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The Role of Synovium and Synovial Macrophages in Experimental Post-Traumatic Knee Osteoarthritis

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Abstract

In osteoarthritis (OA), synovitis is associated with symptom severity. As synovium secretes both catabolic and anabolic factors into the joint, the impact of synovitis in OA remains unclear. We developed a novel co-culture system using tissues from an established rat model of post-traumatic knee OA (PTOA) to study signaling between synovium and chondrocytes. We found that synovium from early stage but not later stage PTOA joints caused an overall protective effect in chondrocytes. We then selectively treated synovial macrophages with liposomal drugs causing depletion, STAT1 inhibition, or STAT6 inhibition in early PTOA joints. We found cartilage damage *in vivo* was not affected, but chondrocyte responses to treated synovium *in vitro* indicate that STAT1 inhibition could be protective, while STAT6 inhibition is potentially pro-inflammatory. Thus, we demonstrate that synovial signals affect chondrocyte physiology in the OA knee joint and that targeting synovial macrophages as a potential therapeutic strategy warrants further investigation.

Keywords

Animal model of disease, cartilage, chondrocyte, inflammation, liposomal drug delivery, osteoarthritis, synovial macrophages, synovium, STAT inhibitors, tissue culture.

Lay Summary

Osteoarthritis is a serious joint disease that causes pain and disability with no direct treatment. Patients manage symptoms with pain medication and exercise. We still do not completely understand the mechanisms behind osteoarthritis, but we do know that the disease involves the cells in cartilage on the ends of bones changing from their normal state to send out inflammatory signals and enzymes that destroy the tissue around them. The joint lining, or synovium, which wraps around the joint space, also changes by becoming inflamed. In my project, we investigated how the synovium signals to cartilage cells and how the condition of the synovium affects osteoarthritis in the joint.

Our experiments used young adult male rats following ethical guidelines. We surgically injured the rats' knees to induce osteoarthritis. We collected cartilage and synovium and grew them in artificial conditions to isolate the signals exchanged between these tissues. Because inflamed synovium is correlated with human disease severity, we hypothesized that growing early osteoarthritic synovium with healthy cartilage cells would cause them to become inflammatory and destructive. To our surprise, we found a protective effect with indications of increased production of tissue molecules by cartilage cells. We then grew synovium from joints at a later stage of osteoarthritis with cartilage cells and found that this protective effect was completely absent. From this, we concluded that the synovium undergoes a change over time, which has implications for our understanding of osteoarthritis, although these findings need to be confirmed in humans.

We then investigated the impact of macrophages in the synovium in osteoarthritis. Macrophages are a diverse immune cell population with important roles in the joint, especially signaling. We packaged different drugs into small lipid particles and injected them into pre-osteoarthritic knees, where only macrophages could consume the drugs. For the three drugs we tested, we found different patterns of reduced inflammation in the synovium and mixed effects in cartilage cells grown with the synovium, but no differences in cartilage damage or pain. Our future work will use knees at a later stage of osteoarthritis, where we expect to see significant results in osteoarthritis-related outcomes.

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List of Abbreviations

Acan	Aggrecan (*gene*)
ACLT	Anterior cruciate ligament transection
ADAMTS4	A disintegrin and metalloproteinase with thrombospondin motifs 4
ADAMTS5	A disintegrin and metalloproteinase with thrombospondin motifs 5
Adamts5	A disintegrin and metalloproteinase with thrombospondin motifs 5 (*gene*)
ANOVA	Analysis of variance
Arg1	Arginase 1 (*gene*)
CCL2	C-C motif chemokine ligand 2
Ccl2	C-C motif chemokine ligand 2 (*gene*)
CCR2	C-C chemokine receptor type 2
CD68	Cluster of differentiation 68
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
COL2A1	Collagen type II, alpha-1 subunit (*gene*)
Col2a1	Collagen type II, alpha-1 subunit (*gene*)
Col10a1	Collagen type X, alpha-1 subunit (*gene*)
COMP	Cartilage oligomeric matric protein (*gene*)
Cq	Quantification cycle
DAB	3,3'-diaminobenzidine
DAMP	Damage-associated molecular patterns
ddPCR	Droplet digital polymerase chain reaction
DMEM	Dulbecco's Modified Eagle Medium
DMM	Destabilization of the medial meniscus
DMMB	Dimethylmethylene blue
DMSO	Dimethyl sulfoxide
DNase I	Deoxyribonuclease I
EDTA	Ethylenediaminetetraacetic acid
ERG	Erythroblast transformation-specific-related gene
EVF	Electronic Von Frey
F12	Ham's F12 medium

FBS	Fetal bovine serum
FOXO1	Forkhead box protein O1
GDF5	Growth differentiation factor 5 (*gene*)
H&E	Hematoxylin and eosin
HMGB1	High mobility group box 1
Hprt1	Hypoxanthine-guanine phosphoribosyltransferase (*gene*)
IFN-γ	Interferon-gamma
IL-1β	Interleukin 1 beta
IL-17	Interleukin 17A
Il17a	Interleukin 17A (*gene*)
IL-4	Interleukin 4
Il6	Interleukin 6 (*gene*)
IL-8	Interleukin 8
Mϕ	Macrophage
M-CSF	Macrophage colony-stimulating factor
MCP1	Monocyte chemoattractant protein 1
MIQE	Minimum information for publication of quantitative real-time polymerase chain reaction experiments
MMP	Matrix metalloproteinase
MMP1	Matrix metalloproteinase 1
MMP13	Matrix metalloproteinase 13
Mmp13	Matrix metalloproteinase 13 (*gene*)
MMP3	Matrix metalloproteinase 3
Mmp3	Matrix metalloproteinase 3 (*gene*)
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
Nfatc1	Nuclear factor of activated Tcells, cytoplasmic 1 (*gene*)
Nfatc2	Nuclear factor of activated Tcells, cytoplasmic 2 (*gene*)
NFATc4	Nuclear factor of activated Tcells, cytoplasmic 4
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
P	Cell passage

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E2
pMMx	Partial medial meniscectomy
Pop4	Ribonuclease P protein subunit p29 (*gene*)
Prg4	Proteoglycan 4 (*gene*)
PTOA	Post-traumatic osteoarthritis
QALY	Quality-adjusted life year
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640 medium
Runx2	Runt-related transcription factor 2 (*gene*)
S100a8	S100 calcium-binding protein A8
S100a8	S100 calcium-binding protein A8 (*gene*)
S100a9	S100 calcium-binding protein A9
S100a9	S100 calcium-binding protein A9 (*gene*)
sGAG	Sulfated glycosaminoglycan
SMALGO	Small animal algometer
Sox9	Sex-determining region Y-box transcription factor 9 (*gene*)
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor beta
Tgfb1	Transforming growth factor beta 1 (*gene*)
TGFBR1	Transforming growth factor beta receptor I
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumour necrosis factor alpha
Tnf	Tumour necrosis factor alpha (*gene*)

Chapter 1

1 Introduction

1.1 The Synovial Joint

The synovial joint is evolution's solution to the requirements of complex motion in vertebrates to traverse and manipulate their environment. From the large load-bearing joints of the lower body to the fine joints of the hand, each point of articulation allows two or more bones to glide, rotate, or pivot against each other with very little friction. Whereas fibrous joints tightly sew bones together and cartilaginous joints allow only semi-flexibility, synovial joints are complex organs comprised of multiple tissue types working together to lubricate and facilitate movement between bones, transmit biomechanical forces, and cushion external impact, including bone, cartilage, synovium, muscle, tendon, and ligament. When disease affects these joints, the resulting pain and disability reduce quality of life, compound comorbid diseases, and threaten survival.

1.1.1 Articular Cartilage

Articular cartilage is the specialized tissue that makes smooth, painless contact between bones possible. Cartilage is able to withstand high cyclic loads of mechanical force, exhibits viscoelasticity, and is resistant to compression and shear forces, with very low surface friction[1,2]. These properties are due to its biphasic composition and structure. The fluid phase of the extracellular matrix, consisting of water and electrolytes, constitutes 65-80% of the wet weight of cartilage. The solid phase includes proteoglycan aggregates, which make up 3-10% of the wet weight, and the network of collagen, responsible for 10-30% of the wet weight[3]. Proteoglycan aggregates consist of branching chains of polysaccharides and proteoglycans; each long chain of hyaluronan, the non-sulfated glycosaminoglycan, links to a high number of proteoglycan molecules, mostly aggrecan, which itself is linked to many small sulfated glycosaminoglycans, and it is the strong osmotic force of these sugar chains that maintains the water content of cartilage and enables it to resist compression[4]. The collagen network is predominantly type II collagen, with small amounts of types III, VI, IX, X, XI, XII and XIV as well[5]. Proteoglycan aggregates are not directly bound to the collagen network, but instead physically trapped within.

Articular cartilage is categorized into the superficial, transitional, and deep zones (Figure 1.1). The superficial zone is higher in water content and has collagen fibres aligned parallel to the articulating surface with many small, dense chondrocytes. The deep zone has lower water content due to the increased collagen and proteoglycan content, with fibres and columns of chondrocytes aligned perpendicular to the articular surface as they enter the calcified zone. The transitional zone acts as the functional gradient in between these morphologically distinct zones.

The matrix also differs with proximity to the chondrocytes. A chondrocyte and its pericellular matrix form a morphologically and mechanically distinct unit called a chondron, which contains higher proteoglycan content[6,7]. The territorial matrix surrounds the pericellular matrix and contains finer type VI collagen fibrils, while the more distant interterritorial matrix has thicker collagen fibers and different associated molecules[6,8,9].

In healthy cartilage, adult chondrocytes are responsible for producing the molecules that make up the matrix. They are relatively quiescent, as they do not migrate and rarely undergo mitosis[10]. Matrix turnover is low, with aggrecan in healthy cartilage having a half-life of 25 years and collagen type II of 117 years[11,12]. Unusually, cartilage contains no innervation or vasculature. Chondrocytes rely on the extracellular matrix for mechanical protection and on diffusion through the fluid phase for nutrition. Thus, they are also dependent on adjacent synovium and subchondral bone.

1.1.2 Synovium

The outer layer of the joint capsule is composed of dense irregular connective tissue continuous with the periosteum. The outer joint boundary is continuous with the bone, and the capsule provides strength and stability to the point of articulation. The inner lining of the joint capsule, called the synovium, lines all non-cartilage surfaces in the joint cavity. The synovial membrane itself can be divided into two major layers, the intima and the subintima (Figure 1.2). The subintima is composed of loose connective tissue and contains fibroblasts, relatively few immune cells, adipocytes, vasculature, lymphatics,

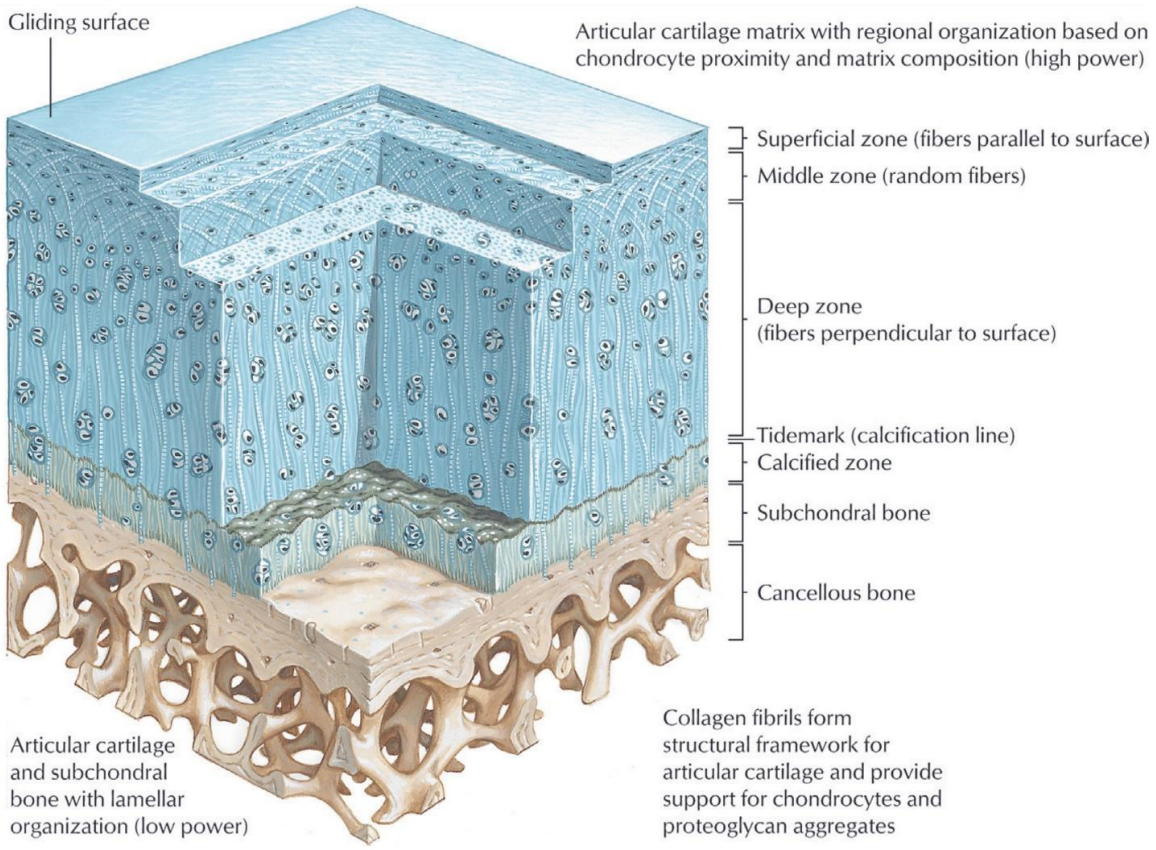

Figure 1.1. Zonal organization of articular cartilage.

Representation of the articular cartilage matrix with zones of chondrocyte and collagen organization. Image courtesy of Netter Images © Elsevier Inc.

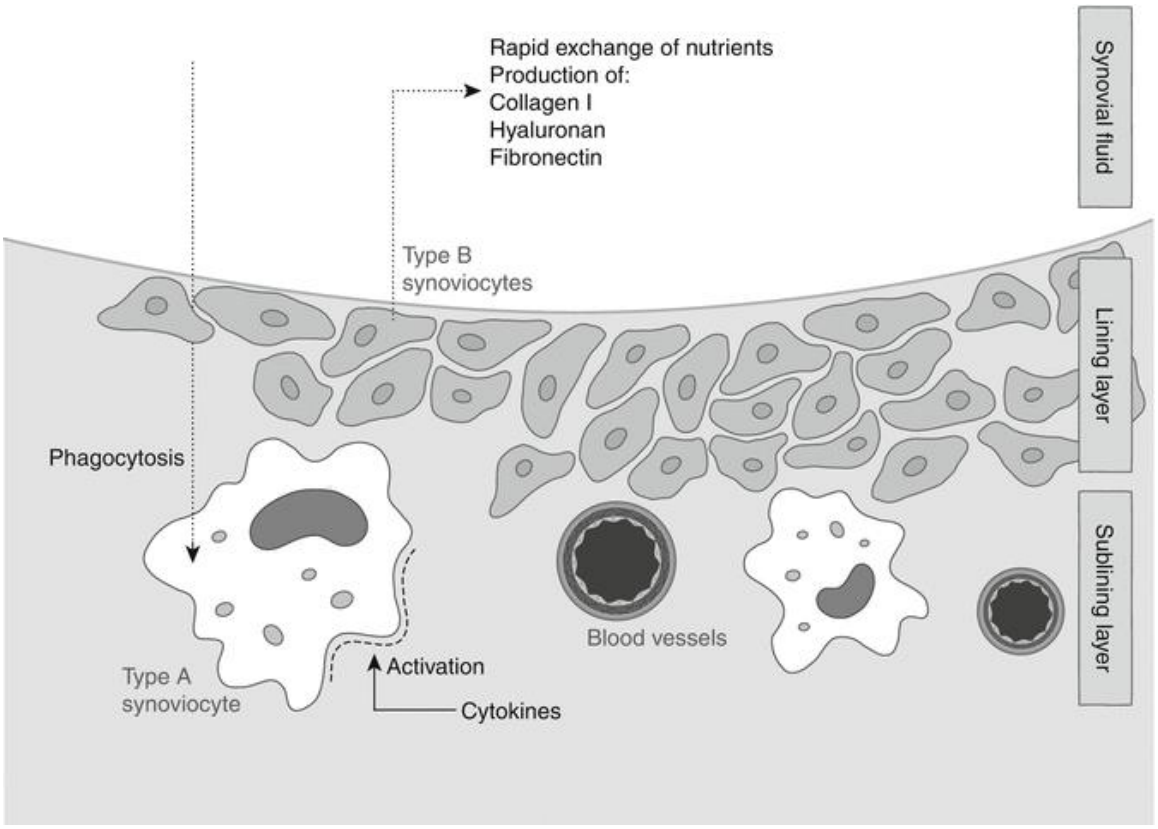

Figure 1.2. Organization of synovium.

Representation of joint synovium with lining and sublining layers. Image reproduced from Bertrand and Hubert 2014[13].

and peripheral nerve terminals. The intima is the continuous lining layer directly in contact with synovial fluid, which may contact but does not adhere to other joint surfaces during motion. Although the lining is continuous with gap junctions, desmosomes, and tight junctions, there is no basal lamina and the intima does not constitute a true basement membrane[14]. Therefore, synovium is not a true epithelium, although it functions as a barrier between the joint and the rest of the body. Nutrients, gases, and signaling molecules from the circulation are filtered through endothelium and synovium, collectively termed the blood-joint barrier, to reach synovial fluid and the cartilage beyond.

Healthy synovial intima chiefly contains two major cell types: synovial macrophages and synovial fibroblasts (classically named type A and type B synoviocytes, respectively)[15]. Synovial fibroblasts contribute to joint lubrication through the production of hyaluronan, long glycosaminoglycan polymers which maintain synovial fluid viscosity and water content through local fluid forces[16]. Synovial macrophages, as part of the innate immune system, monitor the joint environment and phagocytose the products of tissue turnover, thereby maintaining homeostasis in the synovial joint. Within the synovial intima and subintima, the total synovial macrophage population is composed of two distinct populations: resident tissue macrophages and infiltrating macrophages. Resident macrophages, like other tissue macrophages, arise from embryonic progenitors, are present in the prenatal joint, and are capable of self-renewal[17]. Infiltrating macrophages are derived from bone marrow precursors, first appear in the fetal joint perinatally, and are terminally differentiated. In the healthy joint, resident macrophages predominate relative to infiltrating macrophages from the periphery, with small populations of other immune cells such as dendritic cells and T cells.

1.2 Osteoarthritis

Due to the complexity of the synovial joint, there are numerous causes of synovial arthropathy. Problems with individual or multiple joint tissues, in particular the subchondral bone, cartilage, or synovium, or with systemic factors, can affect the health and therefore the function of the joint. Osteoarthritis (OA) is the most common synovial joint disease worldwide. Classically, OA is known as a disease resulting in the destruction of articular cartilage, leading to permanent loss and pathological changes to other joint tissues, with clinical manifestation of joint pain and disability[18]. With rising prevalence and no disease-modifying treatment currently available, OA is a serious global health problem and was elevated to the status of a "Serious Disease" by the United States Food and Drug Administration in 2018.

1.2.1 Etiology

OA is most commonly a primary condition, but may also be secondary to anatomic malalignment, trauma, or metabolic disorder. This classification system, originally developed in the 1980s, is under review by the American College of Rheumatology in light of new information about genetic and environmental factors[19,20]. Risk factors vary by joint, but commonly include age, female sex, genetics, previous injury, and obesity with or without additional components of metabolic syndrome[21,22]. Age is a particularly strong risk factor, to the point where OA is commonly misperceived as a disease of aging. The effect of aging is ascribed to a combination of increased chondrocyte senescence, accumulated damage to the matrix, muscle weakness, hormonal changes (e.g. menopause), and the long latency period of the disease[23]. Genome-wide association studies have pointed to heritable risk in genes related to synovial joint formation (e.g. *GDF5*), musculoskeletal structure and alignment (e.g. *FRZB*), and the molecular components of the matrix itself (e.g. *COL2A1*, *COMP*)[24]. Obesity increases risk through additional biomechanical strain on load-bearing joints and through metabolic syndrome, which has many putative mechanisms including the induction of a pro-inflammatory systemic state and altered adipokine signaling[25].

1.2.2 Pathobiology

Current theory suggests that OA results from either an acute insult to synovial joint tissues or accumulated stress on articular cartilage. Through mechanical and/or molecular signaling, chondrocytes are stimulated from their relatively quiescent state and respond in various ways, including the production of inflammatory and catabolic factors (Figure 1.2).

Chondrocyte-mediated inflammatory responses include the induction of gene expression and activation of intracellular processes that lead to production and release of fatty acid mediators (e.g. prostaglandin E_2 (PGE_2)), reactive oxygen and nitrogen species (e.g. nitric oxide), chemokines (e.g. interleukin-8 (IL-8)), and cytokines (e.g. interleukin 1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α))[22]. OA chondrocytes also produce, release, and activate catabolic enzymes including matrix metalloproteinases (MMPs) and aggrecanases. Matrix proteases degrade both aggrecan (e.g. a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS) 4, 5) and collagen (e.g. MMP1, 3, and 13)[26]. Particularly in early OA, these catabolic processes are accompanied by anabolic responses from chondrocytes such as the induction of aggrecan and type II collagen gene expression. However, the response is either inappropriate or insufficient to adequately compensate, and the protein network is eventually permanently damaged[8,27]. Early surface fibrillation of the matrix and proliferation of chondrocytes in the superficial zone progresses to larger areas of matrix fissures and degradation, with loss of chondrocytes[21]. This releases products (e.g. small leucine-rich proteoglycans, type II collagen or aggrecan fragments) that further stimulate catabolic processes[8,28].

Within deeper cartilage zones, chondrocytes undergo processes similar to those of endochondral ossification. In this developmental program for bone formation, chondrocytes die in an organized and controlled manner, leaving behind mineralized matrix for osteoblasts to populate. In osteoarthritis, articular chondrocytes become more hypertrophic, increase rates of apoptosis, and produce type X collagen, with the matrix showing ectopic calcification and tidemark advancement and duplication[29]. Subchondral bone, as a dynamic tissue, undergoes substantial remodelling throughout the course of

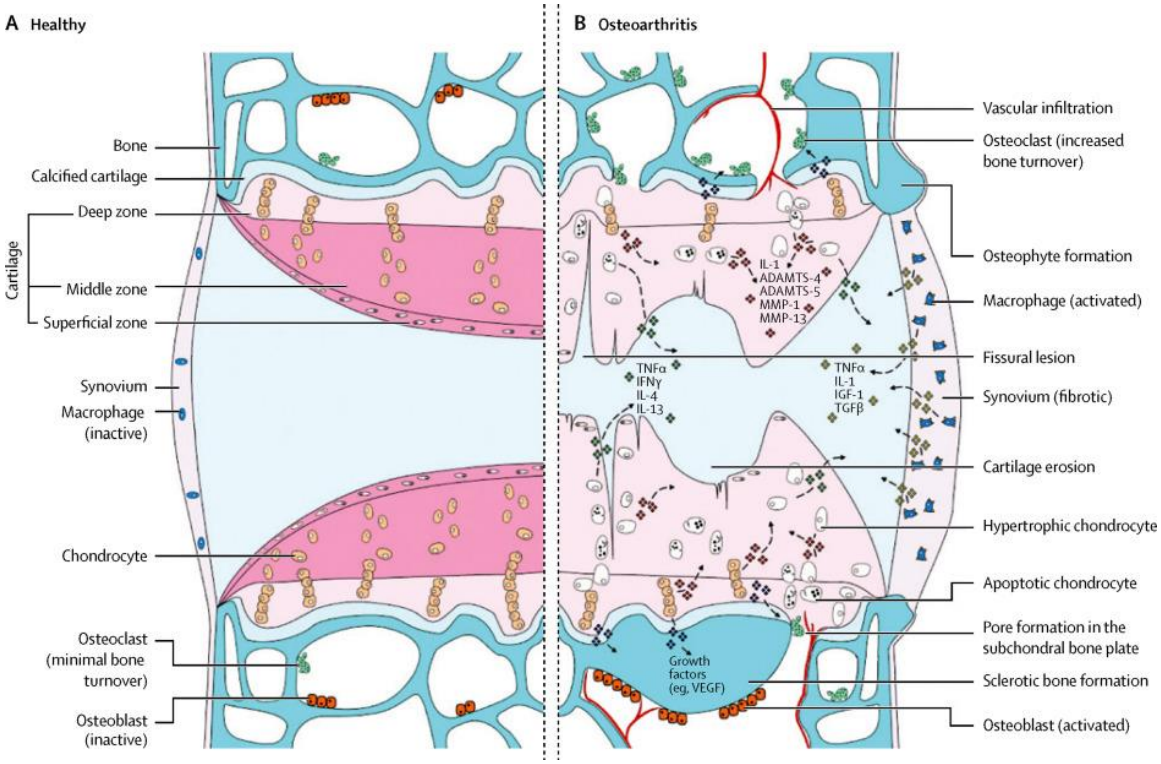

Figure 1.3. Physiological changes in the osteoarthritic joint.

Diagram of a healthy versus osteoarthritic synovial joint with labels indicating changes to joint tissue composition and common signaling molecules. Image reproduced from Glyn-Jones *et al*. 2015[22].

OA, with sclerosis, subchondral cyst formation, and osteophyte formation being the most recognizable features[30].

Synovium also plays a key part in OA pathogenesis. Even in early disease, ultrasonography and arthroscopy can detect increased blood flow and synovial fluid effusions[20]. Microscopic examination of biopsies reveals hyperplasia and villous growth of the lining (fronding), infiltration by mononuclear leukocytes (macrophages, T cells, B cells), angiogenesis, fibrosis, and edema[31]. Synovitis is undetectable in some cases, but for the majority of OA patients, low-grade synovial inflammation persists[31,32]. The presence of synovitis in OA has recently been identified as being clinically important. In the knee and hand, synovitis generally becomes more prevalent with disease progression[33,34,35]. In these joints, the presence of synovitis is also predictive of worse radiographic damage and is strongly associated with greater joint pain[35,36,37,38,39,40]. Like chondrocytes, synovial macrophages and fibroblasts also produce proteases (e.g. MMP3, 13) and various pro-inflammatory cytokines (e.g. IL-1β, interleukin-17 (IL-17), TNF-α), chemokines (e.g. IL-8, monocyte chemoattractant protein 1), lipids (PGE_2), and damage-associated molecular pattern molecules (DAMPs, e.g. S100a8, 9, high mobility group box 1 protein (HMGB1))[21,32]. On the other hand, synovial cells can release counter-regulatory and protective molecules such as anti-inflammatory factors (e.g. interleukin-4, interleukin-10, interleukin-1 receptor antagonist) and tissue inhibitors of metalloproteinases (TIMPs, e.g. TIMP1, 2, 3)[41]. Finally, synovium produces growth factors such as insulin-like growth factor 1, nerve growth factor, vascular endothelial growth factor, and transforming growth factor-β (TGF-β) which are known in the developmental context, but less so in disease. For example, TGF-β signalling is necessary for articular cartilage homeostasis and preventing chondrocyte death, but is also involved in synovial fibrosis and osteophyte formation in OA[42,43]. As there is relatively less research available on the role of synovium in OA, the overall effect of synovitis on OA is unclear.

Beyond the scope of this thesis, other joint tissues also appear to have roles in OA; weakening of tendons and ligaments lead to laxity which may affect the balance of biomechanical forces in the joint. Certain joints such as the knee have additional

stabilizing structures. The meniscus, or articular disc, is a fibrocartilaginous structure with its own biomechanical alignment and matrix physiology which can contribute to OA[44,45]. The infrapatellar fat pad has been noted to express both pro- and anti-inflammatory factors in knee OA, warranting further investigation in the field[46,47].

1.2.3 Disease Burden

OA is an enormous health burden which grows every year due to rising disease incidence and increasing rates of comorbidities that are themselves risk factors for OA. In the 2010 Global Burden of Disease Study, hip and knee OA was ranked as the 11th highest contributor to global disability, more so due to pain than immobility[48,49]. In Canada, OA currently affects 14% of adults over age 20 and is projected to affect over 10.4 million people by 2040[50,51]. Over the next two decades, direct costs on the healthcare system are estimated to total $390 billion, and indirect costs $660 billion. Direct costs of health care include physician visits, hospitalization, laboratory tests, drugs and mobility aids, caregivers, and community resources, while indirect costs include lost work productivity and unemployment for both patients and caregivers[52].

OA is a major cause of reduced quality of life. A recent study in the United States estimated 1.857 quality-adjusted life-years (QALY) lost per knee OA patient, which nearly doubled to 3.501 QALY for patients who were also obese[53]. The difference may be greater still for patients with OA in other joints and other comorbidities. In addition to physical pain, fatigue, and physical limitations, OA patients may experience distress, low mood, sleep difficulties, and anxiety[54,55,56]. For people living with OA, losing the ability to live with autonomy and free of pain is the greatest threat from OA.

1.2.4 Disease-Modifying Treatment for OA

There is currently no disease-modifying treatment approved for human patients. OA treatment focuses on symptom management to maintain participation in activities of daily living[57,58,59,60]. Patient self-management and education, as highly accessible and cost-effective modalities, are recommended for all patients[20,61,62]. Exercise for musculoskeletal strength and weight management has been shown to improve pain, aching, and stiffness, thereby reducing disability[63]. Non-steroidal anti-inflammatory drugs and acetaminophen

are the most abundant pharmacological recommendations for OA symptoms. Intraarticular corticosteroids are also effective at reducing pain in knee OA, but effects do not persist, and steroid injections are not recommended for hand OA[64]. In a minority of cases where focal damage is detected early, surgical interventions to replace the matrix with transplanted autologous cartilage, progenitor cell-generated fibrocartilage, or artificial bioscaffolding have been used, but these are not routine options[22]. Rather, arthroplasty is the surgical treatment of choice that is used to relieve severe symptoms at the end stage of disease. Unfortunately, this means that effective therapy is not available to many patients who suffer for many years in wait for joint replacement. The risks of surgical operation, revisions, and implant replacement later in life make this option far from ideal.

Similar to treatment for rheumatoid arthritis, an autoimmune disease that also directly damages synovial joints, pathological processes have been pharmacologically targeted in OA. Several categories of molecular targets, including various MMP inhibitors (e.g. doxycycline), inflammatory mediator blockers (e.g. adalimumab, anakinra), and recombinant growth factors (e.g. fibroblast growth factor 18), have been tested, but no therapy has been approved as a disease-modifying treatment for OA[65]. As repairing tissue damage in a highly inflammatory environment is difficult, a combination of treatments for preventing further damage, repairing existing damage, and inhibiting the progression of inflammation may ultimately be needed.

1.2.5 Animal Models of OA

Synovial joints are conserved in all mammals; synovial joints have a highly similar morphology despite differences in animal size, weight, and number of legs. A wide variety of animal models is available for OA research, including the mouse, rat, rabbit, dog, sheep, cow, and horse, among others. Larger animals, such as the horse and sheep, are advantageous for studying surgical induction and interventions, and they provide more biological material for data collection[66]. Small animals are more difficult to image and may not be as translatable to human patients due to phylogenetic differences in physiology and biomechanical loading. However, they have lower care costs, age more quickly with experimental OA progression, and are highly suitable for use in drug

screening and genetic modeling studies. Pain and gait changes remain challenging to interpret in all species, although recent work in the OA field has advanced some of the available tools for measuring pain-related behavior in small animal models, especially rodents[67]. Primary OA is found to spontaneously develop in the mouse, rat, dog, and horse with age, while secondary OA models can induce disease via surgical insult to the joint, non-invasive impact injury, ovariectomy, or intraarticular injection of toxins or enzymes.

For this thesis, we selected a post-traumatic rat model, with OA secondary to destabilization of the stifle joint via surgical transection of the anterior cruciate and medial meniscotibial ligaments (ACLT-DMM). Animal intervention and care protocols were based off previous studies using a similar model of joint destabilization via anterior cruciate ligament transection and partial medial meniscectomy (ACLT-pMMx), which resulted in progressive damage to articular cartilage of the operated knee (Figure 1.4)[68,69]. We predicted that in the ACLT-DMM model, changes due to early OA would present at 4 weeks post-surgery, and that disease progression would be similar to the ACLT-pMMx model, with moderate to severe disease evident at 12 weeks post-surgery.

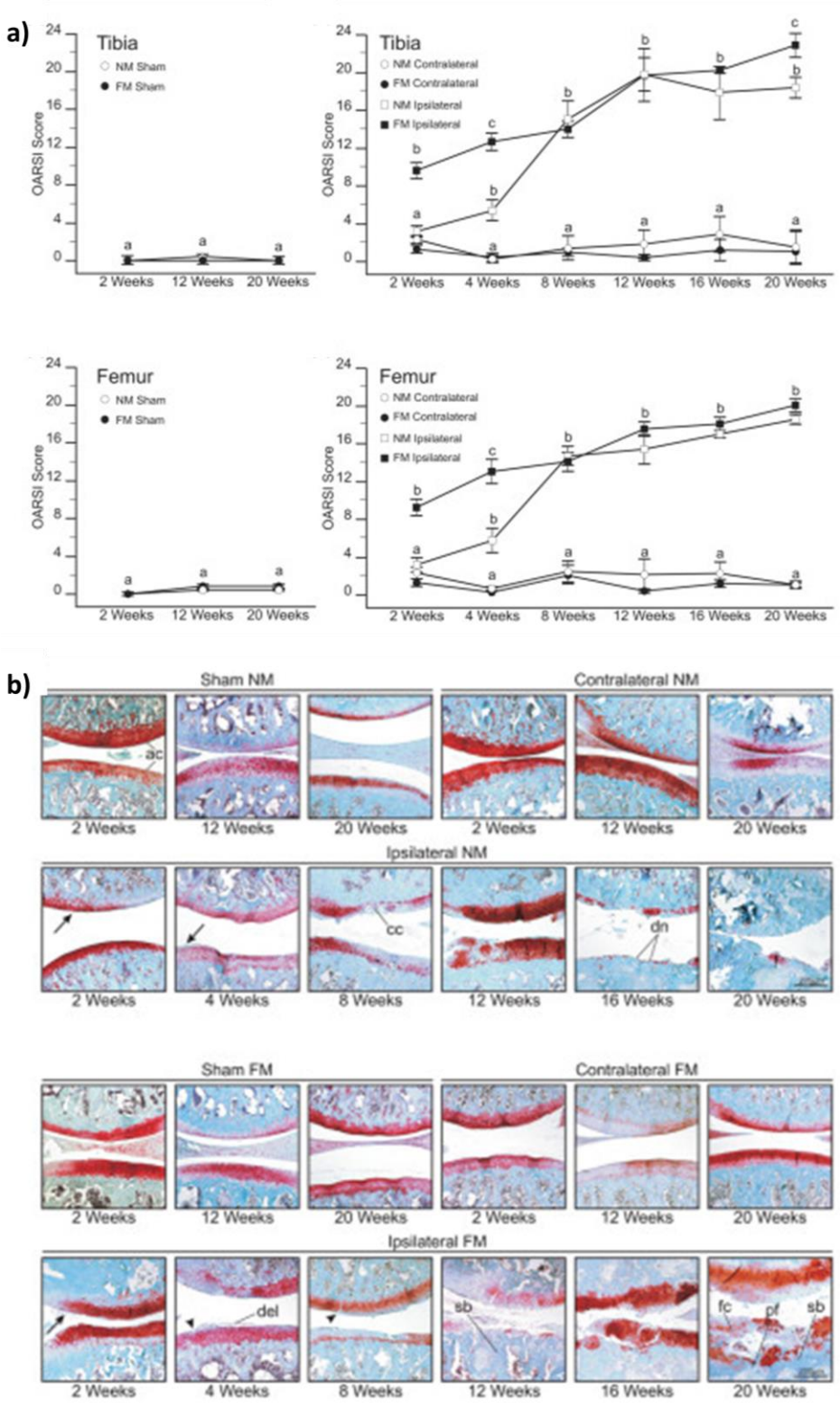

Figure 1.4. Articular cartilage damage increases over time in the ACLT-pMMx rat model.

a) OARSI histopathology scores for tibial and femoral articular surfaces in ipsilateral and contralateral joints from rats given ACLT-pMMx surgery and in ipsilateral joints from rats given sham surgery (control) over 20 weeks post-surgery (mean ± SEM, n = 4). Animals were also subjected to forced mobilization (FM) or nonmobilized (NM) treatment. Significantly different means at each time point are indicated by different letters (one-way ANOVA with Tukey's *post hoc* test, $p < 0.05$). b) Representative micrographs at the same magnification of sagittal sections stained with safranin-O, fast green, and hematoxylin at these timepoints show disruption of the normal articular surface (ac), including surface discontinuity (arrow), vertical fissures (arrowhead), delamination (del), chondrocyte clustering (cc), denudation (dn), sclerotic bone (sb) fibrocartilage-like tissue (fc), and subchondral plate failure (pf). Figure adapted from Appleton *et al*. 2007[66].

1.3 Research Question

Synovitis has recently been recognized as a clinically important feature in patients with OA due to the association of synovitis with pain and structural disease progress, particularly in knee OA. Synovitis results in the production of several inflammatory mediators that can directly impact other joint tissues including cartilage. Synovitis is also present in patients with early OA and becomes more severe with disease progression, making it an attractive target for treatments used in early (pre-surgical) stages of OA. In this thesis, we investigated the role of synovitis in OA and the effect of synovium on cartilage. In particular, we sought to determine if synovitis in early knee OA is able to stimulate articular cartilage and whether the effects of early OA synovium are harmful or protective to articular chondrocyte physiology.

1.3.1 Rationale

The synovium supports cartilage health and function in healthy joints, but its role in early OA development is not well understood. There is urgent need for disease-modifying treatment. Developing an intervention in early OA to prevent joint damage and other pathological changes is preferable. Therefore, studying synovium in early OA will expand our understanding of the disease and possibly provide opportunity for intervention. Synovial macrophages in OA synovium have not been extensively investigated, but their status as effector cells of the innate immune system and the current literature suggest they have important signaling roles. A surgically induced post-traumatic rat model of OA has previously been established and is suitable for evaluating joint physiology and potential interventions for macrophages.

1.3.2 Hypothesis

Early experimental PTOA synovium alters healthy chondrocyte physiology, inducing pro-inflammatory and pro-catabolic effects. Additionally, manipulating synovial macrophages in early PTOA via depletion or inhibition of polarization pathways will modify PTOA outcomes in synovium and cartilage.

1.3.3 Objective

Our overall objective is to investigate the effect of OA synovium on chondrocytes and joint outcomes using a rat model of PTOA (Figure 1.5). Specifically, we will:

1. Establish an *in vitro* co-culture system to study synovium-chondrocyte interactions (Chapter 2)
2. Assess the effect of PTOA synovium on chondrocytes and compare early and later stage PTOA synovium (Chapter 2)
3. Investigate the effect of manipulating synovial macrophages on chondrocyte and joint outcomes (Chapter 3).

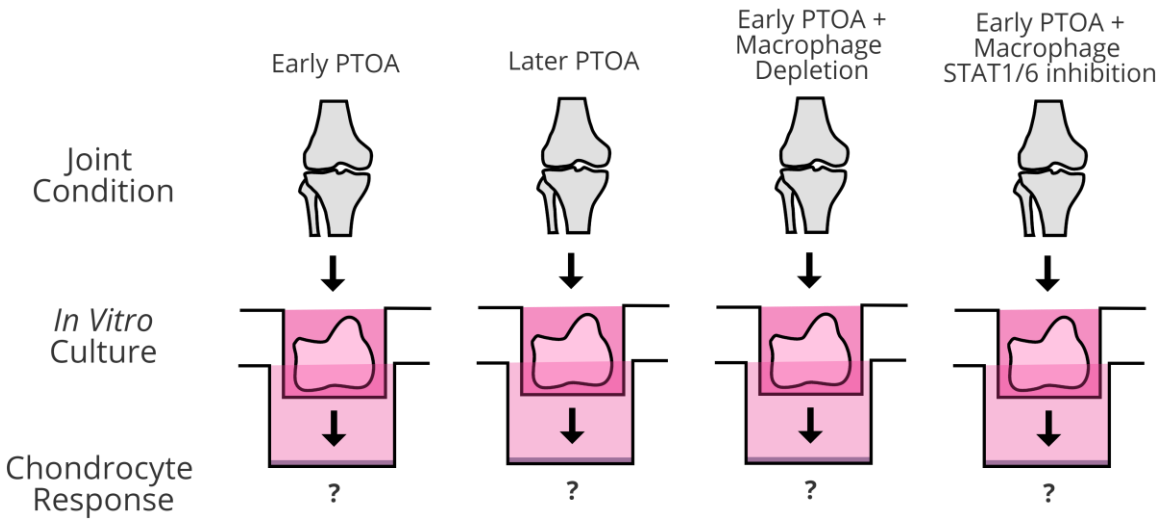

Figure 1.5. Schematic of research objectives.

1.4 References

1. Sophia Fox AJ, Bedi A, Rodeo SA. The basic science of articular cartilage: structure, composition, and function. Sports health. 2009 Nov;1(6):461-8.
2. Furey MJ, Burkhardt BM. Biotribology: friction, wear, and lubrication of natural synovial joints. Lubrication Science. 1997 May;9(3):255-71.
3. Cohen NP, Foster RJ, Mow VC. Composition and dynamics of articular cartilage: structure, function, and maintaining healthy state. Journal of Orthopaedic & Sports Physical Therapy. 1998 Oct;28(4):203-15.
4. Knudson CB, Knudson W. Cartilage proteoglycans. InSeminars in cell & developmental biology 2001 Apr 1 (Vol. 12, No. 2, pp. 69-78). Academic Press.
5. Eyre D. Articular cartilage and changes in arthritis: collagen of articular cartilage. Arthritis Research & Therapy. 2001 Oct 1;4(1):30.
6. Poole CA. Articular cartilage chondrons: form, function and failure. The Journal of Anatomy. 1997 Jul;191(1):1-3.
7. Wilusz RE, Sanchez-Adams J, Guilak F. The structure and function of the pericellular matrix of articular cartilage. Matrix biology. 2014 Oct 1;39:25-32.
8. Heinegård D, Saxne T. The role of the cartilage matrix in osteoarthritis. Nature Reviews Rheumatology. 2011 Jan;7(1):50.
9. Eyre DR, Weis MA, Wu JJ. Articular cartilage collagen: an irreplaceable framework. Eur Cell Mater. 2006 Nov 2;12(1):57-63.
10. Martin JA, Brown T, Heiner A, Buckwalter JA. Post-traumatic osteoarthritis: the role of accelerated chondrocyte senescence. Biorheology. 2004 Jan 1;41(3-4):479-91.
11. Maroudas A, Bayliss MT, Uchitel-Kaushansky N, Schneiderman R, Gilav E. Aggrecan turnover in human articular cartilage: use of aspartic acid racemization as a marker of molecular age. Archives of biochemistry and biophysics. 1998 Feb 1;350(1):61-71.
12. Verzijl N, DeGroot J, Thorpe SR, Bank RA, Shaw JN, Lyons TJ, Bijlsma JW, Lafeber FP, Baynes JW, TeKoppele JM. Effect of collagen turnover on the accumulation of advanced glycation end products. Journal of Biological Chemistry. 2000 Dec 15;275(50):39027-31.
13. Bertrand J, Hubert J. Overview. In: Metabolism of Human Diseases: Organ Physiology and Pathophysiology. 2014 April 11.
14. Dryll A, Lansaman J, Peltier AP, Ryckewaert A. Cellular junctions in normal and inflammatory human synovial membrane revealed by tannic acid and freeze fracture. Virchows Archiv A. 1980 Apr 1;386(3):293-302.
15. Iwanaga T, Shikichi M, Kitamura H, Yanase H, Nozawa-Inoue K. Morphology and functional roles of synoviocytes in the joint. Archives of histology and cytology. 2000;63(1):17-31.
16. Laurent TC, Laurent UB, Fraser J. Functions of hyaluronan. Annals of the rheumatic diseases. 1995 May;54(5):429.
17. Tu J, Guo Y, Hong W, Zhang P, Wang X, Chen X, Lu S, Wei W. The ontogeny of synovial macrophages and the roles of synovial macrophages from different origins in arthritis. Frontiers in immunology. 2019;10:1146.

18. Johnson VL, Hunter DJ. The epidemiology of osteoarthritis. Best practice & research Clinical rheumatology. 2014 Feb 1;28(1):5-15.
19. Arden N, Nevitt MC. Osteoarthritis: epidemiology. Best practice & research Clinical rheumatology. 2006 Feb 1;20(1):3-25.
20. Dieppe PA, Lohmander LS. Pathogenesis and management of pain in osteoarthritis. The Lancet. 2005 Mar 12;365(9463):965-73.
21. Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. Arthritis & Rheumatism. 2012 Jun;64(6):1697-707.
22. Glyn-Jones S, Palmer AJ, Agricola R, Price AJ, Vincent TL, Weinans H, Carr AJ. Osteoarthritis. The Lancet. 2015 Jul 25;386(9991):376-87.
23. Anderson AS, Loeser RF. Why is osteoarthritis an age-related disease?. Best practice & research Clinical rheumatology. 2010 Feb 1;24(1):15-26.
24. Spector TD, MacGregor AJ. Risk factors for osteoarthritis: genetics. Osteoarthritis and cartilage. 2004 Jan 1;12:39-44.
25. Berenbaum F, Eymard F, Houard X. Osteoarthritis, inflammation and obesity. Current opinion in rheumatology. 2013 Jan 1;25(1):114-8.
26. Troeberg L, Nagase H. Proteases involved in cartilage matrix degradation in osteoarthritis. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2012 Jan 1;1824(1):133-45.
27. Karsdal MA, Madsen SH, Christiansen C, Henriksen K, Fosang AJ, Sondergaard BC. Cartilage degradation is fully reversible in the presence of aggrecanase but not matrix metalloproteinase activity. Arthritis research & therapy. 2008 Jun;10(3):R63.
28. Fichter M, Körner U, Schömburg J, Jennings L, Cole AA, Mollenhauer J. Collagen degradation products modulate matrix metalloproteinase expression in cultured articular chondrocytes. Journal of orthopaedic research. 2006 Jan;24(1):63-70.
29. Tchetina EV. Developmental mechanisms in articular cartilage degradation in osteoarthritis. Arthritis. 2010 Dec 29;2011. Burr DB, Gallant MA. Bone remodelling in osteoarthritis. Nature Reviews Rheumatology. 2012 Nov;8(11):665.
30. Weinans H, Siebelt M, Agricola R, Botter SM, Piscaer TM, Waarsing JH. Pathophysiology of peri-articular bone changes in osteoarthritis. Bone. 2012 Aug 1;51(2):190-6.
31. Scanzello CR, Goldring SR. The role of synovitis in osteoarthritis pathogenesis. Bone. 2012 Aug 1;51(2):249-57.
32. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Annals of the rheumatic diseases. 2005 Sep 1;64(9):1263-7.
33. Loeuille D, Chary-Valckenaere I, Champigneulle J, Rat AC, Toussaint F, Pinzano-Watrin A, Goebel JC, Mainard D, Blum A, Pourel J, Netter P. Macroscopic and microscopic features of synovial membrane inflammation in the osteoarthritic knee: correlating magnetic resonance imaging findings with disease severity. Arthritis & Rheumatism. 2005 Nov;52(11):3492-501.
34. Krasnokutsky S, Belitskaya-Lévy I, Bencardino J, Samuels J, Attur M, Regatte R, Rosenthal P, Greenberg J, Schweitzer M, Abramson SB, Rybak L. Quantitative

magnetic resonance imaging evidence of synovial proliferation is associated with radiographic severity of knee osteoarthritis. Arthritis & Rheumatism. 2011 Oct;63(10):2983-91.
35. Roemer FW, Guermazi A, Felson DT, Niu J, Nevitt MC, Crema MD, Lynch JA, Lewis CE, Torner J, Zhang Y. Presence of MRI-detected joint effusion and synovitis increases the risk of cartilage loss in knees without osteoarthritis at 30-month follow-up: the MOST study. Annals of the rheumatic diseases. 2011 Oct 1;70(10):1804-9.
36. Damman W, Liu R, Bloem JL, Rosendaal FR, Reijnierse M, Kloppenburg M. Bone marrow lesions and synovitis on MRI associate with radiographic progression after 2 years in hand osteoarthritis. Annals of the rheumatic diseases. 2017 Jan 1;76(1):214-7.
37. Hill CL, Gale DG, Chaisson CE, Skinner KA, Kazis LE, Gale ME, Felson DT. Knee effusions, popliteal cysts, and synovial thickening: association with knee pain in osteoarthritis. The Journal of rheumatology. 2001 Jun 1;28(6):1330-7.
38. Hill CL, Hunter DJ, Niu J, Clancy M, Guermazi A, Genant H, Gale D, Grainger A, Conaghan P, Felson DT. Synovitis detected on magnetic resonance imaging and its relation to pain and cartilage loss in knee osteoarthritis. Annals of the rheumatic diseases. 2007 Dec 1;66(12):1599-603.
39. Kortekaas MC, Kwok WY, Reijnierse M, Watt I, Huizinga TW, Kloppenburg M. Pain in hand osteoarthritis is associated with inflammation: the value of ultrasound. Annals of the rheumatic diseases. 2010 Jul 1;69(7):1367-9.
40. Haugen IK, Bøyesen P, Slatkowsky-Christensen B, Sesseng S, van der Heijde D, Kvien TK. Associations between MRI-defined synovitis, bone marrow lesions and structural features and measures of pain and physical function in hand osteoarthritis. Annals of the rheumatic diseases. 2012 Jun 1;71(6):899-904.
41. Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. Arthritis research & therapy. 2006 Dec;8(6):R187.
42. Zhai G, Doré J, Rahman P. TGF-β signal transduction pathways and osteoarthritis. Rheumatol Int. 2015 Mar 15;35(8):1283-1292.
43. Blaney Davidson EN, van der Kraan PM, van den Berg WB. TGF-β and osteoarthritis. Osteoarthr Cartil. 2007 Jun;15(6):597-604.
44. Englund M. The role of the meniscus in osteoarthritis genesis. Rheumatic disease clinics of North America. 2008 Aug 1;34(3):573-9.
45. Ashraf S, Wibberley H, Mapp PI, Hill R, Wilson D, Walsh DA. Increased vascular penetration and nerve growth in the meniscus: a potential source of pain in osteoarthritis. Annals of the rheumatic diseases. 2011 Mar 1;70(3):523-9.
46. Gandhi R, Takahashi M, Virtanen C, Syed K, Davey JR, Mahomed NN. Microarray analysis of the infrapatellar fat pad in knee osteoarthritis: relationship with joint inflammation. The Journal of rheumatology. 2011 Sep 1;38(9):1966-72.
47. Ioan-Facsinay A, Kloppenburg M. An emerging player in knee osteoarthritis: the infrapatellar fat pad. Arthritis research & therapy. 2013 Dec 1;15(6):225.
48. Cross M, Smith E, Hoy D, Nolte S, Ackerman I, Fransen M, Bridgett L, Williams S, Guillemin F, Hill CL, Laslett LL. The global burden of hip and knee

osteoarthritis: estimates from the global burden of disease 2010 study. Annals of the rheumatic diseases. 2014 Jul 1;73(7):1323-30.
49. Neogi T. The epidemiology and impact of pain in osteoarthritis. Osteoarthritis and cartilage. 2013 Sep 1;21(9):1145-53.
50. Public Health Agency of Canada. Prevalence of chronic diseases among Canadian adults 2019. https://www.canada.ca/en/public-health/services/chronic-diseases/prevalence-canadian-adults-infographic-2019.html
51. Bombardier C, Hawker G, Mosher D. The impact of arthritis in Canada: today and over 30 years: Arthritis Alliance of Canada 2011. http://www.arthritisalliance.ca/images/PDF/eng/Initiatives/20111022_2200_impact_of_arthritis.pdf
52. Dunlop DD, Manheim LM, Yelin EH, Song J, Chang RW. The costs of arthritis. Arthritis Care & Research: Official Journal of the American College of Rheumatology. 2003 Feb 15;49(1):101-13.
53. Losina E, Walensky RP, Reichmann WM, Holt HL, Gerlovin H, Solomon DH, Jordan JM, Hunter DJ, Suter LG, Weinstein AM, Paltiel AD. Impact of obesity and knee osteoarthritis on morbidity and mortality in older Americans. Annals of internal medicine. 2011 Feb 15;154(4):217.
54. Hawker GA, Stewart L, French MR, Cibere J, Jordan JM, March L, Suarez-Almazor M, Gooberman-Hill R. Understanding the pain experience in hip and knee osteoarthritis–an OARSI/OMERACT initiative. Osteoarthritis and cartilage. 2008 Apr 1;16(4):415-22.
55. Scopaz KA, Piva SR, Wisniewski S, Fitzgerald GK. Relationships of fear, anxiety, and depression with physical function in patients with knee osteoarthritis. Archives of physical medicine and rehabilitation. 2009 Nov 1;90(11):1866-73.
56. Stubbs B, Aluko Y, Myint PK, Smith TO. Prevalence of depressive symptoms and anxiety in osteoarthritis: a systematic review and meta-analysis. Age and ageing. 2016 Jan 20;45(2):228-35.
57. Zhang W, Doherty M, Leeb BF, Alekseeva L, Arden NK, Bijlsma JW, Dinçer F, Dziedzic K, Häuselmann HJ, Herrero-Beaumont G, Kaklamanis P. EULAR evidence based recommendations for the management of hand osteoarthritis: report of a Task Force of the EULAR Standing Committee for International Clinical Studies Including Therapeutics (ESCISIT). Annals of the rheumatic diseases. 2007 Mar 1;66(3):377-88.
58. Zhang W, Moskowitz RW, Nuki G, Abramson S, Altman RD, Arden N, Bierma-Zeinstra S, Brandt KD, Croft P, Doherty M, Dougados M. OARSI recommendations for the management of hip and knee osteoarthritis, Part II: OARSI evidence-based, expert consensus guidelines. Osteoarthritis and cartilage. 2008 Feb 1;16(2):137-62.
59. Fernandes L, Hagen KB, Bijlsma JW, Andreassen O, Christensen P, Conaghan PG, Doherty M, Geenen R, Hammond A, Kjeken I, Lohmander LS. EULAR recommendations for the non-pharmacological core management of hip and knee osteoarthritis. Annals of the rheumatic diseases. 2013 Jul 1;72(7):1125-35.
60. McAlindon TE, Bannuru R, Sullivan MC, Arden NK, Berenbaum F, Bierma-Zeinstra SM, Hawker GA, Henrotin Y, Hunter DJ, Kawaguchi H, Kwoh K.

OARSI guidelines for the non-surgical management of knee osteoarthritis. Osteoarthritis and cartilage. 2014 Mar 1;22(3):363-88.
61. Hochberg MC, Altman RD, Brandt KD, Clark BM, Dieppe PA, Griffin MR, Moskowitz RW, Schnitzer TJ. Guidelines for the medical management of osteoarthritis. Arthritis & Rheumatism. 1995 Nov 1;38(11):1535-40.
62. Gay C, Chabaud A, Guilley E, Coudeyre E. Educating patients about the benefits of physical activity and exercise for their hip and knee osteoarthritis. Systematic literature review. Annals of physical and rehabilitation medicine. 2016 Jun 1;59(3):174-83.
63. Uthman OA, van der Windt DA, Jordan JL, Dziedzic KS, Healey EL, Peat GM, Foster NE. Exercise for lower limb osteoarthritis: systematic review incorporating trial sequential analysis and network meta-analysis. Bmj. 2013 Sep 20;347:f5555.
64. Jüni P, Hari R, Rutjes AW, Fischer R, Silletta MG, Reichenbach S, da Costa BR. Intra-articular corticosteroid for knee osteoarthritis. Cochrane Database of Systematic Reviews. 2015(10).
65. Hunter DJ. Pharmacologic therapy for osteoarthritis—the era of disease modification. Nature Reviews Rheumatology. 2011 Jan;7(1):13.
66. Kuyinu EL, Narayanan G, Nair LS, Laurencin CT. Animal models of osteoarthritis: classification, update, and measurement of outcomes. Journal of orthopaedic surgery and research. 2016 Dec;11(1):19.
67. Teeple E, Jay GD, Elsaid KA, Fleming BC. Animal models of osteoarthritis: challenges of model selection and analysis. The AAPS journal. 2013 Apr 1;15(2):438-46.
68. Appleton CT, McErlain DD, Pitelka V, Schwartz N, Bernier SM, Henry JL, Holdsworth DW, Beier F. Forced mobilization accelerates pathogenesis: characterization of a preclinical surgical model of osteoarthritis. Arthritis research & therapy. 2007 Feb;9(1):R13.
69. Appleton CT, Pitelka V, Henry J, Beier F. Global analyses of gene expression in early experimental osteoarthritis. Arthritis & Rheumatism. 2007 Jun;56(6):1854-68.

Chapter 2

2 A novel *ex vivo* joint co-culture system for investigating synovium-chondrocyte interactions demonstrates protective effects of synovium in early experimental osteoarthritis.

2.1 Chapter Summary

Osteoarthritis (OA) is a degenerative joint disease with rising burden and no disease-modifying drug available. The impact of the synovial tissue on cartilage in OA is not fully understood but may be a strategic therapeutic target. Co-culturing synovial tissue and primary articular chondrocytes obtained from a rat model of post-traumatic knee OA (PTOA) allows us to directly assess chondrocyte responses to synovial signalling. This study optimized conditions of a novel *in vitro* joint model and evaluated the effects of synovium from experimental PTOA joints on naïve chondrocytes.

Whole-joint synovial tissue from early (4 weeks post-PTOA induction) or later (12 weeks) stage OA, or time-matched controls (surgery-naïve or sham surgery), was placed into a transwell system with adult rat primary articular chondrocytes grown in high-density monolayer. Conditioned medium was collected to measure sulfated glycosaminoglycan secretion and chondrocyte RNA was isolated for gene expression analysis.

Early PTOA synovium induced increased sulfated glycosaminoglycan secretion, decreased *Col2a1* expression, and increased *Mmp3* and *Prg4* expression in primary articular chondrocytes compared to controls. These effects were lost with later stage PTOA synovium, which only decreased *Ccl2* expression in chondrocytes.

In conclusion, early PTOA synovium induces changes in chondrocyte physiology that include regenerative responses, whereas later stage PTOA synovium does not induce these effects.

2.2 Introduction

Osteoarthritis (OA) is one of the most urgent global health problems[1,2]. In Canada, OA is projected to affect over 1 in 5 people by 2025[3]. OA typically affects major joints, especially the hip, knee, spine, and hand. As a disease of the whole joint, OA causes pathological changes in all joint tissues, including cartilage, synovium, subchondral bone, meniscus, muscle, tendon, and ligament[4]. OA patients suffer joint pain, reduced function, and disability, but there is no treatment approved to prevent progression to end-stage disease. Evidence-based treatments for OA are only partially effective for management of symptoms, such as pain and stiffness, but cannot slow or reverse disease damage[5,6]. Patients with end-stage disease usually require surgery to replace failing tissues with an implant. While highly effective for a majority of patients, full recovery times from surgery routinely exceed one year and patients experience wide variation in post-surgery outcomes, which are partially influenced by functional ability and comorbid conditions, and some patients require additional revision or implant replacement[6,7,8,9]. Conceptually, early stage medical treatment of OA is therefore an ideal approach to prevent disease progression before radiographic joint damage and loss of function occur[10,11]. Our understanding of the earliest events in OA pathophysiology is limited. Primary OA is idiopathic, while secondary OA follows previous post-traumatic, congenital, biomechanical, or metabolic derangements. Secondary forms of OA are typically easier to model, especially in animals, but share several pathophysiologic and morphologic features with primary OA[12]. Research remains focused on cartilage above other joint tissues.

Cartilage is the site of much change through progression of the disease; when abnormal stresses are applied, chondrocytes rouse from a quiescent state to actively proliferate and produce increased levels of matrix proteins and proteases[13,14]. Catabolic and inflammatory processes damage the matrix over time, with limited regeneration due to the absence of innervation and vasculature that trigger the normal healing sequence in other tissues. As the disease progresses, more chondrocytes become affected and eventually undergo terminal hypertrophy. In late-stage OA, the once-populated cartilage erodes away to underlying bone which articulates poorly.

Synovium is a structurally and functionally heterogenous tissue responsible for maintaining the homeostasis of all joint tissues, including joint cartilage. Synovium provides lubrication by producing synovial fluid, clears debris from the joint cavity, supplies nutrition to cartilage and other joint tissues, and monitors the joint environment for infection and other disturbances[15]. As synovitis has only recently been recognized as a key feature of OA, our understanding of the physiology and roles of the synovium is far from complete[16]. The process by which synoviocytes become activated in early OA is unknown, but likely involves matrix breakdown products and inflammatory signals from cartilage[17]. Synovitis is found in a majority of early and late OA patients, and presents with greater severity in later stages of OA[18,19]. In the knee, clinical studies have shown strong correlation between synovitis and greater pain[16,20,21]. The presence of synovitis is predictive of knee OA incidence and progression and hand OA progression[16,22,23,24,25,26,27,28,29]. Knee synovitis is heterogenous and may appear in any area, but certain patterns are more frequent and have been linked to differences in pain[30]. Synovial production of catabolic and pro-inflammatory factors such as matrix metalloproteinases (MMPs) and tumour necrosis factor-alpha (TNF-α) further suggests a pathogenic role, but anti-inflammatory mediators are also produced by OA synovium. Adding further uncertainty to the role of synovium in OA, clinical trials blocking inflammatory factors such as TNF-α and interleukin-1 beta have not sufficiently shown therapeutic effect[31,32]. Ongoing trials using anti-inflammatory factors such as interleukin-4 or interleukin-10 have shown promising preliminary results[33,34]. Another promising biologic, recombinant fibroblast growth factor 18, produced anabolic effects on cartilage but no effect on pain and stiffness in clinical trials, indicating that treating cartilage damage alone is insufficient[35]. Changes in levels of other growth factors such as nerve growth factor and transforming growth factor-β (TGF-β) superfamily proteins have also been noted in OA[36,37]. TGF-β1, which induces expression of matrix proteins and prevents chondrocyte hypertrophy, is affected by and affects inflammatory signals in the joint[37,38].

Synovitis is clearly an important feature in OA, but its impact on surrounding joint tissues such as articular cartilage is not well understood. Furthermore, there is little contextual information regarding potential differences in the functions of synovium at different stages of OA. Since early OA potentially presents the best strategic window for

introduction of medical treatment for OA, it is particularly important to understand synovium-cartilage interactions during OA development. In this study, we assess the effect of synovium from early and later stage OA joints on primary articular chondrocytes. By integrating *in vitro* chondrocyte cultures with *ex vivo* synovial tissues from an established rat model of experimental post-traumatic OA (PTOA), we establish a new model of intraarticular signaling to test our overall hypothesis that synovium from early PTOA joints induces pro-inflammatory and catabolic changes in chondrocytes.

2.3 Materials and Methods

2.3.1 Reagents

Chemical reagents and histology supplies were purchased from Fisher Scientific or MilliporeSigma unless otherwise specified. Cell and tissue culture media were purchased from Gibco, while tissue culture plastics were from BD Falcon. RNA isolation reagents were purchased from Invitrogen. PCR reagents and equipment, including analysis software, were all supplied by Bio-Rad.

2.3.2 Experimental PTOA rat model

All experiments were performed according to the Animal Use Protocol approved by the institutional Animal Care Committee (Appendix A). Outbred Sprague-Dawley rats (strain code 400) were purchased from Charles River Canada and fed a standard chow diet *ad libitum*. Following a previously established protocol, surgery was performed on the right knees of 12-week-old male rats[39,40]. Female rats were not included in this study due to the known influence of sex hormones on OA, and to simplify experimental logistics. Briefly, animals were given prophylactic ampicillin and buprenorphine for post-procedure analgesia subcutaneously. Anesthesia was induced with isoflurane gas. PTOA was induced by incising the medial aspect of the anterior joint capsule to open the right knee joint, transecting the anterior cruciate ligament and the medial meniscotibial ligament at its attachment to the meniscus, and closing the joint capsule with absorbable sutures. Sham surgery was performed on age-matched animals by opening and closing the joint capsule only. Animals did not undergo forced mobilization and were allowed to recover with monitoring in home cages. Surgery-naïve control animals were also age-matched. Only the operated right knee joints were experimentally compared, as changes in joint tissue physiology occur in the contralateral knee joint and may confound interpretation of results[40]. This protocol induces OA-like disease at a timeline and severity comparable to similar surgical models (data not shown).

2.3.3 Cell and tissue cultures

Knee joint tissues were collected by gross dissection. Overlying skin, muscle, and tendon were removed from legs before opening the joint under sterile conditions.

Primary articular chondrocytes were collected from healthy, surgically-naïve 16-week-old male rats. Femoral and tibial articular surfaces were shaved using a scalpel, carefully excluding contamination by periosteum, meniscus, or bone tissues. Cartilage from multiple joints was pooled in Dulbecco's Modified Eagle Medium, low glucose, with L-glutamine (DMEM), minced into pieces of approximately 1 mm^3, and placed into DMEM with 10% fetal bovine serum (FBS), 1% Antibiotic-Antimycotic, 800 U/mL collagenase II, and 100 U/mL DNase I for 2 hours at 37C with variable agitation on a Miltenyi Biotec gentleMACS Dissociator. The resulting suspension was triturated, passed through a 70 µm cell strainer and washed with primary chondrocyte medium (1:1 DMEM/Ham's F12, low glucose, L-glutamine, with 10% FBS and 1% penicillin-streptomycin). Live cells were counted on a Bio-Rad TC20. P0 chondrocytes were initially seeded at 10% density into 12-well plates, one well per joint, in primary chondrocyte medium, with medium changes every 2-3 days until confluent. For expansion into stocks, P0 chondrocytes were seeded at 2-5% in T75 flasks in primary chondrocyte medium, with medium changes every 2-3 days until confluent. Expanded P0 chondrocytes were trypsinized, gradually frozen to -80C in FBS with 10% DMSO, and stored in liquid nitrogen.

Knee joint synovium was dissected over ice-cold DMEM with care to keep the tissue intact. After opening the joint by cutting around the suprapatellar region, the menisci and patella were removed, and synovium was peeled away from the outer fibrous capsule. Preliminary experiments used the mixed population of synoviocytes isolated by placing minced synovium in DMEM with 10% FBS, 1% Antibiotic-Antimycotic, 100 U/mL collagenase IV, and 100 U/mL DNase I for 1 hour at 37C with variable agitation on the gentleMACS Dissociator. The digest suspension was passed through a 70 µm cell strainer, washed with DMEM with 10% FBS and 1% penicillin-streptomycin, and seeded at 10% in 12-well transwell inserts, 0.4 µm pore size. For subsequent experiments using whole synovial tissue in co-culture, all synovium from a single joint was placed into a

seated 12-well transwell insert with serum-free 1:1 DMEM/F12 with 1% penicillin-streptomycin in both chambers per replicate (n = 1).

To set up co-cultures, P0 chondrocytes were grown as described above, while P1 chondrocyte aliquots were thawed from frozen stocks, washed with primary chondrocyte medium, seeded at 10% density into 12-well plates, and expanded until confluent. Prior to the start of the culture period, chondrocytes were serum-starved overnight (8 to 16 hours) and synovial tissue was placed into serum-free medium for 24 hours. Co-cultures were assembled by transferring the synovium-conditioned medium from the lower chamber onto the serum-starved chondrocytes and placing the transwell insert with synovium and conditioned medium on top (Figure 2.1).

2.3.4 Tissue histology and immunohistochemistry

Synovial tissue from co-cultures were fixed in 4% paraformaldehyde overnight, processed, and embedded in paraplast (Leica Biosystems). Tissue sections were cut at 5 thickness and collected on Superfrost Plus slides (Fisher). H&E slides were stained with Harris Hematoxylin and 0.25% Eosin Y and mounted with Cytoseal XYL (Richard-Allan Scientific) under no.1 coverslips (VWR). Tissues used for immunofluorescence experiments underwent antigen retrieval by immersion in 10 mM Tris, 1 mM EDTA, 10% glycerol buffer heated to 70C and allowed to cool to room temperature, were blocked with 1% bovine serum albumin (GE Healthcare), 0.1% Triton X-100 in PBS, and probed with rabbit anti-cleaved caspase-3 primary antibody (Abcam ab49822) diluted 1:1000 in blocking buffer, followed by AlexaFluor488-conjugated goat anti-rabbit secondary antibody diluted 1:500 in blocking buffer, then mounted with ProLong Gold Antifade Mountant with DAPI. Slides were imaged on a BioTek Cytation 5 multi-mode imager.

2.3.5 Dimethylmethylene blue (DMMB) assay

Conditioned medium from co-cultures was assayed for sulfated glycosaminoglycan (sGAG) content using a protocol based on previously described methods[41,42]. In microwell plates, 200 µL DMMB reagent (38.46 mmol 1,9-dimethyl-methylene blue zinc

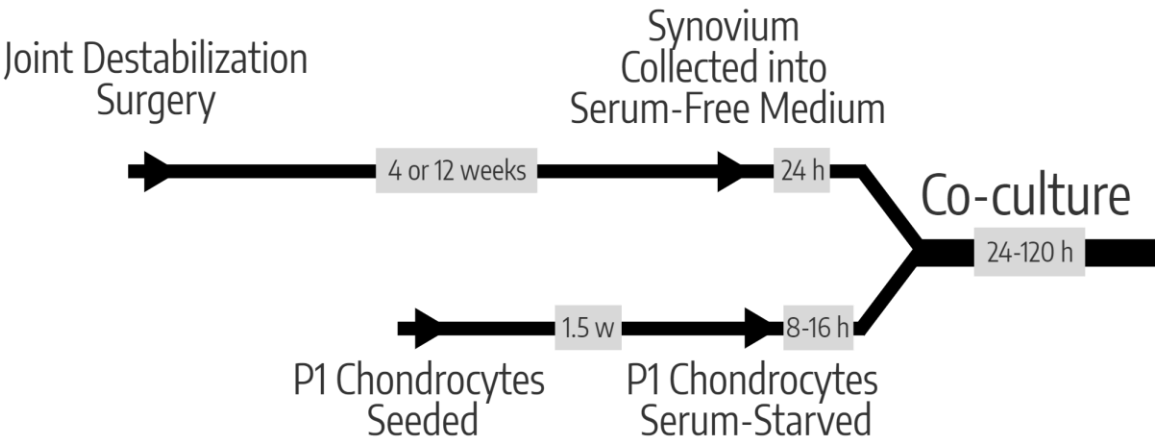

Figure 2.1 Experimental timeline for synovium-chondrocyte co-cultures.

Synovium from joints 4 or 12 weeks post joint destabilization surgery, sham surgery, or no surgery were collected into serum-free medium. Simultaneously, chondrocytes were expanded to confluency. Synovium and chondrocytes were then co-cultured.

chloride, 40.5 mmol glycine, 27.38 mmol sodium chloride, 9.5mmol acetic acid (Bioshop)) was combined with 100 µL of conditioned medium or chondroitin sulfate standards diluted to the appropriate range in serum-free chondrocyte medium in triplicate and read immediately at absorbance wavelength 595 nm with reference wavelength 655 nm on a Safire plate reader (Tecan Life Sciences).

2.3.6 RNA isolation and gene expression analysis

We made efforts to adhere to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines where possible regarding controls for contamination and technical error[43].

Co-cultured chondrocytes were lysed by adding TRIzol to each well and triturating. Samples were transferred to phase separation tubes, combined 5:1 with chloroform, shaken for 50 seconds, and centrifuged at 12000 x *g* for 15 minutes at 4C. The RNEasy Mini Kit (QIAGEN) was used following instructions for subsequent steps. The aqueous phase was combined with an equal volume of 70% ethanol and transferred onto silicaspin columns, followed by on-column DNase I digestion, washes, and elution into nuclease-free water. Eluted RNA yield and purity was measured on a Nanodrop 2000 (Thermo Scientific). Samples were reverse transcribed using iScript Reverse Transcription Supermix following manufacturer thermal cycle recommendations. Quantitative real-time PCR (qPCR) was performed by combining SsoAdvanced Universal SYBR Green Supermix, predesigned primers (Table 2.1), and cDNA template in triplicate. Template per reaction varied by gene. Plates were thermocycled as follows: an initial polymerase inhibitor removal step at 95C for 30 s, a repeating denaturation step at 95C for 15 s followed by an annealing and elongation step at 60C for 30 s for 40 cycles, with an ending melt-curve analysis from 65C to 95C in 0.5 C steps at 2 s/step. Cq values were calculated on CFXMaestro 1.1 and normalized to reference genes *Rpl13a* and *Rplp0*, except where usage of only *Rpl13a* is noted. Reference genes were selected prior to target gene assessment using the Bio-Rad pre-designed rat reference gene panel with representative samples (n = 3) and CFXMaestro Reference Gene Selector tool.

Table 2.1. Reference information for Bio-Rad PrimePCR™ PCR primers.

Gene	Amplicon Context Sequence	Transcription	Amplicon Length
Acan	1:141780455-141787109	intron-spanning	109
Adamts5	11:29043239-29056911	intron-spanning	120
Ccl2	10:69048547-69048678	exonic	102
Col10a1	20:42913429-42916882	intron-spanning	120
Col2a1	7:139647809-139647911	exonic	73
Il6	4:3095863-3097287	intron-spanning	120
Il17a	9:25698119-25699513	exonic	106
Mmp13	8:5531849-5533218	intron-spanning	87
Mmp3	8:5689395-5691167	intron-spanning	66
Prg4	13:72640183-72642005	intron-spanning	118
*Rpl13a**	1:102186223-102186424	exonic	87
*Rplp0**	12:48589001-48589127	exonic	97
S100a8	2:209508182-209508274	exonic	63
Sox9	10:100968094-100968214	exonic	91

* denotes a reference gene.

Initial gene analysis experiments used droplet digital PCR (ddPCR) for its high sensitivity and precision, but unexpected permanent equipment failure necessitated a return to traditional qPCR. ddPCR was performed by combining QX200 ddPCR EvaGreen Supermix, predesigned primers (Table 2.1), and cDNA template. Template per reaction varied by gene and was diluted to allow optimal digital analysis. Droplets were generated from 20 µL reaction mixes on the AutoDG Automated Droplet Generator, then immediately thermocycled with the following steps: 95C for 5 min, 95C for 30 s followed by 60C for 1 min for 45 cycles, 4C for 5min, and 90C for 5 min before holding at 4C, with all steps ramped at 2 C/s. Droplets were read and gated using QuantaSoft™ Analysis Pro 1.0. Absolute copy numbers were normalized to reference genes *Rpl13a* and *Rplp0* for comparison.

2.3.7 TGFBR1 inhibition and FOXO1 inhibition assays

SB 431542 is a well-established selective small molecule inhibitor of the TGF-β type I receptor (TGFBR1)[44]. AS 1842856 is a relatively new small molecule inhibitor of forkhead box O1 (FOXO1)[45]. To determine the minimum effective concentration for both drugs *in vitro*, P1 primary chondrocytes were grown to confluency in a 96-well plate, serum-starved overnight, pre-incubated with inhibitors (dissolved in DMSO vehicle) or DMSO only (control), diluted in serum-free medium for 1 hour, then stimulated with 10 ng/mL TGF-β for 24 hours. Cells were collected in TRIzol for mRNA isolation and qPCR analysis of *Prg4* expression relative to *Rpl13a* as described above.

For inhibition of TGFBR1 signalling in co-culture conditioned medium, P1 primary chondrocytes were again grown to confluency in 96 well plates and serum-starved. Cells were pre-conditioned with 1 µM SB 431542 or DMSO for 1 hour. Conditioned medium samples were thawed to room temperature from -20C storage, combined with inhibitor in DMSO vehicle or vehicle only, and applied to the well with minimal dilution (150 µL neat medium to 5 µL drug in serum-free medium) for 24 hours before collection for gene expression analysis.

2.3.8 Statistics

All data was analyzed using GraphPad Prism 8.2.1. Type I error threshold for all analyses was set at $\alpha = 0.05$. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used for sGAG secretion and gene expression over a timecourse (comparisons between 24h, 48h, 72h, and 96h) in P1 chondrocytes cultured alone in serum-free medium. One-way ANOVA followed by Dunnett's multiple comparisons test was used for the effects of synovium PTOA condition (comparisons between naïve, sham, and PTOA synovium) on chondrocytes and for the effects of TGFBR1 and FOXO1 inhibitors (comparisons between multiple concentrations of inhibitor and DMSO vehicle) on chondrocyte *Prg4* expression. An unpaired two-tailed t-test was used for the effect of TGF-β (comparison between TGF-β and PBS control) on chondrocyte *Prg4* expression. Two-way ANOVA followed by Sidak's multiple comparisons test was used for TGFBR1 inhibitor and conditioned medium treatment (comparisons between TGFBR1 inhibitor and DMSO vehicle with naïve, sham, or PTOA co-culture conditioned medium) on chondrocytes.

2.4 Results

2.4.1 P0 articular chondrocytes express baseline inter-individual variation

Preliminary primary cell co-cultures used P0 articular chondrocytes collected from multiple donor animals, where chondrocytes from a single donor animal were used in a single co-culture replicate. Each chondrocyte culture from one donor animal was grown to 70% confluency before being placed into co-culture with dissociated primary synoviocytes, also from one donor per culture, in serum-free medium for 48 hours. These co-cultures showed variable levels of sGAG secretion within groups, with no significant differences between treatments (Figure 2.2a). Moreover, sGAG levels were below expected values based on previous studies of adult primary chondrocytes in monolayer culture[46]. We revised our approach and grew chondrocytes to 90-100% confluency before co-culture under the same conditions. This resulted in higher sGAG levels than before, but values were still lower than expected (Figure 2.2b). No significant differences between treatments were found for these cultures.

Preliminary 48-hour co-cultures using P0 chondrocytes grown to 70% confluency showed no significant differences in expression of all chondrocyte genes of interest assayed (*Acan*, *Adamts5*, *Ccl2*, *Col2a1*, *Col10a1*, *Il6*, *Mmp3*, *Mmp13*, *S100a8*, *Sox9*) between treatments (Figure 2.3). We switched from standard real-time qPCR to ddPCR to improve detection sensitivity for low-copy genes to assay select genes for revised co-cultures using chondrocytes grown to 100% confluency, but this did not reduce the inter-individual variation between donors observed (Figure 2.4).

2.4.2 P1 articular chondrocytes maintain a chondrocyte-like phenotype

Preliminary experiments (2.4.1) indicated that P0 primary chondrocytes demonstrate a high degree of inter-individual variability in sGAG release and gene expression. Since our experimental design sets the synovium from each PTOA condition as the independent variable with chondrocyte response as the dependent variable, we elected to assess pooled primary articular chondrocytes from multiple donors for all co-cultures instead of using chondrocytes from one joint per co-culture. Thus, we expanded P0 chondrocytes pooled from multiple naïve joints into a large stock of P1 chondrocytes. Morphologically,

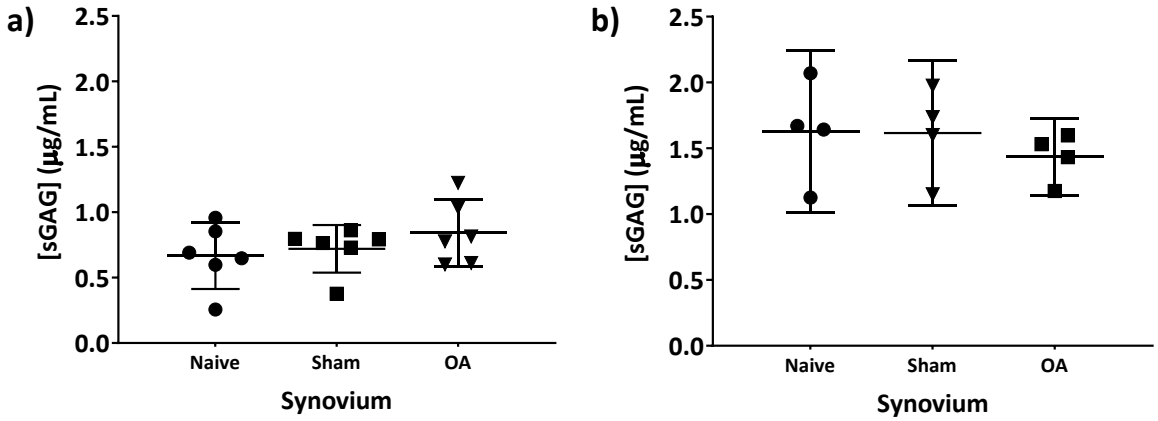

Figure 2.2. sGAG content in conditioned medium from P0 chondrocytes co-cultured with dissociated primary synoviocytes.

P0 primary articular chondrocytes were co-cultured at a) 70% confluency and b) 100% confluency with expanded primary synoviocytes dissociated from age-matched surgery-naïve, surgical sham, and 4W PTOA knee synovium for 48 hours. Conditioned medium was collected and measured for sGAG content by DMMB assay (mean ± 95% CI, n = 4-6).

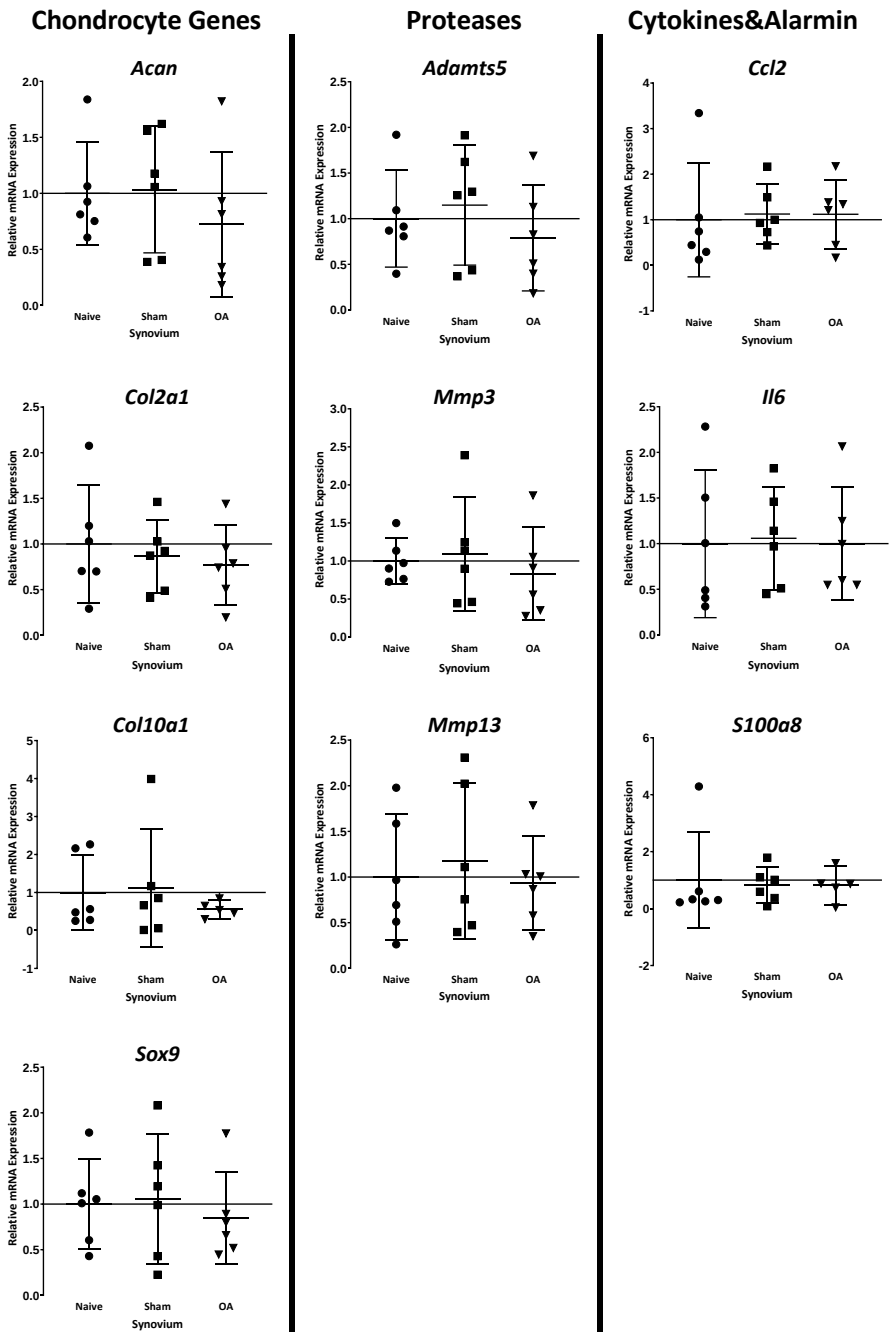

Figure 2.3. Gene expression in P0 chondrocytes co-cultured at 70% confluency with dissociated primary synoviocytes.

P0 primary articular chondrocytes were co-cultured at 70% confluency with expanded primary synoviocytes dissociated from age-matched surgery-naïve, surgical sham, and 4W PTOA knee synovium for 48 hours. qPCR analysis of gene expression levels is displayed as fold change (mean ± 95% CI, n = 6).

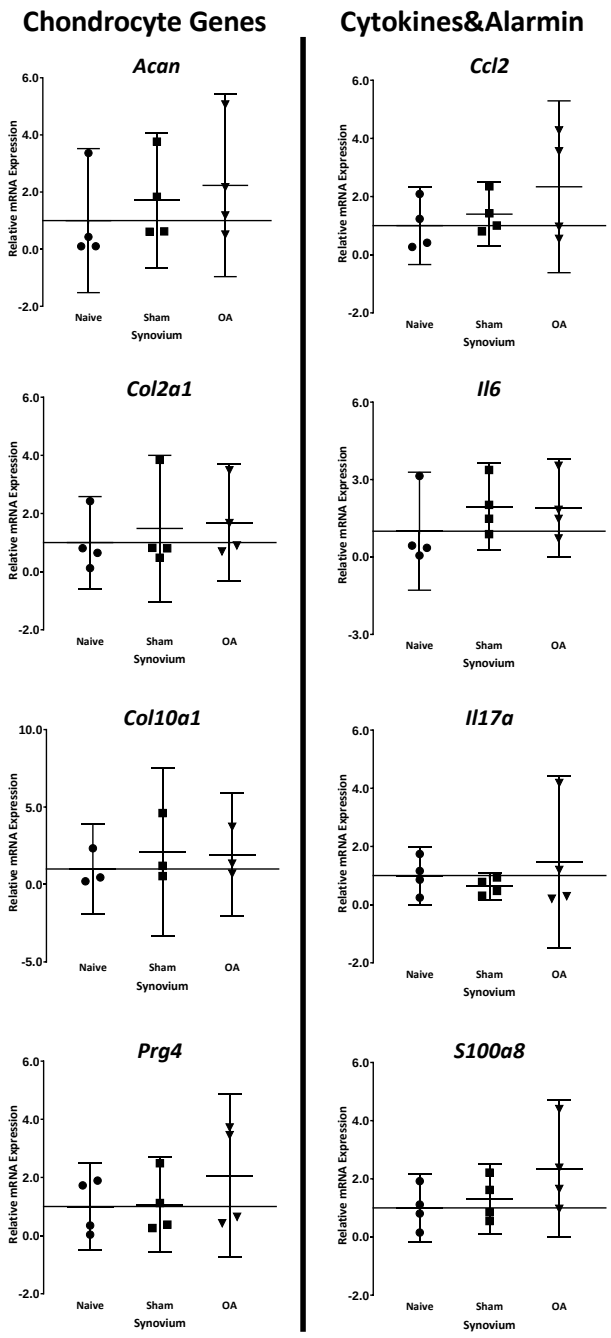

Figure 2.4. Gene expression in P0 chondrocytes co-cultured at confluency with dissociated primary synoviocytes.

P0 primary articular chondrocytes were co-cultured at 100% confluency with expanded synoviocytes dissociated from age-matched surgery-naïve, surgical sham, and 4W PTOA knee synovium for 48 hours. ddPCR analysis of gene expression levels is displayed as fold change (mean ± 95% CI, n = 4).

P1 cells resembled P0 cells, with broad triangular cell bodies during expansion and a more compact, granular "cobblestone" appearance at confluency (Figure 2.5), consistent with the literature[47,48]. Highly confluent monolayer cultures of P1 chondrocytes secreted sGAGs over time in a linear fashion similar to P0 chondrocytes, with significantly higher concentrations in the conditioned medium at 120 hours than at 24 hours (Figure 2.6). P1 chondrocyte expression of *Acan*, *Col2a1*, *Prg4*, *Runx2*, and *Sox9* did not significantly differ between timepoints, although *Prg4* appears to decline over time (Figure 2.7).

2.4.3 Synovial tissue remains viable in serum-free culture

Synovial tissue culture in standard conditions (FBS-supplemented medium, 37C, 5% CO_2) has previously been described[49,50]. As our initial experiments with high density P1 primary articular chondrocytes did not demonstrate a clear response to co-culture with dissociated expanded primary synoviocytes, we decided to use whole synovial tissue from a single knee joint for each co-culture. Rather than isolating synoviocytes, whole synovial tissue culture minimizes cell loss and *ex vivo* selection bias against synovial cell populations that are relatively more sensitive to enzymatic and mechanical tissue dissociation. Co-cultured synovium explants were fixed after 48 hours *ex vivo* and processed for histology to ensure cellular viability during the culture period. Sections stained with H&E found regular synovial tissue organization with no signs of diffuse or focal cell death such as cell swelling and rupture or nuclear condensation and fragmentation[51] (Figure 2.8a). Sections stained with DAPI and anti-cleaved caspase-3 antibody found very few positive apoptotic cells with random distribution through the tissue (Figure 2.8b).

2.4.4 Early PTOA synovium induces changes in primary articular chondrocyte physiology

To test the effects of early PTOA synovium on articular chondrocytes, we co-cultured synovial tissue explants from rat knees with P1 primary articular chondrocytes in monolayer culture. Co-culture for 24 hours with 4W PTOA synovium caused significantly increased sGAG secretion into conditioned medium compared to naïve

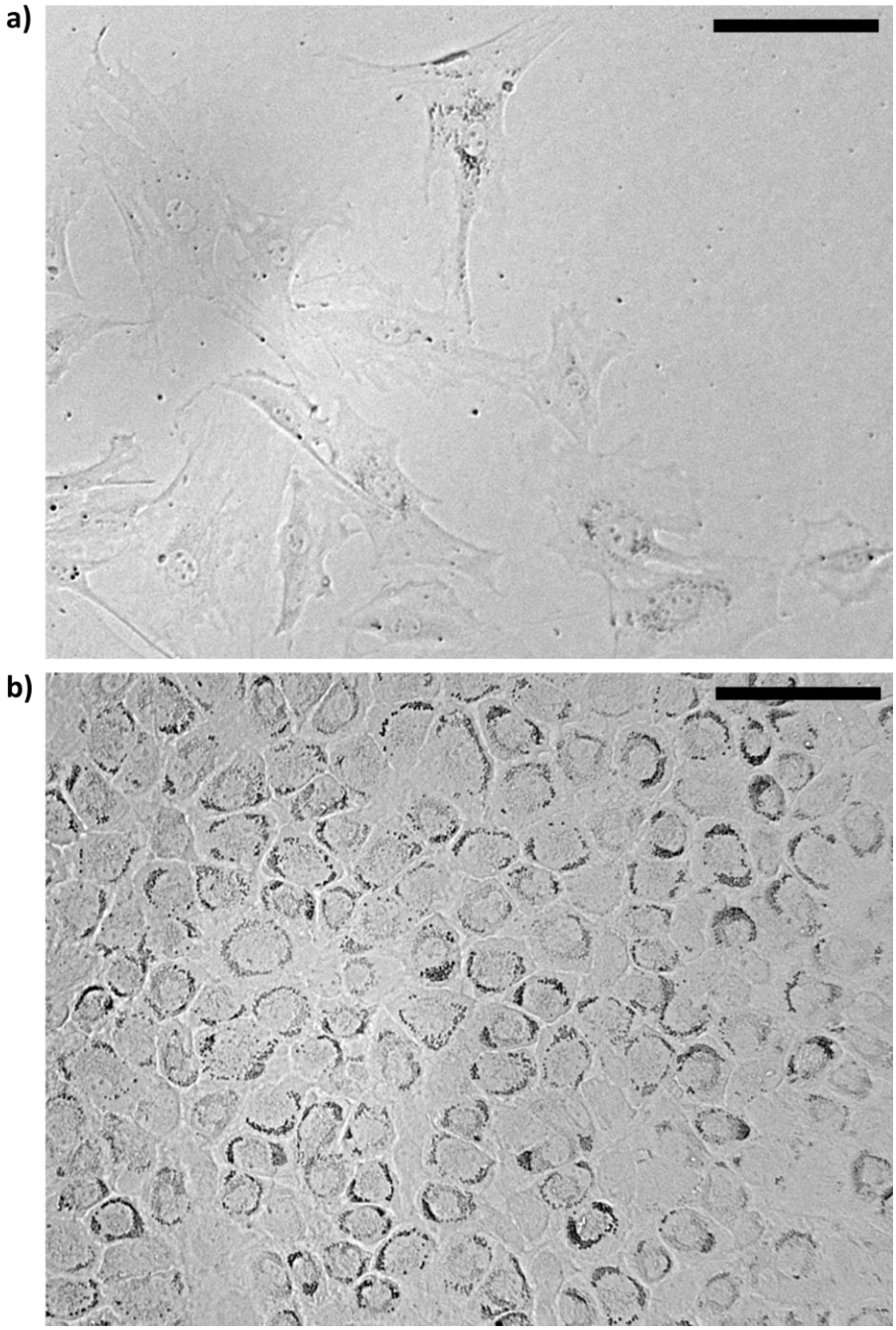

Figure 2.5. Morphology of P1 chondrocytes during expansion in monolayer culture.

Representative phase-contrast micrographs of P1 adult articular chondrocytes expanded in monolayer culture at a) 10% confluency and b) 100% confluency. Bar represents 100 µm.

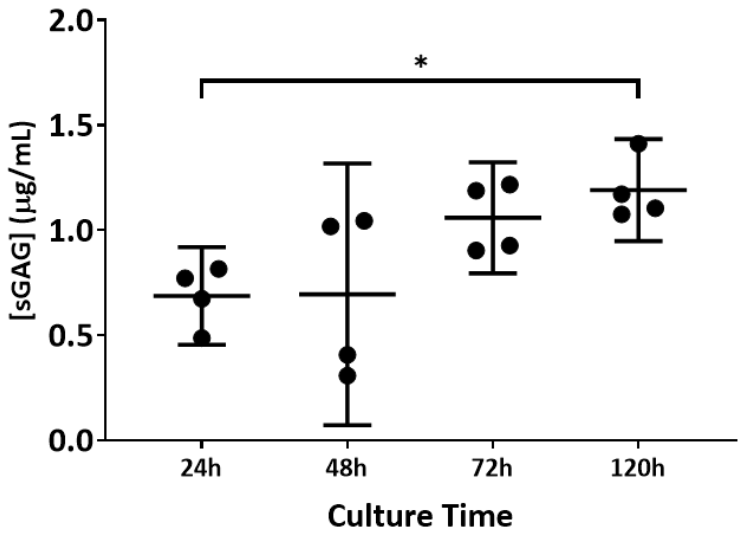

Figure 2.6. sGAG content in conditioned medium from P1 chondrocytes in serum-free conditions without synovial co-culture.

Confluent P1 primary articular chondrocytes were cultured in serum-free medium alone for up to 120 hours. Conditioned medium was collected and measured for sGAG content by DMMB assay (mean ± 95% CI, n = 4, *$p < 0.05$).

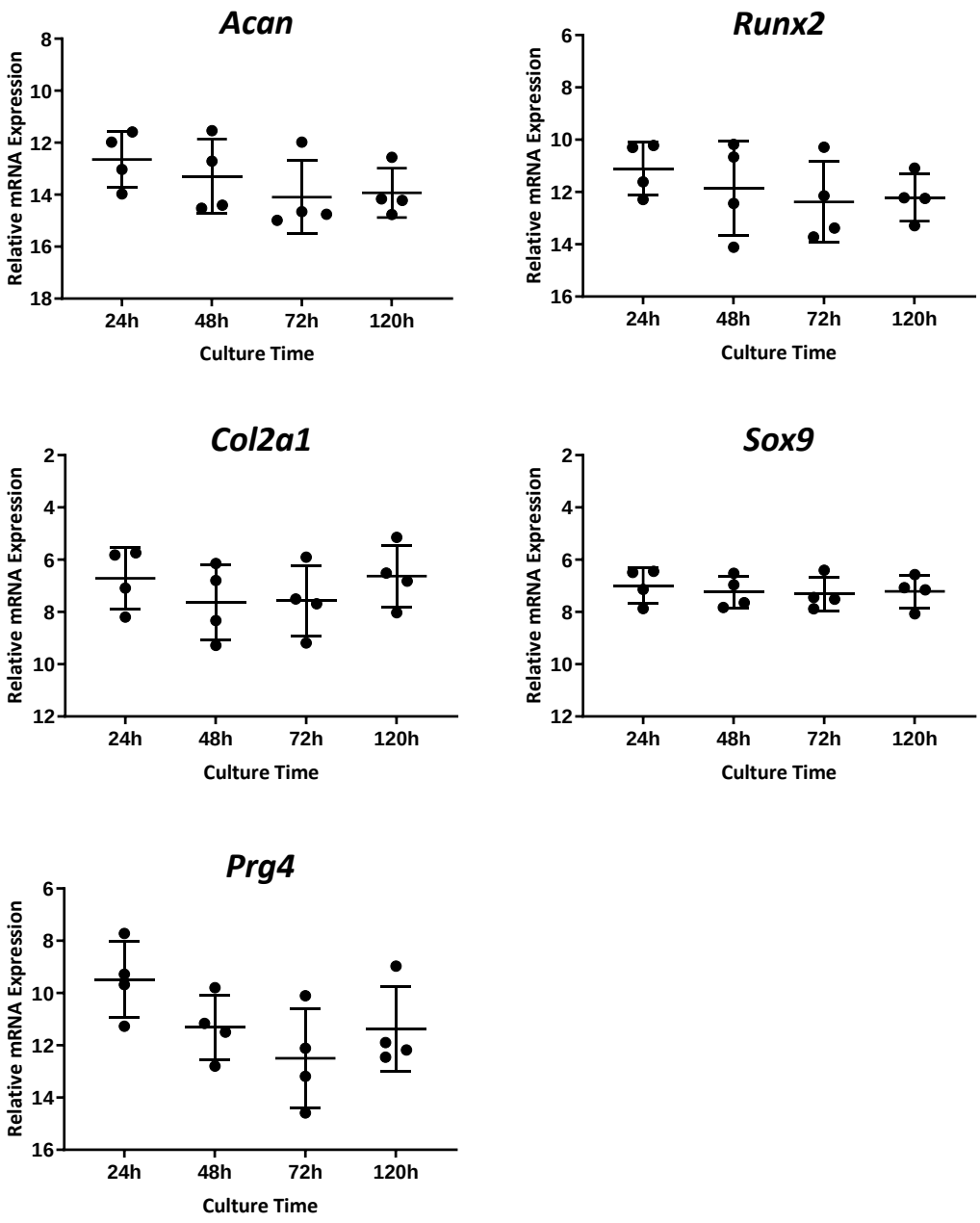

Figure 2.7. Gene expression in P1 chondrocytes in serum-free conditions without synovial co-culture.

Confluent P1 primary articular chondrocytes were cultured in serum-free medium alone for up to 120 hours. qPCR analysis of gene expression levels is displayed as ΔCq (mean ± 95% CI, n = 4).

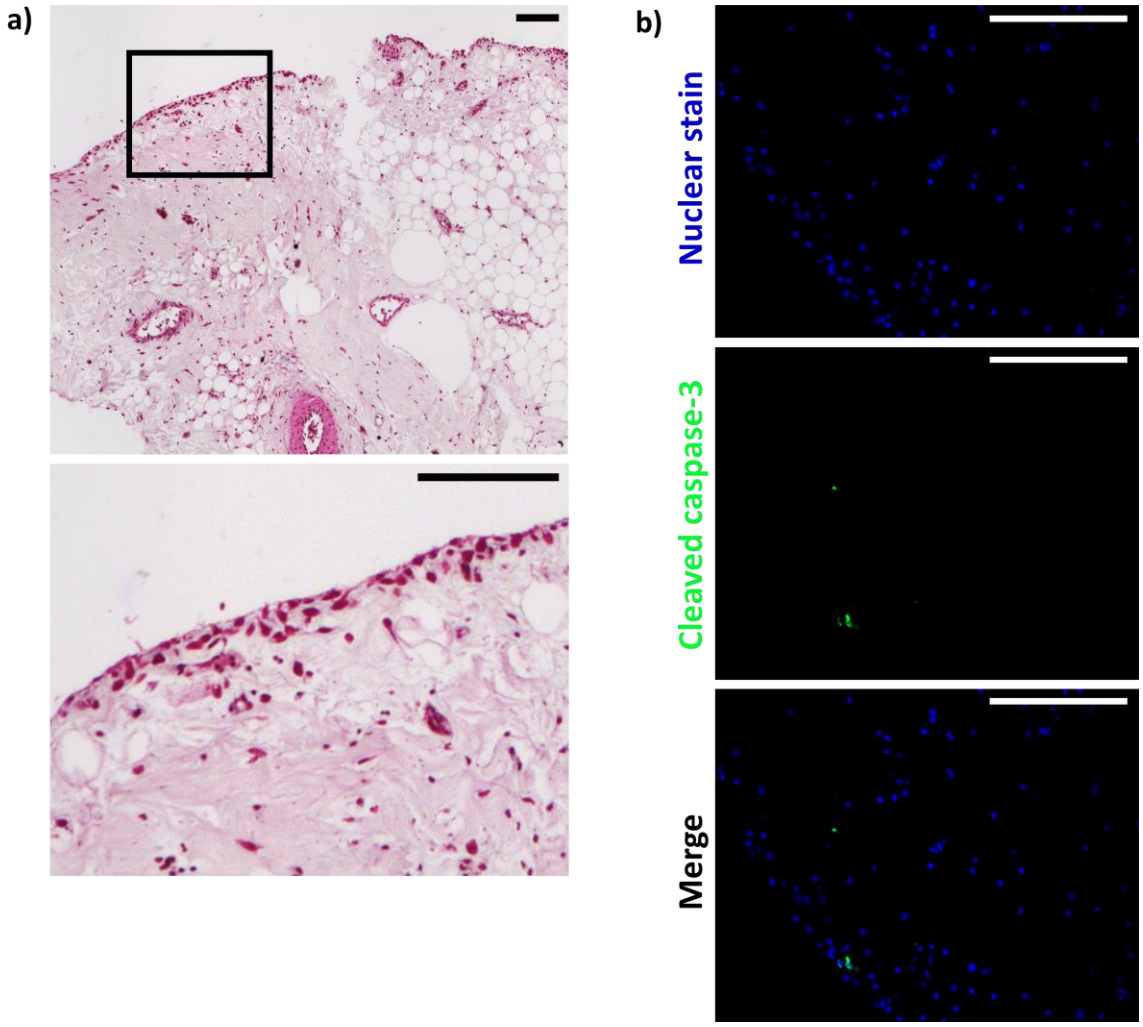

Figure 2.8. Viability of synovial tissue after 48 hours in serum-free co-culture with primary chondrocytes.

Representative micrographs of sections of synovial tissue after 24 h alone in serum-free culture followed by 24 h in co-culture with primary articular chondrocytes, with a) H&E staining with inset for tissue microstructure and general cell appearance and b) immunofluorescence with DAPI and anti-cleaved caspase-3 (n = 5). Bar represents 100 µm.

synovium controls (Figure 2.9a). Sham control synovium values were intermediate but not statistically different from naïve control values.

In gene expression analyses, 4W PTOA synovium also stimulated significantly decreased levels of *Col2a1*, *Il17a, S100a8* levels and significantly increased *Mmp3* and *Prg4* compared to control (Figure 2.10). Sham surgery synovium also caused significantly decreased *Col2a1*, *Il17a*, and *S100a8* levels, as well as decreased *Acan*. These results suggest that surgical effects on synovium persist at 4 weeks post surgery in this model, but PTOA-specific effects are also detectable by this time.

We also measured these effects after a longer co-culture duration of 120 hours to determine whether chondrocytes responses are sustained. At 120 hours, 4W PTOA synovium continued to cause further increases in sGAG secretion (Figure 2.9b). Chondrocyte gene expression changes induced by PTOA appeared to have equilibrated, although *Acan* and *Sox9* levels were significantly lower for sham synovium and appear lower for PTOA, but not significantly different (Figure 2.11).

2.4.5 Later stage PTOA synovium alters *Ccl2* expression in primary articular chondrocytes

The development of PTOA and disease progression result in changes in synovial physiology. To compare the effects of early PTOA synovium on chondrocytes versus synovium from a later stage of PTOA, we co-cultured primary chondrocytes with synovium from PTOA joints collected 12 weeks after surgery, compared with age-matched sham surgery and surgically naïve controls. Interestingly, no significant differences were found between PTOA synovium and controls in sGAG secretion (Figure 2.9c). Similarly, aside from a significant decrease in *Ccl2* expression from naïve synovium to PTOA and sham synovium treatments, no other significant differences in chondrocyte gene expression were found (Figure 2.10). In this experimental model, cartilage damage is well established at 12 weeks PTOA, compared to the minor damage at the 4 week timepoint[39].

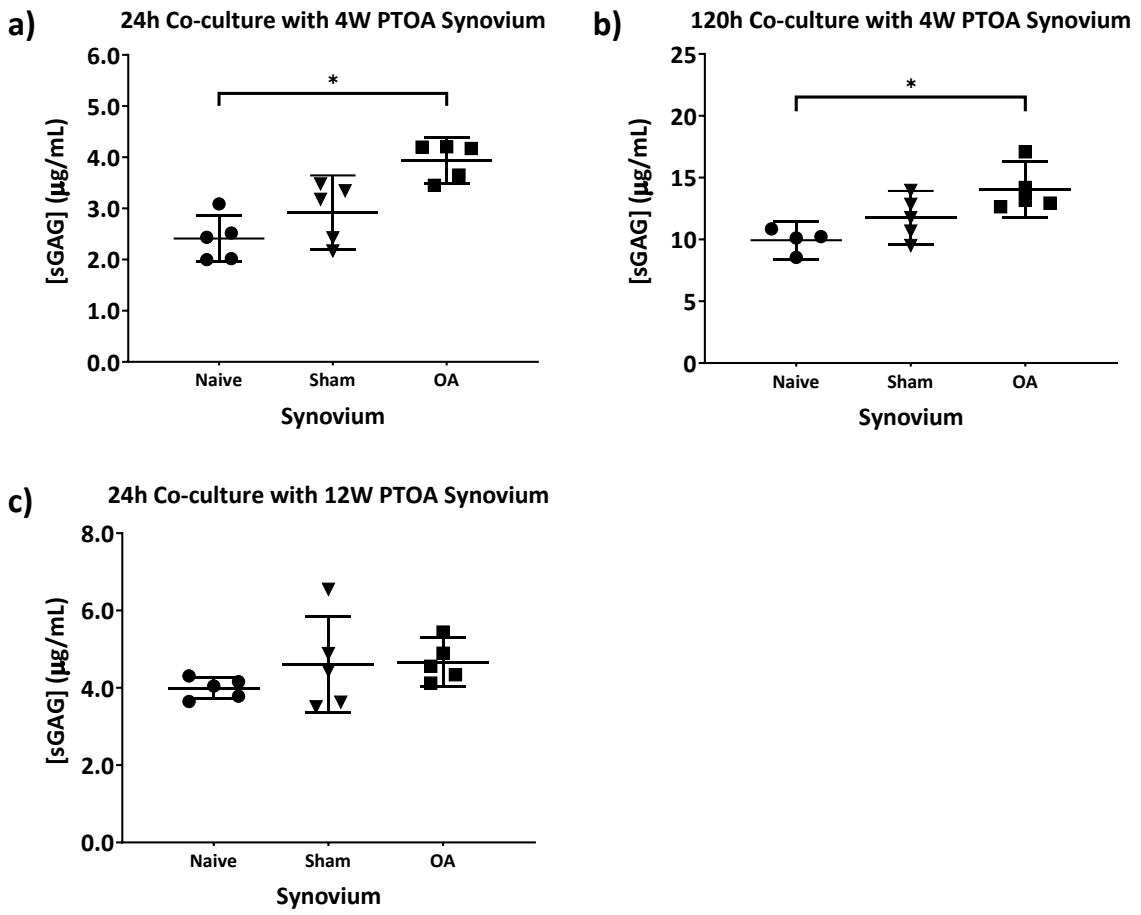

Figure 2.9. sGAG content in conditioned medium from P1 chondrocytes co-cultured with synovial tissue.

P1 chondrocytes were co-cultured a) for 24 hours with 4W PTOA synovium, b) for 120 hours with 4W PTOA synovium, or c) for 24 hours with 12W PTOA synovium. Conditioned medium was collected and measured for sGAG content by DMMB assay (mean ± 95% CI, n = 4-5, *$p < 0.05$).

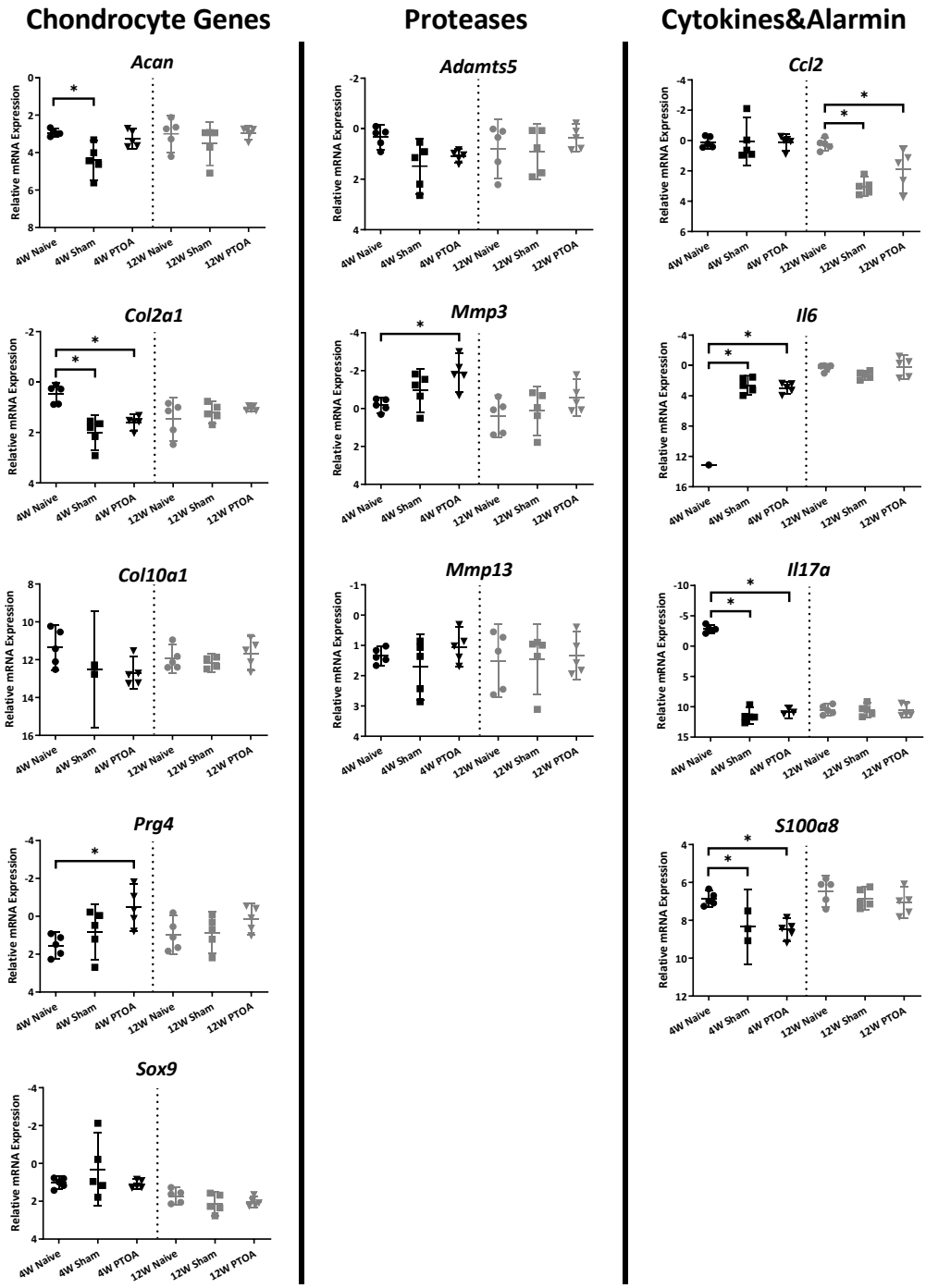

Figure 2.10. Gene expression in P1 chondrocytes co-cultured with synovial tissue for 24 hours.

Confluent P1 articular chondrocytes were co-cultured for 24 hours with synovium from 4W and 12W PTOA joints, sham controls, or surgery-naïve controls. qPCR analysis of gene expression levels is displayed as ΔCq (mean ± 95% CI, n = 5, *p < 0.05).

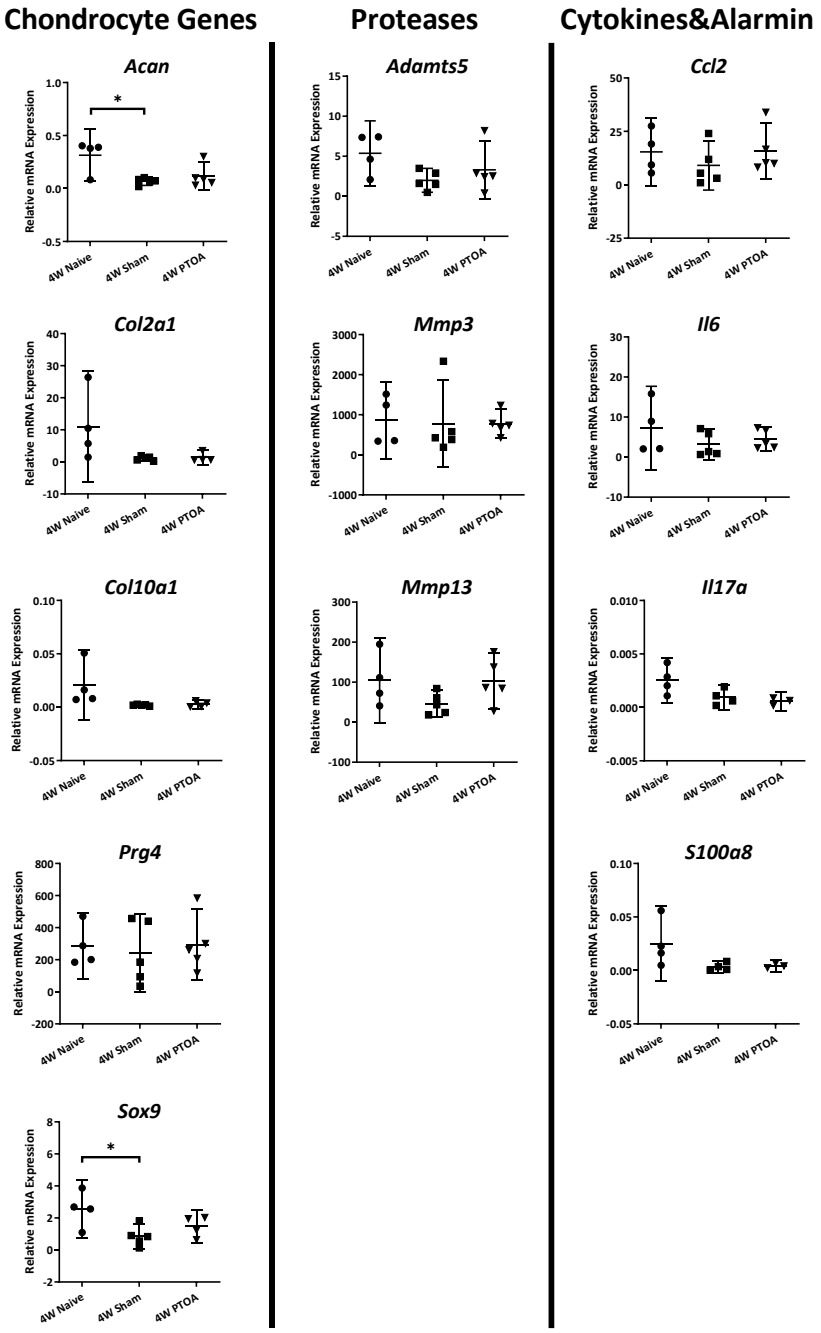

Figure 2.11. Gene expression in P1 chondrocytes co-cultured with synovial tissue for 120 hours.

Confluent P1 articular chondrocytes were co-cultured for 120 hours with 4W PTOA synovium. ddPCR analysis of gene expression levels is displayed as absolute copy number relative to the average of two reference genes per µg of template (mean ± 95% CI, n = 4, *$p < 0.05$).

2.4.6 Effects of early PTOA synovium on primary articular chondrocytes is not fully replicated by conditioned medium transfer

PRG4, also known as lubricin or superficial zone protein, is a lubricating glycoprotein that is protective against OA development when overexpressed in mouse models, partially through its inhibitory effect on chondrocyte catabolism and hypertrophy[52]. Thus, the upregulation of *Prg4* in our co-culture model and the mechanism responsible are potentially beneficial in OA. TGF-β1has previously been shown to induce *Col2a1*, *Acan*, and *Prg4* expression in rat articular chondrocytes and *Prg4* additionally in bovine chondrocytes[38,53,54,55]. Similarly, FOXO1 regulates *Prg4* in murine articular chondrocytes and chondrogenic ATDC5 cells[56]. We wanted to determine if the induction of *Prg4* in chondrocytes stimulated by 4W PTOA synovium was mediated by TGF-β signaling and/or FOXO1 activity. TGF-β (10 ng/mL) was used to stimulate P1 chondrocytes in confluent monolayer culture, which resulted in increased *Prg4* expression (Figure 2.12). Next, the minimal inhibitory concentration of SB 431542, a TGFBR1-selective inhibitor, was determined to be 1 μM for *Prg4* expression using a serial dilution approach (Figure 2.12a). We similarly tested the effects of AS 1842856, a FOXO1-selective inhibitor, on the induction of *Prg4* expression by TGF-β stimulation in P1 chondrocytes (Figure 2.12b). However, FOXO1 inhibition with AS 1842856 did not inhibit *Prg4* induction until 10 μM, the highest dose tested, and this was likely a non-selective effect due to toxicity, as determined by phase contrast microscopy (Figure 2.12c).

To explore the extent of TGF-β signaling in the mechanism of *Prg4* induction in primary articular chondrocytes in co-cultures by co-culture with 4W PTOA synovium, we stimulated naïve confluent P1 articular chondrocytes cultures with stored conditioned media from the same co-culture experiments that had previously demonstrated *Prg4* induction by 4W PTOA synovium and their age-matched sham and naïve synovium controls. Chondrocytes treated with conditioned medium from 4W PTOA, sham, and naïve synovium all increased *Prg4* expression compared to chondrocytes in non-conditioned serum-free medium (Figure 2.13). However, unlike the original co-cultures, conditioned medium from 4W PTOA synovium co-cultures did not significantly increase

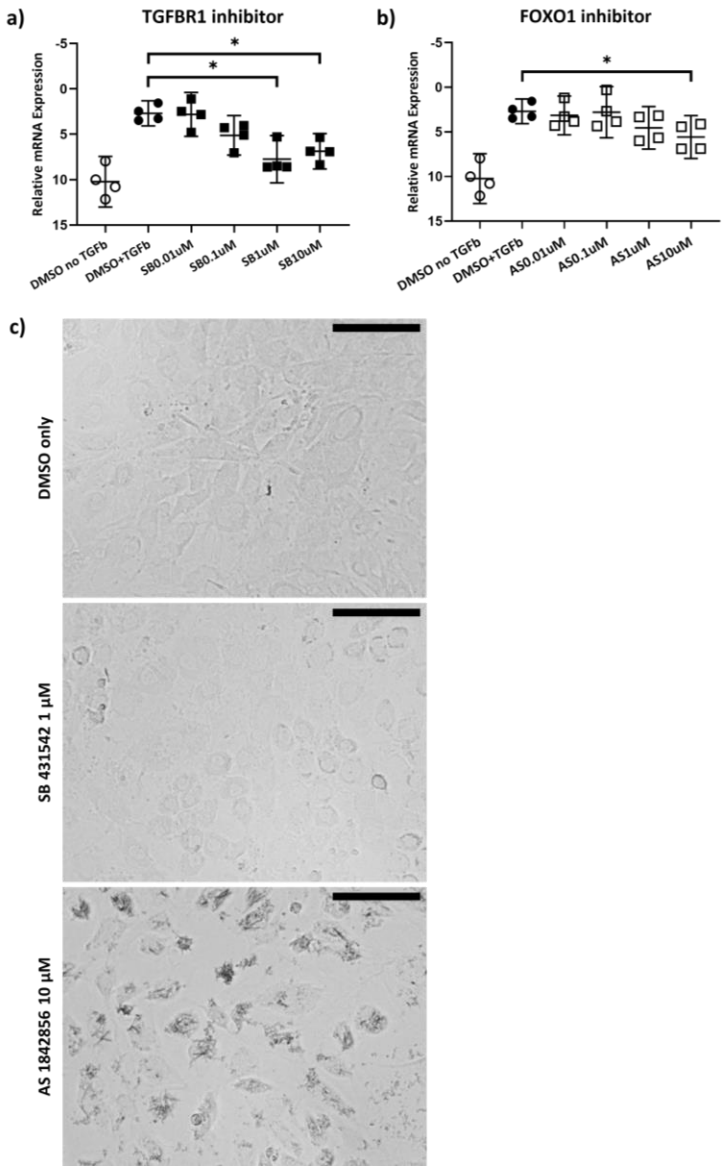

Figure 2.12. P1 articular chondrocytes incubated with inhibitors of TGFBR1 and FOXO1.

Confluent P1 articular chondrocytes were cultured in serum-free medium for 24 hours with DMSO only, 10 ng/mL TGF-β + DMSO, and 10 ng/mL TGF-β + various concentrations of a) TGFBR1 inhibitor SB 431542 or b) FOXO1 inhibitor AS 1842856. qPCR analysis of Prg4 expression levels is displayed as ΔCq relative to reference gene *Rpl13a* (mean ± 95% CI, n = 4, *p < 0.05). c) Representative phase-contrast micrographs of chondrocytes in serum-free culture with DMSO control, 1 µM SB 431542, or 10 µM AS 1842856 (n = 4). Bar represents 100 µm.

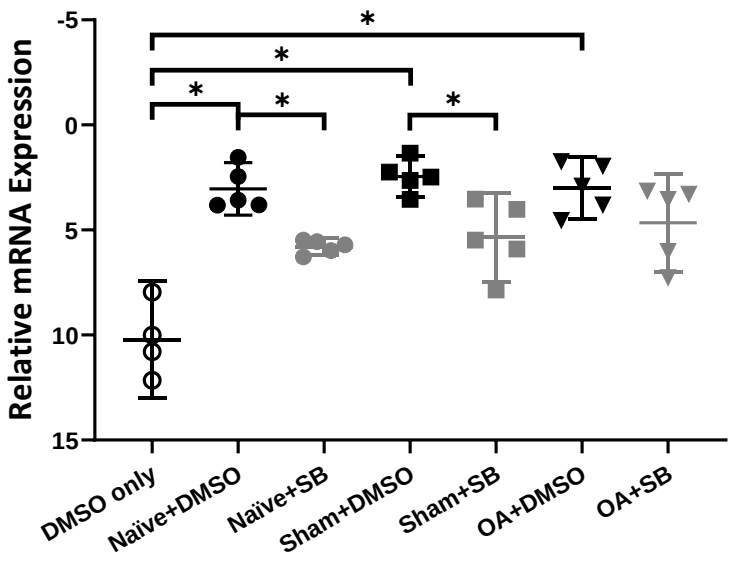

Figure 2.13. *Prg4* expression levels in P1 articular chondrocytes incubated with conditioned medium and TGFBR1 inhibitor.

Confluent P1 articular chondrocytes were incubated with serum-free medium and vehicle (DMSO only), conditioned medium from 4W PTOA, sham, and naïve synovium-chondrocyte co-cultures and vehicle, or with conditioned medium and 1 µM SB 4315421. qPCR analysis of *Prg4* expression levels is displayed as ΔCq relative to reference gene *Rpl13a* (mean ± 95% CI, n = 5, *p < 0.05).

Prg4 expression further compared to conditioned medium from sham and naïve synovium controls. Treatment with 1 μM TGFBR1 inhibitor significantly reduced *Prg4* levels in chondrocytes given conditioned medium from naïve and sham synovium co-cultures compared to DMSO vehicle (inhibitor control). Interestingly, chondrocytes treated with conditioned medium from PTOA synovium co-cultures appeared to decrease *Prg4* expression in response to TGFBR1 inhibitor co-treatment but were not significantly different. Overall, these results show that *Prg4* induction in chondrocytes by co-culture with synovium, regardless of synovium condition, is only partially mediated by TGF-β signaling.

2.5 Discussion

The role of synovium in knee OA is not completely understood, although synovitis is strongly correlated with worse OA symptoms and disease progression. We set out to investigate the effects of synovium from early stage PTOA joints on chondrocytes using a novel *in vitro* co-culture system and to explore whether physiologic changes occur in synovium with progression to later stages of OA development.

We first sought to establish a co-culture system that would allow us to study signalling between synovium and cartilage *ex vivo* without direct contact. A two-chambered transwell insert system with small membrane pore size was selected for this purpose. The biological components consisted of articular chondrocytes that recapitulated the expected physiological phenotype and whole synovial tissue that reconstituted the three-dimensional, population-diverse context of knee synovium.

In developing the chondrocyte component, we determined that sub-confluent young adult rat primary articular chondrocytes did not adequately secrete sGAGs into medium, and that even confluent chondrocyte cultures showed high variation between replicates with one donor per replicate. Since Sprague-Dawley rats are outbred, variation in cartilage homeostasis between individuals likely explains the observed inter-individual variability in chondrocyte sGAG secretion and gene expression. This variability was effectively reduced when we adjusted our chondrocyte model by expanding a pool of naïve chondrocytes from a single animal to allow for many co-cultures to be run with the same biological baseline. We also confirmed reports from the literature that passaging primary chondrocytes from P0 to P1 did not cause significant loss of chondrocyte phenotype, as shown by sGAG release and stable expression of key genes related to chondrocyte function (*Acan*, *Col2a1*, *Prg4*) and phenotypic transcriptional regulators (*Runx2*, *Sox9*)[57,58,59]. Future larger scale experiments could extend these results using cells from multiple animals pooled together and expanded to make stocks of a mixed-source adult primary articular chondrocyte “cell line”. This has significant advantages over transformed cell lines that are chondrogenic but do not behave exclusively similar to articular chondrocytes. Although selective pressures may present a degree of bias in P1

chondrocytes, such effects are likely minimized by the single passage. Our approach also allowed us to demonstrate the utility of this model to directly investigate the physiology of adult rat primary articular chondrocytes, which has not previously been well established in the literature.

In developing the synovium component of our co-culture system, we found that dissociated synovial cells were slow to grow and had less potential to impact chondrocyte physiology when placed into co-culture, with inadvertent selection for more readily expanding synovial fibroblasts. Thus, we elected to use intact synovial tissue, with all dissected tissue from a single donor knee per co-culture replicate. Synovial tissue samples showed no significant signs of apoptosis or necrosis throughout the tissue. We pre-conditioned serum-free medium with isolated synovial tissue for 24 hours after collection to allow for conditioning of the medium prior to transfer into co-culture. Since our preliminary experiments demonstrated that chondrocyte gene expression changes peak between 12 and 48 hours after reception into our co-culture system, we chose 24 hours in our final co-culture model. We adjusted our system to study healthy articular chondrocyte responses to synovium from early and later PTOA knee joints. However, this system could also be used to study other joint diseases where rat models are available, such as rheumatoid arthritis[60]. For our studies, two control conditions were used. Synovium from surgically-naïve joints controlled for animal age, strain, and housing conditions. Synovium from sham surgery joints additionally controlled for surgical effects including procedural stress, drugs given, arthrotomy, hemoarthrosis, and wound healing. Although sham surgery controls are typically the only control used in most surgical PTOA studies, naïve controls allowed us to differentiate which chondrocyte responses were specific to PTOA from effects due to surgical trauma to the synovium.

In the 4W PTOA co-cultures, *Col2a1*, *Il6*, and *Il17a* expression in chondrocytes co-cultured with either sham or PTOA synovium were significantly different from naïve but not from each other, suggesting these changes are due to synovial injury and healing from the surgical procedure, rather than early PTOA. Conversely, *Mmp3* and *Prg4* expression and sGAG release increased in chondrocytes co-cultured with PTOA synovium compared to naïve controls, with sham values trending upward but not significantly, suggesting that

synovial injury and PTOA work together to induce a protective effect. Previous studies of early PTOA cartilage gene expression found that *Mmp3* was upregulated due to increased pro-inflammatory factors such as IL-1β and TNF-α, while *Prg4* has been shown to be downregulated[61,62,63,64,65]. In contrast, our studies demonstrate that early PTOA induces *Prg4* expression. Although PRG4 is widely regarded to have protective effects on joint health, decreased levels of PRG4 in synovial fluid and downregulation of *Prg4* expression in chondrocytes is one of the earliest responses to joint injury and thought to increase the risk of progression to PTOA in patients after traumatic rupture of the anterior cruciate ligament[66]. Therefore, our results point to a possible protective role of synovium in early PTOA development.

Further supporting a potential protective role of early PTOA synovium, synovial injury in the sham synovium control downregulated chondrocyte *Acan* expression at both 24 and 120 hours of co-culture, while PTOA synovium was protective and rescued this effect by restoring *Acan* expression to levels similar to that of the naïve controls. We would not have been able to detect this effect without both naïve and sham surgery controls. Aggrecan breakdown is a critical event in the pathogenesis of early OA, but the evidence describing the direction of *Acan* regulation in early OA is mixed[67,68]. The increase in sGAG secretion, which suggests that chondrocytes increase sGAG synthesis when co-cultured with early PTOA synovium, also contributes to an anabolic effect. Past studies have often conflated proteoglycan content and glycosaminoglycan content in assessing cartilage conditions such as fixed charge density and sulfate metabolism with the reasoning that the two are biochemically and functionally coupled, but their production and composition may not be so in OA. Experimental animals models have found OA cartilage sGAG levels are decreased or not significantly different from healthy controls, but a study of cartilage post-traumatic knee injury in human patients has found increased sGAG levels[69,70,71,72]. Considering the induction of *Mmp3, Prg4,* and *Acan* expression and increased sGAG release in our co-culture system, the effects of early PTOA synovium indicate overall that a regenerative response may be induced in articular chondrocytes. This overall protective effect does not support our original hypothesis that early PTOA synovium would induce pro-inflammatory and catabolic changes in chondrocytes. In contrast, it stands to reason physiologically that an early, endogenous

repair response in the pathogenesis of PTOA might be mediated in part by signaling between the synovium and articular cartilage.

As we identified an overall protective effect of early PTOA synovium on articular chondrocytes, we next contrasted these effects by co-culturing chondrocytes with synovium from a later stage of PTOA to determine if the synovium physiology evolves as experimental PTOA progresses. Our results indicate that the protective effect of early PTOA synovium on chondrocyte physiology is almost completely lost by 12 weeks after PTOA induction. At this timepoint, our hypothesis was refuted, with only a decrease in *Ccl2* expression induced by PTOA and sham synovium compared to age-matched naïve, which may indicate a less inflammatory state induced by surgery. In our PTOA model at 12 weeks, there is significant progression of joint damage as measured by histopathological scoring. Previous studies using various other rat PTOA models have noted early histological changes, including lining layer proliferation, inflammatory infiltrate, and angiogenesis which progress with later disease, with lining layers further increasing, more dense subsynovial infiltrate, and dilation of blood vessels[73,74,75,76,77,78,79,80]. Although some features may progress with later stages of OA, it is difficult to identify stage-specific changes in OA synovium with histology alone. Moreover, little information is reported about the physiology of OA synovium, let alone with respect to disease stage. Studies on synovitis in human patients have compared synovial cell populations in early and late PTOA and found increases in mainly macrophages and T cells, while one recent study in a murine PTOA model showed temporal patterns of macrophage recruitment, activation, and polarization in the synovium following experimental surgery, albeit with no differences between PTOA and sham surgery control[81,82,83]. These results suggest that after injury, a transition in synovial physiology occurs with progression of PTOA, perhaps toward a less regenerative state. In our experiments, both the significant increase in chondrocyte *Il6* expression and the decrease in *Il17a* with injured synovium from both sham and PTOA joints were maintained through 12 weeks post-surgery. As both cytokines have pro-inflammatory functions which synergize with other inflammatory signals and are increased in human OA joints, this outcome is another mixed effect indicative of a complex response[17,18,84,85]. This may have implications for treating PTOA in patients, as early inflammatory

responses after joint injury may in fact be necessary to trigger tissue healing. Treatments for PTOA, as well as other forms of OA, may need to be tailored for early versus later stages of disease development.

The mechanisms resulting in *Prg4* (encoding lubricin) induction in chondrocytes co-cultured with early PTOA synovium remains unclear from our studies, but is most likely an overall net result of multiple contributing pathways. TGF-β has been shown to stimulate increased lubricin production in primary articular chondrocyte cultures[53,55]. Treatment with TGFBR1 inhibitor significantly but only partially reduced the upregulation of *Prg4* by conditioned medium from co-cultures compared to serum-free medium, indicating that TGF-β signaling is at least partially responsible for *Prg4* induction in chondrocytes in a healthy joint context. Although FOXO1 may regulate *Prg4* in addition to synergizing with TGF-β, we were not able to inhibit FOXO1 without toxic effects[56]. Interestingly, although chondrocytes expressed higher *Prg4* levels in co-culture with 4W PTOA synovium compared to naïve synovium, conditioned medium transfer from the co-cultures onto fresh chondrocytes did not significantly increase their *Prg4* levels compared to conditioned medium from naïve synovium co-cultures, contrary to our expectations. This suggests that cross-talk between PTOA synovium and chondrocytes may be required to further induce *Prg4* expression in a PTOA context specifically. Alternatively, unstable signaling mediators may have been lost over time in storage, and further experiments transferring conditioned medium directly may reproduce the difference in *Prg4*. Although we did not measure the impact of TGFBR1 inhibition on *Acan* and *Col2a1* expression, TGF-β has been linked to both in chondrocyte homeostasis and future experiments will include these genes[86]. Other signaling pathways with investigative potential include ERG (erythroblast transformation-specific related gene), which regulates lubricin in the murine joint and has been found to be upregulated in human OA lesions, and members of the nuclear factor of activated T cells (NFAT) family, where loss of *Nfatc1* and *Nfatc2* decreased *Prg4* levels in mice, and NFATc4 has been linked to TGF-β signaling[87,88,89,90].

Our study has several limitations. *Ex vivo* conditions have the potential to influence cell responses, and the process of dissecting and isolating synovial tissue and primary

chondrocytes could be affecting results. However, results from our final experiment with conditioned medium transfers show different results in a static culture system vs a live co-culture system. This observation suggests that the presence of live synovial tissue in our co-culture system is required for maximal effects and produces interactivity between the *in vitro* biological models of synovium and cartilage. Although changes in gene expression do not necessarily translate to changes in protein expression and thus long-term physiology, our co-culture system is capable of demonstrating and testing interactivity between synovium and cartilage to model similar interactions at the joint level *in vivo*. Passage from P0 to P1 and subsequent expansion may alter primary chondrocyte phenotype and select proliferative clones. Furthermore, although we assessed the effects in healthy chondrocytes, we did not examine the responses of OA chondrocytes in co-culture with PTOA synovium, which could differ. However, OA chondrocytes are often more senescent, and we would need to confirm that loss of the OA phenotype does not occur with passage. The rat PTOA model endpoints selected at 4W and 12W post-surgery are based on previous PTOA rat models in the literature, but may not represent the full spectrum of OA pathophysiology or changes that occur earlier or later in disease development. Finally, the pathogenesis of PTOA in rat knee synovium may not reflect human PTOA, or other forms of OA, and thus the protective effect of early PTOA synovium should be confirmed in human joint tissues.

In summary, we have established a responsive co-culture model for investigating the interaction between synovial tissue and articular chondrocyte interaction in rats. Furthermore, this system demonstrated adequate sensitivity to identify disease-specific responses using a rat model of experimental PTOA, which could be adapted further to study other joint diseases where rat models are available. Our PTOA studies demonstrate a surprising but logical protective effect induced by PTOA joint synovium on healthy chondrocytes through mechanisms which are at most only partially dependent on TGF-β signaling. Understanding the mechanism of why synovium loses this effect with disease progression is a critical observation for studies of OA pathophysiology and may provide additional therapeutic insights into the importance of appropriate timing for different

interventions for OA. Enhancement and support of synovial signals, rather than suppression, may prove beneficial, at least in early PTOA.

2.6 References

1. Cross M, Smith E, Hoy D, Nolte S, Ackerman I, Fransen M, Bridgett L, Williams S, Guillemin F, Hill CL, Laslett LL. The global burden of hip and knee osteoarthritis: estimates from the global burden of disease 2010 study. Annals of the rheumatic diseases. 2014 Jul 1;73(7):1323-30.
2. Hunter DJ, Schofield D, Callander E. The individual and socioeconomic impact of osteoarthritis. Nature Reviews Rheumatology. 2014 Jul;10(7):437-41.
3. Bombardier C, Hawker G, Mosher D. The impact of arthritis in Canada: today and over 30 years: Arthritis Alliance of Canada 2011. http://www.arthritisalliance.ca/images/PDF/eng/Initiatives/20111022_2200_impact_of_arthritis.pdf
4. Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. Arthritis & Rheumatism. 2012 Jun;64(6):1697-707.
5. Sinusas K. Osteoarthritis: diagnosis and treatment. American family physician. 2012 Jan 1;85(1).
6. Hermann W, Lambova S, Muller-Ladner U. Current treatment options for osteoarthritis. Current rheumatology reviews. 2018 Aug 1;14(2):108-16.
7. Iannotti JP, Norris TR. Influence of preoperative factors on outcome of shoulder arthroplasty for glenohumeral osteoarthritis. JBJS. 2003 Feb 1;85(2):251-8.
8. Lingard EA, Katz JN, Wright EA, Sledge CB, Kinemax Outcomes Group. Predicting the outcome of total knee arthroplasty. Journal of Bone & Joint Surgery 2004;86(10):2179-86.
9. Bai LB, Lee KB, Song EK, Yoon TR, Seon JK. Total ankle arthroplasty outcome comparison for post-traumatic and primary osteoarthritis. Foot & ankle international. 2010 Dec;31(12):1048-56.
10. Ritter MA, Davis KE, Meding JB, Pierson JL, Berend ME, Malinzak RA. The effect of alignment and BMI on failure of total knee replacement. Journal of Bone & Joint Surgery 2011;93(17):1588-96.
11. Chu CR, Williams AA, Coyle CH, Bowers ME. Early diagnosis to enable early treatment of pre-osteoarthritis. Arthritis research & therapy. 2012 Jun;14(3):212.
12. Kuyinu EL, Narayanan G, Nair LS, Laurencin CT. Animal models of osteoarthritis: classification, update, and measurement of outcomes. Journal of orthopaedic surgery and research. 2016 Dec;11(1):19.
13. Goldring MB, Marcu KB. Cartilage homeostasis in health and rheumatic diseases. Arthritis research & therapy. 2009 Jun 1;11(3):224.
14. Van der Kraan PM, Van den Berg WB. Chondrocyte hypertrophy and osteoarthritis: role in initiation and progression of cartilage degeneration?. Osteoarthritis and cartilage 2012;20(3):223-32.
15. Smith MD. Suppl 1: The Normal Synovium. The open rheumatology journal. 2011;5:100.
16. Mathiessen A, Conaghan PG. Synovitis in osteoarthritis: current understanding with therapeutic implications. Arthritis Research & Therapy 2017;19(1):18.
17. Sellam J, Berenbaum F. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. Nature Reviews Rheumatology. 2010 Nov;6(11):625.

18. Scanzello CR, Goldring SR. The role of synovitis in osteoarthritis pathogenesis. Bone 2012;51(2):249-57.
19. Krasnokutsky S, Belitskaya-Lévy I, Bencardino J, Samuels J, Attur M, Regatte R, Rosenthal P, Greenberg J, Schweitzer M, Abramson SB, Rybak L. Quantitative magnetic resonance imaging evidence of synovial proliferation is associated with radiographic severity of knee osteoarthritis. Arthritis & Rheumatism. 2011 Oct;63(10):2983-91.
20. Riis RG, Gudbergsen H, Henriksen M, Ballegaard C, Bandak E, Röttger D, Bliddal H, Hansen BB, Hangaard S, Boesen M. Synovitis assessed on static and dynamic contrast-enhanced magnetic resonance imaging and its association with pain in knee osteoarthritis: a cross-sectional study. European journal of radiology. 2016 Jun 1;85(6):1099-108.
21. Neogi T, Guermazi A, Roemer F, Nevitt MC, Scholz J, Arendt-Nielsen L, Woolf C, Niu J, Bradley LA, Quinn E, Frey Law L. Association of joint inflammation with pain sensitization in knee osteoarthritis: the Multicenter Osteoarthritis Study. Arthritis & rheumatology. 2016 Mar;68(3):654-61.
22. Felson DT, Niu J, Neogi T, Goggins J, Nevitt MC, Roemer F, Torner J, Lewis CE, Guermazi A, Group MI. Synovitis and the risk of knee osteoarthritis: the MOST Study. Osteoarthritis and cartilage. 2016 Mar 1;24(3):458-64.
23. Atukorala I, Kwoh CK, Guermazi A, Roemer FW, Boudreau RM, Hannon MJ, Hunter DJ. Synovitis in knee osteoarthritis: a precursor of disease?. Annals of the rheumatic diseases. 2016 Feb 1;75(2):390-5.
24. Collins JE, Losina E, Nevitt MC, Roemer FW, Guermazi A, Lynch JA, Katz JN, Kwoh CK, Kraus VB, Hunter DJ. Semi-quantitative imaging biomarkers of knee osteoarthritis progression: data from the FNIH OA Biomarkers Consortium. Arthritis & rheumatology (Hoboken, NJ). 2016 Oct;68(10):2422.
25. de Lange-Brokaar BJ, Ioan-Facsinay A, Yusuf E, Kroon HM, Zuurmond AM, Stojanovic-Susulic V, Nelissen RG, Bloem JL, Kloppenburg M. Evolution of synovitis in osteoarthritic knees and its association with clinical features. Osteoarthritis and cartilage. 2016 Nov 1;24(11):1867-74.
26. Kortekaas MC, Kwok WY, Reijnierse M, Kloppenburg M. Inflammatory ultrasound features show independent associations with progression of structural damage after over 2 years of follow-up in patients with hand osteoarthritis. Annals of the rheumatic diseases. 2015 Sep 1;74(9):1720-4.
27. Haugen IK, Slatkowsky-Christensen B, Bøyesen P, Sesseng S, van der Heijde D, Kvien TK. MRI findings predict radiographic progression and development of erosions in hand osteoarthritis. Annals of the rheumatic diseases. 2016 Jan 1;75(1):117-23.
28. Mathiessen A, Slatkowsky-Christensen B, Kvien TK, Hammer HB, Haugen IK. Ultrasound-detected inflammation predicts radiographic progression in hand osteoarthritis after 5 years. Annals of the rheumatic diseases. 2016 May 1;75(5):825-30.
29. Damman W, Liu R, Bloem JL, Rosendaal FR, Reijnierse M, Kloppenburg M. Bone marrow lesions and synovitis on MRI associate with radiographic progression after 2 years in hand osteoarthritis. Annals of the rheumatic diseases. 2017 Jan 1;76(1):214-7.

30. de Lange-Brokaar BJ, Ioan-Facsinay A, Yusuf E, Visser AW, Kroon HM, van Osch GJ, Zuurmond AM, Stojanovic-Susulic V, Bloem JL, Nelissen RG, Huizinga TW. Association of pain in knee osteoarthritis with distinct patterns of synovitis. Arthritis & Rheumatology. 2015 Mar;67(3):733-40.
31. Calich AL, Domiciano DS, Fuller R. Osteoarthritis: can anti-cytokine therapy play a role in treatment?. Clinical Rheumatology 2010;29(5):451-5.
32. Wenham CYJ, McDermott M, Conaghan PG. Biological therapies in osteoarthritis. Current pharmaceutical design. 2015 May 1;21(17):2206-15.
33. Chevalier X, Eymard F, Richette P. Biologic agents in osteoarthritis: hopes and disappointments. Nature Reviews Rheumatology 2013;9(7):400.
34. Pustjens MF, Mastbergen SC, Steen-Louws C, van Roon JA, Hack E, Lafeber FP. IL4-10 synerkine induces direct and indirect structural cartilage repair in osteoarthritis. Osteoarthritis and Cartilage. 2016 Apr 1;24:S532.
35. Hochberg MC, Guermazi A, Guehring H, Aydemir A, Wax S, Fleuranceau-Morel P, Bihlet AR, Byrjalsen I, Andersen JR, Eckstein F. Effect of Intra-Articular Sprifermin vs Placebo on Femorotibial Joint Cartilage Thickness in Patients With Osteoarthritis: The FORWARD Randomized Clinical Trial. Jama. 2019 Oct 8;322(14):1360-70.
36. Takano S, Uchida K, Inoue G, Miyagi M, Aikawa J, Iwase D, Iwabuchi K, Matsomoto T, Satoh M, Mukai M, Minatani A, Takaso M. Nerve growth factor regulation and production by macrophages in osteoarthritic synovium. Clinical and experimental immunology. 2017 Nov;190(2):235-43.
37. Thielen N, van der Kraan P, van Caam A. TGFβ/BMP Signaling Pathway in Cartilage Homeostasis. Cells. 2019 Aug 24;8(9):969.
38. Zhu Y, Tao H, Jin C, et al. Transforming growth factor-β1 induces type II collagen and aggrecan expression via activation of extracellular signal-regulated kinase 1/2 and Smad2/3 signaling pathways. Mol Med Rep. 2015 Jul 9;12(4):5573-9.
39. Appleton CT, McErlain DD, Pitelka V, Schwartz N, Bernier SM, Henry JL, Holdsworth DW, Beier F. Forced mobilization accelerates pathogenesis: characterization of a preclinical surgical model of osteoarthritis. Arthritis research & therapy. 2007 Feb;9(1):R13.
40. Appleton CT, Pitelka V, Henry J, Beier F. Global analyses of gene expression in early experimental osteoarthritis. Arthritis & Rheumatism. 2007 Jun;56(6):1854-68.
41. Coulson-Thomas V, Gesteira TF. Dimethylmethylene Blue Assay (DMMB). Bio-protocol 2014;4(18): e1236.
42. Müller G, Hanschke M. Quantitative and qualitative analyses of proteoglycans in cartilage extracts by precipitation with 1, 9-dimethylmethylene blue. Connective tissue research 1996;33(4):243-8.
43. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry. 2009 Apr 1;55(4):611-22.
44. Inman GJ, Nicolás FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, Laping NJ, Hill CS. SB-431542 is a potent and specific inhibitor of transforming growth

factor-β superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Molecular pharmacology. 2002 Jul 1;62(1):65-74.
45. Nagashima T, Shigematsu N, Maruki R, Urano Y, Tanaka H, Shimaya A, Shimokawa T, Shibasaki M. Discovery of novel forkhead box O1 inhibitors for treating type 2 diabetes: improvement of fasting glycemia in diabetic db/db mice. Molecular pharmacology. 2010 Nov 1;78(5):961-70.
46. Hunter GK, Rogakou CC, Pritzker KP. Extracellular matrix synthesis by articular chondrocytes and synovial fibroblasts in long-term monolayer culture. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 1984 Aug 17;804(4):459-65.
47. Aulthouse AL, Beck M, Griffey E, Sanford J, Arden K, Machado MA, Horton WA. Expression of the human chondrocyte phenotype in vitro. In vitro cellular & developmental biology. 1989 Jul 1;25(7):659-68.
48. Gosset M, Berenbaum F, Thirion S, Jacques C. Primary culture and phenotyping of murine chondrocytes. Nature protocols. 2008 Aug;3(8):1253.
49. Greenberg DD, Stoker A, Kane S, Cockrell M, Cook JL. Biochemical effects of two different hyaluronic acid products in a co-culture model of osteoarthritis. Osteoarthritis and cartilage. 2006 Aug 1;14(8):814-22.
50. Uitterlinden EJ, Koevoet JL, Verkoelen CF, Bierma-Zeinstra SM, Jahr H, Weinans H, Verhaar JA, Van Osch GJ. Glucosamine increases hyaluronic acid production in human osteoarthritic synovium explants. BMC Musculoskeletal Disorders. 2008 Dec;9(1):120.
51. Elmore SA, Dixon D, Hailey JR, Harada T, Herbert RA, Maronpot RR, Nolte T, Rehg JE, Rittinghausen S, Rosol TJ, Satoh H. Recommendations from the INHAND apoptosis/necrosis working group. Toxicologic pathology. 2016 Feb;44(2):173-88.
52. Ruan MZ, Erez A, Guse K, Dawson B, Bertin T, Chen Y, Jiang MM, Yustein J, Gannon F, Lee BH. Proteoglycan 4 expression protects against the development of osteoarthritis. Science translational medicine. 2013 Mar 13;5(176):176ra34-.
53. Cheng J, Wang Y, Wang Z, Yang M, Wu Y. Differential regulation of proteoglycan-4 expression by IL-1α and TGF-β1 in rat condylar chondrocytes. The Tohoku journal of experimental medicine. 2010;222(3):211-8.
54. DuRaine GD, Chan SM, Reddi AH. Effects of TGF-β1 on alternative splicing of superficial zone protein in articular cartilage cultures. Osteoarthritis and cartilage. 2011 Jan 1;19(1):103-10.
55. Cuellar A, Reddi AH. Stimulation of superficial zone protein/lubricin/PRG4 by transforming growth factor-β in superficial zone articular chondrocytes and modulation by glycosaminoglycans. Tissue Engineering Part A. 2014 Dec 22;21(13-14):1973-81.
56. Matsuzaki T, Alvarez-Garcia O, Mokuda S, Nagira K, Olmer M, Gamini R, Miyata K, Akasaki Y, Su AI, Asahara H, Lotz MK. FoxO transcription factors modulate autophagy and proteoglycan 4 in cartilage homeostasis and osteoarthritis. Science translational medicine. 2018 Feb 14;10(428):eaan0746.
57. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell. 1982 Aug 1;30(1):215-24.

58. Lefebvre, V., Smits, P. (2005) Transcriptional control of chondrocyte fate and differentiation. Birth Defects Res C Embryo Today 75: 200–212.
59. Dong YF, Soung DY, Schwarz EM, O'Keefe RJ, Drissi H. Wnt induction of chondrocyte hypertrophy through the Runx2 transcription factor. Journal of cellular physiology. 2006 Jul;208(1):77-86.
60. Bendele A. Animal models of rheumatoid arthritis. J Musculoskelet Neuronal Interact. 2001 Jun;1(4):377-85.
61. Wei T, Kulkarni NH, Zeng QQ, Helvering LM, Lin X, Lawrence F, Hale L, Chambers MG, Lin C, Harvey A, Ma YL. Analysis of early changes in the articular cartilage transcriptisome in the rat meniscal tear model of osteoarthritis: pathway comparisons with the rat anterior cruciate transection model and with human osteoarthritic cartilage. Osteoarthritis and cartilage. 2010 Jul 1;18(7):992-1000.
62. Shi J, Zhang C, Yi Z, Lan C. Explore the variation of MMP3, JNK, p38 MAPKs, and autophagy at the early stage of osteoarthritis. IUBMB life. 2016 Apr;68(4):293-302.
63. Sieker JT, Proffen BL, Waller KA, Chin KE, Karamchedu NP, Akelman MR, Perrone GS, Kiapour AM, Konrad J, Murray MM, Fleming BC. Transcriptional profiling of articular cartilage in a porcine model of early post-traumatic osteoarthritis. Journal of Orthopaedic Research. 2018 Jan;36(1):318-29.
64. Young AA, McLennan S, Smith MM, Smith SM, Cake MA, Read RA, Melrose J, Sonnabend DH, Flannery CR, Little CB. Proteoglycan 4 downregulation in a sheep meniscectomy model of early osteoarthritis. Arthritis research & therapy. 2006 Apr;8(2):R41.
65. Reesink HL, Watts AE, Mohammed HO, Jay GD, Nixon AJ. Lubricin/proteoglycan 4 increases in both experimental and naturally occurring equine osteoarthritis. Osteoarthritis and cartilage. 2017 Jan 1;25(1):128-37.
66. Elsaid KA, Fleming BC, Oksendahl HL, Machan JT, Fadale PD, Hulstyn MJ, Shalvoy R, Jay GD. Decreased lubricin concentrations and markers of joint inflammation in the synovial fluid of patients with anterior cruciate ligament injury. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 2008 Jun;58(6):1707-15.
67. Lorenz H, Richter W. Osteoarthritis: cellular and molecular changes in degenerating cartilage. Progress in histochemistry and cytochemistry. 2006 Jul 3;40(3):135-63.
68. Zhang Q, Ji Q, Wang X, Kang L, Fu Y, Yin Y, Li Z, Liu Y, Xu X, Wang Y. SOX9 is a regulator of ADAMTSs-induced cartilage degeneration at the early stage of human osteoarthritis. Osteoarthritis and cartilage. 2015 Dec 1;23(12):2259-68.
69. Guilak F, Ratcliffe A, Lane N, Rosenwasser MP, Mow VC. Mechanical and biochemical changes in the superficial zone of articular cartilage in canine experimental osteoarthritis. Journal of Orthopaedic Research. 1994 Jul;12(4):474-84.
70. Elliott DM, Guilak F, Parker Vail T, Wang JY, Setton LA. Tensile properties of articular cartilage are altered by meniscectomy in a canine model of osteoarthritis. Journal of Orthopaedic Research. 1999 Jul;17(4):503-8.

71. Nelson F, Billinghurst RC, Pidoux I, Reiner A, Langworthy M, McDermott M, Malogne T, Sitler DF, Kilambi NR, Lenczner E, Poole AR. Early post-traumatic osteoarthritis-like changes in human articular cartilage following rupture of the anterior cruciate ligament. Osteoarthritis and cartilage. 2006 Feb 1;14(2):114-9.
72. Hosseininia S, Lindberg LR, Dahlberg LE. Cartilage collagen damage in hip osteoarthritis similar to that seen in knee osteoarthritis; a case–control study of relationship between collagen, glycosaminoglycan and cartilage swelling. BMC musculoskeletal disorders. 2013 Dec;14(1):18.
73. Ashraf S, Mapp PI, Walsh DA. Contributions of angiogenesis to inflammation, joint damage, and pain in a rat model of osteoarthritis. Arthritis & Rheumatism. 2011 Sep;63(9):2700-10.
74. Galois L, Etienne S, Henrionnet C, Scala-Bertola J, Grossin L, Mainard D, Gillet P, Pinzano A. Ambivalent properties of hyaluronate and hylan during post-traumatic OA in the rat knee. Bio-medical materials and engineering. 2012 Jan 1;22(4):235-42.
75. Xu Y, Liu Q, Liu ZL, Lim L, Chen WH, Lin N. Treatment with SiMiaoFang, an anti-arthritis chinese herbal formula, inhibits cartilage matrix degradation in osteoarthritis rat model. Rejuvenation research. 2013 Oct 1;16(5):364-76.
76. Wen ZH, Tang CC, Chang YC, Huang SY, Chen CH, Wu SC, Hsieh SP, Hsieh CS, Wang KY, Lin SY, Lee HL. Intra-articular injection of the selective cyclooxygenase-2 inhibitor meloxicam (Mobic) reduces experimental osteoarthritis and nociception in rats. Osteoarthritis and cartilage. 2013 Dec 1;21(12):1976-86.
77. Ferrándiz ML, Terencio MC, Ruhí R, Vergés J, Montell E, Torrent A, Alcaraz MJ. Influence of age on osteoarthritis progression after anterior cruciate ligament transection in rats. Experimental gerontology. 2014 Jul 1;55:44-8.
78. Proffen BL, Sieker JT, Murray MM, Akelman MR, Chin KE, Perrone GS, Patel TK, Fleming BC. Extracellular matrix-blood composite injection reduces post-traumatic osteoarthritis after anterior cruciate ligament injury in the rat. Journal of Orthopaedic Research. 2016 Jun;34(6):995-1003.
79. Ramme AJ, Lendhey M, Raya JG, Kirsch T, Kennedy OD. A novel rat model for subchondral microdamage in acute knee injury: a potential mechanism in post-traumatic osteoarthritis. Osteoarthritis and cartilage. 2016 Oct 1;24(10):1776-85.
80. Xu Y, Dai GJ, Liu Q, Liu ZL, Song ZQ, Li L, Chen WH, Lin N. Sanmiao formula inhibits chondrocyte apoptosis and cartilage matrix degradation in a rat model of osteoarthritis. Experimental and therapeutic medicine. 2014 Oct 1;8(4):1065-74.
81. Lieberthal J, Sambamurthy N, Scanzello CR. Inflammation in joint injury and post-traumatic osteoarthritis. Osteoarthritis and cartilage. 2015 Nov 1;23(11):1825-34.
82. De Lange-Brokaar BJ, Ioan-Facsinay A, Van Osch GJ, Zuurmond AM, Schoones J, Toes RE, Huizinga TW, Kloppenburg M. Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review. Osteoarthritis and cartilage. 2012 Dec 1;20(12):1484-99.
83. Moradi B, Jackson MT, Shu CC, Smith SM, Smith MM, Zaki S, Platzer H, Rosshirt N, Giangreco D, Scanzello CR, Little CB. The role of synovial T-cell

infiltration following knee joint injury in symptoms and progression to osteoarthritis. medRxiv. 2019 Nov 29:19013227.
84. Honorati MC, Bovara M, Cattini L, Piacentini A, Facchini A. Contribution of interleukin 17 to human cartilage degradation and synovial inflammation in osteoarthritis. Osteoarthr Cartil. 2002 Sept 30;10(10):799-807.
85. Stannus O, Jones G, Cicuttini F, et al. Circulating levels of IL-6 and TNF-α are associated with knee radiographic osteoarthritis and knee cartilage loss in older adults. Osteoarthr Cartil. 2010 Sept 3;18(11):1441-1447.
86. Li TF, O'Keefe RJ, Chen D. TGF-beta signaling in chondrocytes. Front Biosci. 2005 Jan 1;10:681-8.
87. Yamamura K, Ohta Y, Mamoto K, Sugama R, Minoda Y, Nakamura H. Effect of eldecalcitol on articular cartilage through the regulation of transcription factor Erg in a murine model of knee osteoarthritis. Biochemical and biophysical research communications. 2018 Jan 1;495(1):179-84.
88. Ohta Y, Okabe T, Larmour C, Di Rocco A, Maijenburg MW, Phillips A, Speck NA, Wakitani S, Nakamura T, Yamada Y, Enomoto-Iwamoto M. Articular cartilage endurance and resistance to osteoarthritic changes require transcription factor Erg. Arthritis & rheumatology. 2015 Oct;67(10):2679-90.
89. Greenblatt MB, Ritter SY, Wright J, Tsang K, Hu D, Glimcher LH, Aliprantis AO. NFATc1 and NFATc2 repress spontaneous osteoarthritis. Proceedings of the National Academy of Sciences. 2013 Dec 3;110(49):19914-9.
90. Tardif G, Pelletier JP, Fahmi H, Hum D, Zhang Y, Kapoor M, Martel-Pelletier J. NFAT3 and TGF-β/SMAD3 regulate the expression of miR-140 in osteoarthritis. Arthritis research & therapy. 2013 Dec;15(6):R197.

Chapter 3

3 Targeting synovial macrophage activation affects chondrocyte and joint outcomes in experimental post-traumatic osteoarthritis.

3.1 Chapter Summary

Osteoarthritis (OA) is the most common joint disease and causes major societal and personal burdens. No disease-modifying drugs have been approved despite extensive cartilage research. Although inflammatory processes in cartilage and synovium are suspected to play a role, the functional impact of synovial macrophages in signalling between synovium and cartilage in OA is not well understood. This study investigated potential roles of synovial macrophages in experimental OA development. Synovial macrophages were selectively manipulated *in vivo* with intraarticular injections of drug-laden liposomes to induce depletion, STAT1 inhibition, or STAT6 inhibition in a rat model of post-traumatic knee OA (PTOA). As we previously determined the effects of PTOA synovium on chondrocytes using our ex vivo co-culture model, we also explored roles played by macrophages in controlling chondrocyte responses to PTOA synovium *in vitro*.

PTOA was surgically induced in young adult rat knees, followed by intraarticular injection of liposomal clodronate (macrophage depletion), fludarabine (STAT1 inhibitor), AS 1517499 (STAT6 inhibitor), or PBS (control), at 14 and 21 days post-surgery. Pain-related behavior was measured at baseline (pre-surgery) and 4 weeks post-surgery prior to histopathological assessment of synovium and cartilage. Synovium was collected from additional animals for co-culture with healthy adult articular chondrocytes. Co-cultures were evaluated for sulfated glycosaminoglycan (sGAG) secretion into medium and chondrocyte gene expression.

In 4 week PTOA joints, depletion of synovial macrophages decreased synovitis scores for lining proliferation, subsynovial infiltration, and fibrin deposition in joints compared to control. Inhibition of STAT1 in synovial macrophages significantly decreased subsynovial infiltration scores. Inhibition of STAT6 decreased scores for lining proliferation, infiltration, and fibrin, similar to macrophage depletion. No changes were found for any treatment in cartilage damage scores or pain-related behavior. In co-cultures, synovium from macrophage-depleted 4 week PTOA joints induced significant downregulation of *Col2a1* expression in chondrocytes. Synovium from STAT1 inhibitor-

treated joints induced significantly increased sGAG secretion and *Acan*, *Adamts5*, and *Mmp13* expression. Synovium from STAT6 inhibitor-treated joints induced upregulation of *Acan* and *Ccl2* and downregulation of *Col2a1*.

In conclusion, all three liposomal drug treatments for synovial macrophages impacted synovial appearance, and each drug had a different pattern of effects. Signalling from synovium on cartilage is impacted by drug treatment, suggesting that future experiments with joints at a later stage of OA may show greater effects on cartilage damage and pain-related behavior.

3.2 Introduction

Osteoarthritis (OA) is the most prevalent joint disease and an increasingly greater health problem. Commonly occurring in the joints of the knee, hip, hand, and shoulder, OA results from pathological changes in articular cartilage, subchondral bone, synovium, and other joint tissues[1]. Patients experience varied pain and loss of mobility resulting in disability. OA is a multifactorial disease, and major components include age, sex, obesity, genetics, previous injury, and joint morphology[2]. The economic and social burden of OA is considerable; in 2010, hip and knee OA were ranked as the 11th highest out of 291 disease categories in disability-adjusted life years lost globally[3]. Current treatments focus on symptom management, as no disease-modifying drugs have been approved for patient use[4]. Novel strategies for treating OA that reduce damage to cartilage and other joint tissues in addition to reducing symptoms are urgently needed.

OA has been discussed as a chronic wound of the synovial joint[5]. Although the exact cause of OA is unknown, current understanding is that catabolic and anabolic processes in chondrocytes become unbalanced, with increasing production of catabolic and pro-inflammatory factors resulting in the destruction and eventual complete loss of articular cartilage, with pain and loss of function of the joint[1]. The role of the synovium in OA pathogenesis is not well understood, but synovitis is present as heterogenous low-grade (relative to other forms of inflammatory arthritis) inflammation in a large proportion of patients with knee OA[6]. Synovitis is correlated with disease progression and greater pain, and knee synovitis is predictive of OA incidence[7,8]. Microscopic examination of OA-associated synovitis shows increased proliferation of the intima, mononuclear cell infiltration, and increased vascularity[9].

Macrophages (Mφ) play central roles in maintaining tissue health through pro-inflammatory signaling, anti-inflammatory signaling, apoptotic cell clearance, and matrix growth and remodeling[10]. Synovial macrophages monitor the joint environment and modulate other cells in the joint (Figure 3.1)[11]. They affect the production of various growth factors, including TGF-β, vascular endothelial growth factor, and nerve growth factor[12,13]. Additionally, a subset of synovial macrophages are known to form an

epithelium-like barrier in the joint lining with tight junctions, which is disrupted in rheumatoid arthritis [14]. In early OA synovium, macrophages from the periphery infiltrate the tissue in significantly greater numbers[15]. Macrophage phenotype is plastic; when activated by different signals, macrophages dynamically polarize into functional subsets[16]. The classical spectrum describes these subsets as pro-inflammatory ("M1-like"), tending to produce catabolic factors such as TNF-α, and anti-inflammatory ("M2-like"), tending to produce anabolic factors such as TGF-β, although polarization is functionally complex with overlapping characteristics[17]. These phenotypes are chiefly driven by the activity of transcription factors signal STAT1 and NF-κB for M1-like and STAT3 and STAT6 for M2-like, with crosstalk between pathways[16,18]. STAT activity in synoviocytes has been implicated in the inflammatory pathways of rheumatoid arthritis, but less is known in the OA context[19]. Recent studies have found that negative regulation of cytokine-activated janus kinase/STAT signalling is reduced in human OA chondrocytes, contributing to cell death, but the implications for synovium are unclear[20].

Our previous work using a rat model of experimental post-traumatic OA (PTOA) has shown that this model develops synovitis and that co-culture of early PTOA synovium induces protective physiologic responses in healthy chondrocytes (Chapter 2). However, synovium from later stage PTOA did not induce these effects, suggesting plasticity of synovial physiology over time as PTOA develops and progresses. Synovial macrophages are a significant effector cell population and play important roles in the joint. Therefore, shifting of macrophage function induced by OA evolution may also be responsible for the less of the protective effect at a later stage of PTOA.

Previously, synovial macrophages have been found to significantly drive inflammatory cytokine and matrix protease production by synovial fibroblasts in human OA synovium and osteophyte formation in murine PTOA[21,22,23]. Systemic depletion of macrophages in a murine post-traumatic and metabolic OA model did not affect OA development but resulted in greater knee inflammation[24]. Although our results support a hypothesis that the synovium provides an initially protective effect in chondrocytes in early PTOA, whether macrophages are required or involved in this effect is unknown.

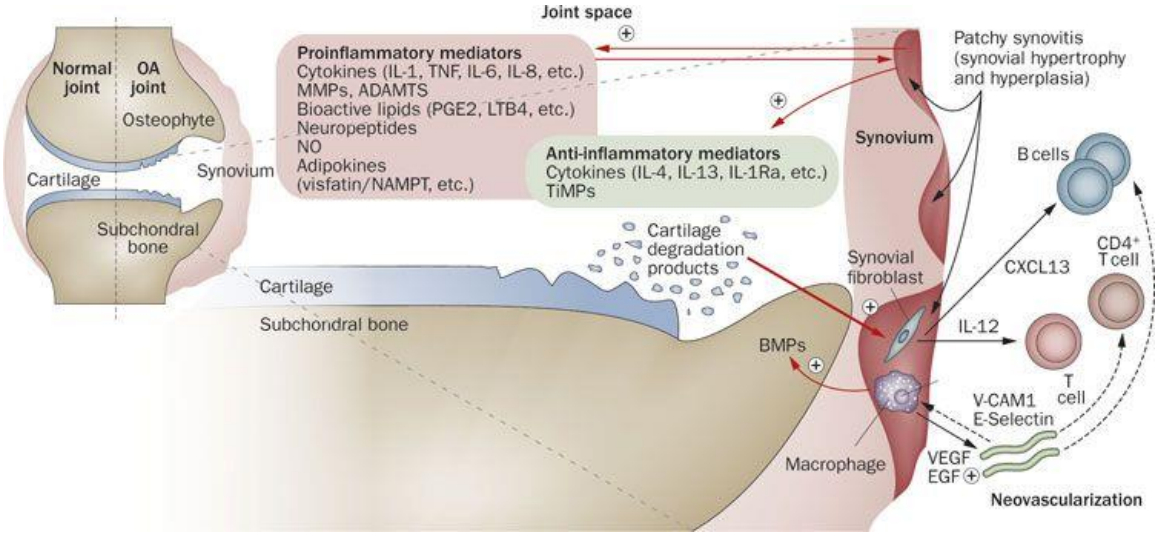

Figure 3.1. Signalling mediators and cells involved in OA synovitis.

Diagram of the OA joint with labels indicating signaling mediators and cell types involved in low-grade inflammation of the synovium. Image reproduced from Sellam & Berenbaum 2010[11].

In this study, we adapt a strategy to deplete macrophages with clodronate liposomes and report a novel strategy for shifting synovial macrophage physiology with selective STAT inhibitor liposomes. Phagocytes recognize large liposomal particles (>0.5 μm) as foreign and engulf them to form phagosomes, triggering breakdown of the particle in phagolysosomes and release of the contents into the cell[25]. Intrarticular injection of drug-laden liposomes therefore selectively deliver drug to synovial macrophages, which form the vast majority of phagocytes in the synovial joint lining. We then explored the effects of manipulating macrophage activation in early PTOA synovium on disease development *in vivo* and on articular chondrocyte physiology *in vitro*.

3.3 Materials and Methods

3.3.1 Reagents

Chemical reagents and histology supplies were purchased from Fisher Scientific or MilliporeSigma unless otherwise specified. Cell and tissue culture media were purchased from Gibco, while tissue culture plastics were from BD Falcon. RNA isolation reagents were purchased from Invitrogen. PCR reagents and equipment, including analysis software, were all supplied by Bio-Rad.

3.3.2 Liposome source and manufacture

Commercial PBS-only liposomes (vehicle-lip) and clodronate liposomes (clod-lip) were purchased from Encapsula Nanosciences. These commercial preparations contained 18.8 mg/mL L-α-phosphatidylcholine and 4.2mg/mL cholesterol in PBS as 1.5-2 μm liposomes, with 18.4 mM clodronate solution encapsulated in clod-lip. To make fludarabine liposomes (STAT1i-lip), a spontaneously formed vesicle dispersion was prepared by rotary evaporation of L-α-phosphatidylcholine and cholesterol mixed to match the commercial preparations (7:3 molar ratio) in chloroform at 30 rev/min and 420 mbar in a 55C water bath, removing remaining traces of chloroform under high vacuum for 24 hours, and hydrating the phospholipid film formed with 2.5 mg/mL fludarabine phosphate in PBS. The vesicle dispersion was then extruded six times through 1.0 μm pore polypropylene filters to form a liposome suspension. To make AS 1517499 liposomes (STAT6i-lip), L-α-phosphatidylcholine, cholesterol, and 1 mg/mL AS 1517499 were mixed in chloroform, evaporated, hydrated in PBS, and extruded as above. Initial batches of liposomes were checked for shape and structure by fixation in 1.5% paraformaldehyde and 1.5% glutaraldehyde in PBS and processing for imaging on a Philips Electronics CM10 Transmission Microscope with a with a Hamamatsu ORCA 2MPx HRL Camera (Figure 3.2a). Size distribution in all batches was checked by dynamic light scattering on a Zetasizer Nano ZS (Malvern Panalytical) to match that of the commercial preparation (Figure 3.2b).

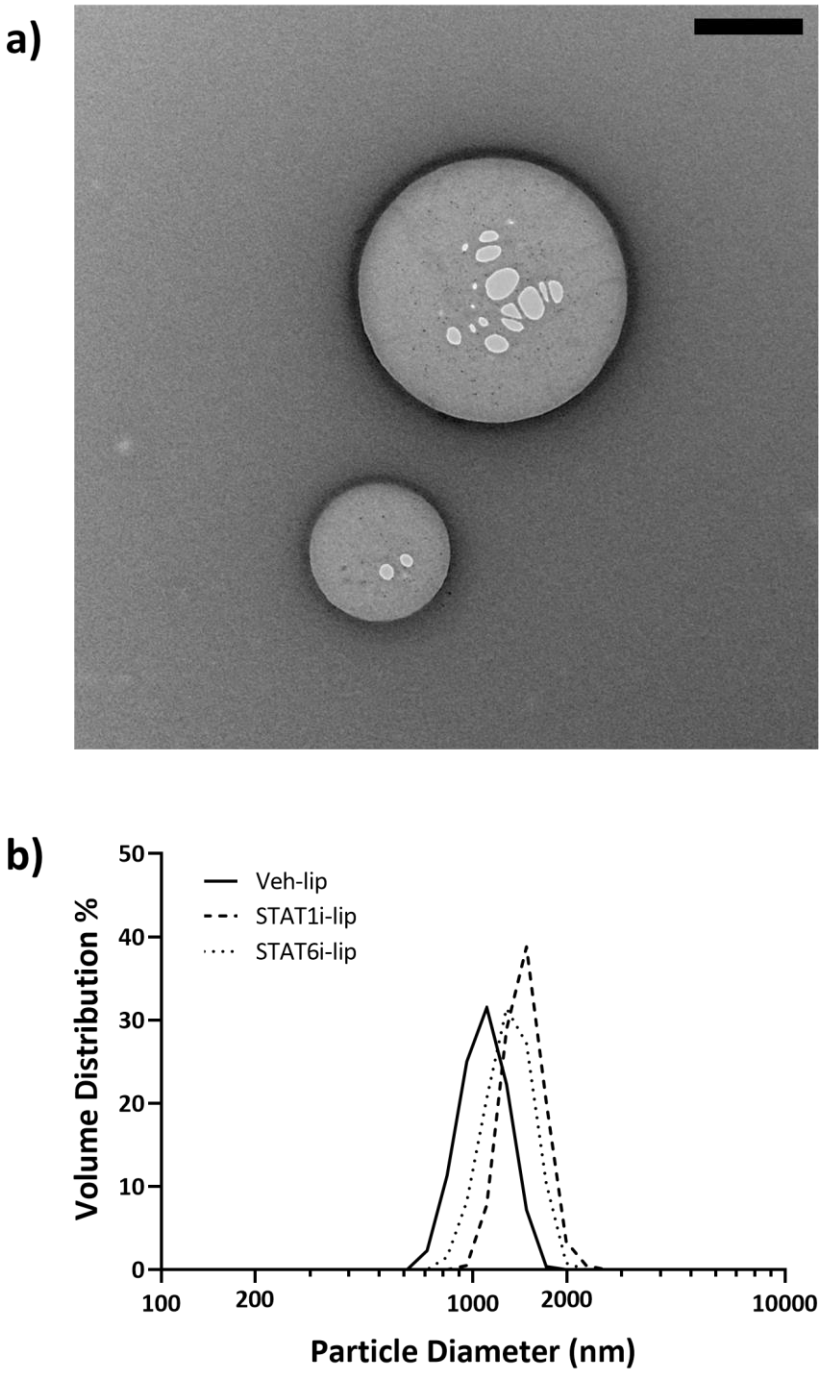

Figure 3.2. Morphology and size distribution of manufactured drug-laden liposomes.

a) Representative transmission electromicrograph of liposomes made by extrusion. Bar represents 200 nm. b) Sample size distributions of two lots of drug liposomes compared to the commercial vehicle liposome preparation.

3.3.3 Experimental PTOA rat model

All experiments were performed according to the Animal Use Protocol approved by the institutional Animal Care Committee (Appendix A). Outbred Sprague-Dawley rats (strain code 400) were sourced from Charles River Canada and fed a standard chow diet *ad libitum*. Following a previously established protocol, surgery was performed on the right knees of 12-week-old male rats[26,27]. Female rats were not included in this study. Briefly, animals were given prophylactic ampicillin and buprenorphine for post-procedure analgesia subcutaneously. Anesthesia was induced with isoflurane gas. PTOA was induced by incising the medial aspect of the anterior joint capsule to open the right knee joint, transecting the anterior cruciate ligament and the medial meniscotibial ligament at its attachment to the meniscus, and closing the joint capsule with absorbable sutures. Animals received a 50 µL intraarticular injection of liposomes in the operated knee on day 14 post-surgery and a second 50 µL injection on day 21. Injections were delivered using a Hamilton syringe through a 30-gauge needle through the patellar tendon to minimize trauma. The primary endpoint for all analyses was 4 weeks post PTOA induction.

3.3.4 Tissue histology and histopathological scoring

Knee joints were fixed overnight at 120° flexion and decalcified with Decal Stat (StatLab for 24 hours, followed by coronal hemisection into anterior (containing infrapatellar fat pad and para-articular tibial bone only) and posterior portions, then further decalcified with Formical-2000 until sufficient tissue pliability was reached (approximately 7 days), and processed for paraffin embedding overnight. Tissues were sectioned in the frontal plane to ensure all four tibiofemoral cartilage articulations were visible, at 5 µm per section with 5° blade bevel angle. Twenty adjacent sections were collected every 600 µm through the joint.

Two adjacent sections every 600 µm were stained with Toluidine blue to score articular cartilage damage (OARSI scale, cartilage degeneration component only[28]); briefly, four articular facets visible in the frontal section (medial and lateral femoral condyle and tibial plateau) were split into inner, middle, and outer thirds, with each third graded 0-5 (0 = no

damage, 1 = minimal damage, 5-10% of cartilage area affected by chondrocyte or matrix loss, 2 = mild, 11-25% affected, 3 = moderate, 26-50% affected, 4 = marked, 51-75% affected, 5 = severe, >75% affected) for a total score out of 15 per articular facet. Two adjacent sections every 600 µm were stained with Harris modified hematoxylin and eosin to assess synovitis (six-component score[29]); briefly, six compartments visible in the frontal section (medial and lateral parapatellar, superior, and inferior) were scored 0-3 (0 = normal, 1 = mild, 2 = moderate, 3 = severe) for a total score out of 18 in each of six categories (synovial lining thickness, synovial infiltration, fibrin deposition, vascularisation, fibrosis, perivascular edema). Slides were imaged on an Olympus BX53 microscope with an Olympus DP73 camera.

Immunohistochemistry was performed on adjacent sections for CD68+ cells as an indicator of macrophage presence. Slides underwent antigen retrieval by immersion in 10 mM Tris, 1 mM EDTA, 10% glycerol buffer at pH 9.0 heated to 70C and gradually cooled to room temperature. Slides were then immersed in 0.2% Triton X-100 in PBS for 10 min to permeabilize cells, followed by 3% hydrogen peroxide in PBS for 15 min to block endogenous peroxidases. Sections were blocked with 1% bovine serum albumin (GE Healthcare), 0.1% Triton X-100 in PBS for one hours. One section on each slide was kept in blocking buffer as a control for nonspecific binding, while the adjacent section was probed with rabbit anti-rat CD68 primary antibody (Abcam ab125212) diluted 1:100 in blocking buffer at 4C overnight. Both sections were probed with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody diluted 1:4000 in PBS at room temperature for 1 hr. Colorimetric detection was performed on slides using the DAB Peroxidase Substrate Kit (Vector Laboratories) following manufacturer instructions. Slides were counterstained with hematoxylin before mounting and imaged as above.

3.3.5 Pain-related animal behavior measurements

Mechanical allodynia and hyperalgesia measures were taken at baseline pre-PTOA induction and 4 weeks post-PTOA induction. The increased sensation of pain in response to non-noxious (allodynia) and noxious (hyperalgesia) stimuli is a feature of OA which is replicated in rat models[30,31,32,33].

Allodynia was measured using the Electronic Von Frey (EVF) unit (Bioseb). Animals were placed in an elevated bar-bottom platform to equilibrate for twenty minutes prior to testing to minimize ambulation. Readings were taken by pressing the tip of the EVF sensor to the plantar surface of the right hind paw at approximately 25 grams/second until paw withdrawal by the animal, with fifteen minutes between repeated measures. The average of two acceptable readings was recorded as the final threshold value.

Hyperalgesia was assessed by pressure application measurement using the Small Animal Algometer (SMALGO) unit (Bioseb). Animals were manually restrained with the right hind leg exposed. Readings were taken by attaching the SMALGO pressure sensor to the experimenter's thumb and pinching the knee with the sensor on the medial side at approximately 200 g/sec. A single threshold value was taken at the point where the animal attempted to withdraw the limb or vocalized discomfort.

3.3.6 Joint tissue cultures and co-culture system

Knee joint tissues were collected by gross dissection. Overlying skin, muscle, and tendon were removed from legs before opening the joint under sterile conditions. Primary articular chondrocytes were collected from two healthy, surgically-naïve 16-week-old male rats. Femoral and tibial articular surfaces were shaved using a scalpel and carefully excluding contamination by periosteum, meniscus, or bone. Cartilage from four joints were pooled together and minced into pieces of approximately 1 mm^3 in DMEM with 10% fetal bovine serum (FBS), 1% Antibiotic-Antimycotic, 800 U/mL collagenase II, and 100 U/mL DNase I, and run for 2 hours at 37C with variable agitation on a Miltenyi Biotec gentleMACS Dissociator. The resulting suspension was triturated, passed through a 70 μm cell strainer and washed with primary chondrocyte medium (1:1 DMEM/Ham's F12, low glucose, L-glutamine, with 10% FBS and 1% penicillin-streptomycin). Live cells were counted on a Bio-Rad TC20. Primary chondrocytes were seeded at 2-5% in T75 flasks in primary chondrocyte medium, with medium changes every 2-3 days until confluent. Expanded P0 chondrocytes were trypsinized, gradually frozen to -80C in FBS with 10% DMSO, and stored in liquid nitrogen.

Knee joint synovium was dissected over serum-free 1:1 DMEM/F12 with 1% penicillin-streptomycin with care to keep the tissue intact. After opening the joint by cutting around the suprapatellar region, the menisci and patella were removed, and synovium was peeled away from the outer fibrous capsule. All synovium from a single joint was placed into a seated 12-well transwell inserts, 0.4 µm pore size, with serum-free medium in both chambers.

For co-cultures, P1 articular chondrocytes were thawed from frozen stocks, washed with primary chondrocyte medium, seeded at 10% density into 12-well plates, and expanded until confluent. Prior to the start of the culture period, chondrocytes were serum-starved overnight (8 to 16 hours) and synovial tissue was placed into serum-free medium for 24 hours to equilibrate. Co-cultures were assembled by transferring the synovium-conditioned medium from the lower chamber onto the serum-starved chondrocytes and placing the transwell insert with synovium and conditioned medium on top.

3.3.7 Dimethylmethylene blue (DMMB) assay

Conditioned medium from co-cultures was assayed for sulfated glycosaminoglycan (sGAG) content using a protocol based on previously described methods[34,35]. In microwell plates, 200 µL DMMB reagent (38.46 mmol 1,9-dimethyl-methylene blue zinc chloride, 40.5 mmol glycine, 27.38 mmol sodium chloride, 9.5mmol acetic acid (Bioshop)) was combined with 100 µL of conditioned medium or chondroitin sulfate standards diluted to the appropriate range in serum-free chondrocyte medium in triplicate and read immediately at absorbance wavelength 595 nm with reference wavelength 655 nm on a Safire plate reader (Tecan Life Sciences).

3.3.8 RNA isolation and gene expression analysis

We made efforts to adhere to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines where possible regarding controls for contamination and technical error[36].

Co-cultured chondrocytes were lysed by adding TRIzol to each well and triturating. Samples were transferred to phase separation tubes, combined 5:1 with chloroform,

shaken for 50 seconds, and centrifuged at 12,000 x *g* for 15 minutes at 4C. The RNEasy Mini Kit (QIAGEN) was used for co-cultured chondrocytes and the Direct-zol RNA Miniprep Kit (Zymo Research) was used for peripheral blood monocyte-derived macrophages. Kit instructions were followed for subsequent steps, including combining the aqueous phase was combined with an equal volume of 70% ethanol and transferred onto silica spin columns, followed by on-column DNase I digestion, washes, and elution into nuclease-free water. Eluted RNA yield and purity was measured on a Nanodrop 2000 (Thermo Scientific). Samples were reverse transcribed using iScript Reverse Transcription Supermix following manufacturer thermal cycle recommendations.

Quantitative real-time PCR (qPCR) was performed by combining SsoAdvanced Universal SYBR Green Supermix, predesigned primers (Table 3.1), and cDNA template in triplicate. Template per reaction varied by gene. Plates were thermocycled as follows: an initial polymerase inhibitor removal step at 95C for 30 s, a repeating denaturation step at 95C for 15 s followed by an annealing and elongation step at 60C for 30 s for 40 cycles, with an ending melt-curve analysis from 65C to 95C in 0.5 C steps at 2 s/step. Cq values were calculated on CFXMaestro 1.1 and normalized to reference genes *Hprt1* and *Pop4* for macrophages and *Rpl13a* and *Rplp0* for chondrocytes. Reference genes were selected prior to target gene assessment using the Bio-Rad pre-designed rat reference gene panel with representative samples and CFXMaestro Reference Gene Selector tool.

3.3.9 Primary peripheral blood-derived macrophage cultures

Rat peripheral blood monocytes were isolated at euthanasia. Following SepMate manufacturer instructions and recommended volumes, whole blood was collected via cardiac puncture from healthy 16-week-old male rats, combined with an equal volume of 2% FBS in PBS, layered over Ficoll-Paque PREMIUM 1.084 in SepMate tubes, and centrifuged at 2200 x *g* for 20 min at room temperature. The top layer containing monocytes was decanted into a new tube and washed with 2% FBS and RPMI 1640 with 10% FBS and 1% penicillin-streptomycin at 300 x *g* for 10 minutes. Cells were plated in macrophage medium (RPMI 1640 with 50 ng/mL recombinant murine M-CSF (PeproTech), 10% FBS, and 1% penicillin-streptomycin) at 200,000 cells per well in 24-

Table 3.1. Reference information for Bio-Rad PrimePCR™ PCR primers.

Gene	Amplicon Context Sequence	Transcription	Amplicon Length
Acan	1:141780455-141787109	intron-spanning	109
Adamts5	11:29043239-29056911	intron-spanning	120
Arg1	1:23010271-23012299	Intron-spanning	68
Ccl2	10:69048547-69048678	exonic	102
Col10a1	20:42913429-42916882	intron-spanning	120
Col2a1	7:139647809-139647911	exonic	73
*Hprt1**	X:153250276-153250384	exonic	79
Il6	4:3095863-3097287	intron-spanning	120
Il17a	9:25698119-25699513	exonic	106
Mmp13	8:5531849-5533218	intron-spanning	87
Mmp3	8:5689395-5691167	intron-spanning	66
*Pop4**	1:95761052-95761187	exonic	106
Prg4	13:72640183-72642005	intron-spanning	118
*Rpl13a**	1:102186223-102186424	exonic	87
*Rplp0**	12:48589001-48589127	exonic	97
S100a8	2:209508182-209508274	exonic	63
Sox9	10:100968094-100968214	exonic	91
Tgfb1	1:83747870-83750404	intron-spanning	109
Tnf	20:6937988-6938129	exonic	112

* denotes a reference gene.

well plates, resulting in 50-70% macrophage differentiation as estimated under phase contrast microscopy after four days incubated at 37C and 5% CO_2. To assess liposome effects *in vitro*, differentiated cells were changed into 0.8 mL fresh macrophage medium with 5 uL of liposome suspension per well, incubated for 24 h, then stimulated with recombinant murine interferon-γ (IFN-γ) 100 ng/mL (PeproTech) or recombinant rat interleukin-4 (IL-4) 10 ng/mL (PeproTech) for 24 h.

3.3.10Statistics

Statistical tests set type I error threshold at $\alpha = 0.05$. One-way ANOVA followed by Dunnett's multiple comparisons test was used for the effects of liposomal drugs (comparisons between veh-lip, clod-lip, STAT1i-lip, and STAT6i-lip) on pain-related behavior, histopathological scores, sGAG secretion, and gene expression. Two-way ANOVA followed by Dunnett's multiple comparisons test was used for the effects of liposomal drugs and activating factor on primary peripheral blood-derived macrophages (comparisons between veh-lip, STAT1i-lip, and STAT6i-lip with M-CSF only, IFN-γ, and IL-4).

3.4 Results

3.4.1 Targeted drug delivery to synovial macrophages alters features of synovitis in early PTOA joints

Sections from 4W PTOA knees injected with drug-laden liposomes were semi-quantitatively scored for the appearance of synovitis in different compartments around the joint. Overall, scores reflect mild to moderate synovitis across all joint compartments, as expected in this animal model (Figure 3.3). Clod-lip effectively reduced CD68+ staining from the intimal lining (Figure 3.4). Clod-lip treatment resulted in a significantly decreased score of the number of intimal lining layers, subintimal inflammatory infiltrate, and fibrin deposition, but increased vascularization scores (Figure 3.5). STAT1i-lip decreased subintimal infiltration and increased vascularization scores. Similar to clod-lip, STAT6i-lip decreased intimal lining layers, subintimal infiltration, and fibrin deposition scores. Overall, it appeared that inhibiting or depleting macrophages resulted in decreased subintimal infiltration and no changes in perivascular edema.

3.4.2 Targeted drug delivery to synovial macrophages does not markedly affect cartilage histopathological scores in early PTOA joints

Sections from the same 4W PTOA knees were semi-quantitatively scored for cartilage damage to articular surfaces following the cartilage degeneration component of the OARSI histopathology grading system[24]. As expected in this rat model of PTOA, PTOA joints treated with veh-lip control had minimal to mild focal cartilage damage, with more damage to the medial articular surfaces (Figure 3.6). Liposome-treated joints scored similarly, with no significant differences found between any treatments and control in damage scores for any joint compartment, nor for the cumulative joint scores (Figure 3.7).

3.4.3 Targeted drug delivery to synovial macrophages does not induce changes in pain-related behavior in early PTOA

Pain-related behavior assessments including mechanical knee hyperalgesia and ipsilateral hindpaw allodynia were performed on rats at baseline and 4W PTOA to compare the effects of clod-lip and STAT1i-lip treatment. Pain-related behavior data from this rat

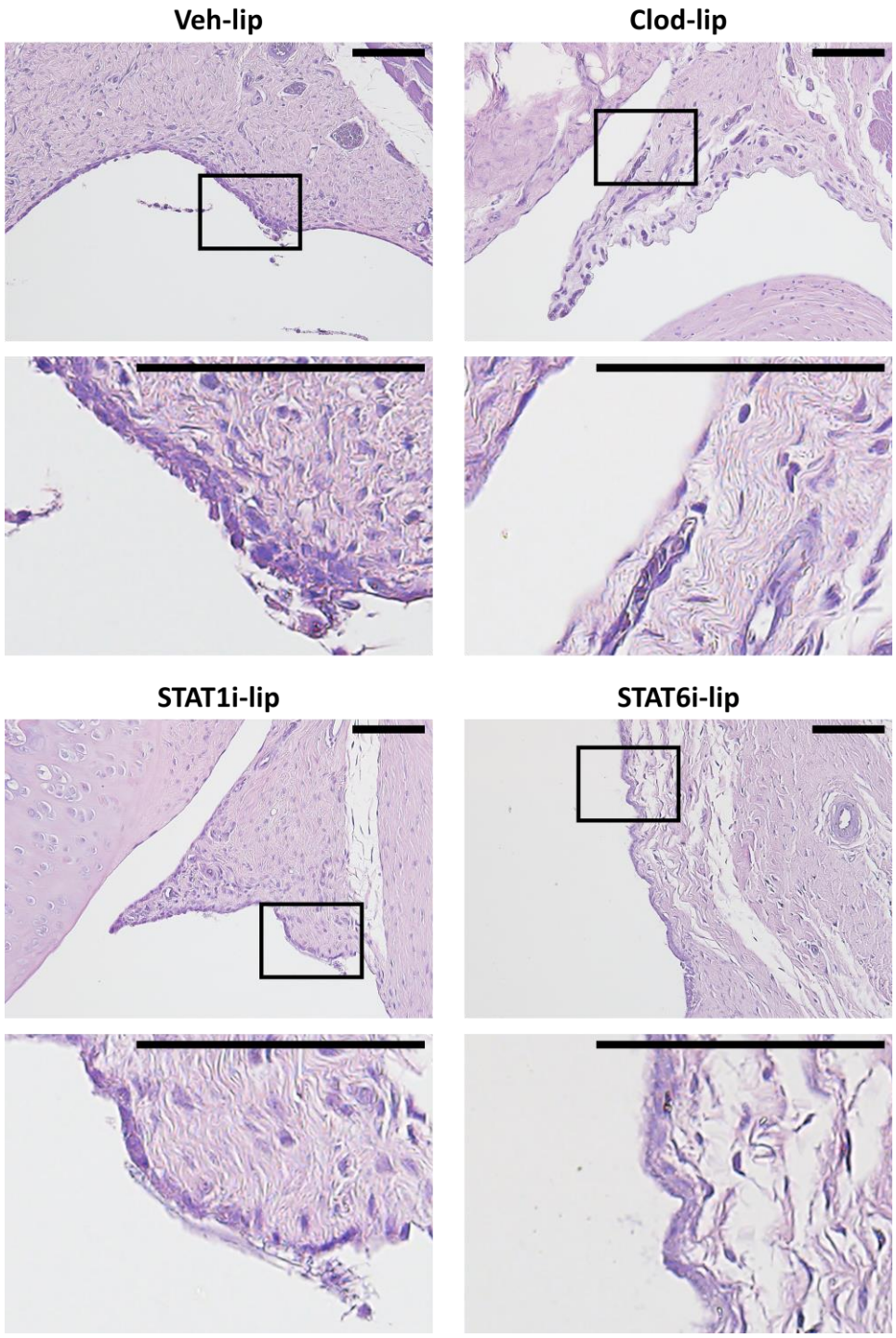

Figure 3.3. Low-grade synovitis present in liposome-treated 4W PTOA joints.

Representative micrographs of H&E stained synovium from 4W PTOA knees injected intraarticularly with vehicle (veh-lip), clodronate (clod-lip), STAT1 inhibitor (STAT1i-lip), and STAT6 inhibitor (STAT6i-lip) treatments (n = 5). Bar represents 100 μm.

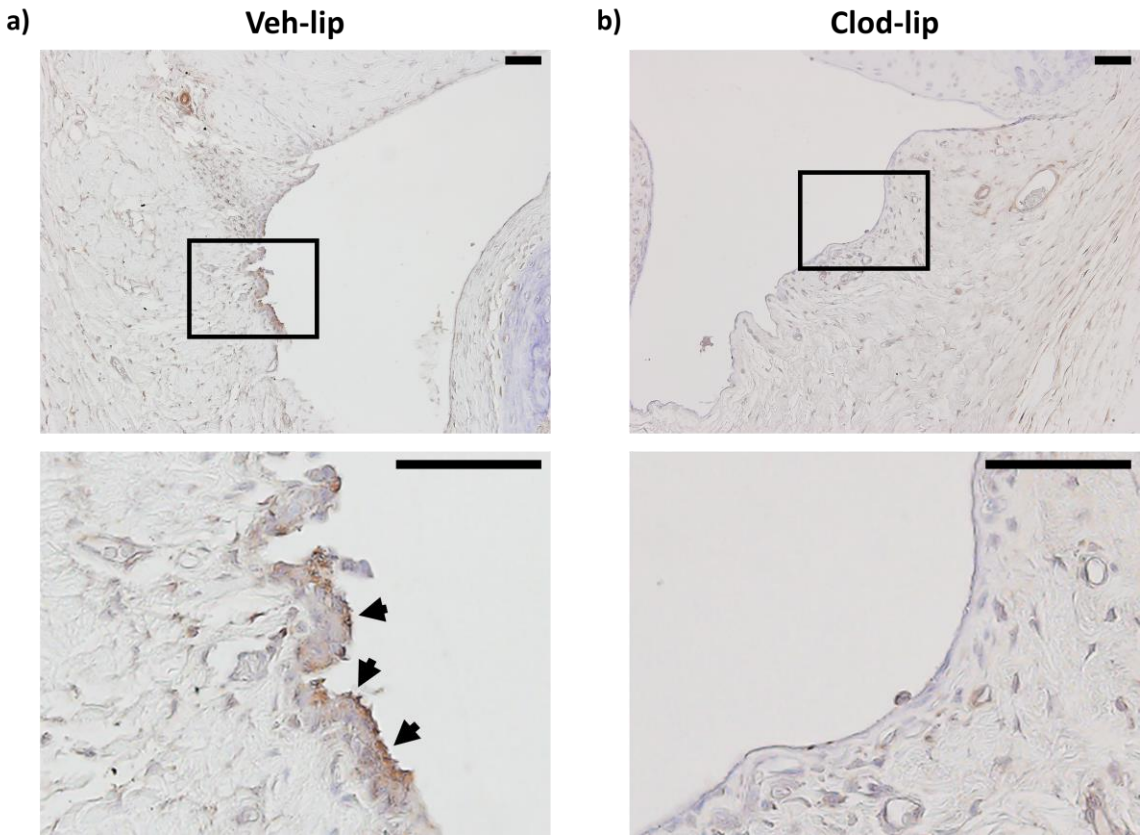

Figure 3.4. CD68+ cells in synovium from clodronate liposome-treated 4W PTOA joints.

Representative micrographs of synovial lining from 4W PTOA knee joints injected intraarticularly with vehicle (veh-lip) and clodronate (clod-lip) treatments (n = 5). Brown stain indicates CD68 with blue hematoxylin counterstain. Arrows indicate CD68+ cells in intimal lining. Bar represents 100 μm.

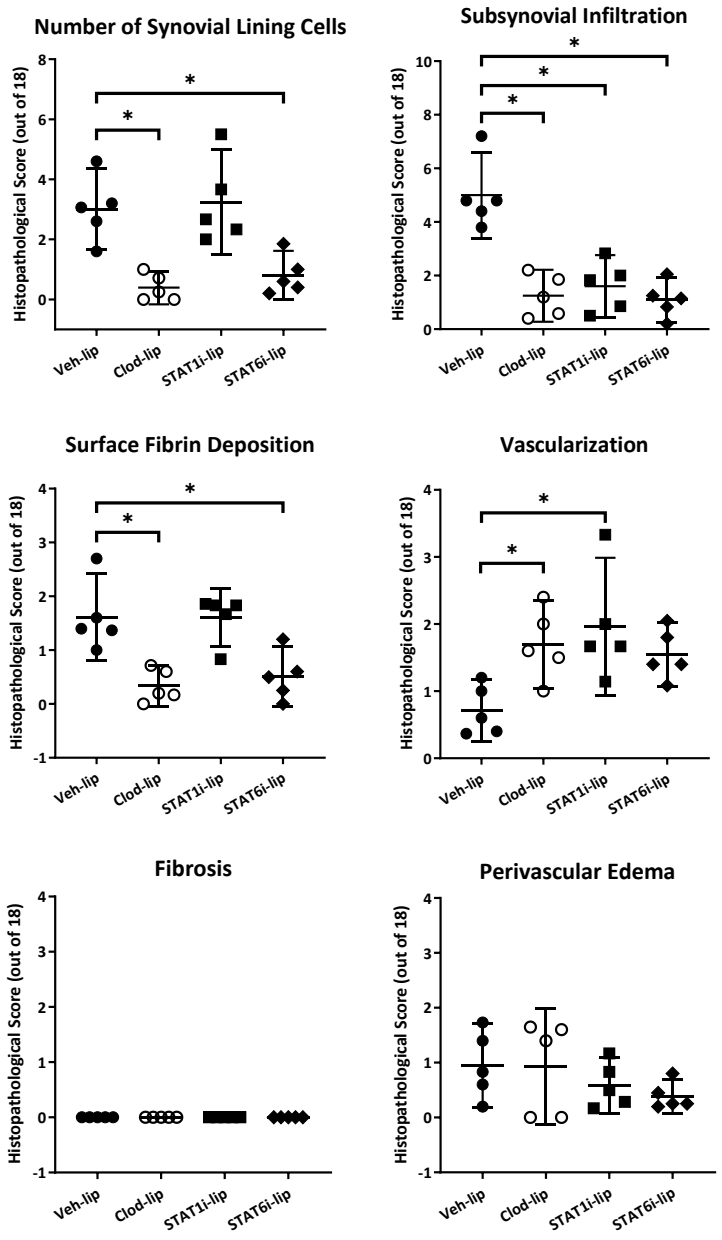

Figure 3.5. Histopathological scoring for features of synovitis in liposome-treated 4W PTOA joints.

Semi-quantitative scoring for features of synovitis in 4W PTOA knee joints injected intraarticularly with vehicle (veh-lip), clodronate (clod-lip), STAT1 inhibitor (STAT1i-lip), and STAT6 inhibitor (STAT6i-lip) treatments (mean ± 95% CI, n = 5, *p < 0.05). For each histopathological feature, scores indicating 0 = normal, 1 = mild, 2 = moderate, and 3 = severe were assigned for each of six compartments, totaling a score out of 18 per compartment; individual scores represent the average of 3-7 slides per joint.

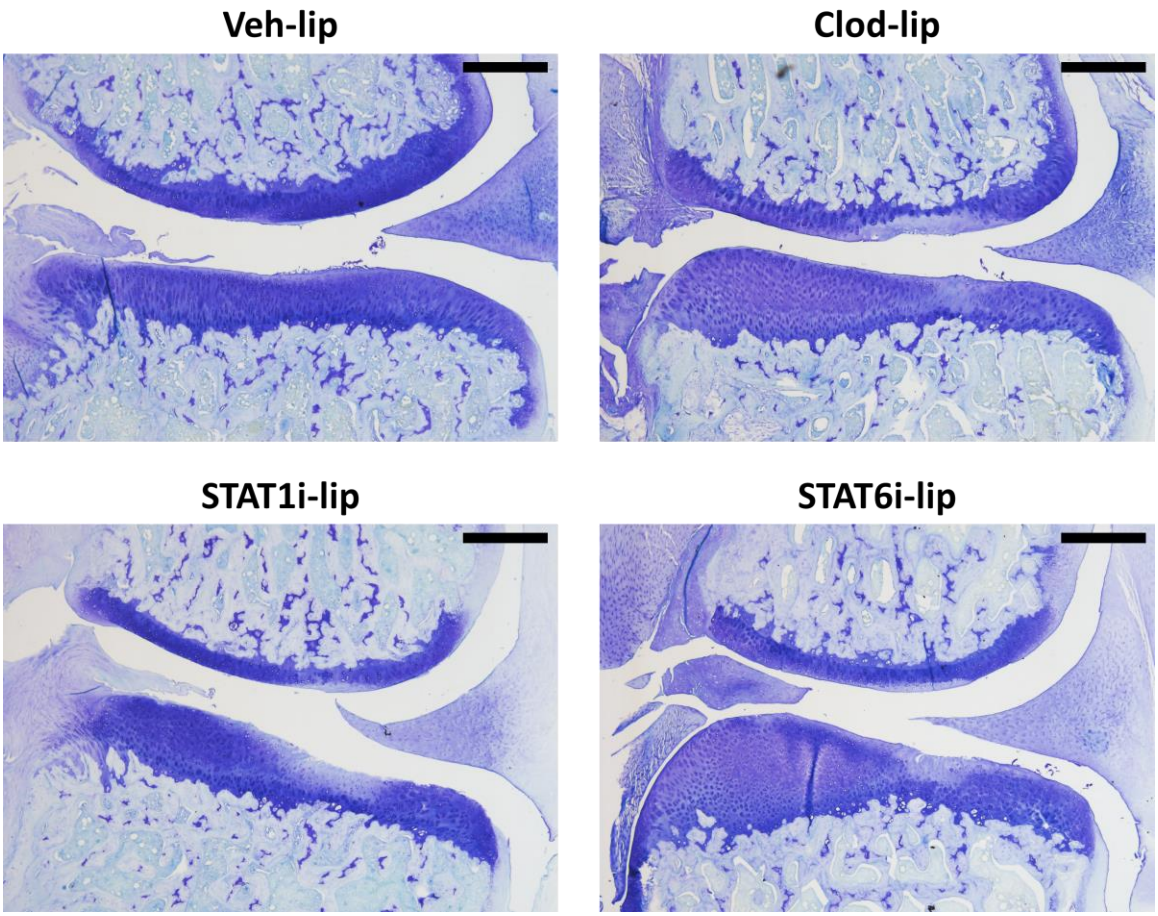

Figure 3.6. Low-grade cartilage damage present in liposome-treated 4W PTOA joints.

Representative micrographs of toluidine blue stained medial joint compartments from 4W PTOA knees injected intraarticularly with vehicle (veh-lip), clodronate (clod-lip), STAT1 inhibitor (STAT1i-lip), and STAT6 inhibitor (STAT6i-lip) treatments (n = 5). Bar represents 500 µm.

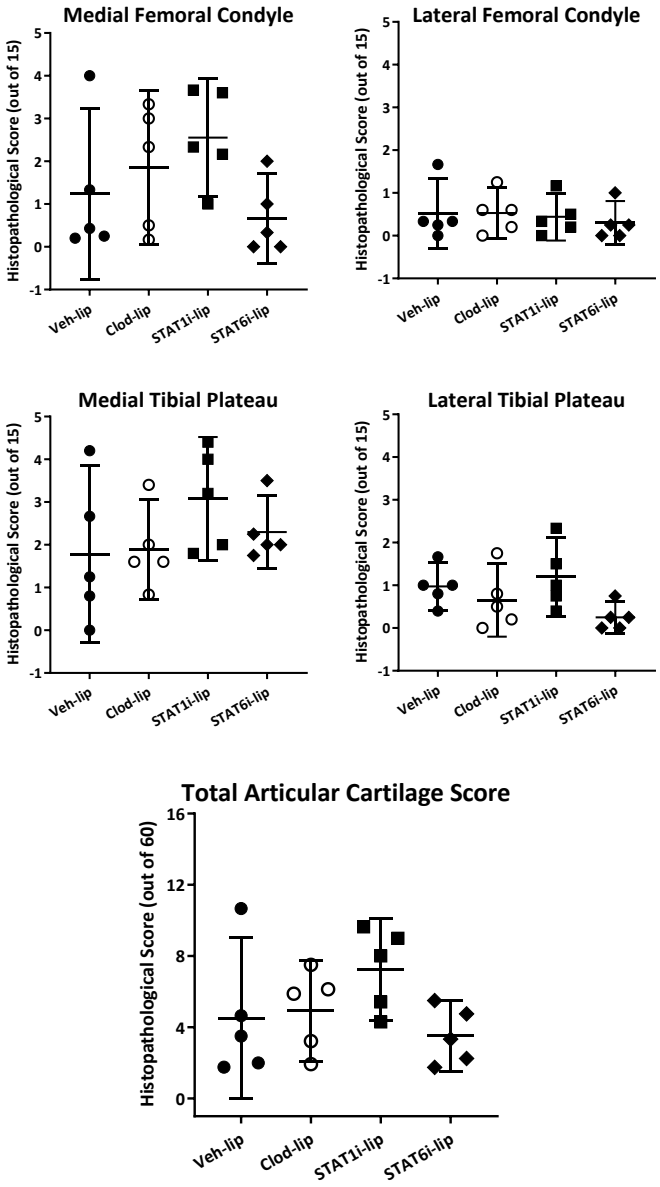

Figure 3.7. Histopathological scoring for cartilage damage in liposome-treated 4W PTOA joints.

Semi-quantitative scoring for chondrocyte and matrix loss in 4W PTOA knee joints injected intraarticularly with vehicle (veh-lip), clodronate (clod-lip), STAT1 inhibitor (STAT1i-lip), and STAT6 inhibitor (STAT6i-lip) treatments (mean ± 95% CI, n = 5, *p < 0.05). Scores indicating 0 = no damage, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe were assigned for each third of an articular facet, totaling a score out of 15 per facet and out of 60 per joint; individual scores represent the average of 3-5 slides per joint.

model of PTOA are not available in the literature. The veh-lip control condition did not suggest that this model develops mechanical hyperalgesia (Figure 3.8) or allodynia (Figure 3.9) at the early 4 week time point in PTOA development. No clear trends were evident for change in limb withdrawal or paw withdrawal thresholds for liposome-injected animals, with animals becoming both more and less sensitive at 4W PTOA compared to baseline for both drug liposomes tested.

3.4.4 Targeted drug delivery to synovial macrophages alters articular chondrocyte physiology

We previously showed that co-culture of whole synovial tissues from early PTOA induces overall protective responses by articular chondrocytes (Chapter 2). Here, we explored the role of synovial macrophages in mediating chondrocyte responses when exposed to synovium using the same co-culture system.

Synovium isolated from 4W PTOA knees injected with drug liposomes differentially induced changes in adult primary articular chondrocyte sGAG release and gene expression, compared with vehicle-lip-treated PTOA synovium. Co-culture of articular chondrocytes with early PTOA synovium from clod-lip-treated (macrophage-depleted) joints did not significantly alter sGAG secretion (Figure 3.10), but significantly decreased *Col2a1* expression (Figure 3.11). STAT1i-lip-treated early PTOA synovium induced further sGAG release beyond the sGAG induction induced by early PTOA synovium alone (Chapter 2). STAT1i-lip also induced significantly increased levels of *Acan, Adamts5,* and *Mmp13,* with strong trends toward increased *Col2a1*, *Mmp3,* and *Prg4.* Synovium from STAT6i-lip-treated joints caused chondrocytes to trend toward increased sGAG secretion, but this difference was not statistically significant. STAT6i-lip-treated synovium induced increased expression of *Acan* and *Ccl2,* and decreased expression of *Col2a1* in chondrocytes.

3.4.5 Liposomal drug delivery alters primary peripheral blood-derived macrophage physiology *in vitro*

Clod-lip depletion of macrophages is well-established in the literature, but the effects of liposomal STAT inhibitor treatments are not reported. To determine STAT1i-lip and

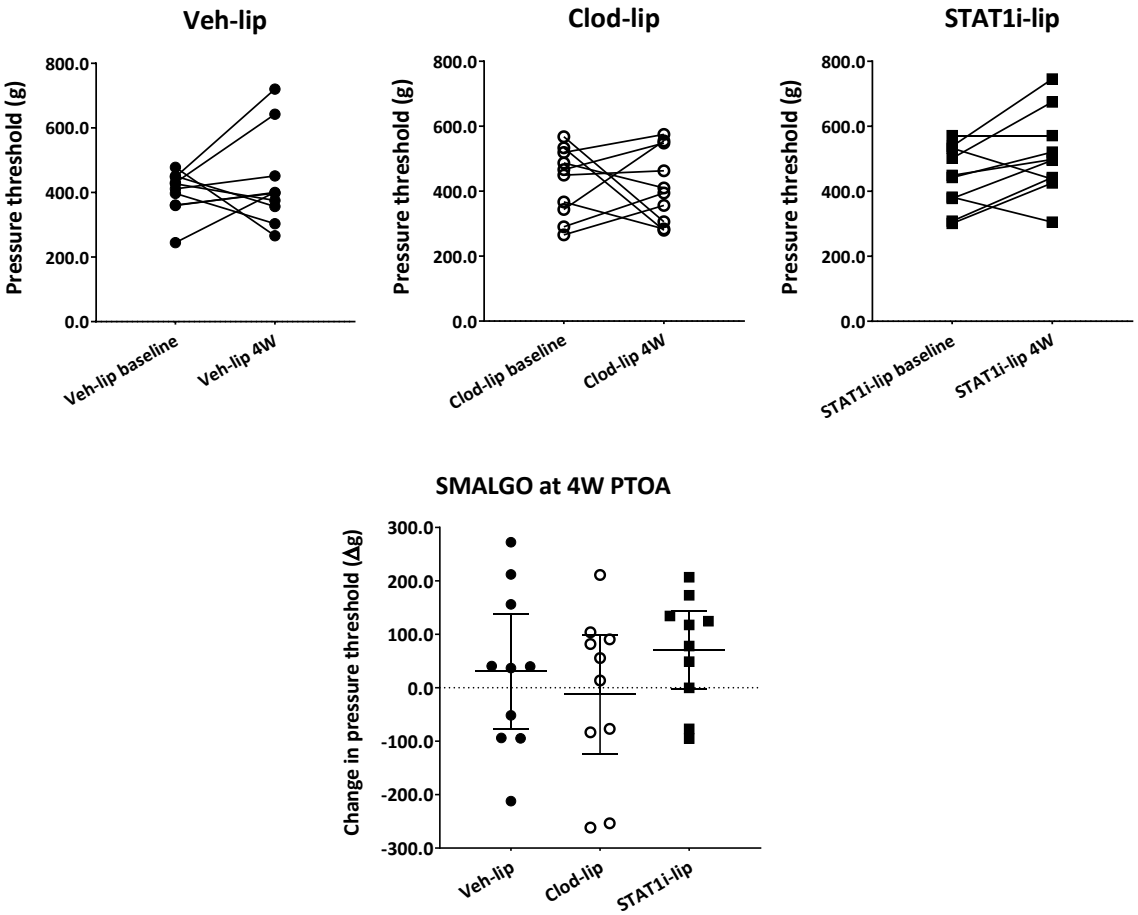

Figure 3.8. Mechanical hyperalgesia in liposome-treated 4W PTOA animals.

Pressure algometry for mechanical hyperalgesia as measured by the SMALGO unit in PTOA knees injected intraarticularly with (veh-lip), STAT1 inhibitor (STAT1i-lip), and STAT6 inhibitor (STAT6i-lip) treatments at 4 weeks post PTOA induction (mean ± 95% CI, n = 10).

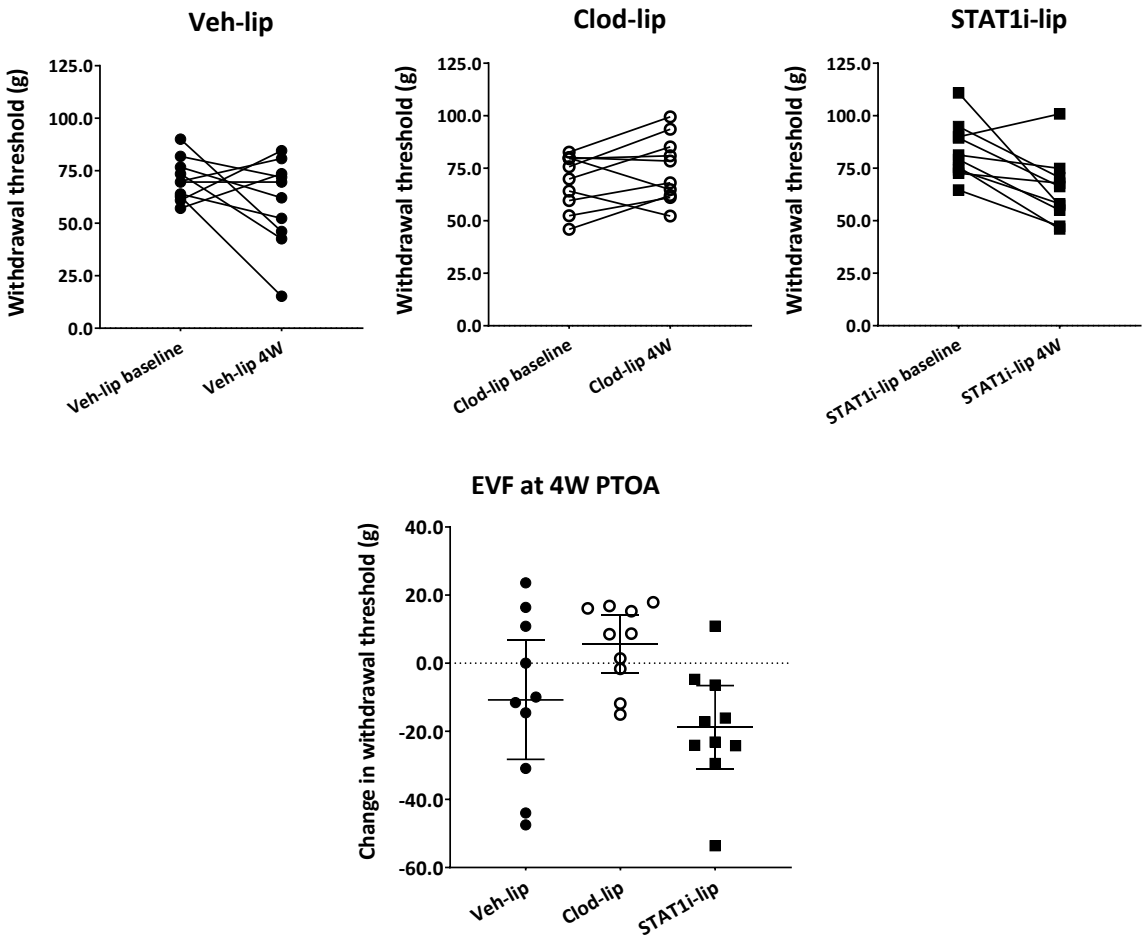

Figure 3.9. Tactile allodynia in liposome-treated 4W PTOA animals.

Pressure algometry for tactile allodynia as measured by the Electronic Von Frey unit in ipsilateral hindpaws of PTOA knees injected intraarticularly with (veh-lip), STAT1 inhibitor (STAT1i-lip), and STAT6 inhibitor (STAT6i-lip) treatments at 4 weeks post PTOA induction (mean ± 95% CI, n = 10).

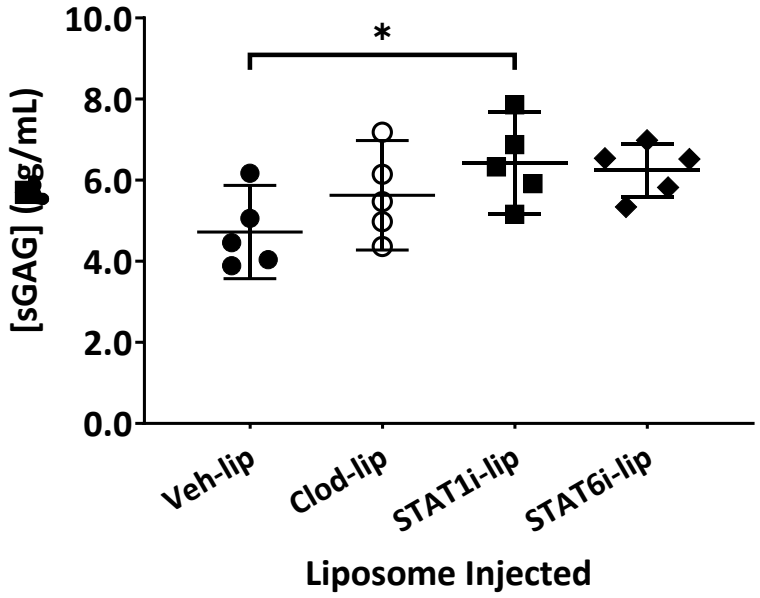

Figure 3.10. sGAG content in conditioned medium from primary articular chondrocytes co-cultured with liposome-treated 4W PTOA synovium.

Synovium was collected from 4W PTOA knee joints injected intraarticularly with vehicle (veh-lip), clodronate (clod-lip), STAT1 inhibitor (STAT1i-lip), and STAT6 inhibitor (STAT6i-lip) treatments. Synovial tissues were co-cultured with healthy adult primary articular chondrocytes for 24 hours. Conditioned medium was collected and measured for sGAG content by DMMB assay (mean ± 95% CI, n = 5, *$p < 0.05$).

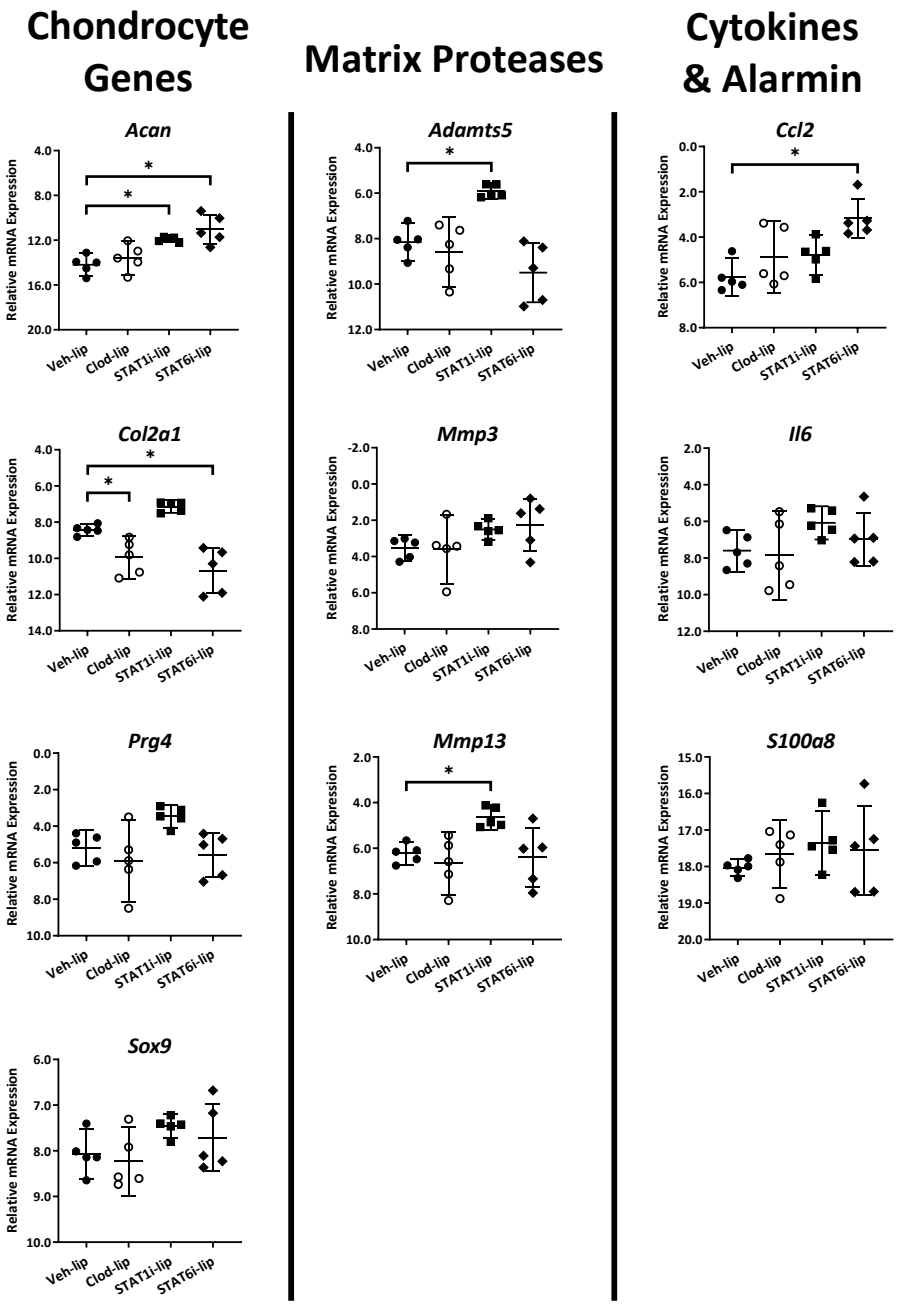

Figure 3.11. Gene expression in primary articular chondrocytes co-cultured with liposome-treated 4W PTOA synovium.

Synovium was collected from 4W PTOA knee joints injected intraarticularly with vehicle (veh-lip), clodronate (clod-lip), STAT1 inhibitor (STAT1i-lip), and STAT6 inhibitor (STAT6i-lip) treatments. Synovial tissues were co-cultured with healthy adult primary articular chondrocytes for 24 hours. qPCR analysis of gene expression levels is displayed as ΔCq (mean ± 95% CI, n = 5, *$p < 0.05$).

STAT6i-lip treatment effects on macrophages physiology, primary peripheral blood-derived macrophages were cultured with each type of liposome in medium alone, with classical activator IFN-γ, or with alternative activator IL-4. STAT6i-lip treatment resulted in higher *Ccl2* expression, while STAT1i-lip did not induce changes in any of the genes selected (Figure 3.12). Stimulation with IFN-γ, regardless of liposome treatment, induced macrophages to increase *Ccl2* levels compared to unstimulated controls. *Ccl2* was downregulated in macrophages given IL-4 with either liposome treatment, but not back to the basal level unstimulated level. No significant differences were found between groups for *Tnf*. Macrophages incubated with IL-4 significantly upregulated *Arg1* with all liposome treatments, but liposomes did not have an additional effect. *Tgfb1* expression significantly decreased in macrophages treated with IL-4 and STAT1i-lip compared to IL-4 and veh-lip.

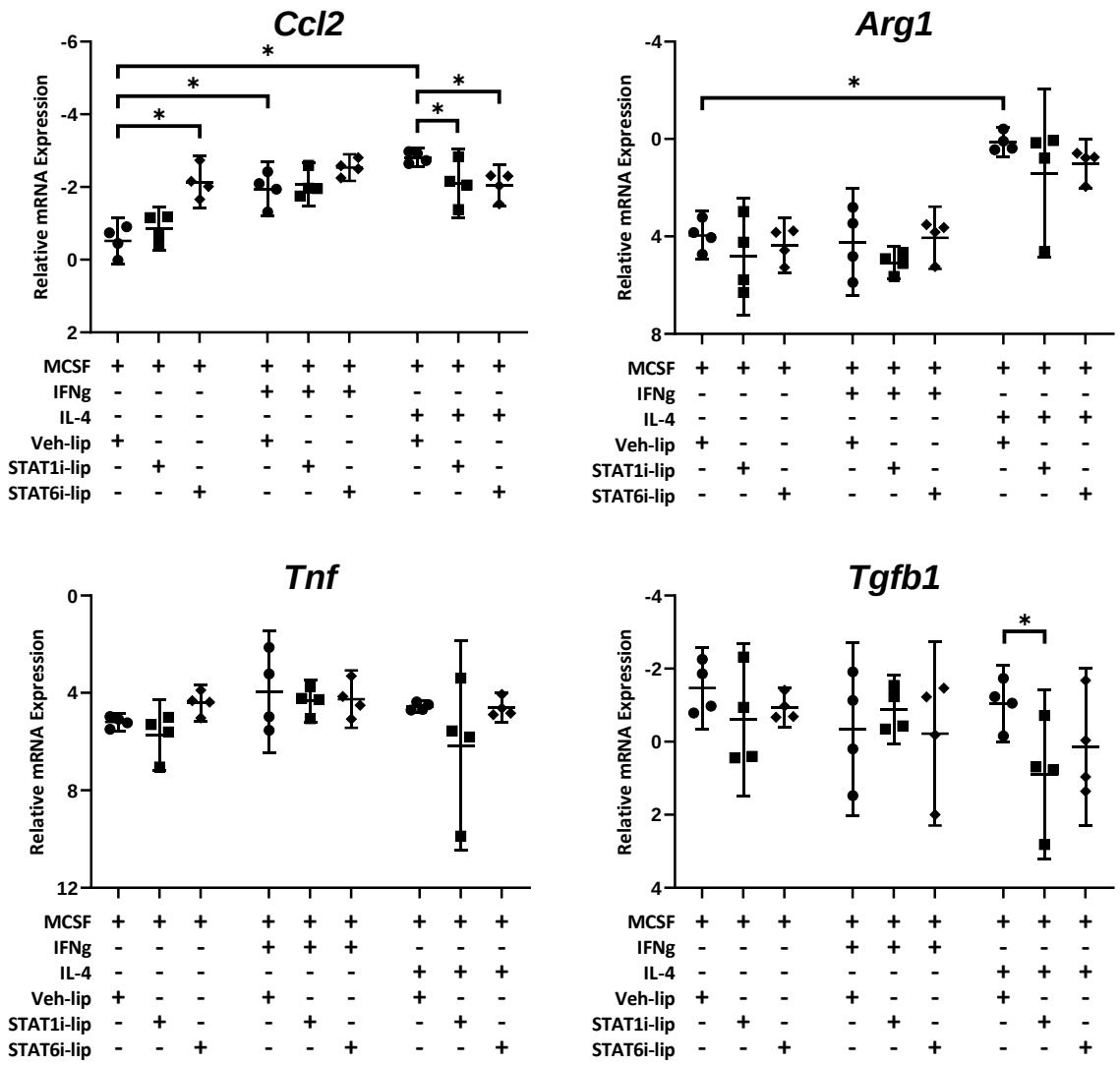

Figure 3.12. Gene expression in primary peripheral blood-derived macrophages treated with liposomes and activating factors.

Primary macrophages were differentiated from peripheral blood monocytes with 50 ng/mL M-CSF and incubated for 24 hours with no factor, 100 ng/mL IFN-γ, or 10 ng/mL IL-4 and treated with vehicle (veh-lip), STAT1 inhibitor (STAT1i-lip), or STAT6 inhibitor (STAT6i-lip). qPCR analysis of gene expression levels is displayed as ΔCq (mean ± 95% CI, n = 4, *p < 0.05).

3.5 Discussion

Synovial macrophages are markedly increased in joints from OA patients and experimental OA models, suggesting that macrophages may play important roles in OA development or progression[15,37]. We investigated the effects of synovial macrophage treatments using drugs to induce depletion or STAT signaling inhibition, targeted to phagocytes through liposomal encapsulation. We previously discovered that chondrocyte physiology is altered by co-culture with synovial tissues and that synovium from early PTOA induces overall protective effects in articular chondrocytes (Chapter 2). To study the effects of synovial macrophage manipulation on synovium and cartilage *in vivo*, we assessed pain-related behavior and histopathological measures of cartilage damage and synovitis. To study the chondrocyte response to treated synovium, we used our novel co-culture model of the synovial joint in combination with macrophage-manipulating treatments. This included whole joint synovium from 4W PTOA animals treated with intraarticular liposomal drugs and healthy primary articular chondrocytes cultured in separate chambers, allowing exchange of signalling molecules.

Our data from clodrosome-injected joints confirmed that these liposomes were effective at depleting macrophages from the synovial intima and reducing synovitis severity, consistent with previous reports[22,38,39,40]. While macrophage depletion reduced subsynovial infiltration and fibrin deposition, sGAG production did not significantly differ, suggesting that macrophages are not directly involved in mediating signals from PTOA synovium to chondrocytes that result in increased sGAG release. However, macrophage depletion did cause downregulation of *Col2a1* expression in chondrocytes, indicating that macrophages are involved in synovium to chondrocyte signaling. Although there was no clear effect on histopathological measures of cartilage damage in the early PTOA, cartilage changes are typically mild at this early stage of experimental PTOA development. Similarly, no difference in mechanical knee hyperalgesia or hindpaw allodynia were detected. Interestingly, depletion of macrophages in these experiments suggest that suppression of *Col2a1* expression in chondrocytes by early PTOA synovium (Chapter 2) may be partially mitigated by synovial macrophages, since *Col2a1* expression was suppressed by PTOA synovium after macrophage depletion.

Activation of STAT family members through various receptors strongly affects macrophage polarization. Specifically, STAT1-mediated effects including activation of NF-κB signaling are known to drive the pro-inflammatory M1-like phenotype, while STAT3 and STAT6 promote the anti-inflammatory M2-like phenotype[16,17]. TGF-β, which induces type II collagen and aggrecan expression in chondrocytes, is found in OA synovium and known to be secreted by M2-like macrophages[23,41,42,43]. This may explain the partially protective effect of mitigating *Col2a1* suppression in chondrocytes exposed to early STATi-Lip PTOA synovium. Moreover, STAT6i-lip treatment further suppressed *Col2a1* expression. M2-like macrophages in PTOA synovium may be responsible for producing TGF-β that helps to sustain chondrocyte *Col2a1* gene expression. This would be congruous with the finding that clod-lip suppresses *Col2a1* expression, as the loss of synovial macrophages would decrease TGF-β levels in the joint environment. Further supporting a protective effect of STAT1 inhibition, synovium from STAT1i-lip-treated joints had decreased subsynovial infiltration and increased vascularization, and co-cultured chondrocytes upregulated matrix molecules *Acan* and *Prg4*. Increased aggrecan expression may compensate for early cartilage damage, while lubricin decreases friction in articulation and inhibits chondrocyte catabolism and hypertrophy[44]. On the other hand, STATi-lip-treated synovium also upregulated matrix metalloproteases *Adamts5*, *Mmp3*, and *Mmp13*. Both major synovial cell types are known to secrete MMPs, and key MMPs are able to cleave the zymogen forms of other family members in an activation cascade[45]. With no significant differences found in cartilage damage and pain-related behavior, it is unclear whether STAT1-lip treatment is beneficial. Further experiments with longer duration of liposomal drug delivery are needed to determine overall joint-level effects, through histology and/or protein analyses.

STAT6i-lip treatment was expected to shift synovial macrophages toward the pro-inflammatory M1-like phenotype. Unexpectedly, STAT6i-lip treated synovium induced chondrocytes to upregulate *Acan* in addition to downregulating *Col2a1* downregulation in chondrocytes, suggesting that these matrix components are regulated by different pathways. Co-cultured chondrocytes also upregulated *Ccl2*, as did peripheral blood-derived macrophages given STAT6i-lip *in vitro*. C-C motif chemokine ligand 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP1), is increased in OA synovial

fluid and associated with symptom severity in patients[37,46,47]. Synovial macrophages and mesenchymal progenitor cells both express corresponding receptor C-C chemokine receptor type 2 (CCR2) and produce CCL2, forming the basis for a positive feedback loop of immune recruitment and inflammation[37,48]. Chondrocytes can also respond to CCL2 by producing MMP3 and MMP13[49,50]. Previous work has found that systemic administration of a CCR2 inhibitor was protective in experimental PTOA, especially when given at an early stage[51,52]. Thus, STAT6 inhibition in synovial macrophages most likely has a overall pro-inflammatory impact on the joint. Histology of STAT6i-lip-treated 4W PTOA joints showed mixed results, with increased vascularization but decreased intimal lining, subsynovial infiltration, and fibrin deposition in the synovium and no significant differences in cartilage damage. As with the previous treatment, results are not conclusive with regard to disease progression. A later PTOA timepoint with prolonged liposome treatment, such as 12 weeks post-induction, is needed to find differences not detectable at 4 weeks PTOA.

To characterize changes in synovial macrophages treated with STAT inhibitor liposomes, peripheral blood-derived macrophage gene expression *in vitro* was assessed after 24 hours of treatment with liposomes and activating factors. In designing the experiment, we assumed based on the literature that classical activation with IFN-γ would cause upregulation of M1-like markers *Ccl2* and *Tnf* and no change or downregulation of M2-like markers *Arg1* and *Tgfb1*, while alternative activation with IL-4 would cause the reverse. Results from these control conditions showed that M(IFN-γ) only upregulated *Ccl2*, while M(IL-4) upregulated both *Ccl2* and *Arg1*. In future experiments, different markers or activating factors should be selected for appropriate phenotyping. Additionally, fludarabine has been noted to cause downregulation of *Stat1* and depletion of STAT1 lasting multiple days *in vitro*[53]. As the pharmacokinetics of liposome-encapsulated drugs differs from that of the free compound, it is unknown whether STAT1i-lip dosage was high enough to cause significant off-target effects[54]. We also predicted that STAT1 inhibition would cause downregulation of *Ccl2* and *Tnf*, in both M0 and M(IFN-γ), but results showed no significant differences in either. Finally, we predicted that STAT6 inhibition would cause downregulation of M2-like markers *Arg1* and *Tgfb1*, in both M0 and M(IL-4), but again no significant differences were detected.

However, as previously discussed, STAT6i-lip did cause significant upregulation of *Ccl2*, which may be an indication of shifting toward the M1-like phenotype. Further characterization of M(STAT1i-lip) and M(STAT6i-lip) phenotype over time is needed, such as protein analyses for marker expression or microscopy for morphology, given that drug effects likely must be sustained *in vivo* to achieve therapeutic effect.

The present study has several limitations. Although fludarabine phosphate and AS 1517499 are selective inhibitors of STAT1 and STAT6, off-target effects cannot be completely eliminated. As a nucleotide analog, fludarabine inhibits certain proteins involved in DNA production and can also inhibit RNA production[55]. Inhibition of DNA synthesis should not be a major problem in mature tissue macrophages, but they do have high transcriptional activity, and an overall effect of transcriptional inhibition cannot be ruled out. AS 1517499 was discovered more recently and its mechanisms of action have not been extensively examined in the literature. However, for both treatments, no overt signs of tissue or cellular necrosis such as nuclear condensation and fragmentation were evident in H&E-stained synovium sections. While our rat model of PTOA has been previously validated, tissue injury from the intraarticular injections may have additional effects on the joint environment, although this was controlled for across conditions. We did not include a no liposome, saline-only control group as a major effect of PBS-only liposomes on PTOA development is not expected. Local effects of injections may be avoided through systemic administration, as seen in the reduction of experimental antigen-induced inflammatory arthritis via systemic administration of clodronate liposomes, but this approach is unlikely to be successful for other drugs or use in patients[39]. Finally, our experiments in investigating physiological differences in chondrocytes induced by liposome-treated synovium used healthy primary articular chondrocytes pooled from the joints of healthy young adult male rats and we cannot rule out the possibility that different responses may occur in OA chondrocytes. As the liposome-treated 4W PTOA joint environment *in vivo* includes signals from early PTOA chondrocytes in addition to the liposomes, use of OA chondrocytes and later PTOA synovium in future experiments may reveal crosstalk effects beyond the co-cultures used in this study.

Our findings conclusively demonstrate that selectively altering synovial macrophage phenotype via drug-laden liposomes induces physiological changes in the joint, including the ability to induce important changes in articular chondrocyte gene expression and synthetic function. Thus, targeting synovial macrophages with liposomal treatments has the potential impact to impact outcomes in OA. Further experiments are needed to characterize longer term effects beyond the immediate effects investigated here. Ongoing work in the lab will address the drug-altered phenotype at later stages of PTOA development and determine whether the STAT inhibitors used here protect against or contribute to inflammation and damage as experimental PTOA progresses.

3.6 References

1. Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. Arthritis & Rheumatism. 2012 Jun;64(6):1697-707.
2. Neogi T, Zhang Y. Epidemiology of osteoarthritis. Rheumatic Disease Clinics. 2013 Feb 1;39(1):1-9.
3. Murray CJ, Vos T, Lozano R, Naghavi M, Flaxman AD, Michaud C, Ezzati M, Shibuya K, Salomon JA, Abdalla S, Aboyans V. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. The lancet. 2012 Dec 15;380(9859):2197-223.
4. Hermann W, Lambova S, Muller-Ladner U. Current treatment options for osteoarthritis. Current rheumatology reviews. 2018 Aug 1;14(2):108-16.
5. Scanzello CR, Plaas A, Crow MK. Innate immune system activation in osteoarthritis: is osteoarthritis a chronic wound?. Current opinion in rheumatology. 2008 Sep 1;20(5):565-72.
6. Neogi T, Guermazi A, Roemer F, Nevitt MC, Scholz J, Arendt-Nielsen L, Woolf C, Niu J, Bradley LA, Quinn E, Frey Law L. Association of joint inflammation with pain sensitization in knee osteoarthritis: the Multicenter Osteoarthritis Study. Arthritis & rheumatology. 2016 Mar;68(3):654-61.
7. Collins JE, Losina E, Nevitt MC, Roemer FW, Guermazi A, Lynch JA, Katz JN, Kwoh CK, Kraus VB, Hunter DJ. Semi-quantitative imaging biomarkers of knee osteoarthritis progression: data from the FNIH OA Biomarkers Consortium. Arthritis & rheumatology (Hoboken, NJ). 2016 Oct;68(10):2422.
8. Felson DT, Niu J, Neogi T, Goggins J, Nevitt MC, Roemer F, Torner J, Lewis CE, Guermazi A, Group MI. Synovitis and the risk of knee osteoarthritis: the MOST Study. Osteoarthritis and cartilage. 2016 Mar 1;24(3):458-64.
9. Scanzello CR, Goldring SR. The role of synovitis in osteoarthritis pathogenesis. Bone 2012;51(2):249-57.
10. Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. Expert reviews in molecular medicine. 2011 Jul;13.
11. Sellam J, Berenbaum F. The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. Nature reviews rheumatology. 2010 Nov;6(11):625.
12. Mapp PI, Walsh DA. Mechanisms and targets of angiogenesis and nerve growth in osteoarthritis. Nature reviews rheumatology. 2012 May 29;8(7):390-8.
13. Takano S, Uchida K, Inoue G, Miyagi M, Aikawa J, Iwase D, Iwabuchi K, Matsomoto T, Satoh M, Mukai M, Minatani A, Takaso M. Nerve growth factor regulation and production by macrophages in osteoarthritic synovium. Clinical and experimental immunology. 2017 Nov;190(2):235-43.
14. Culemann S, Grüneboom A, Nicolás-Ávila JÁ, Weidner D, Lämmle KF, Rothe T, Quintana JA, Kirchner P, Krljanac B, Eberhardt M, Ferrazzi F. Locally renewing resident synovial macrophages provide a protective barrier for the joint. Nature. 2019 Aug;572(7771):670-5.
15. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Annals of the rheumatic diseases. 2005 Sep 1;64(9):1263-7.

16. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. The Journal of clinical investigation. 2012 Mar 1;122(3):787-95.
17. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, Locati M. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 2014 Jul 17;41(1):14-20.
18. Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage M1–M2 polarization balance. Frontiers in immunology. 2014 Nov 28;5:614.
19. Walker JG, Smith MD. The Jak-STAT pathway in rheumatoid arthritis. J Rheumatol. 2005;32(9):1650-1653.
20. de Andrés MC, Imagawa K, Hashimoto K, et al. Suppressors of cytokine signalling (SOCS) are reduced in osteoarthritis. Biochem Biophys Res Commun. 2011 Apr 1;407(1):54-9.
21. Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. Arthritis research & therapy. 2006 Dec;8(6):R187.
22. Manferdini C, Paolella F, Gabusi E, Silvestri Y, Gambari L, Cattini L, Filardo G, Fleury-Cappellesso S, Lisignoli G. From osteoarthritic synovium to synovial-derived cells characterization: synovial macrophages are key effector cells. Arthritis research & therapy. 2016 Dec;18(1):83.
23. Blom AB, van Lent PL, Holthuysen AE, van der Kraan PM, Roth J, van Rooijen N, van den Berg WB. Synovial lining macrophages mediate osteophyte formation during experimental osteoarthritis. Osteoarthritis and cartilage. 2004 Aug 1;12(8):627-35.
24. Wu CL, McNeill J, Goon K, Little D, Kimmerling K, Huebner J, Kraus V, Guilak F. Conditional Macrophage Depletion Increases Inflammation and Does Not Inhibit the Development of Osteoarthritis in Obese Macrophage Fas-Induced Apoptosis–Transgenic Mice. Arthritis & Rheumatology. 2017 Sep;69(9):1772-83.
25. Ahsan F, Rivas IP, Khan MA, Suárez AI. Targeting to macrophages: role of physicochemical properties of particulate carriers—liposomes and microspheres—on the phagocytosis by macrophages. Journal of controlled release. 2002 Feb 19;79(1-3):29-40.
26. Appleton CT, McErlain DD, Pitelka V, Schwartz N, Bernier SM, Henry JL, Holdsworth DW, Beier F. Forced mobilization accelerates pathogenesis: characterization of a preclinical surgical model of osteoarthritis. Arthritis research & therapy. 2007 Feb;9(1):R13.
27. Appleton CT, Pitelka V, Henry J, Beier F. Global analyses of gene expression in early experimental osteoarthritis. Arthritis & Rheumatism. 2007 Jun;56(6):1854-68.
28. Gerwin N, Bendele AM, Glasson S, Carlson CS. The OARSI histopathology initiative–recommendations for histological assessments of osteoarthritis in the rat. Osteoarthritis and Cartilage 2010;18:S24-34.
29. Minten MJ, Blom A, Snijders GF, Kloppenburg M, Van den Hoogen FH, Den Broeder AA, Van der Kraan PM, Van den Ende CH. Exploring longitudinal associations of histologically assessed inflammation with symptoms and

radiographic damage in knee osteoarthritis: combined results of three prospective cohort studies. Osteoarthritis and cartilage. 2019 Jan 1;27(1):71-9.
30. Neogi T. The epidemiology and impact of pain in osteoarthritis. Osteoarthritis and cartilage. 2013 Sep 1;21(9):1145-53.
31. Fernihough J, Gentry C, Malcangio M, Fox A, Rediske J, Pellas T, Kidd B, Bevan S, Winter J. Pain related behaviour in two models of osteoarthritis in the rat knee. Pain. 2004 Nov 1;112(1-2):83-93.
32. Bove SE, Laemont KD, Brooker RM, Osborn MN, Sanchez BM, Guzman RE, Hook KE, Juneau PL, Connor JR, Kilgore KS. Surgically induced osteoarthritis in the rat results in the development of both osteoarthritis-like joint pain and secondary hyperalgesia. Osteoarthritis and Cartilage. 2006 Oct 1;14(10):1041-8.
33. Piel MJ, Kroin JS, van Wijnen AJ, Kc R, Im HJ. Pain assessment in animal models of osteoarthritis. Gene. 2014 Mar 10;537(2):184-8.
34. Coulson-Thomas V, Gesteira TF. Dimethylmethylene Blue Assay (DMMB). Bio-protocol 2014;4(18): e1236.
35. Müller G, Hanschke M. Quantitative and qualitative analyses of proteoglycans in cartilage extracts by precipitation with 1, 9-dimethylmethylene blue. Connective tissue research 1996;33(4):243-8.
36. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry. 2009 Apr 1;55(4):611-22.
37. Raghu H, Lepus CM, Wang Q, Wong HH, Lingampalli N, Oliviero F, Punzi L, Giori NJ, Goodman SB, Chu CR, Sokolove JB. CCL2/CCR2, but not CCL5/CCR5, mediates monocyte recruitment, inflammation and cartilage destruction in osteoarthritis. Annals of the rheumatic diseases. 2017 May 1;76(5):914-22.
38. Van Lent PL, Van den Hoek AE, Van den Bersselaar LA, Spanjaards MF, Van Rooijen N, Dijkstra CD, Van de Putte LB, Van den Berg WB. In vivo role of phagocytic synovial lining cells in onset of experimental arthritis. The American journal of pathology. 1993 Oct;143(4):1226.
39. Richards PJ, Williams AS, Goodfellow RM, Williams BD. Liposomal clodronate eliminates synovial macrophages, reduces inflammation and ameliorates joint destruction in antigen-induced arthritis. Rheumatology. 1999 Sep 1;38(9):818-25.
40. Barrera P, Blom A, Van Lent PL, Van Bloois L, Beijnen JH, Van Rooijen N, De Waal Malefijt MC, Van De Putte LB, Storm G, Van Den Berg WB. Synovial macrophage depletion with clodronate-containing liposomes in rheumatoid arthritis. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 2000 Sep;43(9):1951-9.
41. Zhu Y, Tao H, Jin C, Liu Y, Lu X, Hu X, Wang X. Transforming growth factor-β1 induces type II collagen and aggrecan expression via activation of extracellular signal-regulated kinase 1/2 and Smad2/3 signaling pathways. Molecular medicine reports. 2015 Oct 1;12(4):5573-9.
42. Tsuneyoshi Y, Tanaka M, Nagai T, Sunahara N, Matsuda T, Sonoda T, Ijiri K, Komiya S, Matsuyama T. Functional folate receptor beta-expressing macrophages

in osteoarthritis synovium and their M1/M2 expression profiles. Scandinavian journal of rheumatology. 2012 Mar 1;41(2):132-40.
43. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity. 2010 May 28;32(5):593-604.
44. Ruan MZ, Erez A, Guse K, Dawson B, Bertin T, Chen Y, Jiang MM, Yustein J, Gannon F, Lee BH. Proteoglycan 4 expression protects against the development of osteoarthritis. Science translational medicine. 2013 Mar 13;5(176):176ra34-.
45. Murphy G, Stanton H, Cowell S, Butler G, Knäuper V, Atkinson S, Gavrilovic J. Mechanisms for pro matrix metalloproteinase activation. Apmis. 1999 Mar;107(1-6):38-44.
46. Li L, Jiang BE. Serum and synovial fluid chemokine ligand 2/monocyte chemoattractant protein 1 concentrations correlates with symptomatic severity in patients with knee osteoarthritis. Annals of clinical biochemistry. 2015 Mar;52(2):276-82.
47. Cuéllar VG, Cuéllar JM, Kirsch T, Strauss EJ. Correlation of synovial fluid biomarkers with cartilage pathology and associated outcomes in knee arthroscopy. Arthroscopy: The Journal of Arthroscopic & Related Surgery. 2016 Mar 1;32(3):475-85.
48. Harris Q, Seto J, O'Brien K, Lee PS, Kondo C, Heard BJ, Hart DA, Krawetz RJ. Monocyte chemotactic protein-1 inhibits chondrogenesis of synovial mesenchymal progenitor cells: an in vitro study. Stem Cells. 2013 Oct;31(10):2253-65.
49. Xu YK, Ke Y, Wang B, Lin JH. The role of MCP-1-CCR2 ligand-receptor axis in chondrocyte degradation and disease progress in knee osteoarthritis. Biological research. 2015 Dec;48(1):64.
50. Borzì RM, Mazzetti I, Cattini L, Uguccioni M, Baggiolini M, Facchini A. Human chondrocytes express functional chemokine receptors and release matrix-degrading enzymes in response to C-X-C and C-C chemokines. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 2000 Aug;43(8):1734-41.
51. Appleton CT, Usmani SE, Pest MA, Pitelka V, Mort JS, Beier F. Reduction in disease progression by inhibition of transforming growth factor α–CCL2 signaling in experimental posttraumatic osteoarthritis. Arthritis & rheumatology. 2015 Oct;67(10):2691-701.
52. Longobardi L, Temple JD, Tagliafierro L, Willcockson H, Esposito A, D'Onofrio N, Stein E, Li T, Myers TJ, Ozkan H, Balestrieri ML. Role of the CC chemokine receptor-2 in a murine model of injury-induced osteoarthritis. Osteoarthritis and cartilage. 2017 Jun 1;25(6):914-25.
53. Frank DA, Mahajan S, Ritz J. Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. Nature medicine. 1999 Apr;5(4):444.
54. Allen TM, Hansen CB, de Menezes DE. Pharmacokinetics of long-circulating liposomes. Advanced Drug Delivery Reviews. 1995 Sep 1;16(2-3):267-84.
55. Gandhi V, Plunkett W. Cellular and clinical pharmacology of fludarabine. Clinical pharmacokinetics. 2002 Feb 1;41(2):93-103.

Chapter 4

4 Summary and General Discussion

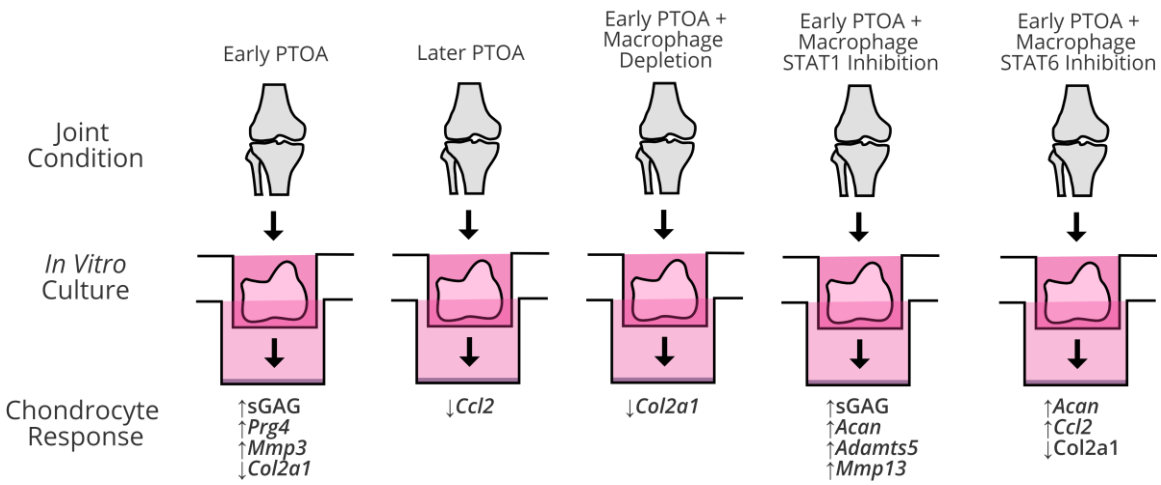

Figure 4.1. Schematic of research outcomes.

We set out to investigate the effect of synovium on chondrocytes in knee OA and the role of synovial macrophages. Cartilage degradation is a major component of OA and a driver of pro-inflammatory changes in the joint. Synovitis has recently become recognized as being an important feature, and evidence for synovial macrophages as key effector cells is building. The work completed here contributes to the body of knowledge on synovium in a rat model of experimental post-traumatic knee OA.

4.1 Establishment of a synovial joint model *ex vivo*

Chondrocytes are the sole cell population in cartilage. Unlike chondrocytes in healthy cartilage, OA chondrocytes actively produce catabolic enzymes and inflammatory signaling molecules. Many attempted interventions have focused on changing chondrocyte physiology to restore the balance between anabolic and catabolic processes. However, such an approach ignores the existence of cartilage as part of a complex joint environment, where unaddressed chronic inflammation in other tissues such as synovium likely contributes to the disruption of homeostasis, reducing the effectiveness of cartilage-selective treatments. Therefore, we focused on measuring chondrocyte gene expression and sGAG release responses upon exposure to OA synovium. Our intention was to test the effects of manipulating chronic synovial inflammation on chondrocyte outcomes in an OA model.

To model the phenotype of adult articular chondrocytes in the joint, we chose to grow primary articular chondrocytes from healthy knee joints, rather than previously described cell lines. We also initially chose to use a monolayer culture, rather than bioscaffold or micromass culture, for ease of use. We used a established rat model of post-traumatic knee OA, as it reliably induces the onset of OA-like changes in joint tissues in a short timeframe, and many molecular biology tools are available for rat tissues. However, rats are small animals, and the number of chondrocytes in a rat knee is limited. Preliminary experiments initially addressed this limitation by using chondrocytes from a single experimental joint, and thus animal, for each co-culture replicate. After a high degree of inter-individual variation was noted in chondrocyte gene expression, we validated the use of expanded and passaged P1 primary chondrocytes to reduce baseline variation *in vitro*.

We focused on gene expression as an early indicator of changes in chondrocyte physiology over protein expression, which would require further validation. This work provides the field with a new monolayer culture model using adult rat articular chondrocytes, which have received less attention relative to human and mouse model cultures.

To model synovium in the joint, we chose to co-culture whole-joint synovial tissue to account for the diversity of cells present and minimize the effects of cell isolation for *in vitro* culture. No necrosis was detected in tissues after several days in culture, consistent with previous papers describing extended synovial tissue cultures, but experiments using longer timelines should validate additional timepoints.

We did not model other joint tissues such as subchondral bone, tendon, ligament, and articular disc, as their contributions in OA are more likely to be biomechanical than through signalling. When the components of our co-culture system are put together, the synovial joint adequately models major signals between synovium and cartilage. The major strength of our work is the isolation of signaling between these crucial joint tissues, allowing us to expand our understanding of chondrocyte physiology in context with synovium, beyond culturing cartilage alone in various forms. Furthermore, the isolation of synovium-cartilage interactions away from the other tissue-tissue interactions taking place in the joint environment is another strength, as this would be very difficult to assess *in vivo*. Our model is suitable for investigating different experimental OA conditions of both synovium and cartilage in future work.

4.2 Effects of PTOA synovium condition on chondrocytes

Supporting the observation that this system provides important insights into synovium-cartilage interactions, we showed that even healthy synovium has a very strong effect on chondrocyte physiology. Compared with chondrocyte gene expression in the absence of synovium co-culture, we observed marked increases in many chondrocyte genes and sGAG release. This is a key observation since it demonstrated that studying chondrocytes in monolayer culture alone may not reflect the nature or magnitude of the responses to various stimuli that occur in the three-dimensional joint environment.

Early PTOA synovium induces differences in chondrocyte responses compared to healthy control. Although changes were found in expression of both anabolic and catabolic genes, we believe the overall response is protective. Unexpectedly, this response was entirely absent when chondrocytes were co-cultured with later stage PTOA synovium. The synovium likely undergoes a phenotypic shift from 4 weeks to 12 weeks post-induction of PTOA, resulting in the induction of *Ccl2*, which likely indicates pro-inflammatory change in gene expression in chondrocytes. Further work is required the understand these biological changes with gene or protein expression analysis in synovial cell populations. Although we were able to assess chondrocyte responses to PTOA synovium, a major limitation is our use of healthy chondrocytes, as OA chondrocytes may respond differently. Future work may address this by establishing a co-culture model for OA chondrocytes using cells from the same rat PTOA model.

Given that synovium exerts a significant biological effect on chondrocytes, we postulate that synovium may therefore be a useful target in the management of cartilage-damaging diseases such as OA. Regardless of whether chronic synovial inflammation in OA is harmful or protective, our work demonstrates that the ability of synovium to influence cartilage outcomes may be a key leverage point in OA management strategies. Therefore, additional work should target comprehensive assessment of synovial pathophysiology in OA and deeper investigation into how biological processes in the synovium can be exploited to achieve a therapeutic benefit. As a proof of concept, the final experiments in this thesis addressed early PTOA joint responses when synovial macrophages were altered.

Liposomal drug-treated early PTOA joints showed the histological appearance of synovitis was significantly affected, confirming a treatment effect. Although cartilage damage was not apparently affected, treatment was only administered for two weeks, while most cartilage changes evolve over longer periods in rodent OA models. Moreover, changes in chondrocytes gene expression likely precede and cause morphologic changes at the tissue level. *In vitro* co-cultures showed that synovium from liposome-treated joints did induce different responses in chondrocytes, suggesting that differences in cartilage damage and animal pain-related behavior may become apparent at a later stage.

Synovium-chondrocyte co-cultures and primary macrophage cultures indicate that STAT1 inhibition may be protective, while STAT6 inhibition may be pro-inflammatory. As we predicted, synovial macrophage manipulation was able to produce changes in chondrocyte physiology, and further development and understanding of selectively altering synovial macrophage behavior could produce therapeutic benefit for OA and other joint diseases. Although drugs used to treat inflammatory rheumatoid arthritis have not been shown to be effective for OA, OA-specific anti-inflammatory and anabolic factors are a current topic of interest. Recent studies have shown that synovial macrophages drive the production of matrix proteases and their inhibitors by synovial fibroblasts. Characterization of the synovium as a whole tissue would improve our understanding of the processes at work. We plan to quantify expression of M1-like, M2-like, and pan-macrophage markers in sections from liposome-treated joints for both early and later stage PTOA.

4.3 Future directions

Ongoing work in the lab will address the limitations of the experiments presented here and follow new courses of inquiry. We are completing a course of liposome-treated 12W PTOA joints for analysis by histology and for co-culture of isolated synovium with chondrocytes to determine longer term effects. We will also use our co-culture model to examine other treatments for synovium and altering synovial macrophages, such as drugs affecting other proteins in the polarization dynamic (e.g. STAT3, SOCS, NF-κB). To continue investigating the protective effect of early PTOA synovium on chondrocytes, other specific inhibitor assays or wider proteomic studies may be completed on co-culture medium to determine the signal or signals responsible.

The work completed here is limited by our use of the animal model; these findings remain to be replicated and expanded upon by studies using female animals, other methods of OA induction, or human tissue samples. While human tissues are more directly translatable in physiology and patient diversity, animal studies are still needed to sufficiently replicate the interaction of local and systemic factors, as well as biomechanical signalling, which is especially relevant to processes in joint

homeostasis.Finally, the relative proportions of macrophage subpopulations remain to be determined, by flow cytometry of isolated synoviocytes or microscopic *in situ* quantification. This information would help to interpret our findings and inform future research questions about synovitis.

Appendix A

Appendix A: Animal Use Protocol Approval.

From: eSirius3GWebServer <esirius3g@uwo.ca>

Subject: eSirius3G Notification -- 2017-042 Annual Renewal Approved
Date: November 1, 2019 at 12:26:33 PM EDT
To: <[redacted]>, <[redacted]>

Western

2017-042:7:

AUP Number: 2017-042
AUP Title: Inflammatory, structural, and pain features of rodent models of osteoarthritis.
Yearly Renewal Date: 11/01/2020

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2017-042 has been approved by the Animal Care Committee (ACC),
and will be approved through to the above review date.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:
   a) Western's Senate MAPPs 7.12, 7.10, and 7.15
      http://www.uwo.ca/univsec/policies_procedures/research.html
   b) University Council on Animal Care Policies and related Animal Care Committee procedures
   http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.html
2) As per UCAC's Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC. http://uwo.ca/research/services/animalethics/animal_use_protocols.html
3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
   a) be made familiar with and have direct access to this AUP;
   b) complete all required CCAC mandatory training (training@uwo.ca); and
   c) be overseen by me to ensure appropriate care and use of animals.
4) As per MAPP 7.15,

a) Practice will align with approved AUP elements;
b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
c) UCAC policies and related ACC procedures will be followed, including but not limited to:

i) Research Animal Procurement
ii) Animal Care and Use Records
iii) Sick Animal Response
iv) Continuing Care Visit

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets, http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care

The University of Western Ontario
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Appendix B

Appendix B: Animal Research: Reporting In Vivo Experiments (ARRIVE) Guidelines Checklist

ARRIVE

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

Carol Kilkenny[1], William J Browne[2], Innes C Cuthill[3], Michael Emerson[4] and Douglas G Altman[5]

[1]The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, [2]School of Veterinary Science, University of Bristol, Bristol, UK, [3]School of Biological Sciences, University of Bristol, Bristol, UK, [4]National Heart and Lung Institute, Imperial College London, UK, [5]Centre for Statistics in Medicine, University of Oxford, Oxford, UK.

	ITEM	RECOMMENDATION	Section/ Paragraph
Title	1	Provide as accurate and concise a description of the content of the article as possible.	Title
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.	Abstract
INTRODUCTION			
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.	Chapter 1, Section 2.2, Section 3.2
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.	Sections 1.3.2, 2.2, 3
METHODS			
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.	Sections 2.3.2, 3.3.3
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.	Sections 2.3.2, 3.3.3
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).	Sections 2.3.2, 3.3.3
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.	Sections 2.3.2, 3.3.3

The ARRIVE guidelines. Originally published in *PLoS Biology*, June 2010[1]

Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.	
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.	Sections 2.3.2, 3.3.3
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.	
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).	Sections 2.3, 3.3
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.	Sections 2.3.8, 3.3.10
RESULTS			
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing. (This information can often be tabulated).	
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.	Sections 2.4, 3.4
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).	Sections 2.4, 3.4
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.	
DISCUSSION			
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.	Sections 2.5, 3.5
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.	Sections 2.5, 3.5
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.	

References:
1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.

Curriculum Vitae

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Post-secondary Education:	University of Western Ontario London, Ontario, Canada 2013-2017 Bachelor of Medical Sciences, Honours Double Major
Honours and Awards:	Ontario Graduate Scholarship 2018-2019
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	Dean's Undergraduate Research Opportunities Program 2016
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Related Work Experience	Graduate Teaching Assistant University of Western Ontario 2017-2019
Publications:	Gibson, C., Lai-Zhao, Y., Tan, V., Bartling, M., & Fung, A. (2018). Cybersecurity (Professional Practice Brief 0045.18). Canadian Health Information Management Association.
	Thompson, J. L., Lai-Zhao, Y., Stathopulos, P. B., Grossfield, A., & Shuttleworth, T. J. (2018). Phosphorylation-mediated structural changes within the SOAR domain of STIM1 enable specific activation of distinct Orai channels. Journal of Biological Chemistry.
	Choi, Y. J., Zhao, Y., Bhattacharya, M., & Stathopulos, P. B. (2016). Structural perturbations induced by Asn131 and Asn171 glycosylation converge within the EFSAM core and enhance stromal interaction molecule-1 mediated store operated calcium entry. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research.
Poster Presentations:	Department of Physiology and Pharmacology Charles W. Gowdey Distinguished Lecture and Research Day November 2019

London Health Research Day
April 2019

Department of Physiology and Pharmacology Charles W. Gowdey Distinguished Lecture and Research Day
November 2018

Canadian Connective Tissue Conference
May 2018

Schulich School of Medicine Department of Medicine Resident Research Day
May 2018

Department of Physiology and Pharmacology Charles W. Gowdey Distinguished Lecture and Research Day
November 2017