Inflammation in osteoarthritis – a focus on the role of interleukin-17



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Declaration

I certify that this thesis is a true and honest account of my own research, which was conducted ethically and the results obtained are genuine. Where I have drawn on the work, ideas, and results of others, this has been appropriately acknowledged in this thesis.



Jolet Yvette Mimpen

Abstract

Osteoarthritis (OA) is increasingly accepted as a whole joint disease, with an important role for synovial inflammation (synovitis) in its pathophysiology. However, there is limited knowledge about underlying OA biology, including the influx of immune cells, the activation status of fibroblasts, and the critical cytokine mediators of disease. One family of cytokines that has received increasing interest is interleukin (IL)-17. IL-17A and its family members IL-17F and IL-17AF are known to play an important role in inflammatory (musculoskeletal) diseases such as psoriasis and ankylosing spondylitis. Initial studies have suggested a role for IL-17A in OA, but there is still limited knowledge on the expression of IL-17 cytokines and receptors in OA tissue, the effect of IL-17 on cells from the OA joint, and how this relates to the synovitis seen in OA patients. Therefore, this thesis aimed to advance the understanding of inflammation, and specifically the role of IL-17 cytokines, in OA pathophysiology.

This thesis uses flow cytometry to show that there is significant infiltration of immune cells, especially T cells and macrophages, in the synovium from patients with end-stage OA. Fibroblasts in end-stage OA synovium show an activated phenotype, with a predominant immune effector population, which might be an important contributor to the synovitis seen in many patients. In patients that have a high risk of developing post-traumatic OA, immunohistochemistry showed that macrophage markers positively correlated with a histological synovitis score, which supports the hypothesis that macrophages are a key driver of synovitis. These findings do not only highlight the importance of inflammation in OA, but also stress the importance of synovium in OA, thereby providing further evidence for OA as a disease of the whole joint.

This thesis also supports that IL-17 cytokines can play an important role in OA. A potential Th17 subset (CCR6+ T cells) was found in a subset of end-stage patients. In synovium from patients at high risk of developing post-traumatic OA, IL-17A and its receptors IL-17RA and IL-17RC were expressed. In addition, expression of IL-17RA positively correlated with a histological synovitis score, potentially making this tissue more susceptible to respond to IL-17 cytokines. Gene expression of *IL17RA* and *IL17RC* in chondrocytes and synovial fibroblasts derived from end-stage OA patients confirms the ability of these cells to respond to IL-17 cytokines. *In vitro* treatment of these cells from end-stage OA patients with IL-17A, and its family members IL-17F and IL-17AF, induced changes in gene expression in several OA-related pathways including matrix destruction, angiogenesis and inflammation. The chronic, low-grade inflammation seen in OA fits well with these effects of IL-17A and further supports a role for IL-17A in OA pathophysiology. As there are currently no disease-modifying OA drugs, anti-IL-17 therapy could be investigated as a potential therapeutic option in OA patients.

In conclusion, this thesis highlights the importance of inflammation in OA, proposing that IL-17A could be an important mediator in sustaining joint inflammation. In addition, it further demonstrates a role for the synovium in OA, which emphasizes that OA should be viewed as a disease of the whole joint. Future studies should apply modern omics techniques to a larger, well-phenotyped cohort of OA patients to identify subtypes of disease in synovium and other OA joint tissues by unravelling the cellular, molecular, and genetic signatures of disease in a longitudinal study.

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I never thought of a PhD as a massive obstacle; it was just the next step in the journey to become a researcher. Well. It certainly was not easy and it included many more highs and lows than I had expected, but it was definitely worth it. I may not have completely unravelled how osteoarthritis works, but I hope I was able to contribute a small piece of the puzzle that will help get us there eventually. However, I would not have been able to do any of this without the support from so many lovely people.

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Abbreviations

ACL	Anterior cruciate ligament
ACTB	β–actin
ADAM	A disintegrin and metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AIA	Antigen-induced arthritis
AP-1	Activator protein 1
BMI	Body mass index
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CBAD	C-terminal C/EBPβ activation domain
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CD	Cluster of Differentiation
C/EBP	CCAAT-enhancer-binding proteins
CIA	Collagen-induced arthritis
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
CyToF	Cytometry by time of flight
D0	DMEM-F12 media with 1% P/S (serum-free)
D10	DMEM-F12 media with 10% FBS and 1% P/S
DAMP	Damage-associated molecular patterns
dds	DESeq2 data set
DMEM-F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMM	Destabilisation of the medial meniscus
DMOAD	Disease modifying OA drug
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal–regulated kinase 1 and 2
FACS	Fluorescence-activated cell sorting
FAP	Fibroblast activation protein
FBS	Foetal bovine serum

FGF	Fibroblast growth factor
FSC-A	Forward scatter area
G-CSF	Granulocyte colony-stimulating factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSEA	Gene Set Enrichment Analysis
GSK3β	Glycogen synthase kinase 3 beta
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IC50	Half maximal inhibitory concentration
IFN	Interferon
IgG	Immunoglobulin
IL	Interleukin
IL-17A/F	Combined name for IL-17A, IL-17F, and IL-17AF cytokines
IL-17R	IL-17 receptor
KL grade	Kellgren Lawrence grade
LFC	Log2 fold change
LPS	Lipopolysaccharide
LRT	Likelihood ratio test
MAP	Mitogen-activated protein
MMP	Matrix metalloproteinases
MS	Mass spectrometry
MSD	Meso scale discovery
NES	Normalised enrichment score
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NOS	Nitric oxide synthase
OA	Osteoarthritis
OKS	Oxford Knee Score
OxKIC	Oxford Knee Injury Cohort
P/S	Penicillin-Streptomycin
padj	Adjusted p-value
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PC	Principle component

PCA	Principle component analysis
PCR	Polymerase chain reaction
PDPN	Podoplanin
PMA	Phorbol 12-myristate-13-acetate
R10	RPMI media with 10% FBS and 1% P/S
RA	Rheumatoid arthritis
RNA-Seq	RNA Sequencing
RPMI	Roswell Park Memorial Institute
RT-PCR	Real-time quantitative polymerase chain reaction
SAPK/JNK	Stress-activated kinase/c-Jun N-terminal kinase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEFIR	Similar expression to fibroblasts growth factor/IL-17R
SSC-A	Side scatter area
SSC-H	Side scatter height
SSC-W	Side scatter width
SNP	Single nucleotide polymorphisms
STAT	Activator of signal transducer and activator of transcription
TBS	Tris-buffered saline
TGF	Transforming growth factor
Th cell	T-helper cell
TIMP	Tissue inhibitors of metalloproteinase
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TRAF	TNF receptor associated factor
TSG	TNF-stimulated gene
UK	United Kingdom
USA	United States of America
VCAM	Vascular cell adhesion protein 1
VEGFA	Vascular endothelial growth factor A
WB	Western blot
WOMAC	Western Ontario and McMaster Universities Osteoarthritis Index

Chapter 1

Introduction

1.1 Introduction

Osteoarthritis (OA) is a progressive and disabling musculoskeletal disorder that affects joints. Patients suffer from pain, loss of function, and a consequent reduction in life quality[1]. OA is the most prevalent joint disease: it is estimated that globally 9.6% of men and 18.0% of women over the age of 60 have symptomatic OA. Increase in life expectancy and aging populations are expected to make OA the fourth leading cause of disability by 2020[2]. Risk factors for OA include age, sex, genetics, physical trauma, joint loading, obesity, female sex, and co-morbidities such as diabetes, cardiovascular disease, and cancer[3]. In addition, recent research has highlighted the potential effect of lifestyle, diet, and consequent alterations of the microbiome on the onset and progression of OA[4]. Even though the search for disease modifying OA drugs (DMOADs) has been substantial, an effective way of inhibiting or slowing down OA-related damage has not yet been identified. Therefore, in patients with early stage disease, medical care focusses on controlling pain and improving health-related quality of life. During end-stage disease, pain or cartilage damage becomes so severe that surgical joint replacement is the only viable treatment option[5]. However, these joint replacements come with a limited life-span, are associated with 1% mortality, and at least 10% of total hip or knee replacements report no improvement of worsening of symptoms 1 year after surgery. This creates an urgent need for better treatments to prevent or limit OA progression[6,7]. Therefore, it is essential to get a better understanding of OA pathophysiology to help develop these much-needed treatments.

1.2 Joints – compilated multi-tissue organs

OA affects synovial joints, which are the most common joints in the human body. Synovial joints have a joint cavity which is filled with fluid where the bones meet but are not in direct contact, allowing smooth movement and increased joint mobility. There are many different tissues that comprise the joint. The knee joint – the main focus of this thesis – includes (subchondral) bone, cartilage, capsule, synovium, synovial fluid, an intra-articular fat pad, meniscus, and several tendons, muscles, and

ligaments. The tissues most commonly described in this thesis, cartilage and synovium, will be discussed in more detail below.

1.2.1 Cartilage

Cartilage is a smooth connective tissue that covers and protects the ends of bones in joints. Cartilage is made up by two elements: 1) the resident cells, chondrocytes, which account for 5% of the cartilage volume, and 2) a thick extracellular matrix (ECM), which accounts for 95% of the cartilage volume[8]. The ECM is a complex network of proteoglycans, collagens, minerals, fibrous proteins, and water that confer the biomechanical properties upon the cartilage. Although chondrocytes only make up 5% of the cartilage, they are essential in maintaining the homeostasis of the ECM as they are responsible for the secretion and turnover of ECM components[9].

1.2.2 Synovium and synovial fluid

In a healthy synovial joint, the joint capsule seals the synovial cavity and synovial fluid from the surrounding tissue. The inner layer of the joint capsule is called the synovial membrane or synovium and is responsible for maintaining the volume and composition of synovial fluid by producing lubricin and hyaluronic acid. Synovial fluid is a viscous fluid that provides lubrication in order to reduce the friction between the bones of the joint. As articular cartilage does not have intrinsic vascular or lymphatic supply, the synovium works together with subchondral bone to supply chondrocytes with nutrition through synovial fluid[10].

Healthy synovium consists of 2 layers. The outer layer (sub-lining) is up to 5 mm thick and consists of fibrous, adipose, and areolar connective tissues. The sub-lining is relatively acellular, is rich in type I collagen, and has a microvascular blood supply which is accompanied by lymphatic vessels and nerve fibres. The inner layer (lining) lies next to the joint cavity and consists of a 1-4 cells (20-40 μm) thick

layer. The resident cells in this layer are mainly synovial fibroblasts, but there are also macrophages present[11].

1.3 Osteoarthritis – a disease of the whole joint

Since the first description of loss of articular cartilage in OA by J.B. Morgagni in 1741, OA has been regarded as a degenerative disease with loss of cartilage caused by 'wear and tear'. Even though cartilage is still seen as the central tissue of the disease, the contribution of other joint tissues is increasingly accepted. In addition to joint space narrowing due to the loss of cartilage, the formation of osteophytes, subchondral cysts, and subchondral sclerosis are the main radiographic features of OA[12,13]. Mechanical stress is one mechanism thought to contribute to changes in bone homeostasis, as it can modify the contour and shape of the subchondral bone through bone remodelling[14,15]. Ligament weakness and particularly meniscal damage in the knee is common. As patients with anterior cruciate ligament or meniscus injury have a 50% chance of developing OA[16], these features are especially common in patients with post-traumatic OA. Inflammation is increasingly accepted as a feature of OA. Inflammation of the synovium (synovitis) and hypertrophy of the joint capsule are often seen, and increased concentrations of inflammatory mediators have been detected in OA synovial fluid[17]. In addition, the infrapatellar fat pad has recently been identified as a potential source of inflammation in knee OA[18].

OA is now widely accepted as a disease of the whole joint with multiple tissues contributing to its aetiology. As a result of biomechanical and cytokine-driven coupling (paracrine signalling), there is significant cross-talk between cartilage and other joint tissues (Figure 1-1). However, the chronicity of tissue involvement is still unclear, as well as the initial site from which OA propagates, which may or may not be cartilage.

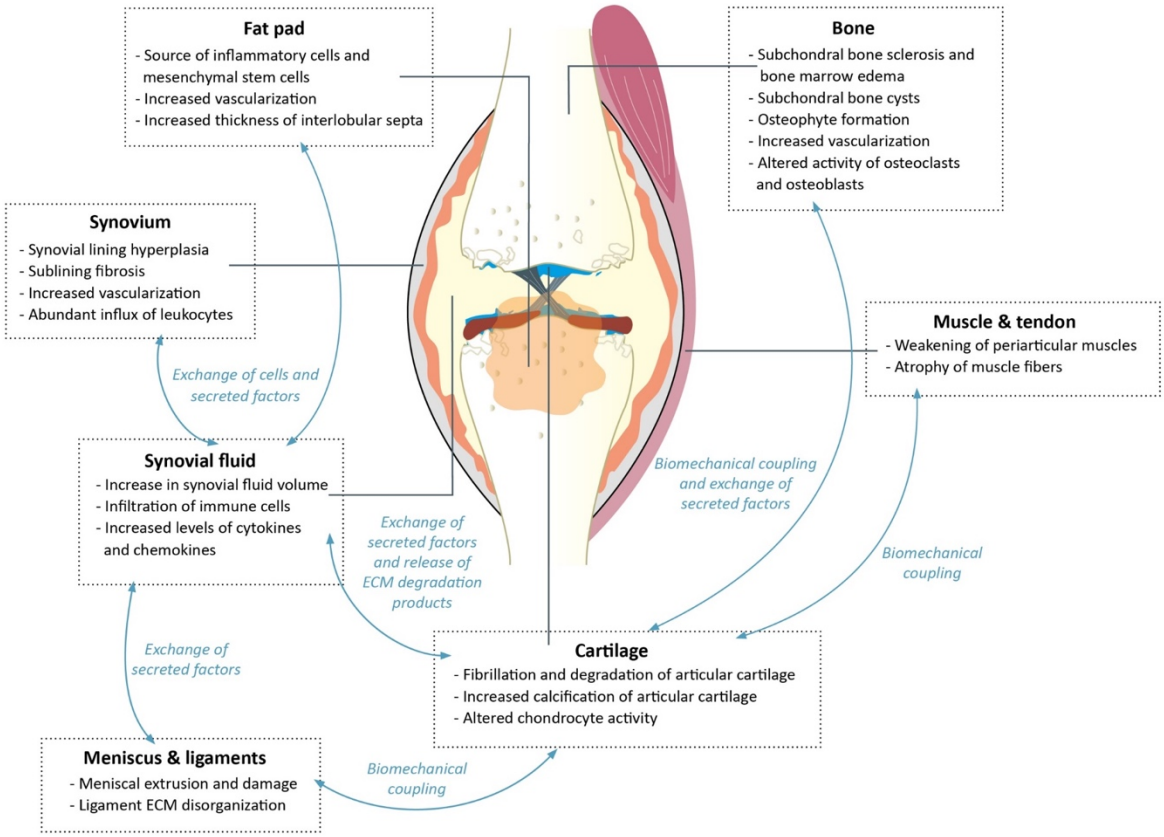


Figure 1-1. *A representation of an osteoarthritic knee, including the main tissues that contribute to the disease and their interactions. Loss of articular cartilage is the main characteristic of OA, but the exact balance of tissue involvement and interaction is dependent on the anatomical site of the joint, as well the subtype and stage of disease. (Re-print with permission from authors, originally published in review by Mimpen & Snelling, 2019[19])*

1.4 OA pathophysiology – what do we know?

As the extend of the involvement of the different joint tissues in the disease process continues to become clear, OA subtypes have been proposed as “bone-driven”, “cartilage-driven”, and “inflammation- or synovium-driven”[20]. Although this fails to consider other joint tissues such as fat pad, synovial fluid, as well as meniscus and other muscles and ligaments that could have a significant contribution to the disease[17,21], this does attest to the fact that different diseases on the OA spectrum could be driven by different joint tissues. In order to unravel the underlying mechanisms of disease, it is important to understand the tissue-specific mechanisms that underlie OA. While some

of these molecular signatures might be unique to an individual tissue, others might be shared between different joint tissues and even different articular joints. Therefore, it is essential to understand the contribution of each joint tissue in isolation, including cartilage and synovium, before starting to understand the interactions between all these tissues.

1.4.1 Resident fibroblasts

The contribution of resident fibroblasts of the joint is an important starting point to understand the onset and progression of OA as they are the predominant cell type in every joint tissue. Using a combination of single-cell RNA sequencing (RNA-Seq), computational analyses, and histological assays, different populations of chondrocytes in human OA cartilage were identified, including their associated cartilage zone and their hypothesized involvement in disease[22]. Bulk RNA-Seq was used to analyse intact knee cartilage from OA patients and control patients, which revealed two major subgroups of OA. While both subgroups showed an increased ribosomal and matrix protein expression compared to control, the groups significantly differed in gene clusters involved in complement activation, innate immune response, and Wnt and transforming growth factor (TGF)-β signalling[23]. Interaction between different joint tissues was shown by Lewallen *et al.* who revealed that RNA-Seq expression profiles from human chondrocytes from an intact part of cartilage differed between patients with different histopathological OA grades[24]. This suggests that there is a significant cross-talk between cartilage and the other tissues in the joint, such as synovium and synovial fluid. The infiltration of inflammatory cells into the synovium and synovial fluid may partially influence the cross-talk with chondrocytes due to release of inflammatory cytokines. However, the specific subsets of immune cells and fibroblasts in synovium and their function in OA, as well as this cross-talk between different joint tissues, remains understudied.

What is known is that resident fibroblasts can produce, or even overproduce, many inflammatory mediators in OA that are characteristic for inflammatory arthritides[25], including damage-associated

molecular patterns (DAMPs), cytokines, chemokines, complement proteins, and adipokines[25,26]. Indeed, studies have found increased levels of these mediators in OA synovial fluid[27–33], which are hypothesized to be involvement in mechanisms underlying OA pathophysiology, including ECM degradation, angiogenesis, complement activation, nitric oxide metabolism, and activation of inflammatory signalling pathways.

1.4.2 Extracellular matrix degradation

Despite the loss of articular cartilage being hallmark feature of OA, the mechanisms underlying this OA-related cartilage degradation are poorly understood. In the healthy joint, the synthesis of new ECM components and the degradation of old ECM components is perfectly in balance. In OA, cartilage breakdown is characterised by a downregulation of aggrecan, a proteoglycan that attracts water and provides tensile support for cartilage, and an increased rate of collagen synthesis. Whereas healthy cartilage mainly contains collagen type II, in OA there is an increase in type I collagen which is normally found in subchondral bone. Compared to type I collagen, type II collagen contains a higher number of hydroxylysine, glucosyl, and galactosyl residues which mediate the interactions with proteoglycans. Therefore, the shift in collagen content in OA affects the integrity of the ECM networks formed by collagen and proteoglycan[34,35].

Dysregulation of ECM homeostasis is a complex process. One of the first groups of proteins that was connected to cartilage degradation in OA are matrix metalloproteinase (MMPs), which are enzymes that degrade all components of the ECM. MMP-1, -2, -3, -8, -9, -13, and -14 have been found to be expressed in cartilage: MMP-1, -2, -13, and -14 have important roles in tissue turnover and are found to be upregulated in disease, while the expression of MMP-3, -8, and -9 has only been found in pathological circumstances[36,37]. Inflammatory cytokines and proteases are key contributors to these processes, because they can stimulate chondrocytes to produce these MMPs, eventually leading to cartilage degradation[35].

1.4.3 Angiogenesis

Angiogenesis has regularly been associated with OA pathophysiology, as it is increased in OA cartilage, subchondral bone, synovium, and menisci[38,39]. OA cartilage has a reduced resistance to angiogenesis due to imbalance in the production of pro-and anti-angiogenic mediators, which allows blood vessels from the subchondral bone to grow into the non-calcified cartilage. This formation of blood vessels leads to ossification of the cartilage surrounding the vessels, which contributes to the thickening of subchondral bone. In addition, the formation of blood vessels and nerves share multiple common pathways, and as the growth of sensory nerves along new blood vessels have been observed in OA joints, these might contribute to pain in OA[38,40]. Angiogenesis and inflammation work together as inflammation promotes angiogenesis, and angiogenesis can sustain an inflammatory micro-environment. The increased vascularity seen in OA synovium is thought to facilitate the chronic inflammation of synovitis due to the influx of immune cells into the synovium[41,42].

1.4.4 Complement

Complement is an integral part of the innate immune system due its role in defence against pathogens, but it also contributes to scavenging cellular debris and immune complexes, and the guidance of adaptive immunity by stimulation of B and T lymphocytes. There is accumulating evidence that activation of the complement pathway is involved in OA pathogenesis[33,43]. Studies have shown that in patients after knee injury there is an increased concentration of complement complexes that result from complement activation in synovial fluid compared to healthy controls[33]. A proteomic analysis of synovial fluid using mass spectrometry showed that 131 proteins were differentially expressed between end-stage OA and traumatic knee injury patients. In this study, complement C1r was the most significantly upregulated protein in end-stage OA, suggesting an important role for the complement system in disease pathogenesis[44]. In addition, gene and protein expression of complement components have been found in OA cartilage and synovial membrane, as

well as cultured chondrocytes and synovial fibroblasts[45,46]. Furthermore, in OA synovium there is a higher expression of complement effectors and lower expression of complement inhibitors compared to healthy synovium[47]. Complement is activated by both catabolic and inflammatory mediators that are hypothesized to play a key role in OA, and by DAMPs that are present in the OA joint, including cartilage ECM proteins, calcium crystals, and components of apoptotic debris[25,43]. Therefore, complement activation is hypothesized to be critical for the development of OA, because of its involvement in ECM degradation, inflammatory processes in chondrocytes and synovial fibroblasts, synovitis, and cartilage angiogenesis[43].

1.4.5 Inflammation as a central player in osteoarthritis pathophysiology

While all these molecular and cellular processes such as the degradation of ECM, angiogenesis, and the activation of complement, are integrated, there is one clear link between these different systems: inflammation. Even though the inflammatory changes in the OA joint are subtle compared to those in autoimmune "inflammatory" arthritis such as rheumatoid arthritis (RA), it is now accepted that inflammation plays a central role in the disease pathogenesis. However, the inflammation is distinct from that in RA and other autoimmune diseases as it is chronic, and comparatively low-grade.

1.4.5.1 Pro-inflammatory mediators

The most commonly investigated cytokines in OA are the pro-inflammatory cytokines IL-1β and TNF-α: several studies have shown increased levels of these cytokines in OA tissues[48–50], and *in vitro* studies have linked their roles to OA[51–54]. However recent clinical trials with antibodies against these cytokines have failed[55,56]. One particular group of cytokines that is increasingly hypothesized to play a role in OA is IL-17. IL-17A is increased in patients with OA compared to control in serum[57,58], and IL-17A concentration in synovial fluid positively correlate with KL grade[57] or WOMAC pain score[59]. When comparing tissue-conditioned media from inflamed and non-inflamed synovium, higher levels

of IL-17A were associated with the inflamed tissue[60]. Another study identified IL-17A in a subpopulation of OA patients, which showed higher median synovial fluid concentrations of cytokines and adipokines such as IL-6 and leptin, and were clinically marked by faster cartilage loss, reduced osteophytes, and reduced minimum joint space width[30]. While the role of IL-17A in OA is still poorly understood, it has been shown to have an important role in several inflammatory (musculoskeletal) disease, including psoriasis, ankylosing spondylitis, and psoriatic arthritis[61].

1.4.5.2 Anti-inflammatory mediators

The classical anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13, are implicated in OA. IL-4, IL-10, and IL-13 do not only antagonise the effects of pro-inflammatory cytokines, but also regulate chondrogenesis, chondroprotection, stimulation of regulatory T cells, and activity of growth factors and bone morphogenetic proteins (BMPs)[26]. Several studies are exploring IL-4-IL-10 fusion proteins as possible DMOADs[62,63]. IL-6 is a cytokine with both pro- and anti-inflammatory qualities. IL-6 protein levels are elevated in serum and synovial fluid OA patients[64,65], and are associated with pain[31], increased MMP levels, and severe radiographic changes[66]. An IL-6 receptor inhibitor tocilizumab showed improvement in pain and morning stiffness in early studies[67], and results from a phase 3 trial completed in February 2019 are expected to be published soon. Interestingly, the activity of IL-6 has been linked to IL-17 in several ways: IL-17 is a strong inducer of IL-6 production[68], IL-6 is a key Th17 polarizing cytokine[69,70], and IL-17 and IL-6 can synergise to enhance each other's signalling [71,72]. In addition, it was shown that the IL-6/IL-17A axis plays an important role of the induction of arthritis in a mice model of arthritis[73]. It appears that IL-6, IL-17A, and the transcription factor STAT3 are functionally linked so that when one of these are upregulated, it leads to a dysregulation of the others, which has been linked to the development of several autoimmune disorders[68]. Together, this strengthens the evidence for a role of both IL-6 and IL-17 in arthritis.

1.4.6 IL-17: a potential contributor to arthritic tissue inflammation

IL-17A is one of the 6 members of the IL-17 family: IL-17A - IL-17F. IL-17A and IL-17F are most studied and can function as homodimers or as the heterodimer IL-17AF. The founding member of the family IL-17A was originally discovered to be produced by Th17 cells, but can also be produced by many other cell types, including cytotoxic T cells, γδ-T cells, natural killer (NK) cells, NKT cells, mast cells, neutrophils, and different types of resident stromal cells[74–80]. Although the main roles of IL-17A and IL-17F are in host defence fighting bacterial and fungal infection, the pro-inflammatory qualities of these cytokines have made them important in several chronic inflammatory diseases by inducing innate-like acute immune defences[81]. IL-17A can contribute to inflammatory arthritis by facilitating tissue inflammation and matrix destruction[82], and by potentially playing an important role in arthritic pain through increasing the excitability of nociceptors and potentiating hyperalgesia[83–86]. In addition, the presence of IL-17 in synovial fluid may point to an OA subtype with a rapid cartilage loss, reduced number of osteophytes, and increased adipokines[30].

1.5 OA – a spectrum of pathologies

OA is a disease that involves many tissues with a complex interplay between clinical, molecular, and genetic factors. Therefore, it might be better to view OA as a spectrum of pathologies that all lead to the loss of articular cartilage at end-stage disease[87]. Although this might not be surprising due to the number of molecular mechanisms that are hypothesized to play a role in OA pathophysiology and the varied clinical manifestations of the disease, disease subtypes are currently not regularly incorporated in study design of clinical or laboratory-based studies. Especially as there are currently no DMOADs available, the ability to group patients on based on specific characteristics of their disease subtype would be the first step towards personalized treatment. Therefore, to improve treatment development in OA, the most important characteristics by which to stratify OA patients must be considered. Stratification factors could be risk factors, clinical signs and symptoms, and molecular signatures (Figure 1-2).

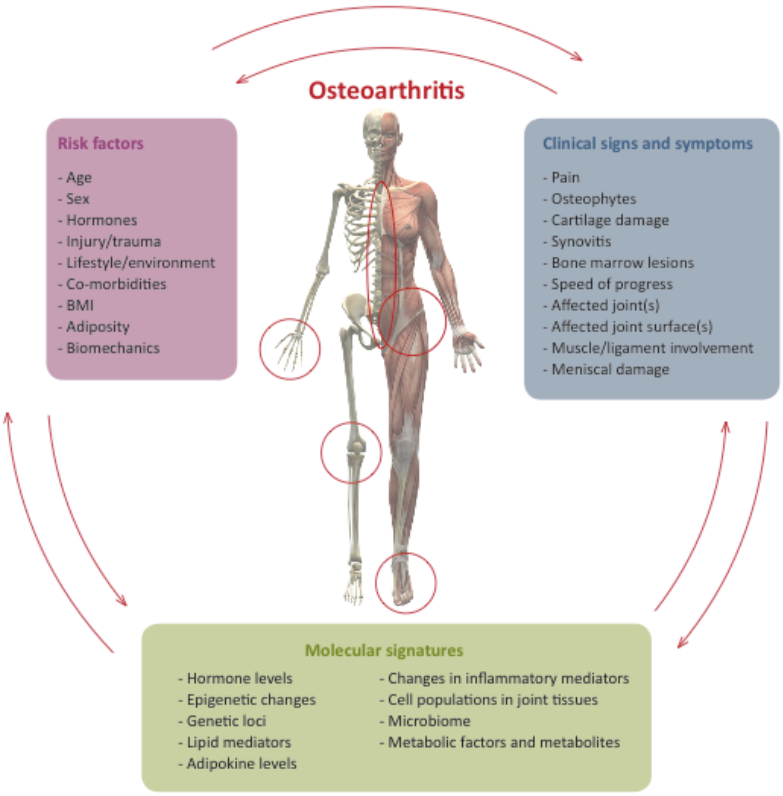


Figure 1-2. *An overview of risk factors, clinical signs and symptoms, molecular signatures, and their interactions, which can be used as stratification categories of OA. The combination of different factors from each of these categories will define distinct subtypes and stages of OA. (Re-print with permission from authors, originally published in review by Mimpen & Snelling, 2019[19])*

1.5.1 Clinical phenotypes and risk factors

When patients present in the clinic, the first step in classifying OA might be to identify the affected joint(s) and joint surface(s), stage of disease, and whether the disease has developed as a consequence of trauma. Clinically adopted imaging, pain, and function scores can be readily acquired in the clinic and do not require invasive testing. These scores can provide information on important clinical signs and symptoms including, pain, the presence or absence of osteophytes, cartilage damage, synovitis, bone marrow lesions, meniscal damage, and muscle/ligament involvement. While the accessibility of these scores makes them ideal to stratify OA patients, patients mostly do not present to the clinic until their pain or function is severely affected at end-stage disease. Therefore,

clinical data might be useful to help distinguish between different subtypes of end-stage disease, but this might only give limited insight in the mechanism of disease that led them to this stage of disease.

Risk factors might also be a broadly applicable stratification step which could help to identify potential underlying mechanisms. In contrast to clinical measurements, risk factors can be identified prior to clinical presentation of OA, which could be an advantage to identify people for prophylactic treatment. Up to this point, no (combination of) risk factors have been found that can adequately and reliably predict the occurrence or prognosis of OA. However, even if we can identify such a (combination of) risk factors, it is still the question how these people can be found or contacted, and how these patients can be treated.

Although both clinical signs and symptoms as well as risk factors could be a good start to stratify patients, there is potentially a lot of overlap between different conditions on the spectrum of OA as most patients only present to the clinic at end-stage disease. However, molecular signatures have the potential to point towards underlying mechanisms of disease. Therefore, it is essential to improve understanding of OA pathophysiology, so that the differences between these subtypes of disease can truly be understood and can be used to develop appropriate treatments for these patients.

1.6 Aim of this thesis

The understanding of OA pathophysiology has rapidly grown during the last decade. While there is a growing body of evidence that inflammation plays an important role in OA, the understanding of the inflammatory environment in OA is still lacking compared to inflammatory arthritides such as RA. As there are currently no DMOADs available, it is critical to increase the understanding of inflammatory processes and to identify key contributing mediators in the OA joint. Therefore, the overarching aim of this thesis is to advance the understanding of the role of inflammation in the pathophysiology of

knee OA. Specifically, this thesis aims to study the immune cells and fibroblasts in synovium and their contribution to synovitis seen in many OA patients, and aims to study the role of the pro-inflammatory cytokine IL-17 in driving or sustaining inflammation in human knee OA.

To achieve this aim, this thesis is split into three chapters. First, Chapter 2 aims to characterise the cellular and molecular environment of synovial tissue derived from end-stage knee OA patients. It is hypothesized that there are immune cell populations as well as fibroblasts with an activated phenotype in end-stage OA synovium, and that associations could be found between cell subsets themselves, as well as with patient characteristics and clinical scores. Chapter 3 aims to identify the expression of macrophage markers and IL-17A and its receptors in tissue from patients at high risk of developing OA after knee injury. It is hypothesized that IL-17A and its receptors and macrophage markers are expressed in synovium of these patients, and that the expression of macrophage markers correlates with a histological synovitis score. Finally, Chapter 4 aims to determine the effect of IL-17 cytokines IL-17A, IL-17F, and IL-17AF on OA-related gene expression of inflammation and matrix destruction in chondrocytes and synovial fibroblasts derived from end-stage OA patients. It is hypothesized that IL-17A causes the strongest changes in gene expression and activation of intracellular signalling pathways, followed by IL-17AF, and finally IL-17F.

Chapter 2

Cellular and molecular characterisation of end-stage osteoarthritis synovium

2.1 Abstract

2.1.1 Introduction

Osteoarthritis (OA) is increasingly accepted as a whole joint disease, with an important role for synovial inflammation (synovitis) in its pathophysiology. Infiltration of immune cells is reported in OA synovium, but the presence, identity, and function of these cells remain understudied. In addition, synovial fibroblasts, the predominant cell type in synovium, have received increased attention due to their potential pathogenic role in joint inflammation. Therefore, this study aims to characterise the cellular composition of end-stage OA synovium and investigate the association between different cell types and clinical scores. Proteomic analysis will be used on matched synovial fluid to investigate if differences in synovial fluid proteins can be used to further specify subsets of disease.

2.1.2 Methods

Synovium from 10 end-stage knee OA patients obtained during total knee replacement surgery was collagenase digested and 4 panels of antibodies were used to study immune and fibroblast populations by flow cytometry. Along with age, gender, and affected knee, clinical scores including body-mass index (BMI), Kellgren-Lawrence (KL)-grade, and Oxford Knee Score (OKS) were collected and used in the analysis. Matched synovial fluid from 8 out of these 10 patients was hyaluronidase-treated and protein composition was evaluated using mass spectrometry.

2.1.3 Results

Macrophages and T cells together were the dominant immune cell populations (76%) in OA synovium. Age showed a significant positive association with macrophages as a proportion of immune cells, and negative association with T cells. A clear CCR6+ T cell population was found in 6 out of 10 patients. These CCR6+ patients had a larger proportion of macrophages, a higher KL-grade, and a higher OKS than the CCR6- patients. Fibroblasts in OA synovium expressed a range of fibroblast (activation) markers that are able to distinguish fibroblast subsets, including CD34, CD90 (THY1), FAP (fibroblast activation protein), and PDPN (podoplanin). Previously published fibroblast subsets (CD45-PDPN+) with unique functions based on the expression of CD34/CD90 or FAP/CD90 were identified in all patient samples. Proteomic analysis of matched synovial fluid samples did not reveal any obvious separation of subsets of patients.

2.1.4 Conclusions

This hypothesis-generating study has shown that there are significant differences in the numbers and subsets of immune cells in OA synovium, which might be associated with age, clinical scores of disease severity, disease subtypes, or fibroblast subsets. The immune cell subset that is most different between patients are the CCR6+ T cells, which are only present in a subset of patients. This suggests a subtype of disease with an important role for CCR6+ T cells and its produced cytokine IL-17A. Previously identified fibroblast subsets were found, with a large immune effector (FAP+CD90+) population, which might be an important contributor to the synovitis seen in many patients. Future studies should investigate if macrophages can be confirmed as are a driver of the synovitis seen in many OA patients. In addition, the presence of IL-17A and its receptors should be further studied in OA tissue, and the effect of IL-17A on the predominant cell subset in cartilage and synovium needs to be tested. This will not only advance our understanding of disease, but may also lead us to the identification of targets for potential disease modifying therapies.

2.2 Background

2.2.1 Introduction

Newly gained knowledge on the pathophysiology of osteoarthritis (OA) has shifted the idea of OA as a degenerative disease of "wear-and-tear" of the articular cartilage to hypothesis that OA is a whole joint disease that involve all joint tissues. The knowledge of the involvement of these joint tissues indicates a multifactorial disease with a complex pathophysiology [21,88].

Although OA was initially referred to as "non-inflammatory" arthritis (in contrast to rheumatoid arthritis (RA), which is referred to as "inflammatory" arthritis), many patients with OA report symptoms related to joint inflammation, including morning stiffness, warmth, pain, and joint effusion. These symptoms can be attributed to synovial thickening and/or synovial fluid effusion, pointing to synovial inflammation (synovitis)[25].

2.2.2 Changes from healthy synovium to synovium in OA

Synovium, consisting of a fibrous sub-lining layer and a thin cellular lining layer, seals the synovial cavity and synovial fluid from the surrounding tissue, and maintains the volume and composition of synovial fluid by producing lubricin and hyaluronic acid[10].

Synovium of OA patients is characterised by hyperplasia of the synovial lining, fibrosis of the synovial sub-lining, and stromal vascularisation[89]. Research from the late 1950s already showed an increase in immunoglobulins in blood and synovial fluid of patients with OA[90]. However, more recent studies of OA synovium and synovial fluid have found an increase in several mediators, including complement components and cytokines[48,91]. Many of these inflammatory mediators, which are characteristic of inflammatory arthritides, are expressed by OA chondrocytes and synovial fibroblasts as well as immune cells[92,93]. Therefore, it is now accepted that chronic, low-grade inflammation is a critical role in OA pathophysiology[25].

2.2.3 Immune cells in OA synovium

The increase in synovial vascularisation in OA is thought to facilitate the influx of leukocytes in response to factors secreted in the OA joint[94]. The predominant immune cell types in OA synovium are reported to be macrophages and T cells. However, many other types of immune cells have been found, including B cells, natural killer (NK) cells, dendritic cells, mast cells, monocytes, neutrophils, and plasma cells, although these are mostly present in small numbers[93–95].

2.2.3.1 Lymphocytes: T cells, B cells, plasma cells, and NK cells

T cells are lymphocytes that have been developed in the thymus and they play a central role in adaptive immunity. The two major types of T cells are T helper cells (identified through the surface marker cluster of differentiation (CD)4) which activate other cells in the immune system; and cytotoxic T cells (CD8) which can release cytotoxins that leads to the death of virally infected cells and tumours. Studies have reported that T cell represent 22% of the infiltrating immune cells in OA synovium[96]. Ishii *et al.* found CD4+ and CD8+ T cells in synovial tissue from OA patients, mostly in synovial aggregates. From immunohistochemistry analysis, it seems that these different T cells are mainly present in the sub-lining and to a lesser extent in the deeper layer. In addition, they found an increased number of CD4+ T cells in the synovium of OA patients compared to healthy controls, and of those cells there was a higher number of IFN-γ+ (Th1) cells than IL-4+ (Th2) cells[97]. Saito *et al*. showed that T cells positive for CD4 seemed to be predominant and more highly present on the medial side than on the lateral side of the synovium[98]. In addition, studies report increases of CD4/CD8 ratio, which Saito *et al.* reported to go from 2:1 in healthy synovium to 5:1 in OA synovium[98]. Another study has found CD3+ cells in the synovium of 65% of the 30 OA patients investigated, and cells positive for activation antigens CD25, CD38, CD43, CD45RO, CD69, and HLA class II were identified[99]. The synovial aggregates in OA express CD80, an inducible co-stimulatory ligand that is involved in T-cell activation, which suggests that these aggregates are areas in which

there is antigen recognition and T-cell activation[100,101]. Other studies have found Th17 cells[102], regulatory T cells (Treg)s[103], memory T cells[104], γδ T cells[105–107], and NKT cells[108].

Although the numbers found are generally low, both B cells and plasma cells have been identified in OA synovium[95]. B cells are lymphocytes that help the adaptive immune response by secreting antibodies, while plasma cells are fully differentiated to only produce one type of antibody. Although B cells and plasma cells are often reported to be less abundant in OA synovium compared to RA synovium[96], a recent study found more B cells in OA than in RA synovium[109]. It was shown that infiltrating B cells are oligoclonal, suggesting that an antigen driven immune response may play a role in OA pathophysiology[110]. Analysis of B cells from OA patients with pronounced synovial B cell infiltration confirmed that the clonal and oligoclonal expansions of these cells is common, that most these cells were activated through their antigen-receptor, and that local antigens could promote their proliferation. Therefore, it is likely that the homing and expansion of B cells in OA synovium is a selective process, and that these B cells could contribute to disease[111].

NK cells are lymphoid cells that are part of the innate immune system, playing an important role in host-rejection of tumour cells and virally-infected cells. However, in diseases such as RA, they have hypothesized to play a role in bone destruction[112]. NK cells have been reported to comprise up to 30% of the mononuclear cells in end-stage OA synovium[113]. A study by Jaime *et al.* have shown that the NK cells in OA synovial fluid express high levels of the pro-inflammatory enzyme granzyme A compared to NK cells in peripheral blood, which is a key modulator of inflammation in mouse models. The presence of the granzyme A positive NK cells correlated with increased levels of pro-inflammatory cytokines in synovial fluid, and is thus hypothesized to contribute to the establishment of chronic inflammation in these patients[114].

Not only are these T and B cell subtypes understudied in OA, but many studies date back to the early 2000 or before. Since then, not only have the methods and reagents available for the analysis of cells improved immensely, but our understanding of T-cell biology has evolved, including the identification of new T cell subsets as well as extra- and intracellular markers for these subsets. Therefore, these results, especially the proportions of cells identified in patient tissue, need to be confirmed and expanded on with the use of new knowledge and new techniques.

2.2.3.2 Macrophages

Macrophages are monocyte-derived cells that have an important role in the detection, phagocytosis and destruction of microbes, cellular debris, and foreign substances, as well as cancer cells and other cells or organisms that do not have proteins specific to healthy body cells on its surface. They can alter their phenotype between pro- and anti-inflammatory depending on the environmental stimuli. In health, the macrophages in synovium remove joint debris from synovial fluid, clear bacterial infections, and control the balance of pro- and anti-inflammatory cytokines in synovial fluid[115]. However, in OA synovium and synovial fluid increased numbers of macrophages have been found[95,115]. Macrophages in OA synovial fluid have an activated phenotype (HLA-DR+) and show an association with knee OA patient reported outcome measures[116], and in OA synovium macrophage infiltration positively correlates with histological infiltration grade[117]. Macrophages in OA synovium are mainly located in the synovial lining, but also scattered through the sub-lining. In addition, studies have found that macrophage numbers are highest at sites of cartilage damage[115]. In contrast to other immune populations found in OA synovium such as T cells, macrophages have been fairly well characterised and several recent functional studies have provided insight into their role in OA pathophysiology. It is now thought that tissue resident macrophages are important for homeostasis of the joint, while macrophages differentiated from blood monocytes have a more inflammatory function[118]. Macrophages can be activated by immune mediators and components released by damaged cartilage[43,119]. Activation of these macrophages contributes to OA synovitis through

secretion of pro-inflammatory cytokines that activate other immune cells, by releasing matrix metalloproteinases (MMPs) that mediate cartilage destruction, and by direct and indirect induction of the formation of osteophytes[120–125].

Although many different subsets of macrophages exist, a crude subdivision of extremes would be the classically activated M1-macrophages, which are involved in Th1 stimulation and release of IL-12, IL-23, TNF-α, IL-1β, and nitric oxide, and the alternatively activated M2-macrophages, which stimulate Th2 cells and release IL-10, CCL18, TGF-β and IL-1 receptor antagonist. Recent studies have shown that the M1/M2 macrophage division is outdated, and that macrophage activation and polarisation is better viewed on a spectrum. Although a new system for *in vitro* experiments has been proposed for macrophages isolated from animals, there are still many questions surrounding the categorisation of human macrophages[126]. Because the M1/M2 division has been used in historical studies, this crude subdivision will also be used in this study for comparative purposes.

Both M1 and M2 macrophages are present in OA synovium[115], although studies report contradicting results whether there is an increase in M1- or M2-type macrophages[127,128]. In addition, there have been several reports of OA synovial macrophages that express a combination of M1- and M2-markers, and there seems to be a large inter-patient variability in the expression of these synovial markers in OA synovium[115].

Recent studies have tried to identify specific patient subtypes based on the presence of macrophage subtypes. A recent study by Wood *et al.* was able to distinguish two subgroups of patients with knee OA: an inflammatory OA macrophage type that is similar to those seen in inflammatory arthritis, and classic OA macrophages which show cartilage remodelling capabilities. Such stratification could help identify patients for targeted disease-modifying treatment[129].

2.2.3.3 Mast cells

In recent years, the evidence and the attention for a potential role of mast cells in OA pathophysiology has been increasing. Mast cells are a type of granulocyte that contain granules rich in histamine and heparin. Many studies have identified mast cells in OA synovium[95]. In contrast to the many other infiltrating immune cells, most studies report that the percentages of infiltrating mast cells in OA have been found to be as high or higher than in RA. Degranulated mast cells, indicating an active state, have mainly been found in the most superficial layers of the synovium. A histological study by de Lange-Brokaar *et al.* found that there are significantly higher numbers of mast cells in OA synovial samples than in RA samples. In addition, the number of mast cells significantly correlated with synovitis score, and a trend was observed towards an association between number of mast cells and increased KL-grade independently of synovitis[130].

In a mouse model of OA, mast cells contributed to the release of PGD2, which resulted in mechanical hypersensitivity in the OA joint[131]. Results from a recent study by Wang *et al.* suggest that mast cells are transcriptionally active in OA synovium. In addition, using an animal model of post-traumatic OA they show that IgE-mediated mast cell activation promotes inflammation and cartilage destruction, leading to a vicious cycle of tissue damage, inflammation, and mast cell activation, resulting in the development and progression of OA. Therefore, they hypothesize that mast cells or tryptase could be used as a disease-modifying treatment for OA[132]. Clinically, the use of histamine H1-receptor antagonists is associated with a decrease in knee OA prevalence, again suggesting a role for mast cells[133].

2.2.3.4 Neutrophils and dendritic cells

Neutrophils are the most abundant type of granulocytes and the most abundant immune cells in blood as they are an essential part of the innate immune system that gets rapidly recruited in response to injury and stress. While neutrophils are among the most prevalent cell types in RA

synovium, most studies have detected no or only small numbers of neutrophils in OA synovium[95]. However, neutrophils are known to play important roles in virtually all inflammatory disease, not only acute, but also in chronic, autoimmune, infectious, and non-infectious diseases. In RA, neutrophils are activated in the joint, which leads to the release of reactive oxygen species and extracellular granule contents due to frustrated phagocytosis. In RA, increases in degranulation, production of reactive oxygen species, and delayed apoptosis of neutrophils correlate with synovial inflammation and the destructive capacity of neutrophils in the RA joint[134]. When a recent study looked at biomarkers in OA synovial fluid, they found that these indicate the presence of activated neutrophils[27]. In OA there is pronounced tissue damage as there is a breakdown of cartilage, but also fibrosis of synovium, which suggests that neutrophils could play a role. However, more research is needed to get a better understanding of the potential role of neutrophils in OA synovium.

Dendritic cells are monocyte-derived antigen-presenting cells that are part of the adaptive immune system. Low numbers of dendritic cells have been described in OA synovium[95]. In an early study from Pettit *et al.* dendritic cells were found in lower levels in synovium from OA than in RA or spondyloarthritis patients[135]. Again, only a few papers were found that looked at dendritic cells in (a model of) OA. In a post-traumatic rabbit OA model, markers for dendritic cells were detected that were especially present in large numbers in the early stages after surgery, suggesting that they could participate in the development of OA[136]. In a mouse model of OA, the transfer of suppressive oligodeoxynucleotide-induced dendritic cells significantly controlled the OA process by regulating immune responses and possibly cartilage destruction due to the downregulation of inflammatory cytokines and MMP-13[137].

2.2.4 Fibroblasts

Fibroblasts are the most common cell type in connective tissue, providing the structural tissue framework. The functional requirements and activity of fibroblasts differs between anatomical sites,

enabling them to support biophysical requirements of each tissue, production of matrix proteins, and to act as immunomodulators. In healthy synovium, synovial fibroblasts express CD55, VCAM1, ICAM1, CD44, and β1 integrins, and produce matrix components, including hyaluronan, collagens, and fibronectin, that provide strength and structure to the synovium, and maintain the volume and composition of the synovial fluid[11,138]. With the knowledge of the versatility of fibroblast functions, it is not surprising that it is now clear that several subtypes of fibroblasts exist within each tissue[139].

2.2.4.1 Fibroblasts can contribute to inflammation

In disease, not only immune cells, but also synovial fibroblasts can contribute to inflammation, fibrosis and tissue destruction[140]. There is an active cross-talk between immune cells and fibroblasts: both the innate and the adaptive immune system can trigger fibroblast activation, and fibroblasts themselves can modulate the behaviour of immune cells by influencing the microenvironment through the secretion of cytokines and chemokines[139]. However, in recent years, research has shown that these cells can also undergo phenotypic changes as a consequence of inflammation. This activation of fibroblasts was first seen in cancer, but has now been shown in musculoskeletal diseases such as rheumatoid arthritis (RA) and tendinopathy[141]. Fibroblasts isolated from RA synovium or diseased tendon also maintain their phenotype in culture and express several fibroblast activation markers. Interestingly, compared to healthy cells, these cells have an increased capacity to respond to inflammatory stimuli[142–144]. While the innate memory in immune cells has long been accepted, 'inflammatory memory' in tissue-resident cells is now slowly gaining acceptance, with a potential link between fibroblast memory and an altered responsiveness of NF-κB to an inflammatory stimulus[145].

2.2.4.2 Fibroblast activation markers can distinguish functionally distinct fibroblast subsets in disease

There is now a growing list of markers that seem to be highly expressed in inflammatory conditions including several papers that use specific combinations of these markers to specify subsets of cells with specific effector functions[146–149]. Fibroblast activation markers that are highly expressed in inflammatory conditions include CD44, CD55, CD90 (also called THY1), cadherin 11, VCAM1 (also known as CD106), endosialin (CD248), fibroblast activation protein (FAP), and podoplanin (PDPN)[147,150–155]. Two recent papers in particular have used these markers to distinguish different fibroblast subsets in arthritis.

Mizoguchi *et al.* described three functionally distinct disease-associated fibroblasts subsets in RA synovium. Using single-cell transcriptomics and immunohistochemistry, they were able to broadly divide PDPN+ fibroblasts from RA and OA patients into three subsets: CD34+ cells which are both present in the lining and sub-lining, CD34-CD90- cells which are mainly present in the lining, and CD34-CD90+ cells, which formed a perivascular zone in RA, while in OA they observed that these cells form a layer around the blood vessels. They found that on average 15% of fibroblasts in synovium of 16 RA patients were CD34-CD90-, 22% of cells were CD34-CD90+, and 36% were CD34+. Although the main focus of this study was on RA, in most experiments OA was studied as a control. After studying 26 patients with OA, they found that 48% of fibroblasts were CD34-CD90-, 8% of cells were CD34-CD90+, and 35% were CD34+[149]. Using single-cell transcriptomics, it was found that there was a significant enrichment of mitotic and proliferative genes in CD34-CD90+ cells, increased migratory and invasive behaviour in CD34-CD90+ and CD34+ cells, CD34-CD90+/- cells promote osteoclast differentiation, and CD34+ cells show an enhanced secretory phenotype that can recruit a high number of monocytes.

A recent paper by Croft *et al.* identified two distinct fibroblasts subsets that drive inflammation and tissue damage in RA using single-cell transcriptional analysis. FAP was more highly expressed in synovium of RA patients than in those whose joint inflammation resolved. These FAP+ (pathogenic) fibroblasts co-localised with PDPN in both the lining and sub-lining of the synovium. FAP+CD90+ (sub-lining) cells correlated with the severity of joint inflammation and severity of bone erosion in an arthritis mouse model, while FAP+CD90- (lining) cells correlated with the extent of cartilage damage. Croft *et al.* used CyToF analysis to study the PDPN+FAP+CD90+/- cells in synovium of 15 OA patients and 8 RA patients. Single-cell transcriptomics analysis show that FAP+CD90+ cells had an increased expression of cytokines and chemokines, while FAP+CD90- had a higher expression of osteoclast inducing mediators, as well as an increase in MMPs involved in cartilage degradation. In humans, PDPN+FAP+CD90+ cells were found to be increased in the synovium of RA patients compared to OA patients, and in RA these cells positively correlated with markers of systemic and tissue inflammation[148]. Although these studies mainly focus on role of these fibroblasts in RA, this data can be used to inform hypotheses of the potential roles of these fibroblast subsets in OA.

2.2.5 Aim of this study

Synovitis is increasingly recognised as important pathological feature of OA. However, the immune cells that infiltrate into the synovium remain understudied. In addition, recent studies have highlighted the importance of (pathogenic) fibroblasts in joint inflammation, but no studies have specifically studied fibroblasts in OA synovium or have looked at the association of fibroblast subsets with immune cell infiltration. Improved understanding of the pathophysiology of synovitis in OA has the potential to enable development and testing of treatments for (subgroups of) patients. Therefore, the overarching aim of this chapter is to characterise the cellular subsets and proteome in end-stage OA synovium and synovial fluid, respectively.

2.3 Objectives

Specifically, the objectives of this chapter are to:

1. Identify the proportion of different immune cell populations in synovium from end-stage OA patients
2. Study the expression of fibroblast activation markers on fibroblasts in synovium from end-stage OA patients
3. Investigate whether fibroblast subtypes or immune cell populations in end-stage OA synovium are associated with each other, with patient characteristics, or with clinical scores.
4. Investigate whether there are IL-17-producing cells in end-stage OA synovium
5. In a pilot study, establish whether the proteome of synovial fluid reveals any subgroups of end-stage OA patients

2.4 Hypotheses

It is hypothesized that:

1. There are populations of immune cells in synovium from end-stage OA patients
2. Fibroblasts in end-stage OA synovium express activation markers, showing an inflammatory phenotype
3. Associations can be seen between the proportion of activated fibroblasts and proportion of immune cells, as well as between predominant immune cell subsets and clinical scores
4. There are IL-17-producing cells in in end-stage OA synovium
5. The synovial fluid proteome informs distinct subsets of patients with end-stage OA.

2.5 Methods

2.5.1 Ethics

Ethical approval for the Oxford Knee Injury Cohort (OxKIC) study was granted in October 2014 (14/WA/1108), and amendments were accepted in July 2015 by the Research Ethics Committee (Wales REC 7). Ethical approval for the Oxford Musculoskeletal Biobank (09/H0606/11) and (19/SC/0134) was granted by the local research ethics committee (Oxford Research Ethics Committee B) for all work on human cartilage and human synovium, and informed consent according to the Declaration of Helsinki was obtained from all patients.

2.5.2 Flow cytometry analysis

2.5.2.1 Patient inclusion and exclusion criteria

Synovial tissue was collected during total knee replacement surgery from patients with end-stage knee OA. Patients that were identified as having rheumatoid arthritis or secondary OA due to trauma were excluded from the study. In addition, tissue samples that yielded less that 1.1 million cells after digestion were excluded, as there were too few cells to run all the antibody panels and isotype controls.

2.5.2.2 Collection of patient characteristics

At the time of collection of synovial tissue, the age of patient at time of operation, sex, and affected knee were collected. After collection of tissue, further information was collected. Kellgren Lawrence (KL)-grade[156] for each of the three knee compartments (medial, lateral, patellar) was scored by Dr Mathew Baldwin based on X-rays and the mean KL-grade of the three compartments was used in this study. Oxford Knee Score (OKS)[157] was obtained from the Electronic Patient Record. If the OKS was not recorded, a new OKS questionnaire was sent to the participants within a year of the surgery to ask if they could recall their functional before their operation[158]. Body mass index (BMI), co-

morbidities, medication, smoking status, and alcohol were collected from the Electronic Patient Record. Due to the wide range of co-morbidities and medication taken, we were unable to use this information with the small patient cohort used in this study. Smoking status and use of alcohol were not available for all patients, and therefore not used in this study.

2.5.2.3 Tissue collection, digestion, and stimulation

Fresh synovial tissue was collected from patients in R10 media (RPMI (Gibco, supplied by Fisher Scientific, Loughborough, UK) with 10% Foetal Bovine Serum (FBS) (Labtech International, Heathfield, UK) and 1% Penicillin/Streptomycin (P/S) (Gibco)) After collection, the tissue was washed in media to wash off any blood, and fat was trimmed if needed. Tissue was put in a new tube with fresh R10 media with the lid untightened and left overnight. On the next morning, the tissue was cut into very small pieces with a scalpel. Digestion media was made by adding 10mL of R0 (serum-free RPMI media with 1% P/S) to a bottle of filtered type II collagenase (Worthington Biochemical Corporation, supplied by Thermo Fisher Scientific, Waltham, MA, USA) to obtain 10 mL of 5 mg/ml of type II collagenase to be used for each cm^3 of tissue. Digestion media and tissue were added to a 50 mL tube, and tissue was left to digest for 2 hours at 37°C with gentle agitation (±80rpm). After two hours, ±20 mL of cold R10 media was added to stop the reaction, after which the digestion mixture was filtered using a 70 μm cell strainer. The strained mixture was centrifuged for 6 minutes at 400g, reconstituted in 5 mL of R10 media before the cells were counted. 1mL of media with 100,000-2,000,000 cells was added to 11 pre-labelled tubes; if there were less than 1.1 million cells (100,000 per tube), the patient was excluded from final analysis. Each tube was either left unstimulated or was stimulated according to Table 2-1 below with Cell Stimulation Cocktail with phorbol 12-myristate-13-acetate (PMA) and Ionomycin to 1X concentration (BioLegend), 100 ng/mL LPS (Thermo Fisher Scientific) or 10 ng/mL IL-1β (BioLegend) Every tube was briefly vortexed before leaving the tubes to incubate for an hour at 37°C. After 1 hour of incubation, Brefeldin and Monensin (both BioLegend,

London, UK) were added to every tube, the tubes were briefly vortexed, and left in the incubator to incubate for another 4 hours.

Table 2-1. *Overview of the 11 samples that were used for each patient in the flow cytometry study.*

Tube	Panel number	Simulated?	Stimulant	Antibody/isotype
1	1	Unstimulated	-	Isotype
2	1	Unstimulated	-	Antibody
3	2	Stimulated	PMA/Ionomycin	Isotype
4	2	Stimulated	PMA/Ionomycin	Antibody
5	2	Unstimulated	-	Antibody
6	3	Stimulated	LPS (100 ng/mL)	Isotype
7	3	Stimulated	LPS (100 ng/mL)	Antibody
8	3	Unstimulated	-	Antibody
9	4	Stimulated	IL-1β (10 ng/mL)	Isotype
10	4	Stimulated	IL-1β (10 ng/mL)	Antibody
11	4	Unstimulated	-	Antibody

2.5.2.4 Extracellular staining and fixation

After incubation, the tubes were washed twice by adding 2 mL of FACS buffer (BioLegend), centrifuging for 5 minutes at 500g, removing the supernatant, and vortexing the cell pellet. Fc-blocker was added and incubated for 10 minutes at 4°C in the dark. A mixture of extracellular antibodies for each panel as well as a mixture of their corresponding isotype controls was prepared in 100 μL FACS buffer according to the Table 2-2, added to each tube, and incubated for 20 minutes in the dark on ice (full specification of the markers used can be found in Appendix A1, and specification of antibodies and isotype control can be found in Appendix A2. After incubation, each tube was washed twice as previously described, Fixation Buffer was added (BioLegend), and incubated for 20 minutes in the dark at room temperature. After fixing the cells, each tube was washed once (now centrifuging for 5 minutes at 800g), after which FACS buffer was added, tubes were covered and left in the dark at 4°C until intracellular staining.

Table 2-2. *Overview of antibodies used in each antibody panel during extracellular staining of flow cytometry samples. Grey spaces are held for antibodies that will be used for intracellular staining. Blue slots are not used.*

Fluochrome	Detector	Laser	Panel 1: Mixed	Panel 2: T-cells	Panel 3: Granulocytes	Panel 4: Fibroblasts
eFluor 450	Blue	Violet	-			
BV650	Red		CD15	CCR6	CD15	-
BV711	Far Red		CD45	CD161	CD45	PDPN
BV786	Infrared		CD34	CD56	CD40	CD34
FITC	Green	Blue	IL-17RC			
PE	Yellow		IL-17RA	GDTCR	CD11c	FAP
PE-CF594	Orange		CD19			
PerCP/Cy5.5	Red				CD117	VCAM
PE-Cy7	Infrared		CD3	CD3		CD90
APC	Red	Red	CD8	CD8	CD206	CD248
AlexaFluor 700	Far Red		CD14	CD45	CD14	CD45
eFluor 780	Infrared		Viability	Viability	Viability	Viability

2.5.2.5 Permeabilization and intracellular staining

Tubes were centrifuged for 5 minutes at 800g, supernatant was removed, and tubes were vortexed. 10X Permeabilization buffer (Invitrogen, supplied by Thermo Fisher Scientific) was diluted in MilliQ water; 1X permeabilization buffer was added to each tube and incubated for 30 minutes at 4°C. After incubation, tubes were washed twice with permeabilization buffer. Mixture of intracellular antibodies for each panel as well as mixtures of their corresponding isotype controls was prepared in 100 μL of permeabilization buffer according to Table 2-3. 100 μL of the mixture was added to each tube and incubated for 60 minutes at 4°C in the dark. Permeabilization buffer was used to wash the tubes twice after which 300 μL of FACS buffer was added for analysis.

Table 2-3. *Overview of antibodies used in each antibody panel during intracellular staining of flow cytometry samples. Grey spaces are held for antibodies that were used for extracellular staining. Blue slots are not used.*

Fluochrome	Detector	Laser	Panel 1	Panel 2	Panel 3	Panel 4
eFluor 450	Blue	Violet	-	IL-17AF	IL-17AF	IL-17AF
BV650	Red					-
BV711	Far Red					
BV786	Infrared					
FITC	Green	Blue		IL-17A	IL-17A	IL-17A
PE	Yellow					
PE-CF594	Orange			IL-17F	IL-17F	IL-17F
PerCP/Cy5.5	Red		CD4	CD4		
PE-Cy7	Infrared				CD68	
APC	Red	Red				
AlexaFluor 800	Far Red					
eFluor 780	Infrared					

2.5.2.6 Preparing compensation controls

Compensation controls were made up with OneComp eBeads compensation beads (Invitrogen, supplied by Thermo Fisher Scientific) and flow cytometry antibody in the used concentration and incubate on ice. Wash the Beads were washed twice with FACS buffer before adding Fixation Buffer (BioLegend) and incubated for 20 minutes in the dark at room temperature. After fixation beads were washed twice before resuspending in 300 μL FACS buffer for analysis.

2.5.2.7 Flow cytometry run

Flow cytometry machine LSR Fortessa Cell Analyzer (BD Biosciences) with FACS Diva version 8.0.1 (BD Biosciences) was used. If the machine was used for the first time that day, CST was ran using FACS Diva CS&T Research Beads (BD Biosciences). Each vial of compensation controls first used to manually check for any overlap with other fluochromes. If any overlap was detected, voltages were adjusted accordingly (Appendix A3). Then compensation controls were run and compensation was analysed and applied. Each sample was used up completely during analysis.

2.5.2.8 Flow cytometry analysis

FlowJo version 10.6.1 (BD Biosciences) was used to analyse every sample. For each sample viable cells were selected through three different gates (Figure 2-1). First, all cells were gated to separate the cells from debris (SSC-A vs FSC-A), then single cells were gated out (SSC-H over SSC-W), and finally viable cells were selected as cells negative for the viability dye (viability dye over SSC-A). The average number of events analysed, along with the percentage of cells (as % of events), single cells (% of cells), and viable cells (% of single cells and % of all cells) are reported (Table 2-4). Gating for all other markers used in this study can be found in Appendix A4.

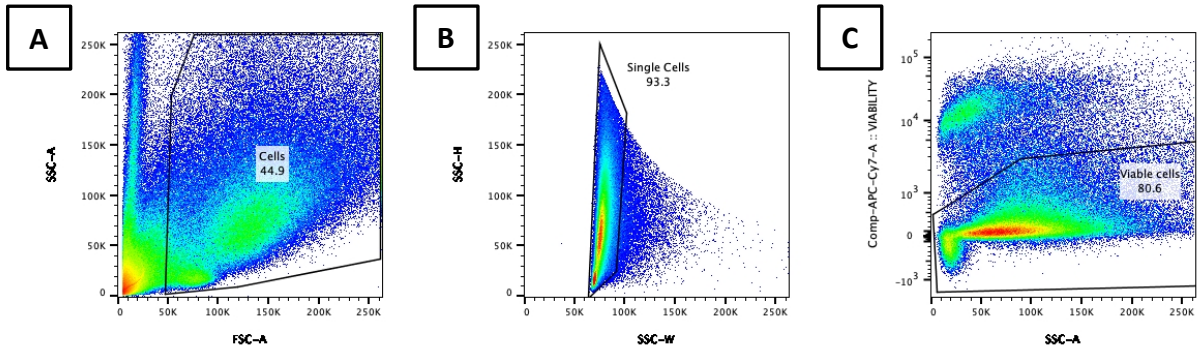


Figure 2-1. *Flow cytometry gating strategy to select viable cells. First, cells were separated from debris (A), then single cells were selected (B), and finally the viable cells which are negative for the viability dye were selected (C).*

Table 2-4. *Overview of analysed events of each sample in flow cytometry analysis. Data includes mean number of analysed events per patient sample, as well as number of events in the gates for all cells, single cells, and viable cells (as per Figure 2-1).*

Patient #	# Events (mean)	Cells (% of events)	Single cells (% of cells)	Viable cells (% of single cells)	Viable cells (% of cells)
1	98,284	41.5	92.3	66.2	25.4
2	83,567	43.4	92.1	80.1	32.0
3	411,036	60.3	92.9	83.2	46.6
4	158,841	46.2	93.6	82.4	35.6
5	296,111	59.6	92.0	68.4	37.6
6	175,666	9.1	89.7	82.5	6.7
7	242,033	62.3	92.3	80.0	46.0
8	341518	45.4	94.2	78.2	33.5
9	148,931	46.2	95.3	77.9	34.3
10	537,429	67.1	95.1	85.9	54.8

2.5.3 Acquisition and initial processing of synovial tissue from patients with high risk of developing OA (OxKIC cohort)

The OxKIC cohort is a cohort of patients that presented with anterior cruciate ligament (ACL) and/or meniscal injuries that have a high risk of developing OA. Synovial tissue samples from patients with knee injury and/or early-stage OA were collected as part of this study. Tissue samples from patients with knee injury and early-stage OA were obtained either 1) with a minimally invasive ultrasound-guided knee lining biopsy at 6 to 12 months after knee injury, or 2) during intraoperative knee synovial biopsy if patients were surgically treated for their knee injury at any point.

Samples were immersed in 10% buffered formalin for 0.5 mm/hour, embedded in paraffin wax before cutting 5μm tissue sections using a RM2135 microtome (Leica Microsystems, Wetzlar, Germany) and baking onto adhesive glass slides at 60°C for 30 min and 37°C overnight.

2.5.4 Immunofluorescence

On the day prior to staining, synovial sections obtained from the OxKIC study were baked at 60°C for 60 minutes and left at room temperature overnight to cool down. Deparaffinisation and antigen retrieval procedure was performed using a PT Link machine (Dako, Glostrup, Demark) by submerging slides in FLEX TRS antigen retrieval fluid.

Immunofluorescence staining was performed as published by Dakin *et al*[159]. In short, after antigen retrieval, slides were left in buffer for 5 minutes, after which an ImmEdge pen (Thermo Fisher Scientific) was used to draw around the section to be stained. Specified sections were blocked for 1 hour in PBS containing 5% normal goat serum (Sigma) in a humid chamber. After blocking solution was removed, sections were incubated with primary antibody in PBS containing 5% normal goat serum (Sigma) and 0.05% saponin (Sigma) for 2 hours. Primary antibodies were used as specified in

Table 2-5. Sections were washed three times in PBS-T (0.2% Tween 20), and incubated in secondary antibody cocktail (Table 2-6) in PBS with 5% equine serum and 0.05% saponin for 2 hours in a humid chamber shielded from the light. After incubation, sections were washed three times with PBS-T and incubated with 2μM POPO-1 nuclear counterstain (Invitrogen) diluted in PBS with 0.05% saponin for 20 minutes. After three washes in PBS-T, autofluorescence of the tissue was quenched using a 0.1% Sudan Black B solution (Applichem, Darmstadt, Germany) in 70% ethanol for 5 minutes. Sections were then washed three times with PBS-T before being mounted using fluorescent mounting medium (VectaShield, Vector laboratories, Peterborough, UK), sealed with nail polish, and stored at 4°C until image acquisition. Synovial tissue that was stained with either universal mouse or universal rabbit negative control antibodies is shown in Appendix A5. High-resolution versions of the confocal images used in this chapter can be found in Appendix A6.

Table 2-5. *Overview of antibodies used for immunofluorescence, including their target, isotype, company, reference number, and dilution used during staining.*

Target	Isotype	Supplier	Ref number	Dilution
Human CD90/Thy1	Monoclonal mouse IgG1	Abcam, Cambridge, UK	ab181469	1:500
Human podoplanin (PDPN)	Monoclonal mouse IgG2a	LSBio, supplied by Stratech, Ely, UK	LS-B16214	1:50
Human fibroblast activation protein (FAP)	Polyclonal rabbit IgG	Abcam	**Ab28244**	1:100

Table 2-6. *Overview of secondary antibodies used for immunofluorescence staining.*

Target	Supplier	Ref number	Dilution
Goat anti-mouse IgG1 (Alexa Fluor 488)	Southern Biotech (supplied by Cambridge Bioscience Ltd, Cambridge, UK)	1070-02	1:200
Alexa Fluor 568 goat anti-mouse IgG2a (γ2a)	Invitrogen, Carlsbad, CA, USA	A21134	1:200
Alexa Fluor goat anti-mouse IgG2b (γ2b)	Invitrogen	A21144	1:200
Alexa Fluor 633 goat anti-rabbit IgG (H+L)	Invitrogen	A21070	1:200

2.5.5 Immunofluorescence image acquisition

Images for the immunofluorescence staining were acquired on a Zeiss LSM 710 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) using a 40x objective with oil immersion (numerical aperture, 0.95). The fluorophores POPO-1, Alexa Fluor 488, Alexa Fluor 568, and Alexa Fluor 633 were excited with lasers lines at 405, 488, 561, and 633 nm, respectively. All channels were acquired sequentially to minimise bleed-through. Averaging was set to 2 and the pinhole was set to ±1 airy unit. ZEN 2009 software (Zeiss) was used to reconstruct two-dimensional images.

2.5.6 Preparation of synovial fluid for proteomic analysis

Where possible, matched synovial fluid was collected from the patients whose synovial tissue was used for flow cytometry experiments. Synovial fluid was spun down at 2500g for 20 minutes at room temperature. Supernatant was aliquoted in 500 μL or 1000 μl volumes into cryovials and fluid was stored at -80°C until further analysis. Synovial fluid was then thawed to room temperature and an equal volume of synovial fluid and 4 mg/ml type I-S hyaluronidase at 618.4 U/mg (Sigma) in sterile water was added to a low-bind Eppendorf vial. The solution was vortexed for 5 seconds before incubation on a shaker for 1 hour at room temperature. Samples were then centrifuged for 5 minutes at 1000g and supernatant was collected. Samples were then processed and analysed by research staff at the Target Discovery Institute (University of Oxford, Oxford, UK) using adapted in-house protocols. Briefly, proteins were digested directly from washed materials by the addition of 1 μg of trypsin (Promega) in 50 mM ammonium bicarbonate overnight at 37°C. The resulting peptides were purified using SOLA C18 HRP (Thermo Fisher Scientific) desalting cartridges. Eluted peptides were dried, re-suspended in 2% acetonitrile, 0.1% trifluoroacetic acid and stored at -20°C prior to analysis.

2.5.7 Proteomics run and analysis

Peptides were detected by nano-UPLC-MS/MS over a 60 min gradient of 0.1% formic acid in 5% DMSO to 0.1% formic acid to 35% acetonitrile in 5% DMSO using a Dionex Ultimate 3000 nano-UPLC with an EASY-Spray column (75 um x 500 um, 2um particle size (Thermo Fisher Scientific) coupled to a Q-Exactive Mass Spectrometer (Thermo Fisher Scientific). MS1 spectra were acquired between 375-1500m/z with a resolution of 70,000. MS2 spectra of HCD fragmented precursors were acquired with a resolution of 17,500. Selected precursors were excluded from repeated analysis for 27s. Raw MS data files were analysed using MaxQuant 1.6.7.0 using the 'match between runs' option, Oxidation (M) and Deamidation (N,Q) were set as variable modifications, two missed cleavage sites were allowed and peptides and proteins were filtered to a 1% false discovery rate threshold. Protein quantitation was performed using the MaxLFQ algorithm within MaxQuant. Post processing was performed in Perseus version 1.6.6.0. Principle component analysis was performed using ClustVis tool developed by Metsalu & Vilo[160].

2.5.8 Statistical analysis

All statistical analyses for flow cytometry data were performed using in GraphPad Prism 8.1.2 (GraphPad Software, La Jolla, CA, USA). Correlation tests using the Pearson's correlation coefficient were used for all associations tested with two continuous variables. If one of the variables was on an ordinal scale, a non-parametric Spearman correlation was used. For the trend lines, data sets were tested for a normal or log-normal distribution using the Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests and the results were used to model a linear, a semi-log or log-log line. Two-way ANOVAs with Sidak's multiple comparisons test was used to test differences between CD45, immune, and fibroblasts subsets as well as clinical scores between groups. A trend was noted when the p-value was lower than 0.1, while differences were considered to be significant when p-value was lower than 0.05. Data is shown as mean ± SD unless otherwise stated.

2.6 Results

2.6.1 Patient cohort

Synovial tissue from end-stage OA patients was collected during total knee replacement surgery. In the end, 10 patients were included in the study (Table 2-7). The gender, age, and affected knee of each patient were provided.

Table 2-7. *Overview of patient information obtained during total knee replacement surgery. Details include gender, age, and affected knee. For the age at time of surgery the average is stated along with the range of ages, and proportions are stated for gender and affected knee.*

Patient #	Gender	Age	Affected knee
1	Female	64	Right
2	Female	58	Left
3	Female	80	Left
4	Male	62	Right
5	Female	72	Left
6	Female	61	Right
7	Female	71	Right
8	Male	75	Right
9	Female	68	Right
10	Female	77	Left
Mean (range)	20% male / 80% female	68.8 (58-80)	40% left / 60% right

2.6.2 Flow cytometry panel development

In order to study the immune and fibroblast populations in synovium of these end-stage OA patients, new flow cytometry panels were developed. The system was limited to the use of 12 different fluochromes, therefore four different panels of markers were used, focussed on 1) general markers + IL-17 receptors, 2) T cells, 3) granulocytes, and 4) fibroblast activation markers. In addition, all panels included an antibody for CD45 and a viability dye, and panels 2, 3, and 4 included antibodies for IL-17A, IL-17F, and IL-17AF.

T cells markers were tested on PBMCs before and after collagenase digestion, to determine whether the collagenase treatment cleaved any cell surface markers. Both CD4 and CD56 were (partly) cleaved, and results for these markers will not be reported. As some cells for the markers tested are present in low proportions in PBMCs, it was not possible to test whether these markers were (partly) cleaved or were not present in the tested tissue. However, previous experience from colleagues suggests that CD117, CD248 and VCAM can be cleaved by digestion. As we were unable to check whether these markers were cleaved or if the expression was low, the results for these markers are not reported.

As the overarching aim of this thesis is to get a better understanding of the role of IL-17 in OA, one of the objectives of this chapter was to investigate whether there are IL-17-producing cells in end-stage OA synovium. However, expression of IL-17, even with stimulation of the cells, was hard to determine due to the high amount of non-specific expression seen in every isotype control. The results of these experiments will briefly be reported in the results, and further discussed in the section 2.7.2.3 of the discussion. Therefore, all the results shown in this chapter are from unstimulated cell populations, unless further specified.

2.6.3 There is a large variation in numbers of immune cells in end-stage OA synovium

After identifying the viable cells in each sample, CD45 was used to separate all viable cells into three categories: 1) CD45+ small cells, which mainly contains T cells and B cells; CD45+ large cells, which mainly contains monocyte-derived cells and granulocytes; and 3) CD45- cells, which mainly contains fibroblasts (Figure 2-2A). The percentage of immune (CD45+) cells in end-stage OA synovium differed between patients, especially in the proportion of CD45+ large cells (Figure 2-2B). While there is a similar proportion of CD45+ small cells for 8 out of 10 patients (apart from patient 1 and 7), there is large variation in the amount of CD45+ large cells with percentages ranging from 4.3% to 27.8% of the total viable cells (Table 2-8).

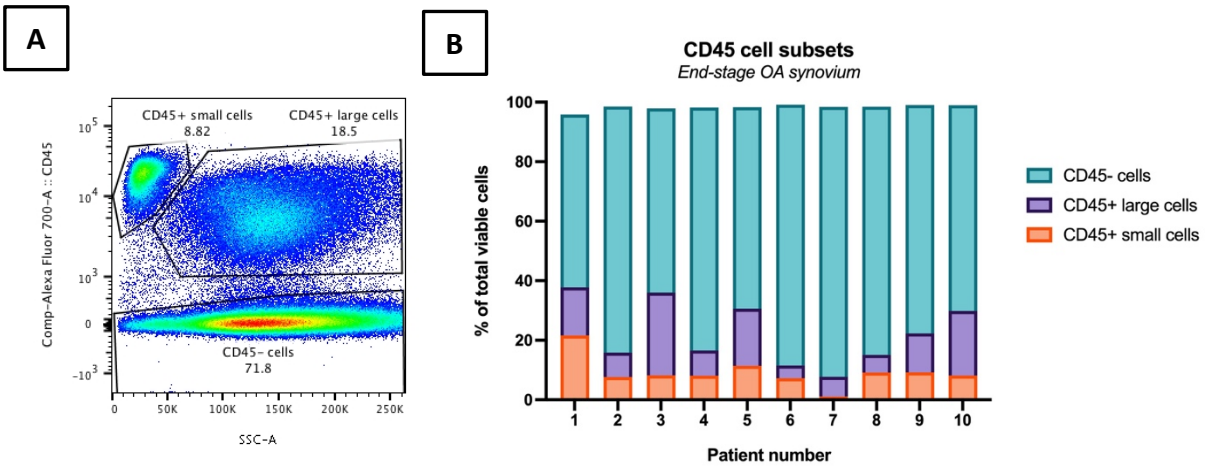


***Figure 2-2**. Identification of three CD45 subsets in end-stage OA synovium. (A) Example of flow cytometry plot gating CD45+ small cells, CD45+ large cells, and CD45- cells using SSC-A vs CD45. (B) CD45 subsets per patients as percentages of all viable cells.*

***Table 2-8**. Percentages of CD45 subtypes (as explained in Figure 2-2) as a percentage of viable cells in end-stage OA synovium.*

Patient #	CD45+ small (%)	CD45+ large (%)	CD45+ total (%)	CD45- (%)
1	21.6	16.2	37.8	58.0
2	7.7	8.2	15.9	82.7
3	8.2	27.8	36.0	61.9
4	8.1	8.5	16.6	81.6
5	11.4	19.2	30.6	67.7
6	7.2	4.3	11.5	87.6
7	1.1	6.6	7.7	90.7
8	9.1	5.9	15.1	83.4
9	9.2	13.1	22.3	76.7
10	8.2	21.7	29.9	69.1
Mean (range)	9.2 (1.1-21.6)	13.2 (4.3-27.8)	22.3 (7.7-37.8)	75.9 (58.0-90.7)

Previous studies have reported that the main immune cells found in OA synovium are T cells and macrophages. Therefore, the prevalence of the markers CD3 (T cells) and CD68 (macrophages) was studied in CD45+ cell population (Figure 2-3, Table 2-9). In this study, these two cell types combined on average make up 76% of all immune cells in OA synovium – 43% macrophages, 33% T-cells. Only 4 patients had a higher proportion of macrophages than T cells, but these patients had 2.3-6.8 times

more macrophages than T cells, while the other 6 patients mostly had similar amounts of both cell types or slightly more T cells.

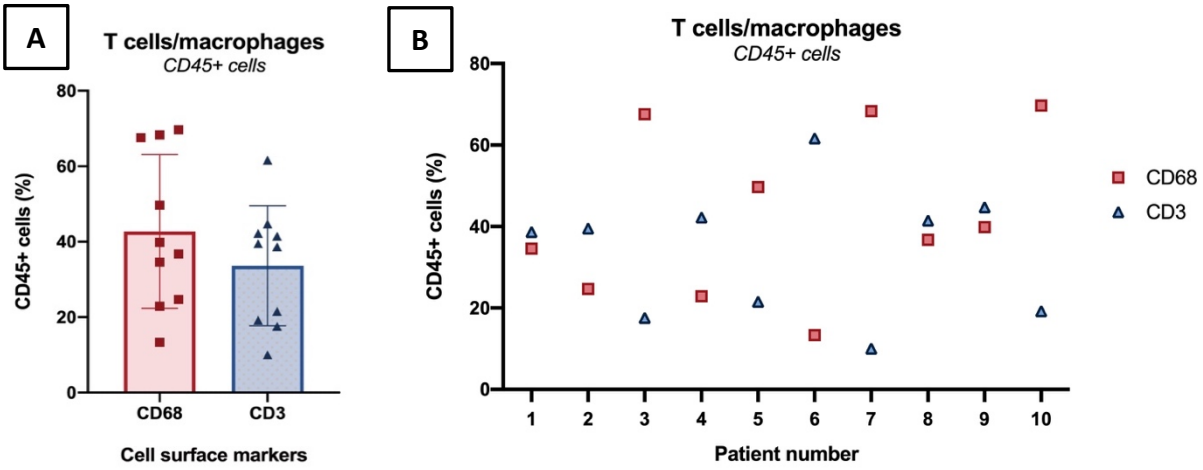


***Figure 2-3**. The proportions of macrophages (CD68) and T cells (CD3) cells in end-stage OA synovium as a percentage of CD45+ cells. (A) Bar graph of average percentages of macrophages and T cells. (B) Percentage of macrophages and T cells for each individual patient. n=10*

***Table 2-9.** Percentages of CD68+ and CD3+ cells (as a percentage of total CD45+ cells) in end-stage OA synovium. Ratio of CD68 to CD3 cells is calculated: higher numbers of macrophages (**red**) and higher numbers of T cells (**blue**).*

Patient #	CD68+	CD3+	CD68/CD3
1	34.6	38.6	0.89
2	24.7	39.5	0.62
3	67.6	17.6	3.85
4	22.9	42.2	0.54
5	49.7	21.5	2.31
6	13.3	61.7	0.22
7	68.3	10.0	6.83
8	36.7	41.4	0.89
9	39.8	44.7	0.89
10	69.7	19.2	3.63
Mean (range)	42.7 (13.3-69.7)	33.6 (10.0-61.7)	-

2.6.3.1 T- and B-cells are the main CD45+ small cell subsets in end-stage OA synovium

T cells (CD3+) and B cells (CD19+) are the major populations in the CD45+ small population. CD45+ small cells were mainly CD3+, while the population of CD19+ cells ranged from 0.34% to 31.1% of CD45+ cells (Figure 2-4, Table 2-10).

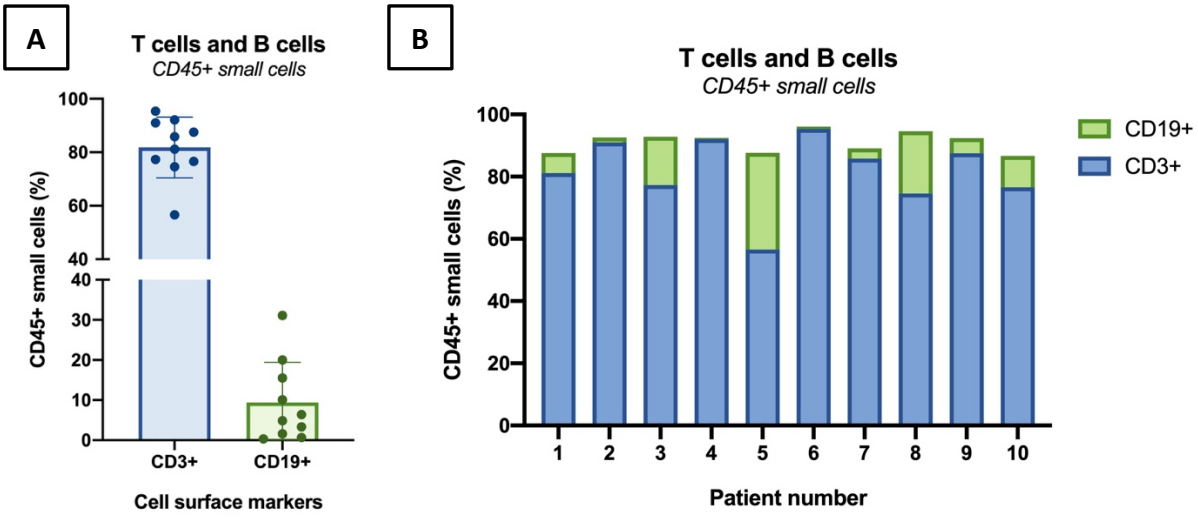


Figure 2-4. *CD3+ and CD19+ cells in CD45+ small cells population of end-stage OA synovium. (A) Bar graphs of the CD3+ and CD19+ populations. (B) Overview of the percentages of distribution of CD3+ and CD19+ cells as a percentage of CD45+ small cells population for each patient. n=10*

Table 2-10. *Overview of the percentages of CD3+ and CD19+ cells as a percentage of CD45+ small cells population in end-stage OA synovium, including isotype controls.*

Patient #	CD3+		CD19+	
	Antibody	Isotype	Antibody	Isotype
1	81.2	0.2	6.4	0.0
2	91.0	0.0	1.6	0.0
3	77.3	0.0	15.5	0.0
4	92.1	0.1	0.3	0.0
5	56.6	0.0	31.1	0.0
6	95.4	0.2	0.7	0.5
7	85.8	0.0	3.3	0.1
8	74.6	0.0	20.0	0.0
9	87.5	0.0	4.9	0.0
10	76.6	0.0	10.1	0.0
Mean (range)	81.9 (56.6-95.4)	0.0 (0.0-0.2)	9.4 (0.3-31.1)	0.1 (0.0-0.5)

2.6.3.2 Presence of CCR6+ cells might indicate a subgroup of patients

To establish the different T cell subsets in end-stage OA synovium, the expression of the cell surface markers CCR6, CD8, GD TCR, and CD161 was studied in the CD3+ population (Figure 2-5, Table 2-11). Percentages of CD3+CD8+ cells range from 11.7% to 42.4% of CD3+ cells. In addition, small CD3+ GD TCR+ and CD3+CD161+ populations were present. However, the most striking difference was in CCR6 expression: while 6 patients had a CD3+CCR6+ population, in which between 19.2% and 36.2% of CD3+ cells are positive for CCR6, 4 patients had no CD3+CCR6+ cells in the synovium. This suggests that the presence or absence of CD3+CCR6+ cells could represent a subgroup of patients.

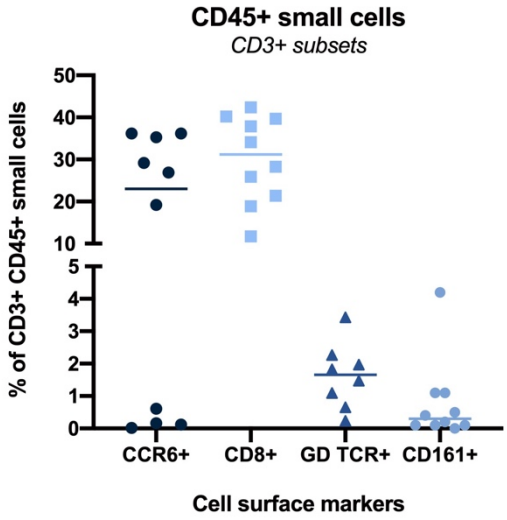


Figure 2-5. *Subsets of CCR6, CD8, CD161 or GDTCR positive as a percentage of CD3+ CD45+ small cells in end-stage OA synovium. CCR6/CD8/CD161: n=10; GD TCR: n=8.*

Table 2-11. *Overview of the percentages of CCR6, CD8, CD161, and GDTCR subsets as a percentage of CD3+ CD45+ small cells in end-stage OA synovium.*

Patient #	CCR6+	CD8+	CD161+	GDTCR+
1	0.1	25.9	4.2	1.5
2	0.6	37.9	1.1	1.1
3	29.2	34.1	0.5	2.0
4	36.2	40.2	0.4	3.4
5	0.0	18.9	0.1	N/A
6	0.2	11.7	0.0	N/A
7	35.3	39.7	1.1	1.8
8	36.2	42.4	0.1	2.3
9	19.2	21.4	0.2	0.2
10	26.9	28.3	0.1	0.7
Mean (range)	18.4 (0.02-36.2)	30.1 (11.7-42.4)	0.8 (0.0-4.2)	1.6 (0.2-3.4)

2.6.3.3 CD68+ cells are the predominant CD45+ large cell population in end-stage OA synovium

The CD45+ large cell population was studied for the expression of monocyte, macrophage, and granulocyte markers (Figure 2-6, Table 2-12). The majority (71%) of the CD45+ large cells expressed the macrophage marker CD68. After CD68, the most prominently expressed cell surface markers were CD206 (56.0%), CD14 (28.7%), and CD40 (12.2%). In addition, a small proportion of cells expressed CD11c or CD15.

It is notable that the CD14+ population is 3 times smaller than the CD68+ population despite being an established marker for monocytes and macrophages. This suggests CD14 could have been (partly) cleaved during the collagenase digestion.

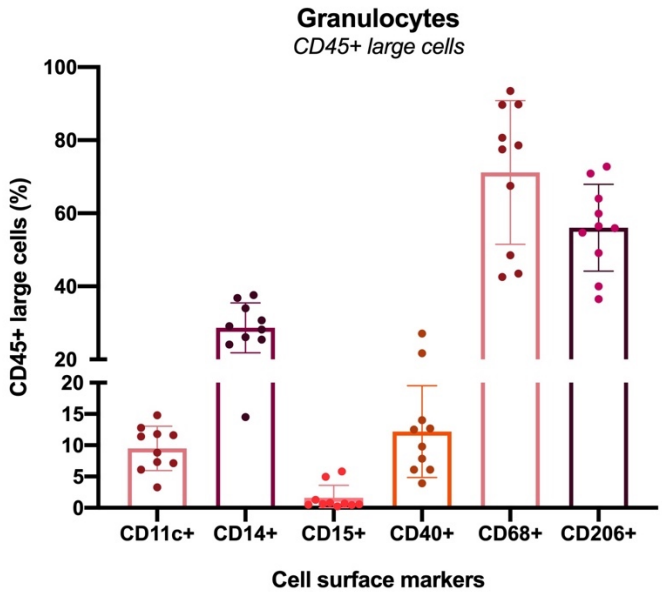


Figure 2-6. *Subsets of CD45+ large cells populations positive for CD11c, CD14, CD15, CD40, CD68, or CD206 in end-stage OA synovium. n=10*

Table 2-12. *Overview of cells in end-stage OA synovium positive for different granulocyte markers as a percentage of CD45+ large cells in end-stage OA synovium. Granulocyte and monocyte markers include CD11c, CD14, C15, CD40, CD68, and CD206, with cells positive for the antibody (AB) or isotype control (ISO).*

Patient #	CD11c+		CD14+		CD15+		CD40+		CD68+		CD206+	
	AB	ISO	AB	ISO	AB	ISO	AB	ISO	AB	ISO	AB	ISO
1	11.8	0.7	25.4	0.5	5.8	0.0	27.1	1.7	67.5	1.3	55.9	1.8
2	14.8	1.1	26.1	0.6	1.3	0.2	7.8	1.0	48.5	0.9	59.9	0.5
3	3.3	1.2	29.1	0.5	0.6	0.1	3.9	1.2	89.8	1.3	70.9	1.0
4	11.6	1.4	36.8	0.8	0.5	0.1	14.0	1.3	42.6	1.5	72.8	0.4
5	7.3	0.9	24.1	1.0	0.7	0.0	6.1	0.7	77.5	0.5	40.0	0.8
6	8.8	4.0	14.5	1.2	5.0	2.4	12.7	4.0	43.5	5.9	64.0	2.0
7	11.4	2.8	37.6	1.2	0.5	0.1	6.1	1.7	80.7	2.9	56.5	1.0
8	12.8	1.9	28.2	1.4	0.8	0.2	12.5	1.8	89.7	2.1	54.7	1.1
9	7.2	2.7	34.0	0.9	0.2	0.0	21.7	2.6	78.6	2.0	49.1	1.1
10	6.1	1.0	30.7	1.0	0.5	0.0	9.8	0.7	93.5	0.6	36.5	0.4
Mean (range)	9.5 (3.3-14.8)	1.8 (0.7-4.0)	28.7 (14.5-37.6)	0.9 (0.5-1.4)	1.6 (0.2-5.8)	0.3 (0.0-2.4)	12.2 (3.9-27.1)	1.7 (0.7-4.0)	71.2 (42.6-93.5)	1.9 (0.5-5.9)	56.0 (36.5-72.8)	1.0 (0.4-2.0)

CD40 and CD206 can further specify macrophage populations into classical M1 (CD40) or M2 (CD206) subtypes. Of all CD68+ cells, 48.5% were CD206+, 12.0% were CD40+, and 9.4% of were CD40+CD206+ (Figure 2-7, Table 2-13).

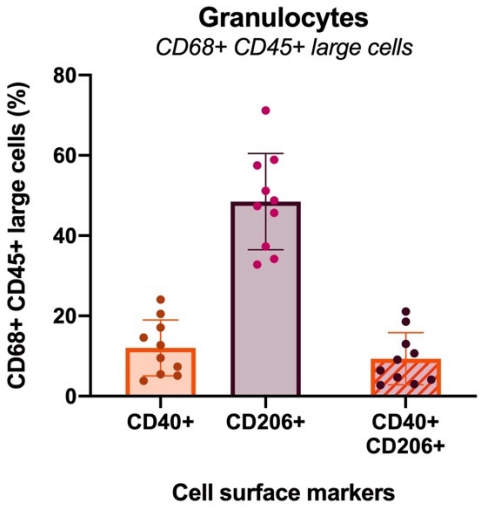


Figure 2-7. *Subsets of CD68+ CD45+ large cells detected in end-stage OA synovium. Cells were positive for CD40, CD206, or both CD40 and CD206. n=10*

Table 2-13. *Overview of the percentages of macrophages positive for CD40 (M1), CD206 (M2) or both CD40 and CD206 in end-stage OA synovium.*

Patient #	CD40+	CD206+	CD40+ CD206+
1	24.1	47.4	21.1
2	7.4	34.2	6.4
3	3.8	71.2	3.0
4	14.6	58.9	13.0
5	5.1	32.8	4.7
6	17.1	48.8	4.1
7	5.5	51.2	2.8
8	12.7	57.5	10.7
9	20.5	45.8	18.6
10	9.5	37.3	9.1
Mean (range)	12.0 (3.8-24.1)	48.5 (32.8-71.2)	9.4 (2.8-21.1)

2.6.4 Fibroblast activation in end-stage OA synovium

The CD45 negative population, mainly comprising fibroblasts, was studied for the expression of CD34 and the fibroblast activation markers CD90, FAP, and PDPN, which can all be used to distinguish fibroblast subsets. FAP and PDPN were expressed on 78% and 77% of CD45- cells, respectively (Figure 2-8). Expression of all fibroblast activation markers was notably lower in patient 6, and patient 4 had a notably small proportion of CD34+ cells (Table 2-14).

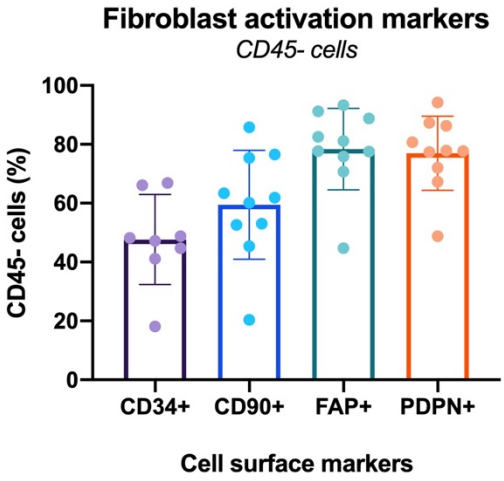


Figure 2-8. *CD34, CD90, FAP, or PDPN single positive cells as a percentage of CD45- cells of end-stage OA synovium. CD34: n=8; CD90/FAP/PDPN: n=10.*

Table 2-14. *Overview of the percentages of CD34, CD90, FAP, or PDPN single positive cells (as a percentage of the CD45- cell population) in end-stage OA synovium. Cell proportions are reported to be positive for the antibody (AB) and isotype control (ISO). CD34: n=8; CD90/FAP/PDPN: n=10.*

Patient #	CD34+		CD90+		FAP+		PDPN+	
	AB	ISO	AB	ISO	AB	ISO	AB	ISO
1	41.1	0.12	45.4	0.072	77.7	0.77	77.9	0.33
2	66.1	0.049	85.8	0.0	82.5	1.37	80.7	0.73
3	48.8	0.32	52.6	0.14	91.2	3.27	87.3	3.08
4	18.1	0.36	76.5	0.045	75.9	1.56	67.3	2.76
5	N/A	N/A	53.0	0.069	81.1	0.43	72.0	0.26
6	N/A	N/A	20.4	0.71	44.7	2.58	48.8	1.51
7	48.2	0.3	63.4	0.076	77.5	1.91	77.4	2.05
8	44.7	0.46	60.1	0.15	70.7	1.54	77.7	0.81
9	66.9	0.22	75.4	0.2	88.8	1.17	86.3	1.28
10	47.2	0.11	61.9	0.061	93.3	1.33	94.2	0.47
Mean (range)	47.6 (18.1-66.9)	0.2 (0.0-0.5)	59.5 (20.4-85.8)	0.2 (0.0-0.7)	78.3 (44.7-93.3)	1.6 (0.4-3.3)	77.0 (48.8-94.2)	1.3 (0.3-3.1)

2.6.4.1 Fibroblasts subtypes based on CD34 and CD90 expression

Mizoguchi *et al.* described different fibroblast subsets with non-overlapping effector functions in PDPN+ fibroblasts from RA and OA patients based on the expression of CD34 and CD90[149]. In the patient population in this study, 23% of CD45- cells were double negative (CD34- CD90-), 32% were CD34- CD90+, and 45% were CD34+ (Figure 2-9, Table 2-15).

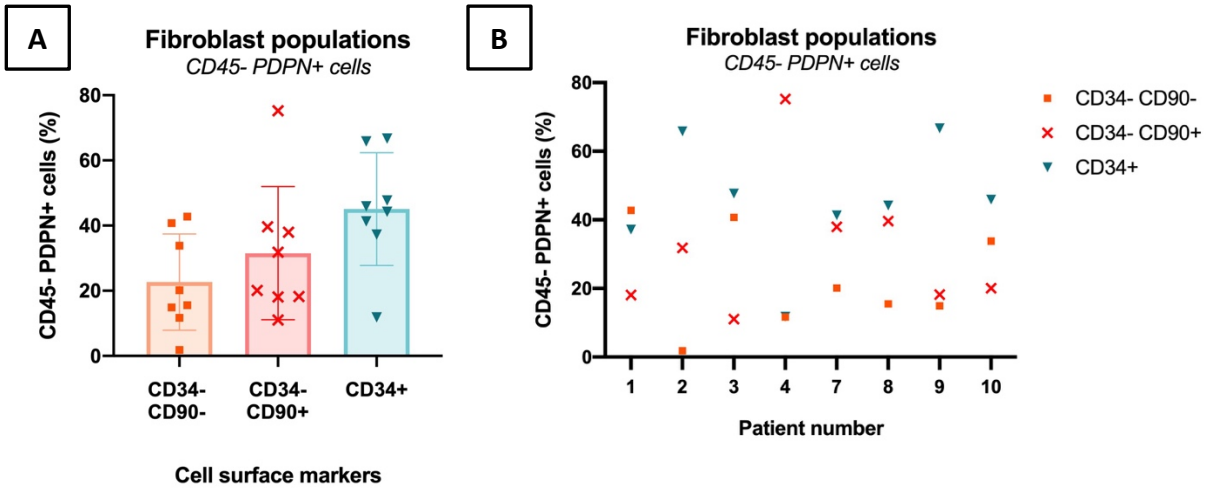


Figure 2-9. *CD34/CD90 fibroblast populations as a percentage of CD45- PDPN+ cells in end-stage OA synovium. (A) Bar graph of the CD34- CD90-, CD34- CD90+, and CD34+ populations. (B) Overview of the percentages of each CD34/CD90 population per patient. n=8*

Table 2-15. *Overview of the percentages of CD34/CD90 fibroblasts (as a percentage of the CD45-PDPN+ cell population) in end-stage OA synovium. n=8*

Patient #	CD34- CD90-	CD34- CD90+	CD34+
1	42.8	18.1	37.2
2	1.8	31.8	65.8
3	40.7	11.0	47.7
4	11.7	75.3	11.8
5	N/A	N/A	N/A
6	N/A	N/A	N/A
7	20.1	37.9	41.3
8	15.5	39.6	44.2
9	14.9	18.2	66.7
10	33.8	20.1	45.9
Mean (range)	22.7 (1.8-42.8)	31.5 (11.0-75.3)	45.1 (11.8-66.7)

2.6.4.2 Fibroblasts subtypes based on FAP and CD90 expression

Croft *et al.* identified 2 distinct fibroblasts subsets based on the expression of FAP and CD90 on PDPN+ fibroblasts[148]. On average 65% of CD45-PDPN+ fibroblasts of the end-stage OA patients in this study had the described FAP+CD90+ immune effector phenotype, while on average 29% of cells had

FAP+CD90- destructive phenotype (Figure 2-10, Table 2-16). When looking at individual patients, 8 of 10 patients had more FAP+CD90+ immune effector cells than FAP+CD90- destructive cells, one patient had an equal amount (patient 1) of both cell types, and one patient had more FAP+CD90- destructive cells than FAP+CD90+ immune effector cells (patient 6).

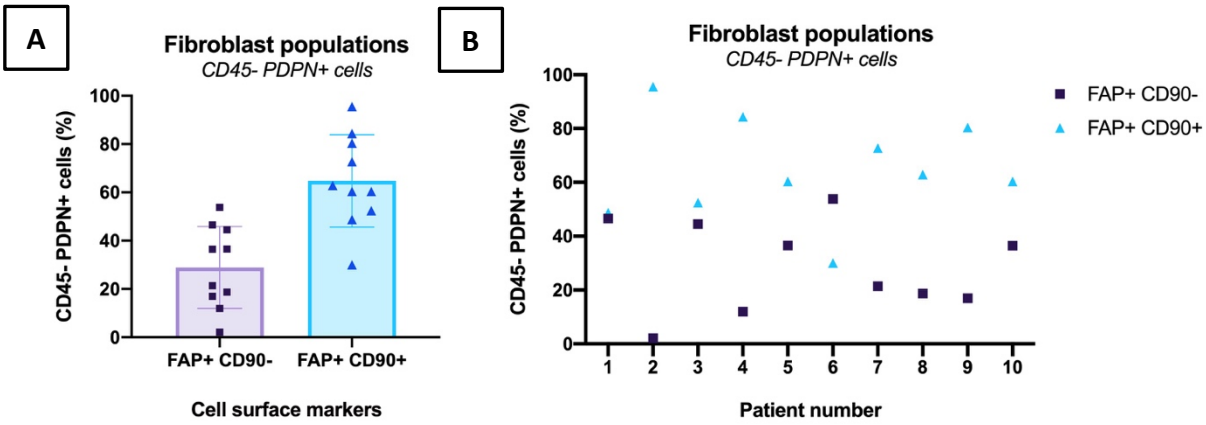


Figure 2-10. *Immune effector phenotypic fibroblasts (FAP+ CD90+) and destructive phenotypic fibroblasts (FAP+ CD90-) as a percentage of CD45-PDPN+ fibroblasts in end-stage OA synovium. (A) Bar graph of FAP+ CD90+ and FAP+ CD90- cells. (B) Overview of the percentage of FAP+ CD90+ and FAP+ CD90- cells in each patient. n=10.*

Table 2-16. *Overview of the percentages of FAP/CD90 fibroblasts (as a percentage of the CD45-PDPN+ cell population) in end-stage OA synovium. n=10*

Patient #	FAP+ CD90-	FAP+ CD90+
1	46.6	48.8
2	2.1	95.6
3	44.6	52.5
4	12.0	84.3
5	36.6	60.4
6	53.8	30.1
7	21.4	72.7
8	18.7	62.9
9	16.9	80.4
10	36.5	60.3
Mean (range)	28.9 (2.1-53.8)	64.8 (30.1-95.6)

2.6.5 Fibroblast activation markers are already expressed in synovium of patients with high risk of developing OA

To study if these fibroblast activation markers are already expressed in synovium from patients after anterior cruciate ligament or meniscus injury (who have a 50% chance of developing OA[16]), synovial tissue sections from a patient in the OxKIC cohort were stained for CD90, PDPN, and FAP antibodies (Figure 2-11). While CD90, PDPN, and FAP were found throughout the tissue, CD90 was also markedly expressed around vessels, which was not the case for PDPN and FAP (Figure 2-11B-D). In addition, the expression of CD90 and especially FAP seems to be more prominent throughout the tissue than the expression of PDPN.

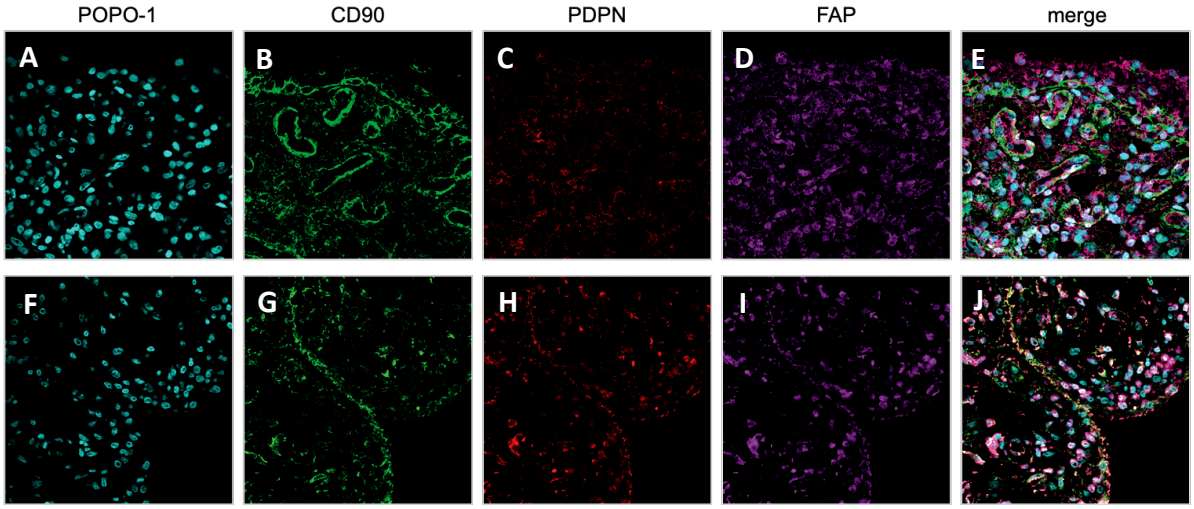


Figure 2-11. *Expression of fibroblast activation markers CD90, PDPN, and FAP in two parts of a synovial section of a patient with high risk of developing OA. Confocal images show expression of CD90 (**green**), PDPN (**red**), and FAP (**purple**), and were counterstained with POPO-1 (**blue**) to visualise nuclei. A merged image is provided to look at the co-expression of markers. Images were taken at 40x magnification with oil.*

2.6.6 Investigating the presence of IL-17 producing cells in end-stage OA synovium

No clear IL-17+ populations were detected by flow cytometry due to the unspecific staining in isotype controls, however a few patients that did appear to have CD3+IL-17+ populations. As the IL-

17A antibody that we used has been used in several previously published studies that looked at IL-17A+ T cell populations[161–163], we focussed on CD3+IL-17A+ populations. Especially after stimulation with PMA and ionomycin for 4 hours, a distinct but small CD3+IL-17A+ population can be seen in some patients (representative plots, Figure 2-12A-F), while no convincing populations were found in other patients (representative plot, Figure 2-12G-I).

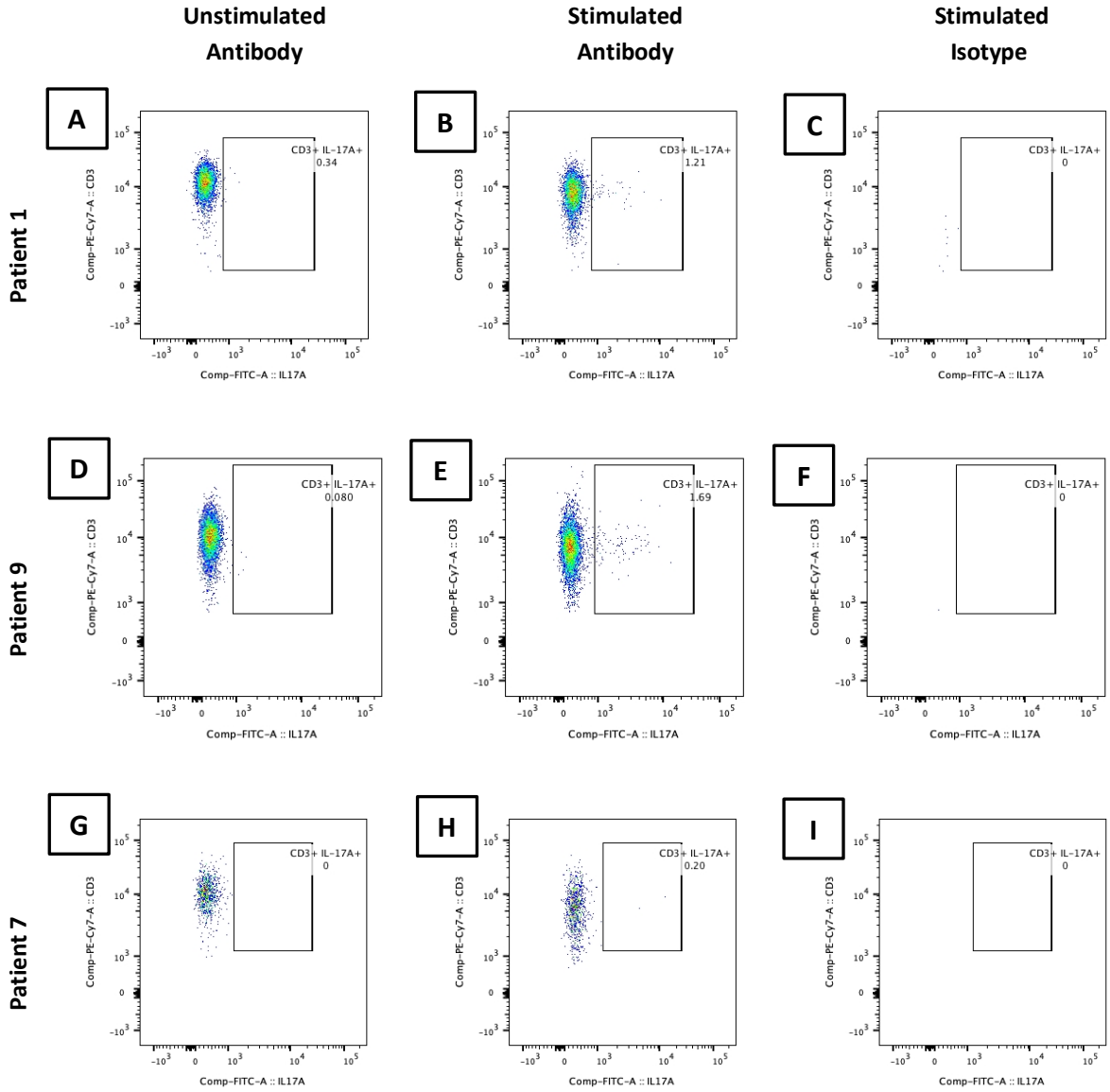


Figure 2-12. *CD45+ small CD3+IL-17+ populations in end-stage OA synovium. Cells were either unstimulated and stained with IL-17A antibody (A, D, G), stimulated with PMA/Ionomycin for 4 hours and stained with IL-17A antibody (B, E, H), or stimulated with PMA/ionomycin for 4 hours and stained with isotype controls (C, F, I).*

2.6.7 Correlation of cell subsets with the other cell subsets or clinical scores

After the collection of the synovial tissue, retrospective clinical information was gathered to see whether any of the differences in cell populations could be explained by differences in body mass index (BMI), Kellgren Lawrence (KL)-grade, Oxford Knee Score (OKS), or other patient characteristics previously gathered (Table 2-17).

Table 2-17. *Patient characteristics obtained during and after surgery. In addition to gender, age, and affected knee previously reported, the body-mass index (BMI) before surgery, the Kellgren-Lawrence (KL)-grade, and Oxford Knee Score (OKS) was collected.* ***Green*** *= OKS obtained retrospectively up to a year after surgery.*

Patient #	Gender	Age	Affected knee	BMI	KL-grade	OKS
1	Female	64	Right	34.8	2.67	17
2	Female	58	Left	33.9	2	9
3	Female	80	Left	34.0	2.33	25
4	Male	62	Right	40.8	4	*not available*
5	Female	72	Left	40.2	2.33	20
6	Female	61	Right	24.5	1.33	16
7	Female	71	Right	28.5	3.33	*not available*
8	Male	75	Right	37.9	3.33	31
9	Female	68	Right	28.5	4	26
10	Female	77	Left	33.9	2	21
Mean (range)	20% male / 80% female	68.8 (58-80)	40% left / 60% right	33.7 (24.5-40.8)	2.73 (1.33-4)	21 (9-31)

2.6.7.1 Macrophage or T cells as predominant cell subset

It was previously identified in 3.6.4 that in 4 out of 10 patients there were significantly more macrophages than T cells, while the other 6 patients had similar proportion of both cells or more T cells than macrophages. Therefore, the patient cohort was split into two groups depending on the predominant immune cell population either being macrophages (red) or T cells (blue). Four categories were studied: CD45 subsets, immune cells, fibroblasts, and clinical markers (Figure 2-13).

In CD45 subsets, patients with a predominant macrophage immune cell population seem to have a higher percentage of CD45+ large cells in their synovium than the T-cell dominant subset, although this was not significant. Patients with macrophages as the predominant immune cell population had a significantly smaller proportion of CD3+ T cells (p=<0.0001), a higher proportion of CD68+ macrophages (p=<0.0001), and a higher proportion of CD68+CD206+ macrophages (p=0.0001) than patients with T cells are the predominant immune cell populations. However, no differences were seen in CD68+CD40+ macrophages, the proportions of fibroblasts, or the clinical scores.

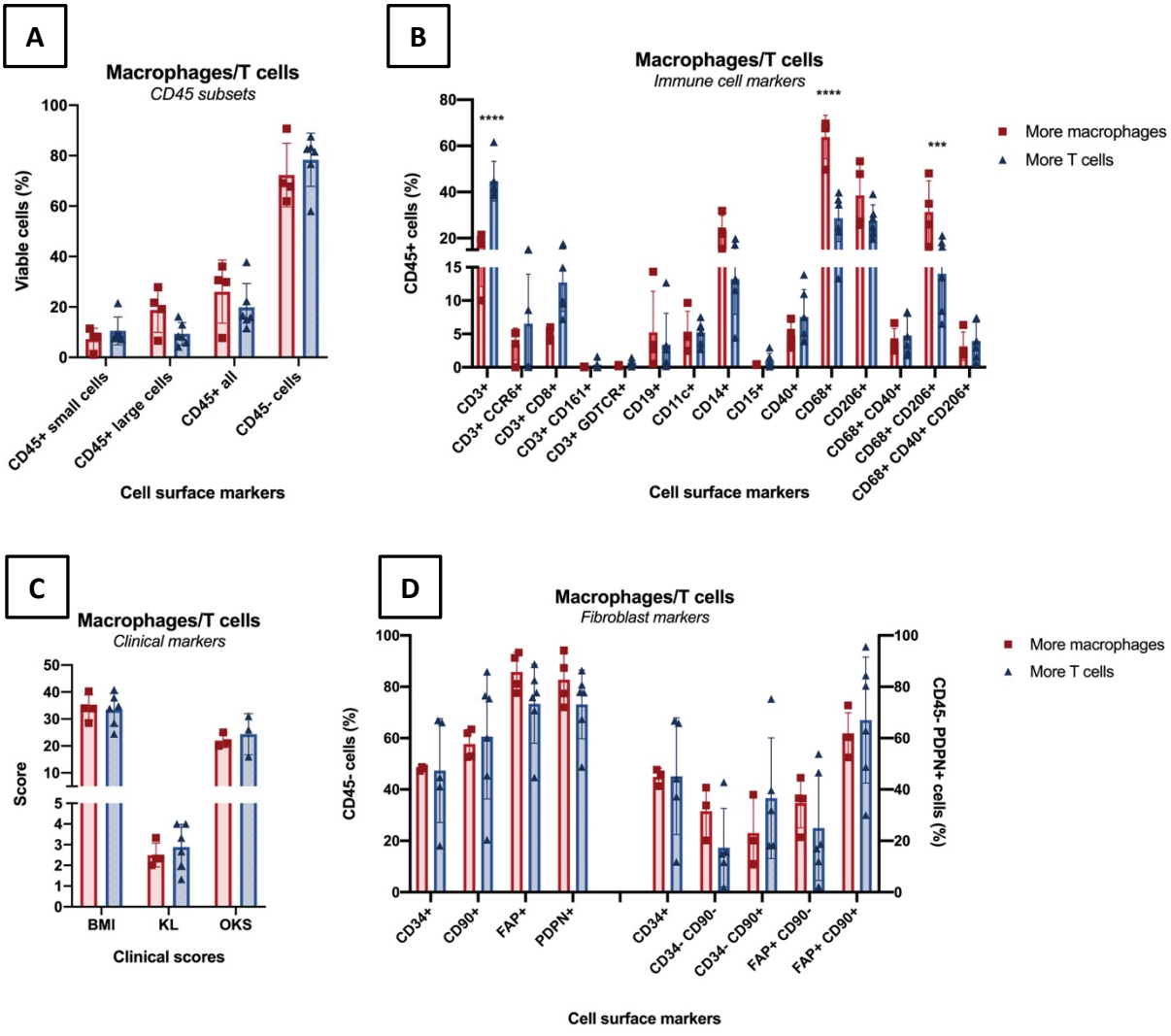


Figure 2-13. *Comparison between end-stage OA patients with either macrophages or T cells as the predominant immune cell population in synovium. (A) CD45 subsets as percentage of viable cells, (B) immune cells as percentage of CD45+ cells, (C) clinical scores, and (D) fibroblast activation markers as percentage of CD45- cells (left) or CD45-PDPN+ cells (right).* ***Squares*** *= more macrophages (n=4),* ***triangles*** *= more T cells (n=6). Two-way ANOVAs. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.*

Upon further investigation it was found that macrophages showed a significant positive correlation with age (r = 0.840, p=0.0024), while T cells were negatively correlated with age (r = -0.674, p=0.0327) (Figure 2-14).

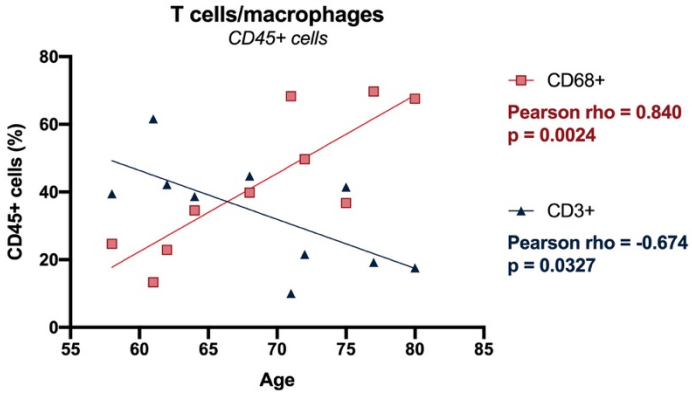


***Figure 2-14**. The proportions of macrophages (CD68) and T cells (CD3) cells in end-stage OA synovium as a percentage of CD45+ cells. Correlation of the proportion of CD68+ and CD3+ cells with age of patient (Pearson's correlation). n=10*

2.6.7.2 Presence or absence of CCR6+ T cells in end-stage OA synovium

When the different T cell subsets were studied in 3.6.4.2, CCR6+ T cells were only found in 6 patients, while these cells were completely absent in the other 4 patients. This suggests that the presence of these cells could present a subtype of patients. Therefore, the patient cohort was split into two groups: patients with absence of CCR6+ T cells (CCR6-, orange), and patients with the presence of CCR6+ T cells (CCR6+, blue) (Figure 2-15).

No differences were found in the proportions of CD45 subsets. A significantly higher proportion of CD68+ cells were present in the CCR6+ patients (p=0.0025). Although no significant differences were found, CCR6+ patients tended to have more CD90+, FAP+, and PDPN+ CD45- cells. In addition, CCR6+ patients might have more FAP+CD90+ fibroblasts and less FAP+CD90- fibroblast than patients

without CCR6+ T cells. For clinical markers, no differences were seen in age (Appendix A7) or BMI, but CCR6+ patients seem to have a higher KL grade (not significant) and a significantly higher OKS than CCR6- patients (p=0.0056).

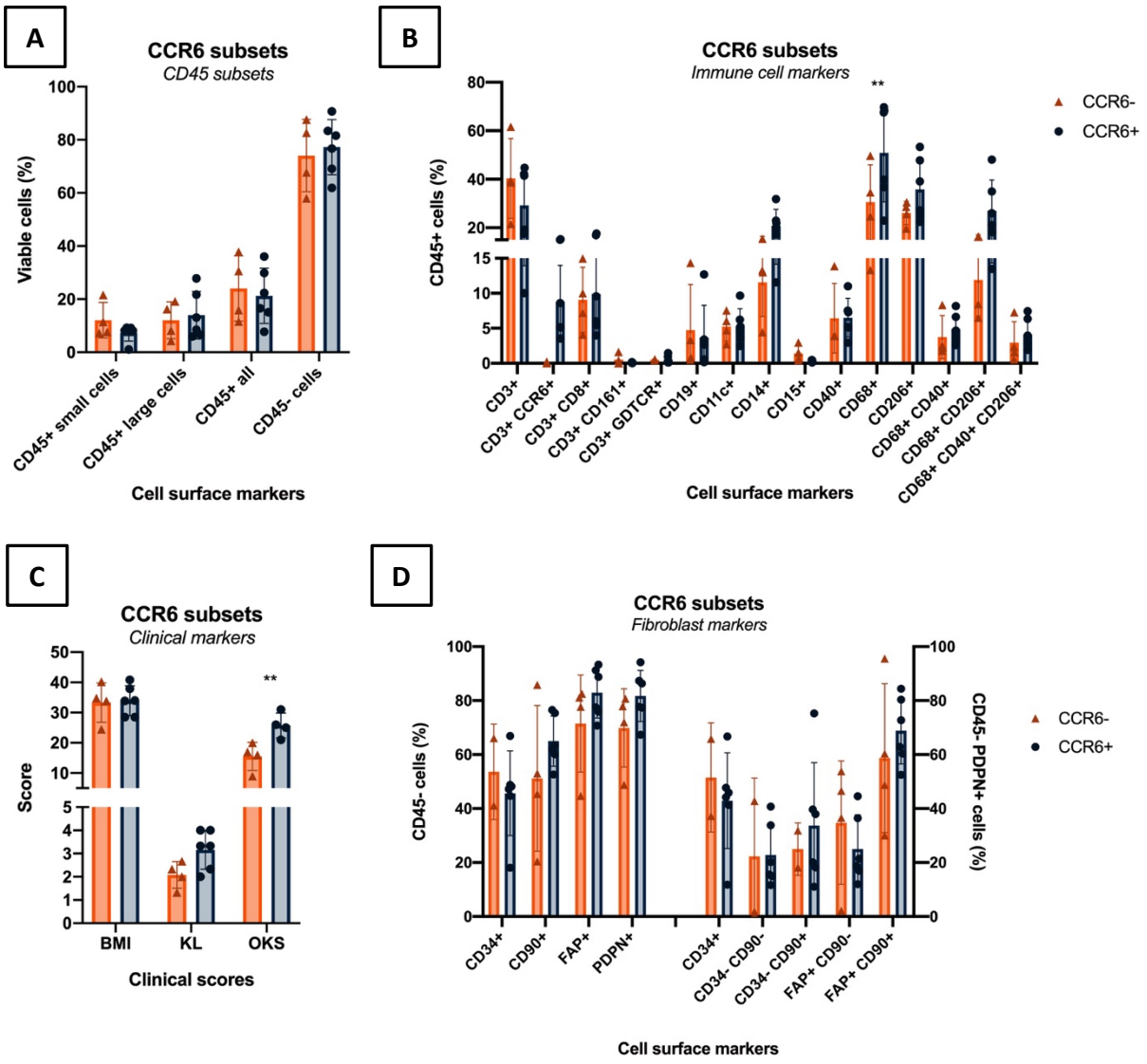


Figure 2-15. *Differences between patients with the absence or presence of CCR6+ T cell population. Differences between the two groups in (A) CD45 subsets as percentage of viable cells, (B) immune cells as percentage of CD45+ cells, (C) clinical scores, and (D) fibroblast activation markers as percentage of CD45- cells (left) or CD45-PDPN+ cells (right). Data points shown as* ***triangles*** *= absence of CCR6+ T cells (n=4),* ***circles*** *= presence of CCR6+ T cells (n=6). Two-way ANOVA. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.*

A trend towards a correlation was found between CD3+CCR6+ cells and KL-grade (r=0.582, p=0.082) and a significant correlation was found with OKS (r=0.738, p=0.046) (Figure 2-16).

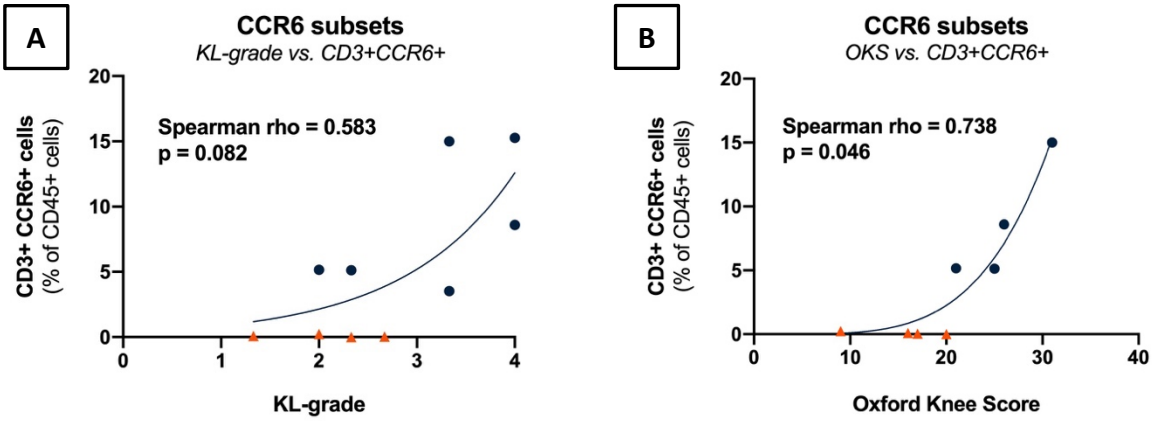


Figure 2-16. *Correlation between CD3+CCR6+ cells (% of CD45+ cells) with clinical markers (A) KL-grade and (B) Oxford Knee Score. Data points shown as* ***triangles*** *= absence of CCR6+ T cells,* ***circles*** *= presence of CCR6+ T cells. KL-grade: n=10, Oxford Knee Score: n=6.*

2.6.7.3 Correlation of fibroblast subsets with proportion of immune cells and KL-grade

In addition to the different subtypes of fibroblasts based on the expression of CD34 and CD90, Mizoguchi *et al.* showed that the proportion of CD34-CD90+ cells found in RA synovium positively correlated with number of infiltrating leukocytes (CD45+ cells). Therefore, this study also investigated possible associations between the different CD34/CD90 and FAP/CD90 fibroblast subsets and the proportion of CD45+ cells (Figure 2-17).

Significant positive correlation (r=0.83, p=0.011) was seen between the proportion of CD45+ viable cells and CD34-CD90- fibroblasts. A trend towards a negative correlation (r=-0.73, p=0.028) was found between the proportion of CD45+ viable cells and CD34-CD90+ fibroblasts. No correlation was found between the proportion of CD45+ viable cells and CD34+ fibroblasts. In addition, no

correlation was found between FAP+CD90- fibroblasts (r=0.49, p=0.151) or FAP+CD90+ fibroblasts (r=-0.289, p=0.419) and CD45+ viable cells.

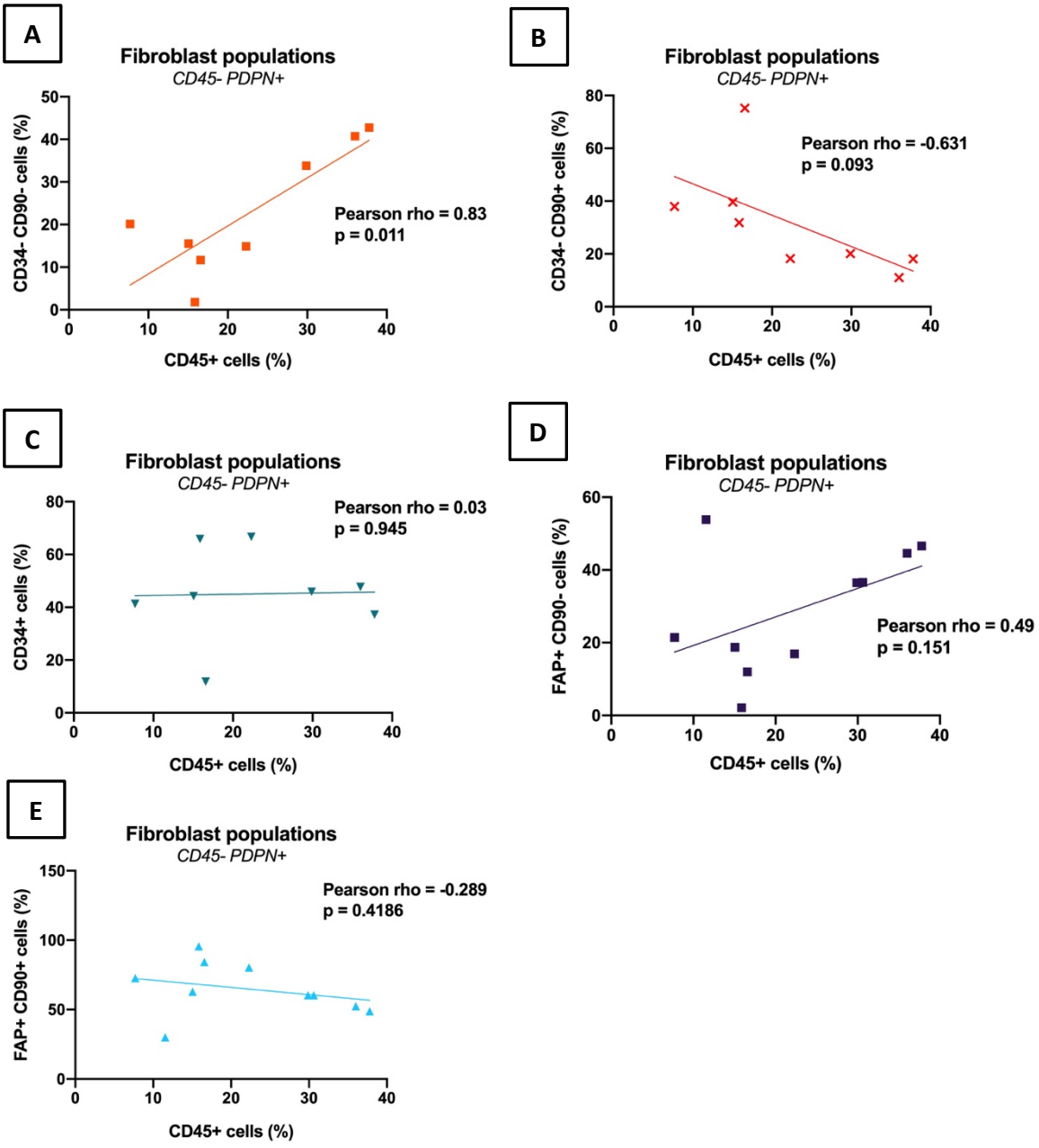


Figure 2-17. *Correlation between CD45+ cells (as a percentage of all viable cells) and fibroblast populations (as a percentage of CD45- PDPN+ cells) in end-stage OA synovium. (A) CD34- CD90- cells, (B) CD34- CD90+ cells, (C) CD34+ cells, (D) FAP+CD90- cells, and (E) FAP+CD90+ cells (A, C) Correlation test using the Pearson correlation efficient; (B) Correlation test using nonparametric Spearman correlation. (A-C) n=8, (D,E) n=10.*

There appears to be a negative association between KL-grade (cartilage damage score) and the cartilage destructive FAP+CD90- fibroblast subset, although this is not significant (r=-0.472, p=0.170). No correlation was found between FAP+CD90- fibroblasts and OKS (r=-0.238, p=0.582) (Figure 2-18). No associations were found between the other fibroblast subsets and KL-grade or OKS (Appendix A7).

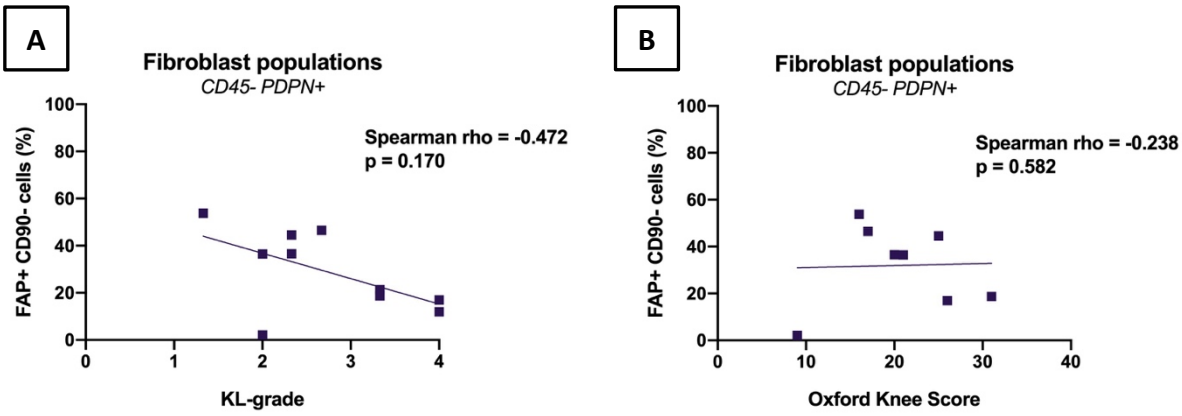


Figure 2-18. *Correlation between and FAP+CD90 fibroblast population and clinical markers of disease in synovium from patients with end-stage knee OA (as a percentage of CD45-PDPN+ viable cells). (A) Kellgren-Lawrence (KL)-grade, and (B) Oxford Knee Score. (A,B) Non-parametric Spearman correlation. (A) n=10, (B) n=8.*

2.6.8 Proteomic analysis of synovial fluid from patients with end-stage OA

From the 10 end-stage OA patients that were included for the investigation of cellular subsets in synovium, matched synovial fluid was collected from 8 out of 10 patients for proteomic analysis. A list of log2 transformed protein quantity values was obtained for each sample, which was used for further analysis.

Principle component analysis (PCA) showed that the PC1/PC2 split the samples in two groups along PC2: patients 7, 5, and 10 and patients 9, 3 ,8, 2, and 4. However, the clearest division between

patient samples can be seen in PC3/PC4 in which patient 2 is clearly separated along the PC3 axis from the other 7 samples (Figure 2-19). In addition to PCA plots, a heatmap was constructed with the protein quantities for each patient (Figure 2-20). A full list of intensity of detected proteins for each sample can be found in Appendix A8.

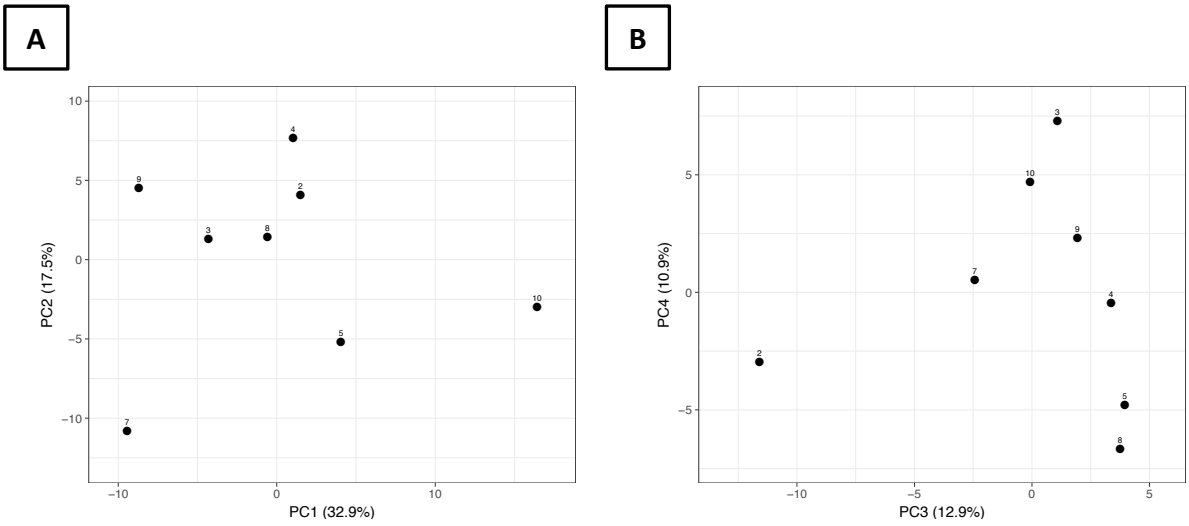


Figure 2-19. *Principle component analysis (PCA) plots for proteomic results of synovial fluid samples. Singular value decomposition with imputation was used to calculate the principle components (PCs). (A) PCA plot of PC1 and PC2. (B) PCA plot of PC3 and PC4. n=8*

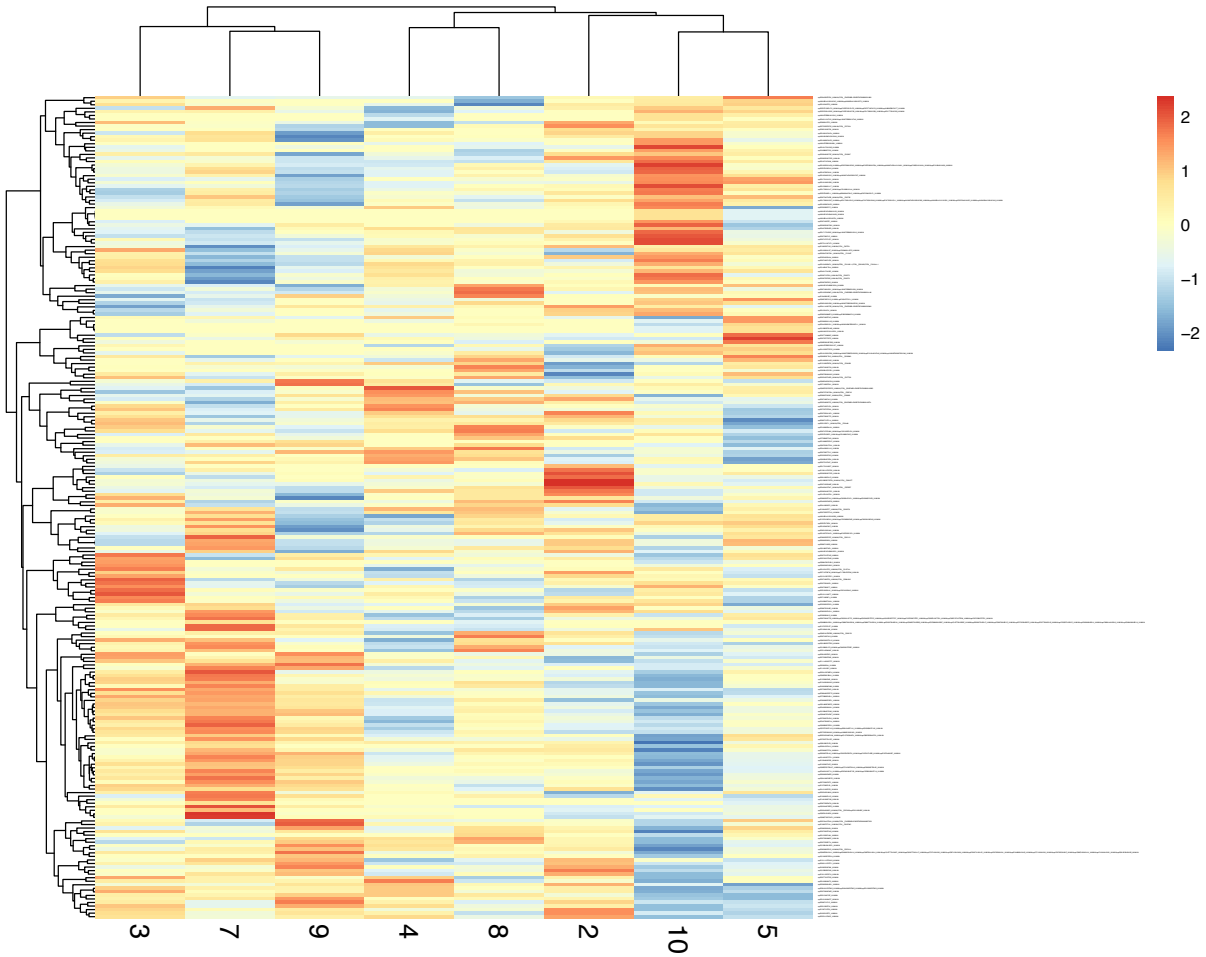


Figure 2-20. *Heatmap of differences in protein expression in synovial fluid between 8 patients with end-stage OA. Rows were centred and unit variance scaling was applied. Both rows and columns are clustered using the correlation distance and average linkage.*

2.7 Discussion and conclusions

2.7.1 Aims of this chapter

Over the last few decades, our understanding of OA has progressed from it being a cartilage-centric disease to a whole joint disease. Synovial inflammation (synovitis) is increasingly recognised as an important part of OA pathophysiology, but the immune cells and fibroblast subtypes involved are still unclear. Therefore, this chapter set out to explore the cellular and molecular environment of end-stage OA synovium in order to get a better understanding of the differences in cellular composition between patients and the potential relationship between cell subsets, as well as cell subsets and clinical scores.

2.7.2 The proportion of immune cells and the subsets of immune cells in synovium varies largely between patients with end-stage OA

Previous studies have focussed on specific cell subsets or only used a few general markers to look at cells in end-stage OA synovium. This more in-depth combination of panels enabled the relationship between different immune cells as well as between immune and fibroblast subsets to be studied. The cells in synovium from 10 OA patients were studied and on average 22.3% of all cells in OA synovium were CD45+, indicating immune cells. However, between the 10 patients, there were large differences between the total number of CD45+ cells, but also in the number of small CD45+ cells and large CD45+ cells. Overall, CD45+ cells were mainly T cells and macrophages, but presence of B cells, neutrophils, and dendritic cells were also identified.

It has been previously reported that the infiltrating immune cells in OA synovium are made up of 65% macrophages and 22% T cells[96]. In this study, the infiltrating immune cells were mostly comprised of macrophages and T cells, but different proportions were found: 43% macrophages and 34% T cells. These differences might be due to the use of different techniques and potential different

patient populations: the study by Pessler *et al.* used the synovial biopsies of OA patients (stage of disease not specified) to study immune cells by immunohistochemistry, while this study used flow cytometry with end-stage OA synovium. In addition, it is unclear how the total number of mononuclear inflammatory cell population was determined by Pessler *et al.*, which might have led to differences.

2.7.2.1 Dominant immune cell types: macrophages and T cells

While on average the proportion of macrophages was higher than T cells in synovium of the 10 patients studied, only 4 patients had a significantly higher number of macrophages than T cells in their synovium. This might be due to an active innate immune response in the joint in response to inflammation, leading to the secretion of monocyte/macrophage attracting CCL-chemokines. The other 6 patients either had similar numbers of macrophages and T cells, or more T cells than macrophages.

When looking at the association between these 2 cell types and age, it was found that the proportion of macrophages positively correlated with age, while the proportion of T cells negatively correlated with age. Although disease generally gets worse with age, age did not show a correlation with KL-grade (Appendix A5). Therefore, it needs to be investigated whether these differences are indeed associated with age, or if having either macrophages or T cells as the predominant immune cell type points to a different subtype of disease.

2.7.2.2 Presence of CCR6+ T cells cell could represent a subset of patients

T cells were further specified with cell surface markers that help specify subset of T cells. A large number of T cells (30.1%) were cytotoxic (CD8+) T cells. Although CD8+ T cells have repeatedly been found in OA synovium, little research has investigated the role of these cells in OA. One study found that knocking out CD8+ T cells in a mouse model of OA slowed cartilage degeneration and that the

expression of TIMP-1-expressing CD8+ T cells correlated with disease severity, suggesting a role in disease[164]. Studies into CD8+ T cells in RA have suggested that these cells may be involved in modulating inflammation[165,166], and articular destruction[166]. In our study CD8+ T cells (as % of CD45+ cells) did not correlate with the proportion of immune cells (CD45+ cells as % of viable cells), KL-grade, or OKS (Appendix A5).

γδ-T cells are a recently discovered subgroup of T cells that express gamma/delta T-cell receptor rather than the alpha/beta T cell receptor that is present on CD4+ and CD8+ T cells. These cells recognise a broad range of antigens, which they can attack through the production cytokines (such as IFN-γ, TNF-α, and IL-17) and chemokines (including CCL5, CXCL10, and lymphotactin), or indirectly through the activation of other immune cells. CD161, which was originally found on NK cells, has been found to be expressed on CD4+, CD8+, GDTCR, and NK-T cells. As the IL-17-secreting CD4+ cells in peripheral blood all express CD161, CD161 is considered as a hallmark for Th17 cells[167]. Several papers have shown changes of these T cell subsets in (chronic) inflammatory diseases, which indicates that this might have a role in OA[168–170]. This chapter identifies both GD TCR+ and CD161+ T cell subsets in the majority of OA patients. Future work should investigate what the role of these cells is OA pathophysiology and whether these affect the onset and/or progression of OA.

Finally, the expression of CCR6 was studied in the T cell population of end-stage OA synovium. CCR6+ T cells were found in 6 out of 10 patients, in which CCR6 was present on ±30% of T cells. Surprisingly, these CCR6+ T cells were completely absent from the synovium of the other 4 patients analysed, suggesting a potential subset of patients. Upon further analysis, it was found that there was not only was there a significant difference in CD68+ macrophages between patients with presence or absence of CCR6+ T cells, patients with CCR6+ T cells tended to have higher KL-grade (higher degree of joint damage) but a significantly higher OKS (less functional impairment). Therefore, these cells might present a subset of patients with faster structural progression but limited impact on pain/function.

CCR6 is a surface receptor for CCL20 and is present on IL-17-producing Th17 T cells and Tregs[171]. Results from clinical, genetic, and animal studies have increasingly implicated the IL-17 cytokine family in the pathophysiology of OA (discussed in more detail in Chapter 4). Increased expression of CCR6 has been found in OA cartilage compared to healthy donor cartilage[172]. Interestingly, mRNA levels of the IL-17 inducible genes *IL6*, *PTGS2* and *NOS2* were elevated in OA chondrocyte cultures treated with recombinant human CCL20[172,173]. To our knowledge, this is the first study to identify CCR6+ T cells in OA synovium. However, the subtype of these CCR6+ T cells was not further specified. It is critical that future studies include markers for these subsets as Th17 and Tregs have opposing functions[174]. In addition, synovial fluid of these patients should be screened for Th17 cytokines. Subsequently, functional studies should be performed to clarify how this cell type and its secreted proteins, especially IL-17A, could affect OA pathophysiology.

In conclusion, this study confirms that T cells are one of the two main immune cell subsets in OA synovium. A third of T cells were identified as cytotoxic T cells, while only small proportions of γδ-T cells and CD161+ T cells were found. This is the first study to identify CCR6+ T cells in end-stage OA synovium. As this cell type was only found in a subset of patients, this might identify a subset of disease with an important role for CCR6+ T cells and its produced cytokine IL-17.

2.7.2.3 IL-17A-producing cells might be present in a subset of end-stage OA patients

The overall aim of this thesis is to get a better understanding of the role of IL-17 in OA. As IL-17 has been increasingly associated with OA but the source of this cytokine in OA is unclear, one of the objectives of this chapter was to identify the IL-17-producing cells in OA synovium. Although markers such as CD161 and CCR6 are often associated with IL-17-producing cells, this study specifically looked at the expression of IL-17A itself to identify IL-17A-producing cells. IL-17A-producing T cells were identified in subset of patients of the cohort used in this study. Due to unspecific staining and low

expression, IL-17F and IL-17AF were not used in the final analysis and IL-17A expression was only analysed in T cells. The fact that IL-17A-producing cells, as identified by presence of IL-17A, were only identified in a subset of patients is unsurprising as previous studies have also only been able to identify IL-17A in a small proportion of patients[175].

However, during this process, multiple problems were encountered. Firstly, many aspects of IL-17-biology are still unclear, including which specific cell subset can produce IL-17, and how these cells can be stimulated to do so. Therefore, general stimulants (PMA/Ionomycin for T cells, LPS for monocytes/granulocytes, and IL-1β for fibroblasts) were used in this study in an attempt to stimulate these cells to produce IL-17. However, it is unclear if these stimuli are able to induce the production of IL-17 in these cells. Therefore, future studies might need to use a cocktail of different cytokines, chemokines, and other stimulants that could induce IL-17-production. Second, there was a significant unspecific binding of IL-17 antibodies seen in isotype controls, which increased the difficulty of identifying this small cell population. Therefore, future studies should include additional controls such as fluorescence-minus one (FMO) samples or positive controls during each flow cytometry run, in order to be able to conclude the presence or absence of IL-17+ cells with more confidence.

2.7.2.4 CD206+ M2 macrophages are predominant macrophage subset the in end-stage OA synovium

Macrophages are one of the two predominant immune cell types, but there was a large variability in the proportion of these cells between patients. This study found that on average 42.7% of all immune cells (CD45+ cells) were positive for CD68 (macrophages).

As discussed before, although this nomenclature is outdated, the macrophages can crudely be divided into M1 and M2 macrophages. Both M1 and M2 macrophages have been found in OA

synovium, but studies are contradictory regarding the relative proportions of each subtype. In addition, several studies report macrophages that express both M1 and M2-type markers, which has been hypothesized as macrophages that are "trapped in transition". Studies have shown that macrophages can both have beneficial effect but also harmful functions in OA. However, it is unclear if these effects are seen due to the presence of different subsets of macrophages or if other factors affect these functions. This study has identified macrophages as one of the two predominant immune cell types in end-stage OA synovium, with half of macrophages expressing M2-marker CD206 and only a small proportion expressing M1-marker CD40. Surprisingly almost all macrophages positive for CD40 also expressed CD206, thus showing further support for M1/M2 macrophages that could be trapped in transition in OA.

A significant positive correlation was found between CD68+ cells and age. More specifically the CD68+CD206+ subset showed a statistically significant correlation with and age as well as with OKS (Appendix A5). It has been reported that monocytes and macrophage have an important role of chronic inflammation in age-related diseases, with a culmination of monocytes/macrophage in the development of these diseases[176]. Therefore, future research should confirm whether changes in macrophage populations are indeed associated with age in OA synovium.

2.7.3 ***Fibroblasts in end-stage OA synovium have an activated phenotype***

This study confirmed the expression of fibroblast activation markers CD34, CD90, FAP, and PDPN on CD45- cells in end-stage OA synovium. This is the first study in which fibroblast activation markers were studied together with patient characteristics and immune cells populations. FAP and PDPN were broadly expressed, with these cells being present on 78% and 77% of CD45- cells, respectively. Several recent papers have identified specific subsets of synovial fibroblasts with non-overlapping effector functions using these fibroblast activation markers. The fibroblast subtypes that were identified in the papers by Mizoguchi *et al.* (2018) and Croft *et al.* (2019) were used to investigate if

these populations could be found in OA patients and if the proportions of these cells found corresponded to the proportions reported in these studies.

2.7.3.1 CD34+ fibroblasts are the dominant fibroblasts subset based on CD34/CD90 expression in end-stage OA synovium

Mizoguchi *et al.* used single-cell RNA sequencing and immunohistochemistry to identify three subsets of PDPN+ RA and OA fibroblasts based on the expression of CD34 and CD90. Although the same markers were used, different proportions of these cells were found in our patient population compared to their population of 26 end-stage OA patients: in our study almost half of fibroblasts were CD34+, after which CD34-CD90+ were most common, closely followed by CD34-CD90- fibroblasts. However, Mizoguichi *et al.* found that in their OA cohort CD34-CD90- cells were most common, followed by CD34+, and only found few CD34-CD90+ cells. Interestingly, the relative proportions of fibroblast subsets in the end-stage OA patients in this study was in accordance to the RA cohort in Mizoguichi *et al.* However, while CD34-CD90+ fibroblasts from RA patients showed a significant positive correlation with the CD45+ population (data was not published for correlations with OA data), in this study CD34-CD90+ fibroblasts showed a significant negative correlation with the CD45+ population. This might suggest that the more of these CD34-CD90+ fibroblasts are present, which were shown to have an enrichment in migratory, invasive, and proliferative genes, the fewer immune cells are present in end-stage OA synovium.

It is important to note that there are fundamental differences in these studies. Firstly, there is a difference in the markers used to select the fibroblasts used in the analysis. While this study defined fibroblasts as CD45-PDPN+ cells which will likely contain cells other than fibroblasts such as endothelial cells, the study by Mizoguchi *et al.* selected their fibroblasts by excluding cells that were positive for CD45, CD235a (glycophorin A (present on red blood cells)), CD31 (present on endothelial cells), and MCAM/CD146 (present on pericytes). Therefore, differences seen between these studies

could be due to the inclusion of cells positive for CD235a, CD31, and CD146 in this chapter. In order to make future comparisons, future studies should include these markers to be able to select a fibroblast population without endothelial cells, red blood cells, and pericytes. Another difference is that the current study used collagenase type 2 for 2 hours to digest the synovium, while Mizoguchi *et al.* used either collagenase type 4 with Dispase II and DNase I for 4 successive periods of 15 minutes or a combination of Liberase and DNase I to digest the synovial tissue obtained during total knee replacement (the precise method used could not be determined from the methods section of Mizoguchi *et al*.), which could have released cells differently from the tissue.

2.7.3.2 Immune effector (FAP+CD90+) fibroblasts are the dominant fibroblast subset in end-stage OA synovium

Croft *et al.* (2019) identified 2 main fibroblast populations in RA patients using CyToF based on the expression of FAP and CD90: FAP+CD90- fibroblasts which have a tissue destructive phenotype, and FAP+CD90+ fibroblasts which have an immune effector phenotype. Although no specific percentages are stated, from the graphs an estimated that there were about 4 times more FAP+CD90- fibroblasts in OA than FAP+CD90+, while in RA there were twice as many FAP+CD90+ than FAP+CD90-. In this study with synovium from 10 OA patients, just over twice as many fibroblasts were FAP+CD90+ than FAP+CD90, which again is in accordance to their RA subset rather than OA subset.

When looking more specifically at the two fibroblast populations in each patient used in this study, it becomes clear that most patients have a higher proportion of FAP+CD90+ immune effector cells than FAP+CD90- destructive cells. One patient had an equal amount of both cell types, and one patient had more FAP+CD90- destructive cells than FAP+CD90+ immune effector cells. Future studies should investigate whether the relative proportions of these two subsets of fibroblasts are an indicator of a subtype of disease.

Croft *et al.* used CD45-PDPN+ cells from end-stage OA patients to look at the expression of FAP and CD90, and did not exclude cells based on other markers as done in Mizoguchi *et al*. Differences in results between this chapter and the study by Croft *et al.* may be due to differences in tissue digestion of synovium, which in Croft *et al.* was digested in Liberase and DNase I and then frozen before being stained and analysed with CyToF. As only few CD45-PDPN+ cells were found to be FAP- in this study compared to over half of the fibroblast population in the paper by Croft *et al*., the freeze/thaw cycle, the digestion method, and the machine used for analysis (CyToF vs. flow cytometry) might have all influenced the different proportion of fibroblast subsets seen between these two studies, especially the potential cleavage or loss of FAP on the cell surface.

2.7.4 Proteomic analysis did not identify OA patient subsets

The protein fingerprint of synovial fluid was studied to investigate if there were differences between these patients, and if any subsets of patients could be identified through this analysis. There were many immunoglobulin-related proteins, matrix- and complement proteins present. The biggest difference between two subsets of patients was found to be the expression hemoglobulin subunit alpha and beta. However, the sample size was too small to draw any conclusions about the many small differences between patients in the large set of proteins.

This analysis method was unable to identify chemokines or cytokines as these were under the limit of detection. As differences in these proteins might give more insight in the differences in the proportions of immune cells present in synovium of different end-stage OA patients, a different processing or analysis method might need to be used in the future to be able to determine the presence and concentration of cytokines and chemokines, such as enzyme-linked immunosorbent assays (ELISAs), meso scale discovery (MSD) platform, or multiplex assays. Combining proteomic data with matched data from another ‘omics’-platform such as metabolomics could also increase the amount of information that can be extracted from this.

2.7.5 Limitations

This exploratory study shows that immune cells are present in the synovium of end-stage OA patients, with a potential for distinct subsets based on dominant immune cells type or presence/absence of specific subsets. Fibroblasts is this tissue express a range of activation markers, which correspond to previously published subsets identified in RA. However, given the exploratory nature of this study, a number of limitations need to be considered to improve study design for future work, including the challenges of working with human samples, digestion method, positive controls, unspecific binding of antibodies, and the use of additional markers to identify specific monocyte/granulocyte and fibroblast subsets.

2.7.5.1 Logistical challenges of obtaining human samples

The use of human OA tissue is a major strength of this study, but it also results in some (logistical) challenges. First, the synovial samples were taken by different surgeons which could have created a bias in which part of the synovium was taken. From previous literature it is known that especially certain subsets of fibroblasts are only found in either the lining or sub-lining of the synovium[141,148,149,154]. Therefore, differences in the distribution of lining/sub-lining of each piece of tissue might have introduced bias in the relative proportions of these populations that were identified. In addition, the size of the tissue that was removed during the total knee replacement surgery significantly differed between patients. The smaller the sample, the lower the number of cells that can be obtained. Not only does this make it the harder to identify small cell populations, this also makes the identified percentage of cell subsets more variable as the false positive cells can have a large effect on the identified cell populations when there is only a small number to start with.

Second, the tissue was acquired during time of surgery which ranged between 9AM and 6PM. The full protocol from digestion of tissue to fixation of cells was approximately 11 hours, which was not

possible to do on days that tissue only arrived late in the afternoon or early evening. Therefore, the tissue was washed to remove blood, fat was cut off, and the remaining tissue was stored in media in an incubator overnight until the full protocol could be performed the following day. Although the tissue was held overnight in an attempt to handle the tissue in a similar way for each patient, the cell population in the tissue could have slightly changed overnight as several immune cells might have died during this time, influencing the identification of cell subsets as well as the relative proportions of these cells.

2.7.5.2 Collagenase digestion

The digestion of tissue is a critical step in the identification process of cells from a tissue as surface proteins get easily cleaved in the process. In this study, a recommended method used previously by the group. However, during digestion different cell surface markers were cleaved, making it impossible to use these for analysis. Therefore, different digestion enzymes and different durations of digestion should be tested to get the optimal cell yield with minimal cleavage of cell surface proteins.

2.7.5.3 Lack of positive control

During the optimisation of the antibody panels, peripheral blood mononuclear cells were used as a positive control for the expression of several T cell, B cell, and monocyte markers. This was to ensure these antibodies worked at the concentration used, but also to check whether cell surface proteins were cleaved by collagenase digestion. However, for several cell surface makers, especially those present on macrophages and fibroblasts were not tested due to a lack of positive control. Therefore, when no expression of these markers was found (CD117, VCAM, CD248, IL-17RA, IL-17RC), it was impossible to conclude whether these markers were not present on the tested cells, or if this marker was cleaved or internalised during the digestion, stimulation, or staining procedure.

2.7.5.4 Panels should be expanded to further distinguish subsets

This study confirmed that the infiltrating immune cells in OA synovium are mainly T cells and macrophages, and that fibroblasts show an activated phenotype. Therefore, future studies should aim to include specific markers to study different subtypes of these main immune cell types in order to get a better insight into their potential function in disease.

For T cells, the currently used panel (CD3, CD4, CD8, CD56, CD161, GD TCR) was unable to use the expression of CD4 and CD56 due to cleavage of these markers significantly weakened the strength of this. In addition to optimising the digestion protocol to minimise cleavage of markers, a marker for regulatory T cells, such as CD25 or FOXP3, a marker for memory T cells, such as CD45RO, or a marker for T cell activation, such as CD69 or CD154 could be added.

Granulocytes and monocyte-derived cells share the expression of several cell surface markers, making it difficult to distinguish these cells using only one of these markers. Therefore, additional markers need to be used to be able to positively identify these cells. In addition, a range of M1 and M2 markers, as well as markers of activated tissue macrophages such as CX_3CR1 could be used to further specify macrophage subsets.

As a limited number of markers could be used in each panel, not all markers that can distinguish different fibroblasts subsets were used in this study. Future studies should add antibodies for CD55, which is present on synovial fibroblasts in the synovial lining where it might contribute to the maintenance and amplification of inflammation, and cadherin 11, which has been shown to be associated with pathological behaviour in previous *in vitro* and animal studies[177,178]. After further optimisation of digestion method, more research is required to identify if VCAM and CD248 were cleaved or not expressed on these cells used in this study in order to clarify whether these markers could be used in future studies.

2.7.6 Future studies

Opportunities for further studies that have already been described include the investigation of the potential subtypes of disease, such as the presence or absence of IL-17-producing CCR6+ T cells, and the relationship between cell subsets and patient characteristic. Additional ideas for future studies are described in more detail below.

2.7.6.1 Macrophages as a driver of synovitis

Synovitis has become a more accepted as having a role in OA pathophysiology. Previous studies have hypothesized that macrophages are one of the major drivers of synovitis in these patients[179]. This study has shown while there are many different types of immune cells present, macrophages are one of the two predominant immune cell subsets in end-stage OA synovium. Therefore, future studies should look at potential associations of macrophages and markers of synovitis is OA tissue.

2.7.6.2 IL-17A in OA

Flow cytometry results show that there are potentially subsets of patients with IL-17A-producing cells based on the expression of CCR6 and IL-17A on T cells. Although especially clinical and genetic studies have shown a potential role for IL-17A in OA, little research has been done to investigate this. Future studies need to use flow cytometry alongside other techniques such as immunohistochemistry to further investigate the presence of these IL-17A-producing cells. However, first the expression of IL-17A cytokine and its receptors IL-17RA and IL-17RC needs to be confirmed in tissue, to confirm that the tissue has the capability to respond to IL-17A. If both these IL-17 receptors are indeed expressed throughout tissue, and on fibroblasts which are the predominant cell type in tissues such as cartilage and synovium, studies should investigate how these cells respond to IL-17A and its closely related cytokines IL-17F and IL-17AF.

2.7.7 Conclusions

In conclusion, this hypothesis-generating study has shown that there are significant differences in the number and subsets of immune cells in OA synovium, which might be associated with age, clinical scores of disease severity, subtypes of disease, or fibroblast subsets. Macrophages and T cells were the two predominant immune cells subsets. However, the immune cell subset that is most different between patients is CCR6+ T cells, which is only present in a subset of patients, thus suggesting a subtype of disease with an important role for CCR6+ T cells and its produced cytokine IL-17A. Previously identified fibroblast subsets were found, with especially high proportions of immune effector fibroblasts. Proteomic analysis of matched synovial fluid did not result in any obvious separation of subsets of patients. Future work should investigate if macrophages are one of the main drivers of synovitis seen in many patients. In addition, as IL-17A could be an important mediator in (a subset of) OA patients, this should be studied in more detail. Therefore, further studies should investigate the expression of IL-17A and its receptors in tissue, and study the effect of IL-17A on synovial fibroblasts and chondrocytes, the predominant cell subset in synovium and cartilage.

2.7.8 Key findings

- Immune cells were identified in end-stage OA synovium.
- Macrophages and T cells are the two predominant immune cell types, suggesting a role for these cells in synovitis.
- The presence of CCR6+ T cells might represent a subset of patients with an important role for CCR6+ T cells and its produced cytokine IL-17A
- Fibroblasts are the predominant cell type in end-stage OA synovium have an activated phenotype

Chapter 3

IL-17RA and macrophage markers are increased in patients at risk of OA with synovial inflammation

3.1 Abstract

3.1.1 Background

Chronic, low-grade inflammation is now generally accepted to play a critical role in the pathophysiology of osteoarthritis (OA). Chapter 2 has shown a clear infiltration of immune cells into end-stage OA synovium, with T cells and macrophages as the predominant immune cell subsets involved. Molecular interrogation has shown an increase of inflammatory mediators in synovial fluid. The interleukin (IL)-17 family of cytokines is receiving increased attention in OA with IL-17A concentrations having been shown to be elevated in the serum and synovial fluid of a number of OA patient cohorts. IL-17A concentrations are also positively correlated with Kellgren-Lawrence grade and WOMAC pain scores. Animal studies have demonstrated potential roles for IL-17A and its IL-17RA/IL17-RC receptor complex in inflammatory arthritis, including affecting pain and synovial inflammation. However, little is known about the expression of IL-17A and IL-17 receptors in human OA tissue, and how this relates to synovitis. This chapter aims to study whether IL-17A and its receptors are expressed in tissues from OA patients and how this relates to macrophage infiltration.

3.1.2 Methods

Synovial tissue was collected from a cohort of patients with knee injury who have a high risk of developing post-traumatic OA. Synovitis was scored from 0 (no inflammation) to 3 (severe inflammation) using H&E-stained slides. Formalin-fixed, paraffin-embedded sections were stained for IL-17A, IL-17 receptors IL-17RA and IL-17RC, and macrophage markers CD68 and CD206, and staining quantified. Primary chondrocytes and synovial fibroblasts from end-stage OA patients were used to study the effect of FGF, IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, TGF-β1, TNF-α, and VEGFA on *IL17RA* and *IL17RC* mRNA expression with RT-qPCR.

3.1.3 Results

IL-17RA and IL-17RC were expressed in all tested tissue samples and *IL17RA* and *IL17RC* mRNA were expressed in fibroblasts from end-stage OA patients. Treatment with single cytokines did not moderate *IL17RA* or *IL17RC* mRNA expression in cells isolated from end-stage OA patients. Expression of IL-17A was found in each section tested. Expression of IL-17RA, and the macrophage markers CD68 and CD206 significantly increased with synovitis score, while IL-17RC and IL-17A did not show significant differences between patients with low or high synovitis scores.

3.1.4 Conclusions

The protein expression of IL-17 receptors in synovium of patients at risk of developing OA, and mRNA expression in cells from end-stage OA patients confirms that this tissue can respond to IL-17A. However, no OA-relevant cytokine tested was able to increase mRNA expression of these receptors in end-stage OA cells. The IL-17A was present in synovium of patients at risk of developing OA, confirming a potential role of this cytokine in disease. Macrophage markers CD68 and CD206 significantly increased with synovitis, pointing towards macrophage infiltration in these patients. IL-17RA also increased with synovitis score; although further studies are needed to confirm whether this increase occurs on resident cells or is due to presence of IL-17RA on infiltrating macrophages.

3.2 Background

3.2.1 Introduction

Chronic, low-grade, innate immune-driven inflammation is now accepted to play a critical role in the pathophysiology of OA. Many OA patients report symptoms related to joint inflammation, such as morning stiffness, warmth, pain, and joint effusion, which is partly due to synovial thickening or synovial fluid effusion. Histological analyses have shown a clear infiltration of inflammatory cells into the synovium, and molecular research has shown an increase of inflammatory mediators in synovial fluid, many of which can are hypothesized to be (over)produced by the resident stromal cells of the joint[25].

3.2.2 Immune cells in OA synovium

OA synovium is characterised by hyperplasia of the synovial lining, fibrosis of the sub-lining, and stromal vascularisation[94]. In Chapter 2, several different immune cells subsets were identified in end-stage OA synovium, including T cells, B cells, macrophages, low numbers of dendritic cells, and neutrophils, which is in accordance with previous histological studies although these also identified natural killer cells and mast cells[94]. Macrophages were found to be one of the two predominant immune cell population in OA synovial tissue (together with T cells), and previous studies have found that their infiltration is positively correlated with OA progression and disease severity[93,180–182]. Innate immune cells such as macrophages can be activated by the complement system and damage-associated molecular patterns (DAMPs), including extracellular matrix (ECM) components released by damaged cartilage[43,119]. Activation of these macrophages contributes to OA synovitis through secretion of pro-inflammatory cytokines that activate other immune cells. Furthermore, macrophages can contribute to OA by releasing matrix metalloproteinases (MMPs) that mediate cartilage destruction, and induce formation of osteophytes directly through to the release of growth factors such as transforming growth factor (TGF)-β, and indirectly through cartilage matrix

remodelling due to MMP release[120–125]. As macrophages can release mediators as MMPs and TGF-β itself, but they can also stimulate resident stromal cells to release these mediators, it is likely that these effects on cartilage destruction and osteophyte formation are both directly and indirectly affected by macrophages[115,124,125,183]. The activation of macrophages by cartilage breakdown products and its subsequent effect of inflammation and cartilage breakdown could indicate that this is part of a vicious cycle in the OA disease process[94,115,121,124].

Macrophages can modify their phenotype and function by responding to stimuli in their microenvironment. Although many different subsets of macrophages exist, a crude subdivision of extremes would be the classically activated M1-macrophages which are more pro-inflammatory and the alternatively activated M2-macrophages, which are more anti-inflammatory[126]. Both M1 and M2 macrophages have been shown to be present in OA synovium[115]. Previous studies have found that CD80-positive M1 macrophages are mainly found in the synovial lining, while CD206-positive M2 macrophages are pre-dominantly located in the sub-lining of OA synovium[184]. Although some studies report that most synovial macrophages in OA have an M2 phenotype[127], others report an increase in M1/M2 ratio that is associated with OA severity[128]. Interestingly, there have been several reports of OA synovial macrophages that express a combination of M1- and M2-markers, and there seems to be a large inter-patient variability in the expression of these synovial markers in OA synovium[115]. In Chapter 2, almost half of the macrophages were CD206+ while only a small subset (±10%) was CD40+. In addition, almost all CD40+ macrophages also expressed CD206. Therefore, more investigations are needed on the mechanisms underlying the activation of macrophages in OA. As previously discussed in Chapter 2, it is known that the M1/M2 division is outdated, but this crude division will be used to compare results to previous studies. However, future studies should use more specific subtyping including a range of markers in order to get more insight into the exact role of macrophages.

3.2.3 Inflammatory mediators in OA

Given that synovitis and the presence of immune cells is an accepted component of OA pathophysiology, it is important to understand the role of the inflammatory mediators secreted by these immune cells and resident stromal cells. Many projects are currently ongoing to unravel the role of both pro- and anti-inflammatory mediators in the pathophysiology of OA. One of the cytokines that has recently received a lot of attention for its potential role in OA is IL-17.

IL-17 cytokines signal through the IL-17 receptor family (IL-17RA-IL-17RE)[185]. IL-17A, IL-17F, and IL-17AF all act through a receptor complex made out of IL-17 receptors IL-17RA and IL-17RC[186]. Whereas IL-17RA can form a complex with different IL-17 receptor subunits to facilitate signalling of different cytokines from the IL-17 family, IL-17RC seems to be specific for the signalling of IL-17A, IL-17F, and IL-17AF[187]. IL-17RA is ubiquitously expressed, with especially highly expression in haematopoietic tissues and cells including macrophages and T cells, while IL-17RC is mainly expression in non-haematopoietic tissues. Although IL-17RA is most highly expressed in haematopoietic tissues, based on the more restricted expression of IL-17RC, IL-17A responses mainly occur in epithelial, endothelial and fibroblasts cells. However, macrophages and dendritic cells are also known to respond[185,188,189].

Several animal studies that have looked at the function of IL-17 and its IL-17 receptors in inflammatory arthritis. In a study using an antigen-induced arthritis (AIA) model, IL-17A did not have an effect on disease severity, but significantly contributed to pain[190]. This was confirmed another studies using the AIA model, in which neutralisation of IL-17A did not lead to a decrease in joint swelling, but significantly reduced secondary mechanical hyperalgesia at the paw in an AIA-model[83], and IL-17A mediated articular hyper-nociception through neutrophil migration and induction of several inflammatory mediators[85]. IL-17RA signalling was found to serve as an amplifier of the effector phase of AIA mice model by direct activation of synovial fibroblasts through IL-17RA to

produce multiple inflammatory mediators, including chemokines that act on neutrophils[191]. Another study showed that IL-17RA knockout mice were completely protected from collagen-induced arthritis (CIA), showing increased levels of Th2-like cells in the spleen compared to normal CIA mice, suggesting that IL-17RA signalling is involved in the suppression of Th2 cytokines[192]. Even though these do give some insight in the role of these receptors in inflammatory arthritis, it is important to be cautious of results from murine models as in mice IL-17A and IL-17F both bind to IL-17RA while IL-17RC only binds to IL-17F, meaning that IL-17RC is not necessary for IL-17A/F/AF signalling in mice[193].

3.2.4 Aim of this chapter

The clinical human data and animal studies of OA clearly suggest a critical role for synovitis, which is characterised by a strong infiltration of macrophages. In addition, IL-17A and its receptors are increasingly indicated to affect inflammation and tissue destruction in OA. Although some preliminary studies have been conducted, little is known about the expression of IL-17A and its receptors in OA tissue, and how this could relate to the synovitis seen in many patients. Therefore, this chapter aims to study the expression of macrophage markers in synovial tissue from patients with knee injury who have a high risk of developing post-traumatic OA to see if the expression of these markers correlates with their synovitis score. Then the expression of IL-17A and its receptors will be studied in this cohort of patients, and investigated whether there is a correlation between these IL-17 markers and synovitis score or macrophage marker expression. Finally, we will determine whether OA-relevant cytokines are able to affect the gene expression of IL-17 receptors IL-17RA and IL-17RC in chondrocytes and synovial fibroblasts from end-stage OA patients, which could make patients more responsive to IL-17A, IL-17F, and IL-17AF.

3.3 Objectives

The objectives of this chapter are to:

1. Investigate the expression of macrophage markers in human synovium in a cohort of knee injury patients who have a high risk of developing OA (knee injury/early OA) and establish whether these markers are correlated with synovitis score
2. Determine if IL-17A and IL-17 receptors A and C (IL-17RA and IL-17RC) are expressed in synovial tissues of patients who have a high risk of developing post-traumatic OA
3. Identify if OA-relevant cytokines modulate *IL17RA* and/or *IL17RC* mRNA expression in chondrocytes and synovial fibroblasts isolated from patients with end-stage OA

3.4 Hypotheses

It is hypothesized that:

1. Macrophage markers CD68 and CD206 are expressed in synovial tissue sections from patients who have a high risk of developing post-traumatic OA and these markers correlate with synovial inflammation (synovitis score).
2. IL-17RA and IL-17RC are expressed in synovial tissue from patients at risk of developing OA
3. Cytokines that are potent inducers of intracellular signalling, such as IL-1β, induce mRNA expression of *IL17RA* and/or *IL17RC* in cells isolated from end-stage OA patients

3.5 Methods

3.5.1 Ethics

Ethical approval for the Oxford Knee Injury Cohort (OxKIC) study was granted in October 2014 (14/WA/1108), and amendments were accepted in July 2015 by the Research Ethics Committee (Wales REC 7). Ethical approval for the Oxford Musculoskeletal Biobank (09/H0606/11) and (19/SC/0134) was granted by the local research ethics committee (Oxford Research Ethics Committee B) for all work on human cartilage and human synovium, and informed consent according to the Declaration of Helsinki was obtained from all patients.

3.5.2 Acquisition and initial processing of synovial tissue of patients with high risk of developing OA (OxKIC cohort)

Synovial tissue samples from patients from the OxKIC cohort were obtained and processed as described in 2.5.3.

3.5.3 Clinical scores for OxKIC cohort

Age was recorded on the day of synovial biopsy. Pain was scored using the Numerical rating scale (NRS-11) on a scale of 0 (no pain) to 10 (worst pain imaginable). Synovial hypertrophy (greyscale synovitis) and suprapatellar effusion were scored during an ultrasound of the knee at time of the synovial biopsy. Both the synovial hypertrophy and effusion were scored as number of mm on the ultrasound, and later subdivided in grades: 0 (<4mm), 1 (4 ~ <8mm), 2 (8 ~ <11mm), and 3 (≥ 11mm).

3.5.4 Immunohistochemistry

On the day prior to staining, slides were baked at 60°C for 60 minutes and left at room temperature overnight to cool down. Deparaffinisation and antigen retrieval procedure was performed using a PT

Link machine (Dako, Glostrup, Demark) by submerging slides in FLEX TRS antigen retrieval fluid. Immunostaining was performed using an Autostainer Link 48 machine using the EnVision FLEX visualisation system (Dako). The primary antibodies used can be found in Table 3-1.

Table 3-1. *Overview of antibodies used for immunohistochemistry including their target, isotype, company, reference number, and dilution used during staining.*

Target	Isotype	Supplier	Ref number	Dilution for IHC
Human IL-17RA	Monoclonal mouse IgG2B	R&D systems, Abingdon, UK	MAB1771	1:300
Human IL-17RC	Monoclonal mouse IgG2B	R&D	MAB22691	1:2,000
Human IL-17A	Monoclonal mouse IgG1	ProteinTech Group Inc, Rosemont, IL, USA	66148-1-Ig	1:6,000
Human CD68	Monoclonal mouse IgG1	Dako	M0814	1:3,000
Human mannose receptor (CD206)	Polyclonal rabbit IgG	Abcam	ab64693	1:10,000
FLEX Negative Control Mouse	Cocktail of mouse IgG1, IgG2a, IgG2b, IgG3 and IgM	Dako	IR750	Ready to use
FLEX Negative Control Rabbit	Immunoglobulin fraction of serum from non-immunized rabbits, solid phase absorbed.	Dako	IS600	Ready to use

Antibody binding was visualized by FLEX 3,3'-diaminobenzidine (DAB) substrate working solution and haematoxylin counterstain (Dako) following the protocols provided by the manufacturer. Antibodies were validated in-house to determine the concentration of antibody needed for positive staining with minimal artefact from the tissue. After the staining procedure, slides were dehydrated in 90% and 100% alcohol for 3 minutes each and twice in xylene for 5 minutes before mounted using Pertex mounting medium (DPX) (Histolab, Gothenburg, Sweden). Synovial tissue that was stained with either universal mouse or universal rabbit negative control antibodies is shown in Appendix B1.

3.5.5 Quantitative analysis of DAB staining with CellProfiler

Each tissue section was imaged using an Olympus BX51 inverted microscope (Olympus, Southend-on-Sea, UK) using DPManager and DPController software (Olympus) at 40x magnification. Images were collected in a systematic fashion to ensure that an accurate overview of the section was created and pieces of sections were not imaged twice.

The program CellProfiler was used to facilitate semi-quantitative analysis of the amount of staining in each image. A previously created pipeline was used called "DAB and nuclei measurements". Before analysis, each image was cropped to ensure that tissue covered most of each image and that no or limited fatty and non-fibrous tissue was present on each image in order to facilitate a representative comparison between sections. A set of 10 images ranging from unstained to heavily stained was then used to confirm the accuracy of the nuclei count and area of the image stained with DAB. The threshold for DAB staining was tested for each antibody by using 10 randomly selected images and manually changing the threshold until a number was reached that gave a representative staining for each image. After this, the threshold was used to run all the images through the pipeline. Percentage area stained (area%) was calculated by dividing the pixels stained with DAB over the total number of pixels in each image, converting to percentage, and then taking the median area% for each patient. A full representation of this analysis pathway can be found in the Appendix B2.

3.5.6 Histological synovitis score for OxKIC cohort

Synovitis was scored by two independent readers using a histological synovitis score based on Haywood and Walsh ranging from 0 (normal) to 3 (severe inflammation) using haematoxylin and eosin (H&E)-stained slides; if no synovial lining was identified, no score was given (Table 3-2). 2 to 4 fields were scored, and later averaged.

***Table 3-2**. Grade of microscopic synovitis based on the Haywood and Walsh scoring system. Modified with permission from thesis Dr Stefan Kluzek.*

Grade	Criteria		
	Synovial lining	**Cellularity**	**Inflammatory cells**
0: normal	<4 cells thick	Sparse cellular distribution	No or few inflammatory cells
1: mild inflammation	4-5 cells thick	Increased cellularity	Few inflammatory cells
2: moderate inflammation	6-7 cells thick	Dense cellularity	Inflammatory cells; no lymphoid aggregates
3: severe inflammation	>7 cells thick	Dense cellularity	Inflammatory cell infiltration; may contain perivascular lymphoid aggregates
-: no synovial lining	No synovial lining was identified, thus was not able to be scored		

3.5.7 Acquisition of end-stage OA tissue

Tissue from end-stage OA patients was collected during total knee replacement surgery. Tissue that was removed and not further needed was collected for research purposes. Cartilage was collected in a sterile pot, synovium was collected in sterile culture medium, and synovial fluid was collected in a sterile tube. All tissue was brought over from the hospital to the research facility as soon as the surgical procedure was completed.

3.5.8 Isolation of primary OA chondrocytes and synovial fibroblasts for in vitro culture

Tissue from end-stage OA patients was collected during total knee replacement surgery. Cartilage was dissected from the subchondral bone of the tibial plateau, washed in D10 media (DMEM-F12 (Gibco, supplied by Fisher Scientific, Loughborough, UK) with 10% Foetal Bovine Serum (FBS) (Labtech International, Heathfield, UK) and 1% Penicillin/Streptomycin (P/S) (Gibco)), and minced before being collagenase digested overnight in collagenase digestion media (1mg/mL collagenase (Sigma, supplied by Merck, Darmstadt, Germany), 0.4% HEPES (Sigma), in DMEM-F12 with 1% P/S and no serum (D0)). Synovium was washed in D10 media, fat was cut off where possible, and fibrous synovium was minced before being collagenase digested for 2 hours. After collagenase digestion, the

collagenase media with digested tissue was filtered through a 70μm cell strainer, and cells were plated out in 10-cm dishes in D10. Once 95% confluent, chondrocytes dishes were scraped and cells were frozen down, while synovial fibroblasts dishes were scraped and cells passaged to p=1 in 10-cm dishes in which they were grown to 95% confluence, after which they were scraped and frozen.

3.5.9 Cell experiments in 96-well plates for cells-to-cDNA analysis of gene expression

Chondrocytes were grown to p=2 and synovial fibroblasts to p=3 in D10, after which they were plated out at 8,000 cells per well into a 96-well flat-bottom plate. Cells were left to attach for 24 hours in D10, then media was replaced with D0 for 24 hours. On the day of the experiment, stimulation media and vehicle control media were made up in D0 to final concentrations shown in Table 3-3. Old media was removed from the cells, media for the experiments was added, and cells were left for 24 hours.

Table 3-3. *Overview of recombinant human proteins used to induce IL17R mRNA expression, including concentration used and supplier.*

Recombinant human protein	Concentration	Supplier
FGF	10 ng/mL	R&D systems, Inc.
IFN-γ	20 ng/mL	Biolegend UK Ltd, London, UK
IL-1β	10 ng/mL	Sigma-Aldrich,
IL-4	10 ng/mL	Biolegend UK Ltd
IL-6	10 ng/mL	Biolegend UK Ltd
IL-8	10 ng/mL	Biolegend UK Ltd
IL-10	10 ng/mL	Biolegend UK Ltd
TGF-β1	4 ng/mL	Biolegend UK Ltd
TNF-α	5 ng/mL	Biolegend UK Ltd
VEGFA	10 ng/mL	R&D

3.5.10 cDNA synthesis using Cells-to-cDNA methodology

Cells were treated for 24 hours before being washed with PBS and harvested in Cells-to-cDNA II Cell Lysis Buffer (Ambion Inc, Foster City, CA, USA), and transferred to a PCR plate for cells-to-cDNA synthesis. cDNA was prepared using a cells-to-cDNA kit following the manufacturer's instructions (Ambion).

3.5.11 Real time quantitative PCR

Samples for real-time quantitative polymerase chain reaction (RT-qPCR) were prepared using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, California, USA). Primers (Table 3-4) were purchased from Primerdesign Ltd (Primerdesign Ltd, Eastleigh, UK). cDNA samples, Fast SYBR Green Master Mix, nuclease-free water, and primers were added to each well of a 384-well plate to get to give a final reaction volume of 10 μL. All RT-qPCRs were performed using a ViiA7 (Life Technologies, Paisley, UK), which included 40 cycles and a melt-curve. The housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and β-actin (*ACTB*) were used (Primerdesign Ltd). Samples were analysed using both housekeeping genes with the delta-delta Ct method[194]. The mean of the ddCT outcomes of the two housekeeping genes was taken and used for all further analyses.

Table 3-4. *Overview of primers used in this chapter, including the source of the primers, and the forward and reverse primer sequences where available.*

Target	Supplier	Forward	Reverse
GAPDH	Primerdesign Ltd	*Proprietary sequence*	*Proprietary sequence*
ACTB	Primerdesign Ltd	*Proprietary sequence*	*Proprietary sequence*
IL17RA	Primerdesign Ltd	TGAGCACATGCACCACATAC	TTAAGGTTGCGTAGAGTGAGTG
IL17RC	Primerdesign Ltd	AGTCGTGCTCTCCTTCCAG	AGATTCGTACCTTCACTCCCTAG
IL17A	Primerdesign Ltd.	CCTCAGATTACTACAACCGATCC	CACTTTGCCTCCCAGATCAC
IL17F	Primerdesign Ltd.	TCAGTAACATCGAGAGC	GATGTCTTCCTTTCCTTGAG

3.5.12 Immunofluorescence staining and image acquisition

Synovial sections obtained from the OxKIC study were used for immunofluorescence staining as described in 2.5.4. The primary antibodies used for immunofluorescence staining in this chapter are specified in Table 3-5. High-resolution versions of the confocal images used in this chapter can be found in Appendix B3.

Table 3-5. *Overview of antibodies used for immunofluorescence including their target, isotype, company, reference number, and dilution used during staining.*

Target	Isotype	Supplier	Ref number	Dilution for IF
Human IL-17RA	Monoclonal mouse IgG2B	R&D systems	MAB1771	1:300
Human IL-17RC	Monoclonal mouse IgG2B	R&D	MAB22691	1:2,000
Human IL-17A	Monoclonal mouse IgG1	ProteinTech Group Inc	66148-1-Ig	1:3,000
Human CD3	Monoclonal mouse IgG2a	Abcam	ab699	1:50
Human CD45	Polyclonal rabbit IgG	Abcam	ab40763	1:800
Human CD68	Monoclonal mouse IgG1	Dako	M0814	1:3,000
Human mannose receptor (CD206)	Polyclonal rabbit IgG	Abcam	ab64693	1:5,000
FLEX Negative Control Mouse	Cocktail of mouse IgG1, IgG2a, IgG2b, IgG3 and IgM	Dako	IR750	Ready to use
FLEX Negative Control Rabbit	Immunoglobulin fraction of serum from non-immunized rabbits, solid phase absorbed.	Dako	IS600	Ready to use

3.5.13 Statistical tests

Statistical analyses were performed using in GraphPad Prism 8.1.2 (GraphPad Software, La Jolla, CA, USA). The Mann-Whitney test was used to determine differences in immunopositive staining between low and high synovitis groups. To test if two types of immunopositive staining were correlated, a correlation test was performed. Correlation tests using the Pearson's correlation coefficient were used for all associations tested with two continuous variables. If one of the variables was on an ordinal scale, a non-parametric Spearman correlation was used. Differences between

mRNA expression of cells treated with vehicle control media or media with a cytokine was tested using a Friedman test with a Dunn's Multiple Comparisons test. A trend was noted when the p-value was lower than 0.1, while differences were considered to be significant when p-value was lower than 0.05. Data is shown as mean ± SD, unless otherwise stated.

3.6 Results

3.6.1 Macrophage markers CD68 and CD206 are increased in patients with high synovitis

As macrophages are considered to be the key drivers of synovitis and are associated with OA progression[115], this chapter investigated whether macrophages are present in synovium from patients with knee injury or early OA. Specially, specimens were stained for CD68, which is a pan marker for macrophages, and CD206, which is mainly present on STAT6-polarised M2 macrophages[195].

Synovial tissue sections from a cohort of knee injury and early OA patients was used to study the expression of CD68 and CD206, and study whether differences in expression could be correlated with a histological score for synovial inflammation. Qualitative analysis showed clear visual differences in the intensity of DAB staining between patients for both CD68 and CD206. When splitting the patients in two groups depending on a low (0.0-1.45) or high (1.46-3.0) synovitis score, it was clear that the expression of both these markers was visibly lower in patients with lower synovitis scores than in patients with high synovitis scores (Figure 3-1).

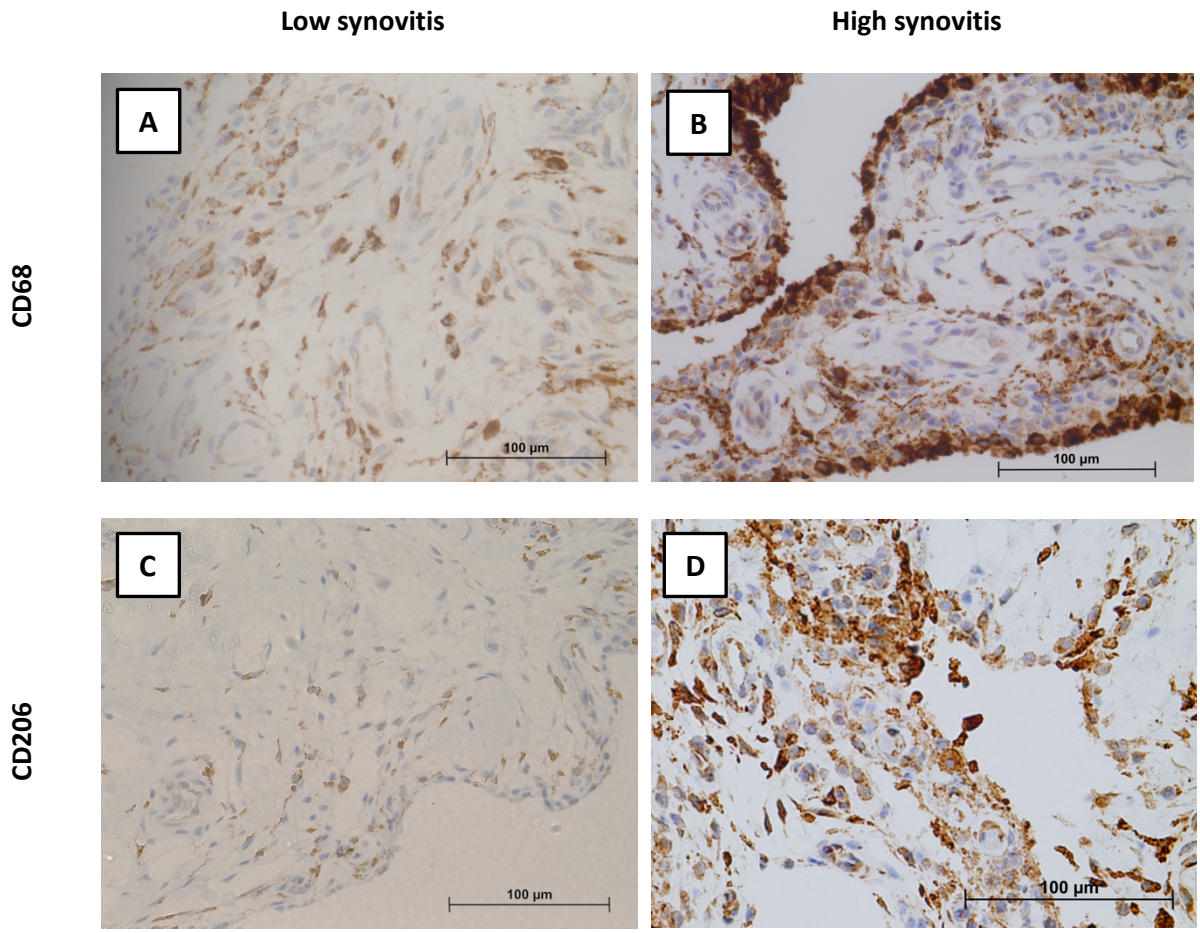
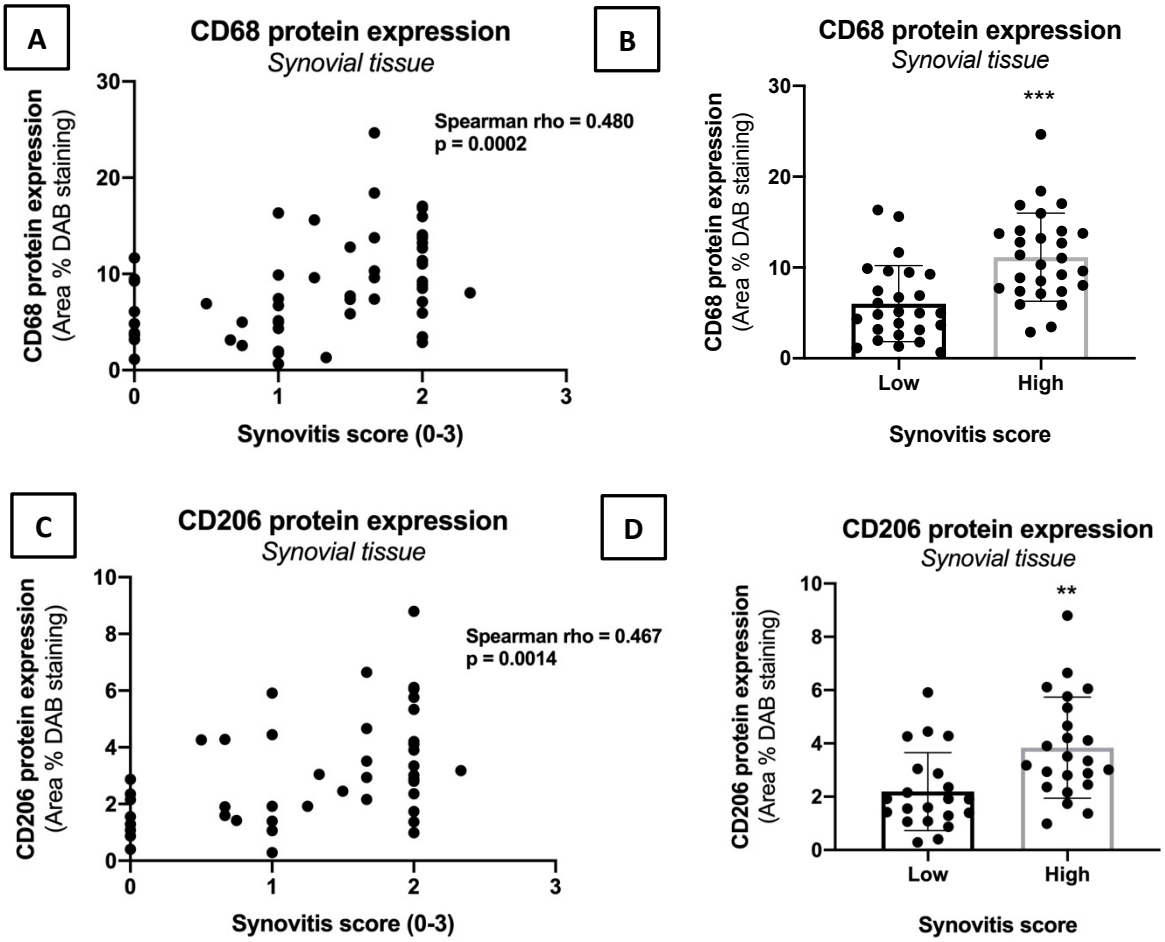


Figure 3-1. *Protein expression of CD68 (A, B) and CD206 (C, D) in patients with knee injury who have a high risk of developing post-traumatic OA with either a low or high inflammatory (synovitis) score. Antibodies were visualised with DAB (brown) and counterstained with haematoxylin (blue). Images were taken at 40x magnification.*

In order to facilitate semi-quantitative analysis of the staining, the digital image analysis pipeline in CellProfiler was used to quantify the protein expression as the area of the tissue that was stained by the antibody. CD68 expression was significantly correlated with synovitis score (p=0.0009). When splitting the patients in two groups depending on a low or high synovitis score, there was a statistically significant difference in CD68 expression (p=0.0001) (Figure 3-2A,B). CD206 expression showed a smaller but still significant correlation with synovitis score (p=0.0014), and was significantly different between groups with low and high synovitis score (p=0.0017) (Figure 3-2C,D). As both CD68 and CD206 correlated with synovitis scores, the relationship between these two markers was

investigated (Figure 3-2E), which was also shown to be significant (p=0.0377). This increase in CD68 and CD206 expression with increasing synovitis score suggests that in patients with synovitis there is increased infiltration of macrophages, including macrophages that express the M2-type marker CD206.



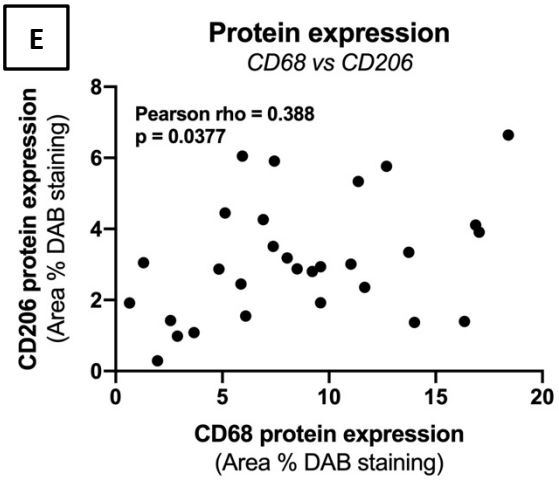


Figure 3-2. *Overview of the percentage of area stained with DAB for CD68 (A, B) and CD206 (C, D). (A, C) Protein expression for CD68 (A) and CD206 (C) for each patient plotted against their synovitis score. (B, D) Protein expression for CD68 (B) and CD206 (D) for each patient grouped by low (0-1.45) or high (1.46-3.0) synovitis score. (E) CD68 protein expression plotted against CD206 protein expression. * = p<0.05, ** = p<0.01, *** = p<0.001. (A, C) Correlation test; (B, D, E) Mann-Whitney test. CD68: total n=52; low n=28, high n=24. CD206: total n=44; low n=21, high n=23. CD68/CD206 pairs: n=29.*

As both macrophage markers correlated with synovitis score, the relationship between the expression of CD68 and CD206 with age and other clinical scores was studied. These clinical scores included pain score, effusion in mm, effusion grade (0-3), delta effusion (difference between effusion of affected knee and effusion of healthy knee), synovial hypertrophy in mm, synovial hypertrophy grade (0-3), and delta synovial hypertrophy (difference between hypertrophy of affected knee and hypertrophy of healthy knee).

CD68 only showed a trend towards a correlation with delta synovial hypertrophy (p=0.0646), while no other clinical scores showed any significant correlation with CD68 (Figure 3-3). CD206 protein expression significantly correlated with both the grade of effusion (p=0.0293) and the grade of synovial hypertrophy (p=0.0236) (Figure 3-4).

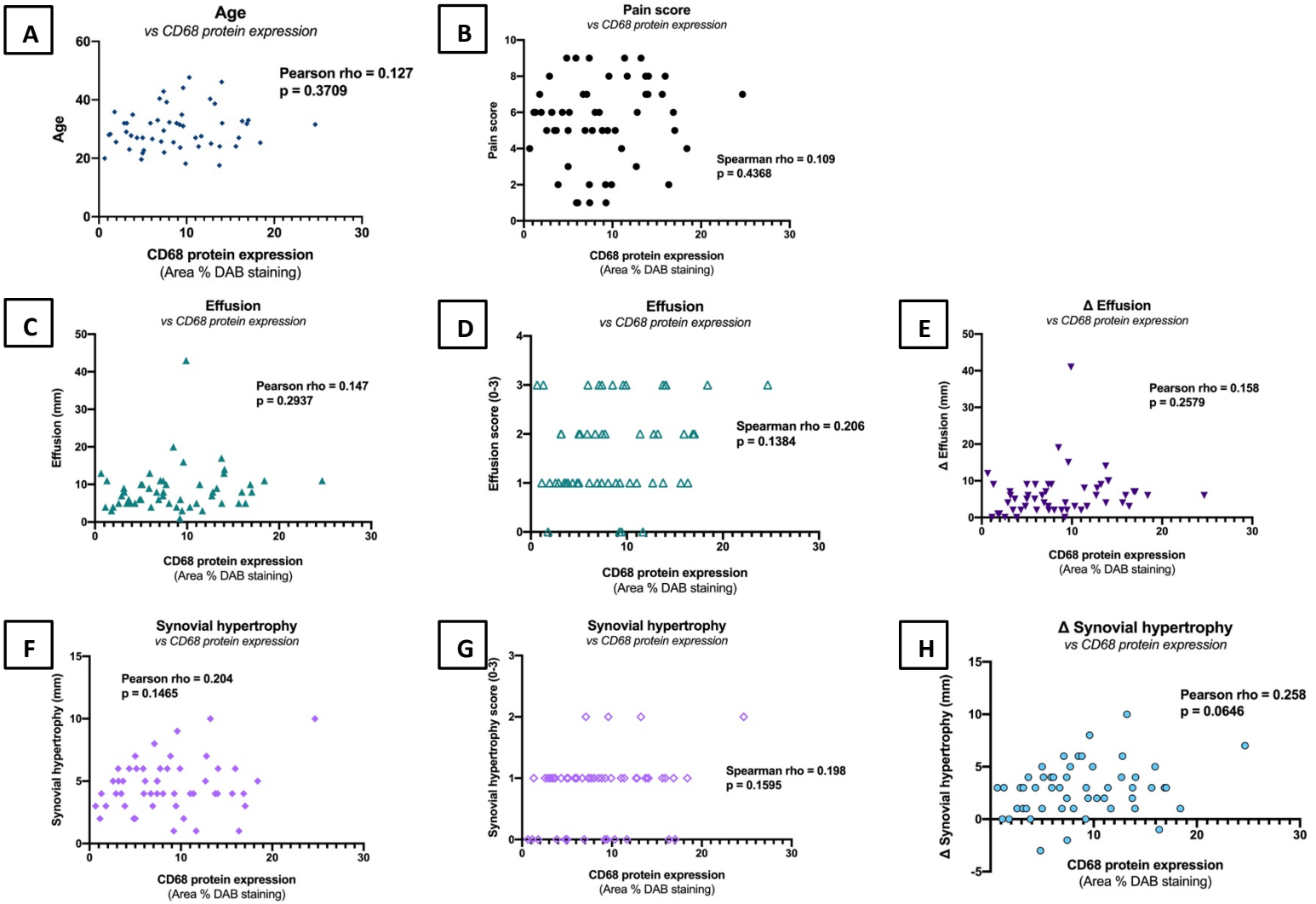


Figure 3-3. *Correlation of clinical scores with CD68 protein expression. (A) Age, (B) Pain Score, (C) Effusion (mm), (D) Effusion score (0-3), (E) Delta Effusion, (F) Synovial hypertrophy (mm), (G) Synovial hypertrophy score (0-3), and (H) Delta synovial hypertrophy. (A,C,E,F,H) Parametric correlation test. (B,D,G) Non-parametric correlation test. (A-E) n=53; (F-H) n=52.*

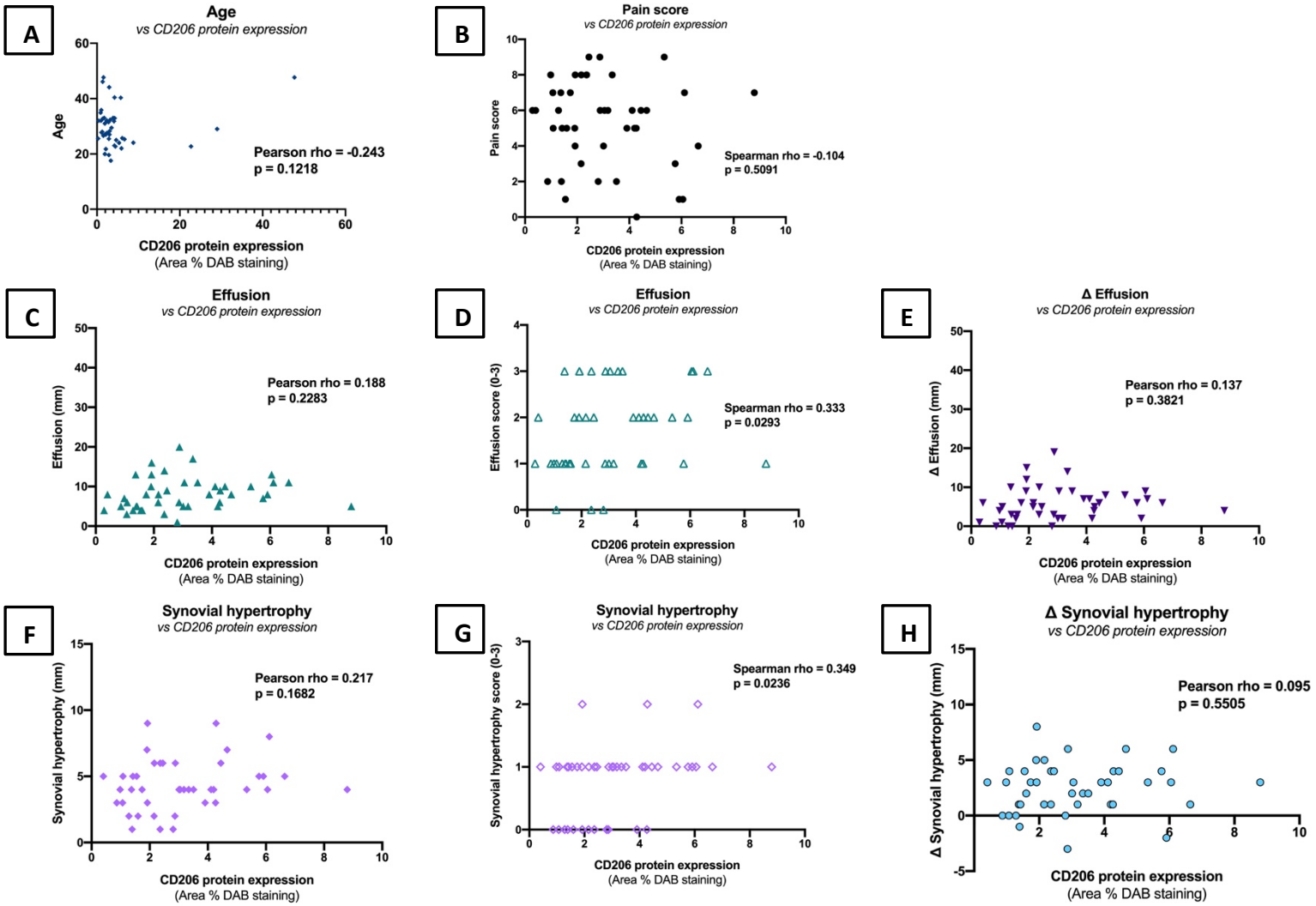


Figure 3-4. *Correlation of clinical scores with CD206 protein expression. (A) Age, (B) Pain Score, (C) Effusion (mm), (D) Effusion score (0-3), (E) Delta Effusion, (F) Synovial hypertrophy (mm), (G) Synovial hypertrophy score (0-3), and (H) Delta synovial hypertrophy. (A,C,E,F,H) Parametric correlation test. (B,D,G) Non-parametric correlation test. (A-E) n=43; (F-H) n=42.*

3.6.2 IL-17A cytokine is expressed in synovial tissue but does not correlate with synovitis score

IL-17A has been detected in serum and synovial fluid from OA patients, but its expression in synovial tissue remains understudied. Therefore, we aimed to study the expression of the cytokine IL-17A in the cohort of patients at high risk of developing post-traumatic OA as used for the macrophage marker staining. Due to limited availability of specific antibodies against IL-17F and IL-17AF, we were unable to study the expression of these related cytokines.

IL-17A was expressed in all synovial tissue specimens sampled, but no visual differences were observed between representative images of patients with low (Figure 3-5A) or high (Figure 3-5B) synovitis. Although background staining is visible, positive cells (white arrows) are clearly distinguishable from negative cells (black arrows), thereby confirming the presence of IL-17A in synovium.

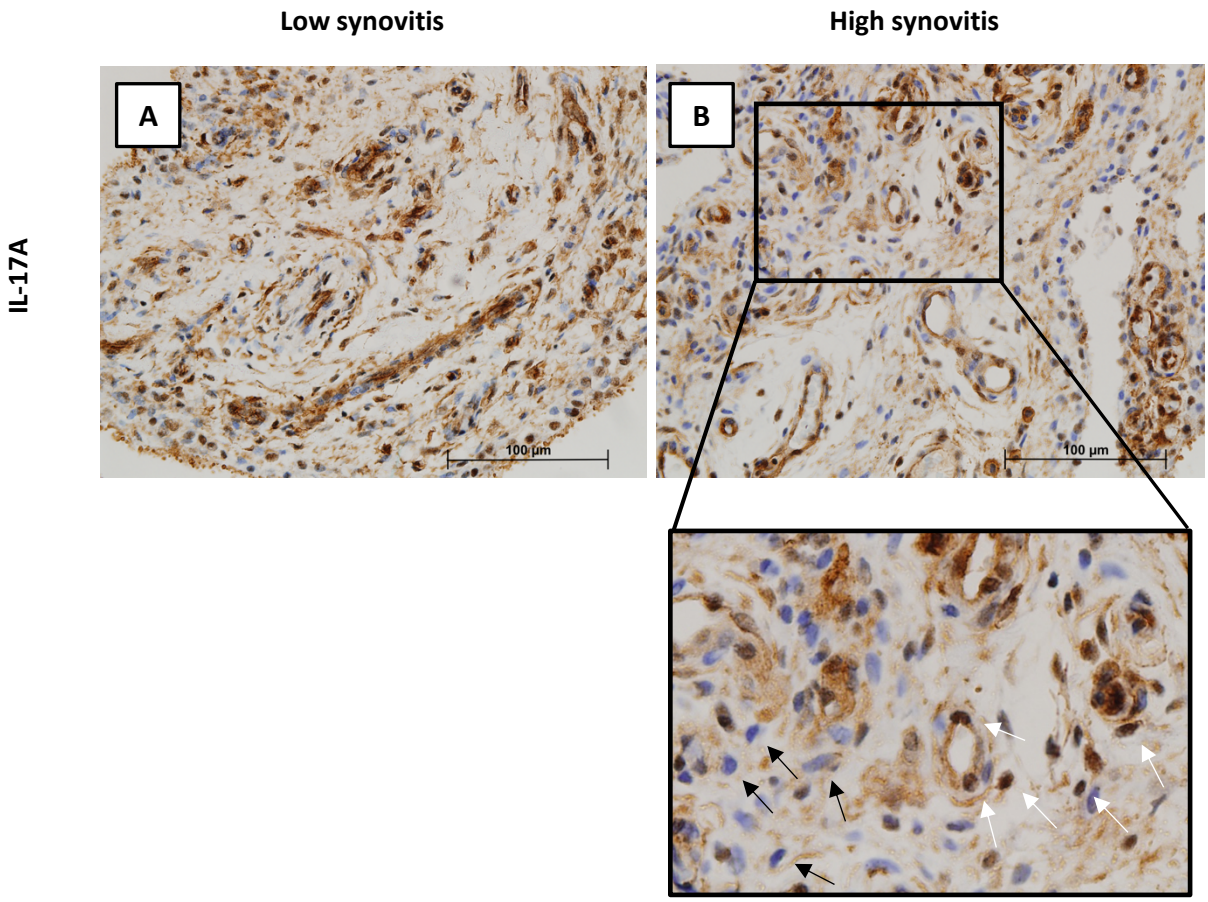


Figure 3-5. *Protein expression of IL-17A in patients with knee injury who have a high risk of developing post-traumatic OA with either a low or high inflammatory (synovitis) score. Antibody was visualised with DAB (brown) and counterstained with haematoxylin (blue). Images were taken at 40x magnification.*

When the IL-17A protein expression was plotted against their corresponding synovitis scores, no correlation was seen between these two scores (p=0.6127), and no difference was detected in IL-17A protein expression between the low and high synovitis groups (p=0.6058) (Figure 3-6). These results suggest that the expression of IL-17A is not dependent on synovitis.

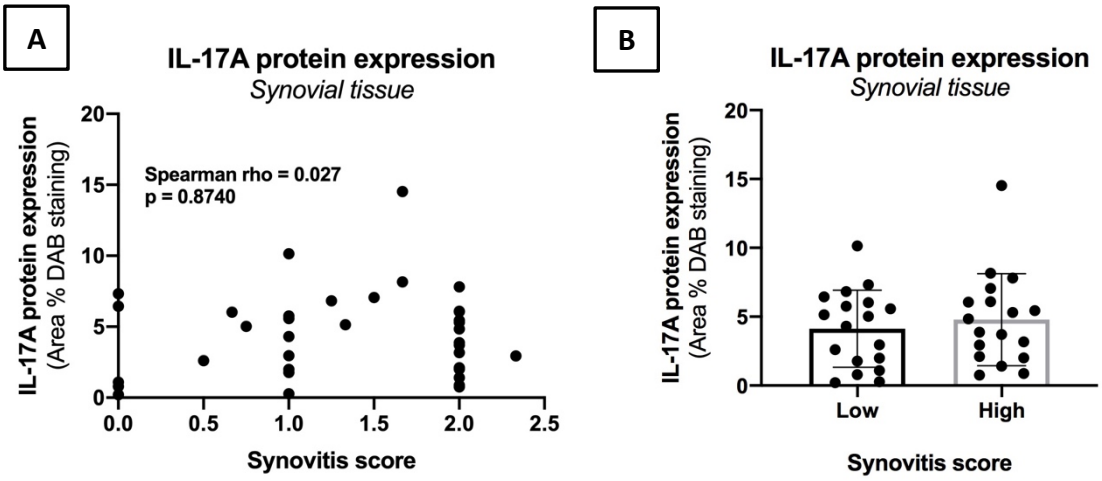


Figure 3-6. *Overview of the percentage of area stained with DAB for IL-17A. (A) Protein expression for IL-17A for each patient plotted against their synovitis score. (B) Protein expression for IL-17A for each patient grouped by low (0-1.45) or high (1.46-3.0) synovitis score. * = p<0.05, ** = p<0.01, *** = p<0.001. (A) Correlation test; (B) Mann-Whitney test. Total: n=36; low n=18; high n=18.*

The protein expression of IL-17A was used to study whether this would correlate with any of the clinical scores that were acquired. However, no trends or significant correlations were seen between IL-17A and any of the clinical scores (Figure 3-7).

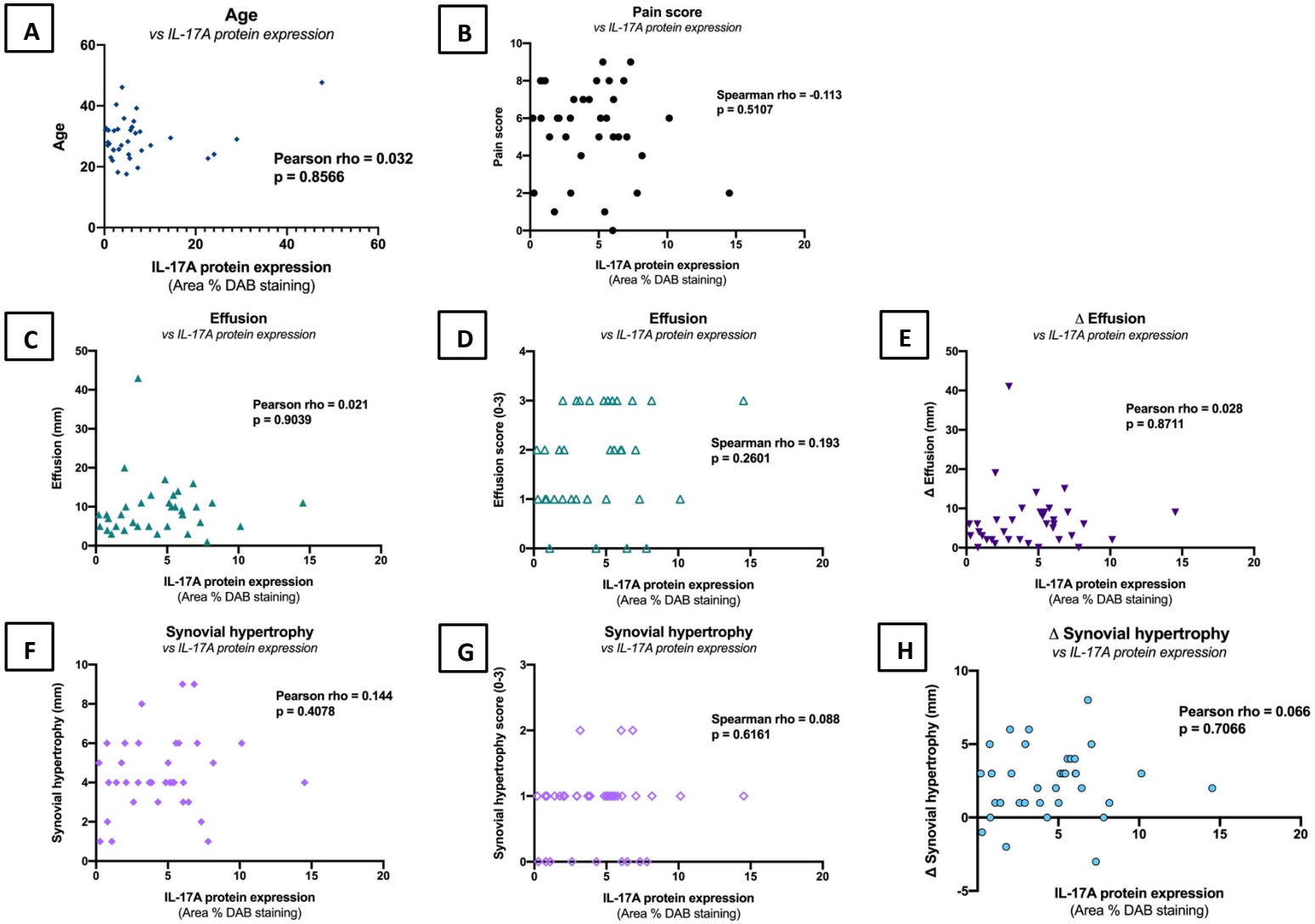


Figure 3-7. *Correlation of clinical scores with IL-17A protein expression. (A) Age, (B) Pain Score, (C) Effusion (mm), (D) Effusion score (0-3), (E) Delta Effusion, (F) Synovial hypertrophy (mm), (G) Synovial hypertrophy score (0-3), and (H) Delta synovial hypertrophy. (A,C,E,F,H) Parametric correlation test. (B,D,G) Non-parametric correlation test. (A-E) n=36; (F-H) n=35.*

Immunofluorescent staining was used to investigate if IL-17A staining co-localises with markers for the main IL-17-producing cells: T cells. A synovial section was stained with a combination of IL-17A, CD3, and CD45. Although clear co-localisation of CD3 and CD45 can be observed, IL-17A staining did not show apparent co-localisation with these markers (Figure 3-8).

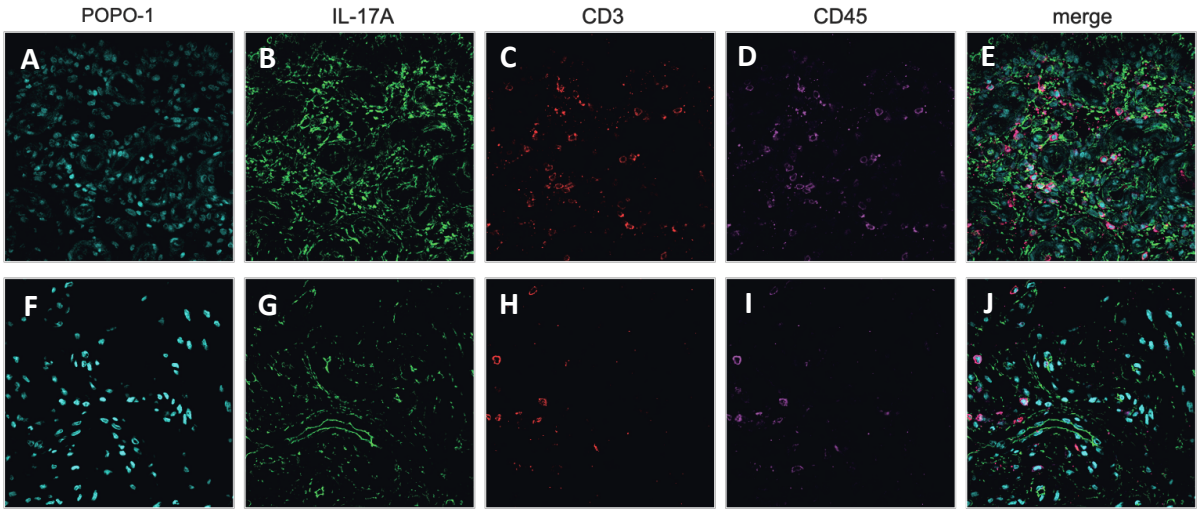


Figure 3-8. *Expression of cell surface markers CD3 and CD45 and the cytokine IL-17A in two parts of a synovial section of a patient with high risk of developing OA. Confocal images show expression of IL-17A (**green**), CD3 (**red**), and CD45 (**purple**), and were counterstained with POPO-1 (**blue**) to visualise nuclei. A merged image is provided to look at the co-expression of markers. Images were taken at 40x magnification with oil.*

3.6.3 Expression of IL-17 receptor A but not IL-17 receptor C is increased in patients with high synovitis

After confirming the presence of IL-17 cytokine in sections of synovial tissue from patients at high risk of developing post-traumatic OA, a selection of slides from the same cohort were stained with an antibody against IL-17 receptor IL-17RA or IL-17RC to investigate whether there were differences in receptor expression between patients with low or high synovial inflammation (synovitis). Expression of both IL-17RA and IL-17RC was found in every tissue section, although expression of IL-17RA was less abundant in synovium with low synovitis than IL-17RC. Together, this does confirm that

synovium of all these patients is able to respond to IL-17A, IL-17F and IL-17AF. Visibly bigger differences were seen in DAB staining between slides that were stained with IL-17RA than with IL-17RC. When comparing patients with low to patients with high synovitis score, there was a visible difference in IL-17RA staining, while differences in IL-17RC between these patients were not apparent (Figure 3-9).

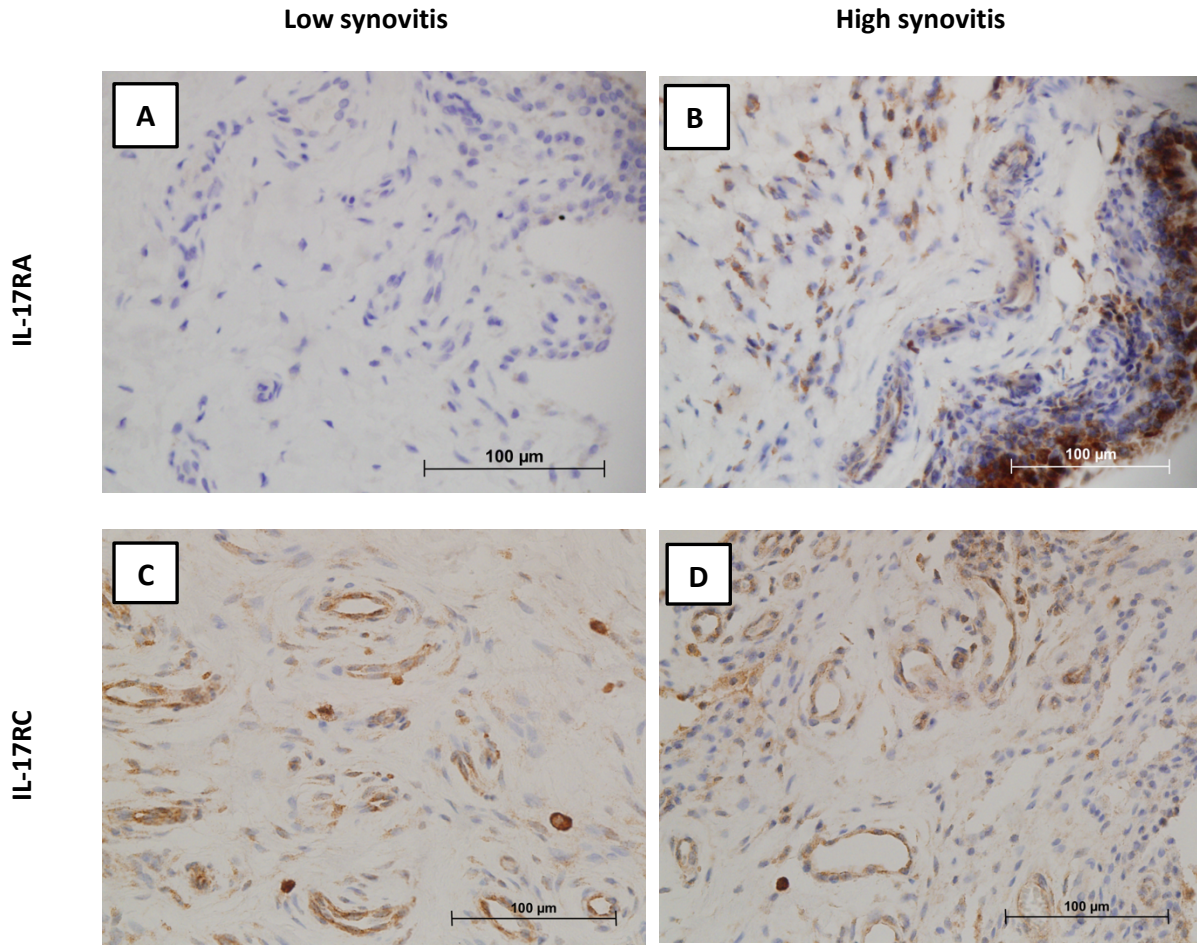


Figure 3-9. *Protein expression of IL-17RA (A, B) and IL-17RC (C, D) in patients with knee injury who have a high risk of developing post-traumatic OA with either a low or high inflammatory (synovitis) score. Antibodies were visualised with DAB (brown) and counterstained with haematoxylin (blue). Images were taken at 40x magnification.*

Using a semi-quantitative analysis of the staining, we found that IL-17RA protein expression (Figure 3-10A,B) was significantly correlated with synovitis score (p=0.036), and there was a statistically

significant difference between the IL-17RA expression of patients with a low and high synovitis score (p=0.0146). However, as visual inspection suggested, IL-17RC protein expression (Figure 3-10C,D) was not correlated with synovitis score (p=0.38), and there was no difference between patient with low or high synovitis when looking at IL-17RC expression (p=0.88).

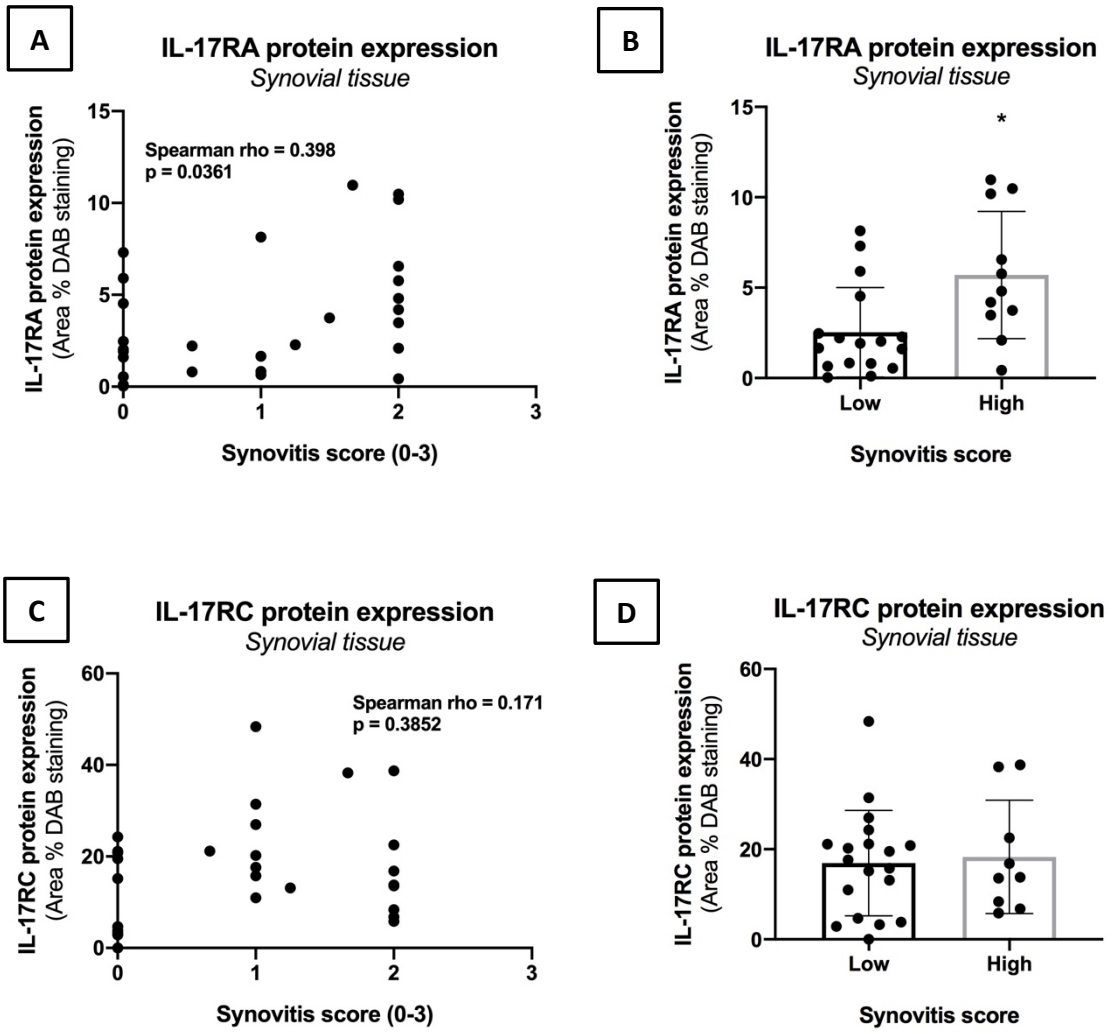


Figure 3-10. *Overview of the percentage of area stained with DAB for IL-17RA (A, B) and IL-17RC (C, D). (A, C) Protein expression for IL-17RA (A) and IL-17RC (C) for each patient plotted against their synovitis score. (B, D) Protein expression for IL-17RA (B) and IL-17RC (D) for each patient grouped by low (0-1.45) or high (1.46-3.0) synovitis score. * = p<0.05, ** = p<0.01, *** = p<0.001. (A) Correlation test; (B) Mann-Whitney test. IL-17RA: total n=28; low n=18, high n=10. IL-17RC: total n=28; low n=19, high n=9.*

The protein expression of IL-17RA and IL-17RC were plotted against clinical scores from these patients to study any possible correlation between the expression of these receptors and the clinical scores of the patients. However, neither IL-17RA (Figure 3-11) nor IL-17RC (Figure 3-12) did not show any trends or signficant correlations with any of the clinical scores tested.

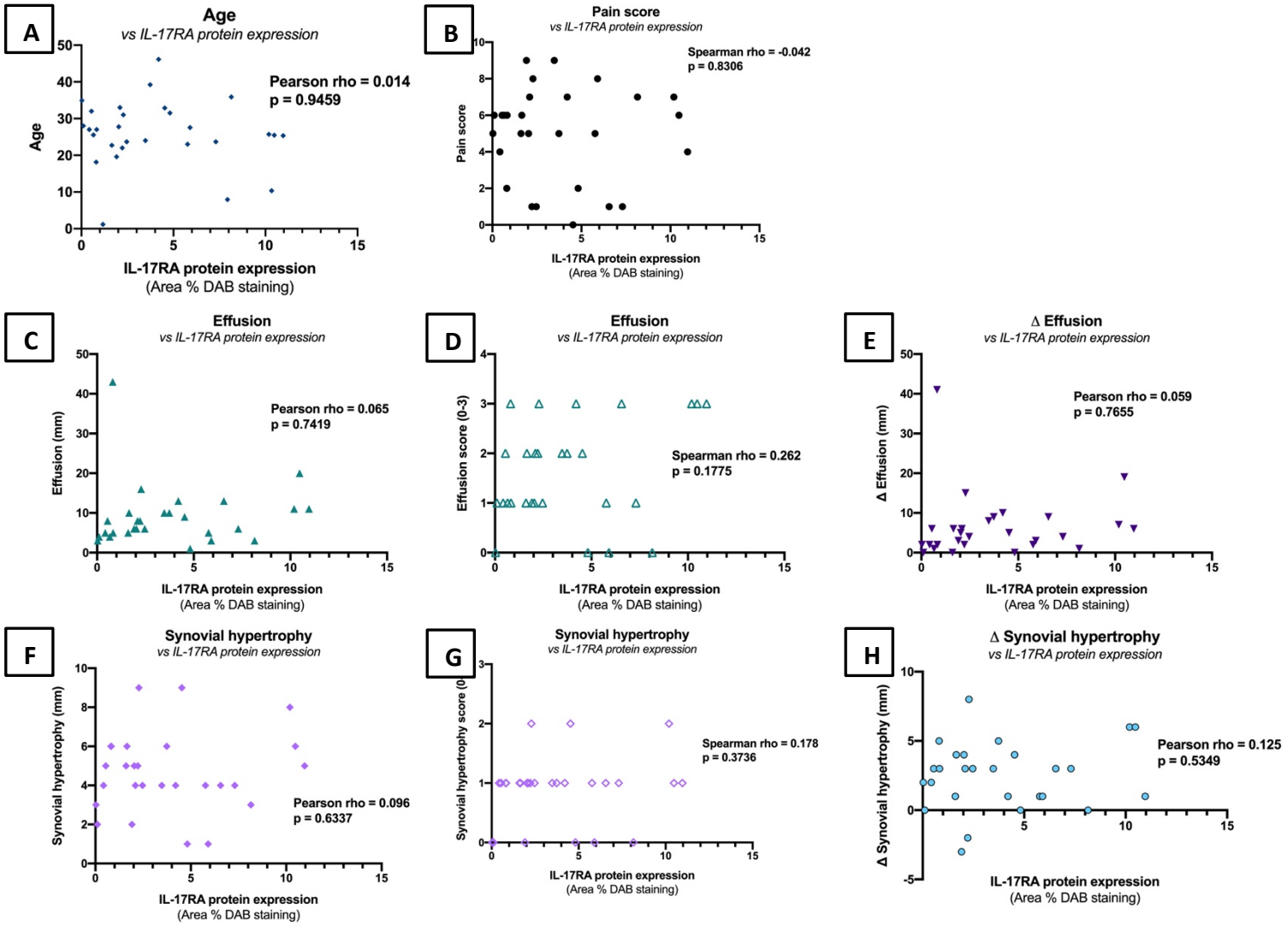


Figure 3-11.
Correlation of clinical scores with IL-17RA protein expression. (A) Age, (B) Pain Score, (C) Effusion (mm), (D) Effusion score (0-3), (E) Delta Effusion, (F) Synovial hypertrophy (mm), (G) Synovial hypertrophy score (0-3), and (H) Delta synovial hypertrophy. (A,C,E,F,H) Parametric correlation test. (B,D,G) Non-parametric correlation test. (A-E) n=28; (F-H) n=27.

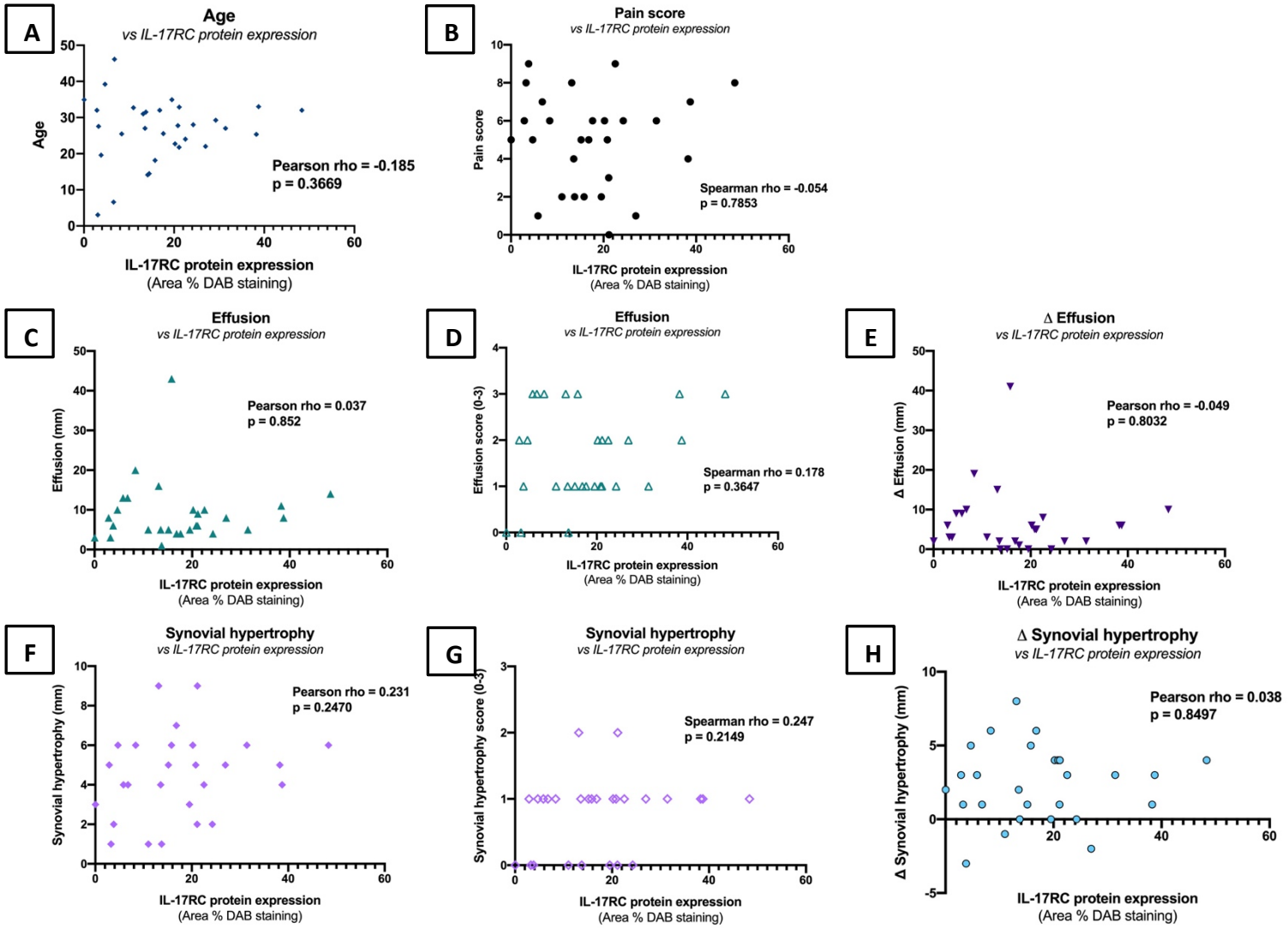


Figure 3-12.
Correlation of clinical scores with IL-17RA protein expression. (A) Age, (B) Pain Score, (C) Effusion (mm), (D) Effusion score (0-3), (E) Delta Effusion, (F) Synovial hypertrophy (mm), (G) Synovial hypertrophy score (0-3), and (H) Delta synovial hypertrophy. (A,C,E,F,H) Parametric correlation test. (B,D,G) Non-parametric correlation test. (A-E) n=28; (F-H) n=27.

3.6.4 Treatment with a single cytokine does not increase IL-17R mRNA expression

To confirm that chondrocytes and synovial fibroblasts from end-stage OA patients can express IL-17RA and IL-17RC, these cells were cultured in D10 media and mRNA expression of *IL17RA* and *IL17RC* was determined by RT-qPCR. mRNA expression of both *IL17RA* and *IL17RC* was found in chondrocytes and synovial fibroblasts, which indicates that these cells are able to produce IL-17RA and IL-17RC. It is worth noting that in these end-stage OA cells *IL17RA* was more highly expressed than *IL17RC* (Figure 3-13) with a difference of 2.03 CT (n=5, 0.84-3.18) in chondrocytes and 3.39 CT (n=4, 2.79-4.20) in synovial fibroblasts. As the expression of *IL17A* or *IL17F* mRNA could not be detected with the RT-qPCR methods used, it was not possible to study the expression of these genes using this method.

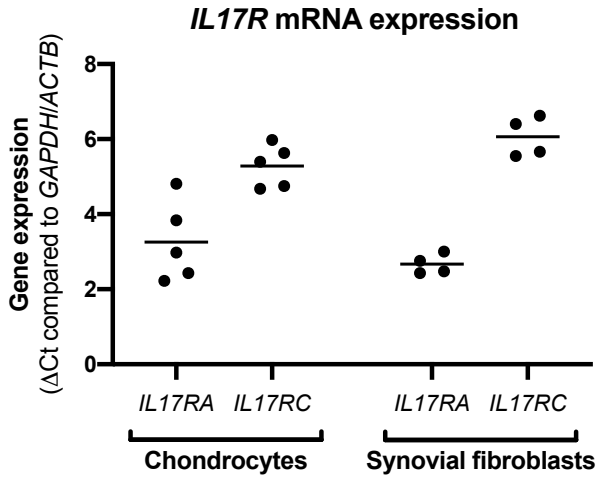


Figure 3-13. *Relative mRNA expression of IL17RA and IL17RC in chondrocytes and synovial fibroblasts from end-stage OA patients. Expression of IL17RA and IL17RC mRNA was measured by RT-qPCR using GAPDH and ACTB as reference genes. Chondrocytes: n=5, synovial fibroblasts: n=4.*

Given the increase in IL-17RA expression in patients with high synovitis, we treated chondrocytes and synovial fibroblasts with OA-associated cytokines to study their ability to regulate the mRNA expression of *IL17RA* and *IL17RC* (Figure 3-14). Although some cytokines induced small changes in expression (IFN-γ decreased and IL-1β increased expression of *IL17RA*; and IL-4 and TGF-β1

decreased expression of *IL17RC*), no statistically significant differences were found in the expression of *IL17RA* or *IL17RC* between vehicle control treated cells and cells treated with one of the cytokines. Only treatment with IL-4 in chondrocytes trended towards significance with a small downregulation in *IL17RC* expression (p=0.057).

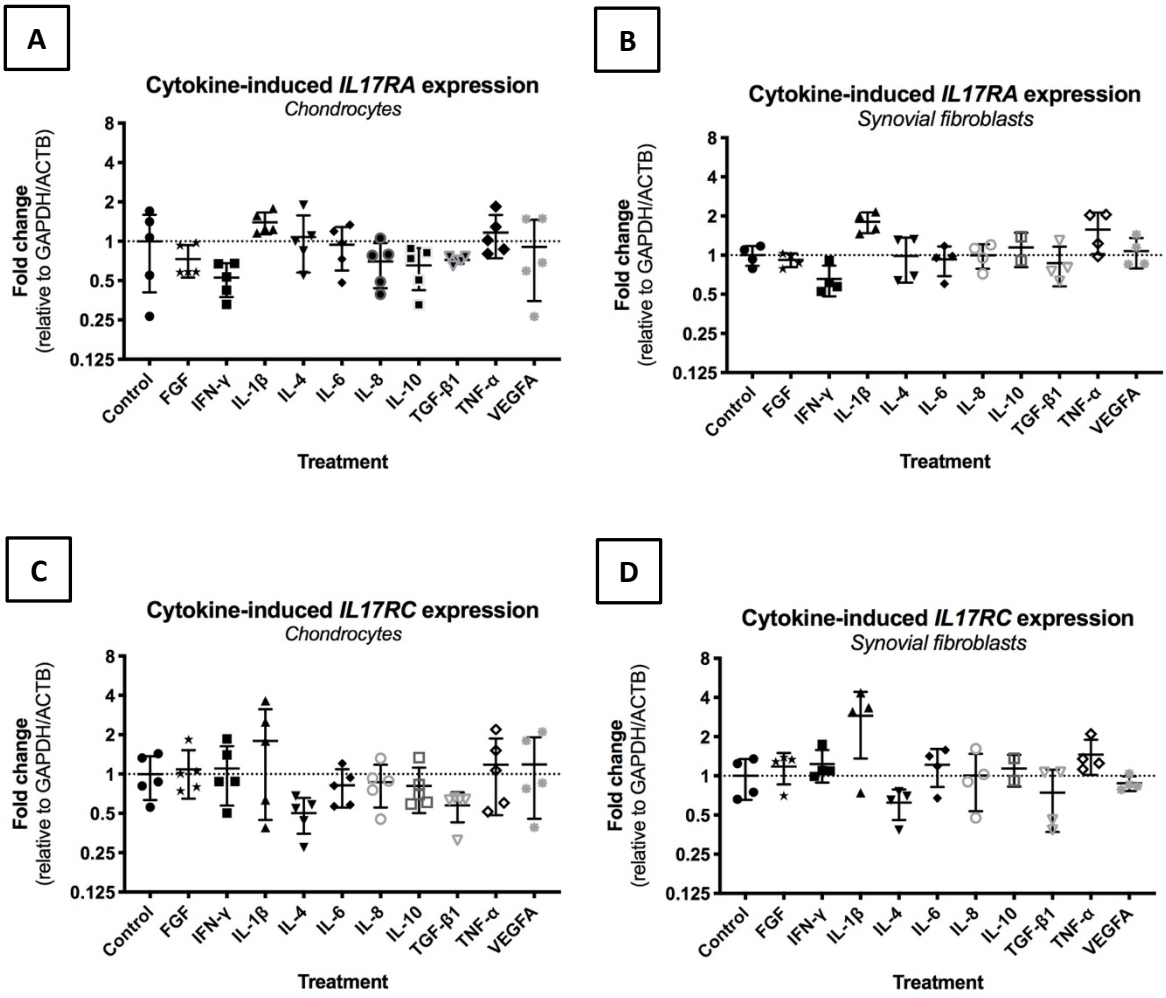


Figure 3-14. *mRNA expression of IL17RA (A, B) and IL17RC (C, D) in chondrocytes (A, C) and synovial fibroblasts (B, D) from end-stage OA patients after treatment with OA-associated cytokines. Cells were treated with vehicle control, FGF (10 ng/ml), IFN-γ (20 ng/ml), IL-1β (10 ng/ml), IL-4 (10 ng/ml), IL-6 (10 ng/ml), IL-8 (10 ng/ml), IL-10 (10 ng/ml), TGF-β1 (4 ng/ml), TNF-α (5 ng/ml), or VEGFA (10 ng/ml) in serum-free media for 24 hours. Expression of IL17RA and IL17RC mRNA was measured by RT-qPCR using GAPDH and ACTB as reference genes. * = p<0.05, ** = p<0.01, *** = p<0.001. (A-D) Friedman test. Chondrocytes: n=5, synovial fibroblasts: n=4.*

3.6.5 IL-17RA shows a trend towards a correlation with CD68 and CD206

As IL-17RA, CD68, and CD206 all correlate with synovitis scores, the correlation between IL-17RA and the expression of these macrophage markers was studied. Correlation between IL-17RC and IL-17A was assessed a control as these proteins were not found to be correlated with synovitis score. As for each antibody staining a selection of the full set of specimens was stained due to limited availability of each specimen, not every specimen was stained with all the antibodies used in this study. IL-17RA was significantly correlated with both CD68 (p=0.0281) and CD206 (p=0.0073) (Figure 3-15A). While, no trend was seen with IL-17RC and CD68 (p=0.8812), it did show a trend with CD206 p=0.0528). IL-17A did not shown any trends with the macrophage markers (CD68 p=0.9443, CD206 p=0.3439) (Figure 3-15B,C). It should be emphasized that correlation does not provide any information on a possible causal relationship between these markers.

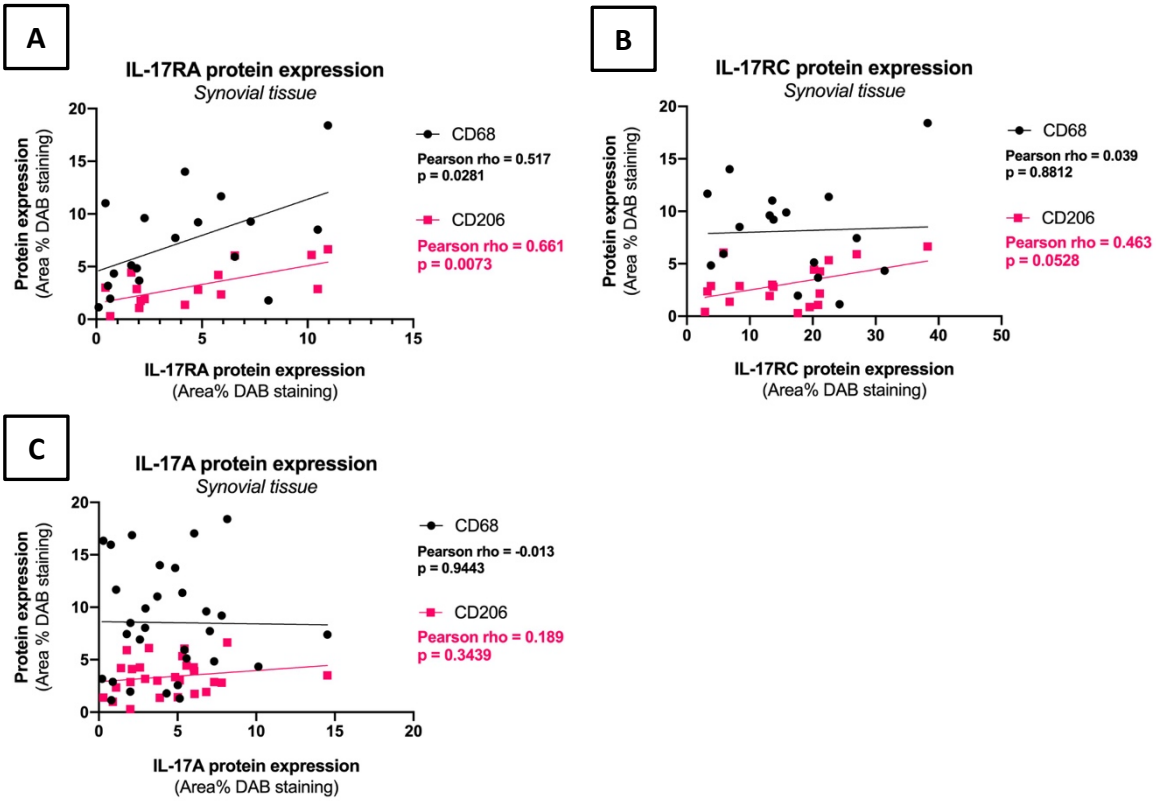


Figure 3-15. *Correlations between the percentage area stained with DAB (area% DAB staining) of IL-17 receptors and macrophage markers. Protein expression of IL-17RA (A), IL-17RC (B), or IL-17A (C), was plotted against the protein expression of CD68 (**black**) and CD206 (**pink**).(A-C) Correlation test. (A) CD68: n=18, CD206: n=15; (B) CD68: n=17, CD206: n=18; (C) CD68: n=30, CD206: n=27.*

3.6.6 IL-17 receptors can co-localise with macrophage markers

As IL-17RA shows a trend towards correlation with macrophage markers CD68 and CD206, which was not the case for IL-17RC, a pilot study was carried out to assess the potential co-localisation of these markers. Two synovial sections from the same patient (synovitis score = 2) were stained with a combination of CD68, IL-17RA or IL-17RC, and CD206. Although IL-17RA did show definite co-localisation with CD68 and CD206 individually (Figure 3-16), co-localisation of IL-17RA was especially obvious with cells that expressed both CD68 and CD206. However, expression of IL-17RA was not limited to the cells that express these macrophage markers. The expression of IL-17RA in the absence of these markers was especially obvious on a vessel (Figure 3-16H), which did not show any overlap with CD68 or CD206.

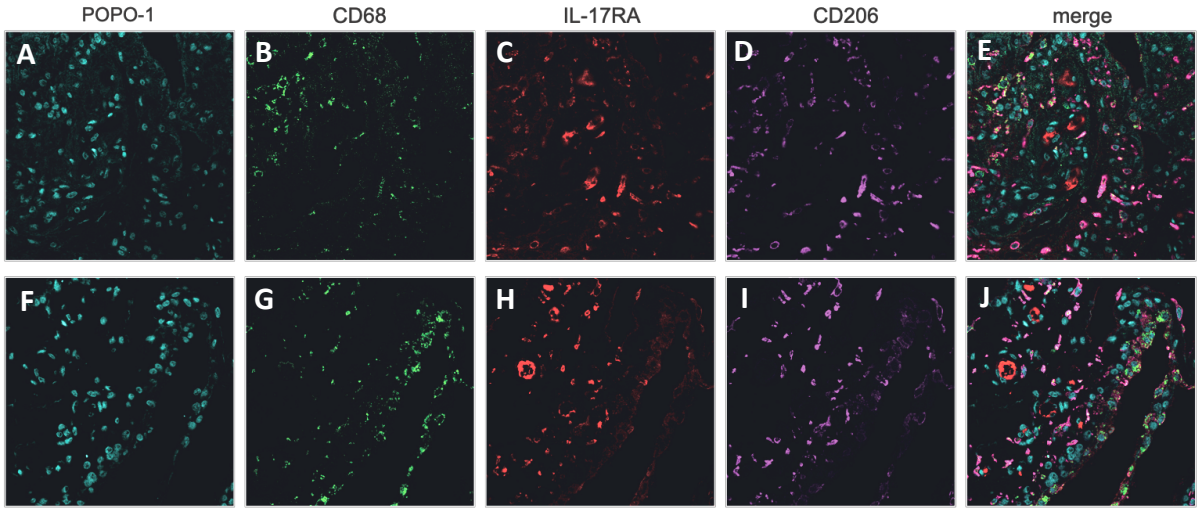


Figure 3-16. *Expression of macrophage markers and IL-17RA in two parts of a synovial section of a patient with a knee injury who has a high risk of developing post-traumatic OA (synovitis score = 2). Confocal images show expression of CD68 (**green**), IL-17RA (**red**), and CD206 (**purple**), and were counterstained with POPO-1 (**blue**) to visualise nuclei. A merged image is provided to look at the co-expression of markers. Images were taken at 40x magnification with oil.*

IL-17RC expression was also found to be co-localised with both CD68 and CD206 (Figure 3-17). Similar to IL-17RA, IL-17RC co-localised especially with cells that expressed both CD68 and CD206, and the expression of IL-17RC was not limited to these cell types.

Another noticeable feature in both sets of staining is that while CD68 is expressed in both the lining and sub-lining of the synovial tissue, CD206 expressions seems to localise primarily to the sub-lining, which is especially clear in Figure 3-16G,I and Figure 3-17B,D. This finding is in accordance with previous studies.

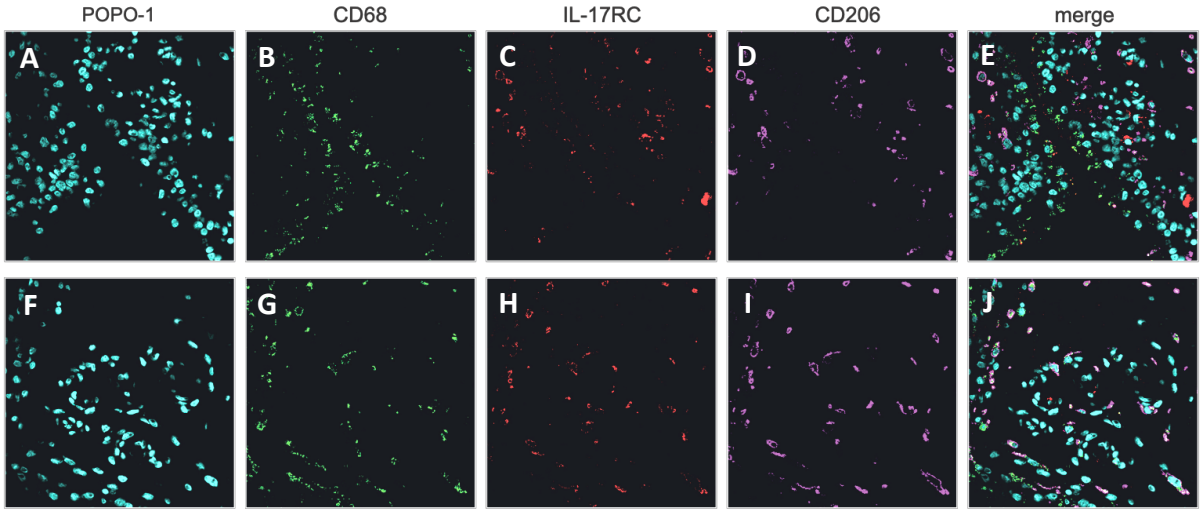


Figure 3-17. *Expression of macrophage markers and IL-17RC in two parts of a synovial section of a patient with a knee injury who has a high risk of developing post-traumatic OA (synovitis score = 2). Confocal images show expression of CD68 (**green**), IL-17RA (**red**), and CD206 (**purple**), and were counterstained with POPO-1 (**blue**) to visualise nuclei. A merged image is provided to look at the co-expression of markers. Images were taken at 40x magnification with oil.*

3.7 Discussion

3.7.1 Aim of the chapter

Clinical and animal studies have implicated synovitis, which is characterised by an infiltration of macrophages, as well as IL-17A and its receptors in the pathophysiology of OA. However, little is known about the expression of IL-17A and its receptors in OA and how this could relate to synovitis. Therefore, this chapter aimed to study whether the expression of macrophage markers in synovial tissue from patients with knee injury who have a high risk of developing post-traumatic OA correlates with synovitis or clinical scores. Subsequently, this chapter characterised the expression of IL-17A, IL-17RA, and IL-17RC in synovium from patients with knee injury who have a high risk of developing post-traumatic OA and investigated whether the expression of these IL-17-related proteins correlates with synovitis score, macrophage marker expression or clinical scores. Finally, this study aimed to investigate whether OA-related cytokines were able to increase the mRNA expression of IL-17 receptors in cells from end-stage OA patients.

3.7.2 Macrophage markers correlate with synovitis score

The macrophage markers CD68 and CD206 were expressed in the synovium of all patients tested in this study. CD206 and especially CD68 were highly correlated with synovitis score. To our knowledge, this is the first time that macrophages markers have been shown to significantly increase with synovitis score in a cohort of knee injury patients who have a high risk of developing post-traumatic OA. In addition, CD68 trended towards correlation and CD206 was significantly correlated with clinical synovial hypertrophy score. As macrophages are large granular cells, it is likely that the infiltration of these cells could significantly contribute to the hyperplasia and hypertrophy of synovial tissue, which was already hypothesized 30 years ago[196].

Immunofluorescence co-staining revealed that while CD68 was present in both the lining and sub-lining of the synovium, CD206 was mainly present in the sub-lining. Both the infiltration of

macrophages with OA synovitis as well as the presence of CD206 in the sub-lining of OA synovium are in accordance with previous studies[127,184]. However, more research with additional markers should be done to confirm the presence of macrophages with M2-type markers in the sub-lining as well as to identify the specific subtype of macrophages present in the lining of this tissue.

3.7.3 IL-17A and its receptors are expressed in synovium from patients at risk of developing OA

The immunohistochemistry staining confirmed that IL-17A was expressed throughout the tissue in each synovial section tested. The expression of IL-17A did not correlate with histological synovitis score or any clinical scores, and did not noticeably co-localise with CD3 and CD45 staining. Expression of IL-17RA and IL-17RC, the IL-17 receptors that facilitate the signalling of IL-17A, were found in each section of synovium, confirming that this tissue is able to respond to IL-17A, and the related cytokines IL-17F and IL-17AF. While there was a relatively constant expression of IL-17RC, the expression of IL-17RA was more variable, increasing with synovitis score. Therefore, a rise in IL-17RA due to inflammatory or mechanical cues in OA could lead to more IL-17RA/IL-17RC complexes being formed, thereby having the potential to make the tissue from patients with high synovitis more responsive to IL-17-cytokines, which could in turn lead to a stronger inflammatory response in these patients. In short, the changes in IL-17RA due to mechanical or inflammatory stimuli could contribute to the development of the inflammatory environment in OA synovium.

However, to increase the number of IL-17RA/IL-17RC complexes that can form, these receptors need to be expressed by same cells. As CD68- and CD206-expression both correlated with synovitis score, and both the macrophage markers show a trend towards a correlation with IL-17RA, it could be that the increase in IL-17RA is a result of macrophage infiltration. Immunofluorescence co-staining of CD68, IL-17RA or IL-17RC, and CD206 in synovium from patients who have a high risk of developing post-traumatic OA revealed that IL-17 receptors co-localised with CD68 and CD206, but that the

expression of these receptors was not limited to these areas. However, as IL-17 signalling mainly occurs in resident stromal cells, future research should focus on the role of IL-17 in the predominant stromal cells – fibroblasts – in cartilage and synovium derived from patients with OA.

3.7.4 Single OA-relevant cytokines do not increase IL17R mRNA expression

Both chondrocytes and synovial fibroblasts derived from end-stage OA patients express *IL17RA* and *IL17RC* mRNA, confirming their ability to respond to IL-17A and its family members IL-17F and IL-17AF. As cartilage breakdown in the cardinal feature of OA, it is important to understand that chondrocytes can be affected by IL-17 cytokines in order to understand if IL-17 could have a role in the breakdown in cartilage seen in OA. Therefore, the mRNA expression of IL-17 receptors in chondrocytes confirms that IL-17 produced in synovium could affect the cartilage, potentially through paracrine signalling of synovially produced IL-17 cytokines.

To determine whether the inflammatory environment could drive the increased expression of IL-17RA seen in fibroblasts from OA synovium, we studied the effect of several OA-relevant cytokines on the *IL17RA* and *IL17RC* mRNA expression in cells from end-stage OA patients. Even though many OA-relevant cytokines were tested, none significantly altered *IL17RA* or *IL17RC* mRNA expression by themselves. However, as these cytokines are never present in isolation, these cytokines instead are likely to work synergistically. An important example is IL-17A itself, which, in combination with other cytokines including IL-1β and TNF-α, can work synergistically to enhance inflammation by stabilising mRNA[81,197]. Furthermore, we observed an increase in IL-17RA protein expression in patients who have a high risk of developing OA, while the cytokine treatment was done on cells from patients with end-stage OA. These cells already have a higher mRNA expression of *IL17RA* than *IL17RC* and might have become resistant to further changes in the *IL17RA* mRNA expression. Future work could treat cells with OA synovial fluid to replicate the inflammatory environment more accurately. In addition, ideally cells from patients with high risk of developing OA (such as patients post knee injury) or

patients diagnosed with early OA should be used in this experiment as they might be more responsive than cells from end-stage OA patients who have been in a chronic disease environment.

3.7.5 Limitations

As with all studies, this investigation has several limitations in addition to those already mentioned. Therefore, this section will describe some further limitations of this study in detail.

3.7.5.1 The use of histological and clinical scores

Both a strength and weakness of this study is the use of histological synovitis score and clinical scores. Patients with OA mainly present to the clinic due to symptoms including pain and stiffness. As clinical scores through questionnaires and ultrasound are the easiest and least invasive scores to obtain, it is important that to use these scores in biological research to study their relationship with biological and molecular signatures, as thoroughly discussed in Mimpen *et al.*[19]. However, there are several limitations to these scores. Although some studies have revealed correlations between clinically adopted imaging, function and pain scores, few studies have been able to predict clinical outcomes due to the variability between patients[198]. In addition, scores from questionnaires, especially pain scores could be heavily influenced by physiological fluctuation, environmental factors, and motivation, while imaging scores can vary between physicians and equipment used[199]. However, due to ethical constraints and the number of patients that present to the clinic with OA it is currently not feasible to get more advanced longitudinal imaging data or biopsies from these patients. Therefore, it remains important that currently utilised clinical scores are intensively studied for their potential relationship with disease pathophysiology.

3.7.5.2 Immunohistochemistry: antibody specificity and semi-quantitative analysis

Although immunohistochemistry enables the study of the expression and localisation of proteins, it has several limitations. The first limitation concerns the specificity of antibody staining. Even though

several antibodies were tested, the best antibody was optimised, and negative controls were used for each protein of interest, some antibodies still showed unspecific staining. Especially in the IL-17A-stained tissue there was strong unspecific staining of the matrix in many sections, while there were a few other sections that showed significantly weaker staining throughout the whole tissue than most other sections.

Second, while immunohistochemistry methods are mainly designed to be qualitative, different methods have been developed to use these images for a semi-quantitative analysis of the staining. While these analyses have been very helpful tool in providing a more quantitative analysis of the data, these outputs need to be used with caution. The first problem is that there will always be images that include parts with no tissue due to the natural shape of tissue biopsies, which will be seen as non-stained tissue. Although a lot of time was invested to crop the images in this study such that only fibrous tissue was present on the image, images that include parts with no tissue or fatty tissue will remain and might influences the results of these analyses. The second problem is that unspecific staining can heavily influence the results. For example, due to the unspecific staining of the matrix in most sections stained for IL-17A, the threshold for DAB staining for semi-quantitative analysis for IL-17A was set very high in an attempt to limit the counted area stained with DAB to the specific IL-17A staining. However, this creates the risk that due to slight differences in intensity of staining between sections, in some sections the background staining might be counted as real staining, while in other sections the actual positive staining might not reach the high threshold. As the background staining is often a problem with cytokine staining, it might be beneficial to count cells with cytoplasmic staining, thereby giving an idea of the percentage of cells express/produce the cytokine of interest. The disadvantage of this is that cytokines that are secreted into the extracellular matrix would not be considered in this quantification. Although this staining gives useful information about the localisation of IL-17A+ cells, other methods such as FACS, RNA-scope, ELISA, or proteomics, are more suitable if quantification of IL-17A in the tissue is desired.

Finally, as it was not possible to use sequential sections of each piece of synovial tissue, the sections that were stained for each protein of interest could vary in cellular and protein expression between sections. In the future, (immunofluorescent) co-staining could be used to confirm correlations between the expression of different proteins that were found in this study.

3.7.6 Future work

In addition to the future work mentioned in the previous sections of this discussion, some findings from this study would benefit from further research.

3.7.6.1 Expression of IL-17A and its receptor on macrophages

Even though the main responses to IL-17A occur in epithelial, endothelial and fibroblasts cells, macrophages have been reported to be able to respond to IL-17A and express IL-17RA and IL-17RC[200]. There are several studies that have looked at the effect of IL-17A on macrophages in different tissues. A study that looked at the effect of IL-17A on mouse macrophages showed that subcutaneous IL-17A injection in mice caused an activation of macrophages, increasing the number of CD206-positive cells on histology and total CD206 protein expression using Western blots. When using an atopic dermatitis mouse model, which has a chronic skin inflammation dependent on IL-17A, IL-17A neutralisation did not decrease CD206+ cells but did decrease CD206 protein expression, showing a potential role for IL-17A in the regulation of CD206[201]. A study using macrophages derived from the human leukemic cell line THP-1 found that IL-17A caused a dose-dependent increased the expression of macrophage markers CD163 and CD206, which was dependent on NF-κB p65[202]. The effect of IL-17A on cytokine production by macrophages was studied in RAW 264.7 cells. IL-17A induced the dose- and time-dependent production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, through MAP kinases ERK1/2 and p38, NF-κB and AP-1 pathway[203]. Therefore, if the

increase of IL-17 receptors would be due to the influx of macrophages, the presence of IL-17A would likely be able to contribute to OA synovitis.

Thus, future research should investigate which cells in synovium express both IL-17RA and IL-17RC, and are thus able to respond to IL-17A. As the main responses to IL-17A occur in epithelial, endothelial and fibroblasts cells, these cells will each require focus in future research into the role of IL-17 in OA synovium[200].

3.7.6.2 Expression of IL-17A and its receptor on blood vessels

A notable feature in this study is the clear expression of both IL-17 receptors IL17RA and IL17RC and IL-17A in cells surrounding vessels in synovium. Expression of these proteins were seen in virtually every vessel in each tissue section, whereas CD68 and CD206 staining was not seen at all in vessels. Previous studies have also noted this and show that IL-17A can promote neutrophil recruitment to sites of inflammation by activating endothelial cells through the p38 MAP kinase and STAT3 pathways[204,205], although it is also postulated that it is the microvascular pericytes and not endothelium that gets activated[206]. Other studies further emphasize a role for IL-17 in the endothelium by showing that IL-17A can promote the production of angiogenic factors, drive vascular inflammation, endothelial dysfunction, and angiogenesis[207–209]. Pickens *et al.* have shown that IL-17A contributes to angiogenesis in RA. The high number of vessels that seen in the synovial sections in this chapter together with the presence of IL-17RA, IL-17RC, and IL-17A suggests that further studies should look into angiogenesis in OA including the potential role of IL-17A in this.

3.7.6.3 Effect of IL-17A on OA fibroblasts

The observed increase in IL-17RA protein expression will most likely lead to increased IL-17A signalling in this tissue. However, currently very little is known about the effects of IL-17A, as well as IL-17F and IL-17AF who also signal through IL-17RA/IL-17RC, in OA synovium. As previous research

has shown that IL-17 signalling mainly occurs in resident stromal cells, the main focus of this should be on these cells. Although we know that IL-17A can induce the production of inflammatory mediators in fibroblasts, it is important to study the effects on the specific target cells, as there are different studies emphasizing the importance of cell-type-specific responses to IL-17A[81]. Ideally cells would be used from patients after knee injury (at risk of developing OA), early-stage OA, and end-stage OA to understand the role of IL-17A and potential disease-stage specific effects throughout disease. However, as it is challenging to obtain tissue from knee injury or early-stage OA patients, cells from end-stage OA patients obtained during total knee replacements can be a good starting point. As both cartilage and synovium can be obtained during this time, it would be interesting to see if IL-17A affects resident cells from these two tissues differently.

3.7.7 Conclusions

This chapter aimed to study the expression of macrophage markers CD68 and CD206, as well as IL-17A and its receptors IL-17RA and IL-17RC in synovium of patients who have a high risk of developing post-traumatic OA. Although macrophage infiltration and IL-17A are both implicated to contribute to the inflammatory environment in the OA knee, little research has been done to confirm these hypotheses. This study confirms that the macrophage markers CD68 and CD206 are expressed in synovium from patients at risk of developing OA, and shows that these markers are positively correlated with synovitis score, which concurs with the notion that macrophages are a key driver of synovitis. In addition, macrophages showed a trend toward correlation with clinical hypertrophy scores, indicating that they might contribute to the synovial hypertrophy seen in these patients.

Furthermore, this chapter confirms that the cytokine IL-17A and its receptors IL-17RA and IL-17RC are expressed in human synovial tissue. No significant differences were seen in IL-17A expression between patients with low or high synovitis in a cohort of patients who have a high risk of developing post-traumatic OA. While the expression of receptor IL-17RC in synovium was relatively stable, it was

shown for the first time that IL-17RA expression increases with synovitis score, suggesting that this can increase sensitivity of this tissue to IL-17. Interestingly, IL-17RA expression was found to trend towards correlation with CD68 and CD206. This study showed that IL-17RA expression could co-localise with these macrophage markers, but was not limited to these areas.

Finally, this study found that both chondrocytes and synovial fibroblasts from end-stage OA patients are able to make *IL17RA* and *IL17RC* mRNA, but any single OA-relevant cytokine tested in this study did not change IL-17R mRNA expression in these cells. As IL-17-signalling mainly occurs in resident stromal cells, it is important that the main focus of further research investigates whether the inflammatory environment in the OA joint can affect IL-17RA expression and studies what effect IL-17 can have on these resident stromal cells.

In conclusion, this study shows that protein expression of IL-17 receptors is present in synovium from patients at risk of developing OA. In addition, mRNA expression of these receptors is detected in synovial fibroblasts and chondrocytes from patients with end-stage OA cells, confirming that these resident joint cells can respond to IL-17A. IL-17A was present in synovium from patients at risk of developing OA, confirming that this cytokine could play a role in disease. Expression of macrophage markers CD68 and CD206, as well as expression of IL-17RA, was positively correlated with synovitis score in these patients. Future research should focus on the role of IL-17A and its related cytokines, not only in OA synovium, but also in other important joint tissues including cartilage, to understand its potential role in disease.

3.7.8 Key findings

- Macrophage markers CD68 and CD206 are expressed in synovium from patients at risk of developing, and expression of these markers positively correlates with synovitis score.
- IL-17 receptors IL-17RA and IL-17RC are expressed in synovium of patients at risk of developing OA, and IL-17RA expression positively correlates with synovitis score.
- IL-17A was present in synovium from patients at risk of developing OA, confirming that this cytokine could play a role in disease.
- Chondrocytes and synovial fibroblasts from end-stage OA patients express *IL17RA* and *IL17RC* mRNA, confirming that they can respond to IL-17A

Chapter 4

IL-17 treatment increases expression of OA-related inflammatory and matrix genes in end-stage OA chondrocytes and synovial fibroblasts

4.1 Abstract

4.1.1 Background

IL-17 is a family of 6 cytokines from which the homodimers IL-17A and IL-17F, and the heterodimer IL-17AF are most well-studied. These IL-17-cytokines have a pivotal function in host defence, but have also been linked to several inflammatory diseases due to their ability to stimulate resident fibroblasts to release mediators that contribute to inflammation, angiogenesis, and matrix destruction. Results from clinical, genetic, and animal studies suggest a role for IL-17A in OA. In this thesis, a potential Th17 cell population (CCR6+ T cells) was identified in a subset of end-stage OA patients. In synovium of patients at risk of OA, expression of IL-17A and its receptors IL-17RA and IL-17RC was identified, and IL-17RA positively correlated with synovitis score. However, it is still unclear how IL-17A, and its family members IL-17F and IL-17AF, can contribute to OA pathophysiology. Thus, this chapter aims to identify changes in gene expression and activation of intracellular signalling pathways induced by IL-17 cytokines in fibroblasts derived from patients with end-stage OA.

4.1.2 Methods

Chondrocytes and synovial fibroblasts derived from 6 end-stage OA patients were treated with 10 ng/ml IL-17A, IL-17F, or IL-17AF. RNA was isolated and libraries were made for RNA-Seq analysis. Data was analysed using DESeq2 in R. End-stage OA chondrocytes and synovial fibroblasts (n=3) were treated with IL-17A, IL-17F, or IL-17A/F for western blot analysis for the activation of ERK1/2, p38, and p65 NF-κB. End-stage OA chondrocytes and synovial fibroblasts were treated with IL-17A with or without the p38 inhibitor SB203580 (n=4) and NF-κB inhibitor JSH-23 (n=4), or clinically-used IL-17A antibody secukinumab (n=5) and gene expression assessed with RT-qPCR.

4.1.3 Results

Treatment with IL-17A, IL-17AF, or IL-17F significantly changed the expression of 856, 188, and 39 genes in chondrocytes and 330, 55, and 17 genes in synovial fibroblasts, respectively ($p<0.05$, log2 fold change +/-1). Pathway analyses showed that IL-17A (and to a lesser extent IL-17AF and IL-17F) regulated several OA-related pathways including immune-, matrix-, angiogenesis-, and complement-pathways. Secukinumab significantly inhibited IL-17A-induced gene expression in both chondrocytes and synovial fibroblasts. Western blot results confirmed that IL-17A induced significant activation of p38 and p65 NF-κB. IL-17AF caused a significant but less profound activation of ERK1/2, p38, and p65 NF-κB, while IL-17F did not induce activation of these pathways. However, p38 and NF-κB inhibitors were unable to inhibit IL-17A-induced gene expression.

4.1.4 Conclusions

This study shows that IL-17A (and to a lesser extent IL-17F and IL-17AF) induced changes in gene expression in OA-related pathways. The chronic, low-grade inflammation seen in OA fits well with the effects of IL-17A on inflammation; its effects on matrix destruction and angiogenesis further supports a role for IL-17A in OA pathophysiology. Secukinumab significantly inhibited IL-17A-induced gene expression, confirming that IL-17A is responsible for the changes in gene expression. Inhibitors of IL-17A-activated signalling pathways p38 and NF-κB did not inhibit IL-17A-induced gene expression, emphasizing the complexity of IL-17-signalling. As there are currently no disease-modifying OA drugs available, anti-IL-17 therapy could be investigated as a potential therapeutic option in selected patients with OA.

4.2 Background

4.2.1 Introduction

IL-17A and the first IL-17-binding receptor (IL-17RA) were discovered in the mid-1990s[210–212]. However, it was not until 2005 that IL-17A was found to be the signature cytokine of a new class of T-helper cells, Th17 cells. Subsequently it was shown that IL-17A was not uniquely produced by Th17 cells, but could also be produced by several other cell types including mast cells, neutrophils, macrophages, B cells, several types of T cells, as well as possibly by resident stromal cells[74–80]. One of the first functional studies of IL-17A, which was done with fibroblasts from rheumatoid arthritis (RA) synovial fibroblasts (resident stromal cells in synovium), showed that IL-17A could induce the production of IL-6 and IL-8, suggesting it directly linked inflammation and host defence. Since then, it has become clear that IL-17A is not only a key mediator in innate immunity, but can also increase inflammation and recruit immune cells contributing to chronic inflammation in diseases such as psoriasis and ankylosing spondylitis. In recent years, evidence from published clinical, animal, and genetic studies, have indicated a role for IL-17A in the pathophysiology of osteoarthritis (OA). In addition, this thesis has identified a potential Th17 population (CCR6+ T cells) in a subset of end-stage OA patients, and in patient at risk of developing post-traumatic OA, IL-17A and its receptors IL-17RA and IL-17RC were identified, and IL-17RA positively correlated with a histological synovitis score.

4.2.2 IL-17: a family of cytokines

Using homology-based cloning, 5 homologous genes to IL-17A were discovered, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F, which exist as homodimers and show conservation in their c-terminal region[213,214]. IL-17F is the most closely related to IL-17A as it shares 50% of its amino acid sequence, is localised in the same chromosomal region, and is co-produced by cells including Th17 cells and $\gamma\delta$ T cells[214–216]. IL-17A and IL-17F can act both as homodimers or as the heterodimer IL-17AF, and are the most well-studied members of the family[217].

IL-17A, IL-17F, and IL-17AF (from now on collectively called IL-17A/F) all signal through the same heterometric receptor complex, which consists of IL-17 receptor A (IL-17RA) and IL-17RC. From binding studies, it appears that IL-17RA has a 100-fold stronger affinity for IL-17A than IL-17F and 10-fold stronger than IL-17AF, while IL-17RC binds all three cytokines with a similar affinity, with a slight preference for IL-17F. When looking at changes in gene expression using microarray in RA synovial fibroblasts, these differences in binding affinity result in IL-17A being able to produce a 10-30 more potent induction of gene expression than IL-17F, with IL-17AF thought to induce an intermediate signalling strength[193,217–220]. While IL-17RA is expressed by nearly every cell type of the body with particularly high expression on immune cells, IL-17RC is mostly expressed by non-immune cells with lower expression by myeloid cells. Therefore, differences in binding affinities of IL-17A/F to IL-17RA and IL-17RC combined with differences in expression patterns of these receptor subunits could underlie potential differences in biological functions[221]. Relative concentrations of IL-17A, IL-17F, and IL-17AF in a tissue together with the expression of IL-17 receptor subunit might help elucidate which of these cytokines is involved in onset and/or progression of OA. As we have seen in Chapter 3 that there is an increase in IL-17RA expression in patients with increasing synovitis, IL-17A/F might become more important in patients in which the synovium is highly inflamed.

4.2.3 Role of IL-17 in host defence

IL-17A/F are highly evolutionary conserved, suggesting that they are important for survival. Indeed, it has been found that IL-17A/F have important roles in innate immunity, as they protect against fungal and bacterial infections particularly at epithelial barrier sites such as the skin, lungs, oral cavity, and the gastrointestinal tract[222]. This has been clearly demonstrated in mouse models, where mouse lacking *Il17a*, *Il17ra*, and *Il17f* genes are susceptible to a broad range of bacterial and fungal infections at mucosal surfaces. Patients with a genetic defect in a component of IL-17A/F signalling pathway have a narrower spectrum of pathogen susceptibility than in mice, but do show susceptibility to chronic mucocutaneous candidiasis and in some cases to cutaneous *Stapylococcus*

aureus infections, emphasizing the importance of IL-17A/F in immunity[223]. In addition, IL-17A/F has been implicated in tissue healing, which is at least partly due to the IL-17A/F-mediated promotion of keratinocyte proliferation after injury of the skin, as well as in the intestinal epithelium where IL-17A/F-signalling can promote intestinal healing[224–227].

Upon the loss of epithelial barrier integrity, cytokines including IL-6, IL-23, and IL-1β are released, which subsequently drive the release of IL-17A/F[228–230]. When IL-17A/F bind to its receptor, the receptor undergoes a conformational change to enable the interaction between the "similar expression to fibroblasts growth factor/IL-17R" (SEFIR) domain of the receptor and of the signalling adaptor Act1, also known as CIKS, which is required for all IL-17 signalling. IL-17A/F can then act through the canonical or non-canonical intracellular pathway (Figure 4-1)[231].

The canonical pathway relies on the E3 ligase activity of Act1, which mediates the ubiquitylation of TRAF6, which then leads to the activation of canonical NF-κB and MAP kinase pathways, including ERK, p38, and JNK, as well as C/EBPβ and δ transcription factors[231]. These different transcription factors can drive the transcriptional activation of IL-17A/F target genes, which leads to the induction of chemokines (such as CXCL1, CXCL2, and CXCL8) that attract myeloid cells to the site of infection or injured tissue, and increases the production of cytokines (such as IL-6 and G-CSF) that promote myeloid-driven innate inflammation. However, the effects of IL-17A/F on gene expression have been shown to differ depending on target cells, site, and, in case of host defence, pathogen[231,232]. In addition to the canonical pathway, binding of IL-17A/F to its receptor can cause the activation of second, non-canonical, pathway. Rather than the ubiquitylation of Act1, Act1 gets phosphorylated, which leads to TRAF2 and TRAF5 recruitment to the receptor complex. TRAF2 and TRAF5 in turn recruit several molecules that control mRNA turnover to enhance the half-life of post-transcriptional mRNAs: they sequester mRNA destabilizing factor SF2, and recruit the mRNA stabilizing factor HuR. The mRNA of chemokines and cytokines is inherently unstable to ensure that genes that promote

inflammation do not accumulate inappropriately and are eliminated when the threat is eliminated[233,234]. Therefore, these transcripts benefit from stabilization[221,231,235].

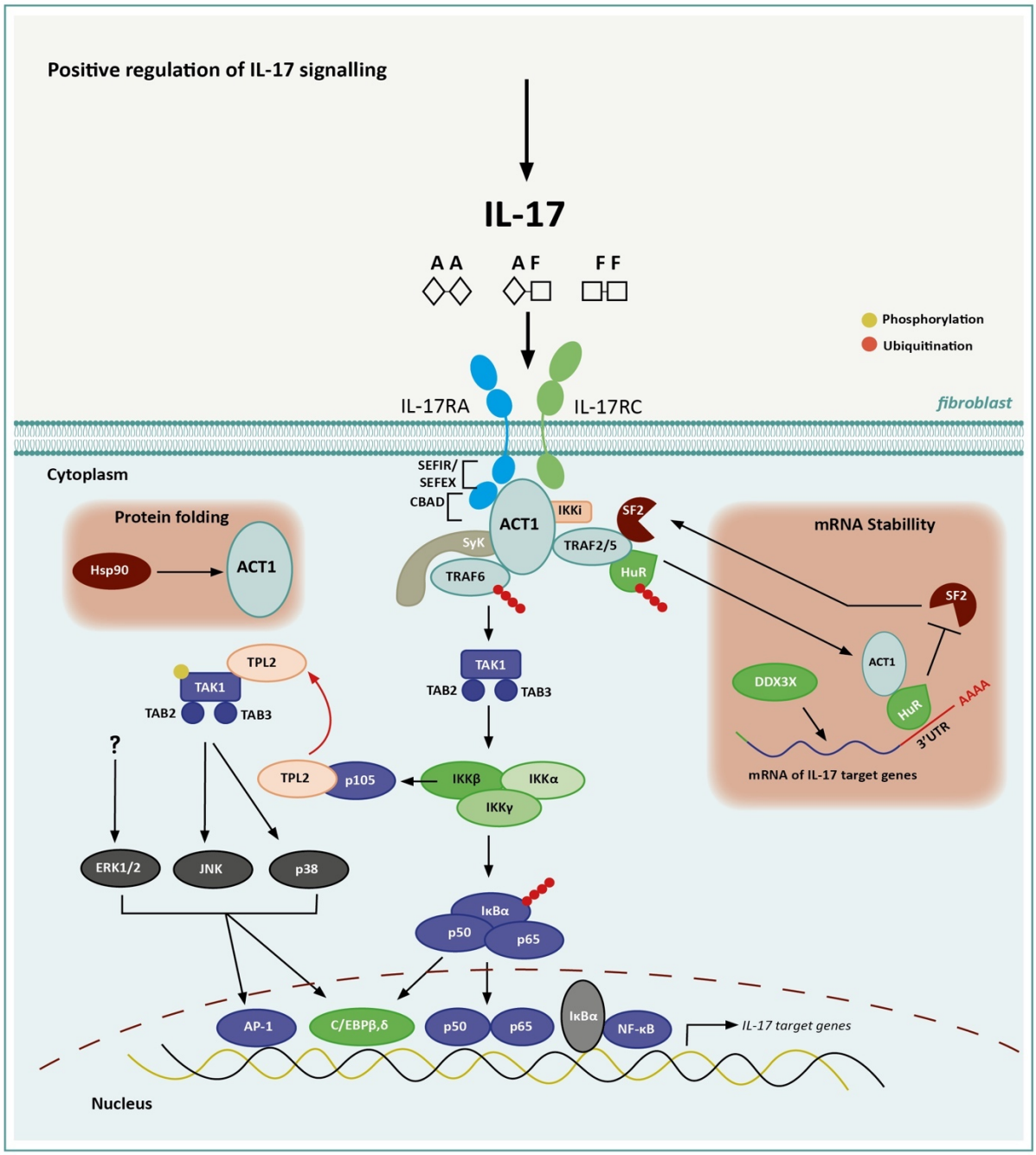


Figure 4-1. *Positive regulation of IL-17 signalling. Upon binding of IL-17A/F to the receptor complex IL-17RA/IL-17RC, a conformational change of the receptor enables the interaction between SEFIR and Act1. The canonical pathway relies on the ubiquitylation of TRAF6, which leads to the activation of canonical NF-κB and MAP kinase pathways, ultimately leading to transcriptional activation of IL-17A/F target genes. In the non-canonical pathway, Act1 gets phosphorylated, leading to the recruitment of TRAF2 and TRAF5 recruitment, which sequester mRNA destabilizing factor SF2, and recruit the mRNA stabilizing factor HuR. Modified from Amatya et al. (2017)*[236].

The outcome of activation of IL-17 receptor is influenced by both the cell type that is activated and the accompanying signals from other cytokines. This is in part due to synergistic interactions between IL-17A/F and other cytokines. Although the best-studied example of synergic effects is between IL-17A and the potent NF-κB activator TNF-α[237], many other mediators that work through a wide range of signalling pathways have been found to synergise with IL-17A/F. These mediators include interferon (IFN)-γ (activator of signal transducer and activator of transcription 1 (STAT1)), IL-13 (activator of STAT6), TGF-β (activator of SMADs), and other NF-κB activators such as IL-1β and B cell activating factor[61,238–242]. A recent study using different cell types (RA synovial fibroblasts, psoriasis skin fibroblasts, human umbilical vein endothelial cells, myoblasts from the muscle femoral quadriceps, and a hepatocyte cell line) has found that the effect of IL-17A or IL-17F alone, and the synergistic effects of IL-17A or IL-17F with IL-1β or TNF-α are dependent on cell type. In addition, while IL-17A alone induced stronger responses than IL-17F alone, this difference is reduced when acting in synergy with other cytokines[243]. While the mechanisms through which these synergistic effects are orchestrated are still unclear, it is likely that it is related to the modulation of the expression of positive and negative regulators of IL-17A/F signalling, such as Hsp90 which maintains the integrity of Act1, A20 (also called TNFAIP3) which deubiquitinates TRAF6, or downstream events pre- or post-transcription, especially IL-17A/F-induced RNA stabilisation (Figure 4-2)[81]. Although more research is needed to further understand the mechanism(s) through which this synergism occurs, it is interesting to hypothesize that changes in the environment in which IL-17A/F act could affect its role in health and disease.

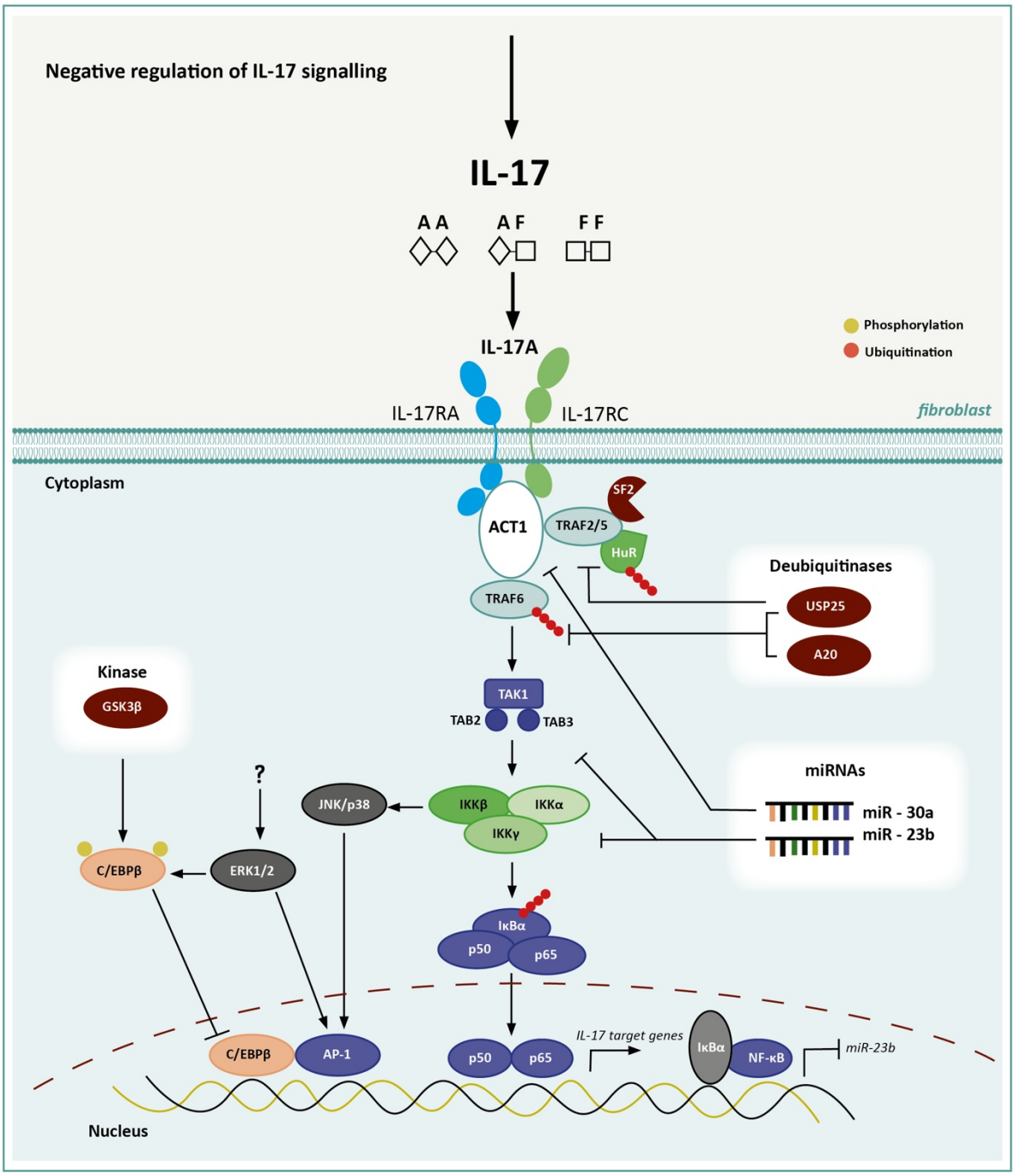


Figure 4-2. *Negative regulation of IL-17 signalling. A selection of negative regulators of IL-17 are A20 (which deubiquitinates TRAF6), GSK3β (which works together with ERK1/2 to phosphorylate C/EBPβ), and miRNAs miR-23b (which induces the degradation of TAB2, TAB3, and IKKα) and miR-30b (which induces degradation of the gene that encodes for Act1). Modified from Amatya et al. (2017)*[236].

4.2.4 Differentiating roles of IL-17A and IL-17F

As IL-17A/F all bind to the same receptor complex, they are assumed to have similar functions and trigger qualitatively similar signalling pathways[228,229]. However, IL-17A induces a more efficient cytokine production (such as IL-6 and IL-8 protein production as demonstrated in RA synovial fibroblasts)[244], it is implicated more strongly in driving autoimmunity than IL-17F. In contrast, while IL-17A and Il-17F are equally important in controlling the bacterial burden at an early stage of in *C. rodentium* infection, IL-17F is more critical in late stage than IL-17A[188]. Although research into the distinct roles of IL-17A and IL-17F in humans are extremely limited, studies in animal models have shown that these cytokines can have non-overlapping, or even opposite effects in mouse models of immune diseases. In a mice model of experimental autoimmune encephalomyelitis (EAE), knock-outs of IL-17A but not IL-17F showed a decrease in disease score, indicating that only IL-17A is required for the induction of EAE. In a mouse model of asthma, IL-17A knockout mice showed a reduction of eosinophil infiltration, while IL-17F knockout mice had a higher Th2 cytokine and eosinophil infiltration, suggesting that IL-17F has a protective role whereas IL-17A promotes inflammation. Lastly, in the dextran sulphate sodium-induced acute colitis mice model, IL-17A is protective, while IL-17F is pathogenic[245]. Although these results should be interpreted with caution as there are differences in IL-17 signalling between mouse and humans, these studies do suggest that the differences can be found between the biological functions of IL-17A and IL-17F. More research is needed to determine the differences in function between IL-17A and IL-17F in humans in health and disease, and what the role of IL-17AF is.

4.2.5 IL-17 in chronic inflammatory and autoimmune diseases

Even though IL-17A/F is vital for protection against bacterial and fungal infections, these host-protective functions can also cause give rise to immunopathology in autoimmunity, cancer, and other inflammatory diseases[81]. Veldhoen (2017) hypothesizes *"The efficient IL-17-mediated recruitment of immune cells after bacterial invasion can equally contribute to the initiation of chronic*

inflammation and autoimmunity. However, IL-17 might not be essential to sustain chronic inflammation. After inflammation has been established, when the tissues are highly susceptible to immune-cell recruitment, the role of IL-17 may be more limited[230]*."*

Reports in the early 2000s already hinted that IL-17A could play a role in inflammatory or autoimmune disease[246–249]. In the last two decades, evidence for a role of the "IL-23-IL-17 axis" as a critical driver of chronic inflammation and autoimmunity has been building. However, IL-17A has been shown to have functions far beyond the production of pro-inflammatory cytokines and the attraction of immune cells. IL-17A can stimulate human airway epithelial cells and RA synovial fibroblasts and chondrocytes to produce matrix metalloproteinases (MMPs)[250–252]. Angiogenesis can be affected by IL-17A due to the promotion of proangiogenic factors in fibroblasts and tumour cells[253]. Although the role that IL-17A can have in early inflammation is clear, the role of IL-17A/F in chronic inflammation is less well-understood. It is thought that high levels of IL-17A/F in early phase inflammation induces molecular changes in fibroblasts, with *in vitro* studies reporting an increase in expression of anti-apoptotic genes. This could mean that the survival of cells that were modified early in disease can contribute to chronicity of disease, even in the absence of inflammation[61,254].

There is a long, growing list of diseases in which there is strong evidence for a role of IL-17A, including arthritis, brain inflammation, kidney disease, and cancer[81]. However, as this is beyond the scope of this thesis, three (auto)-immune diseases have been picked to discuss further due to the musculoskeletal involvement in disease: psoriasis, ankylosing spondylitis, and RA. Psoriasis is a chronic autoimmune disease which is characterised by the rapid increase of skin cells. Ankylosing spondylitis is characterised by long-term inflammation of the joints in the spine. RA, the second most common type of arthritis after osteoarthritis, is characterised by highly inflamed and painful joints. In all these diseases, high levels of IL-17A mRNA, protein, and IL-17-producing cells have been found at the site of the disease compared to control tissue[74,255–262]. In addition, genetic risk loci involved in IL-

23/IL-17-signalling have been found in both psoriasis and ankylosing spondylitis[258,263]. *In vitro* studies using fibroblasts from psoriasis, ankylosing spondylitis, and RA patients have shown that IL-17A can act on stimulate resident fibroblasts to produce proinflammatory mediators, chemokines, angiogenetic factors, and MMPs, thereby inducing inflammation, stimulating the recruitment of more inflammatory cells, and contributing the extracellular matrix destruction[252,264–266]. Therefore, it is thought that IL-17A not only promotes innate immunity but also pathogenic/chronic inflammation by stimulating the secretion of pro-inflammatory factors by resident cells and by causing the influx of more inflammatory cells. Not only IL-17A, but also IL-17F and IL-17AF have been found in psoriasis and RA, but IL-17A remains the main focus of research[267–269].

After the inhibition of IL-17A showed to be successful in *in vitro* and *in vivo* models of disease [270–272], inhibitors were tested for clinical use. Inhibitors for IL-17A (secukinumab and ixekizumab) or its receptor IL-17RA (brodalumab) have shown remarkable results. As 40-80% of psoriasis patients go into complete resolution of all disease, these anti-IL-17 drugs are now one of the most effective treatments available for psoriasis[81]. In addition, anti-IL-17 treatment decreased the disease activity score by at least 20% for around 60% of ankylosing spondylitis patients, with up to 20% of patients going into partial remission[273]. However, IL-17A blockade was not more effective than standard-of-care biologics in RA[81], which might be explained by the variability that is seen in the expression of IL-17 cytokines and receptors in RA[268].

4.2.6 IL-17A/F in osteoarthritis

Osteoarthritis (OA) is a whole joint disease with the involvement of different joint tissues. The inflammation in OA is chronic, low-grade and primarily driven by the innate immune system[25]. Many inflammatory mediators are currently being investigated for their potential role in OA pathophysiology. However, one cytokine that has been increasingly reported to have a role in OA is IL-17A.

4.2.6.1 *Clinical reports of IL-17A in osteoarthritis*

IL-17A was found in increased concentrations in serum and synovial fluid from OA patients in multiple studies when comparing OA patients with healthy controls, showing several positive correlations with different pain, function, and disease severity scores[58,274–276]. When comparing inflamed with non-inflamed synovium from OA patients, an increased concentration of IL-17A was found in inflamed OA tissue, which correlated with the release of IL-6, IL-23, and TGF-β1[60]. IL-17RA was found to be expressed in synovial tissue and chondrocytes by Honorati *et al*.[277], and van Baarsen *et al.* found expression of IL-17A, IL-17F, IL-17RA, and IL-17RC in OA synovial tissue[268]. Another study identified a subgroup of patients with high IL-17 in their synovial fluid, which had higher concentrations of inflammatory mediators (including IL-6, leptin, resistin, CCL7, and NGF), and reduced osteophytes, sclerosis and minimum joint space width, thereby describing a potential inflammatory OA phenotype[30].

4.2.6.2 *Genetic association between IL-17A/IL-17F and osteoarthritis*

Genetic associations between polymorphisms in IL-17 genes and OA have also been reported. In a Chinese population, patients with single nucleotide polymorphisms (SNPs) in the genes for IL-17A or IL-17F were found to have a higher susceptibility to knee OA after adjustment for BMI, sex, age, smoking, and drinking status[278]. In a Han Chinese population, a SNP in IL-17A (rs2275913) was associated with an increased risk of OA among males, younger patients, smokers, and drinkers, while there was no association between a SNP in IL-17F and OA risk[279]. A study in a Croatian Caucasian population reported that a three-SNPs haplotype including one SNP in IL-17A and two in IL-17F (rs2275913-rs763780-rs1889570), was associated with a higher risk of developing knee but not hip OA. Interestingly, they found another SNP that associated with protection of development of hip OA[280]. However, a British study was unable to find a relationship between SNPs in IL-17 genes in a cohort of hip OA patients[281].

4.2.6.3 IL-17A in animal models

Several animal models have studied the role of IL-17A in OA. In a rabbit model, OA induced by Hulth's method of post-traumatic OA, which involves severing the ligaments surrounding the knee joint and transecting the meniscus, was compared to the effect of IL-17A injection in three different concentrations into the knee. After 12 weeks, X-ray results, histological scores, and mRNA results revealed that the IL-17 groups displayed the same typical manifestations of OA as seen in after using the validated Hulth's method, thus concluding that IL-17A can induce OA in a similar matter as induced by the Hulth's method[282]. A study by Richter *et al.* investigated the role of IL-17A in arthritic pain. A single injection of IL-17A into the rat joint caused a long-lasting sensitization of nociceptive C fibres to mechanical stimuli. When they used an antibody against IL-17A in an antigen-induced arthritis model, this blockade improved guarding score and reduced secondary mechanical hyperalgesia at the ipsilateral paw. Therefore, these results indicate IL-17A could be involved in arthritic pain, and that targeting IL-17A might serve to treat OA pain[83].

4.2.6.4 Effects of IL-17A in vitro

Although there is a growing number of reports in clinical, animal, and genetic studies that support a role for IL-17 in OA, limited *in vitro* studies have been done, all done in the early 2000s. The benefit from these *in vitro* studies is that cells and tissues from OA patients can be used to look at the actual state of these tissues/cells and their responses to specific stimuli. In cells from end-stage OA patients, it was shown that IL-17A was able to increase the gene or protein expression of several inflammatory mediators, including IL-1β, IL-6, IL-8, CCL2, CXCL1, COX-2, and iNOS[173,283]. The effect of IL-17A on the ECM has also been reported, showing that IL-17A inhibited proteoglycan synthesis in OA chondrocytes due to the IL-17A-induced increase in MMP production[284]. To our knowledge, no studies have performed a broad gene expression screen to look at the effect of IL-17A/F in cells

derived from OA patients, and no studies have looked at or compared the effects of IL-17A with those of IL-17F and IL-17AF *in vitro* or *in vivo* models of OA.

4.2.6.5 Evidence in this thesis for a role of IL-17A/F in osteoarthritis

In addition to the published work, this thesis adds further strength to a role for IL-17A/F in OA. In Chapter 2, a subset of end-stage OA patients was identified with the presence of CCR6+ T cells. As CCR6 is used as a marker for IL-17A/F-producing cells, this suggests that IL-17A/F could be important mediators in this subset of patients. In addition, in Chapter 3 it was shown that IL-17A cytokine was broadly expressed in synovium of patients at risk of OA. Both protein expression of IL-17RA and IL-17RC were found to be expressed in this tissue, and *IL17RA* and *IL17RC* mRNA was shown to be produced by end-stage OA chondrocytes and synovial fibroblasts, confirming that this tissue can respond to IL-17A/F. Interestingly, IL-17RA protein expression increased with synovitis score in patients at risk of OA, suggesting that this tissue could become more responsive to IL-17A/F with increasing synovitis.

4.2.7 Aim of this chapter

The overwhelming number of reports of increased concentrations of IL-17A in (at least a subgroup of) patients strongly suggest a role for IL-17A in OA. Characteristics of the disease, including the loss of articular cartilage due to ECM degradation, the increased vascularity in subchondral bone and synovium, together with the low-grade, chronic, innate inflammatory character of the disease fit well with the IL-17A-induced effects reported in other diseases. In addition, the findings from this thesis further support a role for IL-17A/F in OA. The overarching aim of this study is to investigate and compare the role of IL-17A/F in driving inflammation and matrix destruction in OA, including the signalling pathways through which these cytokines act. This will be used to establish the potential of inhibiting the most active IL-17 cytokine as a therapeutic option in OA.

4.3 Objectives

Specifically, the objectives of this chapter are to:

1. Identify and compare the effect of IL-17A/F on gene expression of OA-related genes in end-stage OA chondrocytes and synovial fibroblasts
2. Investigate and compare the effect IL-17A/F on the activation of the ERK1/2, p38, or NF-κB pathway in end-stage OA chondrocytes and synovial fibroblasts
3. Investigate whether the inhibition of IL-17A protein or IL-17A-activated intracellular signalling pathways p38 or NF-κB can modulate IL-17-induced gene expression in end-stage OA chondrocytes and synovial fibroblasts

4.4 Hypotheses

It is hypothesized that:

1. In end-stage OA chondrocytes and synovial fibroblasts, IL-17A will cause the most potent changes in the gene expression of several immune- and matrix-related genes that are implicated in OA, followed by IL-17AF and IL-17F. However, there will be subtle differences in the genes changed by IL-17A, IL-17F, and IL-17AF.
2. IL-17A will induce activation of ERK1/2, p38, and NF-κB pathways more potently than IL-17AF and IL-17F.
3. Inhibitors of p38 and NF-κB pathways and clinically-used IL-17A antibody secukinumab will attenuate the IL-17A-induced gene expression *in vitro* in end-stage OA chondrocytes and synovial fibroblasts.

4.5 Methods

4.5.1 Ethics

Ethical approval was granted for the Oxford Musculoskeletal Biobank (09/H0606/11) and (19/SC/0134) by the local research ethics committee (Oxford Research Ethics Committee B) for all work on human cartilage and human synovium, and informed consent according to the Declaration of Helsinki was obtained from all patients.

4.5.2 Isolation of primary chondrocytes and synovial fibroblasts for cell experiments

Primary cells were isolated and cultured as described in 3.5.8.

4.5.3 Cell culture for RNA Sequencing experiment

Human articular chondrocytes were grown to p=2 and synovial fibroblasts to p=3 in D10 (DMEM-F12 (Gibco, supplied by Fisher Scientific, Loughborough, UK) with 10% Foetal Bovine Serum (FBS) (Labtech International, Heathfield, UK) and 1% Penicillin/Streptomycin (P/S) (Gibco)) after which they were plated out in 95,000 cells per well in a 12-well plate. Cells were left to attach for 24 hours in D10, before medium was changed to serum-free DMEM-F12 with P/S (D0) and left for 24 hours. On the day of the experiments, vehicle control, IL-17A, IL-17F, and IL-17AF (all from Biolegend UK Ltd, London, UK) were diluted in D0 medium to 10ng/ml. Old D0 medium was removed and stimulation medium was added. After 24 hours of treatment, medium was removed, cells were washed with PBS, before harvesting in Trizol and storing at -80°C.

4.5.4 Bulk RNA Sequencing library preparation and run

In brief, for each sample, around 96,000 cells were lysed in 300μL of TRIzol (Invitrogen, supplied by Thermo Fisher Scientific, Waltham, MA, USA). An equal volume of ethanol was added to the lysed cells. RNA was then extracted using a Direct-zol MicroPrep kit with DNase treatment (Zymo

Research, Irvine, CA, USA) following the manufacturer's instructions. RNA concentration was measured in eluted samples using a NanoDrop spectrophotometer. RNA quality of eight randomly chosen samples was assessed using High Sensitivity RNA ScreenTapes (Agilent) on an Agilent 2200 TapeStation. After confirming good quality for all samples, all 48 samples were diluted to 100ng in 50μL in a LoBind 96-well plate.

Library preparation was done using a NEBNext Ultra II Directional RNA Library Prep Kit for Illumina with poly-A selection (Illumina, San Diego, CA, USA) following the manufacturer's instructions. In short, RNA was isolated, fragmented and primed, after which cDNA is synthesized. The synthesized double-stranded cDNA is then purified and ligated with an adaptor sequence before enrichment using PCR. The prepared libraries are then purified and the quality assessed. After the Library Prep, every library was quantified for DNA content with High Sensitivity DNA ScreenTapes (Agilent). Libraries from 24 samples with unique identifiers were pooled and quantified again. On the day of sequencing, one pool was thawed and denatured using 0.2M NaOH and diluted to a 1.8pM concentration in 1.3mL for sequencing. The pool was added to the NextSeq High Output kit (75 cycles) and the kit was run on an Illumina NextSeq 500.

4.5.5 Bulk RNA Sequencing analysis

Raw FASTQ files containing reads were generated by the Illumina software CASAVA v1.8. The raw FASTQ files were processed using CGAT-flow readqc and mapping workflows (https://github.com/cgat-developers/cgat-flow)[285]. The quality of the reads was assessed using FASTQC and ReadQC. Raw reads were aligned to the GRCh38 reference genome using HiSat2 (v2.0.5). The mapped reads were visualised using IGV (v2.3.74) to further assess quality of mapping. The quantification of mapped reads against GCRh38 reference genome annotation was carried out using FeatureCounts (v1.5.0). FASTQC, ReadQC, and quantification was done with the help of Dr Adam Cribbs. Downstream analyses were performed using R version 3.5.1 (R Foundation, Vienna,

Austria), and Rstudio version 1.1.456 (RStudio, Boston, MA, USA). Differential expression analysis was performed using the DESeq2 package[286] using 'apeglm' method to apply the shrinkage of logarithmic fold change[287]. The adjusted p-value (padj) and significance of changes in gene expression were determined by applying the Bonferroni-Hochberg correction of 5% false discovery rate. PCA plots were generated using the package ggplot2, heatmaps were generated using the package pheatmap, and volcano plots were created using the package Enhancedvulcano. Gene-set enrichment analysis for hallmark, immune, and matrix-related pathways were performed with the help of Dr Mathew Baldwin, using the fgsea package on genes that were significantly changed compared to control (padj < 0.05) ranked by the sign of the fold change multiplied by the inverse log10 of the padj. An overview of the genes that contribute to significant changes in pathways tested using the enrichment analysis can be found in Appendix C4. Protein coverage was calculated using 'RNA-seq Data Analysis A Practical Approach' by Korpelainen *et al*.[288].

4.5.6 Cell culture for western blot

Chondrocytes were grown to p=2 and synovial fibroblasts to p=3 in DMEM-F12 with 10% FBS and 1% P/S (D10), after which they were plated out in 40,000 cells per well in a 24-well plate. Cells were left to attach for 24 hours in D10, before medium was changed to serum-free DMEM-F12 with P/S (D0) and left for 24 hours. Western blot (WB) Cell lysis buffer was made by 1 part of WB buffer A with 1 part WB buffer B and 0.01 part protease inhibitor (Table 4-1). On the day of the experiments, vehicle control, IL-17A, IL-17F, and IL-17AF were diluted in D0 medium to 10ng/ml. Old D0 medium was removed and stimulation medium was added. When treatment was started, cells for 0 min control were harvested by washing with WB buffer A after which 300μL of WB Cell lysis buffer was added, after which the cells in WB Cell lysis buffer were harvested and frozen at -20°C until analysis. After each incubation period (10 mins, 30 mins, 1 hour, and 8 hours), cells were washed in WB buffer A, harvested in 300μL WB Cell lysis buffer, and frozen at -20°C until analysis.

***Table 4-1**. Overview of different western blot (WB) buffer that together make up the WB Cell lysis buffer, including buffer name, components of the buffer, and additional notes.*

Buffer	Components	Notes
WB buffer A	9.2mg of Na_3VO_4 (Sigma) in 50mL D-PBS	Made on day of use
WB buffer B	0.389g Tris base (Fisher Scientific, Loughborough, UK), 5mL glycerol (Sigma), 10mL 20% SDS (Sigma), 35mL water	pH adjusted to 6.8 with HCl after which solution was autoclaved
Protease inhibitor	Protease Inhibitor Cocktail Set III, EDTA Free (Calbiochem)	-

4.5.7 SDS-PAGE and Western blot

All buffer for the SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and WB protocols were made as described in Table 4-2. The proteins in WB cell lysis buffer were separated by gel electrophoresis in a 10% Mini-PROTEAN TGX Precast gels (Bio-Rad Watford, UK) with a 1-to-1 mixture of sample in WB Cell lysis buffer and 2x Laemmli Sample Buffer (Bio-Rad). After gel electrophoresis, proteins were blotted on PVDF membrane (Bio-Rad) using a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked in blocking buffer and subsequently stained with loading control (vinculin) and phosphorylated-protein antibody overnight in antibody buffer on a shaker in the cold room (Table 4-3). The blots were washed in TBS-T and secondary antibody in antibody buffer was incubated for 2 hours at room temperature on a shaker. Blots were washed again in TBS-T and proteins were visualized with ECL (electrogenerated chemiluminescence) solution (GE Healthcare, Chicago, IL, USA). Due to differences in intensity between the vinculin and phospho-antibodies, the blots were imaged twice in an ALLIANCE 6.7 Chemiluminescence Imaging System (UVITEC, Cambridge, UK): one short exposure for vinculin, and one longer exposure for the phospho-protein. The membranes were subsequently stripped (Takara BioInc, Kusatsu, Japan), blocked again, and stained with total-protein antibody in antibody buffer overnight in the cold room on a shaker. Blots were then washed, stained with secondary antibody, washed again, and imaged. ImageJ was used to analyse the intensity of the stained bands for semi-quantitative analysis using the 0h control for each patient as a reference, which was ran on every blot. The final graphs show the amount of

phosphorylated ERK1/2, NF-κB, or p38, over the amount of total ERK1/2, NF-κB, or p38, normalised to the non-treated 0 hours control. Representative western blots for each antibody using protein lysate from either end-stage OA chondrocytes or synovial fibroblasts can be found in Appendix C5.

Table 4-2. *Buffers used during SDS-PAGE and western blot experiments.*

Buffer	Components	Notes
Running buffer	25mM Tris, 192mM Glycine, 0.1% (w/v) SDS, pH8.3	10X Running buffer (BIO-RAD) was diluted 10 times in distilled water
TBS	10X TBS (BIO-RAD)	TBS was diluted 10 times in distilled water
TBS-T	TBS with 0.1% Triton X-100 (Sigma)	
Blocking buffer	10% milk powder and 2% BSA (Sigma) in TBS-T	
Antibody buffer	5% BSA (Sigma), 1% Tween 20 (Fisher Scientific) in TBS-T	

Table 4-3. *Primary and secondary antibodies used for western blot experiments, including target, company, ref number, and dilution of each antibody.*

Target	Supplier	Ref number	Dilution
Vinculin (reference protein)	Cell Signalling Technology, Danvers, MA, USA	13901	1:1000
phospho-ERK1/2	Cell Signalling Technology	4370	1:2000
ERK1/2	Cell Signalling Technology	4695	1:1000
phospho-p65 NF-κB	Cell Signalling Technology	3033	1:1000
p65 NF-κB	Cell Signalling Technology	8242	1:1000
phospho-p38	Cell Signalling Technology	4511	1:1000
p38	Cell Signalling Technology	8690	1:1000
phospho-SAPK/JNK	Cell Signalling Technology	4668	1:1000
SAPK/JNK	Cell Signalling Technology	9252	1:1000
Anti-rabbit IgG, HRP-linked	Cell Signalling Technology	7074	1:1000 (phospho-proteins) 1:2000 (total-protein)

4.5.8 Cell culture for inhibitor treatment and cells-to-cDNA synthesis

Chondrocytes and synovial fibroblasts were cultured and plated out in 96-well plates as described in 3.5.9. On the day of the experiment, cells were pre-treated for 2 hours with JSH-23, SB204580, or vehicle control (Table 4-4) before the addition of 10 ng/mL concentration of IL-17A for 24 hours. SB203580 inhibits p38 signalling by suppressing the activation of MAPKAP kinase-2 and inhibiting the phosphorylation of heat shock protein 27. JSH-23 inhibits NF-κB signalling by interfering with the nuclear localisation signals of p65. Secukinumab, a specific monoclonal antibody against IL-17A, was mixed with 10ng/ml IL-17A or vehicle control and directly added to the cells and left for 24 hours. After 24 hours, cells were washed with PBS before being harvested in cells-to-cDNA Cell Lysis Buffer (Ambion Inc, Foster City, CA, USA), and transferred to a PCR plate for cells-to-cDNA synthesis. cDNA was prepared using a cells-to-cDNA kit following the manufacturer's instructions (Ambion).

Table 4-4. *Overview of inhibitors used in cell experiments, including used concentrations, time of pre-treatment and supplier.*

Treatment	Concentrations	Time of pre-treatment	Supplier
JSH-23 (NF-κB inhibitor)	0.5, 5.0, and 50 μM	2 hours	Sigma
SB203580 (p38 inhibitor)	0.1, 1.0, and 10 μM	2 hours	Sigma
Secukinumab	0.5, 5.0, and 50 μg/ml	N/A	Kindly provided by Dr. Ash Maroof at UCB

4.5.9 Real time quantitative PCR

Samples for quantitative real-time polymerase chain reaction (qRT-PCR) were prepared as described in 3.5.11. All primers used in this chapter (Table 4-5) were purchased from Primerdesign Ltd (Primerdesign Ltd, Eastleigh, UK), Qiagen (Qiagen, Crawley, UK) or designed in-house and synthesized by Sigma.

Table 4-5. *Overview of primers used in this chapter, including the source of the primers, and the forward and reverse primer sequences where available.*

Target	Supplier	Forward	Reverse
GAPDH	Designed in-house	GAAGGTTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
ACTB	Designed in-house	CCTGGCACCCAGCACAAT	GCCGATCCACACGGAGTACT
IL6	Primerdesign Ltd	GCAGAAAACAACCTGAACCTT	ACCTCAAACTCCAAAAGACCA
CXCL1	Designed in-house	GGCGGAAAGCTTGCCTCAATCCTG	GCCTCCTTCAGGAACAGCCACCA
MMP1	Primerdesign Ltd	GCACTGAGAAAGAAGACAAAGG	CTAAGTCCACATCTTGCTCTTG
MMP3	Primerdesign Ltd	ATGATGAACAATGGACAAAGGATAC	AGTGTTGGCTGAGTGAAAGAG
NFKBIZ	Qiagen	*Proprietary sequence*	*Proprietary sequence*
MAP3K8	Designed in-house	ATGGAGTACATGAGCACTGGA	GCTGGCTCTTCACTTGCATAAAG
PTGS2	Primerdesign Ltd	CAGGCTTCCATTGACCAGAG	TTTCTCCTGTAAGTTCTTCAAATGAT
NOS2	Qiagen	*Proprietary sequence*	*Proprietary sequence*
CSF3	Designed in-house	GCTGCTTGAGCCAACTCCATA	GAACGCGGTACGACACCTC
PDPN	Primerdesign Ltd	CCGAAGATGATGTGGTGACTC	CGATGCGAATGCCTGTTACA
CCL20	Designed in-house	TGCTGTACCAAGAGTTTGCTC	CGCACACAGACAACTTTTTCTTT

4.5.10 Statistical analysis

Statistical analyses for bulk RNA-Seq were performed using R and RStudio as described in sections 5.5.5. All other statistical analyses were performed using in GraphPad Prism 8.1.2 (GraphPad Software, La Jolla, CA, USA). For treatment with IL-17A/F for western blot analysis, 2-way ANOVAs with Dunnett's multiple comparisons test was used and data is shown as mean+SD. For all experiments with IL-17A and inhibitors, differences between treatment with IL-17A alone and other treatments Friedman test with Dunn's test to correct for multiple comparisons, expect when there was missing data in which Kruskal-Wallis test with Dunn's multiple comparisons test was used. All data for inhibitor experiments is shown as the individual measurements with mean. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

4.6 Results

4.6.1 RNA Sequencing of IL-17-treated OA chondrocytes and synovial fibroblasts

The transcriptome of sets of samples (24 samples of chondrocytes and 24 samples of synovial fibroblasts) were analysed using poly-A tail selected, mRNA sequencing. Samples from each cell type were from 6 different donors and treated for 24 hours with vehicle control, or IL-17A, IL-17F, or IL-17AF, all at 10 ng/ml. A full overview of the samples can be found in Appendix C1.

All quality data and reads data were found on the Illumina BaseSpace account that was connected to the run. In total 489,256,512 reads were made during the chondrocytes run, of which 95.8% of reads were identified. Similar number of reads were identified for each sample of the run (percentage of number of reads identified for each sample from the total identified reads was between 2.7% and 6.8%, with the median of 3.7% of total number of reads). Quality of identification of each read is expressed by the Q-score, with the reads that reach at least a Q-score of 30 being at least 99.9% accurate, which was 81.9%. For the run with synovial fibroblasts samples, there were 495,048,984 reads, from which 94.6% were identified. Distribution of the sample identification was between 2.4% and 5.0%, with a median of 4.0%. The quality of samples identified for this run was even higher than the last with 91.2% of samples having a Q-score$\geq$30.

After sequencing, all samples were analysed using a FastQ screen to check all samples for potential contaminants that could have been introduced. However, no obvious alignment with other species were detected (Appendix C2). For chondrocytes, sample reads were between 13.3 and 32.7 million, thus having at least 13.3 times protein coverage of the chondrocytes. For synovial fibroblasts, sample reads were between 23.6 and 49.2 million reads, thus having at least 23.6 times protein coverage of the synovial fibroblasts.

4.6.2 RNA Sequencing analysis of IL-17-treated OA chondrocytes and synovial fibroblasts with DESeq2

4.6.2.1 Clustering of samples

After a count matrix was created, differences in expression between control and IL-17A/F-treated samples were tested in R using the DESeq2 package. A DESeq2 data set (dds) was created using the count matrix, and a likelihood ratio test (LRT) was used to account for donor variability.

4.6.2.1.1 Chondrocytes

Heatmaps of the counts-matrix (Figure 4-3A) and the sample-to-sample distance (Figure 4-3B), and principle component analysis (PCA) plot (Figure 4-3C) was created using the analysed dss. From the heatmaps, it becomes clear that the samples mainly cluster based on their corresponding donors, and no clear pattern can be seen in the effect of different treatments. Principle component analysis shows that PC1 accounts for 61% of the variability, and seems to reflect donor variability. There are three clusters, each with samples from two different donors. In each case, the cells from clustered samples were treated on different days and with different plate layouts excluding the possibility that this is due to similarities in treatment day or interaction with neighbouring wells in each plate. As all 6 donors were treated at the same time of the day (between 9.38AM and 9.44AM), differences due the circadian rhythm are also unlikely. Along the PC2 axis, samples treated with control or IL-17F for each donor are often close together, with IL-17AF-treated samples are a little separated or close to IL-17F, while IL-17A was often seen further away the other three samples of each patient. This suggests that the PC2 axis, which accounts for 12% of the variance, could explain the changes in expression due to differences in response to IL-17 cytokines.

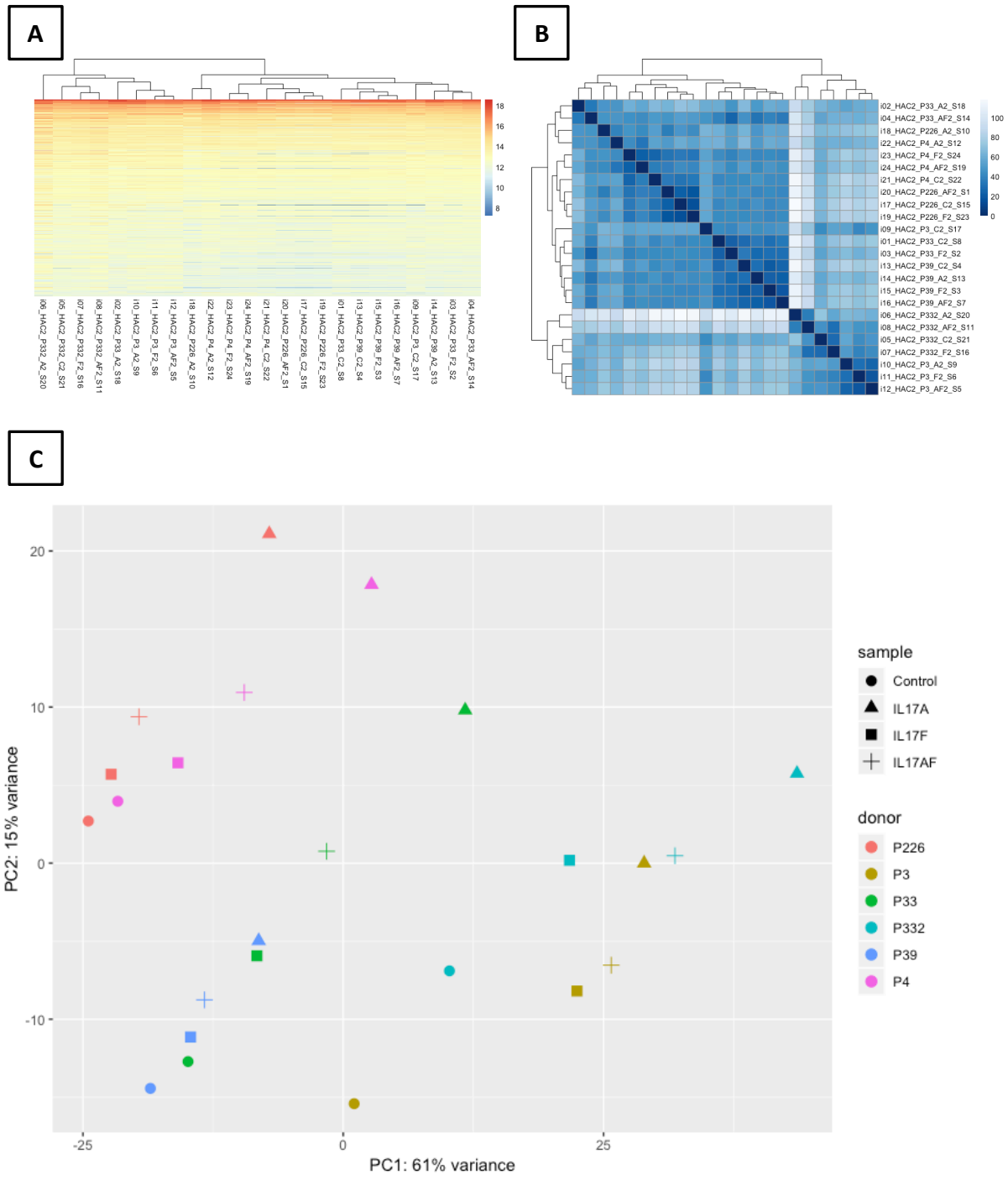


Figure 4-3. *Overview of different plots showing clustering of the chondrocytes. (A) Heatmap of the counts matrix for chondrocytes samples, blue shows low counts while red shows high counts. (B) Heatmap of the sample-to-sample distances, dark blue represents no to little difference while white represents large differences between samples. (C) Principle component (PCA) plot for chondrocytes samples. Different sample types are represented with different shapes (circle = control, triangle = IL-17A, square = IL-17F,* plus sign *= IL-17AF) and different donors are represented with different colours (P226 = red, P3 = mustard, P33 = green, P332 = turquoise, P39 = blue, P4 = pink).*

4.6.2.1.2 Synovial fibroblasts

Similar to the differences seen in the analysed chondrocyte samples, from the heatmaps it becomes clear that samples mainly cluster based on the donors (Figure 4-4A and B). PC1, which accounts for 69% of the variance, in the PCA plot (Figure 4-4C) suggests that this variability is due to donor variability. It looks like two sets of samples of two donors cluster together, while the last two sets of samples are distinct. Samples from patient 299 and patient 330 were cultured on the same day, and although they cluster together the samples from patient 317 and patient 377, which were also cultured on the same day as each other, are clearly separated. This suggests that clustering of 299 and 330 is not due to day of treatment effect. Plate layout of each patient were varied to exclude similarities due to similarity in neighbouring wells, and all samples were treated at a similar time a day (between 9.49AM and 9.53AM) minimising the chance of variability due to circadian rhythm.

Along the axis of PC2, the synovial fibroblast samples spread based on type of treatment. For each donor, the samples treated with control medium are at the lowest point of the axis, followed by IL-17AF and IL-17F-treated samples which often appear close together. IL-17A-treated samples are farthest away from the control samples of each donor.

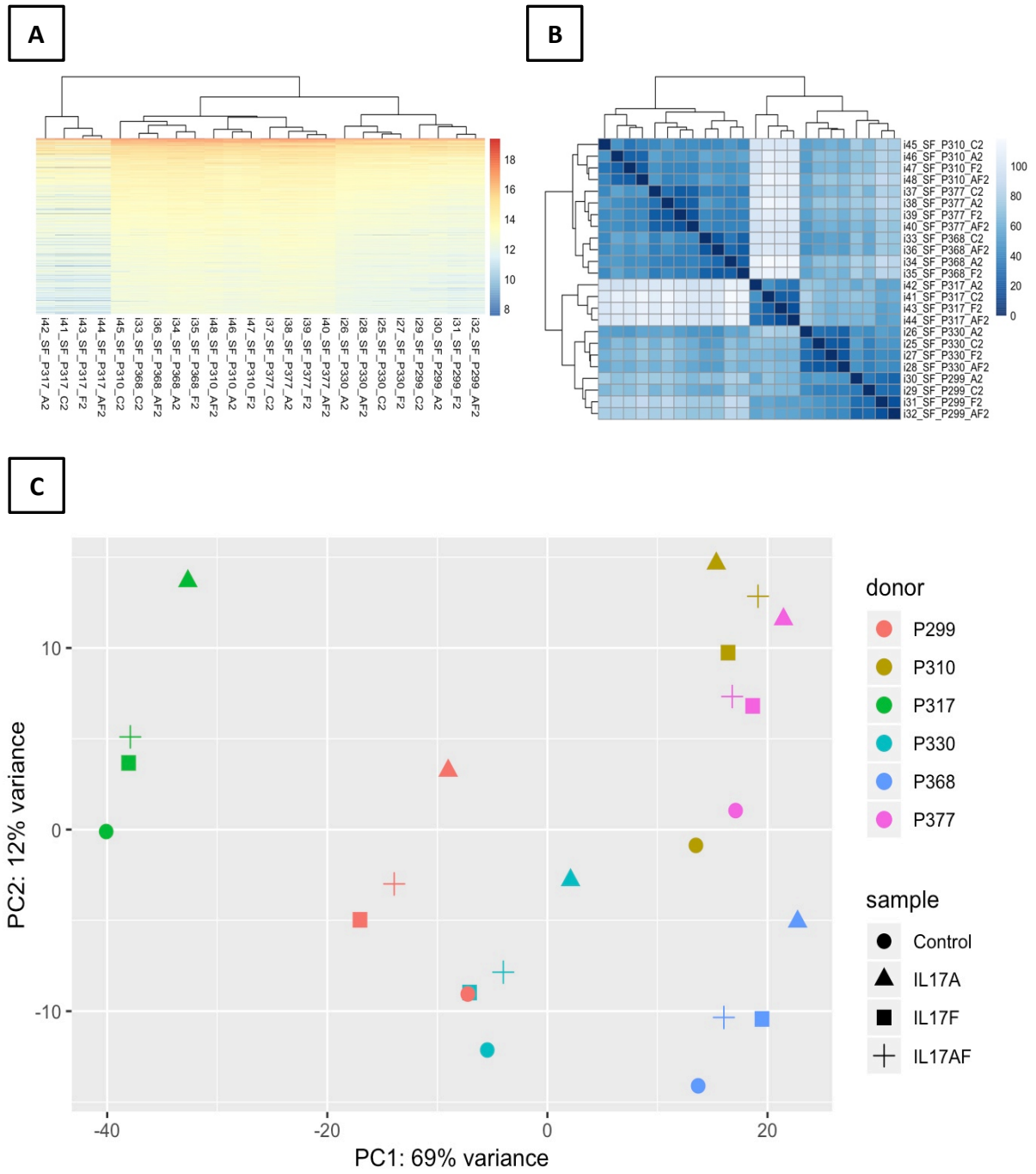


Figure 4-4. *Overview of different plots showing clustering of the synovial fibroblasts. (A) Heatmap of the counts matrix, blue shows low counts while red shows high counts. (B) Heatmap of the sample-to-sample distances, dark blue represents no to little difference while white represents large differences between samples. (C) Principle component analysis (PCA) plot showing the first and second principle components (PC) and their variance. Different sample types are represented with different shapes (circle = control, triangle = IL-17A, square = IL-17F,* plus sign *= IL-17AF) and different donors are represented with different colours (P299 = red, P310 = mustard, P317 = green, P330 = turquoise, P368 = blue, P377 = pink).*

4.6.2.2 Differential gene expression

In order to identify differentially expressed genes between control and different IL-17 treatments, log2 fold changes (LFC) were calculated using the apelgm test with the adjusted p<0.05 as a threshold. A similar number of genes across all IL-17 treatment types showed an LFC higher or lower than zero for both chondrocytes (Table 4-6) and synovial fibroblasts (Table 4-7). However, IL-17A/F-cytokines caused a change in almost twice as many genes in chondrocytes compared to synovial fibroblasts, suggesting that chondrocytes are more prone to changes by IL-17A/F than synovial fibroblasts.

When only including significantly changed genes (adjusted p-value (padj) < 0.05) with an LFC of <1 or <-1, the number of changed genes vary by IL-17 cytokine, with the highest number changed by IL-17A (856 genes for chondrocytes, 330 for synovial fibroblasts), followed by IL-17AF (188 genes/55 genes), and finally IL-17F (39 genes/17 genes). Volcano plots clearly visualise this shift in number of regulated genes for chondrocytes (Figure 4-5) and synovial fibroblasts (Figure 4-6). A full list of differentially expressed genes between control and IL-17-treated samples with an LFC >1 or <-1 can be found in Appendix C3.

Table 4-6. *Overview of results for chondrocytes samples treated with IL-17 compared to control. Data includes number of genes upregulated (LFC > 0) or downregulated (LFC < 0), outliers, genes with low counts, and number of genes with at least a 1 log2 fold change in- or decrease in expression. LFC = log2 fold change. n=6.*

	Chondrocytes		
	IL-17A	**IL-17F**	**IL-17AF**
LFC > 0	2786 (17%)	2732 (17%)	2723 (17%)
LFC < 0	2644 (16%)	2698 (17%)	2707 (17%)
Outliers	0 (0%)	0 (0%)	0 (0%)
Low counts	1578 (9.7%)	1578 (9.7%)	1578 (9.7%)
LFC > 1 or < -1	856	39	188

Table 4-7. *Overview of results for synovial fibroblasts samples treated with IL-17 compared to control. Data includes number of genes upregulated (LFC > 0) or downregulated (LFC < 0), outliers, genes with low counts, and number of genes with at least a 1 log2 fold change in- or decrease in expression. LFC = log2 fold change. n=6.*

	Synovial fibroblasts		
	IL-17A	**IL-17F**	**IL-17AF**
LFC > 0	1418 (8.8%)	1454 (9%)	1467 (9.1%)
LFC < 0	1654 (10%)	1618 (10%)	1605 (9.9%)
Outliers	0 (0%)	0 (0%)	0 (0%)
Low counts	2506 (16%)	2506 (16%)	2506 (16%)
LFC > 1 or < -1	330	17	55

While the potency of these IL-17-cytokines from most to least potent generally is IL-17A, IL-17AF, and IL-17F, there were a few genes that did not follow this pattern (Table 4-8). In chondrocytes, IL-17F caused a 0.5 LFC stronger decrease in expression of *MOB3B*, the gene encoding for MOB kinase activator 3B, than IL-17A and 1.0 LFC stronger decrease than IL-17AF. IL-17AF caused a 0.5 LFC stronger decrease in *ATP8A2* and *XK*, a slightly stronger decrease in expression of *AKAP12* and *ROBO2*, a lightly stronger increase in *SPINK1* than IL-17A. In synovial fibroblasts, IL-17F was able to cause an increase in *MX2*, while no increase was caused by IL-17AF. An even strong difference was seen in the expression of *PSTPIP1*, which was significantly increased by IL-17AF, but not by IL-17A or IL-17F.

Table 4-8. *Overview of differentially expressed genes in chondrocytes synovial fibroblasts between control or IL-17 treated samples in which IL-17AF caused a stronger response than IL-17A or IL-17F causing a stronger response than IL-17A and/or IL-17AF. Green boxes note logarithmic fold changes (LFC) of at least +1 and red boxes note LFC of at least -1, while blank boxes show an LFC between -1 and 1. Genes are sorted alphabetically on SYMBOL gene name.*

Chondrocytes					
Ensemble gene name	**SYMBOL gene name**	**Adjusted p-value**	**Log2 Fold Change (LFC)**		
			IL-17A	**IL-17F**	**IL-17AF**
ENSG00000131016	*AKAP12*	1.66E-14	-1.242	-0.704	-1.268
ENSG00000132932	*ATP8A2*	1.27E-02	-0.792	-0.019	-1.255
ENSG00000120162	*MOB3B*	2.94E-04	-0.715	-1.202	-0.217
ENSG00000185008	*ROBO2*	1.81E-07	-0.969	-0.279	-1.007
ENSG00000164266	*SPINK1*	2.27E-02	1.480	0.019	1.844
ENSG00000047597	*XK*	8.54E-03	-0.855	-0.025	-1.347
Synovial fibroblasts					
ENSG00000183486	*MX2*	6.01E-03	2.070	1.135	0.034
ENSG00000140368	*PSTPIP1*	2.99E-02	0.150	0.014	1.586

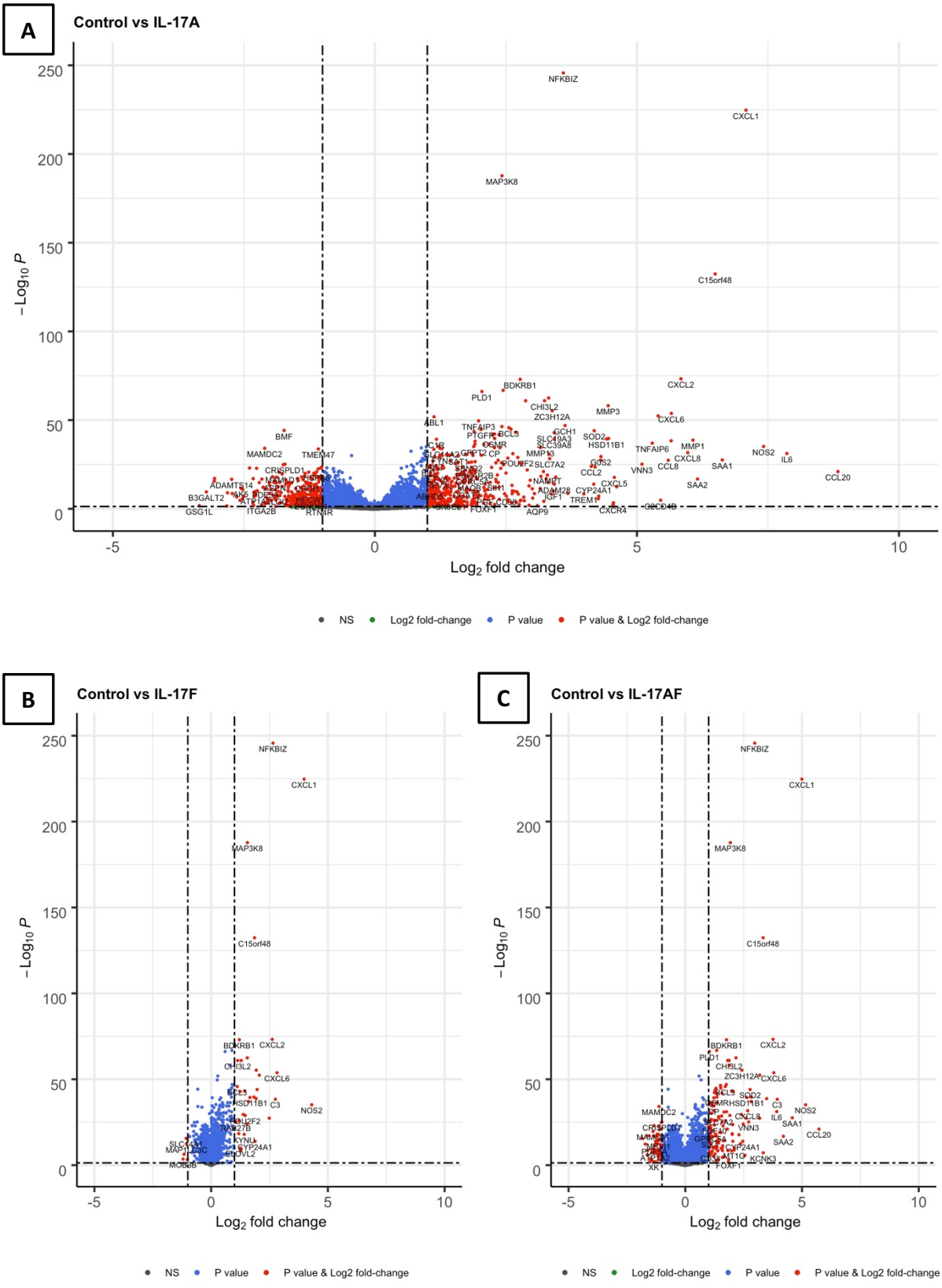


Figure 4-5. *Volcano plots showing the changes in gene expression after treatment with IL-17A (A), IL-17F (B), or IL-17AF (C) in chondrocytes. **Grey** = not significant (NS); **green** = Log2 fold change of at least 1 or -1, but p>0.05; **blue** = p-value < 0.05, but log2 fold change >-1 and <1; **red** = p-value<0.05 and log2 fold change of at least -1 or +1. n=6.*

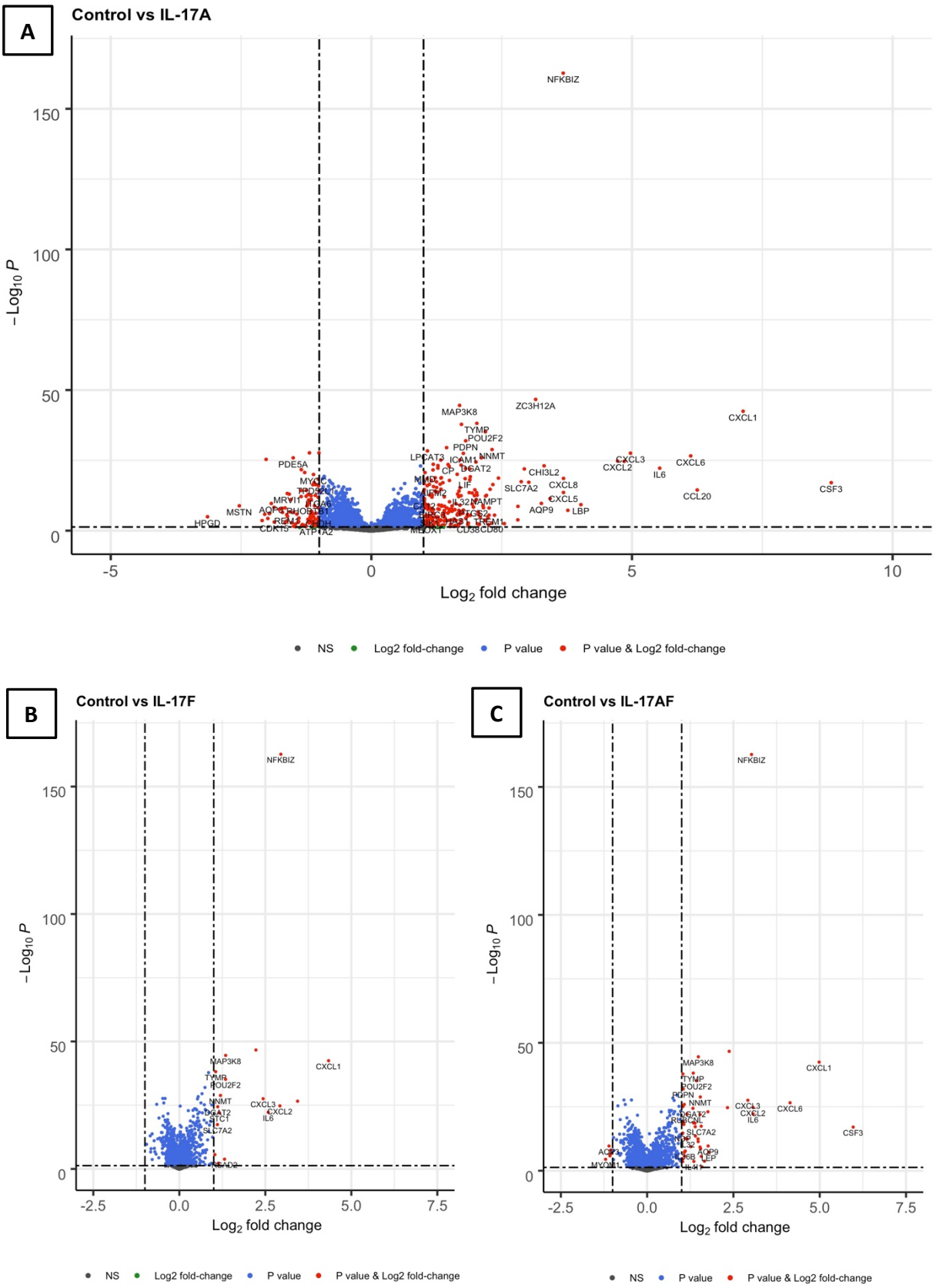


Figure 4-6. *Volcano plots showing the changes in gene expression after treatment with IL-17A (A), IL-17F (B), or IL-17AF (C) in synovial fibroblasts. **Grey** = not significant (NS); **green** = Log2 fold change of at least 1 or -1, but p>0.05; **blue** = p-value < 0.05, but log2 fold change >-1 and <1; **red** = p-value<0.05 and log2 fold change of at least -1 or +1. n=6.*

4.6.3 IL-17A treatment causes changes in major biological pathways

As all the IL-17-treatment caused changes in similar genes but IL-17A caused the largest changes in gene expression, IL-17A will be the main focus of the analysis for the remainder of this section. However, the volcano plots for the results of each enrichment analysis for IL-17F and IL-17AF are shown in Appendix C4.

To elucidate the biological processes involved that are affected by the genes regulated by IL-17A/F-treatment, the dataset was analysed using the Gene Set Enrichment Analysis (GSEA) hallmark gene set, which is a collection of 50 hallmark gene pathways which condense information from over 4,000 original overlapping gene sets from specific collections[289]. IL-17A caused significant changes in 17 of these pathways in chondrocytes and 16 pathways in synovial fibroblasts. However, the changes in normalised enrichment score by IL-17A in synovial fibroblasts were generally a bit higher than those in chondrocytes. IL-17AF induced significant changes in 7 pathways in chondrocytes and synovial fibroblasts, while IL-17F only significantly affected 1 pathway in chondrocytes and 3 in synovial fibroblasts (Appendix C4). While IL-17AF and IL-17F changed less pathways than IL-17A, the normalised enrichment score for these pathways was often higher for IL-17AF than IL-17A (Table 4-9).

Volcano plots are shown to illustrate the genes that were changed by IL-17A treatment in chondrocytes (Figure 4-7) and synovial fibroblasts (Figure 4-8), with the genes that contributed most to the changes in the hallmark pathway in red and gene symbols provided. Interestingly, many pathways that are significantly changed have been linked to OA, including inflammatory responses and cytokines, complement, hypoxia, angiogenesis, and glycolysis. However, as this only gives insight into the broad biological pathways that were changed, the following sections will focus more on specific collections of genes. Two main features of OA pathophysiology are matrix destruction in cartilage and synovial inflammation. Therefore, the effect of IL-17A/F on inflammation- and matrix-

related genes was studied in more detail using enrichment analyses with a collection of genes that focussed on 1) regulation of the (innate) immune system, 2) regulation of cytokines, chemokines and growth factors, and 3) regulation of extracellular matrix and the cytoskeleton.

Table 4-9. *Overview of hallmark pathways affected by IL-17A, IL-17F, or IL-17AF-treatment in chondrocytes and synovial fibroblasts. **Blue** = increase in normalised enrichment score (NES), **red** = decrease in NES, **grey** = no significant change.*

Pathway	Chondrocytes			Synovial fibroblasts		
	IL-17A	IL-17F	IL-17AF	IL-17A	IL-17F	IL-17AF
Allograft Rejection	1.91			2.18		
Angiogenesis	2.02					
Complement	2.41		2.56	2.38		2.53
Estrogen Response Early	1.68					
Glycolysis	1.94			1.84		
Hypoxia	2.36		2.40	2.24	2.38	2.49
Il2 Stat5 Signaling	2.27		2.69	2.21		2.84
Il6 Jak Stat3 Signaling	2.46		2.94	2.71		2.40
Inflammatory Response	2.74		2.74	2.96		
Interferon Alpha Response	2.16			2.24		2.62
Interferon Gamma Response	2.65		2.84	2.75		
Kras Signaling Up	1.97			2.19		
Mitotic Spindle				-1.70		
Mtorc1 Signaling	1.82			2.10		
Myc Targets V1	1.68			1.92		
Myc Targets V2				2.00		
Myogenesis	-1.54			-2.21	-2.25	-2.16
Tnfa Signaling Via Nfkb	3.14	2.37	3.47	3.23	2.58	2.98
Unfolded Protein Response	1.95					

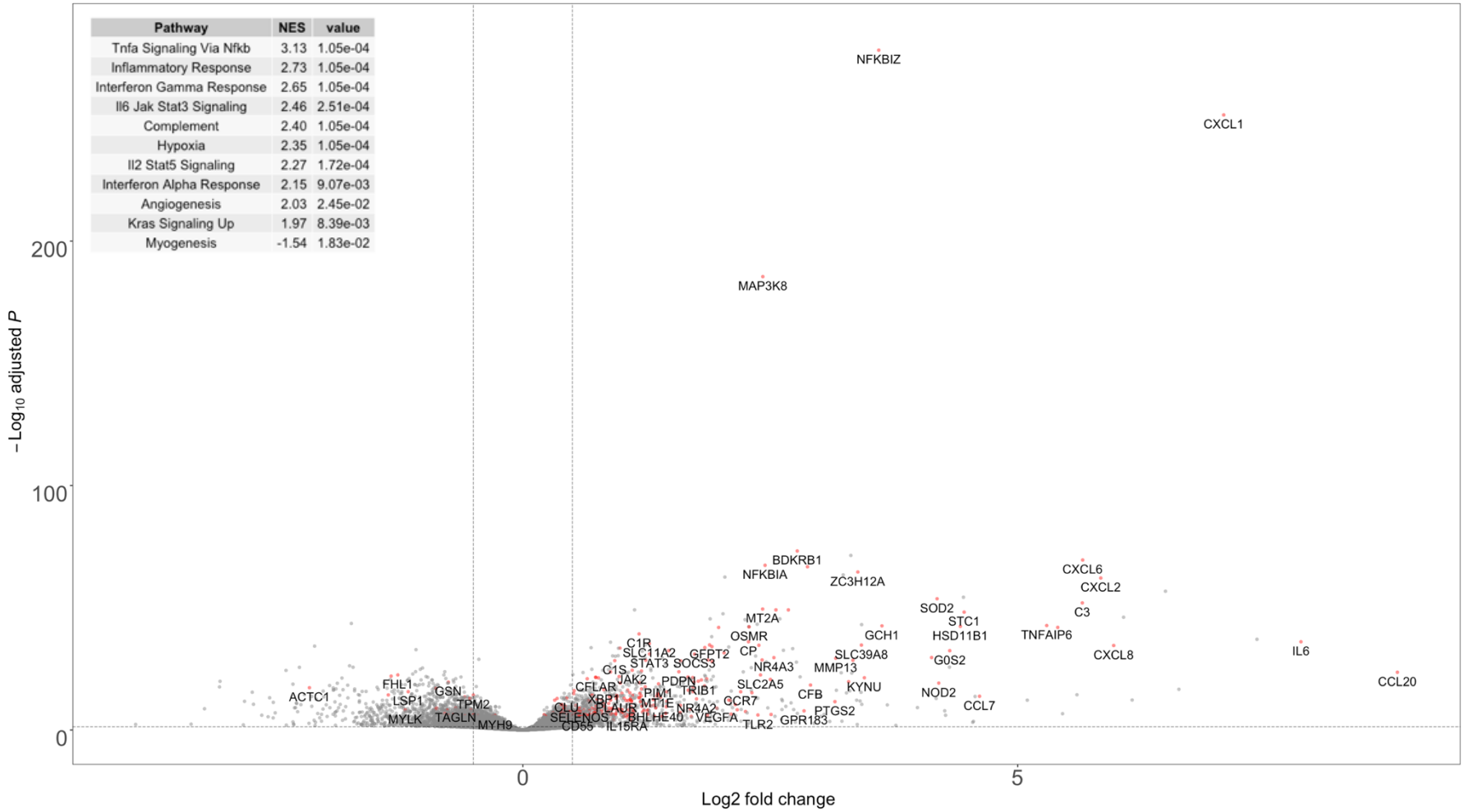


Figure 4-7*. Volcano plot representing genes changed by IL-17A treatment in chondrocytes, with the most important genes that correspond to the changes in GSEA Hallmark pathways highlighted by name and red dots.*

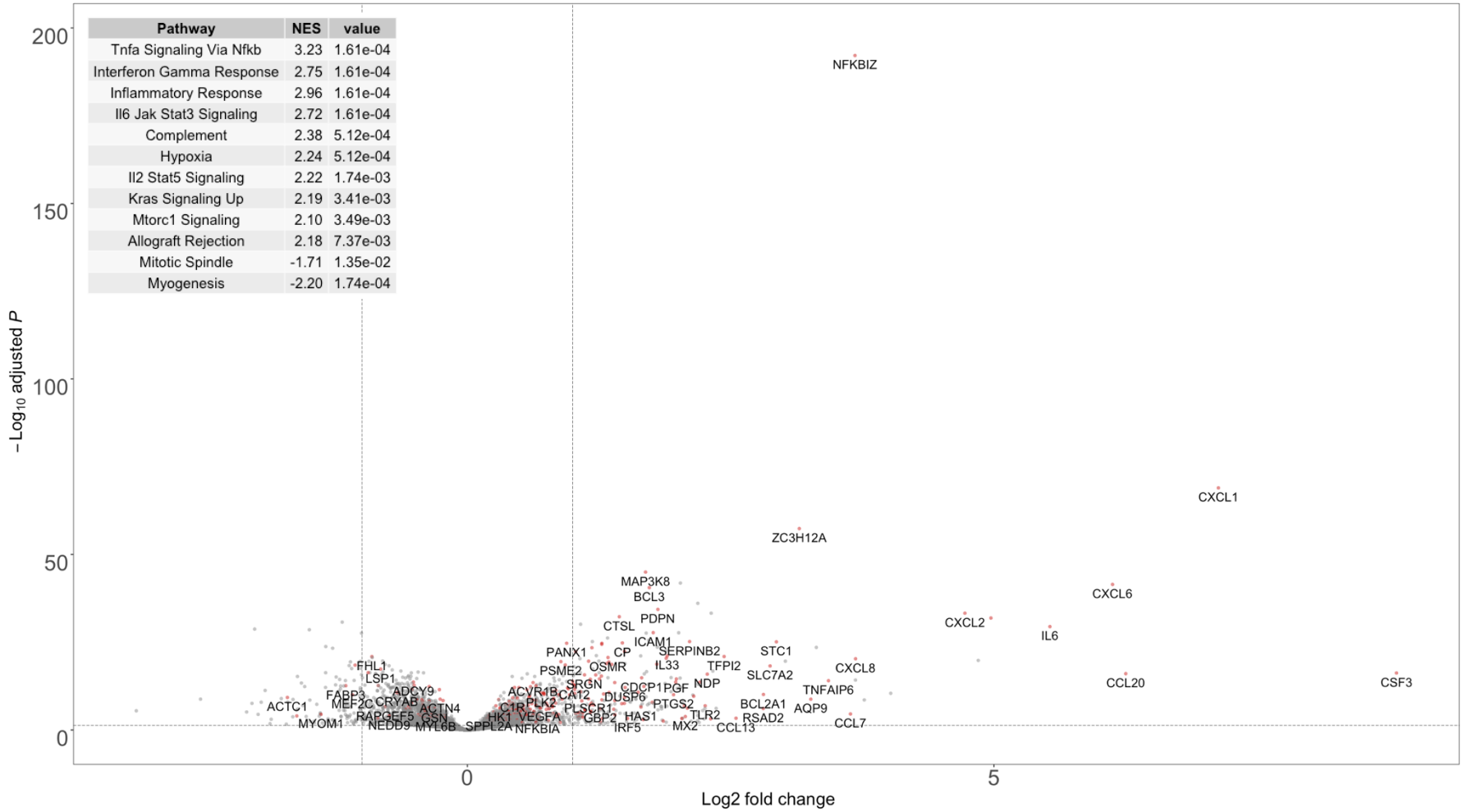


***Figure 4-8**. Volcano plot presenting genes changed by IL-17A treatment in synovial fibroblasts, with the most important genes that correspond to the changes in GSEA Hallmark pathways highlighted by name and red dots.*

4.6.4 IL-17A affects genes related to (innate) immune system

GSEA hallmark pathway analysis of IL17A regulated genes in synovial fibroblasts and chondrocytes showed that several pathways linked to the immune system are affected. When looking more specifically at a gene set that includes pathways that affect immune responses, 10 pathways were significantly upregulated by IL-17A in chondrocytes and 17 pathways were increased in synovial fibroblasts (Table 4-10). In chondrocytes, the most highly increased genes encode for CXCL-chemokines, CCL20, IL-6, and iNOS, among others (Figure 4-9). In synovial fibroblasts, these changes seem to be driven in increases in genes encoding for several CXCL- and CCL-chemokines, interleukins, G-CSF, and leptin, among others (Figure 4-10). Because of the strong increases in cytokines and chemokines, a separate pathway analysis for the regulation of chemokines and cytokines was performed. IL-17A increased the enrichment score for 7 pathways in chondrocytes and for 13 in synovial fibroblasts (Table 4-11). In both these pathway analyses it becomes clear that IL-17A both changes more pathways but also often caused a higher normalised enrichment score in synovial fibroblasts than chondrocytes.

Although similar pathways were regulated by IL-17A in both cell types, the most strongly enriched pathways did differ in both the immune and cytokine/chemokine enrichment analysis. This suggests that while IL-17A has similar effects on these cells, the function of the cell and its environment might be able to influence or regulate these effects.

In addition to the similarities between IL-17A in both cell types, IL-17F and IL-17AF in both cell types significantly activated two pathways in the immune pathway analysis: positive regulation of immune system process and regulation of immune system process. Interestingly, the latter was not activated by IL-17A in chondrocytes but was activated in synovial fibroblasts. For the cytokine/chemokine enrichment analysis, IL-17F was not able to change any pathways in both cell types, while IL-17AF affected the pathways relating to cytokine activity and cytokine receptor binding in both cell types,

and chemokine receptor binding in chondrocytes. Volcano plots for IL-17A, IL-17F, and IL-17AF for cytokine/chemokine pathway analysis can be found in Appendix C4.

Table 4-10. *Overview of immune system pathways affected by IL-17A, IL-17F, or IL-17AF-treatment in chondrocytes and synovial fibroblasts. **Blue** = increase in normalised enrichment score (NES), **red** = decrease in NES, **grey** = no significant change.*

Pathway	Chondrocytes			Synovial fibroblasts		
	IL-17A	IL-17F	IL-17AF	IL-17A	IL-17F	IL-17AF
Cellular Response To Interleukin 1	2.35			2.15		
Negative Regulation Of Lymphocyte Differentiation				1.81		
Positive Regulation Of Immune Effector Process	1.97					
Positive Regulation Of Immune Response	2.01			2.08		
Positive Regulation Of Immune System Process	2.31		2.60	2.46	2.27	2.41
Positive Regulation Of Innate Immune Response	1.98			2.41		
Positive Regulation Of Myeloid Cell Differentiation				1.97		
Positive Regulation Of T Cell Proliferation				1.84		
Regulation Of Immune Effector Process	1.89			1.75		
Regulation Of Immune Response	1.86			1.95		
Regulation Of Immune System Process			2.41	2.22	2.19	2.18
Regulation Of Innate Immune Response	1.97			2.53		
Regulation Of Interleukin 1 Beta Production	1.89					
Regulation Of Interleukin 1 Production	1.96			1.86		
Regulation Of Interleukin 12 Production				1.94		
Regulation Of Interleukin 6 Production	2.06			1.85		
Regulation Of Myeloid Cell Differentiation	1.78					
Regulation Of T Cell Differentiation				1.79		
Response To Interleukin 1	2.31			2.02		
Toll Like Receptor Signaling Pathway				2.15		

Table 4-11. *Overview of cytokine/chemokine pathways affected by IL-17A, IL-17F, or IL-17AF-treatment in chondrocytes and synovial fibroblasts.* ***Blue*** *= increase in normalised enrichment score (NES),* ***red*** *= decrease in NES,* ***grey*** *= no significant change.*

Pathway	Chondrocytes			Synovial fibroblasts		
	IL-17A	IL-17F	IL-17AF	IL-17A	IL-17F	IL-17AF
Chemokine Activity	2.50					
Chemokine Receptor Binding	2.55		2.70	2.57		
Cytokine Activity	2.32		2.59	2.64		2.72
Cytokine Receptor Activity				1.83		
Cytokine Receptor Binding	2.38		2.51	2.42		2.67
Growth Factor Activity				2.14		
Negative Regulation Of Cytokine Production				1.94		
Positive Regulation Of Chemokine Production				1.91		
Positive Regulation Of Cytokine Biosynthetic Process				1.84		
Positive Regulation Of Cytokine Production	2.14			2.12		
Regulation Of Chemokine Production				1.82		
Regulation Of Cytokine Biosynthetic Process				2.02		
Regulation Of Cytokine Production	2.12			2.07		
Regulation Of Cytokine Secretion				1.91		
Regulation Of Vascular Endothelial Growth Factor Production	2.07					

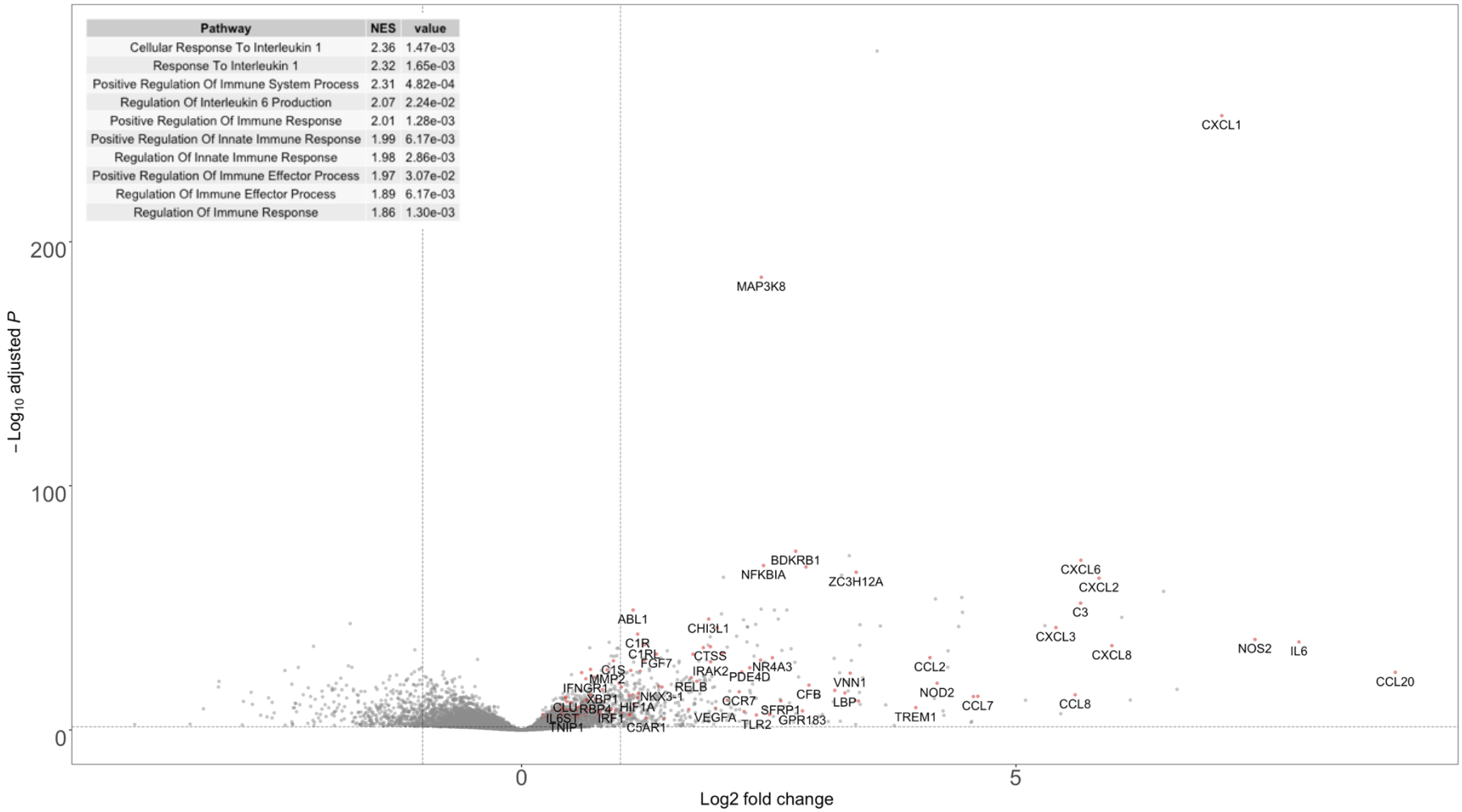


Figure 4-9. *Volcano plot representing genes changed by IL-17A treatment in chondrocytes, with the most important genes that correspond to the changes in immune pathways highlighted by name and red dots.*

Pathway	NES	value
Regulation Of Innate Immune Response	2.53	2.07e-04
Positive Regulation Of Immune System Process	2.46	2.07e-04
Positive Regulation Of Innate Immune Response	2.42	6.47e-04
Regulation Of Immune System Process	2.22	2.07e-04
Toll Like Receptor Signaling Pathway	2.15	3.36e-02
Cellular Response To Interleukin 1	2.15	2.70e-02
Positive Regulation Of Immune Response	2.07	1.49e-03
Response To Interleukin 1	2.02	3.54e-02
Positive Regulation Of Myeloid Cell Differentiation	1.97	4.07e-02
Regulation Of Immune Response	1.95	1.13e-03

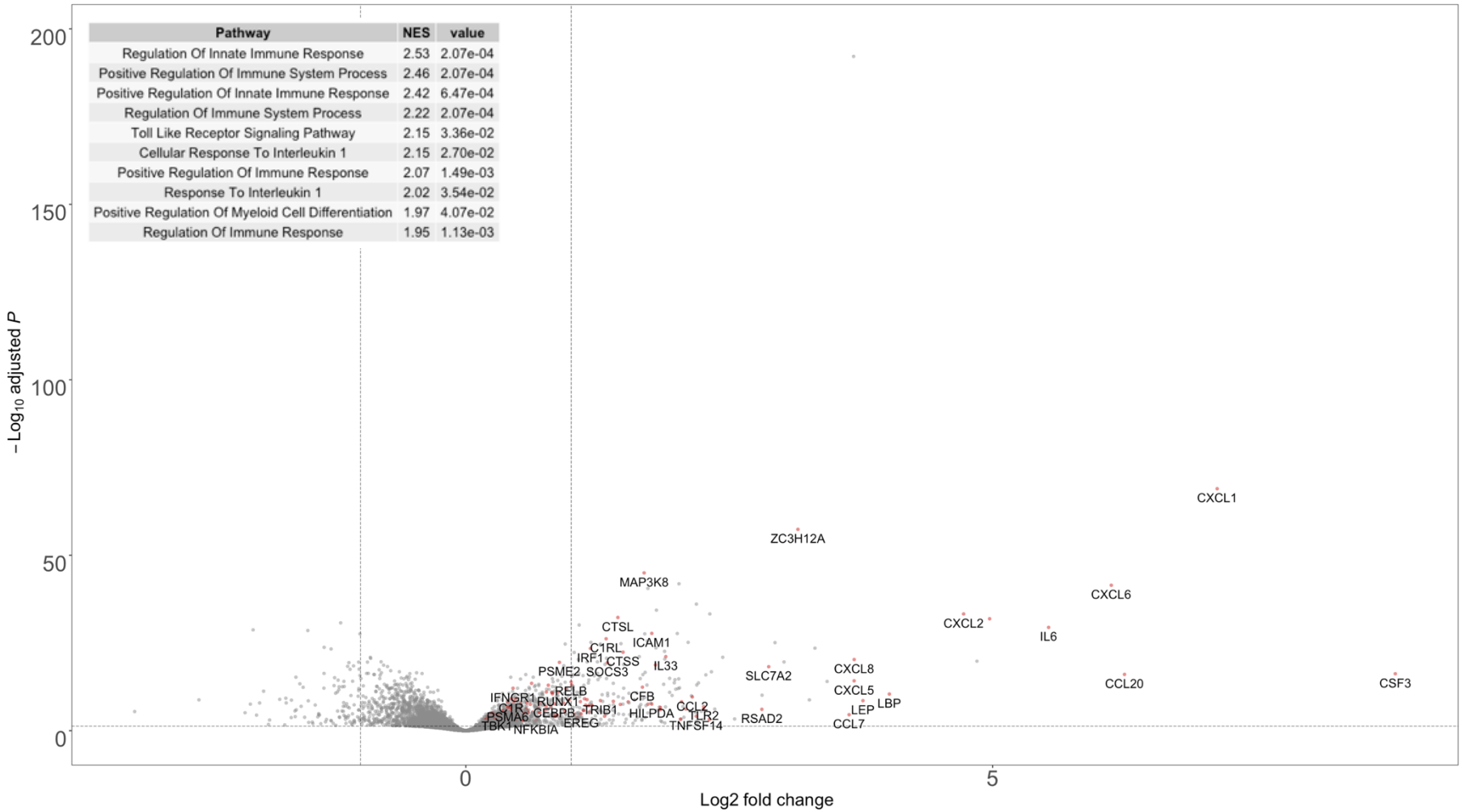


Figure 4-10. *Volcano plot representing genes changed by IL-17A treatment in synovial fibroblasts, with the most important genes that correspond to the changes in immune pathways highlighted by name and red dots.*

4.6.5 IL-17A can contribute to matrix destruction

As cartilage loss is the cardinal feature of OA, increased turnover of extracellular matrix is a pivotal part of OA pathophysiology. Therefore, an enrichment analysis specific for extracellular matrix- and cytoskeleton-related pathways was used to investigate whether IL-17A/F-induced changes in gene expression affects ECM turnover and cytoskeleton organisation. IL-17A treatment led to a significant change in enrichment score in 19 pathways in chondrocytes and 14 pathways in synovial fibroblasts (Table 4-12).

In chondrocytes, IL-17A caused an increase in ECM disassembly, while a decrease was seen in 17 other pathways including organization of the cytoskeleton, actin filaments, and cortical cytoskeleton (Figure 4-11). These changes seem to be driven by a strong increase in genes encoding for the MMP-1, -2, -3, -10, and -13 proteins, and a downregulation in gene coding for actin, receptors regulating the reorganisation of actin cytoskeleton, and the proliferation and differentiation of chondrocytes. In synovial fibroblasts, the strongest decreases in enrichment score were seen in pathways relating to the cortical and actin cytoskeleton, (proteinaceous) ECM, and actin filaments (Figure 4-12). These changes mainly correspond to a decrease in expression of genes encoding for proteins that regulate actin, cell-matrix adhesion, and the cytoskeleton. Although many pathways overlap between those induced by IL-17A in chondrocytes and synovial fibroblasts, over half of the pathways changed were only regulated in one of the two cell types.

IL-17F did not significantly change any of the ECM or cytoskeleton pathways in chondrocytes, while IL-17AF mainly caused changes in actin and cortical cytoskeleton pathways. Importantly, IL-17AF was able to cause changes in two pathways in chondrocytes which were not significantly changed by IL-17A (actin-based cell projection and cytoskeleton). In synovial fibroblasts, IL-17F changed two ECM-related pathways, while IL-17AF changed these ECM pathways as well as pathways related to the

regulation of the cytoskeleton organisation and its components (Volcano plots for IL-17F and IL-17AF can be found in Appendix C4).

Table 4-12. *Overview of extracellular matrix pathways affected by IL-17A, IL-17F, or IL-17AF-treatment in chondrocytes and synovial fibroblasts.* ***Blue*** *= increase in normalised enrichment score (NES),* ***red*** *= decrease in NES,* ***grey*** *= no significant change.*

Pathway	Chondrocytes			Synovial fibroblasts		
	IL-17A	IL-17F	IL-17AF	IL-17A	IL-17F	IL-17AF
Actin Based Cell Projection			-1.97			
Actin Cytoskeleton	-2.02		-2.02	-2.00		-1.89
Actin Filament	-1.62					
Actin Filament Based Movement	-1.86					
Actin Filament Based Process				-1.78		
Actin Filament Bundle	-2.26		-2.09	-1.83		
Actin Filament Bundle Organization	-1.85					
Actin Filament Organization	-1.46			-1.59		
Cortical Actin Cytoskeleton	-2.56		-2.38	-2.30		
Cortical Cytoskeleton	-2.07		-1.82	-2.13		
Cytoskeleton			-1.47	-1.53		
Cytoskeleton Organization				-1.68		
Extracellular Matrix				-1.74	-1.98	-1.71
Extracellular Matrix Component				-1.59		-1.75
Extracellular Matrix Disassembly	1.82					
Intermediate Filament Cytoskeleton	-1.79					
Negative Regulation Of Actin Filament Depolymerization	-1.79					
Negative Regulation Of Actin Filament Polymerization	-2.05					
Negative Regulation Of Cytoskeleton Organization	-1.57			-1.68		-2.04
Positive Regulation Of Actin Filament Bundle Assembly	-1.77					
Positive Regulation Of Cytoskeleton Organization	-1.71					
Proteinaceous Extracellular Matrix				-1.86	-2.23	-1.86
Regulation Of Actin Filament Based Process	-1.52					
Regulation Of Actin Filament Depolymerization	-2.04			-2.16		
Regulation Of Actin Filament Length	-1.86					
Regulation Of Cytoskeleton Organization	-1.60			-1.52		-1.70

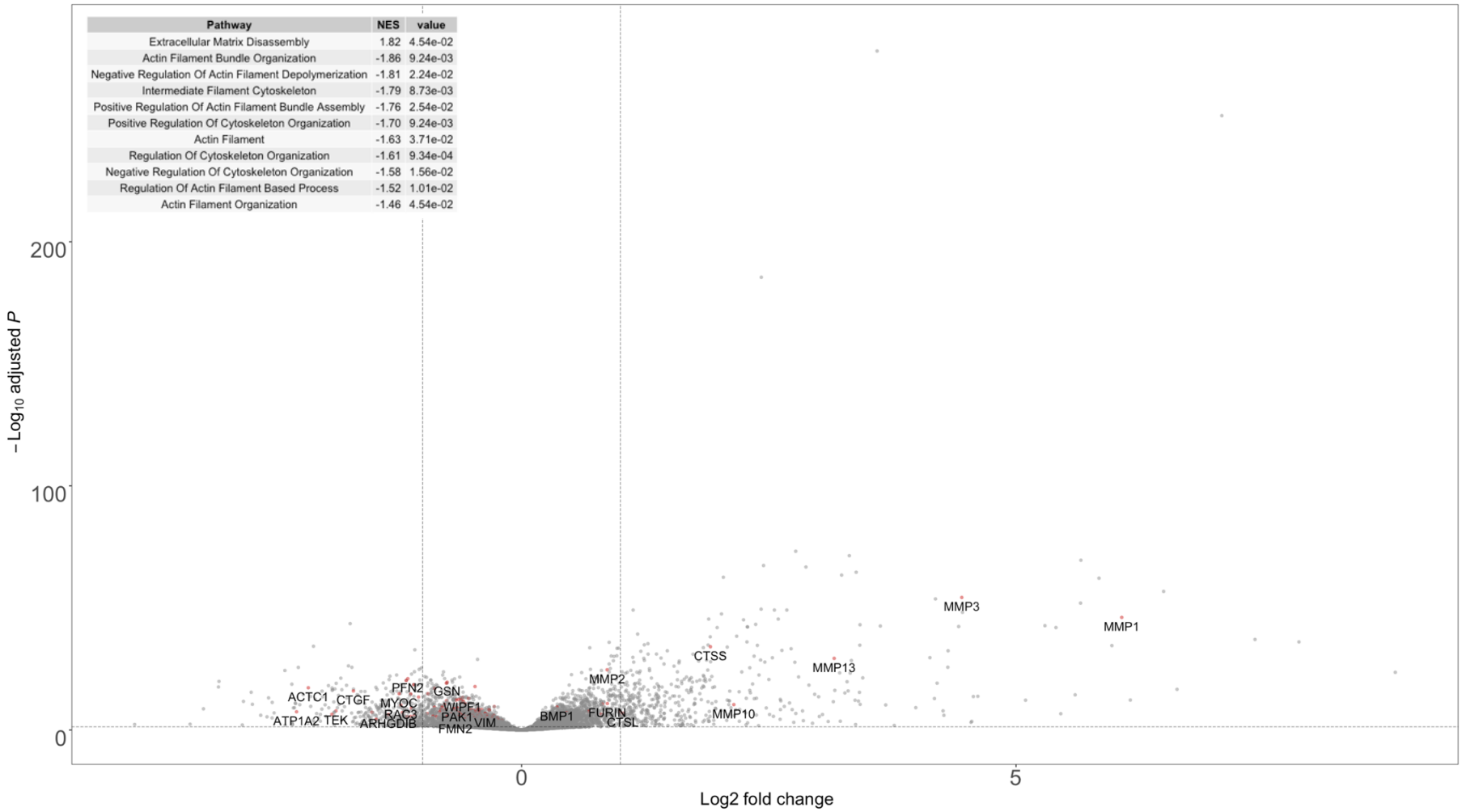


Figure 4-11. *Volcano plot representing genes changed by IL-17A treatment in chondrocytes, with the most important genes that correspond to the changes in matrix pathways highlighted by name and red dots*.

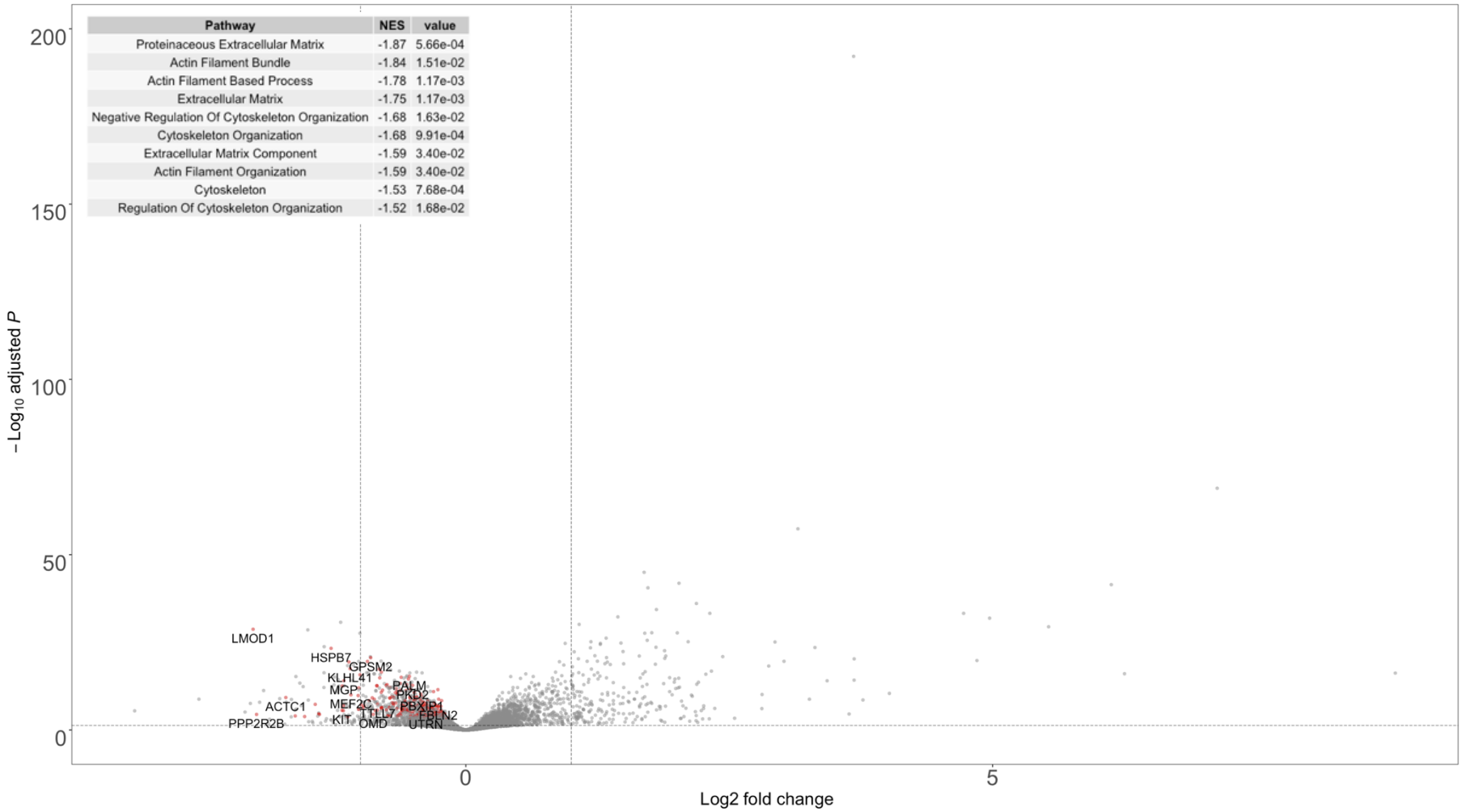


Figure 4-12. *Volcano plot representing genes changed by IL-17A treatment in synovial fibroblasts, with the most important genes that correspond to the changes in matrix pathways highlighted by name and red dots.*

4.6.6 IL-17A activates p38 and NF-κB pathways

To confirm the ability of IL-17A/F to activate key intracellular cell signalling pathways, chondrocytes or synovial fibroblasts from end-stage OA patients were treated with control medium or 10 ng/mL IL-17A/F for 10 minutes, 30 minutes, 60 minutes, or 480 minutes (8 hours). Western blot analysis was used to investigate the activation status of key intracellular signalling mediators p65 NF-κB, and the MAP kinases ERK1/2 (p44/p42 MAP kinases), p38, and JNK. However, due to the low protein expression of SAPK/JNK in these cells, it was not possible to quantify the protein levels of SAPK/JNK before and after treatment. Activation of ERK1/2, p38, and p65 NF-κB is shown as the ratio of phosphorylated protein over the total amount of protein compared to baseline control (Figure 4-13).

IL-17A significantly activated p38 (after 10 and 30 minutes) and NF-κB p65 subunit (after 10, 30, and 60 minutes) in both chondrocytes and synovial fibroblasts. While increases in activation were seen by IL-17AF for p38 and NF-κB p65 in chondrocytes and synovial fibroblasts, these increases were only significant in activation of NF-κB p65 after 30 minutes in chondrocytes, and after 10 and 30 minutes in synovial fibroblasts. IL-17A caused a stronger activation of p38 and NF-κB p65 than IL-17AF. However, IL-17AF, but not IL-17A, significantly increased ERK1/2 activation in synovial fibroblasts. Due to variation in activation level of ERK1/2 in chondrocytes control samples, it was not possible to draw any conclusions from the changes in activation of ERK1/2 in these cells.

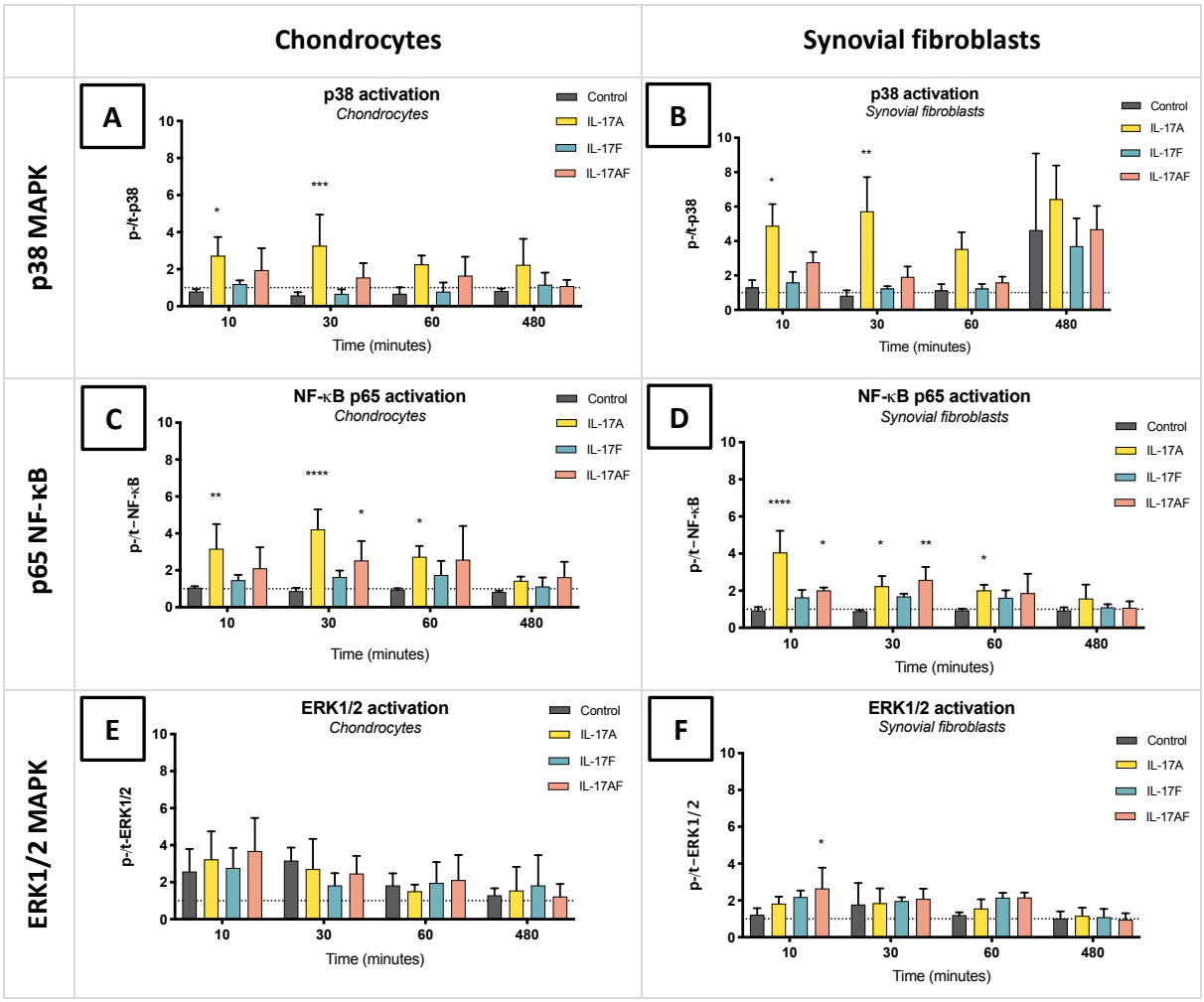


Figure 4-13. *Activation of p38 MAP kinase, p65 NF-κB, and ERK1/2 (p44/p42 MAP kinases) in chondrocytes and synovial fibroblasts by IL-17A, IL-17F, and IL-17AF (all 10 ng/ml). Results show the relative ratio of phosphorylated protein over total protein compared to baseline control. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. 2-way ANOVA, Dunnet's multiple comparisons test. Mean+SD. N=3.*

4.6.7 p38- and NF-κB-inhibitors are not able to decrease IL-17A-induced gene expression

It is known that IL-17 signalling differs between cells, but there are still many questions on how exactly IL-17 signalling is orchestrated within these cells. From the previous section it is clear that IL-17A can cause a strong increase in activation of p38 and p65 NF-κB in end-stage OA chondrocytes and synovial fibroblasts. Therefore, this study set out to test whether IL-17A-induced gene expression could be prevented by blocking the p38- or NF-κB-pathway. To do so, end-stage OA chondrocytes or synovial fibroblasts were treated with 10 ng/ml IL-17A with or without increasing concentrations of p38 inhibitor SB203580 (0.1, 1.0, or 10 μM) or NF-κB inhibitor JSH-23 (0.5, 5.0, or 50 μM). Cells were pre-treated with inhibitor for 2 hours before adding IL-17A to this for 24 hours.

For each cell type, 4 genes were chosen from the RNA-Seq results that were 1) significantly changed by IL-17A, 2) with a baseline sufficient baseline expression for RT-qPCR analysis, 3) genes related to immune, matrix, or intracellular signalling pathways. In chondrocytes, change in expression of inflammatory gene *IL6*, the matrix genes *MMP1 and MMP3*, and the intracellular signalling gene *NFKBIZ* were studied, while *CXCL1*, *IL6*, *MMP3*, and *NFKBIZ* were studied in synovial fibroblasts.

In chondrocytes, treatment with the p38 inhibitor SB203580 alone resulted in a small non-significant decrease in *IL6* and *MMP1* mRNA expression and NF-κB inhibitor JSH-23 caused a small non-significant decrease in *IL6* mRNA expression compared to vehicle control. p38 inhibition caused a small concentration-dependent decrease in IL-17A induced gene expression in chondrocytes, although none of these changes reached statistical significance (Figure 4-14A-D). Treatment with NF-κB inhibitor JSH-23 (Figure 4-14E-H) also did not cause any changes in IL-17A-induced gene expression with 0.5 and 5.0 μM. However, a small, non-significant, decrease occurred in *NFKBIZ* and *IL6* expression after treatment with 50 μM JSH-23.

In synovial fibroblasts, the p38 inhibitor SB203580 alone caused a strong decrease in *MMP3* and *NFKBIZ* mRNA expression compared to vehicle control, although mRNA expression of *IL6* and *CXCL1* was unaffected (Figure 4-15A-D). A decrease in expression of all genes tested was seen after treatment with the NF-κB inhibitor JSH-23 alone compared to vehicle control (Figure 4-15E-H). The p38 inhibitor SB203580 caused a small concentration-dependent decrease in IL-17A-induced *IL6* and *CXCL1* mRNA expression. Although a non-significant decrease in mRNA expression after treatment with inhibitors were seen compared to IL-17A alone, it is likely that this decrease in mRNA expression is due to the effect of these inhibitors on baseline use of these signalling molecules by cells alone rather than the inhibition of IL-17A-induced effects.

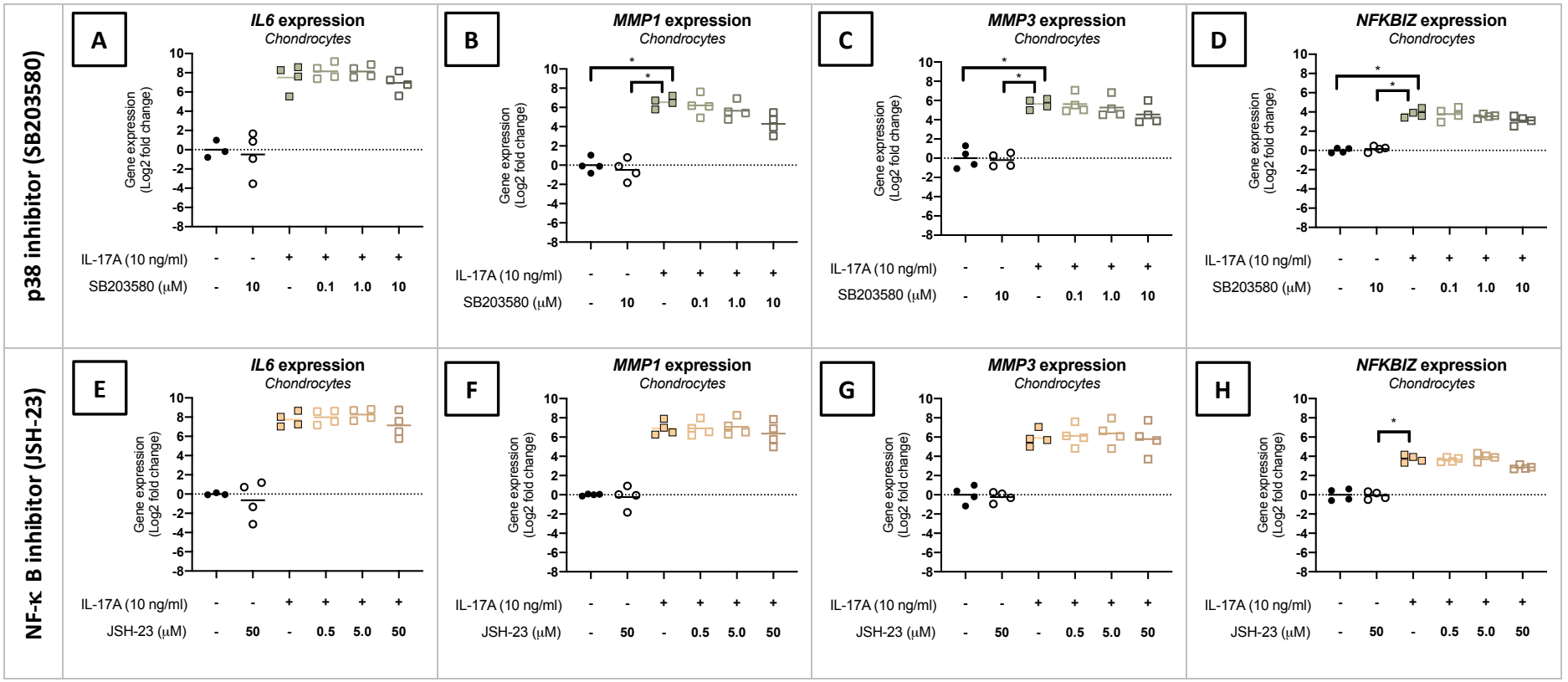


Figure 4-14. *Gene expression of chondrocytes after treatment with IL-17A with or without p38 inhibitor SB203580 (A-D) or NF-κB inhibitor JSH-23 (E-H). Gene expression was calculated using the ddCT-method using both GAPDH and ACTB as housekeepers. Gene expression is expressed as log2 fold change (LFC) compared to control. Changes in gene expression were calculated comparing each treatment to IL-17A treatment alone. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. (A,E) Kruskal-Wallis test with Dunn's multiple comparisons test. (B-D and F-H) Friedman test with Dunn's multiple comparisons test. Individual values and mean. N=4.*

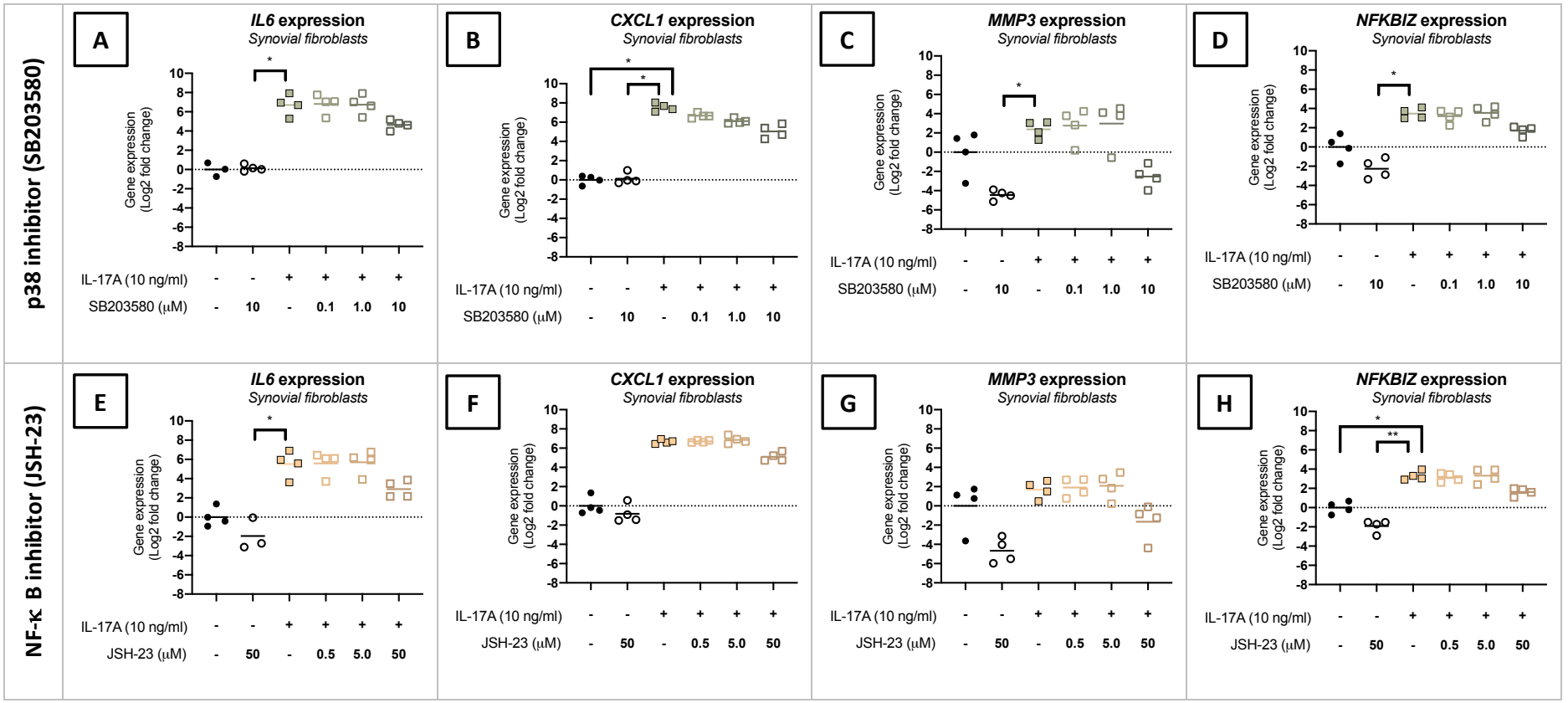


Figure 4-15. *Gene expression of synovial fibroblasts after treatment with IL-17A with or without p38 inhibitor SB203580 (A-D) or NF-κB inhibitor JSH-23 (E-H). Gene expression was calculated using the ddCT-method using both GAPDH and ACTB as housekeepers. Gene expression is expressed as log2 fold change (LFC) compared to control. Changes in gene expression were calculated comparing each treatment to IL-17A treatment alone. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. (A,E) Kruskal-Wallis test with Dunn's multiple comparisons test. (B-D and F-H) Friedman test with Dunn's multiple comparisons test. Individual values and mean. N=4.*

4.6.8 Secukinumab inhibits IL-17A-induced expression of OA-relevant genes in chondrocytes and synovial fibroblasts

The clinically-used IL-17A-antibody secukinumab was tested to confirm that IL-17A is responsible for the changes seen in OA-relevant inflammatory, matrix turnover, fibroblast activation, and intracellular signalling genes. Chondrocytes and synovial fibroblasts from end-stage OA patients were treated with 10 ng/mL IL-17A for 24 hours with or without increasing, clinically-relevant concentrations of secukinumab (0.5, 5.0, and 50 μg/ml).

Changes in the IL-17A-induced effects were investigated in 10 genes that were found to have been significantly changed by IL-17A in the RNA-Seq experiment. In both chondrocytes and synovial fibroblasts, changes in expression of inflammatory genes *CXCL1*, *IL6, CCL20*, and *PTGS2*, the matrix genes *MMP1* and *MMP3*, the intracellular signalling genes *MAP3K8* and *NFKBIZ*, and the fibroblast activation gene *PDPN* were studied. In addition, the expression of *NOS2* mRNA in chondrocytes and expression of *CSF3* mRNA in synovial fibroblasts was studied. However, mRNA expression of *CSF3* and *CCL20* could not be quantified in the used RT-qPCR assay due to a low baseline expression of these genes.

Secukinumab decreased the IL-17A-induced increase in gene expression for almost every gene tested in a dose-related manner. However, this decrease was generally only statistically significant after treatment with the highest dose of secukinumab (50 μg/ml) due to the variation in gene expression between the different patients. For every gene tested, only minimal differences were seen between control-treated samples and samples treated with 50 μg/ml secukinumab alone, pointing to the fact the decrease in gene expression seen was due to the effect of secukinumab on IL-17A, rather than from a decrease of gene expression by secukinumab alone.

In chondrocytes, 5 μg/ml secukinumab caused a statistically significant inhibition of IL-17A induced *MMP1* and *MAP3K8* mRNA expression, and 50 μg/ml secukinumab was able to significantly inhibit IL-17A-induced gene expression for *IL6*, *CXCL1*, *PTGS2*, *MMP1*, *MMP3*, *PDPN*, *NFKBIZ*, and *MAP3K8* mRNA expression (Figure 4-16).

In synovial fibroblasts, 5 μg/ml secukinumab caused a statistically significant decrease in IL-17A-induced *IL6, PDPN,* and *NFKBIZ* mRNA expression, and 50 μg/ml secukinumab decreased the IL-17A-induced gene expression of *IL6*, *CXCL1*, *PTGS2*, *MMP1*, *MMP3*, *NFKBIZ*, and *MAP3K8* with statistical significance (Figure 4-17).

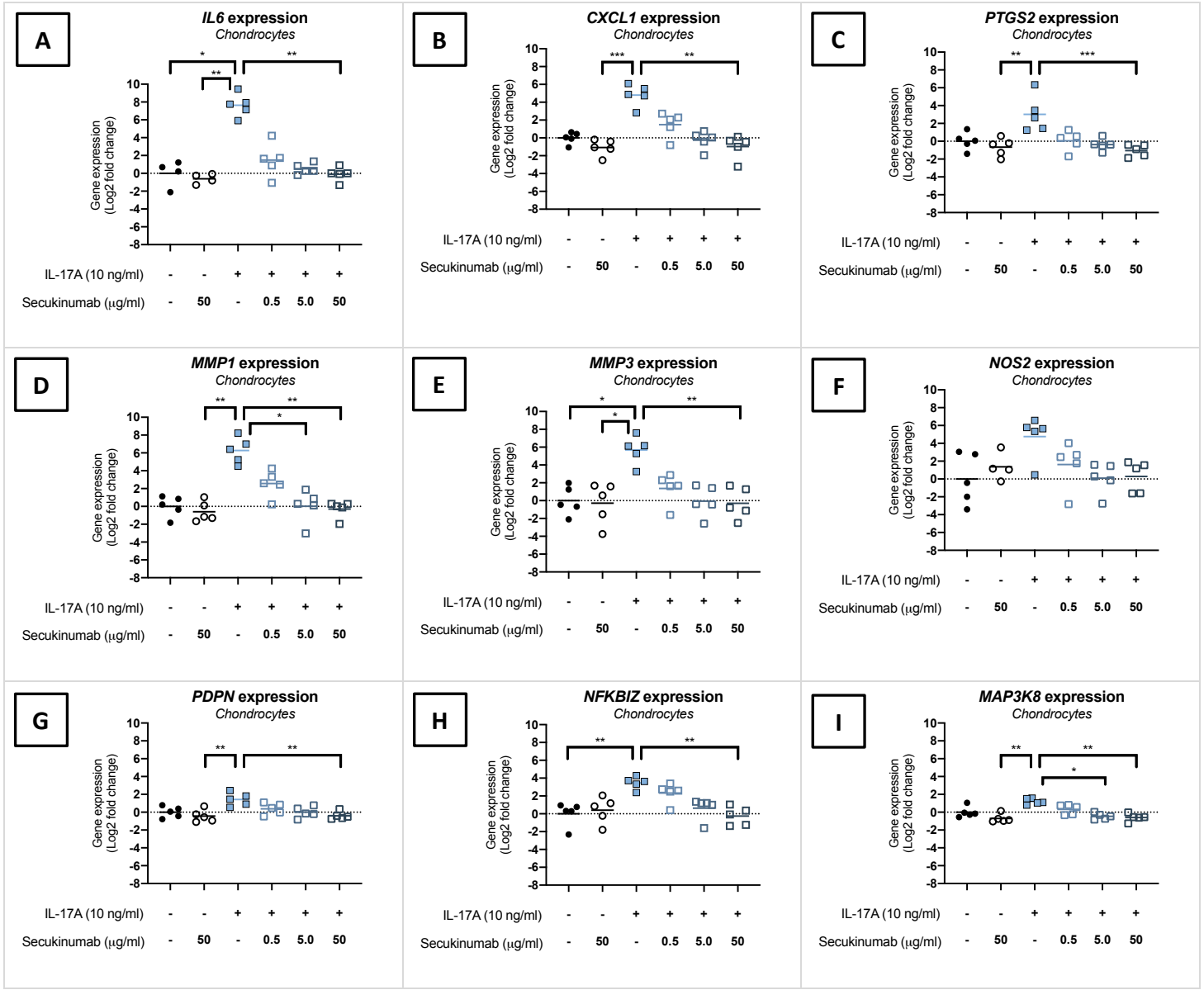


Figure 4-16. *Gene expression of chondrocytes after treatment with IL-17A (10 ng/ml) with or without clinically-used IL-17A-antibody secukinumab in different concentrations (0.5, 5.0, or 50 μg/ml). Gene expression was calculated using the ddCT-method using both GAPDH and ACTB as housekeepers. Gene expression is expressed as log2 fold change compared to control. Changes in gene expression were calculated comparing each treatment to IL-17A treatment alone. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. (A,F) Kruskal-Wallis test with Dunn's multiple comparisons test. (B-E, G-I) Friedman test with Dunn's multiple comparisons test. Individual values and mean. N=5.*

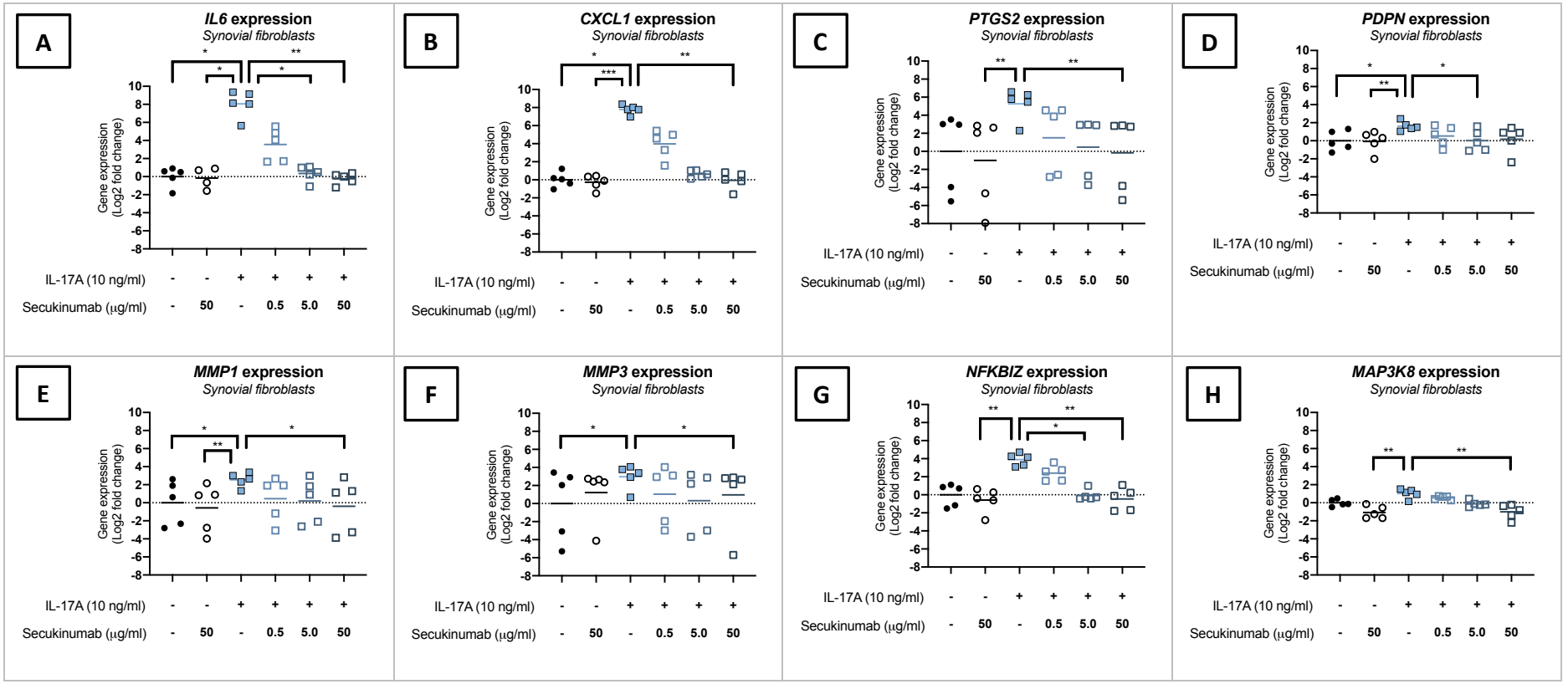


Figure 4-17. *Gene expression of synovial fibroblasts after treatment with IL-17A (10 ng/ml) with or without clinically-used IL-17A-antibody secukinumab in different concentrations (0.5, 5.0, or 50 µg/ml). Gene expression was calculated using the ddCT-method using both GAPDH and ACTB as housekeepers. Gene expression is expressed as log2 fold change (LFC) compared to control. Changes in gene expression were calculated comparing each treatment to IL-17A treatment alone. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. (A) Kruskal-Wallis test with Dunn's multiple comparisons test. (B-H) Friedman test with Dunn's multiple comparisons test. Individual values and mean. N=5.*

4.7 Discussion

4.7.1 Aim of this chapter

The pro-inflammatory cytokine IL-17A is known to play a role in inflammatory diseases such as psoriasis and ankylosing spondylitis, and there is a growing number of reports in clinical, animal, and genetic studies that support a role for IL-17 in OA. In this thesis, a potential Th17 cell population (CCR6+ T cells) was identified in a subset of end-stage OA patients. In synovium of patients at risk of OA, expression of IL-17A and its receptors IL-17RA and IL-17RC was identified, and IL-17RA positively correlated with synovitis score. However, the specific role IL-17A/F could play in OA disease pathophysiology is still unclear. Therefore, the overarching aim of this study was to investigate and compare the role of IL-17A/F in driving inflammation and matrix destruction in OA, including the signalling pathways through which these cytokines act. The results of this were used to establish the potential of inhibiting the most active IL-17 cytokine as a therapeutic option in OA.

4.7.2 IL-17A/F can contribute to OA pathophysiology by promoting inflammation, immune-cell recruitment and matrix destruction and angiogenesis

IL-17A/F induced changes in gene expression in both chondrocytes and synovial fibroblasts from end-stage OA patients. IL-17A/F targeted genes included those encoding signalling molecules, cytokines, and chemokines. Pathway analysis revealed regulation of multiple cellular pathways including those related to immune responses, matrix, complement, and angiogenesis. Many of these pathways have been implicated in the development or progression of OA, supporting a role of IL-17A/F in OA in (a subgroup of) patients that show increased concentrations of these cytokines in the joint. While both chondrocytes and synovial fibroblasts respond in similar ways to IL-17A/F, the most potent cytokine IL-17A seems to activate extracellular matrix destruction more strongly in chondrocytes, while regulation of the immune system including chemokine and cytokine activation

was more strongly induced in synovial fibroblasts, confirming previous reports that the IL-17A/F-signalling does differ between cell types.

Research on IL-17A/F-driven diseases psoriasis and ankylosing spondylitis have shown that IL-17A/F strongly affects the inflammation and extracellular matrix as described in the Background section of this chapter. As synovitis and cartilage damage are two key features in OA, the following section will focus on IL-17A/F-induced expression of genes regulating pro-inflammatory environment, the recruitment of immune cells, and matrix destruction along with key IL-17A/F intracellular signalling proteins that are regulated by IL-17A/F.

4.7.2.1 Intracellular signalling mediators

The most significantly upregulated gene following IL-17A/F treatment in both chondrocytes and synovial fibroblasts was *NFKBIZ.* Other important genes for signalling molecules that will be discussed are *MAP3K8* and *TNFAIP3.*

4.7.2.1.1 NFKBIZ

NFKBIZ encodes for the protein "Inhibitor of NF-κB Zeta" (IκBζ), which is a central regulator of IL-17 signalling. IκBζ transcription factor from the NF-κB family, which acts as a driver of transcription by facilitating IL-17-induced gene expression by activating IL-17A/F target genes in cooperation with NF-κB, and suppressing the activity of miR-23b, a natural inhibitor of IL-17A/F signalling. Although *NFKBIZ* mRNA is intrinsically unstable, the ability of IL-17A/F to stabilize mRNA transcripts can help to stabilize these transcripts, thereby indirectly upregulating the expression of all its target genes[236]. In addition, several studies have shown that IκBζ is a critical signalling molecule in IL-17A/F-driven diseases, as it is known that the expression of some IL-17A/F-target genes is IκBζ-dependent[236]. In a study using human keratinocytes IL-17A/F-induced expression of key psoriasis genes was significantly downregulated after the knockdown of IκBζ, and silencing of IκBζ by siRNA before stimulation with

IL-17A/F significantly reduced protein levels of target proteins, thereby demonstrating that this is a key regulator of IL-17A/F-inducible psoriasis-associated genes[290]. This was confirmed by another study that showed that ablation of *NFKBIZ* transcript in keratinocytes did not affect differentiation or proliferation but did reduce IL-17A/F-mediated expression of pro-inflammatory cytokines and chemokines[291].

IκBζ is not only essential for IL-17A/F signalling but also for IL-36 signalling. Interestingly, it has been shown that there is a positive feedback loop between IL-36 and the IL-23/IL-17 axis, which is hypothesized to be important for the persistent psoriatic phenotype. One recent paper found that IL-36 receptor antagonist attenuated OA development in destabilisation of the medial meniscus (DMM) mice and reduced MMP secretion in chondrocytes *in vitro*. Further studies should investigate whether an IL-36/IL-17 positive feedback loop occurs in (a subset of) OA patients in order to clarity whether the inhibition of IκBζ function is a strategy that could be further investigated for the treatment or prevention of patients with high levels of IL-36 and/or Th17 cytokines in OA.

4.7.2.1.2 MAP3K8

MAP3K8 is upregulated to a similar degree by IL-17A, IL-17F, and IL-17AF in both chondrocytes (5.4-, 2.9- and 3.8-fold increase respectively) and synovial fibroblasts (3.2-, 2.5-, and 2.8-fold increase respectively). *MAP3K8* encodes for the enzyme mitogen-activated protein (MAP) kinase kinase kinase 8, also called tumor progression locus 2 (TPL2). After the initiation of IL-17A/F signalling, IKK can phosphorylate p105, thereby releasing TPL2. After TPL2-mediated phosphorylation and catalytic activation of TAK1 and dynamic mechanism of TPL2-TAK1 interaction, TAK1 mediates activation of NF-κB and MAP kinases JNK and p38, although it is not needed for activation of ERK1/2[236,292]. TPL2 deficiency is associated with a severely attenuated IL-17-stimulated gene expression of several chemokines and proinflammatory genes in astrocytes. In addition, *Tpl2*-knockout mice were resistant to experimental autoimmune encephalomyelitis due to an impaired recruitment of

leukocytes to the CNS. IL-17 signalling in these mice could only be restored by the addition of wild type TPL2, but not control GFP or an inactive TPL2[292]. From these studies it becomes clear that this is a pivotal gene in IL-17 signalling due to its role in the activation of JNK, p38, and NF-κB. This might not only suggest that JNK, p38, and NF-κB are important pathways through which IL-17A/F signals in chondrocytes and synovial fibroblasts, this might also present an intracellular target to inhibit IL-17A/F-induced gene expression. In addition, the small differences in induction of *MAP3K8* expression might contribute to the differences in the number and strength of gene expression between the three IL-17A/F cytokines.

4.7.2.1.3 TNFAIP3

TNFAIP3 was significantly upregulated by IL-17A in chondrocytes (3.9-fold increase) but not synovial fibroblasts. *TNFAIP3* encodes for the protein TNF-α induced protein 3, which is also called A20. A20 has been described as natural inhibitor of TNF-α, TLR, IL-1R, Nod-like receptor pathways. More recently, it has been shown that A20 can inhibit IL-17 signalling by deubiquitinating TRAF6, which ultimately leads to the downregulation of IL-17-induced signalling through MAP kinase and NF-κB pathways[293]. This suggests that IL-17A activates this mechanism in chondrocytes to negatively regulate its own signalling. However, further research is necessary to clarify why this gene was upregulated by IL-17A in chondrocytes but not in synovial fibroblasts, and if this means that IL-17A-induced effects in synovial fibroblasts are more sustained than in chondrocytes.

4.7.2.2 Pro-inflammatory mediators

Not only did the pathway analyses show a strong enrichment in the regulation of (innate) immune system, there was also an enrichment in complement and cytokine activity after treatment with IL-17A/F. As it is accepted that synovitis is a critical feature of OA, it is important to understand which pro-inflammatory mediators are most strongly regulated by IL-17A/F and how these could affect OA pathophysiology.

4.7.2.2.1 Cytokines

IL6, *CSF3*, *PTGS2*, *PTGES*, *TNFAIP6*, and *NOS2* were all upregulated by IL-17A/F treatment of chondrocytes and synovial fibroblasts. The upregulation of *IL6* is in agreement an earlier study showing IL-6 protein is increased by IL-17A in OA chondrocytes[173]. As discussed in Chapter 1, IL-6 can act as a pro- and an anti-inflammatory cytokine as it is hypothesized to be one of the key cytokines in OA[26]. *IL6* expression was increased by IL-17A (233-fold) and IL-17AF (15-fold) in chondrocytes, and by all three cytokines in synovial fibroblasts (46-fold by IL-17A, 8.4-fold increase by IL-17AF, and 6-fold by IL-17F). IL-6 can cause changes in subchondral bone and contribute to the degradation of cartilage, both by itself and by working in synergy with other cytokines. As discussed in more detail in Chapter 1, the activity of IL-6 has been linked to IL-17 in other systems several ways: IL-17 a strong inducer of IL-6 production[68], IL-6 is a key Th17 polarizing cytokine[69,70], and IL-17A and IL-6 can synergise to enhance each other's signalling[71,72]. In addition, it was shown that the IL-6/IL-17A axis plays an important role of the induction of arthritis in a mice model of arthritis[73]. IL-6, IL-17A, and the transcription factor STAT3 are functionally linked so that when one of these are upregulated, it leads to a dysregulation of other members, which has been linked to the development of several autoimmune disorders[68]. Therefore, it is possible that IL-6 and IL-17A/F can separately and together induce OA-related changes in the joint.

PTGES (encoding for the enzyme prostaglandin E synthase) and *PTGS2* (encoding for the prostaglandin-endoperoxide synthase 2 (PTGS2) also called cyclooxynase-2 (COX-2)) are part of the prostaglandin family and contribute to formation of prostacyclin, an important protein that is expressed during inflammation. In both chondrocytes and synovial fibroblasts, *PTGS2* and *PTGES* were most strongly upregulated by IL-17A in chondrocytes (9-fold and 6-fold, respectively) and in synovial fibroblast (4- and 2-fold, respectively); IL-17AF lead to a slight increase in both genes in both cell types (between 1.6- and 1.3-fold increase); IL-17F was unable to increase the expression. A

variant of the *PTGS2* gene has been found to be involved in the risk of developing knee OA. Although COX-2 inhibitors seem to have analgesic effects in human OA[294], these inhibitors are not chondroprotective in an OA mouse model[295]. Therefore, previous studies suggest that PTGES and PTGS2 could contribute to the development of OA due to their role in inflammation and pain, but more research is needed to clarify what their exact roles in disease could be.

The gene *TNFAIP6* that encodes for the protein TNF-stimulated gene 6 (TSG-6), is increased by all three IL-17 cytokines in chondrocytes (39-fold by IL-17A, 7-fold by IL-17AF, and 3-fold by IL-17F) and IL-17A (11-fold) and IL-17AF (3-fold) in synovial fibroblasts. Early studies have found that this protein was found in cartilage and synovium from arthritic joints, but not healthy joints, indicating that it could play a role in OA[296]. Since then, there has been a lot of research into the role of TSG-6 in OA, and TSG-6 is a global biomarker for OA[297]. However, studies have found controversial roles for TSG-6 as it is associated with cartilage degradation and inflammation, but with the majority of studies reporting chondroprotective and anti-inflammatory effects[298–300]. Interestingly, when retinal lesions were studied in CCL2 and CX3CR1-knockout mice, administration of TSG-6 decreased *Il17a* gene expression[301]. Future research should clarify if TSG-6 expression in OA joints is related to IL-17A/F expression and if IL-17A/F-induced *TNFAIP6* expression is a negative feedback mechanism to induce anti-inflammatory effects and reduce IL-17A production.

NOS2, the gene that encodes for inducible nitric oxide synthase (iNOS), is strongly increased upon IL-17A/F stimulation in chondrocytes (171-fold increase by IL-17A, 36-fold by IL-17AF, and 20-fold by IL-17F), but not synovial fibroblasts. iNOS is responsible for production of NO, which is a recognised pro-inflammatory and destructive mediator in OA. Increased concentrations of NO have been found in OA, and it is thought that NO induces the production of cartilage matrix proteoglycans and increased MMP activity, and that it is strongly linked to chondrocyte apoptosis[302]. The inhibition of

the iNOS enzyme has been found to have chondroprotective effects, so iNOS inhibitors have been proposed for the treatment of OA[303].

In contrast to *NOS2*, expression of *CSF3* was induced by IL-17A (453-fold increase) and IL-17AF (63-fold increase) in synovial fibroblasts, but the expression of this gene was not induced in chondrocytes. *CSF3* encodes for the protein granulocyte colony-stimulating factor (G-CSF). In mouse models of inflammatory arthritis, pain and disease severity were ameliorated after prophylactic or therapeutic treatment with G-CSF receptor antibody, which correlated with reduced joint neutrophil numbers without reducing the number of circulating neutrophils, therefore being suggested as a therapeutic option for arthritis and inflammatory pain[304]. However, in rabbits with a cartilage defect systemic administration of G-CSF increased the quality of the repaired cartilage tissue, suggesting that G-CSF could have reparative effects[305]. As IL-17A is a strong inducer of *CSF3*, more research is needed to find whether G-CSF is protective or harmful in OA.

4.7.2.2.2 Complement

There is accumulating evidence that activation of the complement pathway, which is important in host defence, scavenging cellular debris and immune complexes, and the guidance of adaptive immunity by stimulation of B and T lymphocytes, is involved in OA pathogenesis[33,43]. Complement can be activated by three different pathways (classical, alternative, and mannose-activated), which all lead to the cleavage of complement component C3. *C3*, the gene encoding for the protein C3, was increased after IL-17A/F treatment in chondrocytes (50-fold by IL-17A, 15-fold by IL-17AF, and 7-fold by IL-17F) and by IL-17A in synovial fibroblasts (3-fold), which could lead to an increase in protein expression of this central component in the complement system. The increase of C3 itself could by itself lead to an increased activation of complement, as the alternative pathway is activated by a spontaneous hydrolysis of C3[306]. The classical pathway is triggered by the activation of the C1-complex, which is made up of C1q, C1r and C1s molecules. In chondrocytes, IL-17A causes

approximately 2-fold increase in the genes encoding for C1r and C1s, which again could lead to an increase in the formation of C1-complexes. Other complement-related genes that were induced by IL-17A/F include *C1QTNF*-related genes, *C1R*, *C2CD4A*, *C2CD4B*, and *C5AR1*.

The results of the RNA-Seq analysis in this thesis suggest that IL-17A/F could be involved in the regulation of complement. Interestingly, there are several studies that have shown that complement is able to both positively and negatively regulate IL-17 expression[307]. Complement activation product C5a has been shown to be able to inhibit production of IL-17A in macrophages[308], but promote expression of IL-17A from human CD4+ T cells[309]. In addition, a study using a mouse model for severe airway hyperresponsiveness showed that different complement elements contributed to the regulation of IL-23/Th17 axis[310]. Therefore, studies are needed to investigate cross-talk between IL-17A/F and complement in OA, and to establish whether this contributes directly to disease pathogenesis.

4.7.2.3 Chemotactic factors

In Chapter 2, it was confirmed that there was a clear infiltration of immune cells into end-stage OA synovium. However, large differences were seen between patients regarding the proportion of immune cells and subsets of immune cells present. From the enrichment analysis focussed on the regulation of chemokines and cytokines, IL-17A/F caused a clear increase in pathways related to chemokine activity, receptor binding and production. Therefore, it is important to understand the effect of IL-17A/F on chemokine production and how this could affect OA pathophysiology.

4.7.2.3.1 CXCL-chemokines

CXCL1, chemokine (C-X-C motif) ligand 1*,* was significantly increased by IL-17A, IL-17F, and IL-17AF in both chondrocytes and synovial fibroblasts. Increases of at least 2-fold were also seen in *CXCL2*, *CXCL3*, *CXCL5*, *CXCL6*, and *CXCL8* in both cell types. All these are from the ELR-positive CXC motif

family, which induce the migration of neutrophils and bind to CXCR1 (CXCL6 and -8) or CXCR2 (CXCL1, -2, -3, -5, -6, and -8)[311]. A recent network analysis has shown that there is an increased expression of *CXCR1* mRNA in OA synovium, which could mean that this tissue is very responsive to these chemokines[312]. In addition, there are some studies that have looked at CXCR1/2 signalling in OA cartilage. Studies have shown that ELR+CXC chemokines are retained in the cartilage by binding to complex sugar molecules and have an important role in supporting cartilage integrity following injury. However, in osteoarthritic joints, the cartilage gets so badly damaged that these chemokines are lost, thereby losing its protective role in the cartilage and contributing to inflammation by attracting neutrophils into the joint[313]. Another study confirmed these finding, showing that ELR+ chemokines maintain viability and differentiation of chondrocytes, but that disruption of CXCR1/2 signalling induces phenotypic instability of chondrocytes, which is a leading cause of OA[314]. Therefore, Ha *et al.* conclude that maintaining an optimum level of CXCR1/2 signalling is important, as high CXCR1/2 signalling contributes to development of OA, while low signalling might be a protective factor of the development of chronic OA[315]. As IL-17A/F-cytokines lead to an increase in chemokines that act on CXCR1/2 receptors, this could be one of the ways IL-17A/F can contribute to the development of OA. However, future studies need to confirm that not only increase gene expression but also chemokine protein secretion and CXCR1/2 signalling increases in response to IL-17A/F.

Although studies report that neutrophils are generally not found in OA synovial tissue, a small but clear CD15+ population was found in all 10 end-stage OA patients studied in Chapter 2. In addition, a recent study identified six OA-related synovial fluid biomarkers that were significantly associated with OA symptoms that correlated with markers for activated macrophages and neutrophils, thereby concluding that these biomarkers could attest to an inflammatory endotype of OA[27]. Neutrophils might be able to contribute to tissue damage seen in OA as these cells can release damage-associated molecular patterns (DAMPs), leading to the release of catabolic and pro-inflammatory

mediators by chondrocytes and macrophage, which could contribute to destruction of matrix[316]. In contrast, neutrophil extracellular vesicles have been shown to exert powerful protective effects in inflammatory arthritis, including RA, by protecting against cartilage breakdown[317]. IL-17A/F increases the gene expression of several neutrophil-attracting chemokines in synovial fibroblasts and to a lesser extent in chondrocytes, suggesting that these cytokines could contribute to the influx of these immune cells into the joint. Therefore, both the presence and role of neutrophils in OA needs to be further studied to get a better understand of how the induction of neutrophil-attracting chemokines could affect the onset or progression of OA.

4.7.2.3.2 CC chemokines

The mRNA expression of several CC (C-C-motif) chemokines was at least 2-fold increased. In chondrocytes, *CCL2*, *CCL7*, *CCL8*, *CCL13*, *CCL20*, and *CCL27*, as well as the receptors *CCR1* and *CCR7*. For synovial fibroblasts, expression of both *CCR1* and *CCR7*, as well as chemokines *CCL2*, *CCL13*, and *CCL20* were increased by IL-17A. Expression of CCR1, CCL2, CCL7, and CCL8 has previously been found in synovium of inflammatory OA patients[318]. The most highly upregulated chemokine in both cell types was CCL20, which has been shown to induce proinflammatory genes such as those encoding for IL-6, COX-2, and iNOS, and increased the release of MMP-1, MMP-13, PGE2, and GAG fragments in cartilage explants in culture, thereby contributing to matrix degradative responses in cartilage[172]. Serum concentrations of CCL2 are associated with increased odds of presence and progression of radiographic knee OA[319]. In addition, in mouse models of OA CCL2 has been found to mediate monocyte recruitment, inflammation, and cartilage destruction[320], and regulate pain-related behaviour[321]. CCL13 is a monocyte chemoattractant protein, which was found to be significantly higher in serum of OA patients than in healthy controls, and patients with KL grade 4 have significantly higher CCL13 concentration in serum and synovial fluid than those with KL grade 2-3, thus being suggested as a biomarker for OA progression[322]. Although these are just preliminary studies, these do suggest that IL-17A/F-induced increases of chemokines could contribute to

inflammation, matrix degeneration, and potentially pain in OA. As Chapter 2 found varying proportions of granulocytes and monocyte-derived cells in end-stage OA synovium, further studies should investigate whether increased concentrations of IL-17A/F in the OA joint are associated with increased infiltration of these cell subsets.

4.7.2.3.3 Facilitating the influx of immune cells

Angiogenesis and inflammation work hand-in-hand as inflammation can increase angiogenesis, and angiogenesis can facilitate inflammation. Synovitis is an accepted feature of OA pathophysiology, and the increased vascularity seen in OA synovium is thought to facilitate the chronic inflammation due to the influx of immune cells into the synovium[41,42]. Genes encoding for important pro-angiogenic mediators in OA were increased by IL-17A/F, including *VEGF* (by IL-17A and to lower extent IL-17AF in chondrocytes), *ICAM* (by IL-17A and lower extent IL-17AF in chondrocytes and synovial fibroblasts, small increase by IL-17F in synovial fibroblasts), and *VCAM* (by IL-17A in synovial fibroblasts). Therefore, IL-17A/F might not only induce the secretion of chemoattractants, but also facilitate the movement of these immune cells into the joint by stimulating the secretion of angiogenic factors.

4.7.2.4 Extracellular matrix-related genes

The loss of articular cartilage due to the destruction of extracellular matrix is one of the oldest and most studied characteristics of OA. The dysregulation of extracellular matrix is a very complex process, which remains to be poorly understood. From the enrichment analysis of extracellular matrix-related pathways, it became clear that IL-17A/F caused a decrease in regulation and organisation of extracellular matrix and cytoskeletal processes. IL-17A/F treatment significantly increased the expression of genes encoding for MMP-1, -2, -3, -13, and -14 in chondrocytes. In synovial fibroblasts, IL-17A/F treatment caused an increase in the expression of genes encoding for MMP-1, -2, and -3, although these changes were smaller than in chondrocytes.

MMP-13, also known as collagenase-3, is the most studied MMP in cartilage biology. *MMP13* gene expression was 9-fold increased by IL-17A and 2-fold by IL-17AF in chondrocytes. While it is barely detected in healthy cartilage, several studies show that MMP-13 levels are increased in OA[323–326]. Together with the fact that MMP-13 can cleave type II collagen, the major type of collagen present in cartilage[327], this has suggested that MMP-13 could be a major catabolic effector in OA. In mouse models of OA, MMP-13 levels correlate with chondrocytes undergoing hypertrophic differentiation, and overexpression of MMP-13 causes extensive ECM degradation[328,329]. When using MMP-13 knock-out mice in a mouse model of OA, the progression of OA was inhibited as mice were protected from proteoglycan loss and structural damage in cartilage chondrocyte hypertrophy and osteophyte development still occurred[330].

MMP-3, also called stromelysin-1, is another well-studied enzyme in OA as it can not only degrade several types of collagen, but also gelatin, aggrecan, fibronectin, laminin, and elastin. *MMP3* mRNA expression was 22-fold increased by IL-17A, 4-fold by IL-17AF, and 2-fold by IL-17F in chondrocytes, and in synovial fibroblasts increased by 4-fold by IL-17A and 2-fold by IL-17AF. In previous studies it was found that protein expression of proMMP-3 and MMP-3 is significantly higher in synovial tissue of OA patients compared to healthy controls[331,332]. In addition, expression of MMP-3 is positively related to OA severity, which is why this has been suggested to be used as an important indicator of early diagnosis and disease activity of OA[332,333].

MMP-1 is a collagenase that is capable of degrading collagenase type I, II, and III. In this study, strong increases were seen in *MMP1* expression in chondrocytes by IL-17A (67-fold), IL-17AF (11-fold), and IL-17F (4-fold). In synovial fibroblasts, increases were found by all three cytokines, but to a smaller extent: IL-17A (8-fold), IL-17AF (2.6-fold), IL-17F (1.8-fold). Expression levels of MMP-1 are low in healthy cartilage, but significant upregulation is observed in OA cartilage[334]. In addition to MMP-13, -

3, and -1, there are other MMPs that have been hypothesized to play a role in OA. MMP-2 can break down type IV collagen and expression of this has been found to be increased in chondrocytes from OA cartilage[335]. MMP-14 is involved in aggrecan degradation and cadherin cleavage, and is also increased in OA cartilage[336]. Interestingly, MMP-14 appears to have the ability to activate other MMPs, including MMP-2 and MMP-13[327,337,338], which increases the interest for this protein in OA.

MMPs are not the only group of matrix-related proteins that have received attention for their potential role in matrix turnover in OA. Other groups of proteins include ADAM (a disintegrin and metalloproteinase), ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), TIMPs (tissue inhibitors of metalloproteinases), and different proteoglycans such as aggrecan. The IL-17A/F induced increase in expression of *MMP*, *ADAM* and *ADAMTS* genes that encode proteins that cleave matrix, alongside a decrease in the expression of genes encoding for aggrecan and TIMPs can ultimately lead to a dysregulation and breakdown of cartilage in OA.

4.7.3 IL-17A can cause a strong increase in p38 and p65 NF-κB activation in OA chondrocytes and synovial fibroblasts

Western blot revealed that the intracellular signalling proteins p38 and NF-κB p65 were activated by IL-17A/F, and that the extent of the activation reflected the number of genes regulated by each of these cytokines in chondrocytes and synovial fibroblasts in RNA-Seq analysis: IL-17A causing a strong significant activation, IL-17AF causing a modest but occasionally significant activation, and IL-17F causing a small, non-significant activation. However, the results from ERK1/2 activation were more striking as IL-17A was not able in cause a significant increase in ERK1/2 activation, although IL-17A-induced ERK1/2 expression has been previously shown in studies with synovial sarcoma cells, human RA synovial fibroblasts, and human cardiac fibroblasts[339–341]. As it is known that effects of IL-17 do differ between cell types, it could be that this is one of the differentiating effects of IL-17 between

cells which could contribute to the differential effects seen between the different IL-17A/F cytokines (Figure 4-18).

Although IL-17A did not increase ERK1/2 activation, treatment with the IL-17AF heterodimer did lead to increase in ERK1/2 activation. This raised the questions as to why ERK1/2 is not activated in a similar way as p38 and NF-κB p65 by IL-17A, and how IL-17AF could induce a stronger activation of one of these pathways than IL-17A which binds more strongly to the IL-17RA/IL-17RC receptor. It was first thought that all MAPK activation in the IL-17A/F signalling pathway, including p38, ERK1/2, and JNK, were all activated through TPL2-dependent phosphorylation of TAK, which is similar to the signalling seen by toll-like receptor and TNF receptor signalling. However, recent studies have shown that ERK1/2 activation in IL-17A/F signalling works independently from TPL2, and the mechanism that does activate ERK1/2 activation in IL-17A/F signalling is still unclear[236,292,342]. Interestingly, the sequencing data has shown that all three IL-17 cytokines in both chondrocytes and synovial fibroblasts could increase the expression of the gene for TPL2 (*MAP3K8*) as explained in 4.7.2.1.2.

In IL-17 signalling ERK1/2 is not only able to induce transcription of IL-17A/F target genes, but it can also contribute to the negatively regulation of IL-17 signalling as shown in the stromal cell line ST2. One of the negative regulation mechanisms of IL-17 signalling is the binding of phosphorylated C/EBPβ to C-terminal C/EBPβ activation domain (CBAD) on IL-17RA. This association leads to dampening of IL-17-dependent signalling by deubiquitylation of TRAF6, a protein that when ubiquitinylated mediates MAPK and NF-κB signalling. However, the phosphorylation of C/EBPβ is dependent on glycogen synthase kinase 3β (GSK3β) or ERK1/2[231,236,293,343]. Therefore, the activation of ERK1/2 by IL-17AF heterodimer in this study could thus mean that there is a higher chance of ERK-dependent phosphorylation of C/EBPβ, leading to a stronger dampening of IL-17-induced signalling.

However, then the question remains how IL-17AF can induce a different signal than IL-17A while binding less potently to same receptor complex. There is very little known about the differences in IL-17 signalling between IL-17A, IL-17F, and IL-17AF as it has often been assumed that they induce the same signals as they all bind to the same receptor. However, from other signalling molecules such as Type I interferon signalling it becomes clear that a spectrum of functions can be activated by Type I interferons by binding to the same to receptor chains: IFNAR1 and IFNAR2[344]. A first insight in the potential differential signalling of IL-17A/F comes from a recent study that investigated the binding of IL-17AF to its receptor, using IL-17A/F constructs expressed in a human embryonic kidney (HEK) 293S cell line. They revealed that while IL-17RA binds IL-17A with a much higher affinity than IL-17F, IL-17AF can bind to the IL-17RA part of the receptor with both its "A-side" and its "F-side". As IL-17RC does not discriminate between the two sides of the cytokine, IL-17AF can cause the formation of two topologically-distinct heterotrimeric complexes, which they hypothesize could lead to different signalling properties[345]. However, better insight in the differential signalling of the three IL-17A/F cytokines in different cell types is needed to get a better understanding of their potential functions, which can be used to further unravel their potential role in promoting inflammation, influx of immune cells, and matrix destruction in OA.

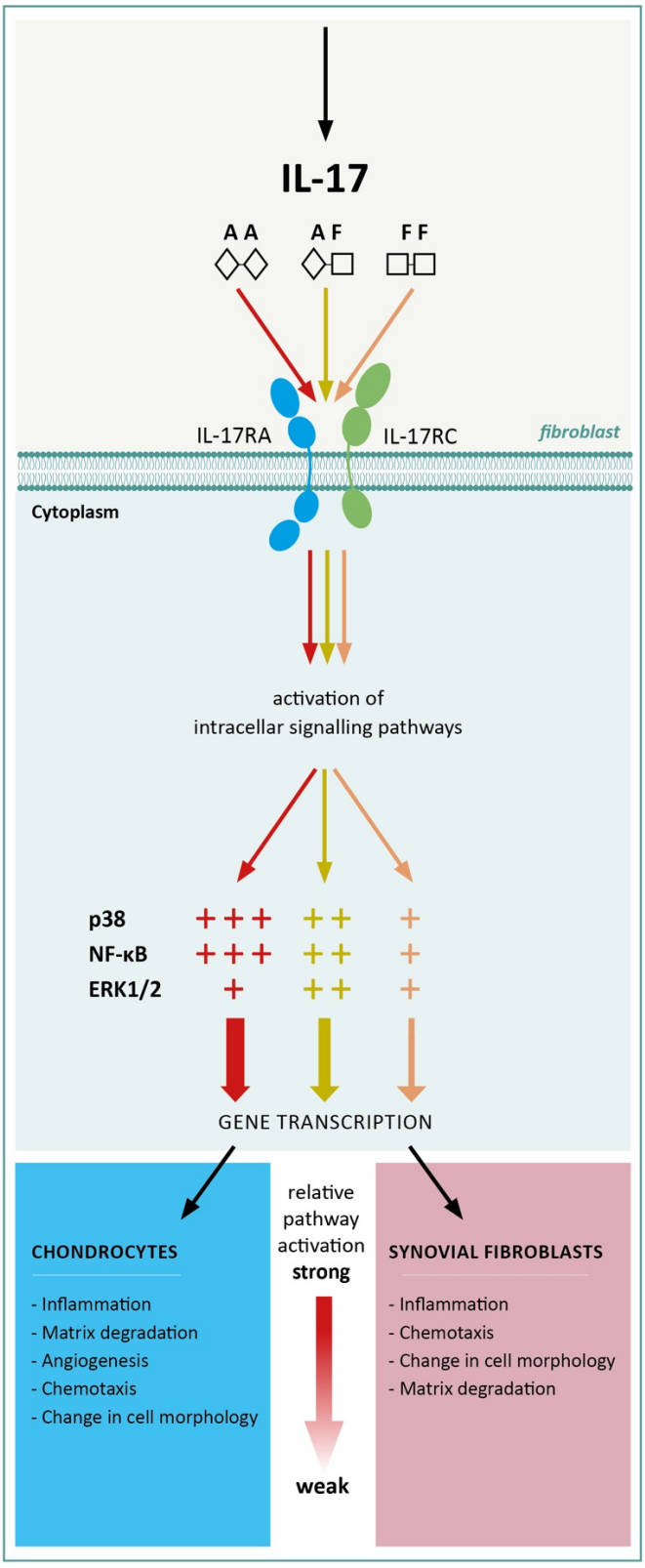


Figure 4-18. *Overview of the effects of IL-17A, IL-17F, and IL-17AF on the activation of intracellular signalling pathways and gene transcription based on the findings of this chapter. IL-17A causes the strongest overall activation of intracellular signalling pathways and strongest increase in gene transcription, followed by IL-17AF, and finally IL-17F.*

4.7.4 Inhibition of p38 and NF-κB does not prevent or inhibit IL-17A-induced gene expression

The data from both RNA-Seq and western blots reveal that out of the three IL-17A/F cytokines at 10 ng/mL, IL-17A is able to cause the largest induction of gene expression and the strongest activation of p38 and p65 NF-κB. As IL-17A is already used as a clinical target in other inflammatory (musculoskeletal) diseases, further work in this chapter focussed on intracellular signalling pathways activated by IL-17A as well as IL-17A itself as potential targets to inhibit IL-17A-induced gene expression in end-stage OA chondrocytes and synovial fibroblasts.

Firstly, a p38 and an NF-κB inhibitor were used to investigate whether these could inhibit IL-17A-induced gene expression. For each cell type, 4 genes related to immune, matrix, or intracellular signalling pathways were chosen from the RNA-Seq results that were significantly changed by IL-17A and had a baseline sufficient baseline expression for RT-qPCR analysis. Although some inhibitors caused a trend to decrease the IL-17A-induced gene expression, none of the concentrations tested of p38 inhibitor SB203580 or NF-κB inhibitor JSH-23 were able to significantly decrease IL-17A-induced mRNA expression of *IL6*, *MMP1*, *MMP3*, or *NFKBIZ* in chondrocytes and *IL6*, *CXCL1*, *MMP3*, or *NFKBIZ* in synovial fibroblasts. While both inhibitors did not affect baseline expression of these genes in chondrocytes, SB203580 decreased baseline mRNA expression of *MMP3* and *NFKBIZ,* and JSH-23 decreased baseline expression of all four genes tested in synovial fibroblasts. This suggests that that there is a much higher baseline p38 and NF-κB-driven expression of these transcripts of the tested genes in synovial fibroblasts than in chondrocytes.

The concentrations of the inhibitors used in this study were based on previous reports of IC50 (half maximal inhibitory concentration). For SB203580, the IC50 in human end-stage OA chondrocytes after stimulation with 10 ng/ml IL-1β for 24 hours was around 1 μM for all genes tested except *NOS2* (10 μM), which is why 0.1, 1.0, and 10.0 μM was used in this study[346]. For JSH-23, no IC50 studies

were found using chondrocytes or synovial fibroblasts, which is why the study by Shin *et al.* was used, who reported a 3 μM (nuclear translocation) and 7.1 μM (inhibition of transcriptional activity) IC50 using LPS-stimulated RAW264.7 cells, which is why 0.5, 5.0, and 50 μM were used[347]. However, the used concentrations might not have be high enough for the cell types or IL-17A signalling might require higher concentrations to be inhibited than that the cytokines used in the available literature. As IL-17 signalling activates multiple intracellular signalling proteins, the inhibition of one pathway might not be sufficient to reduce IL-17A-incuded expression of tested genes if other intracellular pathways can compensate for this. Therefore, the experiment should be repeated with higher concentrations of inhibitors as well as a combination of inhibitors to check if IL-17A-induced gene expression can be prevented by the inhibition of one intracellular pathway or if the intracellular signalling is so dynamic that IL-17A-induced gene expression can only be inhibited by inhibiting IL-17A itself or its receptors. Additionally, other p38 and NF-κB inhibitors should be used to confirm results, and a positive control should be used to confirm inhibitor activity. Lastly, western blot analysis of short-term incubations (10, 30, and 60 minutes) could be done to clarify whether an inhibition of target signalling pathways occurs and whether compensatory activations occurs in other pathways.

4.7.5 Secukinumab inhibits IL-17A-induced gene expression

Secukinumab is a clinically-used IL-17A antibody that is approved for the use in psoriasis, psoriatic arthritis, and ankylosing spondylitis. In a population of moderate to severe plaque psoriasis patients during a clinical trial with secukinumab, it was modelled that the average steady state serum concentration of secukinumab in patients receiving the highest dose was 44.5 μg/ml, while the maximum serum concentration at steady state was 55.3 μg/ml[348]. Therefore, the average of these values – 50 μg/ml – was used as the highest concentration in this *in vitro* model, and two lower concentrations (5.0 μg/ml and 0.5 μg/ml) were tested alongside it.

A concentration-dependent decrease in IL-17A-induced gene expression was seen for all genes tested with the highest concentration of secukinumab often bringing the gene expression back to baseline. This not only confirms that the expression of these genes is indeed increased by IL-17A, it also suggests that this could be a potential therapeutic option for a (sub)group of OA patients with high concentrations of IL-17A. The decrease in IL-17A-induced gene expression was mostly only significant using the highest concentration of secukinumab due to variations in response between the cells from the 5 patients tested. In future experiments, different specific and non-specific IL-17A/F-inhibitors should be tested in *in vitro* or *ex vivo* models to see which candidate would have the most potential in (as subset of) OA patients, before moving *to in vivo* models.

4.7.6 Limitations

In this chapter, it was shown that the strongest changes in gene expression and activation of intracellular signalling proteins were caused by IL-17A, followed by IL-17AF, and lastly IL-17F. These changes in gene expression lead to the enrichment of several key biological pathways including inflammation, angiogenesis, and matrix destruction. While the activation of matrix destruction genes was strongest in chondrocytes, the activation of immune pathways and cytokines/chemokine regulation was strongest in synovial fibroblasts, suggesting that IL-17A/F-induced effects is dependent on cell type (Figure 4-18). The cytokine with the strongest effect on these cells – IL-17A – was further investigated to look at the therapeutic potential of blocking IL-17A or its activated intracellular signalling proteins. While inhibition of its activated signalling molecules p38 or NF-κB did not affect IL-17A-induced gene expression, the IL-17A antibody secukinumab significantly reduced IL-17A-induced gene expression. Although this study has significantly increased the understanding of the effect IL-17A/F in OA chondrocytes and synovial fibroblasts, there are some limitations that should be discussed.

4.7.6.1 Patient-derived end-stage OA fibroblasts

One of the strengths of this study is the use of cells derived from tissue from end-stage OA patients that was obtained during total knee replacement surgery. The use of patient-derived cells will lead to more representative results than using cell lines derived from chondrosarcoma or synovial fibrosarcoma. In addition, these patients-derived cells are a much better representation of the natural variations in responses seen in a patient population. However, due to the limited patient-derived cells available, these variations in responses to treatment often make it harder to draw conclusions from experiments, as statistically significant differences are hard to reach with low patient numbers and large variations. An example of this is the results of the inhibition IL-17A by secukinumab: although IL-17A clearly increased the expression of the genes tested, this was not always statistically significant from control-treated synovial fibroblasts and especially in chondrocytes. In addition, a dose-response could be seen after treatment with increasing concentrations of secukinumab, but often only the highest concentration reached statistical significance. This can largely be attributed to the variation in expression between different patients. As access to human tissue is often limited, only a limited number of patient cells could be used for these experiments. Although this variation does limit the statistical significance that can be reached, the variation also provides a more accurate representation of human variability, which is extremely important for the research into human disease and potential treatment of these diseases.

A great deal of effort is being put into developing highly accurate techniques for biological research that can overcome low patient numbers and large variations. An example of this is RNA-Seq, which is able to measure every transcript in the transcriptome and pick up transcripts with low expression. However, this technique also has several draw-backs including a high false discovery rate due to the high number of transcripts analysed and a bias towards longer transcripts, among other things. Although these “omics” techniques present many possibilities for future research, they are often expensive, making them currently hard to use for routine work.

Another limitation of these cells is that they are derived from patients with end-stage disease. Therefore, it is important to consider that these cells have been exposed to a chronic, low-grade inflammatory environment, which could have induced multiple changes in these cells or could have made them more or less responsive to IL-17A/F. Therefore, the findings of this study only apply to end-stage disease and no conclusions can be made on the potential effects of IL-17A/F in early OA pathophysiology.

4.7.6.2 Concentration-dependent signalling and temporal effects

Although the concentration of IL-17A/F cytokine used in the assay has been chosen with care after dose-response studies measuring specific IL-17A/F-induced genes, due to limited resources only one concentration was studied in RNA-Seq analysis. However, it needs to be considered that it is possible that IL-17A/F-induced effects differ depending on the concentration. An example of concentration-dependent effects is the WNT3A signalling as low concentrations activate the calcium-dependent pathway, whereas high concentrations lead to activation of the β-catenin-dependent pathway, which both very distinct target genes[349]. Therefore, it is important that future studies include a concentration range of IL-17A/F to study potential concentration-dependent effects that may reflect physiological variation in IL-17A/F levels.

In addition to only using one concentration, only one time point was used in this study. In previous work, end-stage OA chondrocytes were treated with IL-17A for 4, 8 or 24 hours and the greatest increase in *IL6* mRNA expression was found after 24 hours of treatment, which is why this time point was used for further studies. However, IL-17F and IL-17AF were not investigated in this study, and no later time points were investigated in the previous or current study to look at the long-term effects of these cytokines. Thus, future work should study the temporal effect of IL-17A/F in order to get a better understanding of early and late responses to these three cytokines.

4.7.6.3 Use of gene expression as an output

Although gene expression analysis using RNA-Seq is a high-throughput method with a highly precise output, it is important to note that changes in gene expression do not necessarily correlate to changes in protein expression. Therefore, it is extremely important that future studies confirm that the IL-17A/F-induced changes in gene expression that were seen in this study are translated into changes in protein expression and functional activity of hallmark pathways. Although treatment times might need to be extended to account for the time that is needed to translate mRNA into proteins, the cell medium from IL-17A/F treated cells could be used to look at changes in the concentrations of proteins secreted by these cells. Especially proteins that have previously been related to OA inflammation, such as cytokines and chemokines, and extracellular matrix proteins that have been connected to matrix destruction in OA, including MMPs and proteoglycans, should be looked at in more detail using a protein quantification method such as enzyme-linked immunosorbent assays (ELISAs), meso scale discovery (MSD) platform, multiplex assays, or proteomics.

4.7.6.4 The use of high-throughput methods to determine clinically-relevant changes

The RNA-Seq data presented in this thesis gives a snapshot of the IL-17A/F induced transcriptome in chondrocytes and synovial fibroblasts from end-stage OA patients. However, the functional relevance of the transcriptomic changes has not been determined. Functional relevance may depend on how stable this gene transcript is, how often it gets transcribed, if there are post-transcriptional changes, and the importance of the protein in a certain pathway or disease process.
Therefore, the functional effect of a highly changed gene might be overestimated, while small changes in important genes, i.e. an aggregation of changes in multiple genes that will together result in a functional change, could easily be overlooked. The development of several pathway analysis programmes has already improved the way that this data can be interpreted, but OA-specific

databases could immensely improve the way this data can be interpreted. In addition, the integration of transcriptomic data with other "omics" outputs, such as single-cell RNA-Seq, chromatin-immunoprecipitation sequencing, proteomics, and metabolomics, could provide further insight. This "multi-omics" or "integrative omics" approach is currently being developed as this offers the opportunity to understand the flow of information that underlies a disease much better than a single omics approach[350].

4.7.7 Future studies

This study significantly increases the understanding of IL-17A/F-induced inflammation and matrix destruction in OA. It is essential that this knowledge is combined with recently published findings to formulate new research questions and explore these effects furthers. Along with the suggestions for future studies that have been discussed earlier in this chapter, future research should understand the effect of chronic IL-17A/F exposure in the inflammatory environment of the OA joint. Therefore, future studies should investigate the effects of IL-17A/F on long-term treatment, the effects of IL-17A/F in combination with other mediators, and ultimately the effects of IL-17A/F in a joint environment.

4.7.7.1 Long-term treatment

While this study characterises relatively short-term effects of IL-17A/F treatment on end-stage OA chondrocytes and synovial fibroblasts, it is important to consider these effects persist into chronic inflammation. Long-term treatment using both mRNA and proteins outputs could give more insight into the potential chronic effects induced by these cytokines in the relatively closed environment of the joint. Ideally, cells from early-stage OA patients would be used for this to be able to study the potential changes chronic exposure to IL-17A/F could induce in these cells before moving into *in vivo* models.

4.7.7.2 Effects of IL-17A/F with other mediators

To understand the role that IL-17A/F could play in OA, it was essential to first elucidate at the effect of IL-17A/F on end-stage OA derived chondrocytes and synovial fibroblasts in isolation. However, it is known that the effects of IL-17A/F are amplified *in vivo* through synergy with other mediators such as TNF-α. Therefore, the next step in research should investigate the effects that IL-17A/F could have in an inflammatory joint environment, as there might both be mediators that can act in an additive or synergistic manner with IL-17A/F and mediators that reduce or inhibit IL-17A/F-induced effects. A next step in this process could be to culture chondrocytes and synovial fibroblasts derived from end-stage OA patients in a similar manner as done in this study (24 hours with 10 ng/mL IL-17A/F), but with or without the addition of synovial fluid from end-stage OA patients in order to better replicate the inflammatory environment in the OA knee.

4.7.7.3 Effect of IL-17A/F in the OA joint

As IL-17A/F-signalling mainly occurs in non-haemopoietic cells and fibroblasts are the predominant cell type in cartilage and synovium, resident fibroblasts were an important starting point to look at the effect of IL-17A/F in OA. However, previous studies have confirmed that IL-17A/F can also act on resident endothelial and epithelial cells, and some immune cells such as macrophages and dendritic cells [185,188,189]. In Chapter 3 it was noted that the expression of IL-17 receptor A and C often co-localised with the expression of macrophage markers CD68 and especially CD206 in the synovium of patients after knee injury or early-stage OA. In addition, Chapter 3 revealed vessels stained positively for IL-17A and its receptors IL-17RA and IL-17RC. Therefore, there is a strong rationale to study the effects of IL-17A/F on tissue-resident and infiltrating macrophages as well as endothelial cells in the OA joint.

Ideally, the effect of IL-17A/F should be tested on different cells in isolation first to understand the direct effect of these cytokines on different cell types. Subsequently, the effects of IL-17A/F should

be tested in more advanced *in vitro* or *ex vivo* model. Firstly, a co-culture model could be used. A direct co-culture model could include different stromal cells (for example chondrocytes and synovial fibroblasts) or combine stromal cells with immune cells to look at the interaction between these different cell types. However, as there are technical challenges to culturing different cell types in the same culture medium, indirect co-culture could also be used to look at the effect of the secretome of one cell type on a second cell type. Another option would be to use synovial explants from end-stage OA patients. In addition to synovial explants for the interaction between immune and stromal cells in synovium, cartilage explants should be used to get a better understanding of how IL-17A/F treatment affects the balance of catabolic and anabolic factors in matrix factors in OA. Other *in vitro* models that could be worth exploring for further work would be an "organ-on-a-chip" approach that include different joint tissues, or "organoids" – a simplified *in vitro* 3D version of an organ. After using these *in vitro* or *ex vivo* systems to understand the interactions between different cell type in response to IL-17A/F and exploring the potential of different inhibitors to inhibit these IL-17A/F-induced responses, studies should move into *in vivo* models to examine the effect of IL-17A/F in OA joints.

4.7.8 Conclusions

This chapter set out to characterise and compare the effect of IL-17A/F on gene expression and intracellular signalling in chondrocytes and synovial fibroblasts from end-stage OA patients, and find whether the inhibition of IL-17A itself or intracellular signalling pathways could inhibit these IL-17-induced effects.

IL-17A/F treatment caused changes in ±5400 genes in chondrocytes and ±3000 in synovial fibroblasts from end-stage OA patients. For the majority of regulated genes, IL-17A caused the largest log2 fold changes, followed by IL-17AF, and IL-17F. Enrichment analysis revealed that IL-17A (and to a lesser extent IL-17F and IL-17AF) caused changes in many OA-related pathways, including those related to

inflammation, matrix degradation, complement activation, angiogenesis, and hypoxia. IL-17A caused stronger activation of immune pathways in synovial fibroblasts than chondrocytes, while it caused more changes in matrix-related genes in chondrocytes than in synovial fibroblasts, suggesting that IL-17A/F-related changes are specific to the cell type and perhaps the cell environment.

The differences in potencies between IL-17A, IL-17F and IL-17AF seen in gene expression were reflected in their ability to activate intracellular signalling pathways: IL-17A induced the most potent changes in gene expression and in activation of p38 and NF-κB p65 signalling activation, IL-17AF lead to a smaller but significant activation of ERK1/2, p38, and NF-κB p65, while no significant activation was seen after IL-17F treatment. Inhibitors of p38 and NF-κB were unable to inhibit IL-17A-induced gene expression in chondrocytes and synovial fibroblasts, showing that this would be an inadequate candidate for inhibition of IL-17-induced effect. The clinically used IL-17A antibody secukinumab was able to significantly inhibit the IL-17A-induced gene expression, almost reducing it to baseline expression. This does not only confirm that IL-17A was indeed responsible for the changes seen in gene expression, but also suggests its potential as a therapeutic target for OA patients.

In conclusion, this study shows that IL-17A (and to a lower extent IL-17F and IL-17AF) induced changes in gene expression in many OA-related pathways. The chronic, low-grade inflammation seen in OA fits well with the effects IL-17A on inflammation and its effects on matrix destruction and angiogenesis further support a role for IL-17A in OA pathophysiology. The clinically-used IL-17A antibody secukinumab was able to significantly inhibit IL-17A-induced gene expression, confirming that IL-17A is responsible for these changes in gene expression. Inhibitors of IL-17A-activated signalling pathways p38 and NF-κB were unable inhibit IL-17A-induced gene expression, emphasizing the complexity of IL-17-signalling. As there are currently no disease-modifying OA drugs, secukinumab should be investigated as a potential therapeutic option in patients with OA.

4.7.9 Key findings

- IL-17A causes the strongest changes in gene expression and activation of intracellular signalling pathways in end-stage OA chondrocytes and synovial fibroblasts, followed by IL-17AF, and finally IL-17F
- IL-17A-induced changes in inflammation, matrix destruction, and angiogenesis pathways support a role for IL-17A in OA pathophysiology
- Secukinumab significantly reduced IL-17A-induced gene expression, confirming that the observed changes in gene expression were caused by IL-17A

Chapter 5

Discussion

5.1 Introduction

Osteoarthritis (OA) has long been viewed as a disease of wear and tear that leads to the breakdown of articular cartilage. However, it has become accepted that OA is better viewed as a spectrum of pathologies that involve different joint tissues with cartilage loss as the cardinal feature. Synovial inflammation (synovitis) is increasingly recognised as an important part of the disease, but there is limited knowledge about underlying biology, including drivers of the influx of immune cells, the identify of immune cells, and the activation status of fibroblasts. From previous studies, it is hypothesized that macrophages are one of the dominant immune cell subsets in OA synovium and could contribute to synovitis in OA. However, specific inflammatory mediators, produced by both immune and stromal cells of the joint, have also been linked to synovitis and OA. One family of inflammatory mediators that has received increasing interest is interleukin (IL)-17. The homodimers IL-17A and IL-17F, and the heterodimer IL-17AF are most thoroughly investigated, and play an important role in autoimmune and chronic inflammatory diseases such as psoriasis and ankylosing spondylitis. Initial studies have shown that IL-17A is present in increased concentrations in the OA joint, and that it can increase the production of several cytokines, chemokines, and MMPs in cells derived from end-stage OA cartilage and synovium. However, there is still limited knowledge on the expression of IL-17 cytokines and receptors in OA tissue, the effect of IL-17 on cells from end-stage osteoarthritis patients, and how this could relate to the synovitis seen in many patients.

While OA affects millions of people in the UK alone, there are still no disease modifying treatments available, emphasizing the requirement for improved understanding of the pathophysiology of the disease. Therefore, this thesis aimed to advance understanding of inflammation, and specifically the role of the pro-inflammatory cytokine interleukin (IL)-17, in human knee OA.

This chapter will discuss the overarching aim, objectives, and hypotheses of each chapter before presenting the key findings and implications of these findings. After discussing the limitations of this work and the opportunities for future work, the overall conclusions of this work will be summarised.

5.2 Aims of this thesis

Chapter 2 aimed to characterise the cellular and molecular environment of OA synovial tissue. The objectives of this chapter were to identify the immune cell and fibroblast subsets in end-stage synovium, investigate the potential association of different cell subsets and clinical scores, and to study the proteomic profile of end-stage OA synovial fluid. It was hypothesized that there are small populations of immune cells as well as fibroblasts with activated phenotype in end-stage OA synovium, that associations could be found between cell subsets themselves and with patient characteristics and clinical scores, and that there are differences in the proteomic profile of synovial fluid from different end-stage OA patients.

Chapter 3 subsequently aimed to map the expression of macrophage markers in tissue from patients at high risk of developing OA after knee injury. The objectives of this chapter were to investigate the expression of macrophage markers, IL-17A, and IL-17 receptors IL-17RA and IL-17RC, and establish whether these markers were correlated with synovitis score in patients at risk of OA. In addition, this chapter aimed to identify if OA-relevant cytokines modulate the mRNA expression of *IL17RA* and *IL17RC* in chondrocytes and synovial fibroblasts from end-stage OA patients. It was hypothesized that macrophage markers were expressed in this synovial tissue and would correlate with synovitis score, that IL-17RA, IL-17RC and IL-17A are expressed in synovium, and that cytokines such as IL-1β can induce the mRNA expression of *IL17RA* and *IL17RC* in cell isolated from end-stage OA patients.

Finally, Chapter 4 aimed to determine the effect of IL-17 cytokines on inflammation and matrix destruction. The objectives of this chapter were to use end-stage OA chondrocytes and synovial

fibroblasts to compare the effect of IL-17 cytokines on the gene expression of immune- and matrix-related genes implicated in OA, investigate and compare the effect of IL-17 cytokines on activation of intracellular signalling pathways, and investigate whether IL-17-induced gene expression could be inhibited by the use of a specific IL-17 antibody or inhibitors of its activated intracellular signalling pathways. It was hypothesized that IL-17A causes the most striking changes in gene expression and activation of intracellular signalling pathways, followed by IL-17AF, and IL-17F, and that both the inhibition of intracellular pathways and the inhibition of IL-17A itself could attenuate IL-17A induced gene expression.

5.3 Key findings of this thesis

Chapter 2 has shown that there is a significant infiltration of immune cells, especially T cells and macrophages, in end-stage OA synovium. However, large differences were found between the proportion of total immune cells and subsets of immune cells between patients. CCR6+ T cell levels showed the strongest difference and were only present in a subset of patients. The proportion of immune subsets might be associated with age, clinical scores of disease severity, subtypes of disease, or the proportion of fibroblast subsets. Fibroblasts in end-stage OA synovium had an activated phenotype, and previously published fibroblast subsets with unique functions based on the expression of fibroblast activation markers were identified in all patient samples. Proteomic analysis of matched synovial fluid did not result in any obvious separation of subsets of patients.

As macrophages were found to be one of two predominant immune cell populations, Chapter 3 further investigated the expression of macrophage markers, although this time in synovium from patients that suffered from anterior cruciate ligament or meniscus injury, who have a 50% chance of developing OA. In this cohort of patients, the expression of both CD68 and CD206 was identified and both markers positively correlated with synovitis score. Furthermore, protein expression of receptors IL-17RA and IL-17RC were found in this tissue and mRNA expression of these receptors was

found in chondrocytes and synovial fibroblasts from end-stage OA patients. Immunofluorescent co-staining showed that expression of IL-17 receptors co-localised with CD68 and CD206, but expression of IL-17 receptors was not limited to cells that expressed these macrophage markers, which is in line with the *IL17R* mRNA expression found in chondrocytes and synovial fibroblasts. While the protein expression of receptor IL-17RC in synovium was relatively stable, the expression of IL-17RA positively correlated with synovitis score, suggesting that this can increase sensitivity of this tissue to IL-17-cytokines. IL-17A was present in synovium from patients at high risk of developing OA, confirming that this cytokine could play a role in disease.

Chapter 4 assessed the effect of IL-17 cytokines IL-17A, IL-17F and IL-17AF in the predominant cell types in cartilage and synovium from end-stage OA patients: chondrocytes and synovial fibroblasts. This study showed that IL-17A (and to a lesser extent IL-17F and IL-17AF) induced changes in gene expression in many OA-related pathways, including immune-, matrix-, angiogenesis-, and complement-pathways. The differences in potencies between IL-17A, IL-17F and IL-17AF seen in gene expression were reflected in their ability to activate intracellular signalling pathways: IL-17A induced the most potent changes in gene expression and in activation of p38 and NF-κB p65 signalling activation, IL-17AF led to a smaller but significant activation of ERK1/2, p38, and NF-κB p65, while no significant activation was seen after IL-17F treatment. Inhibitors of p38 and NF-κB did not reduce IL-17-induced gene expression. However, the IL-17A antibody secukinumab was able to significantly inhibit IL-17A-induced gene expression, almost reducing expression to baseline.

5.4 Implications of these findings

5.4.1 Important role for inflammation in osteoarthritis

Inflammation in OA has long been a controversial subject. OA has often been referred to as “non-inflammatory arthritis” in contrast to RA which is known as “inflammatory arthritis”. However, many patients with OA report symptoms related to joint inflammation, including morning stiffness,

warmth, pain, and joint effusion. These symptoms can be attributed to synovial thickening and/or synovial fluid effusion, pointing to synovitis[25]. Although synovitis is increasingly recognised as an important pathological feature of OA, the immune cells that infiltrate into the synovium remain understudied, and fibroblasts subsets in OA synovium are poorly understood.

This thesis shows that approximately 22% of cells in end-stage OA synovium are immune cells. However, large differences were seen in both the proportion of total immune cells and subset of immune cells between patients, which supports the hypothesis that OA may be a spectrum of pathologies in which the relative involvement of each joint tissue and cell type differs between patients. Macrophages and T cells were the predominant immune cell types, comprising 76% of the total immune cell population. In synovium of patients at risk of developing post-traumatic OA, the macrophage markers CD68 and CD206 both positively correlated with a histological synovitis score, which supports the notion that macrophages are a key driver of synovitis.

Not only immune cells, but also fibroblasts have been shown to be able to contribute to inflammation. In previous studies it has been shown that there are distinct fibroblast subsets that can mediate joint inflammation and tissue damage, and it has been hypothesised that the interaction of these fibroblasts with immune cells can contribute to the persistence of joint inflammation[141]. This study found that fibroblasts in end-stage OA synovium had an activated phenotype, with the presence of several fibroblast activation markers. Subsets with distinct functions that have previously been identified in RA synovial fibroblasts were found in end-stage OA synovium, with the predominant fibroblast subsets being immune effector fibroblasts. In patients with RA, this population correlates with markers of systemic and tissue inflammation, and single cell transcriptomics has shown that these cells had an increased expression of cytokines and chemokines[148]. Therefore, both immune cells and activated fibroblasts could be important contributors to the synovitis seen in many OA patients.

5.4.2 IL-17 could play an important role in OA pathophysiology

IL-17 cytokines have already been shown to play an important role in inflammatory diseases such as psoriasis and ankylosing spondylitis, in which IL-17A induces inflammation, stimulates the recruitment of immune cells, and contributes to extracellular matrix destruction[252,264–266]. Various clinical studies have shown that IL-17A is increased in OA patients[30,58,274–276], but little is known about the role of IL-17A and its related cytokines IL-17F and IL-17AF is OA.

This thesis identifies CCR6+ T cells in synovium of end-stage OA patients. CCR6 is the receptor for CCL20 and it is often used as a marker for IL-17-producing T cells. These CCR6+ T cells were only identified in a subset of patients, suggesting a subtype of disease with an important role for CCR6+ T cells and its produced cytokine IL-17A[171]. In addition, IL-17A-producing T cells were identified in a subset of patients. As a previous paper also only detected IL-17A in a small subset (9.2%) of patients[175], it could be that IL-17 mainly plays a role in a subgroup of OA patients in which IL-17-producing cells are present.

This thesis further showed that mRNA of the IL-17 receptors IL-17RA and IL-17RC are expressed in chondrocytes and synovial fibroblasts from end-stage OA patients, confirming that OA synovium and cartilage can respond to IL-17A, IL-17F, and IL-17AF. Protein expression of these receptors was also found in the synovium of patients with a high risk of developing OA. While the protein expression of receptor IL-17RC in synovium was relatively stable, it was shown for the first time that IL-17RA expression increases with synovitis score in a cohort of patients that suffered from anterior cruciate ligament or meniscus injury, who have a 50% change of developing OA with a high risk of developing OA. This suggests that patients with synovitis become more sensitive to IL-17, meaning that the effect of these cytokines could still be enhanced in an inflammatory state even without changes to the IL-17 concentrations in the joint.

When investigating the effect of IL-17 cytokines on end-stage OA chondrocytes and synovial fibroblasts, it became clear that IL-17A (and to a lesser extent IL-17AF and IL-17F) can act on these cells and cause changes in the expression of a large number of genes. Enrichment analyses showed that particularly IL-17A regulated several OA-related pathways including immune-, matrix-, angiogenesis-, and complement-pathways. The IL-17A antibody secukinumab was able to significantly inhibit IL-17A-induced gene expression, confirming that IL-17A is responsible for these changes in gene expression. The chronic, low-grade inflammation seen in OA fits well with the effects IL-17A has on inflammation, which includes an increase in the production of cytokines and chemokines, and activation of the complement system; and effects of IL-17A on matrix destruction and angiogenesis further supports a role for this cytokine in OA pathophysiology (Figure 5-1). As there are currently no disease modifying drugs available for OA, anti-IL-17 therapy should be further investigated as a potential therapeutic option in selected patients with OA.

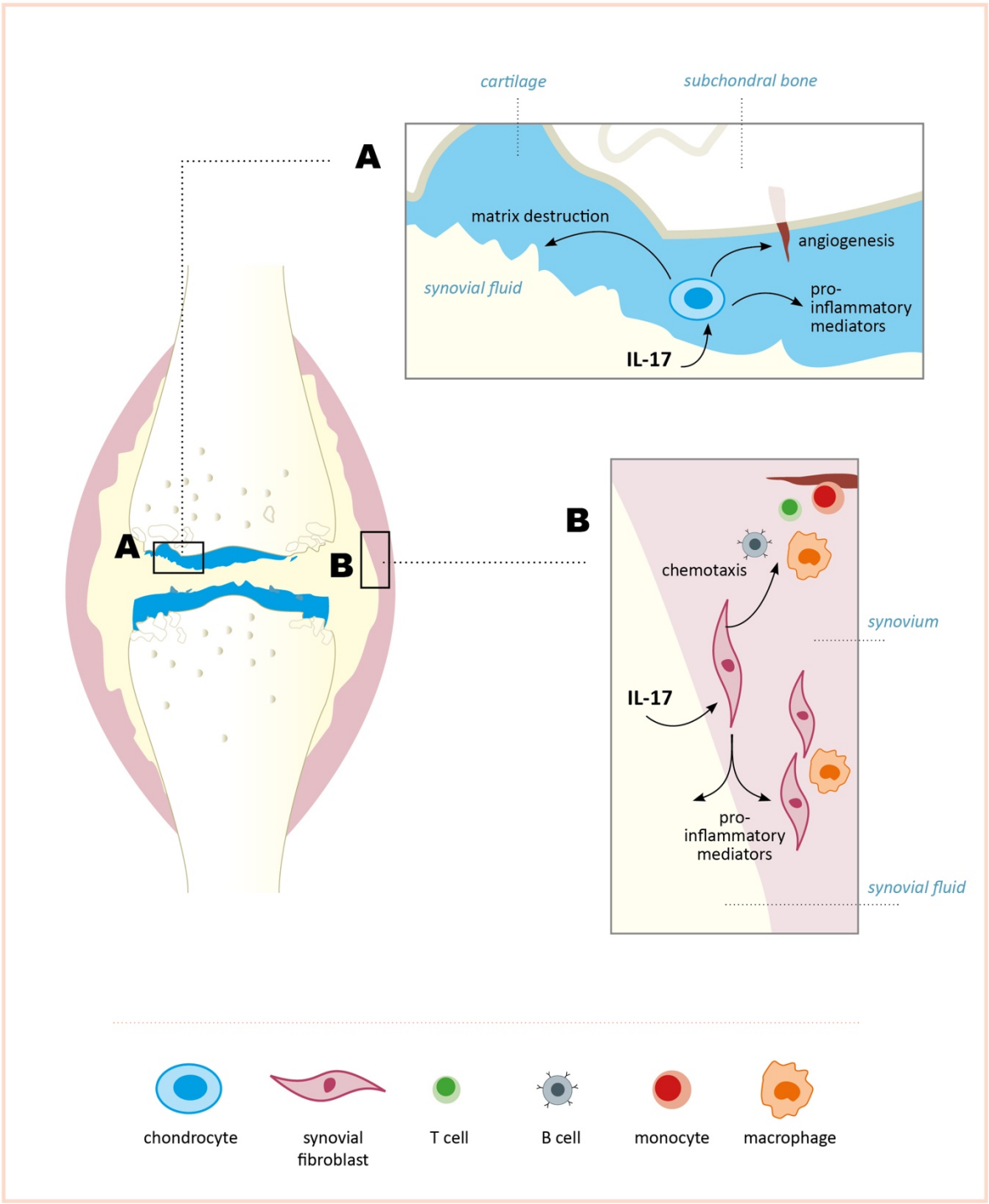


***Figure 5-1**. Overview of the potential role of IL-17 in end-stage OA cartilage (A) and synovium (B) based on the findings of this thesis. In cartilage, IL-17 acts on chondrocytes and can contribute to matrix destruction, angiogenesis, and the production of pro-inflammatory mediators. In synovium, IL-17 acts on synovial fibroblasts which can stimulate chemotaxis and the production of pro-inflammatory mediators.*

5.4.3 Osteoarthritis is a whole joint disease

While cartilage has long been at the centre of attention in OA, the importance of other joint tissues that can contribute to OA aetiology has become more accepted. The infiltration of immune cells and the presence of activated fibroblasts in late stage OA synovium, and the increase of macrophages with synovitis score in patients with a high risk of developing OA adds to the evidence that synovium can play an important role in development and progression of OA.

In addition, the ability of chondrocytes to respond to inflammatory mediators such as IL-17 cytokines shows that the resident cells in the cartilage can respond to inflammatory mediators which are likely produced elsewhere in the joint or the body and that can reach the cartilage through synovial fluid or the subchondral bone. Therefore, this thesis does not only add to the evidence that the synovium can play an important role in OA, but also highlights how the paracrine effect of synovium and the cross-talk between joint tissues can affect disease.

5.5 Limitations

This thesis has significantly improved our understanding of inflammation and the effect of IL-17 in OA. However, there are some limitations and areas that could be improved and extended in future studies.

5.5.1 Human tissue

One of the great strengths of this study is that human tissue was used for all experiments in this thesis. Although this results in a lot more variation between samples than when using immortalised cell lines, this also greatly strengthens the results of this study as it reflects the human variation in physiologically relevant cells from patients seen in the clinic and the need for further stratification of OA. In addition to this, the use of human tissue also comes with several (logistical) challenges. First, the end-stage OA tissue used in this thesis was collected by different surgeons which could have

introduced bias in the size and location of the tissue collected. Especially as it has been shown that fibroblasts subsets with specific effector functions reside in either the lining or sub-lining of the synovium, differences in the ratio of these layers between biopsies could have influenced results. In addition, the location from which the synovium is taken can be important, especially if the cartilage damage was more pronounced on one side of the knee joint than the other, which is often reflected by more pronounced synovitis on the damaged side of the joint.

Second, while end-stage OA tissue is relatively easy to obtain during total joint replacement surgeries, it is difficult to access tissue (particularly cartilage) from patients with early disease and it is especially difficult to access healthy comparator control tissue. In end-stage disease, the phenotype and functionality of the cells could have changed over the time of disease, thus only giving information about the responses of these tissues at end-stage disease. In order to take this research forwards, especially when looking at how certain cells or inflammatory mediators affect the onset and/or progression of disease and potential therapeutic options for this, it is essential that avenues to access tissues from earlier stages of disease are explored.

5.5.2 Osteoarthritis in the knee joint

All the cells and tissues used in this thesis were taken from the knee joint, which is the most common joint to be affected by OA[5]. However, articular joints are comprised of a diverse range of tissues, and every articular joint is made up of differing proportions of each tissue type, has distinct functional requirement, and experiences different loads[19]. As it is now widely accepted that OA is a whole joint disease with the involvement of all joint tissues, it is likely that the disease slightly differs between the different anatomical sites. Therefore, it needs to be considered that the results presented and conclusions drawn in this thesis apply to knee OA, but not necessarily to OA at other joint sites.

5.6 Future work

Throughout this thesis, several suggestions for future work have already been discussed. However, here new directions for future studies are discussed, including a more detailed investigation into the cellular composition of end-stage OA synovium, the involvement of different joint tissues in OA, concentrations of IL-17 cytokines in the OA joint, and the investigation of the effect of IL-17 cytokines in animal models of disease.

5.6.1 Specification of immune and fibroblast subsets in end-stage osteoarthritis synovium

The exploratory study presented in Chapter 2 shows that there are several subsets of immune cells and different activated fibroblast subsets in end-stage OA synovium. Future studies should validate these findings in a larger patient population and further characterise the subset of immune cells and fibroblasts and their functions in OA. Both the proportion and the ratio of fibroblast subsets with specific effector functions that are present in synovium might distinguish the subtype of disease, estimate the speed of disease progression, or identify the right treatment option for each patient. This could not only advance the understanding of disease, but it also has the potential to revolutionize OA treatment by tailoring future therapies to disease subtype.

5.6.2 Different joint tissues contribute to osteoarthritis

With the gradual acceptance of OA as a disease of the whole joint, studies need to expand their focus from one tissue, often cartilage, to also include other joint tissues such as synovium and synovial fluid, as well as other understudied tissues such as the fat pad, muscles, tendons, and ligaments. The proximity in which these tissues co-exist makes it very plausible that there is cross-talk between these tissues. Therefore, future studies should not only focus on cartilage, but also include several other joint tissues to better understand the whole disease process. However, the

knowledge gained studying soft tissues in OA and other joint diseases should also be applied to cartilage. For example, as fibroblast subsets with unique effector functions have been found in synovium, other joint tissues such as cartilage should be investigated to gain further insight into the different subsets of chondrocytes as well.

5.6.3 IL-17 concentrations in OA joint

This thesis gives great insight in the effect IL-17A, IL-17F and IL-17AF have on end-stage OA chondrocytes and synovial fibroblasts gene expression. However, to be able to understand if, how, and which cytokine can have an effect on the onset or progression of OA, future studies should investigate the concentrations of IL-17A, IL-17F, and IL-17AF in the OA joint. As IL-17A elicits the strongest activation of intracellular signalling and strongest increase in gene expression in chondrocytes and synovial fibroblasts, it is logical to focus on this cytokine in future studies. However, if IL-17F or IL-17AF are found in much higher concentrations in the joint than IL-17A, this could make IL-17F or IL-17AF a more plausible target or make it more interesting to focus on all three cytokines. Therefore, instead of looking at inhibitors for IL-17A as potential therapeutic targets (such as secukinumab or ixekizumab), the IL-17RA receptor (brodalumab[351]), which would inhibit the effect of all cytokines that use IL-17RA as a receptor, or IL-17A/IL-17F dual antibody (bimekizumab[352]) could be a more relevant therapeutic option. In addition, future studies should continue to try to identify the IL-17-producing cells in the OA joint, as these cells or mediators that stimulate these cells to release IL-17 might be used as a therapeutic target for patients with high concentrations of IL-17.

5.6.4 Determine the role of IL-17 cytokines using in vivo models

This thesis has significantly increased the understanding of the effect IL-17 has on end-stage OA chondrocytes and synovial fibroblasts in isolation. As these cells are the predominant cells in cartilage and synovium, this gives a good indication of the potential effect of these cytokines in these tissues. However, in order to get a better understanding of the potential contribution of these

cytokines to OA pathophysiology, *in vivo* models, such as STR/ort mice[353], domestic rabbits[354], or destabilisation of the medial meniscus (DMM) model for post-traumatic OA[355], might be needed for several reasons.

First, chondrocytes and synovial fibroblasts live in a complex micro-environment in which there are interactions with different cells, matrix, and mediators within each joint tissue, and interactions and cross-talk between joint tissues, which is something that cannot be tested yet in *in vitro* or *ex vivo* models available. Second, as this study has shown that the expression of IL-17RA can co-localise with macrophage markers, fibroblasts are unlikely to be the only cells in synovium that respond to IL-17 cytokines. In addition, there seems to be an increase in IL-17RA expression in patients with synovitis, which is potentially due to the influx of IL-17RA-expressing macrophages. Therefore, the only way to understand how the inflammatory state of the tissue affects its response to IL-17 would be to test this in an *in vivo* model. Third, as it is very difficult to acquire tissue, especially cartilage, from an early OA disease state, and we have limited understanding of the onset and progression of disease, an *in vivo* model could make it possible to understand the effect that IL-17 cytokines could have throughout the course of the disease, and how it effects symptoms such as pain which cannot be tested in *in vitro* or *ex vivo* models. Fourth, one of the most intriguing features of IL-17A/F is the ability to stabilise mRNA. As transcripts from several inflammatory mediators and their regulators are inherently instable, this mechanism can boost the signals from the environment, which might especially be relevant in OA patients with synovitis. Finally, after exploring the effects of IL-17 *in vivo,* these models can provide the opportunity to test whether antibodies or small molecules against IL-17 cytokines, IL-17RA, or IL-17-producing cells have potential to be a therapeutic option for OA.

5.7 Conclusions

This thesis highlights the importance of inflammation in OA. There is significant infiltration of immune cells, especially T cells and macrophages, in end-stage OA synovium. In addition, the

fibroblasts in end-stage OA synovium have an activated phenotype, with a large immune effector population, which might be an important contributor to the synovitis seen in many patients. In patients that have a high risk of developing post-traumatic OA, the macrophage markers CD68 and CD206 both positively correlated with a histological synovitis score, which supports the hypothesis that macrophages are a key driver of synovitis. Furthermore, the presence of immune cells and activated fibroblasts in synovium highlights the importance of synovium in OA, thereby providing further evidence for OA as a whole joint disease.

Furthermore, findings from this thesis support that IL-17 could play an important role in OA. IL-17-producing cells (CCR6+ T cells) were found in a subset of end-stage patients. In a cohort of patients at high risk of developing post-traumatic OA, the cytokine IL-17A itself and its receptors IL-17RA and IL-17RC were expressed. Chondrocytes and synovial fibroblasts derived from end-stage OA patients expressed *IL17RA* and *IL17RC*, confirming its ability to respond to IL-17 cytokines. Treatment of these cells with IL-17A, and its family members IL-17F and IL-17AF, induced changes in gene expression in OA-related pathways. The chronic, low-grade inflammation seen in OA fits well with the effects IL-17A on inflammation; its effects on matrix destruction and angiogenesis further supports a role for IL-17A in OA pathophysiology.

In conclusion, this thesis highlights the importance of inflammation in OA, and proposes that IL-17A could be an important mediator in sustaining this inflammation. In addition, it further demonstrates a role for the synovium in OA, which emphasizes that OA should be viewed as a disease of the whole joint. Future studies should apply modern omics techniques to a larger, well-phenotyped cohort of OA patients to identify subtypes of disease in synovium and other OA joint tissues by unravelling the cellular, molecular, and genetic signatures of disease in a longitudinal study.

5.8 Key findings

- Osteoarthritis is a disease of the whole joint with an important role for synovium
- Inflammation is important in the pathogenesis of osteoarthritis
- IL-17 is implicated in inflammatory osteoarthritis

Chapter 6

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Appendices

All appendices can be found in volume 2