though informative, methods to specifically image macrophages would be preferable. As the presence of synovial macrophages or their products are correlated with OA progression, *in vivo* imaging of activated macrophages has become of interest. The folate receptor β (FR-β) has become an interesting target ligand for activated macrophages. It was shown that injection of a folic acid analog labeled with a radio nucleotide followed by single-photon emission computed tomography (SPECT) combined with computed tomography (CT) could be used as an imaging tool to assess macrophage activation in experimental OA in rats[261]. One study reported a method to image of FR-β^+ activated macrophages with a receptor specific imaging agent (^{99m}Tc-EC20/Etarfolatide) using SPECT/CT imaging of radiographic OA knees. The association found between the number of activated macrophages, and radiographic knee OA severity also suggested

that drug delivery by specifically targeting activated macrophages via FR-β could be a possibility[262], though targeting specific macrophage phenotypes would be even more preferable (**chapter 5**). However, imaging of these macrophages to actually distinguish a macrophage phenotype remains challenging. For instance, reports regarding FR-β as a marker for a macrophage phenotype are somewhat controversial, as studies have reported that FR-β^+ macrophages can be seen as a marker for pro-inflammatory macrophages[263,264], or anti- inflammatory macrophages[147], as the expression of the receptor seems to be coupled with the expression of both M1 and M2 markers[164], which makes distinction *in vivo* between the phenotypes challenging. In another recent study[265], a tracer (cFLFLF-PEG-^{64}Cu) was developed consisting of a probe that targets formyl peptide receptor-1 (Fpr1), and was used to visualize macrophages via positron emission tomography (PET) in the monosodium iodoacetate (MIA) model of OA in rats. Stronger signal of the tracer was found in the MIA injected knees than in the controls, and the probe had mainly accumulated in the synovial membrane of the MIA knees. Additionally, high binding of the probe was found in RAW264.7-derived M(LPS) suggesting specific binding to pro-inflammatory macrophages, and indicating that the probe could be used to specifically visualize and target Fpr1 expressing macrophages in an OA joint. These studies demonstrate that specific imaging of macrophages is a relative new, yet interesting field. When fully exploited, for instance imaging of a specific phenotype so visualize the balance between subsets within tissue, could considerably contribute to current OA treatment regimens.