

Elucidating the role of inflammatory mediators in Frozen Shoulder



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Abstract

Frozen shoulder is a painful inflammatory fibrotic disease of the glenohumeral joint capsule. While it's frequently self-limiting, patients can be symptomatic for years. The underlying pathophysiology of frozen shoulder is not fully understood, hampering the development of an effective therapy. Increased expression of inflammatory mediators has been identified in frozen shoulder, suggesting a potential role for these mediators in disease. However, the precise cellular interactions and the cell types expressing inflammatory mediators have not been identified.

A single cell atlas revealed that the rotator interval of the glenohumeral joint capsule is comprised of fibroblasts, myeloid cells, lymphocytes, endothelial cells, and mural cells. Clustering of myeloid cells revealed a $CD48^+IL1A^+$ macrophage subset which showed increased expression of several pro-inflammatory genes, including *PTGS2*, *S100A8*, *EREG*, *IL1A*, *IL1B*, *IL1R1*, *IL1RN*. In frozen shoulder, $CD48^+IL1A^+$ macrophages show increased expression of several pro-inflammatory genes, including IL-1 genes (*IL1A*, *IL1B*, *IL1R1*).

$CD48^+IL1A^+$ macrophages were localised to both the lining and sub-lining regions of the rotator interval of the glenohumeral joint capsule. The expression (immunohistochemistry) of CD68, CD48, IL-1 α , IL-1 β , and IL-1R was increased in frozen shoulder relative to comparator patient tissues. As the *IL1R1* gene was mainly expressed by fibroblasts, ligand-receptor interaction analysis predicted several important IL-1 ligand-receptor pairs between $CD48^+IL1A^+$ macrophages and fibroblast populations, including *IL1A-IL1R1* and *IL1B-IL1R1*. In turn, fibroblast populations were predicted to communicate with myeloid cell populations via the *CSF1-CSF1R* pathway.

Fibroblasts derived from frozen shoulder capsular tissues acquire an inflammatory fibrotic phenotype upon IL-1 α and/or IL-1 β stimulation, which was characterised by an increase in inflammation (*CCL2*, *CCL5*, *CCL8*, *CCL20*, *CXCL3*, *CXCL5*, *NFKBIA*, *NFKBIZ*, *IGF1*, *FGF7*, *IL6*, *CSF2*, *CSF3*) and fibrosis related genes (*TGFB1*, *TGFBR1*, *WNT5A*, *COL3A1*, *MMP2*, *TIMP1*), as well as an increased secretion of inflammatory mediators (CXCL-12, GM-CSF, IL-1RA, IL-33, IL-6).

This thesis provides novel insight into the cellular basis of frozen shoulder, revealing a distinct $CD48^+IL1A^+$ macrophage subset enriched for inflammation in advanced-stage frozen shoulder. $CD48^+IL1A^+$ macrophages release IL-1 α and IL-1 β , which activate surrounding macrophages and fibroblasts via IL-1R to release chemokines and other inflammatory mediators, further promoting inflammation and fibrosis. This data supports the hypothesis that macrophage-fibroblast crosstalk contributes to the underlying pathophysiology of frozen shoulder. Importantly, this thesis provides sufficient data to identify the IL-1R pathway as a potentially important therapeutic target.

Për babain dhe nënën, pa përkrahjen e të cilëve nuk do të isha këtu.

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Declaration

I hereby certify that this thesis is the result of my own work. Where I have drawn on the work, ideas, and results of others, this has been appropriately acknowledged in the thesis.

Hamez Gacaferi

COVID-19 disclosure

Regular patient care and (non-COVID) research were significantly affected by the COVID-19 pandemic, including some of the work in this thesis.

As a result of the national lockdown, the Botnar Research Centre was shut down for approximately 3 months (from March to June 2020), which prevented me from going to the lab. In addition, regular patient care and elective surgeries were either postponed or cancelled. This prevented me from collecting tissues from patients undergoing elective surgery during that period of time. It took over a year before elective surgeries went back to pre-pandemic levels of activity.

Finally, I was redeployed to full-time clinical work in the NHS (John Radcliffe Hospital, Oxford University Hospitals) between April and June 2020, and was therefore unable to work on my DPhil during this time.

Abbreviations

2D two-dimensional

3D three-dimensional

AGE advanced glycation end product

BH Benjamini-Hochberg correction

CCL CC chemokine ligand

COX cyclooxygenase

CSF colony-stimulating factor

CXCL C-X-C Motif Chemokine Ligand

DAB diaminobenzidine

DAMP damage-associated molecular pattern

DEG differentially expressed gene

DMEM Dulbecco's Modified Essential Medium

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

DNAse deoxyribonuclease

ECM extracellular matrix

ELISA enzyme-linked immunosorbent assay

FAP fibroblast activation protein

FCS foetal calf serum

FGF fibroblast growth factor

GH glenohumeral

GO Gene Ontology

GRCh38 Genome Reference Consortium Human Build 38

GSDMD gasdermin-D

GSEA geneset enrichment analysis

GWAS genome-wide association study

H&E haematoxylin & eosin

HGF hepatocyte growth factor

HLA human leukocyte antigen

HMGB1 high-mobility group box 1

ICECAP Inflammation and fibrosis: CausEs in adhesive CAPsulitis

IL interleukin

IMS industrial methylated spirits

IRAK interleukin-1 receptor-activated protein kinase

ISH in situ hybridisation

KEGG Kyoto Encyclopedia of Genes and Genomes

LOD limit of detection

log2FC log2 fold change

LRT likelihood ratio test

MAPK mitogen-activated protein kinase

MMP matrix metalloproteinase

MRI magnetic resonance imaging

mRNA messenger ribonucleic acid

MSD Meso Scale Discovery

MSigDB Molecular Signatures Database

MUA manipulation under general anaesthesia

MyD88 myeloid differentiation primary response gene 88

NATMI Network Analysis Toolkit for the Multicellular Interactions

NF- κ B nuclear factor- κ B

NHS National Health Service

NLRP3 NLR family pyrin domain containing 3

NSAID non-steroidal anti-inflammatory drug

OA osteoarthritis

OMB Oxford Musculoskeletal BioBank

p_{adj} adjusted p value

PAMP pathogen-associated molecular pattern

PBS phosphate-buffered saline

PC principal component

PCA principal component analysis

PDGF platelet-derived growth factor

PDPN podoplanin

PRR pattern recognition receptor

QC quality control

RA rheumatoid arthritis

RAGE receptor for advanced glycation endproducts

RCT randomised controlled trial

REC Research Ethics Committee

RNA ribonucleic acid

RNAseq RNA sequencing

ROM range of motion

RT-PCR reverse transcription polymerase chain reaction

scRNAseq single cell RNA sequencing

snRNAseq single nuclei RNA sequencing

TAB TAK1 binding protein

TAK1 transforming growth factor- β activated kinase 1

TGF transforming growth factor

TIMP tissue inhibitors of metalloproteinase

TLR toll-like receptor

TNF tumour necrosis factor

TRAF tumour necrosis factor associated factor

UK United Kingdom

UMAP Uniform Manifold Approximation and Projection

UMI unique molecular identifier

US United States

VCAM vascular cell adhesion molecule

VEGF vascular endothelial growth factor

WHO World Health Organisation

α -SMA α smooth-muscle actin

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Introduction

1.1 Overview: frozen shoulder

Musculoskeletal disorders are a global disease burden in our ageing population and commonly affect the shoulder joint [1, 2]. In the UK, the estimated prevalence of shoulder pain is approximately 7%, increasing to 26% in elderly patients, making it the third most common musculoskeletal complaint presented to General Practitioners [1, 3]. A known cause of shoulder complaints is frozen shoulder, which commonly affects up to 10% of patients of working age [4, 5]. Affected patients experience significant pain and restricted shoulder motion, impairing activities of daily living and work. Frozen shoulder has a sudden onset and is frequently self-limiting, although more severely affected patients can be profoundly symptomatic up to three years prior to the condition resolving, impacting patients' livelihood significantly [4].

The first description of frozen shoulder in literature was by Duplay in 1872 ('peri-arthritis scapulohumeral'), but the term frozen shoulder was not introduced until 1934 by Codman [6, 7]. In 1945 Neviaser introduced a new synonym that would better describe the underlying inflammation and fibrosis of the shoulder capsule: 'adhesive capsulitis' [8]. Over the years, this disease has been given various names in different parts of the world: 'Steife Schulter' in Germany, 'capsulite retractile' in France, and '50-year-old shoulder' ('goju-kata') in Japan. Despite the fact that this syndrome has been described in orthopaedics for over 150 years, the aetiology and pathogenesis remain unknown and successful therapeutic options are yet to be developed.

Frozen shoulder presents a unique opportunity to study an example of a localised

chronic inflammatory fibrotic disease that is ultimately self-limiting, and therefore is a tractable disease to therapeutically target. To understand what drives the underlying inflammation, fibrosis, and ultimately resolution, fundamental research with well-phenotyped capsular tissues from frozen shoulder patients is required. This could inform development of new treatments for frozen shoulder patients during earlier symptomatic stages of the disease. Importantly, understanding how frozen shoulder ultimately resolves has translational ability to inform treatments of other chronic inflammatory fibrotic diseases. This thesis hopes to provide a significant contribution towards these goals.

1.2 Shoulder anatomy and function

1.2.1 The shoulder

The shoulder joint is a complex synovial joint, which has the greatest range of motion (ROM) of all the joints in the human body [9]. The shoulder joint is made up of a ball and socket articulation between the glenoid cavity of the scapula and humeral head. Therefore, it is also known as the glenohumeral (GH) joint (Figure 1.1). The labrum, a cartilaginous structure surrounding the glenoid, and the glenoid form the socket, whereas the humeral head is the ball of the shoulder joint. The bones of the shoulder consist of the humerus, the scapula, and the clavicle (Figure 1.1). The acromion, which is an extension of the scapula, forms the ‘roof’ of the shoulder. The space between the acromion and the humeral head is the subacromial space. Clinically, this is a very important area of the shoulder as it contains the subacromial bursa and the rotator cuff tendons, which are often included in shoulder pathology (i.e., subacromial pain syndrome) [10]. Another clinically important area of the shoulder is the rotator interval: a triangular area located in the anterosuperior aspect of the GH joint capsule [11]. It is formed by the anterior boundary of the supraspinatus, the superior boundary of the subscapularis muscle, and it is bordered by the coracoid process medially (Figure 1.2). Besides the GH joint, there are two other synovial joints in the shoulder girdle: the acromioclavicular joint and the sternoclavicular joint. Lastly, the scapulothoracic surface makes it possible for the scapula to glide across the thorax when moving (i.e., elevating) the shoulder.

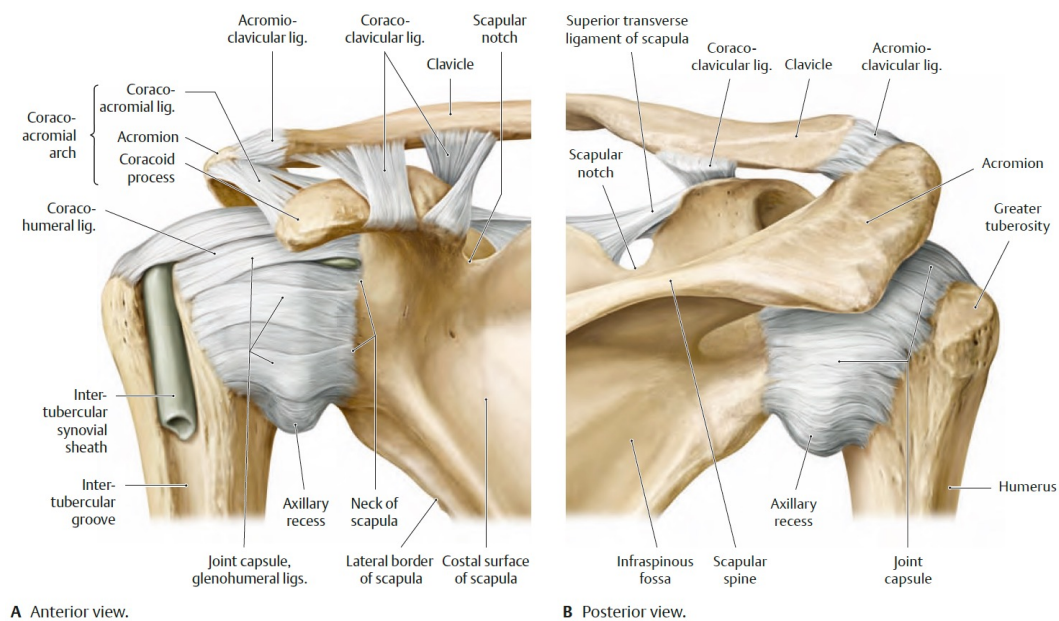


Figure 1.1: The shoulder joint and associated ligaments.

A) Anterior view of the shoulder joint. B) Posterior view of the shoulder joint. *Adapted from Gilroy et al. 2012 (p 285, Fig. 21.13) by Thieme, Stuttgart.*

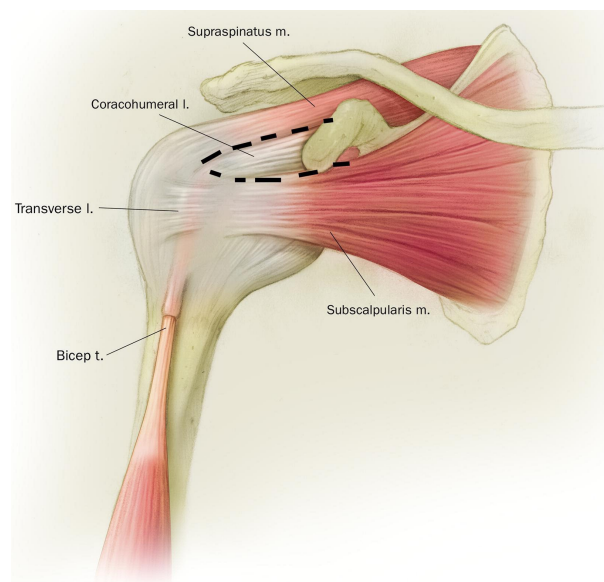


Figure 1.2: The rotator interval.

The rotator interval marked with discontinuous black lines. *Adapted from Provencher et al. (2008).*

1.2.2 Functional anatomy (rotator cuff)

Movements of the shoulder are initiated by contractions of surrounding muscles. The most important muscles of the shoulder are the deltoid muscle and the rotator cuff (Figure 1.3). The rotator cuff consists of four muscles: the supraspinatus, the infraspinatus, the subscapularis, and the teres minor. The main biomechanical function of the rotator cuff is to stabilise the GH joint during movements, by compressing the humeral head against the glenoid. The supraspinatus muscle initiates abduction of the humerus. The supraspinatus rests on the scapula, above the spina scapulae which separates the supraspinatus from the infraspinatus (Figure 1.3). The infraspinatus and the teres minor muscles both have a role in external rotation (i.e. lateral rotation) of the shoulder (humerus) as well as shoulder stabilisation. The infraspinatus and the teres minor are localised on the dorsal surface of the scapula, below the spina scapulae (Figure 1.3). Finally, the subscapularis muscle is the largest and strongest rotator cuff muscle and it causes internal rotation (i.e. medial rotation) of the humerus as well as depression of the humeral head (i.e. stabilisation) (Figure 1.3). The subscapularis is localised on the anterior side of the scapula and it fills the subscapular fossa. Other muscles that attach (close) to the shoulder joint and play important roles in movements of the shoulder and arm are the pectoralis major and minor, the teres major, the biceps brachii, the triceps brachii, the trapezius, and the latissimus dorsi.

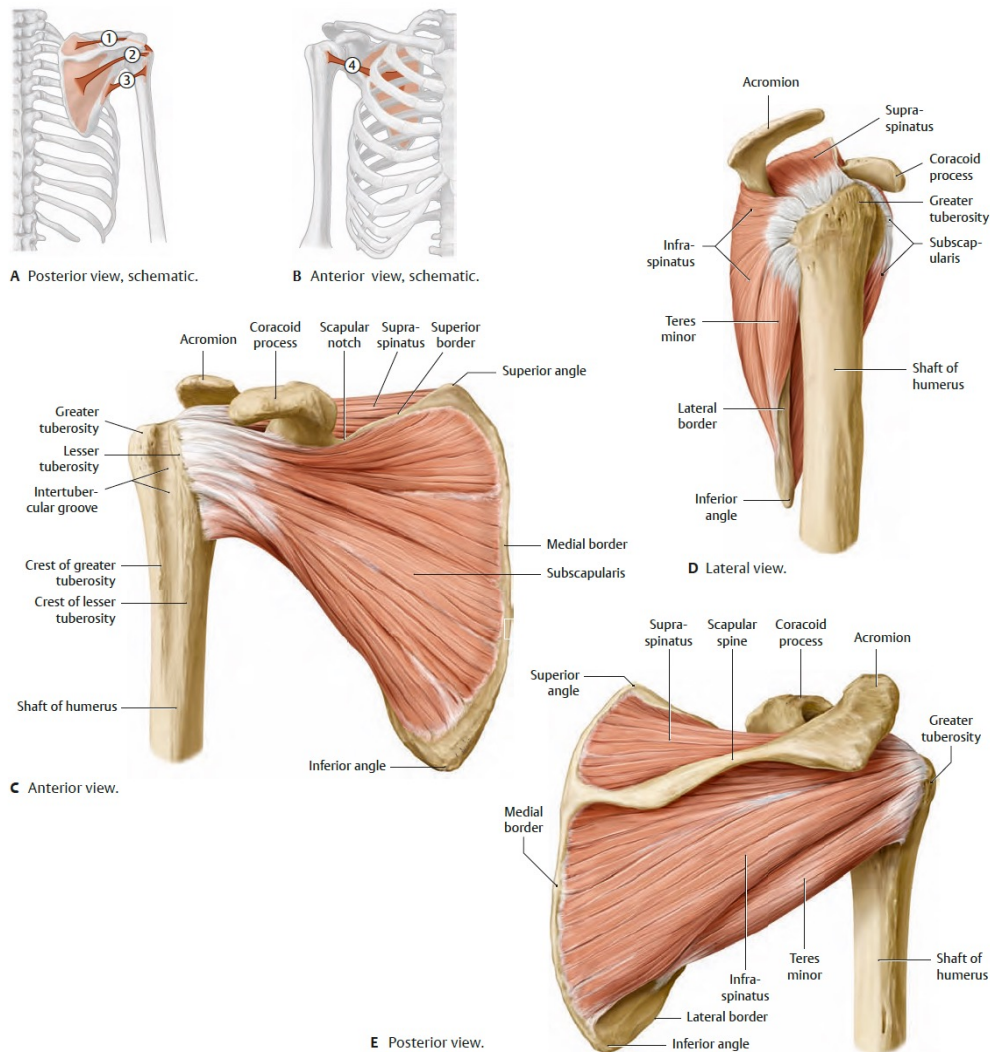


Figure 1.3: The rotator cuff.

The anatomy of the rotator cuff muscles. A) and B) show schematics of the posterior and anterior view respectively, with 1 = supraspinatus, 2 = infraspinatus, 3 = teres minor, and 4 = subscapularis. C) Anterior view. D) Lateral view. E) Posterior view. *Adapted from Gilroy et al. 2012 (p 297, Fig. 21.24) by Thieme, Stuttgart.*

1.2.3 The GH joint capsule

The GH joint is a ball and socket joint of the glenoid and the humeral head. Both bones are covered with hyaline cartilage and are surrounded by a joint capsule, which is reinforced by a group of ligaments that encapsulate the GH joint (Figure 1.1). These ligaments provide stability of the joint (together with the rotator cuff muscles, the long head of the triceps muscle and the long head of the biceps muscle), which is needed since the shoulder joint is extremely lax to facilitate a wide ROM. The joint capsule reaches from just past the circumference of the glenoid cavity to below the anatomical neck of the humerus. The ligaments that connect the glenoid and the humeral head are the superior, middle, and inferior GH ligaments (Figure 1.1). The inferior GH ligament (also known as the inferior GH ligament complex) consists of an anterior band, posterior band, and the axillary recess. The axillary recess (or pouch) is focally reinforced by the spiral GH ligament (also known as the fasciculus obliquus). The coracohumeral ligament connects the coracoid process with the humeral head and can be found in the rotator interval (together with the superior and middle GH ligaments) (Figure 1.2). Furthermore, the transverse humeral ligament holds the long tendon of the biceps brachii muscle in the intertubercular sulcus, which goes into the GH joint and attaches to the superior labrum and glenoid. The GH joint capsule (and any other synovial joint) consists of a fibrous membrane on the outside. This layer is locally thickened with GH ligaments and merged with tendons (e.g., subscapularis tendon). On the inside, the joint capsule is lined with synovium (synovial membrane) which produces synovial fluid, providing the hyaline cartilage with nutrients. Movement stimulates diffusion of synovial fluid across all surfaces of the joint.

1.2.4 The microscopic anatomy of the GH joint capsule

The classic joint capsule is usually depicted as consisting of two layers, the synovium (or synovial membrane) on the inside and the fibrous membrane on the outside. The synovium consists of a lining layer (intima layer with a thickness of usually 2-3 cells) and a bigger sub-lining layer (subintima) [12, 13]. In normal, healthy joints there are two types of cells within the lining layer: synovial fibroblasts and synovial macrophages [13]. Synovial fibroblasts are known for producing hyaluronic acid and a complex of proteins, which cause the high viscosity of synovial fluid and enable lubrication of the joint surfaces [14–16]. The sub-lining of the synovium consists of a collagenous extracellular matrix (ECM) with resident fibroblasts, (infiltrating) immune cells, vascular networks (capillaries, arterioles, venules), lymphatic vessels, and a rich nerve supply [12, 13, 17]. The vascular networks help the influx of macrophages and infiltrating inflammatory cells to the (sub-)lining layers in disease. Although sparse, T cells ($CD4^+$ and $CD8^+$ cells) are also present in normal synovium in small numbers in proximity to blood vessels [12, 13].

While the synovium of the GH joint capsule has been extensively investigated, there is limited research on the histology of the outer fibrous membrane in healthy human subjects. Several histologic studies of cadaveric shoulder specimens showed a thin layer of loose oriented collagen fibres, with predominantly fibroblast-like cells [18, 19]. A histological study of the inferior GH ligament complex found that the capsule consists of a lining layer with three distinct layers of collagen fibres [20]. The study refers to GH ligaments not as actual ligaments, but as thickened areas in the joint capsule with a well-organised collagen distribution [20]. Yamazaki (1990)

also reported that the GH joint capsule consists of three distinct layers of fibrous bundles [21]. Kubo et al. (2020) found that GH joint capsule consists of a synovial membrane, the fibrous membrane, and a distinct layer of elastic fibres in between [22]. In conclusion, the literature on the histology of the GH joint capsule is limited and ambiguous. Yet, the GH joint capsule, particularly at the rotator interval, is involved in frozen shoulder and therefore it is crucial to understand its microscopic anatomy [23].

1.2.5 Embryological origins of the GH joint

The GH joint (and other synovial joints) are made up of mesenchymal tissues, including synovium, ligaments, and joint capsule. Before discussing the different cell types present in the GH joint, it is important to understand the embryological origins of the GH joint as this could inform understanding the diseased adult joint, including frozen shoulder (as discussed later). The mesenchymal tissues (including joint tissues) derive from the mesoderm, a primary embryonic germ layer, which lays between the endoderm (inner layer) and the ectoderm (outer layer) [24, 25]. These mesenchymal tissues consist of stromal cells, including fibroblasts, endothelial cells, and tissue-resident macrophages [24]. While fibroblasts and most endothelial cells derive from the mesoderm, the tissue-resident macrophages and some endothelial cells derive directly from the yolk sac [24, 26]. Rather than coming out of the pool of circulating adult monocytes, which derive from haematopoietic stem cells, most tissue-resident macrophages are thought to become resident during early during embryonic development, persisting into their respective adult tissues (Figure 1.4) [24, 27–30]. Thus, depending on their embryonic origin, macrophages have a different phenotype and may therefore behave differently in health and disease.

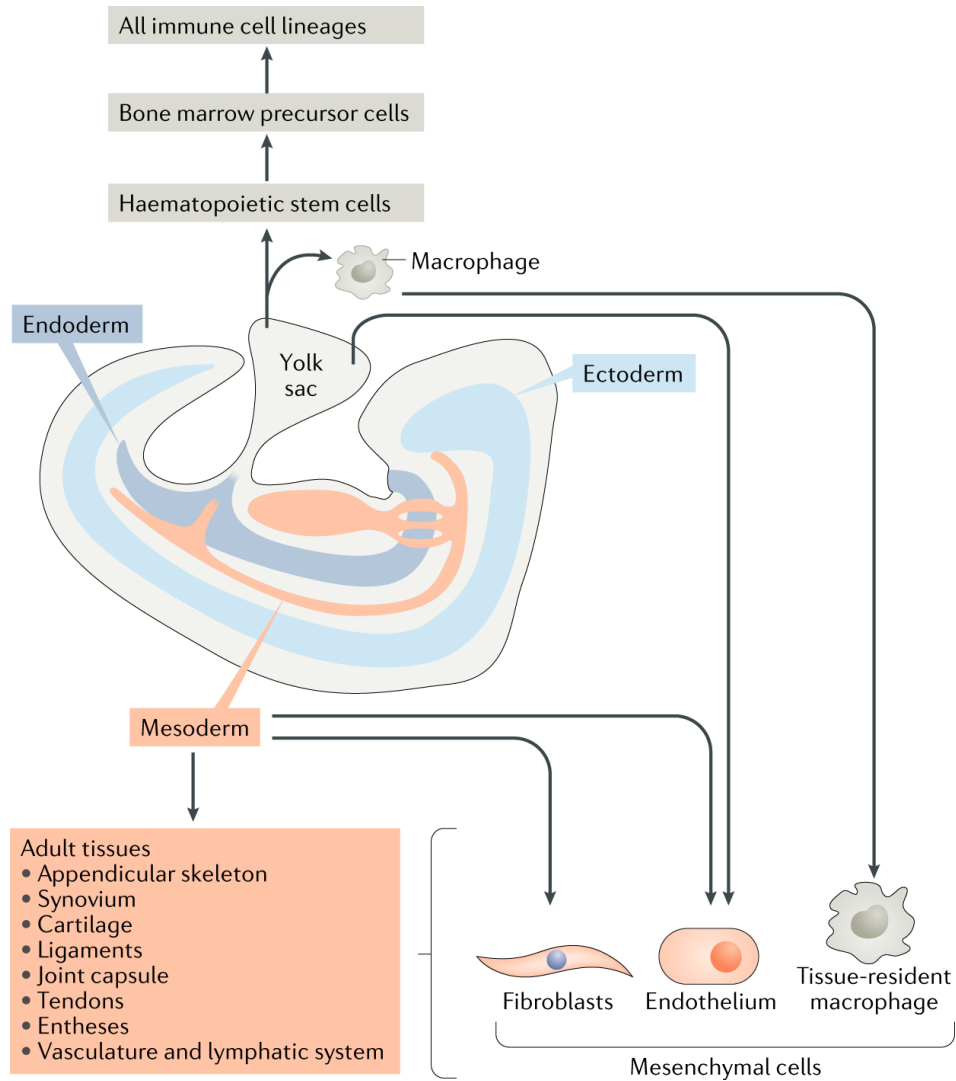


Figure 1.4: Embryological origins of mesenchymal tissues of the joint.

While fibroblasts and most endothelial cells derive from the mesoderm, it is thought that tissue-resident macrophages and some endothelial cells derive directly from the yolk sac. *Adapted from Dakin et al. (2018).*

The GH joint develops in the late embryonic stages [31, 32]. Literature suggests that parts of the GH joint develop as early as 6 post-conception weeks (e.g., the labrum) with the configuration of the GH joint being similar to the adult one by 7-8 post-conception weeks in the final embryonic stages [31, 32].

1.2.6 Stromal cells of the joint

Stromal cells are non-haematopoietic, tissue-resident cells that make up the connective tissue of any organ. Stromal cells, including fibroblasts, tissue-resident macrophages, pericytes, and endothelial cells, support the function of the parenchyma (i.e., the important functional elements of each body system). Fibroblasts and pericytes are the predominant stromal cells in most tissues. Interestingly, stromal cells are also thought to play an important role in the pathophysiology of joint inflammation [24, 33]. Stromal cells adopt a pro-inflammatory phenotype after being exposed to an inflammatory stimulus (stromal cell activation), after which they express distinct inflammatory markers [24, 34–36]. These phenotypic changes can persist so that stromal cells can respond more adequately to future inflammatory stimuli (stromal cell memory). These pathological features have been seen in several diseases, including tendinopathy, cancer, and rheumatoid arthritis (RA) [34–36].

1.2.7 Fibroblasts

In joint connective tissues, fibroblasts are the dominant cell population [33]. Fibroblasts are mostly known to have a structural function in healthy tissues as they are involved in the production of ECM collagen types I, III, V, fibronectin, and the basement membrane (laminin, collagen types IV, VII), which provides tissue structure, mechanical strength and contractility of tissues [37–39]. In addition, fibroblasts release factors involved in turnover of ECM, including degrading enzymes like matrix metalloproteinases (MMPs) [40]. Besides secreting components of the ECM and preserving the structural integrity of human tissues, fibroblasts are also involved in immunomodulation: there is increasing evidence on their role in the pathophysiology

of diseases such as cancer, RA, and tendinopathy [34–36].

Fibroblasts are known to adopt a pro-inflammatory phenotype in disease, and therefore aid the persistence of inflammation in inflammatory diseases like RA [41, 42]. Fibroblasts orchestrate an influx (and maintenance) of immune cell populations in the joint upon (stromal cell) activation. Moreover, they can release a variety of immune-mediating factors including transforming growth factor (TGF) β , MMP-1, MMP-3, C-X-C Motif Chemokine Ligand (CXCL) 12, interleukin (IL) 6, IL-15, and alarmins, stimulating a pro-inflammatory environment [24, 41–45]. Activation of the nuclear factor- κ B (NF- κ B) pathway and involvement of toll-like receptor (TLR) 4 are important features of fibroblasts in pathologic joint tissues. Interestingly, fibroblasts show distinct phenotypes (i.e., are not homogeneous) within connective tissues and the number of populations varies between tissue types [43, 46–50]. In arthritis, five distinct fibroblast subsets have been identified within the synovium, including a $FAP^+ THY1^+$ subset that drives inflammation in RA and a $FAP^+ THY1^-$ subset that drives tissue damage in osteoarthritis (OA) [51]. These findings emphasise the important role of fibroblasts in inflammatory disease. Therefore, it is important that we understand the role of distinct fibroblasts populations in disease as it could inform future strategies to precisely target pathogenic fibroblast populations.

1.2.8 Tissue-resident macrophages

Tissue-resident macrophages are present in most tissues of the human body, including the liver (Kupffer cells), nervous system (microglia), bone (osteoclasts), and the lung (alveolar macrophages) [52, 53]. Tissue-resident macrophages are known to have diverse biological functions, depending on the resident/target tissue and the local

environment [24, 54, 55]. They play a role in immune surveillance, including inducing and resolving inflammation to maintain tissue homeostasis [24, 56–58]. Historically, macrophages have been classified as either having a pro-inflammatory phenotype (M1), or an anti-inflammatory phenotype (M2) [59, 60]. However, recent evidence supports that macrophages are not just extremes of a functional continuum as many activations profiles have been identified [61, 62].

Tissue-resident macrophages play an important role in inducing inflammation in joint disease (e.g., RA) via pattern recognition receptors (PRRs), including the TLRs. They release IL-6, IL-1 α , tumour necrosis factor (TNF) α , alarmins and many other inflammatory mediators, which in turn promote angiogenesis, fibroblast accumulation and proliferation, and leukocyte recruitment [14, 24, 57, 58, 63, 64]. Furthermore, tissue-resident macrophages can be transcriptionally primed due to local tissue stress by disease activity and/or therapies [24, 65]. These biologically diverse cells are also implicated in fibrosis and tissue repair across a variety of tissue types [14, 66–68]. Recently, nine distinct macrophage subsets have been identified in synovial tissues of RA, of which five were tissue-resident macrophages [69, 70]. Alivernini et al. (2020) describe two macrophage subsets that produced pro-resolving lipid mediators and induced a reparative phenotype of synovial fibroblasts *in vitro* [70]. These findings demonstrate that tissue-resident macrophages play an important role in (local) inflammation and resolution of diseased musculoskeletal tissues.

1.3 Frozen shoulder

1.3.1 Classification, clinical signs, and diagnosis

There are many definitions of a frozen shoulder in the literature, including Codman’s 12 criteria for a frozen shoulder (Table 1.1) [7]. It is often described as a shoulder condition characterised by pain and both active and passive restriction of shoulder motion, and a normal radiograph [71]. Despite the lack of an official consensus definition and classification for the frozen shoulder, this disease is often divided into a primary (idiopathic) and a secondary frozen shoulder [71, 72]. A primary frozen shoulder includes all cases without an underlying or associated condition, whereas a secondary frozen shoulder is associated with a comorbidity. This list of comorbidities includes diabetes mellitus, hypo- or hyperthyroidism, Dupuytren’s disease and many more [4, 73–77]. Yet, this classification can be confusing as it suggests a causal relationship between an associated comorbidity and frozen shoulder. Since the underlying pathophysiology of frozen shoulder is not fully understood, and there is no causal link between the comorbidities and frozen shoulder, it is better to speak of risk factors and/or associated comorbidities, and not of primary/secondary frozen shoulder. A list of notable associated comorbidities can be found in Table 1.2.

Table 1.1: Codman's criteria for frozen shoulder.

The 12 criteria of a frozen shoulder as proposed by Codman (1934)
1. The condition comes on slowly
2. Pain is felt near the insertion of the deltoid
3. Inability to sleep on the affected side
4. Painful and incomplete elevation
5. ... and external rotation
6. Restriction of both spasmodic
7. ... and adherent type
8. Atrophy of the spinati
9. Little local tenderness
10. X-rays negative, except for bony atrophy
11. The pain was very trying to every one of them
12. ... but they were all able to continue their daily habits and routines

Table 1.2: Risk factors for frozen shoulder.

A list of notable risk factors (comorbidities) for frozen shoulder
- Age >40 years
- Female sex
- Diabetes mellitus
- Hypo- or hyperthyroidism
- Dupuytren's disease
- Cardiovascular disease
- Cerebrovascular accident
- Shoulder immobilisation
- Trivial upper extremity trauma (post-traumatic)
- Surgically or radio-therapeutically treated breast cancer
- Parkinson's disease

Frozen shoulder is a clinical diagnosis, meaning the diagnosis is made based on a medical history and physical examination. The clinical course is often divided into three phases: the freezing phase, the frozen phase, and the thawing phase [78]. The first phase (the freezing phase) is characterised by a painful inflammation of the joint capsule, during which patients experience a lot of pain during movements and at rest (e.g., night pain). This inflammatory phase is superseded by fibrosis, which causes a serious disabling restriction of movement of the shoulder (frozen phase). Finally, during the thawing phase the contracture of the shoulder will slowly resolve with most patients almost fully recovering. Although there are three distinct overlapping phases of frozen shoulder, it is clinically easier to distinguish between a phase with mainly pain and a phase with mainly stiffness [78]. Moreover, the cellular basis underpinning each clinical phase of frozen shoulder remains unknown.

Physical examination shows a painful restriction of both active and passive shoulder movements. The severity of pain and the degree of restriction depend on the stage of disease at clinical presentation. For example, someone who is in the frozen phase may present with less pain but will have a considerably limited **ROM** of the shoulder. Of all shoulder motions, restriction of external rotation is the most common finding in frozen shoulder patients. Several criteria have been proposed for physical examination, but there is no consensus. More than 50% restriction of passive external rotation compared to the contralateral (healthy) shoulder is usually maintained when diagnosing a frozen shoulder [79].

Diagnosing a frozen shoulder can be very challenging during the freezing phase because pain is the predominant complaint. **GH OA** and subacromial pathology

(e.g., rotator cuff tendinitis) should be considered as differential diagnoses. Not only do the complaints of these conditions align with the symptoms of a frozen shoulder, but the patients with subacromial pathology are often in the same age category. However, frozen shoulder is unlikely in patients older than 70 years, as they are more likely to have OA and/or a torn rotator cuff. In addition, abnormalities during physical examination could be a result of several shoulder diseases and therefore a frozen shoulder diagnosis cannot be based on these findings alone. In patients with subacromial pathology, restriction of shoulder movement is often due to pain, whereas the restriction in frozen shoulder is mechanical and usually found in later stages of the disease. Furthermore, active ROM is painful in subacromial pathology while passive ROM is preserved. In contrast, frozen shoulder patients demonstrate limited active and passive ROM. Therefore, a significant loss of passive external rotation in a later stage of the disease makes frozen shoulder more likely. For these reasons, it is very important to follow the disease course closely.

Additional diagnostic imaging can be performed to exclude other pathology. An X-ray of the shoulder can be performed to rule out other causes such as OA or calcific tendinitis. Furthermore, an ultrasound (or an MRI scan) of the shoulder can exclude rotator cuff pathology. Note that in some cases these pathologies occur simultaneously with or precede a frozen shoulder.

1.3.2 Epidemiology of frozen shoulder

The estimated prevalence of frozen shoulder in the general population lies between the 2-5% [80–82]. The true prevalence and incidence remain unknown and the discrepancies in estimated values are due to the difficulties of diagnosing a frozen

shoulder. In addition, only the recalcitrant frozen shoulder cases are seen by specialist consultants for optional surgery. Frozen shoulder usually affects patients ranging from 40 to 70 years with a peak incidence in the 5th decade [4]. A large primary care study from the UK found that it affects 8.2% of men and 10.1% of women in the working population [5]. It has also been suggested that women are more frequently affected than men [4, 83, 84].

Frozen shoulder is often regarded as a self-limiting condition, with most cases fully resolving within two to three years [4]. However, a significant proportion of patients remain symptomatic in the long term with (mild) pain and restricted ROM [4]. A study with an average follow-up of 4.4 years showed that only 59% of patients recovered completely, and that approximately 6% retain severe symptoms in the longer term [4]. Interestingly, 6 to 34% of affected patients develop a frozen shoulder on the contralateral side [85].

Frozen shoulder is associated with a comprehensive list of comorbidities, including diabetes mellitus, thyroid disease, and cardiovascular disease (Table 1.2) [4, 73–75, 77, 86]. Of these, diabetes mellitus is the most notable one since the incidence of frozen shoulder in diabetics is estimated to be between 10-36% [73–75]. Some literature suggests that diabetic patients have worse outcomes from frozen shoulder: the disease is often more persistent and therapy resistant compared to non-diabetics [73, 75, 87, 88]. Several studies also reported a relatively high incidence of Dupuytren’s disease in patients with frozen shoulder (3-52%) and noted similarities between the two contracture syndromes [4, 74, 76, 89].

1.4 Treatment of frozen shoulder

During the symptomatic period patients experience pain, immobility, and stress associated with uncertainty about the future and/or not being able to work [4, 90, 91]. Therefore, an important part of the treatment for frozen shoulder is patient education, guidance, and management of their expectations. Patients must understand that frozen shoulder is a self-limiting disease (in most cases), and that the disease course can be quite long. The aetiology and underlying pathophysiology of frozen shoulder are poorly understood, hampering the development of successful therapies for symptomatic patients. Yet, there are many treatment options for frozen shoulder, including conservative management and surgery. The evidence on the effectiveness of these treatments is often poor and none of the treatment options seem to alter the total disease duration [85, 92–95]. The most common treatment options are briefly discussed below.

1.4.1 Pharmacological therapies

Pharmacological therapies for frozen shoulder include oral medication and glucocorticoid injections. Oral medication focuses on pain relief based on the World Health Organisation (WHO) analgesic ladder with paracetamol and non-steroidal anti-inflammatory drugs (NSAIDs). While NSAIDs can have a short-lasting effect on the pain, there seems to be no evidence for the efficacy of opioids in frozen shoulder [92–94]. Opioids should be avoided as frozen shoulder is a chronic disease and hence there is a significant risk of opioid dependency. Finally, oral corticosteroids can improve immobility and reduce pain, yet these effects are not maintained in the long term [96, 97].

1.4.2 Corticosteroid injections

Corticosteroid injections have a strong analgesic and anti-inflammatory effect and should therefore be administered in the early stages of frozen shoulder (‘freezing phase’). Corticosteroid injections provide short-term improvements in terms of pain relief and mobility [95, 98–100]. Corticosteroid injections seem to be more effective than NSAIDs [95, 101]. Like with oral corticosteroids these effects are not maintained in the long term, but given the potential adverse effects of oral glucocorticoids, localised injections are preferable [99, 100, 102, 103]. One meta-analysis showed that the effects of intra-articular corticosteroid injections is beneficial and may last up to 26 weeks but does not affect the total duration of disease [104]. There is still debate over the best location for corticosteroid injections (intra-articular and/or sub-acromial), however both seem to have a (short-term) positive effect [105–109].

1.4.3 Physical therapy

Patients with frozen shoulder are very often treated with physical therapy. The goal of physical therapy is to improve shoulder function as well as to prevent further restriction of shoulder motion. Despite the frequent use of physical therapy for the treatment of frozen shoulder, studies on the effectiveness are often of low to moderate quality, and do not show any clinically significant differences between treatment groups [79, 110]. Compared to glucocorticoid injections, physical therapy and exercise are not as effective in the short term [110]. Moreover, one prospective study showed better improvements in shoulder function with exercise (i.e., home exercise within pain limits) when compared to physical therapy [111]. Combination therapy of glucocorticoid injections with physical therapy was effective in one randomised

controlled trial (RCT) when compared to corticosteroid injections or physical therapy alone [112]. Like other conservative treatment options, there is no evidence for a long-term effect of physical therapy for the treatment of frozen shoulder [78, 92].

1.4.4 Invasive treatment options

Hydrodilatation (also known as hydrodistension or capsular distension) attempts to stretch the GH joint capsule by increasing the joint volume via an ultrasound-guided (or arthrographic), intra-articular sodium chloride injection. Hydrodilatation can give a short-lasting symptom reduction [92]. Hydrodilatation in combination with a corticosteroid injection is also effective in reducing pain and improving shoulder function in the short term [113]. However, no proven long-term benefit of hydrodilatation has been demonstrated yet, and moreover, it is not clear whether this is associated with improved patient outcomes compared to conservative treatment options [113].

If conservative management fails, patients are usually referred to an orthopaedic surgeon for either a manipulation under general anaesthesia (MUA) or arthroscopic release. During MUA, the contracted GH joint capsule is gently stretched in a controlled environment, tearing the fibrotic joint capsule in order to improve shoulder motion. This procedure is performed under general anaesthesia, which allows you to move beyond the normal pain thresholds. MUA is often seen as a safe procedure, but serious complications can occur: haemarthrosis, nerve injuries, rotator cuff ruptures, labral injuries and GH dislocations and/or fractures [114]. Few studies showed an improvement in ROM and pain after MUA [78, 92].

During an arthroscopic capsular release, an antero-superior release of the GH

capsule is performed at the rotator interval with a radiofrequency ablator (and/or controlled manipulation). Sometimes, the surgeon can choose to expand the capsular release inferiorly or even do a full 360° release [115]. This, however, might arguably increase the risk of iatrogenic complications of an arthroscopic procedure. Clinically relevant complications that can arise with this procedure are wound infection, axillary nerve injuries, and post-operative stiffness [116]. There is some evidence to support the effectiveness of this procedure, which shows that both the ROM and pain scores significantly improve shortly after arthroscopic release [87, 117–119]. Interestingly, diabetics seem to have significantly worse outcomes compared to non-diabetic frozen shoulder patients [87, 119].

The UK FROST trial (a multicentre, three-arm, RCT), which compared MUA, arthroscopic capsular release, and early physical therapy, found that no intervention was clinically superior after one year [120]. Out of these three treatment options, the authors found a significantly increased risk of complications in the arthroscopic release group [120]. Based on the results of the UK FROST trial, most cases should be treated with conservative management options and surgery should be reserved for recalcitrant cases.

1.4.5 Cost-effectiveness of treatment options

Frozen shoulder frequently affects the working population and therefore it can arguably cause significant individual and societal costs. Until quite recently the economic evidence for the treatment of frozen shoulder was very limited [92]. A recent cost analysis in the US was performed to assess the value of physical therapy and supervised neglect [121]. The authors found that the average patient costs for phys-

ical therapy were \$6,150.46 (range, \$191.52-30,000.00), compared to \$45.00 (range, \$30.00-75.00) for supervised neglect [121]. In the **UK**, the healthcare implications of frozen shoulder have been assessed based on a single general practitioner visit per case [90]. Depending on the prevalence (2-5%), the costs varied from at least £44.1 million to £110.3 million, representing a significant cost for an already burdened **NHS**. Finally, a Dutch study estimated the burden due to a frozen shoulder to be €4,521 per patient, with approximately half of these costs being due to lost earnings (i.e., absenteeism from work) [122]. Finally, the UK FROST trial also compared the cost-effectiveness of arthroscopic release, **MUA**, and early physical therapy, and found that **MUA** was the most cost-effective intervention [120].

Although frozen shoulder is a self-limiting, non-lethal disease, it can be concluded that it comes with significant individual and societal cost. This further affects the life quality of affected patients who already, besides pain and disability, experience confusion and anxiety associated with uncertainty about the implications for the future [90, 91].

1.5 Pathophysiology of frozen shoulder

1.5.1 Macroscopic changes of the GH joint capsule

On radiographic imaging (and during surgery), a tight and thickened GH capsule can be seen with a decrease in joint volume [72, 123]. The contracture of the GH capsule reduces the joint volume to 3-4ml, compared to a normal volume of 10-15ml [81]. An MRI (or arthrogram) sometimes shows loss of the axillary recess, supporting a decrease in joint volume. The rotator interval and the coracohumeral ligament seem to be affected most by the contracture in frozen shoulder [23, 72, 124, 125]. In 1987, Neviaser described four different stages of the frozen shoulder based on the clinical course and the arthroscopic appearance [123]:

1. Synovitis without adhesions or contractures.
2. Synovitis with early adhesion formation and capsular contracture.
3. Significant adhesions with loss of axillary recess and less synovitis.
4. Advanced adhesions with very restricted joint volume and no synovitis.

1.5.2 Genetic factors

Some efforts have been made to investigate genetic factors in frozen shoulder. A study in dizygotic and monozygotic twins found a heritability of 42% for frozen shoulder [126]. Another study showed that 20% of the affected patients had a positive family history of first-degree relatives [127]. Furthermore, shoulder capsule cells from frozen shoulder patients (with a normal karyotype) showed clonal chromosomal changes: numerical trisomies of chromosomes 7 and 8 [128]. Also, higher rates

of human leukocyte antigen (HLA) B27 were found in patients with frozen shoulder compared to controls, suggesting that HLA-B27 is a risk factor for frozen shoulder [129]. Interestingly, the findings above have also been identified in Dupuytren’s contracture [130, 131].

Finally, a recent genome-wide association study (GWAS) of frozen shoulder in the UK found five genome-wide significant loci associated with frozen shoulder [132]. Potential causal genes at associated loci included *WNT7B*, *MMP14*, and *SFRP4* [132]. Using Mendelian randomisation the authors found type 1 diabetes mellitus to be a likely causal risk factor for frozen shoulder [132]. Moreover, three out of five loci associated with frozen shoulder were also significantly associated with Dupuytren’s disease [132]. A GWAS of Dupuytren’s disease identified associated loci that also contained genes *WNT7B*, *MMP14*, and *SFRP4* [133]. This implies that genetic factors are likely to play a role in the aetiology of contracture syndromes, including frozen shoulder.

1.5.3 Microscopic changes of the GH joint capsule during frozen shoulder

There have been many efforts to understand the biological mechanisms underpinning frozen shoulder [23, 74, 134–138]. While macroscopic and microscopic (histologic) changes in frozen shoulder have been well documented, the underlying pathophysiology remains poorly understood. Both the synovium as well as the joint capsule have been studied, particularly at the rotator interval. Initially, there was a discussion amongst researchers about the pathophysiology frozen shoulder being either an inflammatory or a fibrotic process, however it is now generally accepted that it

involves both processes [8, 23, 74, 134, 137]. Thus, there is initially an inflammatory phase, which is eventually superseded by a (reactive) fibrotic phase.

1.5.4 Changes of the **ECM** in frozen shoulder

Several studies have shown a dense collagen matrix of collagen type I and type III with (proliferating) fibroblasts and myofibroblasts in capsular tissue of frozen shoulder patients, which is suggestive of a fibrotic process [23, 74, 134, 136, 139]. High expression of (myo)fibroblasts (vimentin) was seen at the rotator interval and the axillary recess, whereas this was not seen at the posterior capsule of frozen shoulder patients [139]. This suggests that frozen shoulder is a localised problem, rather than a generalised capsular contraction. It also raises the question whether a posterior release or a full 360° capsular release is indicated. Furthermore, new collagen deposition was seen particularly at the rotator interval area of frozen shoulder patients compared to control groups, which is characterised by increased protein expression (immunostaining) of type III collagen [138].

Altered expression of **MMPs** in capsular tissues have also been identified in frozen shoulder [134, 135, 140]. **MMPs** and their natural inhibitors (tissue inhibitors of metalloproteinases (**TIMPs**)) regulate the turnover of **ECM** that fibroblasts produce. The results of studies that investigated **mRNA** expression of **MMPs** in frozen shoulder are heterogenous [141]. However, an absence of **MMP-14** expression was noted in all frozen shoulder tissues when compared to control tissues and palmar fascia tissues from patients with Dupuytren's contracture [134]. **MMP-14** activates **MMP-2**, an important mediator of collagen degradation, and thus in frozen shoulder this likely results in a limited degradation of collagen (and excessive collagen pro-

duction). In addition, there is an increased expression of **TIMPs** in frozen shoulder, including **TIMP-1** and **TIMP-2** [134, 140]. This suggests a potential imbalance of **ECM** turnover in frozen shoulder, which leads to excessive collagen accumulation. While historically **MMPs** have been considered to be mainly responsible for **ECM** degradation and turnover, recent studies show that they are also implicated in the pathogenesis of inflammation and fibrosis in a variety of diseases, including liver, lung, and kidney disease, as well as other joint diseases such as **RA** and **OA** [142–145].

1.5.5 Inflammatory mediators in frozen shoulder

Besides fibroblast proliferation, histological analysis of rotator interval biopsies showed a (chronic) inflammatory infiltrate with mast cells (mast cell tryptase), macrophages (CD68⁺), T-cells (CD3⁺) and B-cells (CD20⁺) [23]. In addition, increased vascularity (CD34⁺, endothelial cells), lymphatics (LYVE1⁺PROX1⁺), and nerve cells (S100 protein) were also seen in frozen shoulder capsular biopsies [23]. The presence of mast cells in these biopsies further supports fibroblast proliferation in frozen shoulder, as they have been shown to regulate fibroblast proliferation and differentiation (into myofibroblasts) [146, 147]. These findings may suggest that frozen shoulder is an immunomodulated process starting with an inflammatory phase which eventually leads to reactive fibrosis, with mast cells potentially acting as an intermediary between these two underlying processes [148].

Recent identification of cytokines and other inflammatory mediators in frozen shoulder suggest that this underlying pathophysiology might be cytokine mediated. Increased expression of growth factors and inflammatory mediators such as **IL-1 β** ,

IL-6, IL-8, TNF- α , TGF- β , vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), and cyclooxygenases (COXs) (COX-1, COX-2) have been identified in frozen shoulder patient tissues [134, 135, 138, 149–151]. Recently, a study also reported an increased expression of several alarmins in frozen shoulder tissues, including high-mobility group box 1 (HMGB1), IL-33, S100A8, and S100A9 [152]. As these findings are mainly based on basic observational studies, the exact mechanisms in which these inflammatory mediators promote and/or sustain inflammatory processes in frozen shoulder remains unclear.

The immune system uses an innate arm (first line, rapid response) and an adaptive arm (chronic, slow response) to respond to any danger stimuli [153]. The host immune system recognises ‘danger signals’, including exogenous molecules from invading microorganisms and endogenous molecules from tissue injury, which in turn ‘alarm’ the innate and subsequently the adaptive immune system [154–157]. Exogenous molecules have been named pathogen-associated molecular patterns (PAMPs), whereas their endogenous counterparts have been named damage-associated molecular patterns (DAMPs) or alarmins [154–159]. Both molecules can act as innate immune stimulators, triggering inflammatory responses through the innate immune system via PRRs, including TLRs and inflammasomes [19, 157, 159–161]. These responses cause inflammation in the concerned tissues by releasing more inflammatory mediators, and although this is supposed to be a protective response to eliminate (foreign) stimuli and initiate tissue repair, it can also progress to cytokine storms (as seen in acute infections/sepsis) or chronic diseases if not adequately regulated (e.g., sepsis, RA, diabetes mellitus, cancer, asthma, cardiovascular disease) [162–165]. This

might also be the case in frozen shoulder, as frozen shoulder tissues showed an increased expression of alarmins compared to control tissues [152].

Based on analysis of frozen shoulder tissue biopsies, we know that a variety of inflammatory mediators are present (and increased) in frozen shoulder. Therefore, it is hypothesised that inflammatory mediators play a key role in the underlying pathophysiology of frozen shoulder. However, the cell types expressing these inflammatory mediators and the exact cellular interactions which drive the underlying inflammation and fibrosis remain unknown.

1.5.6 Fibroblasts and macrophages in frozen shoulder

Growth factors and cytokines orchestrate the function of fibroblasts as well as ECM remodelling via MMPs and their inhibitors [134]. A potential role of fibroblasts in regulating the inflammation and fibrosis in frozen shoulder was suggested from *in vitro* studies from capsular tissue biopsies [165]. In this study, cultured fibroblasts isolated from diseased capsular tissues produced elevated levels of pro-inflammatory mediators compared to control fibroblasts from patients undergoing shoulder stabilisation surgery [165]. Furthermore, upon inflammatory stimuli, fibroblasts showed an activated pro-inflammatory phenotype measured by an increase in stromal activation marker transcript and protein expression (vascular cell adhesion molecule (VCAM), podoplanin (PDPN), CD248) [165]. Stromal cell activation, as mentioned in section 1.2.6, is not exclusive to frozen shoulder as it has been seen in several other (musculoskeletal) diseases [34–36, 51]. This activated phenotype of fibroblasts and associated cytokine dysregulation may drive inflammation and fibrosis in frozen shoulder [165].

Macrophages have also been identified in frozen shoulder (most likely both tissue-resident and circulating monocyte-derived macrophages) [23, 74]. While heavily researched across a wide range of diseases, macrophages have not been extensively studied in frozen shoulder, a self-limiting, inflammatory fibrotic disease. Given the highly heterogenous and diverse phenotype of tissue-resident macrophages, together with their pro-inflammatory and pro-resolving capacities, and being involved in other joint diseases, one could hypothesise that these cells and their potential interactions may be implicated in inflammatory fibrotic processes in frozen shoulder [14, 66–68, 70].

Recent work studying frozen shoulder capsular tissues collected from patients undergoing capsular release suggested these tissues show a resolving inflammatory milieu compared to chronic tendon tears [166]. Frozen shoulder capsular tissues showed reduced expression of **NF- κ B** target genes, and capsular macrophages and fibroblasts highly expressed pro-resolving receptors compared to shoulder tendon tears [166]. Taken together, it can be hypothesised that crosstalk between capsular fibroblast- and macrophage populations plays an important role in the pathophysiology of frozen shoulder.

1.6 DPhil objectives and hypotheses

Based on the literature highlighted in sections 1.5.5 and 1.5.6, I hypothesise that inflammatory mediators play a key role in the pathobiology of frozen shoulder. Furthermore, I hypothesise that inflammatory mediators enable crosstalk between stromal cell subsets, possibly regulating the biological processes of inflammation, fibrosis, and ultimate resolution during frozen shoulder. The over-arching aim of this thesis is therefore to investigate the role of inflammatory mediators in frozen shoulder. This DPhil is comprised of three chapters with multiple objectives, which are briefly outlined below.

1.6.1 First results chapter (Chapter 2)

Objectives

- Use comparator and frozen shoulder patient tissues to identify the major cell types comprising the rotator interval of the GH joint capsule.
- Identify distinct cell types expressing inflammatory mediators in frozen shoulder and comparator capsular patient tissues.

Hypotheses

- The rotator interval of the GH joint capsule consists of distinct cell types including fibroblasts, macrophages, and T-cells.
- Immune cells will express a variety of inflammatory mediators.
- Expression of pro-inflammatory mediators and their cognate receptors is increased in frozen shoulder relative to non-diseased comparator tissues.

Methods

Cells deriving from capsular tissues from frozen shoulder patients and comparator groups will be characterised *ex vivo* with single cell RNA sequencing (scRNAseq). This will generate a cellular atlas of the human shoulder capsule, informing the distinct cell types that comprise the shoulder joint capsule in health and disease. Importantly, it will allow for identification of cell types expressing inflammatory mediators.

1.6.2 Second results chapter (Chapter 3)

Objectives

- Identify the micro-anatomical niche in which the cells expressing inflammatory mediators reside.
- Confirm protein expression of important inflammatory mediators and their cognate receptors.
- Identify potential crosstalk between inflammatory mediators and neighbouring cell types.

Hypotheses

- Protein expression of inflammatory mediators and their cognate receptors is increased in frozen shoulder relative to non-diseased comparator tissues.
- There are complex cellular interactions between macrophages and fibroblasts which are mediated by inflammatory mediators and drive inflammatory processes in frozen shoulder.

Methods

Well-phenotyped patient capsular tissues will be used for histology and immunohistochemistry to spatially map the previously identified inflammatory mediators (Chapter 2) onto GH joint capsular tissues. Furthermore, the single cell atlas generated in Chapter 2 will be computationally interrogated to identify important ligand-receptor pairs across macrophage and fibroblast populations.

1.6.3 Third results chapter (Chapter 4)

Objectives

- Identify differences in levels of secreted inflammatory mediators in tissue digests from comparator and frozen shoulder capsular patient tissues.
- Investigate the effects of specific inflammatory mediators (identified in Chapter 2 and Chapter 3) on patient-derived cells *in vitro*.

Hypotheses

- Frozen shoulder tissue digests will have increased levels of inflammatory mediators as cells in frozen shoulder are expected to secrete more inflammatory mediators relative to those in comparator capsular tissue digests.
- The addition of specific inflammatory mediators will further induce an inflammatory response in patient-derived cells.

Methods

The Meso Scale Discovery (MSD) platform will be used to measure the concentration

of inflammatory mediators in supernatants of freshly digested capsular tissues from both comparator and frozen shoulder patient groups. A recombinant form of a chosen inflammatory mediator will be added to cell cultures of fibroblasts derived from frozen shoulder capsular tissues. Bulk RNA sequencing (**RNAseq**) will be used to identify changes in gene expression in incubated cells. In addition, supernatants of these cell experiments will be analysed with the **MSD** platform to assess the secretion of inflammatory mediators from frozen shoulder fibroblasts.

CHAPTER 2

A single cell atlas of the rotator interval of
the GH joint capsule

2.1 Introduction

Many efforts have been made to understand the biological mechanisms underpinning frozen shoulder, yet the underlying pathophysiology remains poorly understood [23, 74, 134–137]. As a result, current therapies are not always effective and do not address the underlying cellular basis of disease. While often self-limiting, with some cases resolving after one year, a significant proportion of frozen shoulder patients remain symptomatic for several years [4]. Thus, frozen shoulder presents an unmet clinical need for affected patients. To address this, fundamental research with tissues from frozen shoulder patients is needed. Furthermore, we need to identify the key cell types that comprise the GH joint capsule. This could provide a better understanding of the biological mechanisms driving frozen shoulder and help identify new therapeutic targets.

To date, analysis of frozen shoulder patient tissue biopsies from the rotator interval capsule and/or the synovium of the GH joint capsule have shown inflammation and fibrosis with an imbalance of ECM turnover (as mentioned in Chapter 1, section 1.5) [23, 134, 140]. Several studies have suggested the role of inflammatory mediators such as cytokines (IL-6, TNF- α), growth factors (VEGF, PDGF), and alarmins (HMGB1, IL-33, S100 proteins) in frozen shoulder [134, 135, 138, 149, 150, 152]. Furthermore, histological studies have identified stromal cells (fibroblasts) and immune cells (macrophages, T cells, B cells) in the rotator interval capsule and/or the synovium of the GH joint capsule [23, 74]. Particularly, fibroblasts and macrophages have been proposed as key players in orchestrating the underlying biological mechanisms in frozen shoulder [165, 166]. However, the precise cellular interactions

and the cell types expressing above mentioned inflammatory mediators have not been identified.

Recent advances in understanding the pathophysiology of diseases have come forth by analysing patient tissues with **scRNAseq** [167]. This state-of-the-art platform in the field of single-cell biology has helped to identify different cellular subsets in a variety of diseases, understand the cellular profile of tissues, identify important cellular interactions, and even identify new therapeutic targets. An example of this is seen in **RA**, where recently nine macrophage populations were identified in synovial tissues, including pro-resolving macrophages [70].

To identify the cell types and inflammatory mediators involved in frozen shoulder, this chapter will create a single cell atlas of the rotator interval of the **GH** joint capsule comprised of tissue biopsies collected from frozen shoulder and comparator (arthroplasty) patient samples. After identifying the main cell populations present in the rotator interval of the **GH** joint capsule, this chapter will focus on cell types expressing inflammatory mediators.

2.2 Objective and hypotheses

The overarching objective of this chapter is to identify distinct cell types expressing inflammatory mediators in frozen shoulder and comparator capsular patient tissues. This will be accomplished by creating a single cell atlas of the rotator interval of the GH joint capsule using **scRNAseq**. The single cell atlas will then be interrogated to investigate differences in gene expression of inflammatory mediators in tissues from patients with frozen shoulder relative to comparator tissues.

The overarching hypothesis of this chapter is that the rotator interval of the GH joint capsule consists of major cell types including fibroblasts, macrophages, and T cell populations. I hypothesise that mainly macrophages and T cells express a variety of inflammatory mediators. These cell types might therefore be implicated in sustaining inflammatory processes in advanced stages of frozen shoulder. Finally, I hypothesise that the expression of inflammatory mediators and their cognate receptors increases in frozen shoulder relative to non-diseased comparator patient tissues.

2.3 Materials and methods

2.3.1 Ethics statement

A multi-centred, cohort, observational, and laboratory study (Inflammation and fibrosis: CausEs in adhesive CAPsultitis (**ICECAP**)) was used to collect tissue from the rotator interval of the **GH** capsule (<https://www.ndorms.ox.ac.uk/research/research-groups/soft-tissue-joint-disease-dakin-group/clinical-studies/icecap-1>). Ethical approval for this study (and the collection of capsular tissue) was acquired from the **NHS** Health Research Authority - Oxford B Research Ethics Committee (**REC**) on 26/11/2018 (**REC** reference: 18/SC/0649 – IRAS project ID 257757). Oxford University Hospitals Trust Management approval was acquired on 16/01/2019 (R&D reference: 13727). Comparator capsular tissue from the rotator interval was also collected under the Oxford Musculoskeletal BioBank (**OMB**) ethics (reference: 09/H0606/11). This study was conducted according to the principles of the Declaration of Helsinki and in accordance with medical research involving human tissue (Human Tissue Act, 2004). Full written informed consent was obtained from all participants. Each participant had the right to withdraw from the study at any time.

2.3.2 Study population and tissue collection

Patients were recruited from two **NHS** hospitals in the **UK** as part of the **ICECAP** study: Oxford University Hospitals (lead site) and South Tees Hospitals. **ICECAP** patients were split into three groups: one disease group and two comparator groups (Table 2.1). In addition, patients undergoing elective joint replacement surgery for

OA were also included as a comparator group (**OMB** ethics). All potential participants were evaluated by upper limb (orthopaedic) surgeons through clinical examination and radiography during outpatient clinics. In- and exclusion criteria as stated in the **ICECAP** study protocol can be found in Table 2.2. Whilst under general anaesthetic patients underwent a biopsy of a very small quantity of shoulder joint capsule at the site of the rotator interval (size of a grain of rice). Fresh capsular tissues were then collected in Dulbecco’s Modified Essential Medium (**DMEM**)-F12 media (1:1 mixture of **DMEM** and Ham’s F-12 Medium) containing 10% foetal calf serum (**FCS**) and 1% penicillin-streptomycin. The samples were transported to the Botnar Research Centre, University of Oxford. The tissue was then prepared for either **scRNAseq**, tissue sectioning (Chapter 3), or cell culture (Chapter 4).

Table 2.1: **ICECAP** patient groups used for tissue collection.

Group A (biopsy)	Group B* (waste tissue)	Group C (waste tissue)
Patients with shoulder instability undergoing elective surgery for anterior stabilisation	Patients with displaced proximal humeral fractures undergoing elective surgical repair	Patients with frozen shoulder undergoing arthroscopic capsular release

*patients undergoing elective joint replacement surgery for **OA** were also included as a comparator group (**OMB** ethics).

Table 2.2: Inclusion and exclusion criteria of the study population.

Inclusion criteria

- male or female, and over 18 and under 80 years
- participant is willing and able to give informed consent

AND

- on the waiting list for anterior stabilisation procedure

OR

- on the waiting list for surgical repair of a proximal humeral fracture*

OR

- on the waiting list for elective joint replacement surgery for OA or fractures

OR

- on the waiting list for elective capsular release procedure for frozen shoulder
-

Exclusion criteria

- RA
 - Patient is receiving anti-coagulation therapy
 - Significant cognitive impairment or language issues precluding informed consent
 - Other factors that, in the opinion of the responsible clinician, might jeopardise the safe and ethical conduct of participant care and study operation
 - Previous ipsilateral shoulder surgery
-

*Only patients undergoing surgery after a minimum of 3 weeks post-trauma were included as earlier samples showed a high inflammatory response in early pilot studies.

2.3.3 Preparing a cell suspension for scRNAseq

Upon arrival, freshly acquired capsular tissue samples were cut into small pieces (2mm²) in a tissue culture hood (Figure 2.1). Pieces of tendon, fat, and where possible, synovium were excised. A total of n = 6 comparator and n = 4 frozen shoulder capsular tissue samples were used (Table 2.3). The samples were placed in

a 50ml falcon tube and digested in **DMEM-F12** media (without **FCS** or penicillin-streptomycin), containing Worthington type 2 collagenase at 4mg/ml with deoxyribonuclease (**DNase**) (Worthington **DNase** 1, 10mg/ml, Lorne) by adding 100 μ l **DNase** 1 (Lorne) to 5ml of medium. The falcon tubes (with the samples) were incubated at 37°C in a heated shaker for 90 minutes. After digestion, 40ml of **DMEM-F12** media containing 10% **FCS** was added to the sample (gently mixed) to stop the digestion process. To create a single cell suspension, the cells were passed through a cell strainer twice. This suspension was spun at 1700rpm for 5 minutes at room temperature. Afterwards, the supernatant was removed, and the sample was re-suspended in 300 μ l **DMEM-F12** media containing 10% **FCS**. The single cell suspension was put on ice and was taken to the Wellcome Trust Centre for Human Genetics (Moustafa Attar), University of Oxford.

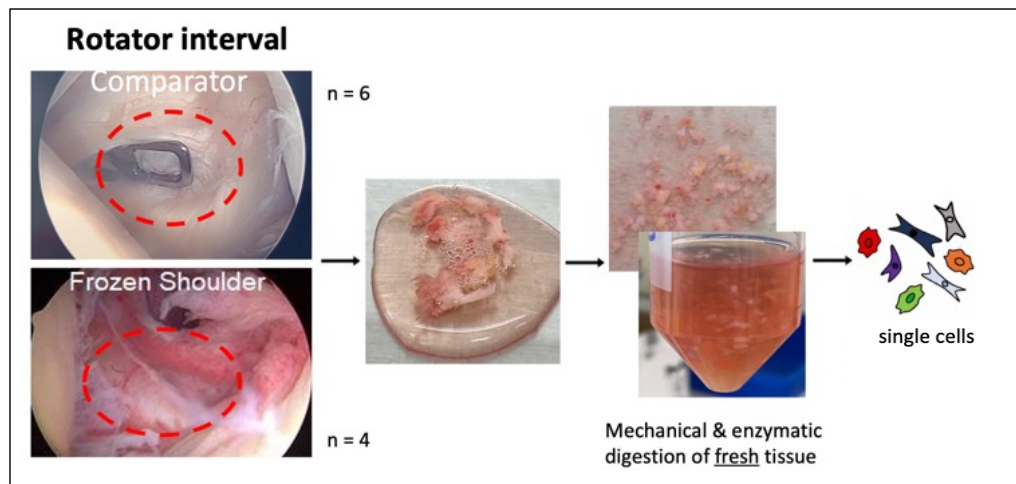


Figure 2.1: Digestion of tissues from the rotator interval of the **GH** joint capsule. Creating a single cell suspension from freshly acquired capsular tissue samples ($n = 10$) for **scRNAseq**. Both mechanical and enzymatic (collagenase + **DNase**) digestion methods were used.

Table 2.3: Demographics of GH joint capsule tissue donors for scRNAseq.

Donor	Gender	Age (years)	Tissue phenotype	Co-morbidities
Comparator 1	Male	63	Arthroplasty for humeral #	Hypothyroidism
Comparator 2	Male	69	Arthroplasty for OA	N/A
Comparator 3	Female	75	Arthroplasty for OA	N/A
Comparator 4	Male	65	Arthroplasty for OA	N/A
Comparator 5	Male	70	Arthroplasty for OA	Hypertension
Comparator 6	Female	75	Arthroplasty for OA	N/A
Frozen shoulder 1	Male	56	Symptoms for 2+ years	Diabetes Mellitus
Frozen shoulder 2	Male	50	Symptoms for 2 years	N/A
Frozen shoulder 3	Male	52	Symptoms for 2+ years	Diabetes Mellitus
Frozen shoulder 4	Female	44	Symptoms for 8 months	N/A

#, fracture; N/A, not applicable.

2.3.4 Cell sorting and scRNAseq

Cells were stained with 7-AAD dye (Bio-Legend 420404) for viability and subsequently underwent live/dead sorting on a cell sorter (SONY SH800 cell sorter, SONY Biotechnology). Low viability samples (<80%) were excluded from analysis (and scRNAseq was not performed). The cell count was performed using the Bio-Rad TC20 Automated Cell Counter, after which approximately 20,000 – 30,000 cells per sample were loaded onto the 10x Genomics Chromium Controller (Chip G). The 10x Chromium Next GEM Single Cell 3' Reagent Kits v3.1 were used to prepare the sequencing libraries following the manufacturer user guide (CG000204). Finally, the single-cell gene expression libraries were sequenced on the NovaSeq6000 sequencing platform (v1.5 chemistry, 28bp/98bp) to a minimum depth of 50,000 reads/cell.

2.3.5 Alignment of the **scRNAseq** data

The **scRNAseq** data was aligned to the Genome Reference Consortium Human Build 38 (**GRCh38**) and Release 32 (GENCODE v32/Ensembl 98) of the Gencode transcripts (2020-A) using Cell Ranger 6.1.1 (10x Genomics). A count matrix containing unique molecular identifier (**UMI**) counts per gene per cell was then generated. Duplicate **UMIs** (e.g., molecules that have been sequenced more than once) were collapsed and were counted as a single read, resulting in only unique **UMIs** being quantified. CellBender and Scrublet were used to remove ambient background **RNA** and to identify doublets respectively [168]. The Seurat vignette from the Satija lab was used for pre-processing steps and downstream analysis [169]. For this reason, the count matrix was imported in R (version 4.2.0) (<https://www.r-project.org>) and transformed into a Seurat object using the CreateSeuratObject function.

2.3.6 Quality control (**QC**) and filtering of cells

The standard pre-processing workflow was performed according to the Seurat vignette on R [169]. This included **QC**, normalisation, and the detection of highly variable genes for downstream analysis. The following **QC** parameters were used: cells with a very low or a very high gene count (<300 or >6500 n_Features), and/or a high mitochondrial gene expression (percent.mt $>10\%$) were removed from the dataset (Figure 2.2). The samples were log normalised using the NormalizeData function with a scale factor of 10,000. The FindVariableFeatures function was used to identify the top 3,000 highly variable genes for principal component analysis (**PCA**) by applying the variance stabilizing transformation method. The data was then scaled (ScaleData) while adjusting for mitochondrial expression.

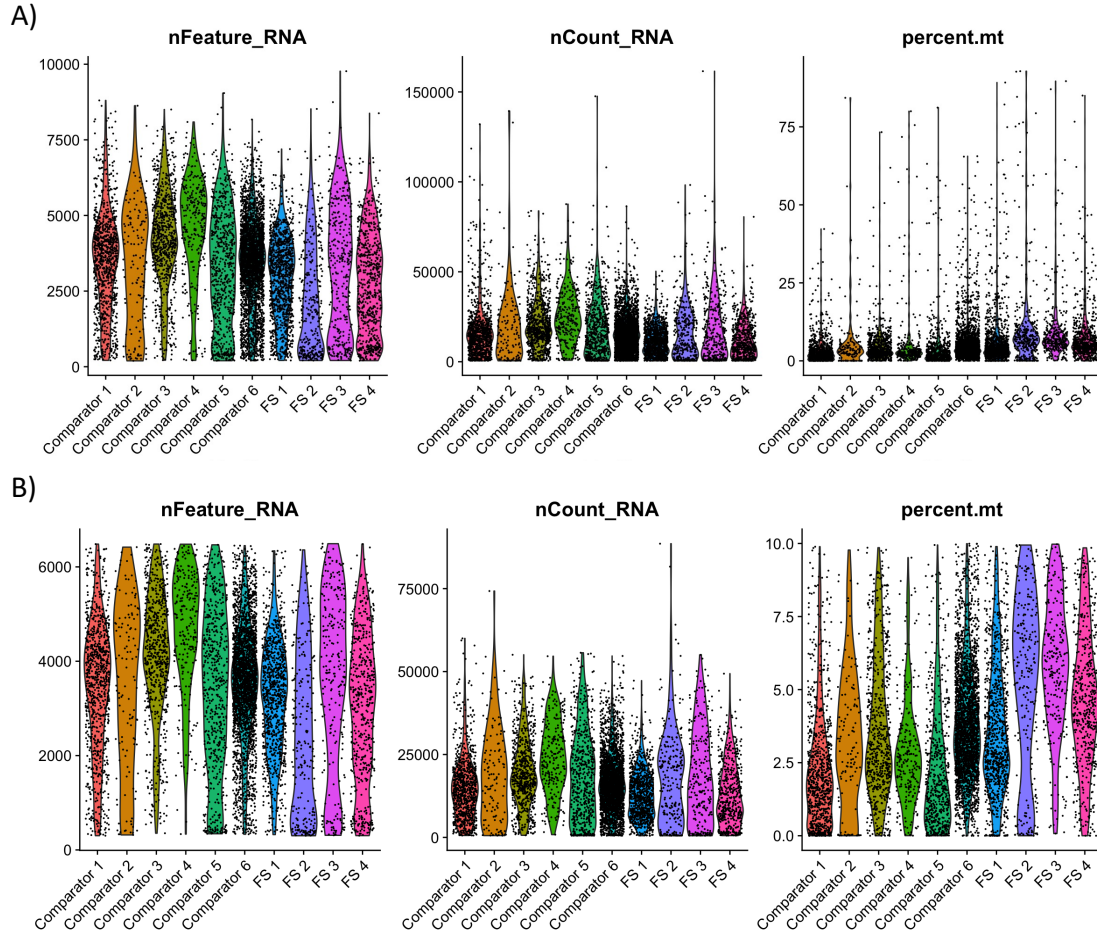


Figure 2.2: QC of scRNAseq data - Filtering of cells.

A) Pre-QC, **B)** post-QC: Violin plots of the unique number of genes (nFeature_RNA), the total number of molecules (nCount_RNA), and the proportion of mitochondrial gene count (percent.mt) per donor sample. FS, frozen shoulder. QC parameters used: nFeature_RNA count <6500 and percent.mt <10%. Each dot represents a unique cell.

2.3.7 Integration and cell clustering

After PCA (RunPCA), integration and batch correction were performed using Harmony by the condition (comparator and frozen shoulder) (Figure 2.3) [170]. Second, after assessing the dimensionality of the dataset with an Elbow plot (Figure 2.4), nine principal components (PCs) were used for the Uniform Manifold Approximation

and Projection (**UMAP**) with the RunUMAP function. The nearest neighbour graph was constructed with the FindNeighbors function using the Euclidian distance metric. Finally, unsupervised clustering (FindClusters) was performed at a resolution of 0.4 using the Leiden clustering algorithm. After clustering, clusters with a low number of molecule ($\text{nCount_RNA} < 10,000$) and gene count ($\text{nFeature_RNA} < 2,000$) were removed from the dataset using the subset function. In addition, clusters highly expressing haemoglobin markers (*HBA*, *HBB*, *HBD*, *HBM*) were also excluded.

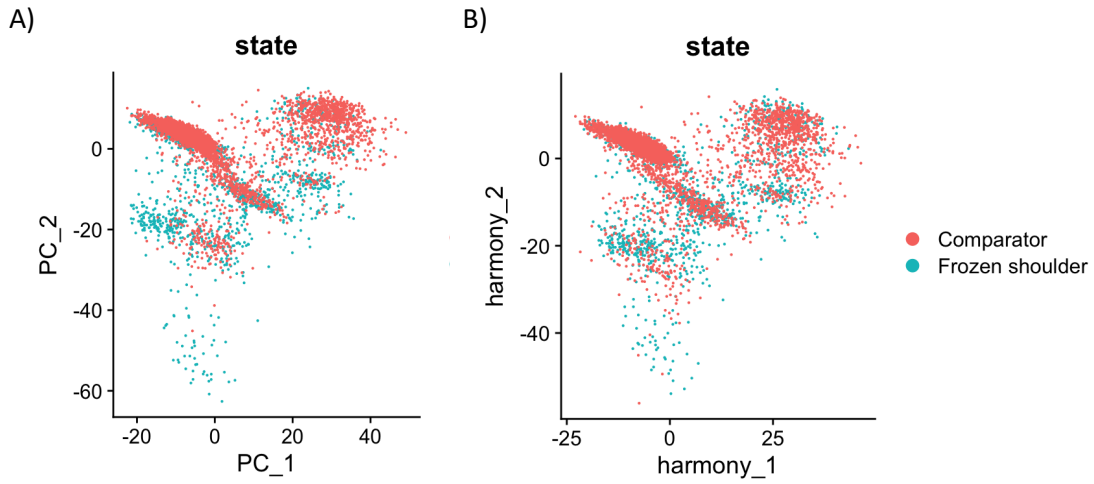


Figure 2.3: **QC** of **scRNAseq** data - Batch correction using Harmony.

A) Reduced dimension plot of first two (uncorrected) **PCs**. **B)** Reduced dimension plot of the first two dimensions after batch correction with Harmony.

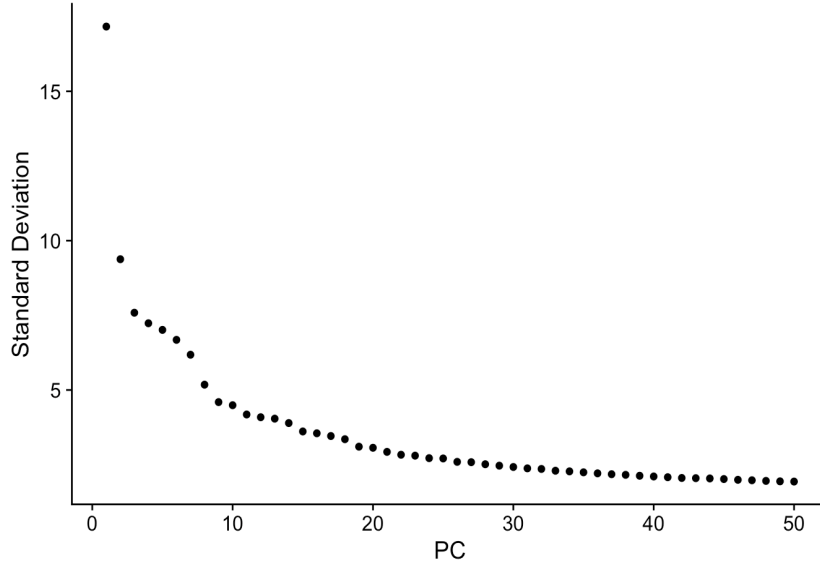


Figure 2.4: Elbow plot of the first 50 PCs.

Visualisation of the standard deviation of each PC. The more PCs are retained, the more variance is captured (i.e., the smaller the standard deviation).

2.3.8 Cluster annotation

After visualising the clusters using UMAP, top negative and positive cluster marker genes were identified using the default Seurat FindAllMarkers function. The Wilcoxon rank sum test with Bonferroni correction was used to determine significance which was set at an adjusted p value (padj) of <0.05 . The average log2 fold change (log2FC) was set at ≥ 1.0 in either direction. To visualise the top differentially expressed genes (DEGs) per cluster an RNA heatmap was created. Key marker genes for annotation were chosen based the top DEGs per cluster, literature selected markers, and SingleR annotation (HumanPrimaryCellAtlasData, DatabaseImmuneCellExpressionData, MonacoImmuneData) [171]. Clusters of interest were computationally isolated and were re-analysed through iterating the same pre-processing workflow described above.

2.3.9 Pseudo bulk differential expression analysis

For pseudo bulk differential expression analysis, a **scRNAseq** raw count data matrix was generated. Counts of individual genes within a specific cell cluster that had a total count greater than 10 were included. Sample level **QC** was performed using **PCA** and hierarchical clustering (sample-to-sample distances) after normalising the raw counts and log transforming (regularised log transform) the normalised counts using DESeq2 [172]. A table of **DEGs** within a specific cell cluster across conditions (comparator and frozen shoulder) was generated. Significantly down- and upregulated **DEGs** were filtered out using a **padj** of <0.05 (Benjamini-Hochberg correction (**BH**)). To visualise the significant **DEGs**, a volcano plot was generated using **padj** values <0.05 (**BH**) and **log2FC** of ≥ 1.0 in either direction. Volcano plots were generated using the EnhancedVolcano package in R [173].

2.3.10 Pathway analysis

To assess the functionality of specific cell clusters, over-representation analysis of top marker genes and genesets identified from the **scRNAseq** data was performed using the Gene Ontology (**GO**) database and the Molecular Signatures Database (**MSigDB**) in combination with the gsfisher and ClusterProfiler packages in R [174–177]. Top marker genes of each cluster were selected and the gene universe was restricted to genes that were detected in a minimum of 10% of cells. Significant pathways were ranked by odds ratio and evaluated by the Fisher Exact test using **BH** correction for multiple testing. **GO** categories with more than 500 genes and **GO** categories that have an over-representation odds ratio of less than 2 were filtered out.

2.4 Results

2.4.1 A single cell atlas of the rotator interval of the **GH** joint capsule

Using **scRNAseq** on comparator ($n = 6$) and frozen shoulder ($n = 4$) capsular tissues from the rotator interval, a single cell atlas was created. Clustering of 6,818 cells generated 10 cell clusters (Figure 2.5A), which were expressed in both conditions (comparator and frozen shoulder), as well as in samples from individual donors (Figure 2.5B&C). An **RNA** heatmap showing the top 10 marker genes for each of the clusters (Appendix A.1) and feature plots with the number of molecules, mitochondrial gene expression, doublet scores, and cell cycle phase were generated (Appendix A.2). In addition, SingleR annotation **UMAPs** and ScoreHeatmaps were generated (Appendices A.3 to A.5).

The single cell atlas included both $CD45^-$ ($n = 7$) and $CD45^+$ ($n = 3$) cell clusters (Figure 2.6A). The single cell atlas revealed five $COL1A1^+PDGFRA^+FAP^+$ fibroblast clusters, two $CD14^+CD68^+$ myeloid cell clusters, a $CD2^+CD3^+$ lymphocyte cluster, a $PECAM1^+VWF^+$ endothelial cell cluster, and a $ACTA2^+NOTCH3^+$ mural cell cluster (Figure 2.6B). Cell clusters with similar key marker genes were merged before further downstream analysis. The five fibroblast clusters were merged into one fibroblast cluster and the two myeloid cell clusters were merged into one myeloid cell cluster. This resulted in five major cell clusters, with fibroblasts being the most abundant cell type (Figure 2.7A&B). The top marker genes per cluster were re-analysed and visualised in an **RNA** heatmap (Figure 2.7C).

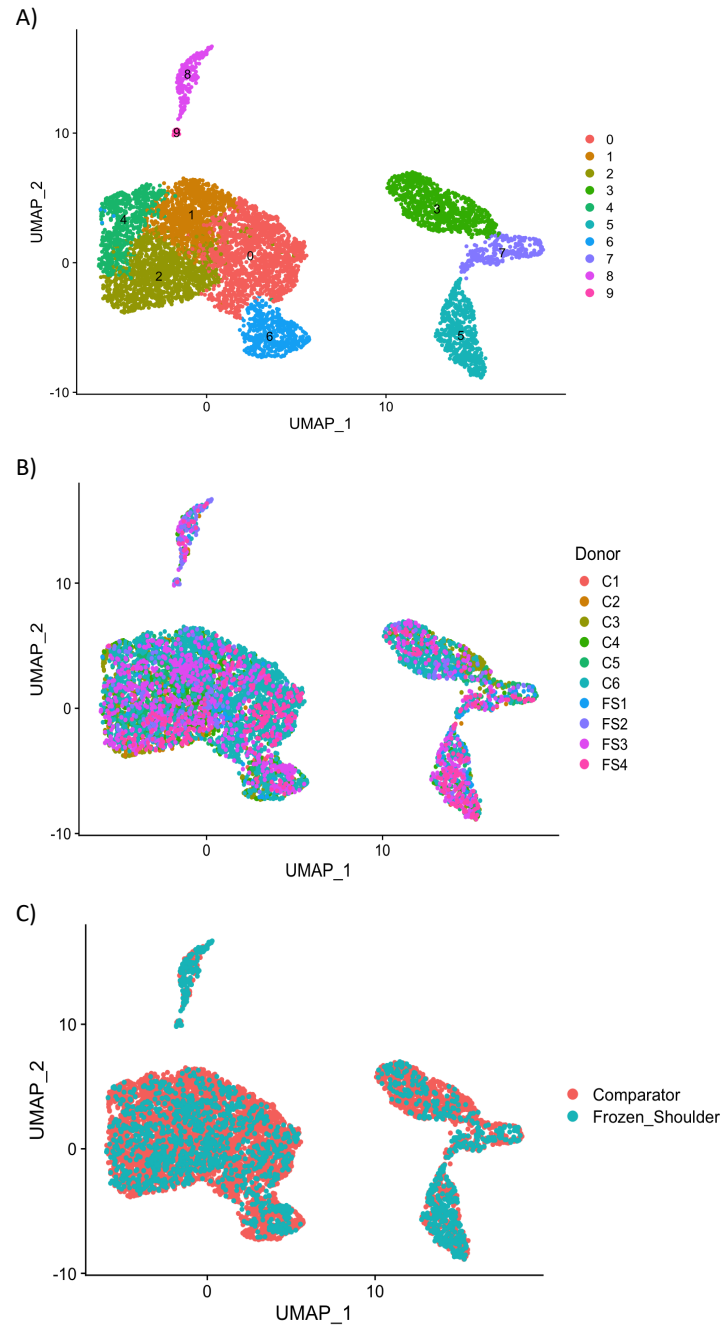
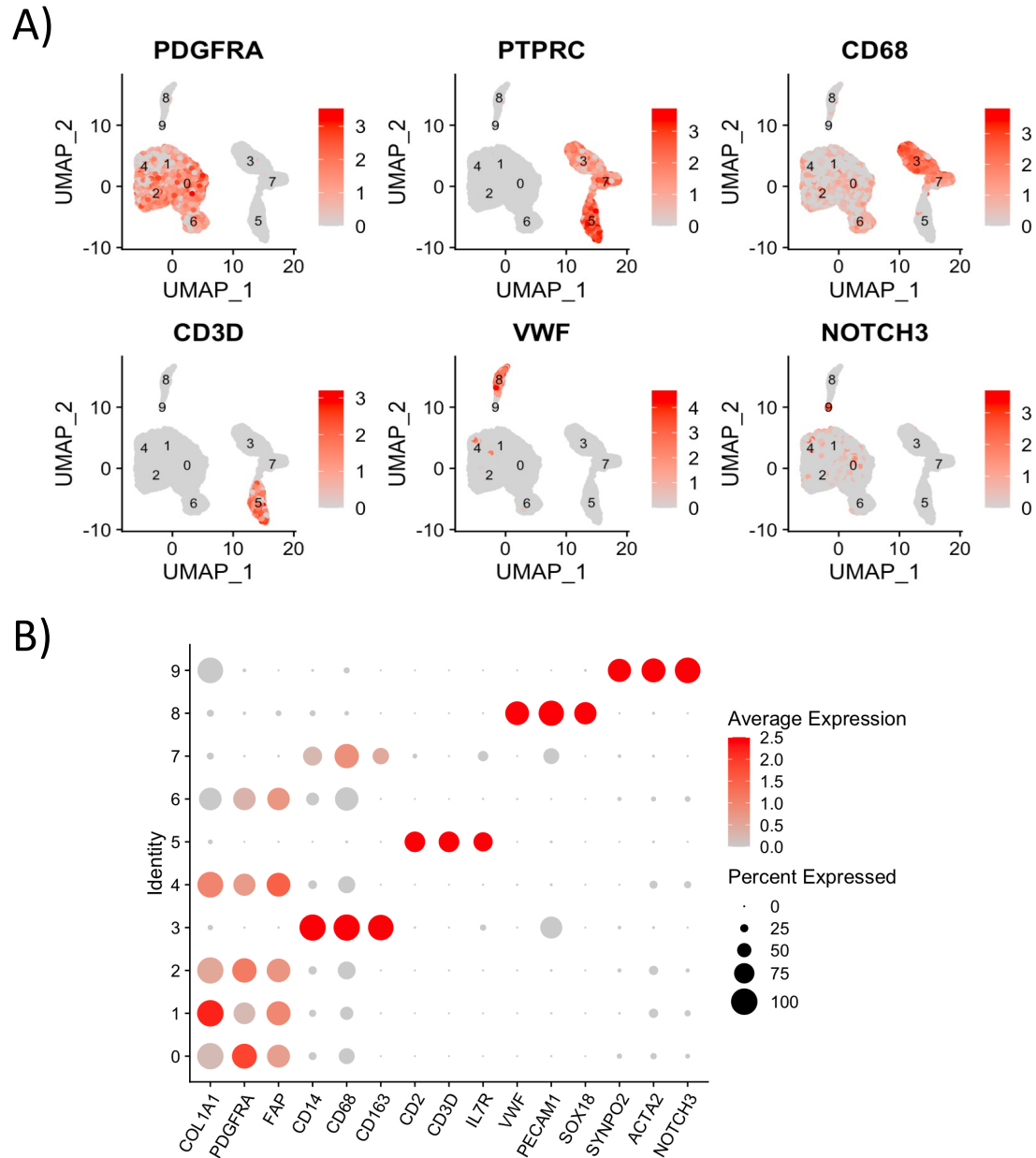


Figure 2.5: Single cell atlas of the rotator interval of the **GH** joint capsule.

A) **UMAP** dimensionality reduction of cells from both comparator ($n = 6$) and frozen shoulder ($n = 4$) samples revealed 10 distinct cell clusters. **B)** **UMAP** showing cells from individual donors (C, comparator; FS, frozen shoulder). **C)** **UMAP** showing cells from comparator and frozen shoulder samples.



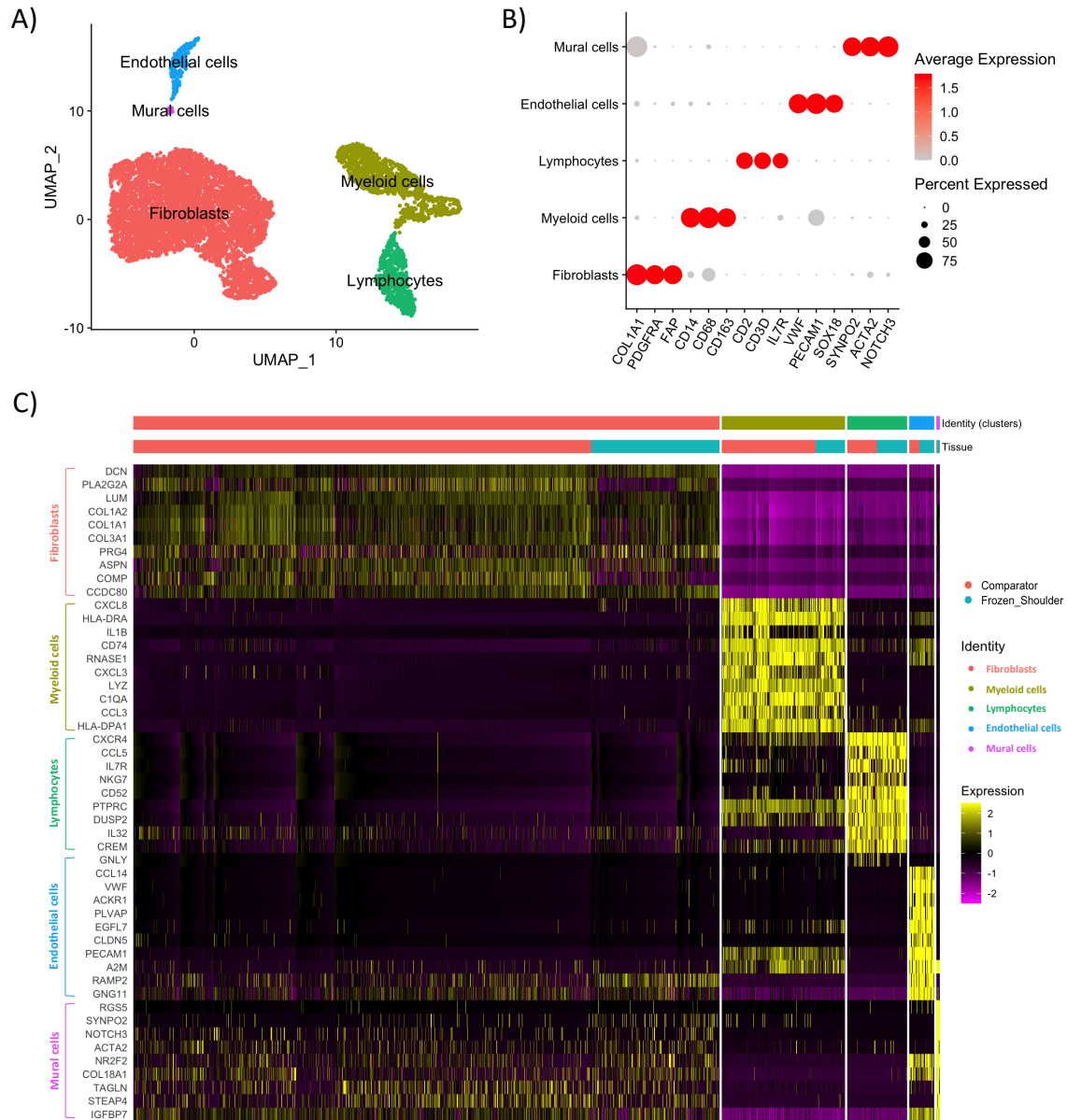


Figure 2.7: Major cell clusters of the rotator interval of the GH capsule.

A) UMAP dimensionality reduction of cells from both comparator ($n = 6$) and frozen shoulder ($n = 4$) samples showing five major cell clusters, including fibroblasts, myeloid cells, lymphocytes, endothelial cells, and mural cells. **B)** Dot plot showing key marker genes for each of the cell clusters. The size of the dots represents the percentage of cells and the intensity of the (red) colour represents the average expression level. **C)** RNA expression heatmap showing the top 10 marker genes (rows) for each of the five clusters (coloured columns) in comparator and frozen shoulder tissue. Yellow indicates increased expression of a particular gene, whereas purple indicates a decrease in expression of a particular gene for each cell.

2.4.2 Single cell atlas of the $CD14^+CD68^+$ myeloid cell clusters

As one of the objectives of this chapter is to identify which cells express inflammatory mediators within the GH joint capsule, the cell atlas was interrogated for a variety of literature selected inflammatory mediators, alarmins and their cognate receptors (Figure 2.8A). The $CD14^+CD68^+$ myeloid cell cluster expressed most of these markers, including *PTGS1*, *PTGS2*, *IL1A*, *IL1B*, *IL6*, *IL10*, *S100A8*, *S100A9*, and *TLR* receptor genes. For this reason, the $CD14^+CD68^+$ myeloid cell cluster was computationally isolated and re-analysed using a resolution of 0.2, to ensure the identification of possible myeloid cell subsets (Figure 2.8B). Clustering of the $CD14^+CD68^+$ myeloid cell cluster revealed four distinct myeloid cell subsets, including three macrophage populations: $MERTK^+LYVE^{high}$ macrophages, $MERTK^+LYVE^{low}$ macrophages, and $CD48^+IL1A^+$ macrophages (Figure 2.8C&D). The fourth subset was a $CD48^+CD1C^+$ dendritic cell population (Figure 2.8C&D). These myeloid cell subsets were expressed in both conditions (comparator and frozen shoulder), as well as in samples from individual donors (Appendix A.6). In addition, Single R annotations of the myeloid cell subsets were generated (Appendices A.7 to A.9).

Looking at the top positive genes per cluster, the $MERTK^+LYVE^{high}$ macrophages were enriched for *MAF*, *DAB2*, *RNASE1*, and *SELENOP*, whereas the $MERTK^+LYVE^{low}$ macrophages were enriched for *THBD*, *MMP9*, *ICAM1*, and *TNF* (Figure 2.9). The $CD48^+IL1A^+$ macrophages showed enrichment for a variety of inflammatory mediators, including *IL1B*, *S100A8*, *IL1RN*, and *PTGS2*. The $MERTK^+LYVE^{low}$ macrophages seem to be a mixed macrophage cluster, expressing markers from the $MERTK^+LYVE^{high}$ and $CD48^+IL1A^+$ macrophages (Figure 2.9).

Finally, the $CD48^+ CD1C^+$ dendritic cell cluster showed enrichment for *AREG*, and a variety of *HLA* genes (Figure 2.9).

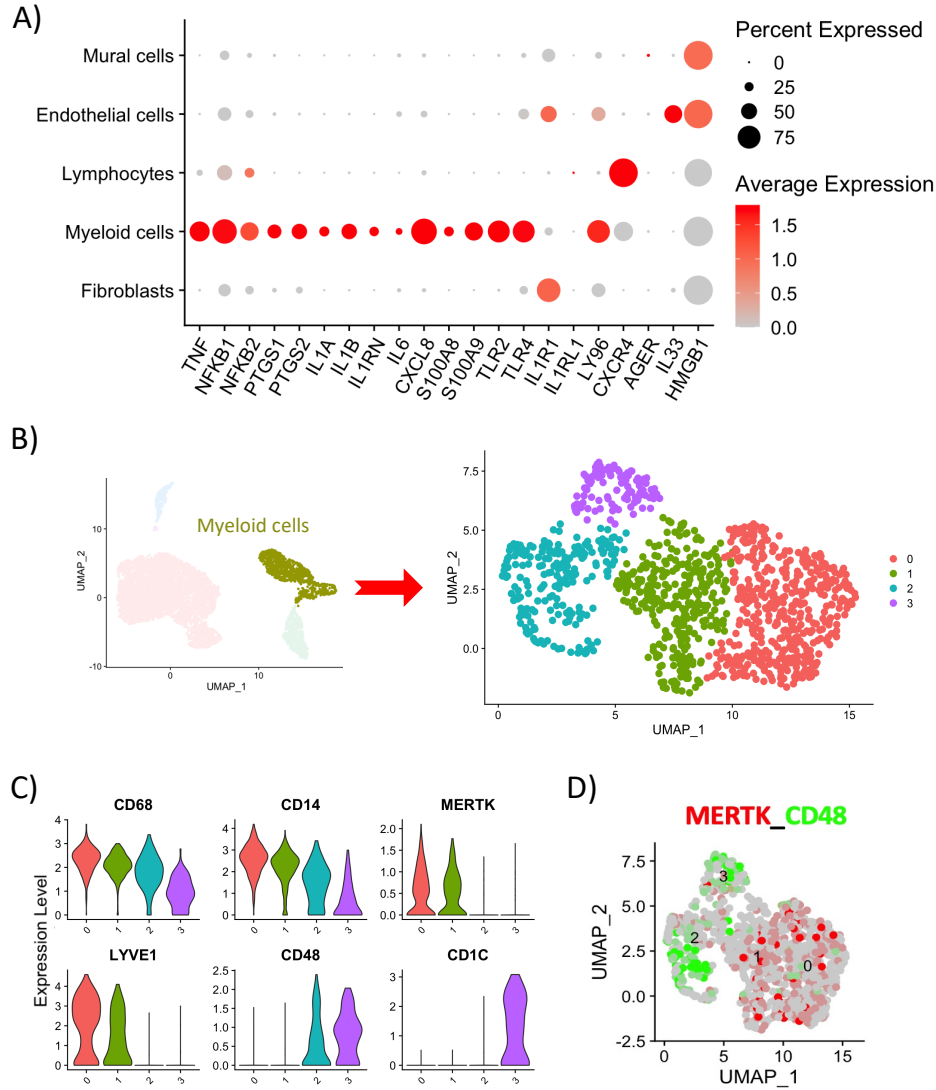


Figure 2.8: Single cell atlas of the $CD14^+ CD68^+$ myeloid cell cluster.

A) Dot plot showing the expression of a variety of literature selected inflammatory mediators per cluster. The size of the dots represents the percentage of cells and the intensity of the (red) colour represents the average expression level. **B)** UMAP dimensionality reduction of the $CD14^+ CD68^+$ myeloid cell cluster reveals four myeloid subsets. **C)** Violin plots of selected marker genes, including *CD68*, *CD14*, *MERTK*, *LYVE1*, *CD48*, and *CD1C*. **D)** Merged feature plot of *MERTK* (red) and *CD48* (green).

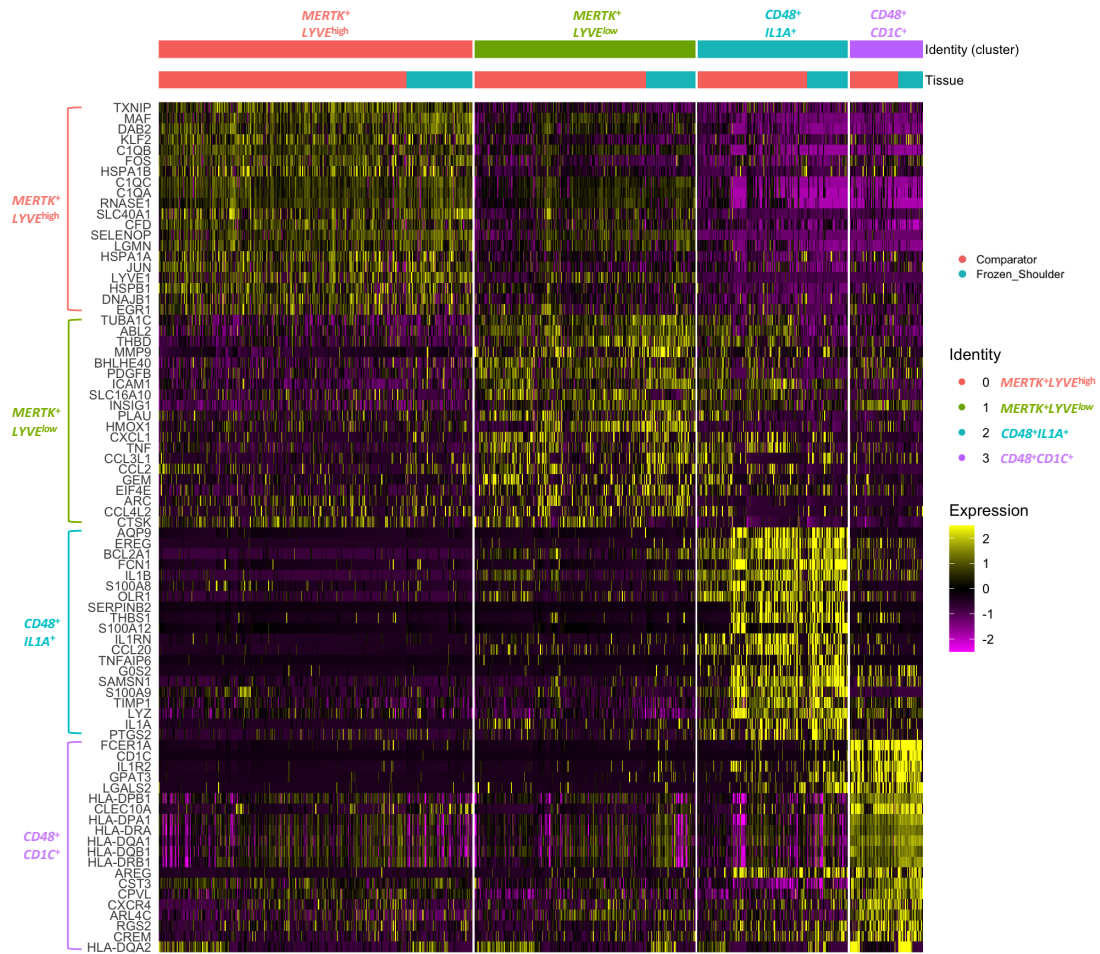


Figure 2.9: RNA heatmap of the myeloid cell clusters.

RNA expression heatmap showing the top 20 marker genes (rows) for each of the four myeloid cell clusters (coloured columns) in comparator and frozen shoulder tissue. Yellow indicates increased expression of a particular gene, whereas purple indicates a decrease in expression of a particular gene for each cell.

2.4.3 Top marker genes of $CD48^+IL1A^+$ macrophages

Looking closer at the $CD48^+IL1A^+$ macrophages, the top 20 positive marker genes consisted of inflammatory mediators and alarmins, including *EREG*, *IL1B*, *S100A8*, *SERPINB2*, *THBS1*, *S100A12*, *IL1RN*, *CCL20*, *TNFAIP6*, *S100A9*, and *PTGS2* (Figure 2.10). Interestingly, the top negative markers genes of this cluster were some of the top positive genes from the $MERTK^+LYVE^{high}$ macrophages (e.g., *MAF*, *LYVE1*, *DAB2*, *C1QB*) (Figure 2.10B). Volcano plots with the top negative and positive marker genes of the other myeloid cell populations can be found in Appendix A.10.

2.4.4 Pathway analyses of $CD48^+IL1A^+$ macrophages

To further assess how the $CD48^+IL1A^+$ macrophages might be implicated in sustaining inflammatory processes in frozen shoulder, unique GO and MSigDB pathway signatures of the different myeloid clusters were interrogated (Figures 2.11 and 2.12). $CD48^+IL1A^+$ macrophages showed enrichment for biological processes related to leukocyte chemotaxis, myeloid leukocyte migration, humoral immune response, and response to lipopolysaccharide (Figure 2.11). In addition, the MSigDB canonical pathways showed that $CD48^+IL1A^+$ macrophages are enriched for IL-10 signalling, IL-1R pathway, IL-1 processing, and NF- κ B pathway (Figure 2.12). Similar results were seen using other GO and MSigDB domains (Appendices A.11 to A.14).

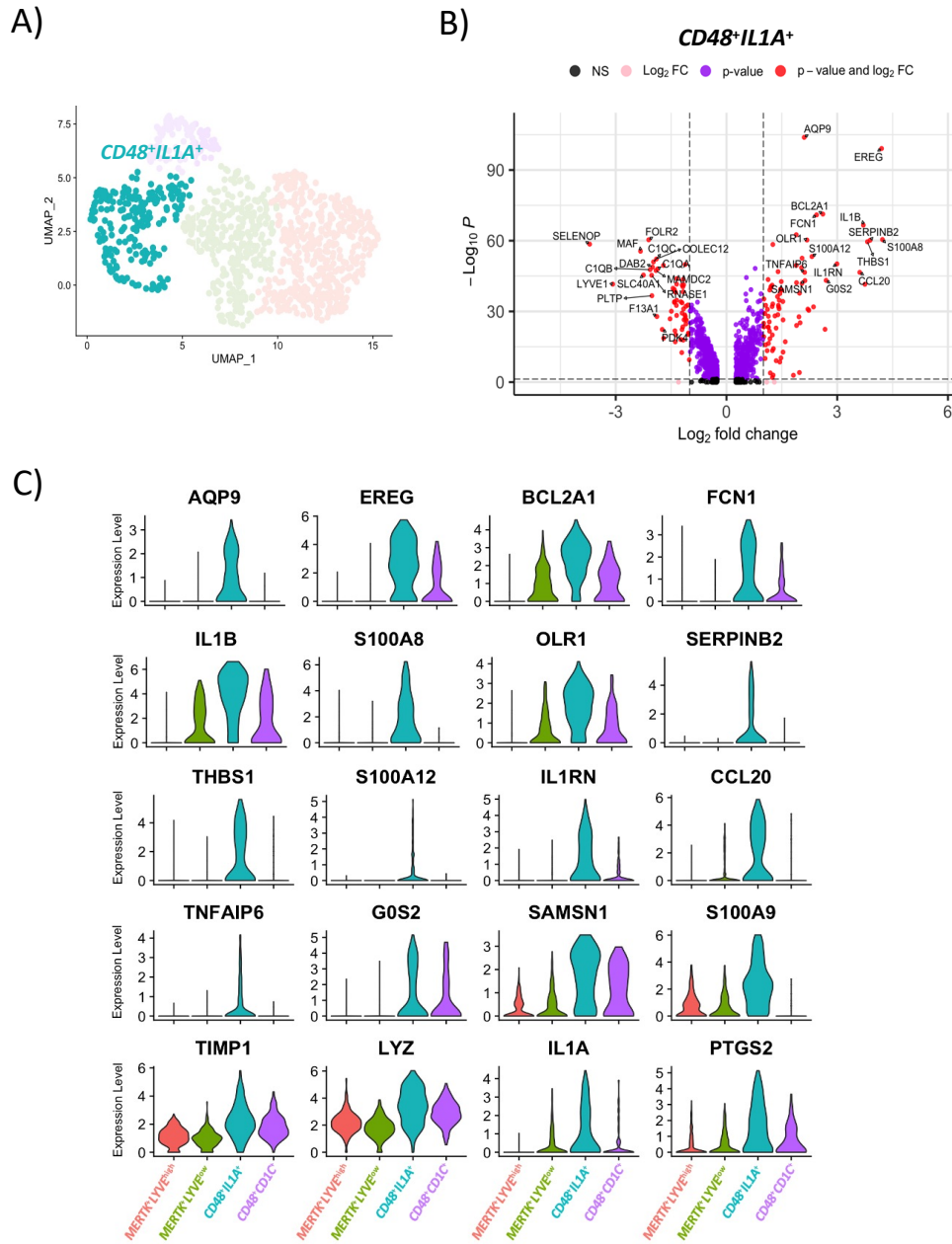


Figure 2.10: Top 20 genes of the *CD48⁺IL1A⁺* macrophage population.

A) UMAP dimensionality reduction of the *CD14⁺CD68⁺* myeloid cell cluster reveals four myeloid subsets. The *CD48⁺IL1A⁺* macrophage population is highlighted. **B)** Volcano plot displaying the top negative (negative log₂FC; left) and positive marker genes (positive log₂FC; right) that characterise the *CD48⁺IL1A⁺* macrophages. *P*_{adj} set at 0.05 and log₂FC ≥ 1 in either direction. **C)** Violin plots showing the expression of the top 20 positive marker genes relative to the other myeloid cell clusters.

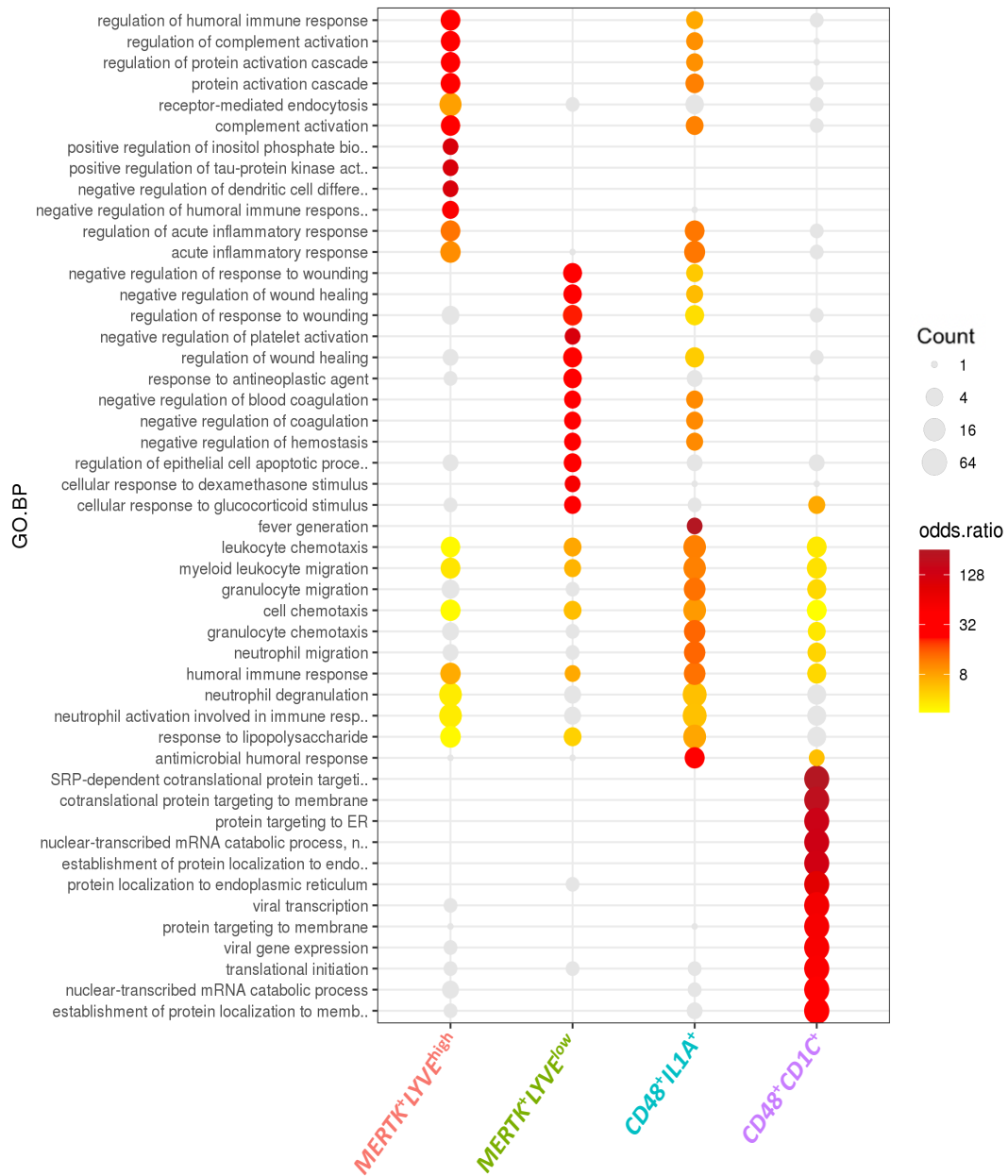


Figure 2.11: Pathway analysis of $CD14^+CD68^+$ myeloid cell subsets (GO). Over-representation analysis reveals the main enriched GO biological process (BP) pathways. The dot size is proportional to the the number of genes in the GO category that were found as cluster markers (Count). The intensity of the (red) colour represents the odds ratio.

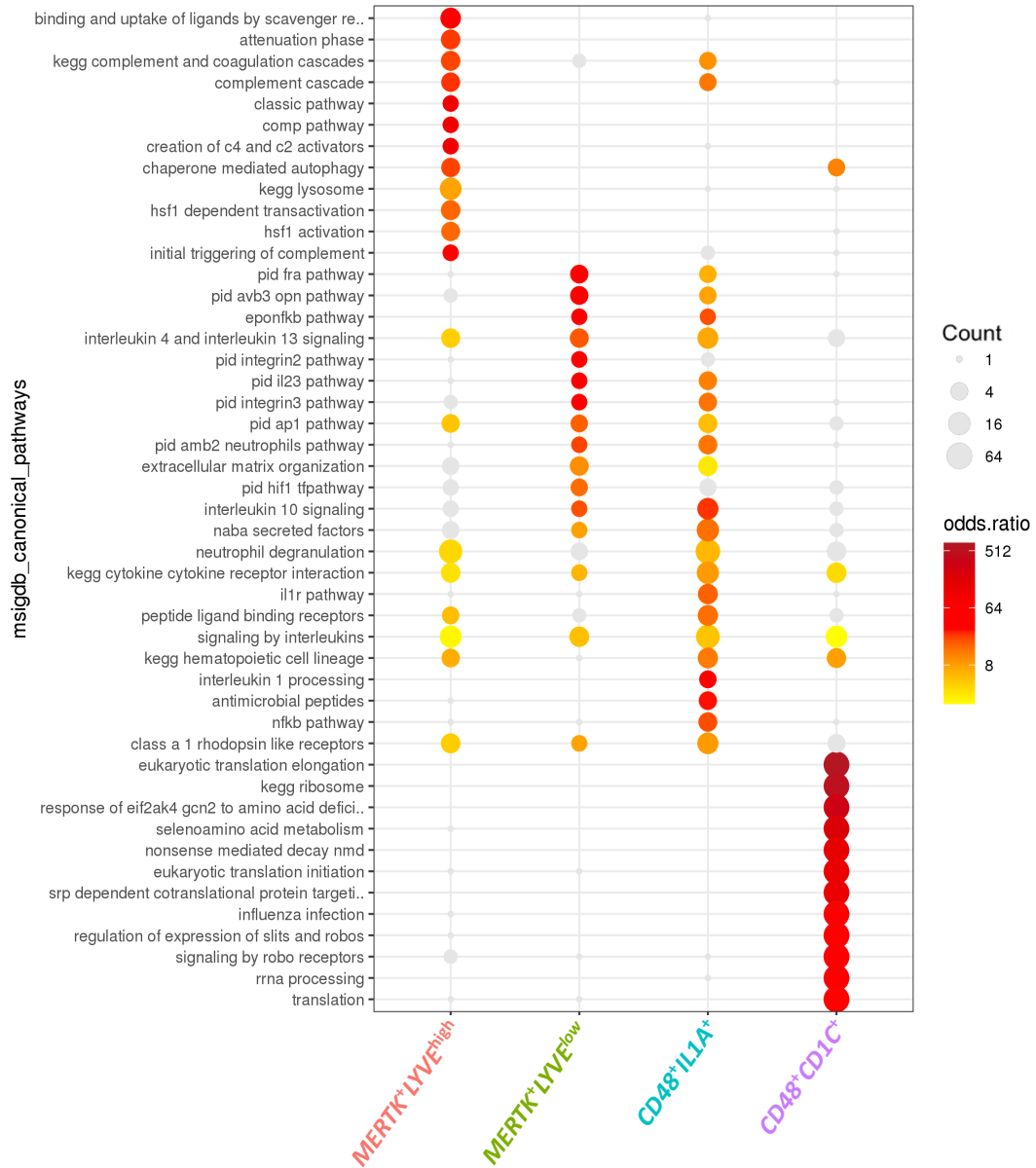


Figure 2.12: Pathway analysis of $CD14^+CD68^+$ myeloid cell subsets (MSigDB). Over-representation analysis reveals the main enriched MSigDB canonical pathways. The dot size is proportional to the the number of genes in the MSigDB category that were found as cluster markers (Count). The intensity of the (red) colour represents the odds ratio.

2.4.5 $CD48^+IL1A^+$ macrophages in frozen shoulder

In frozen shoulder, the proportion of $CD48^+IL1A^+$ macrophages and $CD48^+CD1C^+$ dendritic cells increased relative to comparator capsular tissues, whereas the $MERTK^+$ macrophage populations decreased in proportion (Figure 2.13).

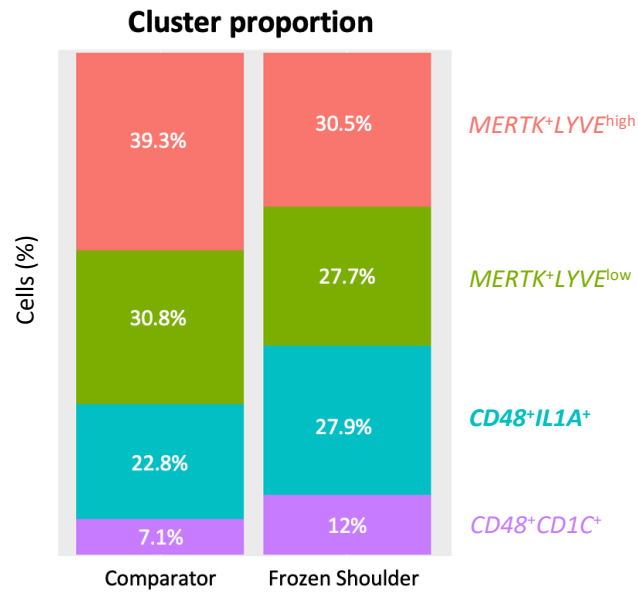


Figure 2.13: Cluster proportions of $CD14^+CD68^+$ myeloid cells across conditions. There is a trend for the proportion of $MERTK^-$ myeloid subsets increasing in frozen shoulder, whereas $MERTK^+$ macrophages decrease in proportion.

Pseudo bulk differential expression analysis was performed to identify differences in gene expression between $CD48^+IL1A^+$ macrophages in frozen shoulder relative to comparator capsular tissues. It was necessary to remove two comparator donor samples due to a low number of cells in the $CD48^+IL1A^+$ macrophage population. Therefore, pseudo bulk differential expression was performed on $n = 4$ comparator and $n = 4$ frozen shoulder capsular tissue samples. There was a clear separation between comparator and the frozen shoulder samples on the first two PCs

(Figure 2.14A). Hierarchical clustering showed high overall correlation between the samples (>0.90) (Figure 2.14B).

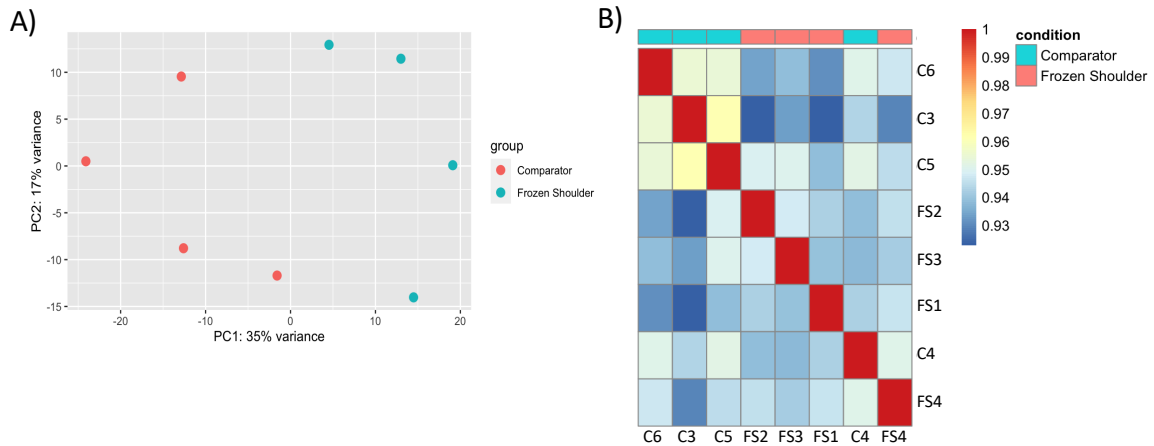


Figure 2.14: Sample level QC: pseudo bulk differential expression analysis.

Pseudo bulk differential expression analysis of $CD48^+IL1A^+$ macrophages. **A)** Projection of comparator ($n = 4$) and frozen shoulder ($n = 4$) samples after dimensionality reduction by PCA. The distribution of variance among the PCs is plotted. **B)** Hierarchical clustering of comparator ($n = 4$) and frozen shoulder ($n = 4$) samples. There is a high overall correlation between samples (>0.90).

The top 20 significantly DEGs included mostly genes that are downregulated in frozen shoulder relative to comparator $CD48^+IL1A^+$ macrophages, including *FN1*, *SPP1*, *MAF*, *ECM1*, *MARCO*, and *TGFBI* (Figure 2.15A). Contrarily, significantly upregulated genes in frozen shoulder $CD48^+IL1A^+$ macrophages (relative to comparator), included *GPAT3*, *TIMP1*, *IL1A*, *ADAM19*, *IL1R1*, *MMP9*, *IL1B*, and *TNFAIP6* (Figure 2.15B&C). Particularly, IL-1 related genes are significantly increased in frozen shoulder $CD48^+IL1A^+$ macrophages. A list of the top 25 significantly down- and upregulated DEGs in frozen shoulder, including their $\log_2FC$ s and p values can be found in Appendices A.15 and A.16.

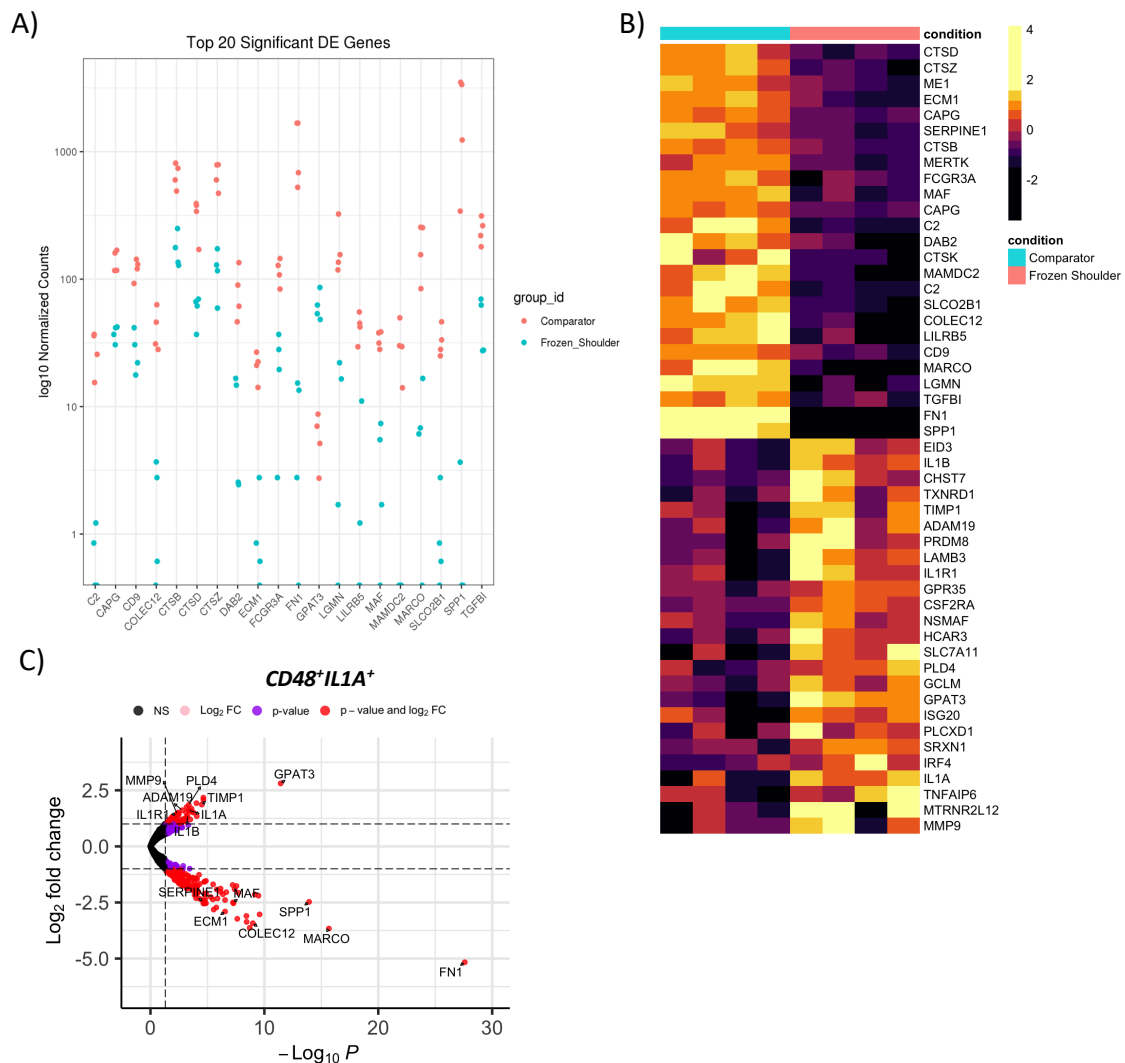


Figure 2.15: **DEGs** in $CD48^+IL1A^+$ macrophages (frozen shoulder vs comparator). **A)** The top 20 significantly **DEGs** plotted by log10 normalised counts across comparator ($n = 4$) and frozen shoulder ($n = 4$) samples. **B)** **RNA** heatmap displaying normalised expression of the top 25 downregulated and the top 25 upregulated genes in $CD48^+IL1A^+$ macrophages in frozen shoulder relative to comparator samples. **C)** Volcano plot displaying the significantly down- (negative **log2FC**; bottom) and upregulated (positive **log2FC**; top) genes of interest of $CD48^+IL1A^+$ macrophages in frozen shoulder relative to comparator samples. P_{adj} set at 0.05 and **log2FC** ≥ 1 in either direction.

2.5 Discussion

The overarching aim of this chapter was to identify distinct cell types expressing inflammatory mediators in frozen shoulder and comparator capsular patient tissues. The first part of this discussion focuses on the single cell atlas of the rotator interval of the **GH** joint capsule and the major cell types present. Secondly, cells expressing inflammatory mediators and specific genes of inflammatory mediators that are significantly upregulated in frozen shoulder relative to comparator tissues are discussed.

2.5.1 The rotator interval of the **GH** joint capsule comprises five major cell types

To my knowledge, this study is the first to generate a single cell atlas comprising all the cell types of the rotator interval of the **GH** joint capsule. **ScRNAseq** revealed five major cell types, with $COL1A1^+PDGFRA^+FAP^+$ fibroblasts being the largest cell population, followed by $CD14^+CD68^+$ myeloid cells, $CD2^+CD3^+$ lymphocytes, $PECAM1^+VWF^+$ endothelial cells, and a small cluster of $ACTA2^+NOTCH3^+$ mural cells. As there are no other **scRNAseq** datasets comprising all the cell types of the **GH** joint capsule, the findings in this chapter (**mRNA** expression) are compared to previous studies using mostly immunohistochemistry (protein expression). Yet, the findings of this chapter are in keeping with previously identified cell types of the rotator interval of the **GH** capsule in existing literature using histology approaches [23, 74, 136, 138, 150, 151, 165, 166, 178–181].

Histological studies often found an abundance of fibroblasts [23, 74, 138, 165, 166, 178–181]. Bunker et al. (1995) reported immuno-positive staining of vimentin,

α smooth-muscle actin (α -SMA), CD45 and CD68 in histological samples of the coracohumeral ligament and the rotator interval capsule, stating there are (proliferating) (myo)fibroblasts and macrophages present [74]. Rodeo et al. (1997) used immunohistochemistry to identify fibroblasts (5B5), synovial cells (CD68), T cells (CD43), and B cells (CD37) in both capsule and synovium [150]. Ryu et al. (2006) reported increased immuno-positive staining of VEGF and CD34 in synovial tissues of diabetic patients with frozen shoulder, suggestive of (vascular) endothelial cells [11]. However, the authors did not look at the GH joint capsule [150]. Hand et al. (2007) found strong immuno-positive staining of vimentin with proliferative cell markers (PC10, MIB1), confirming the dominant cell population to be proliferating fibroblasts [23]. However, very little myofibroblasts (desmin, α -SMA and calponin) were detected [23]. In addition, Hand et al. (2007) also confirmed the presence of CD45⁺ immune cells, including macrophages (CD68), T cells (CD3), B cells (CD20), and mast cells (mast cell tryptase) [23]. Kilian et al. (2007) found increased *COL1A1* (α 1(I)) mRNA expression in frozen shoulder relative to control samples [136]. Histology confirmed the presence of fibroblasts (procollagen I, collagen I) in the rotator interval of frozen shoulder samples, however their expression was decreased relative to control samples [136]. Kanbe et al. (2009) reported CD68 and VEGF positive staining in synovial tissue samples of frozen shoulder, however the authors did not look at the GH joint capsule [151]. Xu et al. (2012) showed fibroblastic proliferation with increased expression of CD34 in frozen shoulder capsular samples relative to control samples [178]. Hagiwara et al. (2012) found mainly fibroblast-like cells in both control and frozen shoulder capsular samples [180]. Hwang et al. (2016) found that capsular tissues from frozen shoulder patients showed fibroblastic proliferation [181]. Hettrich et al. (2016) reported the presence of (myo-)fibroblasts (α -SMA) in

frozen shoulder capsule and synovium, but not in control capsule [179]. Interestingly, capsule sections of patients treated with corticosteroid injections showed a significant decrease of myofibroblasts (α -SMA staining) in both capsule and synovium [179]. The authors also mention positive CD31 staining, however these results were not shown [179]. Yang et al. (2020) reported increased *COL1A1* and *COL3A1* mRNA expression in frozen shoulder fibroblasts compared to control fibroblasts [182].

The findings of this chapter seem to be most in line with the histological findings of Hand et al. (2007): fibroblasts are the dominant cell population and there is a significant presence of immune cells [23]. Likewise, smooth-muscle actin transcript (*ACTA2*) is only found in a small cluster of mural cells (*ACTA2*⁺*NOTCH3*⁺), whereas other histological studies found an abundance of myofibroblasts (α -SMA staining) [74, 179]. The single cell atlas generated in this study also identified myeloid cells (*CD14*⁺*CD68*⁺), lymphocytes (*CD2*⁺*CD3*⁺), and (vascular) endothelial cells (*PECAM1*⁺*VWF*⁺). However, unlike the findings of Hand et al. (2007), no mast cells were found in the single cell atlas generated in this chapter [23]. Besides the fact that mRNA expression does not equal protein expression, differences in findings (e.g., myofibroblasts, mast cells) are possibly explained by several, mostly clinical, reasons.

First, biopsies could have been taken at different stages of frozen shoulder and/or different control samples might have been used. Early-stage frozen shoulder samples might have not developed fibroplasia and/or fibrosis yet. Another explanation, as frozen shoulder is a self-limiting inflammatory fibrotic disease, is that the myofibroblasts in advanced-stage (fibrotic) samples have undergone apoptosis. This is in

line with the findings of Kilian et al. (2007), where advanced-stage frozen shoulder samples showed similar **mRNA** and immunohistochemical staining of collagen III to control samples [136]. This is to the contrary of non-resolving fibrotic disease (e.g., visceral fibrosis), where myofibroblasts seem to develop ways of evading apoptosis and hence fibrosis is sustained [183]. It must also be noted that all the above-mentioned studies have a relatively small sample size with significant biological diversity between the samples (e.g., with or without frozen shoulder related comorbidities). Furthermore, as Hettrich et al. (2016) point out, corticosteroid treatment (and most likely other anti-inflammatory drugs) might influence these results [179]. To add another layer of complexity, the tissue type differs between studies. Studies investigate either **GH** joint capsule, synovium, or both, and sometimes it is not clear. Finally, even if **GH** joint capsule is consistently used across all studies, the localisation of the capsule might also affect the results. For example, Ulthoff and Boileau (2007) found positive vimentin immunostaining at the rotator interval capsule, the coracohumeral ligament, and the axillary fold, but not at the posterosuperior capsule. They concluded that in frozen shoulder therefore is a localised contracture, which mostly affects the anterior **GH** joint capsule [139].

Two additional studies have also confirmed fibroblasts and macrophages in frozen shoulder capsule tissues [165, 166]. Both studies found that fibroblast activation markers (**PDPN**, CD106 (**VCAM**), CD90, fibroblast activation protein (**FAP**), CD248, CD146) were present in samples of frozen shoulder patients [165, 166]. The role of fibroblast activation in disease, as well as a more in-depth analysis of the *COL1A1*⁺ *PDGFRA*⁺ *FAP*⁺ fibroblasts identified in this chapter, will be discussed in Chapter 3. Dakin et al. (2019) also found increased expression of macrophage markers (CD206,

CD163, IRF5) in frozen shoulder capsular tissues relative to shoulder tendon tears [166]. Interestingly, the authors of this study also found that tissues from frozen shoulder patients highly expressed pro-resolving proteins, suggesting that patients with advanced-stage frozen shoulder are on a resolving trajectory [166].

After having identified the cell types present in the rotator interval of the GH joint capsule, the rest of this discussion is focused on the $CD14^+CD68^+$ myeloid cell cluster, as it is expressing most of the inflammatory mediators previously identified in literature [134, 135, 138, 149, 182, 184].

2.5.2 $CD14^+CD68^+$ myeloid cell atlas reveals four distinct myeloid subsets

Clustering of the $CD14^+CD68^+$ myeloid cells revealed four distinct myeloid subpopulations, including two $MERTK^+$ and two $MERTK^-$ myeloid subsets. Three out of four are macrophages: $MERTK^+LYVE1^{high}$ macrophages, $MERTK^+LYVE1^{low}$ macrophages, and $CD48^+IL1A^+$ macrophages. The last subset ($CD48^+CD1C^+$) is a monocyte-derived dendritic cell cluster. The idea of different macrophage subsets is known in literature and is increasingly accepted due to scRNAseq [70, 185, 186].

Looking at the top positive marker genes of each cluster, there is a resemblance between the anti-inflammatory (M2) macrophages and the $MERTK^+LYVE1^{high}$ macrophages, whereas the $CD48^+IL1A^+$ macrophages look more like the traditional pro-inflammatory (M1) macrophages [185]. While the M1/M2 macrophage classification is an oversimplification, scRNAseq did reveal anti-inflammatory and

pro-inflammatory macrophage populations [61]. Moreover, the expression profile of $CD48^+IL1A^+$ macrophages is the exact opposite of $MERTK^+LYVE1^{high}$ macrophages; the top negative markers genes of $CD48^+IL1A^+$ macrophages include *MAF*, *LYVE1*, *DAB2*, and *C1QB* (top positive marker genes of the $MERTK^+LYVE1^{high}$ macrophages) and vice versa. The $MERTK^+LYVE1^{low}$ macrophages seems to be a mixed macrophage cluster, expressing marker genes from both $MERTK^+LYVE1^{high}$ and $CD48^+IL1A^+$ macrophages. Thus, theoretically the $MERTK^+LYVE1^{low}$ macrophages could be polarised towards either $MERTK^+LYVE1^{high}$ or $CD48^+IL1A^+$ macrophages, depending on the exposure of these macrophages to inflammatory and/or anti-inflammatory mediators [61, 185].

The $CD14^+CD68^+$ myeloid subsets found in the rotator interval of the **GH** joint capsule show similarities with the macrophage subsets found in knee synovial tissues of **RA** patients [70]. Aliverni et al. (2020) reported four major subsets: two $MERTK^+$ and two $MERTK^-$ subsets, which the authors further divided into nine smaller clusters [70]. The $MERTK^+LYVE1^{high}$ macrophages described in this chapter are also present in knee synovial tissues of **RA** patients (annotated as $MERTK^+FOLR2^{high}TREM2^-LYVE1^+$) [70]. The authors also identified a $MERTK^-HLA^{high}CD48^+$ dendritic cell subset which shows similar gene expression as the dendritic cell cluster identified in this chapter ($CD48^+CD1C^+$), including *HLA* genes and *CXCR4* [70]. Finally, a $MERTK^-CD48^+S100A12^+$ subset was also identified, highly expressing alarmins and pro-inflammatory mediator genes (*S100A8*, *S100A9*, *S100A12*, *FCN1*) similar to the $CD48^+IL1A^+$ macrophages found in the rotator interval of the **GH** joint capsule [70].

Only one study by Akbar et al. (2021) has used **scRNAseq** on frozen shoulder capsule samples, creating an immune cell atlas ($CD45^+$ cells) [187]. Like the single cell atlas in this study, the authors found both myeloid cells as well as lymphocytes [187]. However, Akbar et al. (2021) found that the macrophage population significantly decreased in frozen shoulder relative to control tissues. In addition, the authors showed that the T cell populations significantly increased, suggesting a T cell driven disease [187]. Akbar et al. (2021) identified five myeloid cell populations in frozen shoulder, including one $LYVE1^+FOLR2^+$ macrophage population and four $CD1C^+$ dendritic cell populations [187]. The $LYVE1^+FOLR2^+$ macrophage population from their study shows great similarity with the $MERTK^+LYVE1^{high}$ macrophages identified in this chapter, as they both express *SELENOP*, *C1QB*, *LGMN*, *C1QC*, *C1QA*, *SLC40A1*, and *RNASE1* [187]. The $CD1C^+IL1R2^+$ dendritic cell population identified by Akbar et al. (2021) is similar to the $CD48^+CD1C^+$ dendritic cell population identified in this chapter as they both express *IL1R2*, *HLA-DQA2*, *AREG*, and *HLA-DQB1* [187]. Furthermore, the inflammatory dendritic cell population ($IL1B^+CXCL8^+$) from their study showed similar expression to the inflammatory $CD48^+IL1A^+$ macrophage population from this chapter (*BCL2A1*, *IL1B*, *TNFAIP6*, *GOS2*) [187]. $CD45^-$ (stromal) cells were not included in their single cell atlas, as the authors mainly focused on the $CD45^+$ cell populations [187].

The differences in myeloid cell populations between the study from Akbar et al. (2021) and the myeloid cell populations identified in this chapter could be explained by several reasons [187]. First, the comparator samples used in this chapter are from patients undergoing either shoulder replacement surgery for **OA** or patients undergoing surgery for a proximal humeral fracture (see Table 2.3). This is

different from the control samples used in the study from Akbar et al. (2021), who use relatively younger patients undergoing arthroscopic stabilisation surgery for GH instability [187]. As mentioned in section 2.5.1, frozen shoulder samples might have been taken at different disease stages, from patients with different comorbidities, and patients who may have had different treatment options prior to surgery. In addition, the single cell atlas generated in this chapter comprises a total of $n = 10$ samples, whereas Akbar et al. (2021) used $n = 20$ capsule samples [187]. Yet, the single cell atlas generated in this chapter consisted of a total of 6,818 cells, whereas the single cell atlas from Akbar et al. (2021) consisted of only 3,347 cells [187]. While both studies have used fresh tissues, differences in digestion protocols may have led to different cell counts and different results. An example of this is the differences between warm-dissociated libraries and cold-dissociated libraries [188]. Finally, differences in read depth and QC metrics could have also contributed to different results. Thus, there many factors that could have influenced both the results of this chapter and the study in question.

2.5.3 $CD48^+IL1A^+$ macrophages express inflammatory mediators and alarmins

The top positive marker genes of $CD48^+IL1A^+$ macrophages found in the rotator interval of the GH joint capsule consist of inflammatory mediators and alarmins, including *EREG*, *IL1B*, *S100A8*, *SERPINB2*, *THBS1*, *S100A12*, *IL1RN*, *CCL20*, *TNFAIP6*, *S100A9*, and *PTGS2*. Most of these inflammatory marker genes expressed by $CD48^+IL1A^+$ macrophages have been identified in literature before, however this is the first study to identify a distinct $CD48^+IL1A^+$ macrophage population which expresses most of them [134, 135, 138, 149, 150, 184]. It is therefore likely that

$CD48^+ IL1A^+$ macrophages might be the key drivers of inflammatory processes in frozen shoulder.

Previous studies have suggested that the underlying pathophysiology of frozen shoulder might be cytokine-mediated [134, 135, 138, 149–152, 180–182, 184, 189–193]. Rodeo et al. (1997) found increased expression of $TGF-\beta$, PDGF, HGF, IL-1 β , and $TNF-\alpha$ in capsule and synovial tissues of frozen shoulder patients [138]. Bunker et al. (2000) reported the presence of *IL1A*, *IL1B*, *IL6*, *TNFA*, and *TNFB* mRNA in frozen shoulder capsule samples [134]. As mentioned in section 2.5.1, Ryu et al. (2006) reported increased immuno-positive staining of VEGF in synovial tissues of diabetic patients with frozen shoulder [150]. Kanbe et al. (2009) confirmed the immuno-positive staining of IL-6, VEGF, NF- κ B in frozen shoulder synovial tissues [151]. However, Ryu et al. (2006) and Kanbe et al. (2009) did not investigate capsular tissues from the rotator interval [150, 151]. Li et al. (2009) reported increased expression of $TGF-\alpha$ protein and mRNA in frozen shoulder capsular tissues compared to control tissues [189]. Kababbe et al. (2010) documented elevated mRNA levels of *IL6* and *CXCL8* (IL-8) in synovial tissue samples from frozen shoulder patients relative to controls, however the authors did not assess the GH joint capsule [135]. Hagiwara et al. (2012) reported increased gene expression of *PDGFB* and *IL1B* in most idiopathic frozen shoulder capsular samples [180]. Lho et al. (2013) found increased mRNA levels of *IL1A*, *IL1B*, *TNFA*, *PTGS1* (COX-1), and *PTGS2* (COX-2) in the GH joint capsule of frozen shoulder patients relative to controls [149]. COX-2 was confirmed with immunohistochemistry in frozen shoulder GH joint capsule [149]. The authors also examined cytokine mRNA levels in the subacromial bursae of patients with frozen shoulder and found increased expression of *IL1A*,

IL6, *TNFA*, and *PTGS2* [149]. In addition, *IL-6* and *TNF- α* were also detected in synovial fluid (enzyme-linked immunosorbent assay (*ELISA*)) of frozen shoulder patients [149]. Kim et al. (2013) reported increased *mRNA* and protein expression of *ICAM1* (CD54) in frozen shoulder capsular tissues compared to control tissues [190]. Hwang et al. (2016) found increased immuno-positive staining of advanced glycation end products (*AGEs*) in frozen shoulder capsular tissue compared to control tissues [181]. Cohen et al. (2016) reported elevated (*mRNA*) expression levels of *TNC* and *FN1* in capsule and synovial tissue samples of the *GH* joint capsule of frozen shoulder patients relative to controls [184]. With (bulk) *RNAseq*, Cui et al. (2017) found a variety of *DEGs* in frozen shoulder relative to control tissues, including increased expression of *IL6* and *AREG* [191]. Cher et al. (2018) reported increased immuno-positive staining of alarmins *HMGB1*, *IL-33*, *S100A9*, and *S100A9* in frozen shoulder capsular tissues relative to control tissues [152]. Akbar et al. (2019) showed that fibroblasts cultured from frozen shoulder capsular tissues produced increased levels of *IL-6*, *IL-8*, and CC chemokine ligand (*CCL*)-20 compared to control fibroblasts [165]. Dakin et al. (2019) found that advanced-stage frozen shoulder tissues showed reduced *mRNA* expression of *TNFA*, *IL6*, and *CXCL8* (*IL-8*) relative to chronic rotator cuff tendon tear tissues, suggesting that these frozen shoulder patients are on a resolving trajectory [166]. Yang et al. (2020) reported increased *mRNA* expression of *TGFB1* and *IL6* in frozen shoulder synovial/capsular fibroblasts relative to control fibroblasts [182]. Transcriptomic analysis by Kamal et al. (2020) revealed an upregulation of *PDGFB* and a downregulation of *TNFA* in frozen shoulder capsular tissues compared to control tissues [192]. Finally, Yano et al. (2020) reported an increased gene expression level of *RAGE*, *HMGB1*, *TLR2*, *TLR4*, and *NFKB* in coracohumeral and inferior *GH* ligaments from frozen shoulder

patients compared to those from rotator cuff patients [193].

This chapter provides a distinct $CD48^+IL1A^+$ macrophage subset which expressed most of the previously identified inflammatory mediators in literature. The role of these macrophages in sustaining inflammation is further emphasised by pathway analyses, which confirmed enrichment for biological processes related to inflammatory processes. While the $CD48^+IL1A^+$ macrophage subset was present in both frozen shoulder and comparator capsule tissue samples, there were some differences between the two conditions (discussed below).

2.5.4 Upregulation of **IL-1** related genes in frozen shoulder $CD48^+IL1A^+$ macrophages

The cluster proportions of $CD14^+CD68^+$ myeloid subsets were compared across conditions. $MERTK^-$ myeloid subsets increase proportionally in frozen shoulder, whereas $MERTK^+$ macrophages decrease in proportion. Statistical analysis was not performed due to a small sample size and relatively low cell count. For differential gene expression analysis across conditions, pseudo bulk expression analysis was performed. Pseudo bulk expression analysis revealed the top significantly **DEGs**, including down- and upregulated marker genes. Downregulated genes in frozen shoulder relative to comparator $CD48^+IL1A^+$ macrophages included: *FN1*, *SPP1*, *MAF*, *ECM1*, *MARCO*, and *TGFBI*. In contrast, significantly upregulated genes in frozen shoulder $CD48^+IL1A^+$ macrophages (relative to comparator) included *GPAT3*, *TIMP1*, *IL1A*, *ADAM19*, *IL1R1*, *MMP9*, *IL1B*, and *TNFAIP6*.

FN1, *SPP1*, *MAF*, *ECM1*, *MARCO*, and *TGFBI* are pro-fibrotic genes that are

usually significantly upregulated in macrophages of chronic, non-resolving fibrotic diseases [103, 194–198]. Interestingly, the findings in this chapter suggest that these pro-fibrotic genes are downregulated in $CD48^+IL1A^+$ macrophages in frozen shoulder, an (inflammatory) fibrotic shoulder condition. These findings should therefore be discussed in consideration of the different phases of frozen shoulder. As most frozen shoulder patients in this study had symptoms for at least 2 years (Table 2.3), it is likely that these patients were on a resolving trajectory (‘thawing’ phase of disease). Downregulation of *FN1*, *SPP1*, *MAF*, *ECM1*, *MARCO*, and *TGFBI* would suggest that the $CD48^+IL1A^+$ macrophages are isolated from frozen shoulder patients who are possibly already in their ‘thawing’ phase, as suggested by Dakin et al. (2019) [166]. This theory is also in line with previous findings of *FN1* (and *TGFB*) expression in frozen shoulder by Cohen et al. (2016), as the patients in this study underwent surgical release of the GH joint capsule with less <1 year of complaints [184]. In addition, Rodeo et al. (1997) also found increased immuno-positive staining of TGF- β in frozen shoulder [138]. Although the duration of symptoms was not specified in this study, it is likely that these patients also underwent surgery at an earlier stage [138]. While *SPP1* (osteopontin), *MAF*, *ECM1*, and *MARCO* have not been previously identified in frozen shoulder, they are extensively investigated in other chronic fibrotic diseases, including liver and cardiac fibrosis [194–197]. In addition, most of these pro-fibrotic genes (and proteins) are known to interact closely with each other, promoting a pro-fibrotic environment in chronic disease [103, 194–198]. For example, the MAF/SPP1-axis has been shown to promote bone marrow fibrosis in patients with primary myelofibrosis [195].

Upregulation of pro-inflammatory genes *IL1A*, *ADAM19*, *IL1R1*, *IL1B*, and *TN-*

FAIP6 in frozen shoulder $CD48^+ IL1A^+$ macrophages support the idea that these macrophages are likely to be involved in driving and/or maintaining an inflammatory micro-environment in frozen shoulder. All these genes are well described in inflammatory fibrotic diseases [199–201]. Despite that these frozen shoulder samples come from patients that are in the later stages of frozen shoulder, these macrophages seem to maintain an inflammatory phenotype. In addition, previous frozen shoulder studies identifying inflammatory mediators consistently found an upregulation of *IL1A* and *IL1B* [134, 138, 149].

Upregulation of matrix associated genes *TIMP1* and *MMP9* are suggestive of an increased **ECM** turnover in frozen shoulder. This is in keeping with the existing literature on frozen shoulder [134, 140, 202]. In addition to the role of **MMPs** and their natural inhibitors **TIMPs** in **ECM** degradation and turnover, recent studies have also suggested that **MMPs** are implicated in inflammation in a variety of diseases, including liver, lung, and kidney disease, as well as other joint diseases such as **RA** and **OA** [142, 144, 145].

GPAT3 was the most significant upregulated gene in frozen shoulder $CD48^+ IL1A^+$ macrophages. Whilst not reported in frozen shoulder patient tissue samples, *GPAT3* has been described to play a role in adipogenesis via insulin-stimulated phosphorylation [203]. *GPAT* genes in general have been studied in metabolic diseases such as diabetes mellitus [203]. This may be upregulated as two out of four frozen shoulder patients had diabetes mellitus (Table 2.3).

While there are discrepancies in expression of other inflammatory and fibrotic

mediators across studies (and likely frozen shoulder stages), increased *IL1A* and *IL1B* in frozen shoulder patient tissues was observed. This suggests that the ongoing inflammation in frozen shoulder might be IL-1-axis driven. Therefore, these inflammatory mediators are further explored in Chapters 3 and 4.

2.5.5 Limitations

This chapter has several limitations which need to be taken into consideration when interpreting the results. Both clinical and technical challenges, as well as limitations are discussed below.

Clinical limitations and challenges

Frozen shoulder research inherently comes with some clinical limitations. The clinical course of frozen shoulder has three phases: an inflammatory (freezing) phase, a fibrotic (frozen) phase, and finally a resolving (thawing) phase (Chapter 1, section 1.3) [78]. Therefore, differences in findings are expected when comparing samples across and within studies. The capsular tissue samples used in this study are collected from patients with advanced-stage frozen shoulder. It would be of significant interest and value to study capsular tissue samples collected from patients with early-stage frozen shoulder, particularly when investigating the role of inflammatory mediators. There are however significant challenges that precluded me from being able to collect tissue from patients with early-stage frozen shoulder. First, a practical challenge: the vast majority of frozen shoulder cases are managed by the patient and with primary care. In these settings there is little or no opportunity for invasive biopsies to be performed. Second, an ethical challenge: discussions with ethicists and with our ethical committee led to the conclusion that obtaining a biopsy at the time of surgery was

the only option likely to receive ethical approval. This approach is what eventually was approved. Inevitably these patients are in the later stages of frozen shoulder. Thus, since I am dependent on acquiring capsular tissues from frozen shoulder patients undergoing a capsular release procedure (as per usual care), I am limited to analysing samples collected from advanced-stage frozen shoulder patients. For these reasons, this study also has a relatively small sample size ($n = 10$), which could have underpowered the analyses. The way forward is to further develop strategies of miniaturising biopsy techniques. This would eventually provide an opportunity to compare samples of frozen shoulder from different disease stages.

Another factor that is likely to affect the analysis of frozen shoulder capsular tissues is comorbidity. It is well documented that frozen shoulder is associated with a variety of comorbidities, including diabetes mellitus and thyroid disease. In addition, it is documented that anti-inflammatory drugs such as corticosteroid injections affect the patient tissue samples [179, 204]. As all patients had corticosteroid injections as part of their usual care pathway, only the inclusion of patients with injections given at least 3 months prior to surgery was allowed. Therefore, previous treatments and comorbidities of included patients have been recorded. Corticosteroid injections have shown to be mainly effective in pain relief and improving short-term mobility in frozen shoulder [98–100]. These effects, however, are not maintained in the long term, and moreover they do not appear to affect the duration of disease [104]. Given their short-term effect, these changes are likely not permanent. Three months post-injection was considered sufficient not to alter the mRNA expression significantly. Patients with previous surgery were sufficiently excluded as per the exclusion criteria (Table 2.2).

Finally, the comparator group is also an important clinical limitation. While patients with OA or a proximal humeral fracture do not have frozen shoulder, their GH joint capsule will also have undergone changes [205]. Early scRNAseq pilot runs with samples from patients with proximal humeral fracture <3 weeks post-trauma showed significant inflammation and did not match the other comparator samples. Another possible comparator group is the younger GH instability group undergoing arthroscopic stabilisation. As these biopsies were often not sufficient for scRNAseq due the relatively small biopsies, this group was not used for scRNAseq, but for other analyses (Chapters 3 and 4). For these reasons, comparator tissues are not referred to as control but rather as comparator, as control often insinuates that these are healthy tissues. However, as the same challenges arise when trying to collect capsular tissue samples from these patient groups as from frozen shoulder patients, I am again dependent on the usual care pathway of OA, proximal humeral fractures, and GH instability.

Technical challenges and limitations

Sequencing of the transcriptome of individual cells (scRNAseq) has almost become the gold standard when investigating cellular heterogeneity of certain tissues. ScRNAseq enables measurement of transcriptome-wide gene expression at single cell resolution, allowing for multiple cell clusters to be identified and distinguished from one another. In addition, scRNAseq allows for identification of rare cell types, regulatory networks, developmental trajectories, cross-condition comparisons, and more. While the opportunities that arise from scRNAseq seem infinite, there are some limitations that are worth considering when interpreting the results from this chapter.

First, there are some general limitations when working with **scRNAseq**. The lack of a reference atlas, especially as this is one of the first single cell atlases of the rotator interval of the **GH** joint capsule, leads to unsupervised clustering approaches which involves manual cluster annotation. While time-consuming it also makes the reproducibility of results limited. Reference cell atlases could fix this; however, this is still in development (e.g., Human Cell Atlas) [206]. To overcome this, I have used a combination of the top marker genes per cluster, a list of literature selected markers, and SingleR (an automated annotation package) for the single cell atlas of the rotator interval of the **GH** capsule. While immune cell annotation with SingleR is often accurate, it can sometimes misclassify certain cells (like fibroblasts in this case, Appendix A.3) [171]. The SingleR developers therefore stress that manual intervention is still necessary. As most of the clusters expressed marker genes that were known in literature and some clusters had already been identified in other (joint) diseases (e.g., macrophage clusters in **RA**), the clustering is satisfactory.

Sequencing of capsular tissues resulted in relatively low cell counts compared to other **scRNAseq** datasets of other human tissue and/or organs [207, 208]. This single cell atlas generated a total of 6,818 cells (from $n = 10$ samples). A reason for this might be that **GH** joint capsule is a matrix heavy tissue which makes it more difficult to isolate cells from whole tissue. Creating single cell suspensions from solid tissues needs optimal dissociation protocols to reach a balance between obtaining cells that are difficult to isolate and damaging fragile cells. Literature states that for these tissues single nuclei RNA sequencing (**snRNAseq**) might be preferable, as this technique minimises the dissociation-induced, artefactual transcriptional stress responses and/or **RNA** degradation that is encountered when creating a single cell

suspension [188]. However, it has been documented that **snRNAseq** results in an under-representation of immune cells when compared to **scRNAseq** [188]. Additionally, only nuclear transcripts will be included for analysis, leaving out cytoplasmic **RNA** and potentially important information. For these reasons, **scRNAseq** was used for generating a single cell atlas of the rotator interval of the **GH** joint capsule, as it was hypothesised that immune cell clusters express inflammatory mediators in frozen shoulder.

In addition to the different sequencing techniques, dissociation protocols can also significantly affect the quality of cells [188, 209, 210]. The capsular tissue samples in this study have been digested at 37°C (warm-dissociated library). Recent studies have reported that warm-dissociated libraries show increased stress responses when compared to cold-dissociated libraries [188, 209, 210]. The reason for this is that the transcriptional machinery of mammalian cells is active at body temperature, leaving room for alterations in gene expression as a result of dissociation [210]. This could have caused more cells from the rotator interval capsule to be filtered out during **QC** due to high stress responses and/or increased mitochondrial expression. Both warm- and cold-dissociated libraries report depletion of certain cell types depending on their sensitivity to either cold- or warm-dissociation [188]. This may have led to an under-representation of certain cell types and/or missing cell types in the rotator interval single cell atlas. Both dissociation methods should be used in the future to analyse the differences in cell clusters in the rotator interval.

In this study, freshly acquired capsular tissue samples were processed and digested, and only viable cells were sorted for **scRNAseq** (section 2.3.3). While this comes

with certain logistical challenges and the inability to run all samples at once to decrease batch effects, it does have the preference above cryopreserved and methanol fixed tissues. Loss of epithelial cells and stress responses have been reported in cryopreserved samples [188]. Methanol fixation damages cells, leading to contaminating single cell droplets with ambient RNA [188].

Differential gene expression analysis was performed with pseudo bulk expression analysis. This is because scRNAseq treats each cell as a separate sample. Single cells within one sample are dependent on each other as they are isolated from the same donor. Differential gene expression analysis across conditions while using each cell as a replicate would cause a significant inflation of p values. Hence, it is necessary to aggregate cells within each donor sample (replicate). This can be done with pseudo bulk expression analysis. Unfortunately, two comparator samples had to be excluded due to low cell count, resulting in an $n = 8$ of capsular tissues for pseudo bulk expression analysis. Yet, there were sufficient samples to detect significantly down- and upregulated genes in frozen shoulder relative to comparator capsular tissues. Moreover, the results were in keeping with previous findings in literature (e.g., increased *IL1A* and *IL1B* expression in frozen shoulder).

Finally, the transcriptome does not necessarily translate to protein expression, particularly when dealing with cytokines that are regulated post-translationally. ScRNAseq also fails to provide spatial information on the location of these cells and pro-inflammatory mediators in the tissues. Therefore, the cell clusters and pro-inflammatory mediators identified with scRNAseq still require validation via histology and other methods. This will be addressed in the next chapters.

2.6 Conclusion

As the first study of its kind generating a comprehensive single cell atlas of the rotator interval of the GH joint capsule, scRNAseq revealed five major cell types: $COL1A1^+PDGFRA^+FAP^+$ fibroblasts, $CD14^+CD68^+$ myeloid cells, $CD2^+CD3^+$ lymphocytes, $PECAM1^+VWF^+$ endothelial cells, and $ACTA2^+NOTCH3^+$ mural cells. Additional clustering of the $CD14^+CD68^+$ myeloid cells, revealed four myeloid subsets, including $MERTK^+LYVE1^{high}$ macrophages, $MERTK^+LYVE1^{low}$ macrophages, $CD48^+IL1A^+$ macrophages, and $CD48^+CD1C^+$ dendritic cells. The $CD48^+IL1A^+$ macrophages highly expressed a variety of inflammatory mediators, most of which were previously identified in frozen shoulder literature.

This chapter provides novel insight into the cellular basis of frozen shoulder, revealing a distinct $CD48^+IL1A^+$ macrophage subset enriched for inflammation resident within the rotator interval of the GH joint capsule. In frozen shoulder this subset shows upregulation of $IL1A$ and $IL1B$. These findings suggest a possible role for $CD48^+IL1A^+$ macrophages in sustaining inflammatory processes in advanced-stage frozen shoulder. Based on the findings in this chapter, chronic inflammation in frozen shoulder might be IL-1-axis driven.

Key findings Chapter 2

- The rotator interval of the **GH** joint capsule consists of $COL1A1^+PDGFRA^+$ FAP^+ fibroblasts, $CD14^+CD68^+$ myeloid cells, $CD2^+CD3^+$ lymphocytes, $PECAM1^+VWF^+$ endothelial cells, and $ACTA2^+NOTCH3^+$ mural cells.
- There are four $CD14^+CD68^+$ myeloid subsets present in the rotator interval of the **GH** joint capsule, including $MERTK^+LYVE1^{high}$ macrophages, $MERTK^+LYVE1^{low}$ macrophages, $CD48^+IL1A^+$ macrophages, and $CD48^+CD1C^+$ dendritic cells.
- $CD48^+IL1A^+$ macrophages in the rotator interval of the **GH** joint capsule highly express inflammatory mediators and alarmins, including *EREG*, *IL1B*, *S100A8*, *SERPINB2*, *THBS1*, *S100A12*, *IL1RN*, *CCL20*, *TNFAIP6*, *S100A9*, and *PTGS2*.
- $CD48^+IL1A^+$ macrophages are enriched for biological processes related to inflammatory processes, including leukocyte chemotaxis, myeloid leukocyte migration, humoral immune response, and response to lipopolysaccharide.
- In frozen shoulder, $CD48^+IL1A^+$ macrophages show increased expression of several pro-inflammatory genes, including **IL**-1 genes (*IL1A*, *IL1B*, *IL1R1*, *IL1RN*).

CHAPTER 3

Spatially mapping inflammatory mediators
to GH capsular tissues and investigating
macrophage-fibroblast crosstalk

3.1 Introduction

Evidence supports the role of inflammatory mediators in the pathophysiology of frozen shoulder [134, 135, 138, 149, 151, 152, 180, 182, 184, 189, 191–193]. Macrophages are known to release inflammatory mediators and regulate inflammation and fibrosis [55, 70, 211]. The previous chapter described an inflammatory $CD48^+IL1A^+$ macrophage population, which could potentially drive inflammation in frozen shoulder. Having identified this inflammatory macrophage population, this chapter seeks to identify the micro-anatomical niche in which these cells reside and neighbouring cells that may be involved in crosstalk that could sustain inflammation in frozen shoulder.

In RA and other arthritic joint diseases, studies have reported an increase of macrophages and inflammatory mediators in the lining layer of synovial tissues [70, 212–216]. Inflammatory macrophages can activate fibroblasts, after which they adopt an inflammatory phenotype, aiding the persistence of inflammation in diseases like RA [41, 42, 70, 211]. Fibroblast activation markers have also been identified in frozen shoulder [165, 166]. It is therefore likely that in frozen shoulder, macrophages are communicating with fibroblasts, driving inflammatory fibrotic processes.

The findings of Chapter 2 revealed a $CD48^+IL1A^+$ macrophage population enriched for inflammation localised to the rotator interval of the GH joint capsule. Since the expression of *IL1A*, *IL1B*, and *IL1R1* was significantly increased in $CD48^+IL1A^+$ macrophages from frozen shoulder patients, it raises the question whether the ongoing inflammation in frozen shoulder might be IL-1 mediated.

3.2 Objectives and hypotheses

This chapter has three objectives. The first objective is to identify the micro-anatomical niche in which the cells expressing inflammatory mediators reside. The second objective is to confirm the protein expression of important inflammatory mediators and their cognate receptors. This will be accomplished by using immunohistochemistry on well-phenotyped patient capsular tissues. The third objective is to identify cellular interactions between the $CD48^+ IL1A^+$ macrophage and fibroblast cell populations. For this, the single cell atlas generated in Chapter 2 will be computationally interrogated to identify fibroblast subsets and important ligand-receptor pairs across macrophage and fibroblast populations. Specifically, the IL-1-axis will be investigated as it might be one of the key pathways active in frozen shoulder as suggested in Chapter 2.

The first hypothesis is that protein expression of the previously identified inflammatory mediators and their cognate receptors are present in GH joint capsular tissues, and that their expression is increased in frozen shoulder samples relative to comparator samples. Secondly, it is hypothesised that the IL-1/IL-1R axis is a key inflammatory pathway between macrophages and fibroblasts in the GH capsule.

3.3 Materials and methods

3.3.1 Ethics statement and tissue collection

Capsular tissues from the rotator interval of the **GH** joint capsule used in this chapter were collected under the same ethical approval described in Chapter 2, section 2.3.1. Tissue biopsies were collected in line with the protocols described in Chapter 2, section 2.3.2. In addition to capsular tissue samples from patients undergoing joint replacement surgery for **OA** (**OMB** ethics), patients undergoing surgery for a proximal humeral fracture (**ICECAP** group B) and patients with shoulder instability (**ICECAP** group A) were also used as comparator groups for histological analyses in this chapter. These groups were compared to the disease group, i.e., patients with frozen shoulder (**ICECAP** group C).

3.3.2 Sectioning of **GH** joint capsule tissues for histology

Samples of capsular tissue were immersed in 10% buffered formalin for a minimum of 48 hours. After formalin fixation, shoulder capsule samples were processed in a tissue processor (Leica ASP300S tissue processor) and finally embedded in paraffin wax. Sections were cut using a rotary RM2135 microtome (Leica Microsystems Ltd) and baked onto adhesive glass slides at 60°C for 30 minutes and 37°C for 60 minutes.

3.3.3 Haematoxylin & eosin (**H&E**) staining

First, the tissue slides were heated for approximately 10 minutes on a hot plate for the wax to melt. The slides were put in xylene for 20-30 minutes to de-paraffinise, in 100% ethanol for 10 minutes, and subsequently in 70% ethanol for 5 minutes.

Continuing, the slides were rinsed with distilled water and were placed in distilled water to stay hydrated. To stain with **H&E**, Gills III haematoxylin was poured on top of the distilled water and the slides were left in for 3 minutes. The slides were then placed in a Coplin jar and were washed with running tap water to increase the staining intensity for 3-5 minutes. Next, alcohol eosin was poured on top of the slides and the slides were left for 40 seconds, after which they were rinsed again with distilled water. Following the rinse, the slides were put in 70% ethanol (for 5 minutes), 100% ethanol (twice for 5 minutes), and finally xylene (for 5 minutes) again. The slides were mounted using mounting medium (DPX Mounting Medium, for Microscopy, Fischer) and were left to set for 24 hours until ready for imaging.

3.3.4 Immunohistochemistry for markers of inflammation

For single immunostaining, tissue slides were first baked in an oven at 60°C for 60 minutes. Antigen/target retrieval was performed by means of heat-induced epitope retrieval with an automated Dako PT Link machine (Dako, Glostrup, Denmark). Tissue slides were then placed in the Dako PT links for 90 minutes. Afterwards, the slides were put into a wash buffer solution for a minimum of 5 minutes. Immunostaining was performed according to a previously published protocol [217]. The next step included antibody staining using the EnVision FLEX visualisation system with an autostainer (Autostainer Link 48 (Dako)). Before immunostaining, the Dako reagents were prepared for the autostainer according to the protocols provided by the manufacturer. Diaminobenzidine (**DAB**) substrate chromogen (Dako) was used to visualise the antibody binding, whereas nuclei were counterstained with haematoxylin. The primary antibodies were diluted in Antibody diluent (Dako) at predefined required concentrations (Table 3.1). Continuing, the slides were placed

onto the autostainer. After staining, the slides were placed in distilled water. The slides were then consecutively taken through industrial methylated spirits (**IMS**) twice (3 minutes each) and through xylene twice (5 minutes each). Finally, the slides were mounted using mounting medium (DPX Mounting Medium, for Microscopy, Fischer Chemical) and were left to set for 24 hours until ready for imaging.

Table 3.1: Primary antibodies used for immunohistochemistry.

Antibody	Dilution	Species	Isotype	Company	Catalogue no.
HMGB1	1/200	Mouse	IgG2b	abcam	ab190377
S100A8	1/1000	Rabbit	IgG	abcam	ab180735
S100A9	1/150	Rabbit	IgG	abcam	ab92507
IL-1α	1/300	Rabbit	IgG	abcam	ab227482
IL-1β	1/200	Mouse	IgG1	abcam	ab156791
IL-33	1/100	Rabbit	IgG	abcam	ab187060
IL-1R1	1/200	Rabbit	IgG	abcam	ab190078
IL-1RA	1/200	Rabbit	IgG	abcam	ab175392
TLR-2	1/250	Rabbit	IgG	abcam	ab209216
TLR-4	1/200	Mouse	IgG2b	abcam	ab22048
CXCR-4	1/200	Rabbit	IgG	abcam	ab181020
CD48	1/200	Rabbit	IgG	biorbyt	orb432138
CD68	1/200	Mouse	IgG1	abcam	ab201340
CD90	1/200	Mouse	IgG1	abcam	ab181469
CD163	1/150	Mouse	IgG2a	LS-Bio	LSB10966
COX-2	1/200	Mouse	IgG2a	LS-Bio	LSC339544
PDPN	1/200	Mouse	IgG1	abcam	ab10288

3.3.5 Multiplex immunofluorescence for co-localisation of inflammatory markers

First, the tissue slides were baked at 60°C for 60 minutes for the first stage of antigen retrieval. Further deparaffinisation and antigen/target retrieval was performed as described above (section 3.3.4). Immunofluorescence (multiplex staining) was per-

formed according to a previously published protocol [217]. After antigen retrieval, a hydrophobic barrier was drawn around the section of each slide with an ImmEdge pen. This way reagents were kept localised onto the tissue sections. A mixture of phosphate-buffered saline (PBS) and 5% normal goat serum were added to the sections for blocking purposes (in a humid chamber for approximately 20-30 minutes). Next, a cocktail of primary antibodies was made (Table 3.1). This cocktail was applied onto the slides, after which the slides were kept in a humid chamber for 2 hours. Then, the slides were rinsed 3 times with PBS Tween (PBS-T) for 5 minutes. A second cocktail, consisting of 300 μ l of 5% normal equine serum and secondary antibodies (Table 3.2), was administered onto the slides for another 2 hours. After rinsing the slides again with PBS-T (three times), the sections were incubated with POPO-1 (Life Technologies, P3580) for nuclear co-staining (20 minutes). A concentration of 1 μ M POPO-1 in 1ml of 0.05% Saponin in PBS (Sigma) was used. Saponin increases the nuclear permeability, resulting in improved nuclear counterstaining. Following additional rinsing (3 times) with PBS-T, the sections were incubated with a solution containing Sudan Black B, after which the sections had to be rinsed again with PBS-T. Finally, the slides were mounted with Vectashield soft mounting medium (H1000). Edges of the coverslip were sealed with clear nail varnish and the slides were stored in a dark box at 4°C until imaging.

Table 3.2: Secondary antibodies used for immunofluorescence.

Antibody	Dilution	Brand
Alexa Fluor Goat anti-Rabbit IgG	1/200	Life Technologies
Alexa Fluor 568 Goat anti-Mouse IgG2a	1/200	Life Technologies
Alexa Fluor Goat anti-Mouse IgG2b	1/200	Life Technologies
Goat anti-Mouse IgG1	1/200	Southern Biotech

3.3.6 RNAscope in situ hybridisation (ISH) protocol

To further visualise the spatial proximity of cells expressing *IL1A* and *IL1B*, RNAscope ISH was performed on capsular tissue sections as described in the manufacturer protocol (RNAscope 2.5 HD Detection Reagent – RED User Manual) [218]. All the reagents used in this protocol were provided by the manufacturer (Table 3.3).

Table 3.3: RNAscope 2.5 HD Reagent Kit – RED and *IL1A/B* Probes

Preatreatment Reagents (Catalogue no. 322300 and 32200)
- RNAscope Hydrogen Peroxide
- RNAscope Protease Plus
- RNAscope Target Retrieval (10X)
RNAscope 2.5 HD Detection Reagents - RED (Catalogue no. 322360)
- RNAscope 2.5 AMP 1
- RNAscope 2.5 AMP 2
- RNAscope 2.5 AMP 3
- RNAscope 2.5 AMP 4
- RNAscope 2.5 AMP 5-RED
- RNAscope 2.5 AMP 6-RED
- RNAscope 2.5 Fast RED-A
- RNAscope 2.5 Fast RED-B
RNAscope Wash Buffer (50X) (Catalogue no. 310091)
RNAscope Probe – Hs-IL1A (Catalogue no. 556791)
RNAscope Probe – Hs-IL1B (Catalogue no. 310361)

Sample pre-treatment

Tissue slides were first baked in an oven at 60°C for 60 minutes. For deparaffinisation, the slides were consecutively incubated in xylene twice (5 minutes each) and in 100% ethanol twice (3 minutes each), after which they were dried for 5 minutes at room temperature. A hydrophobic barrier was drawn around the section of each slide with an ImmEdge pen. Before running the assay, the tissue sections were pre-treated with 5-8 drops of hydrogen peroxide (RNAscope) for 10 minutes. The slides

were then washed three times with distilled water. The second pre-treatment consisted of submerging the slides in boiling RNAscope 10X Target Retrieval solution at 100-104°C for 30 minutes, after which they were washed with distilled water three times. For the last pre-treatment step the slides were treated with 5 drops of RNAscope Protease Plus solution and were put in a humidity tray. The slides were then incubated for 30-60 minutes at 40°C in an oven (HybEZ, Advanced Cell Diagnostics). After flicking the slides to remove the Protease Plus solution, the slides were submerged in distilled water and were washed several times.

Probe hybridisation and RNAscope assay

The RNAscope assay consisted of several steps of hybridisation and signal development as described in the manufacturer protocol (Figure 3.1) [218]. Each step was followed by washing the slides with Wash Buffer (RNAscope) for 2 minutes (twice). Once these steps were completed, signal detection was performed by adding RED solution onto each slide. The RED solution was made up by using a 1:60 ratio of Fast RED-B to Fast RED-A (RNAscope). The slides were incubated in a humidity tray for 10 minutes at room temperature. After incubation, the slides were put in a staining dish with fresh tap water. Once washed with fresh tap water, the slides were put into a staining dish containing 50% haematoxylin for 2 minutes. The slides were then transferred into a staining dish with tap water and were washed with running tap water to increase the staining intensity for 3-5 minutes. The tap water was then replaced with 0.02% ammonia water for 2-3 minutes. Finally, the ammonia water was replaced with tap water and slides were washed for 3-5 minutes.

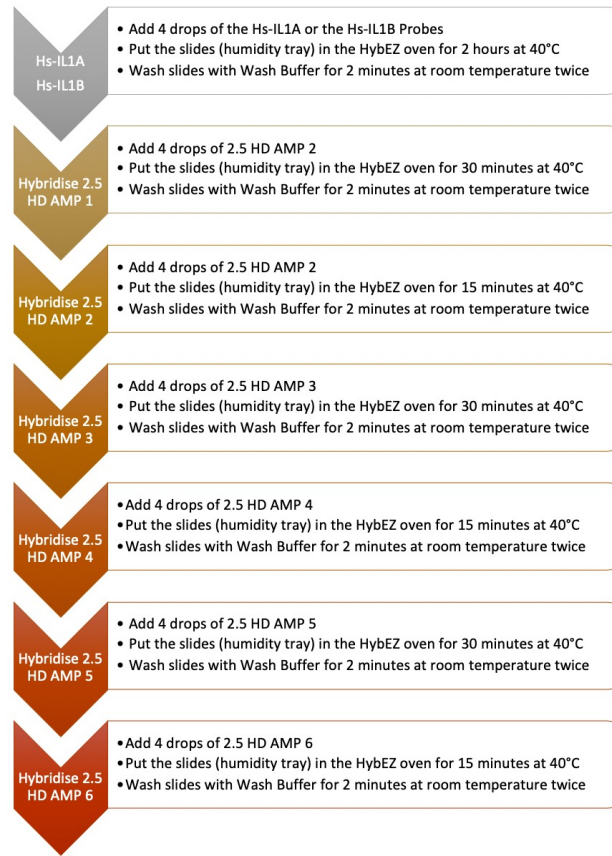


Figure 3.1: Schematic overview of the RNAscope assay.

The slides are treated with the probes provided by the manufacturer (RNAscope). After each step the slides are washed with Wash Buffer for 2 minutes at room temperature while applying frequent agitation. The wash step is then repeated with fresh Wash Buffer. The slides are kept in a humidity tray while in the HybEZ oven.

Mounting and imaging of slides

Once counterstained, the slides were dried at 60°C in a dry oven for at least 15 minutes (until the slides were completely dried). The slides were then dipped into fresh xylene, after which they were mounted with 1-2 drops of EcoMount and coverslips. The slides were left to set for 24 hours until ready for imaging.

3.3.7 Imaging

DAB-stained immunohistochemistry and RNAscope images were acquired on a Motic slide scanner (MoticEasyScan One) using the Motic EasyScanner software at 40x magnification. Multiplex immunostaining images were acquired using a confocal microscope (Zeiss LSM 710) at 40x magnification (immersed with oil). Immunofluorescence images were created using the ZEN 2009 (Zeiss) software. Scale bars were included in all images.

3.3.8 Semi-quantitative image analysis of immuno-positive staining

Semi-quantitative image analysis for single immunostaining images was conducted using a validated ImageJ (National Institutes of Health, version 2.3.0) algorithm to quantify the percentage of **DAB** (nuclei) staining and antibody staining present in each image as described by Franklin et al. (2014) [219–221]. Staining intensity was then calculated by normalising the area of immuno-positive staining to the number of nuclei within the field of view. Statistical analyses were performed with GraphPad Prism (GraphPad Software, version 9.4.0). The Pairwise Mann Whitney U test was used to detect statistically significant differences in immuno-positive staining, with a p value of <0.05 being statistically significant.

3.3.9 Fibroblast single cell atlas and pathway analysis

The single cell atlas of the rotator interval of the **GH** joint capsule was generated in Chapter 2. To explore cellular communication between the $CD48^+IL1A^+$ macrophage subset and fibroblasts identified in Chapter 2, the fibroblast cluster was

computationally isolated and re-analysed through iterating the same pre-processing workflow described in Chapter 2, sections 2.3.6 to 2.3.8. Pathway (enrichment) analysis was performed using the GO database as previously described in Chapter 2, section 2.3.10.

3.3.10 Macrophage-fibroblast ligand-receptor interaction analysis

Important ligand-receptor interactions between macrophages and fibroblasts were identified using NicheNet in R [222]. To investigate macrophage-fibroblast cross talk, NicheNet allowed for the prediction of ligands from the $CD48^+IL1A^+$ macrophages ('sender') which are most likely to affect target gene expression in the fibroblast population ('receiver'), and vice versa. Furthermore, it predicts which specific (fibroblast) target genes are affected by $CD48^+IL1A^+$ predicted ligands. NicheNet's ligand-receptor network and weighted integrated networks were loaded into R. Only genes that were expressed in at least 10% of the cells in one cluster were considered for analysis. 'Frozen shoulder' was set as the condition of interest, whereas 'Comparator' was set as the reference condition. Differential expression between the conditions was calculated with the standard Seurat Wilcoxon test. The ligands were ranked based on their activity using the Pearson correlation coefficient, i.e., based on the presence of their target genes in the 'receiver' population. After, inferring receptors and top-predicted target genes of the ligands of interest, only bona fide ligand-receptor interactions documented in literature (and publicly available databases) were selected. Finally, log fold changes were added to ligands from 'sender' cells to check for up- and downregulation in frozen shoulder versus comparator conditions.

3.3.11 Identifying cell types communicating via the **IL**-1-axis

Network Analysis Toolkit for the Multicellular Interactions (**NATMI**) was used to identify the cell types which are predicted to communicate via the **IL**-1-axis, as it allows for user-supplied ligand-receptor interaction analysis [223]. Using the connectomeDB2020 ligand-receptor pair list, interactions between the $CD48^+IL1A^+$ macrophages and fibroblast clusters were then predicted based on the expression of **IL**-1-related ligands in $CD48^+IL1A^+$ macrophages and the expression of their cognate receptors in fibroblasts. The genes of interest were *IL1A*, *IL1B*, *IL1R1*, *IL1R2*, and *IL1RAP*. A heatmap was then generated to visualise the results.

3.4 Results

3.4.1 Histology of the rotator interval of the GH joint capsule

Histology of the rotator interval of the GH joint capsule reveals a thin lining layer (Figure 3.2A). In comparator patient samples, the lining layer is a few cell layers thick, and the sub-lining area has a low cellularity. In frozen shoulder, the lining layer is expanded, and the sub-lining shows significantly increased cellularity and vascularity (Figure 3.2B).

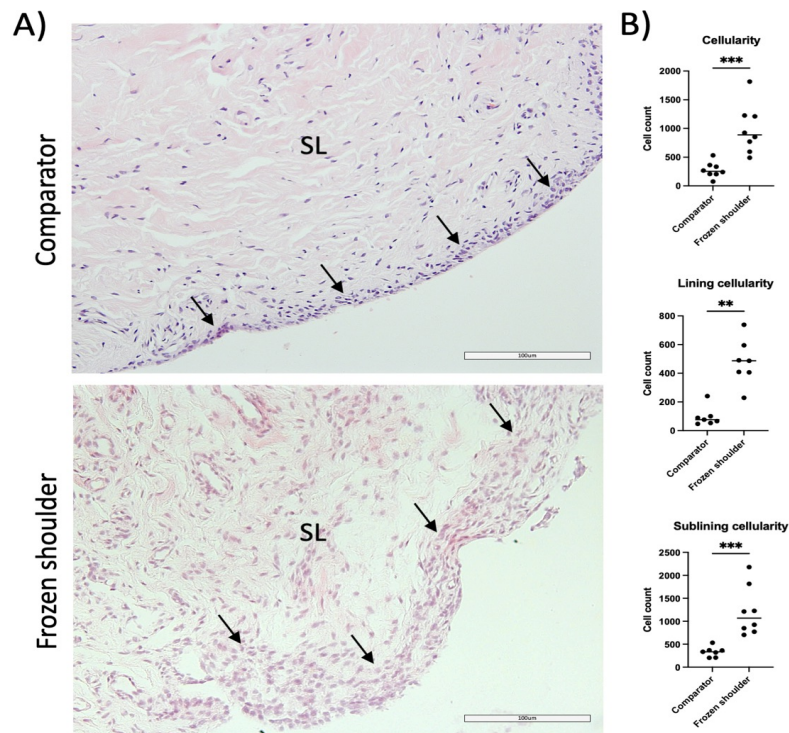


Figure 3.2: H&E staining of the rotator interval of the GH joint capsule.

A) Up = comparator. Down = frozen shoulder. Frozen shoulder tissue shows increased cellularity and vascularity with an expansion of the lining layer. Arrows = lining layer. SL = sub-lining. Haematoxylin was used as nuclear counterstaining. Scale bar = 100µm (white). B) Semi-quantitative image analysis of cellularity in comparator and frozen shoulder capsular tissue. Bar shows median values. Every dot represents a unique donor. Statistically significant differences were calculated using the Mann Whitney U test. ** $p < 0.01$, *** $p < 0.001$.

3.4.2 Spatially mapping inflammatory mediators onto the rotator interval capsule

Immunohistochemistry of both comparator and frozen shoulder capsular tissue was performed (Figure 3.3). Tissues were stained with CD68, a general macrophage marker, and CD48 a marker of previously identified $CD48^+IL1A^+$ macrophages (and $CD48^+CD1C^+$ dendritic cells) (Chapter 2, section 2.4.2). CD68 and CD48 expression was present in both comparator and frozen shoulder tissue. Quantitative image analysis revealed that CD68 and CD48 expression were both significantly increased in frozen shoulder relative to comparator capsular tissue (Figure 3.3).

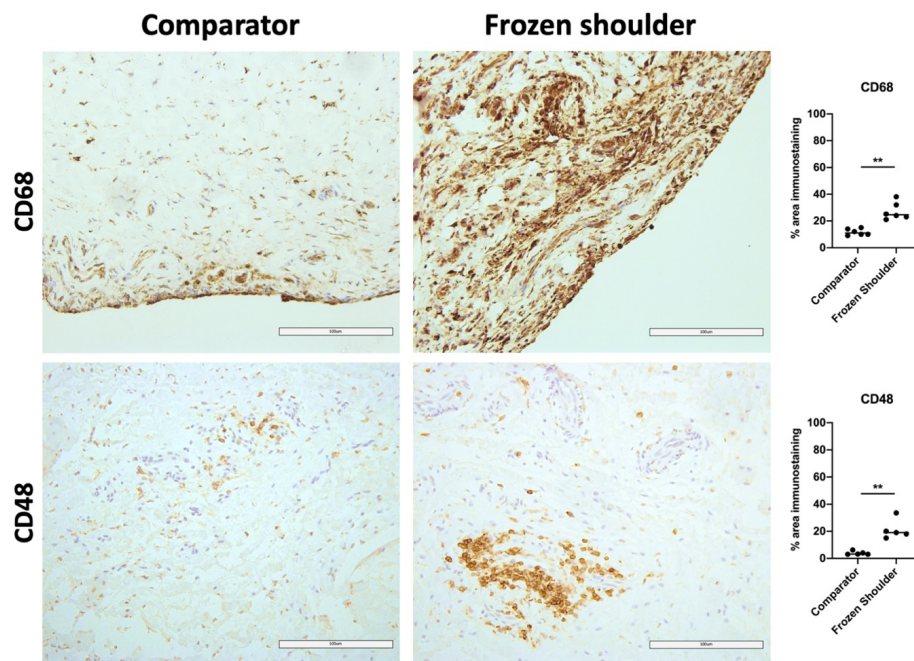


Figure 3.3: Immunohistochemistry of macrophage markers in GH capsular tissue. Left = representative images of CD68 and CD48 single staining (= brown) in frozen shoulder and comparator capsular tissue. Haematoxylin was used as nuclear counterstaining (= purple). Scale bar: 100 μ m (white). Right = semi-quantitative image analysis of CD68 and CD48 immunostaining respectively. Every dot represents a unique donor. Statistically significant differences were calculated using the Mann Whitney U test. ** $p < 0.01$.

To further confirm the expression of inflammatory mediators and their cognate receptors expressed by the $CD48^{+}IL1A^{+}$ macrophages, capsular tissues were stained with $IL-1\alpha$, $IL-1\beta$, S100A8, S100A9, $IL-1R$, and $TLR-4$ (Figure 3.4). All these markers were highly expressed in frozen shoulder capsular tissues.

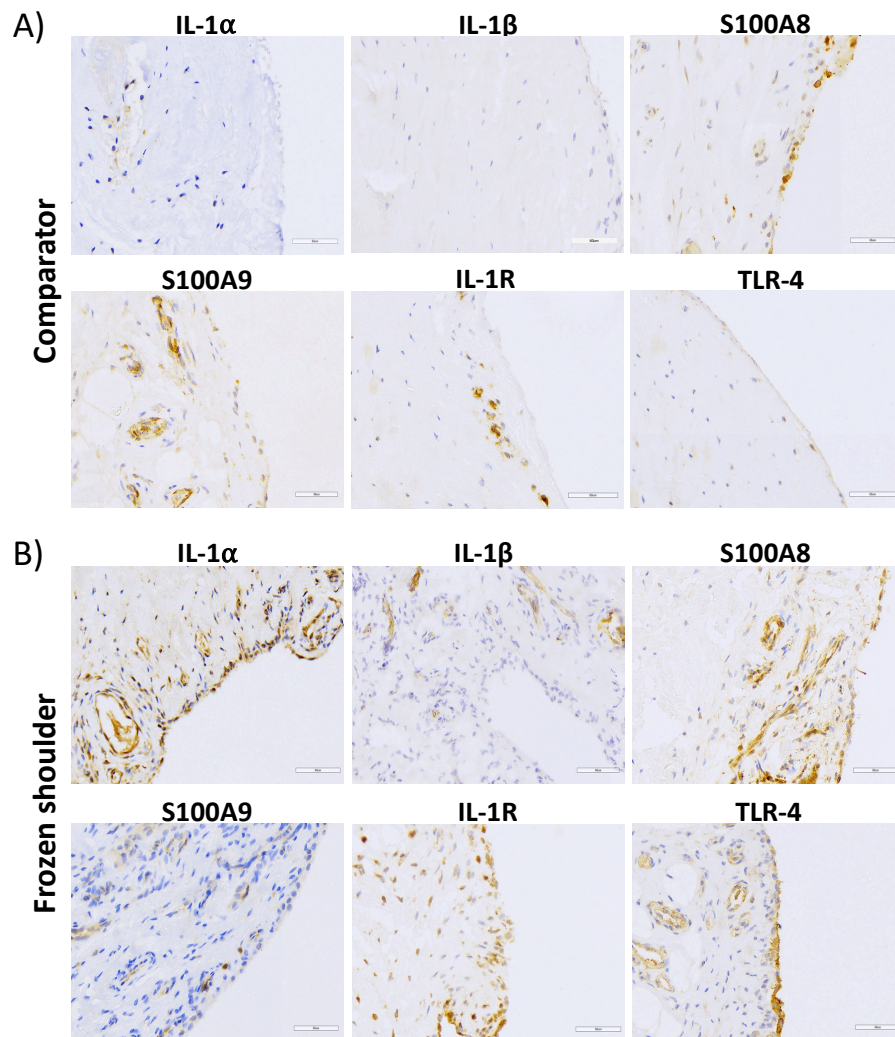


Figure 3.4: Immunohistochemistry of inflammatory mediators in GH capsular tissue. Representative images of $IL-1\alpha$, $IL-1\beta$, S100A8, S100A9, $IL-1R$, and $TLR-4$ single staining (= brown) in comparator (A) and frozen shoulder (B) capsular tissue. Haematoxylin was used as nuclear counterstaining (= purple). Scale bar = $60\mu m$ (white).

Semi-quantitative image analyses of immuno-positive staining reveals that **IL-1 α** , **IL-1R**, and **TLR-4** expression was significantly increased in samples of frozen shoulder relative to comparator capsule, whereas S100A8 and S100A9 expression did not differ across conditions (Figure 3.5).

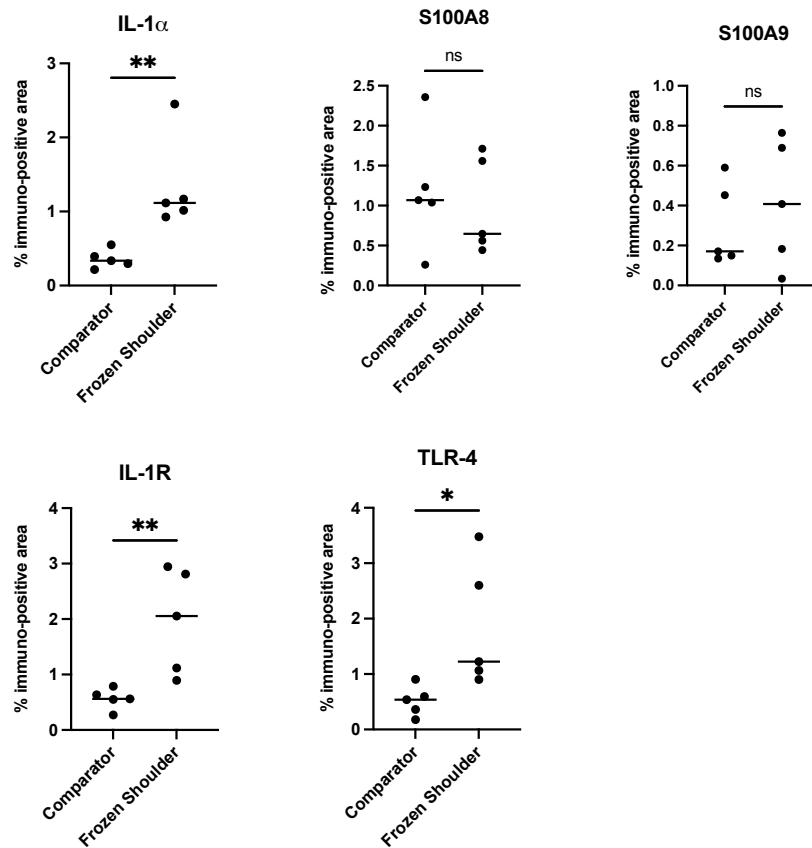


Figure 3.5: Semi-quantitative image analyses of inflammatory mediators in frozen shoulder and comparator capsular tissues.

Graphs showing semi-quantitative image analysis of **IL-1 α** , S100A8, S100A9, **IL-1R**, and **TLR-4** immunostaining respectively. Every dot represents a unique donor. Statistically significant differences were calculated using the Mann Whitney U test. *p<0.05, **p<0.01, ns = not significant.

In addition to these markers, capsular tissues were also stained with alarmins **HMGB1**, **IL-33**, and the **TLR-2** receptor (Appendix B.1). While **HMGB1** and **IL-33** were both significantly increased in frozen shoulder relative to comparator capsular

tissues, there was little to no **TLR-2** expression in both comparator and frozen shoulder capsule (Appendix **B.1**). Isotype control staining can be found in Appendix **B.2**.

3.4.3 Co-localisation of inflammatory mediator markers in rotator interval capsule

Immunofluorescence was performed for co-localisation analysis of inflammatory mediators in frozen shoulder capsular tissue. Tissues were stained with CD90 (*THY1*) and **PDPN** as markers of activated fibroblasts, CD68 (tissue resident macrophage marker), CD163 (anti-inflammatory macrophage marker), **TLR-4**, S100A8, **COX-2** (*PTGS2*), **IL-1R**, **IL-1 α** , **IL-1 β** , and **IL-1RA** (Figure **3.6**). Co-localisation analysis revealed that S100A8 and **TLR-4** were highly expressed in frozen shoulder capsular tissues, predominantly by the cells localised to the lining layer (Figure **3.6A**). CD90 expression was limited to sub-lining peri-vascular cells (Figure **3.6A**). The sub-lining area showed some CD68⁺**COX-2**⁺, CD68⁺S100A8⁺, and CD68⁺**IL-1RA**⁺ macrophages (Figure **3.6B&C**). Co-localisation analysis also revealed cells expressing both **IL-1R** and **IL-1 α** mainly in the lining layer (Figure **3.6D**). Finally, both **IL-1 β** and CD163 were expressed in frozen shoulder capsular tissues, but there was little to no co-localisation of cells expressing both **IL-1 β** and CD163 (Figure **3.6E**).

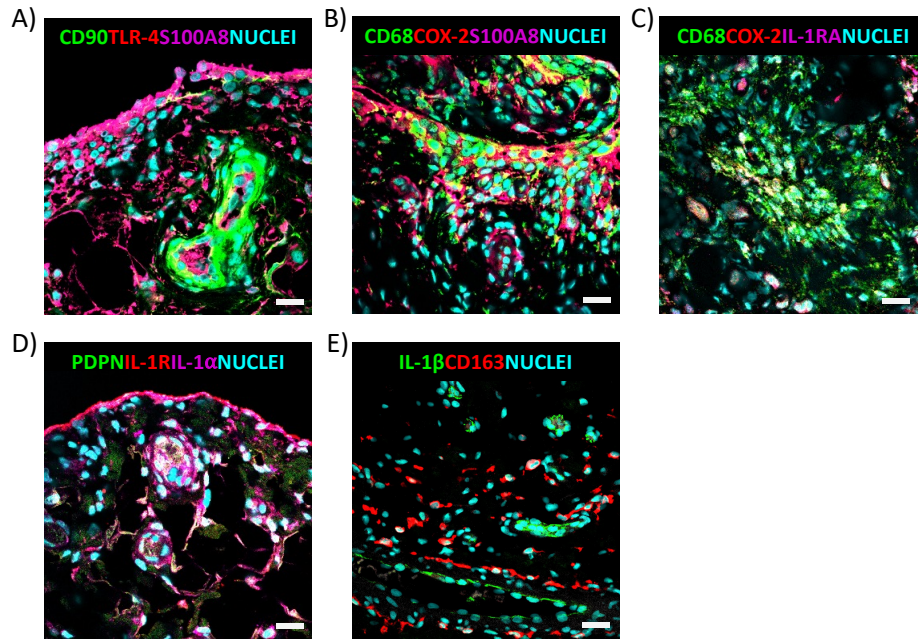


Figure 3.6: Immunofluorescence images of $CD48^+ IL1A^+$ macrophage markers. Representative immunofluorescence images of frozen shoulder capsular tissue stained with = A) CD90 (green), **TLR-4** (red), S100A8 (purple), B) CD68 (green), **COX-2** (red), S100A8 (purple), C) CD68 (green), **COX-2** (red), and **IL-1RA** (purple), D) **PDPN**, (green), **IL-1R** (red), **IL-1α** (purple), E) **IL-1β** (green) and CD163 (red). POPO-1 was used as nuclear counterstaining (= cyan). Scale bar = 20μm (white).

3.4.4 RNAscope **ISH** targeting *IL1A* and *IL1B* in the rotator interval capsule

RNAscope **ISH** with probes targeting *IL1A* and *IL1B* was performed on capsular tissue sections from frozen shoulder and comparator patient groups. Overall, little **mRNA** transcripts (puncta) of *IL1A* and *IL1B* were seen in capsular tissue sections of frozen shoulder (Figure 3.7) and comparator patient groups (Appendix B.3). The *IL1A* and *IL1B* **mRNA** transcripts that were there, were localised in the sub-lining, mostly in the peri-vascular area. Positive and negative control **ISH** images can be found in Appendix B.4.

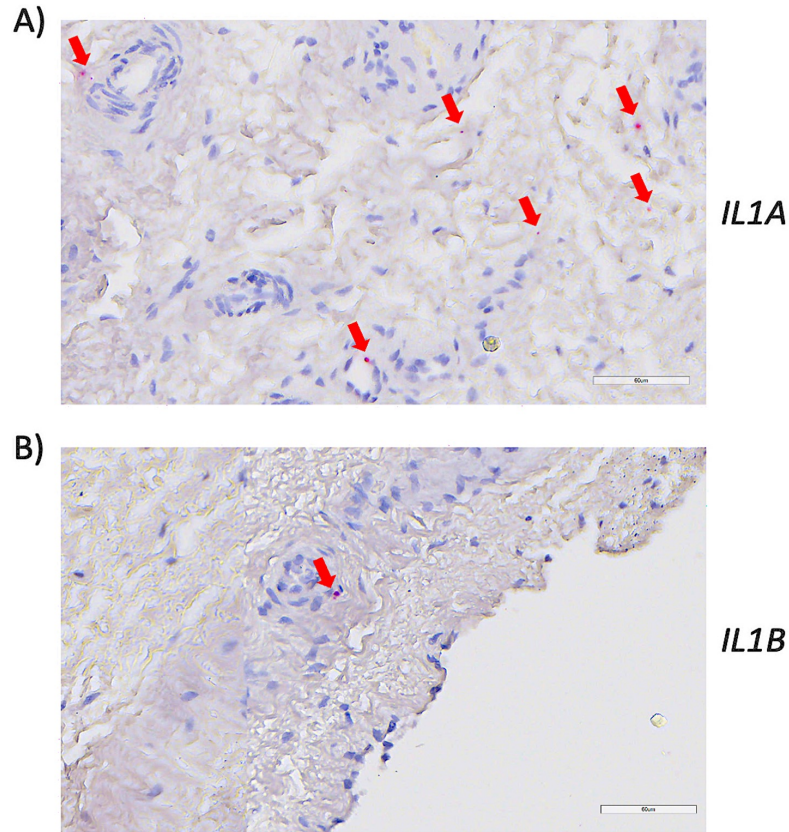


Figure 3.7: **ISH** targeting *IL1A* and *IL1B* in frozen shoulder capsular tissues. Representative images of **ISH** of A) *IL1A* and B) *IL1B* **mRNA** transcripts in capsular tissue sections of frozen shoulder patients. Red arrows showing RNAscope puncta, which represent single **mRNA** transcripts. Scale bar = 60µm (white).

3.4.5 Fibroblast cell atlas of the rotator interval of the **GH** joint capsule

To further investigate the **IL**-1-axis in frozen shoulder, the expression of **IL**-1-related genes was reassessed using the single cell atlas of the rotator interval of the **GH** joint capsule generated in Chapter 2. While most genes (*IL1A*, *IL1B*, *IL1RN*, *IL1RAP*, and *IL1R2*) were mainly expressed by myeloid cells, the **IL**-1 receptor gene (*IL1R1*) was predominantly expressed by fibroblasts and endothelial cells (Figure 3.8).

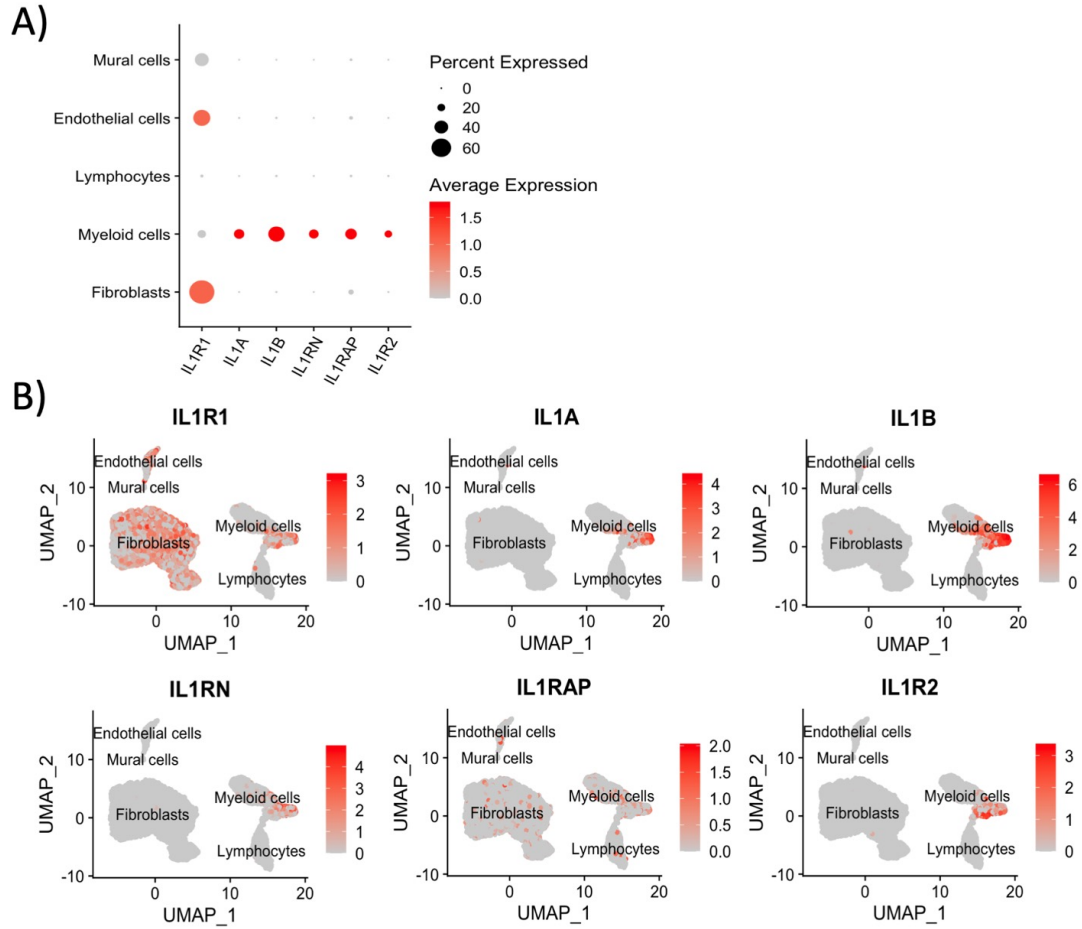


Figure 3.8: Expression of the **IL**-1 machinery in the single cell atlas of the rotator interval of the **GH** joint capsule.

A) Dot plot showing *IL1R1*, *IL1A*, *IL1B*, *IL1RN*, *IL1RAP*, and *IL1R2* expression for each of the cell clusters. The size of the dots represents the percentage of cells and the intensity of the (red) colour represents the average expression level. B) Feature plots showing *IL1R1*, *IL1A*, *IL1B*, *IL1RN*, *IL1RAP*, and *IL1R2* expression. The intensity of the (red) colour represents the average expression level.

As this suggests a potential interaction between myeloid cell clusters and fibroblasts via the **IL**-1-axis, the fibroblast cluster was computationally isolated and re-analysed using a resolution of 0.3, to ensure the identification of possible fibroblast subsets. Clustering of the fibroblasts (*COL1A1*⁺*PDGFRA*⁺*FAP*⁺) revealed six distinct subsets, including five *THY1*⁺ clusters and one *THY1*^{low} cluster (Fig-

ure 3.9). The $THY1^+$ clusters comprised of $FMOD^+DKK3^+$, $CXCL12^+APOE^+$, $POSTN^+CTHRC1^+$, ARC^+CXCL2^+ , and $MFAP5^+PI16^+$ subsets (Figure 3.10). The $THY1^{low}$ cluster was annotated as CLU^+HBEGF^+ (Figure 3.10). These subsets were expressed in both conditions (comparator and frozen shoulder), as well as in samples from individual donors (Appendix B.5). In addition, Single R annotations of the fibroblast subsets were generated (Appendices B.6 to B.8).

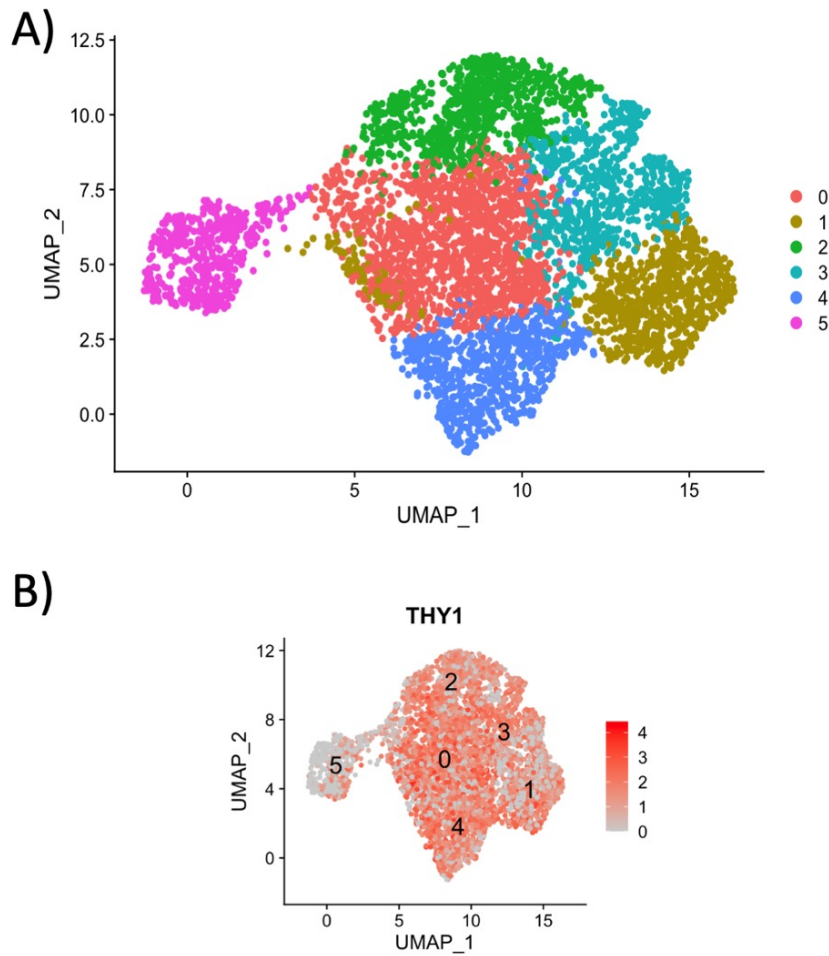
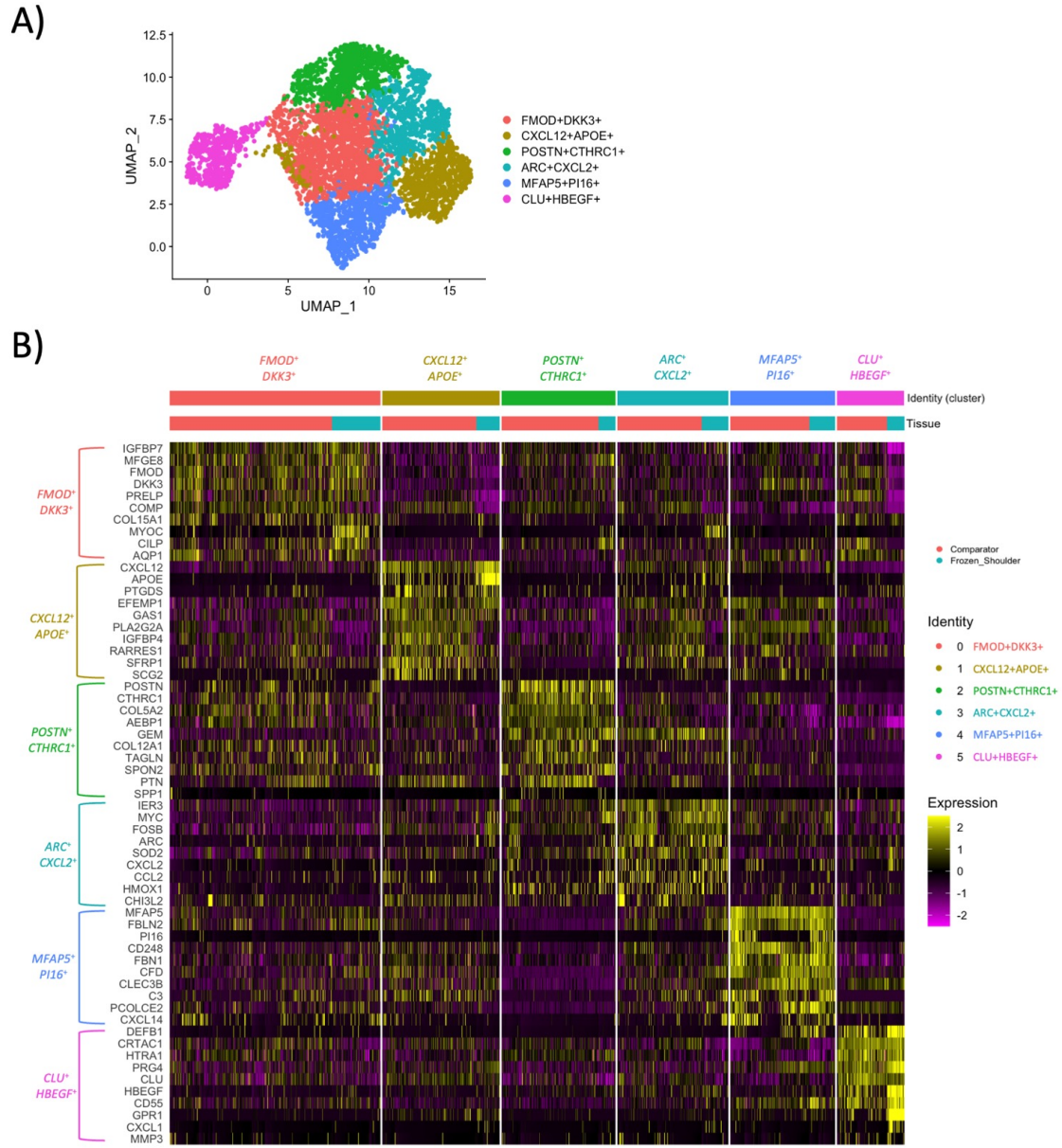


Figure 3.9: Single cell atlas of the $COL1A1^+PDGFRA^+FAP^+$ fibroblasts. A) **UMAP** dimensionality reduction of fibroblasts from both comparator ($n = 6$) and frozen shoulder ($n = 4$) samples showing six fibroblast clusters. B) Feature plots showing $THY1$ expression. The intensity of the (red) colour represents the average expression level.



Looking at the top positive genes per cluster, the $FMOD^+DKK3^+$ and $POSTN^+CTHRC1^+$ fibroblast subsets are enriched for matrix associated and/or collagen genes like *COMP*, *COL15A1*, *CILP*, *COL5A2*, *SPP1*, and *COL12A1* (Figure 3.10; Appendix B.9). Both $CXCL12^+APOE^+$ and ARC^+CXCL2^+ fibroblasts show enrichment for chemoattractant genes, including *CXCL12*, *PTGDS*, *CXCL2*, and *CCL2* (Figure 3.10; Appendix B.9). $MFAP5^+PI16^+$ fibroblasts express immune associated genes, including *C3*, *CFD*, and *CD248* (Figure 3.10; Appendix B.9). Finally, the CLU^+HBEGF^+ fibroblast subset shows enrichment for *PRG4*, *CLIC5*, and *CD55* (Figure 3.10; Appendix B.9).

3.4.6 Pathway analysis of fibroblast subsets

To further assess the role of each fibroblast subset, unique GO biological processes and molecular function pathway signatures were interrogated (Figures 3.11 and 3.12). $FMOD^+DKK3^+$ fibroblasts showed enrichment for biological processes related to collagen fibril organisation and extracellular matrix organisation (Figure 3.11). The $POSTN^+CTHRC1^+$ fibroblast subset is also enriched for these matrix associated GO terms, but also for genes associated with a response to mechanical stimuli and a cellular response to TGF- β (Figure 3.11). In addition, GO molecular function analysis showed that both $FMOD^+DKK3^+$ and $POSTN^+CTHRC1^+$ fibroblasts are enriched for a variety of matrix associated molecular functions (Figure 3.12). $CXCL12^+APOE^+$ fibroblasts are enriched for biological processes related to positive chemotaxis (Figure 3.11). The ARC^+CXCL2^+ subset is enriched for molecular functions related to chemokine activity, chemokine receptor binding, and cytokine activity (Figure 3.12). The $MFAP5^+PI16^+$ fibroblast showed enrichment for humeral immune response and vascular associated biological processes (Figure 3.11).

Finally, the CLU^+HBEGF^+ fibroblast subset showed enrichment for biological processes related to complement activation (Figure 3.11).

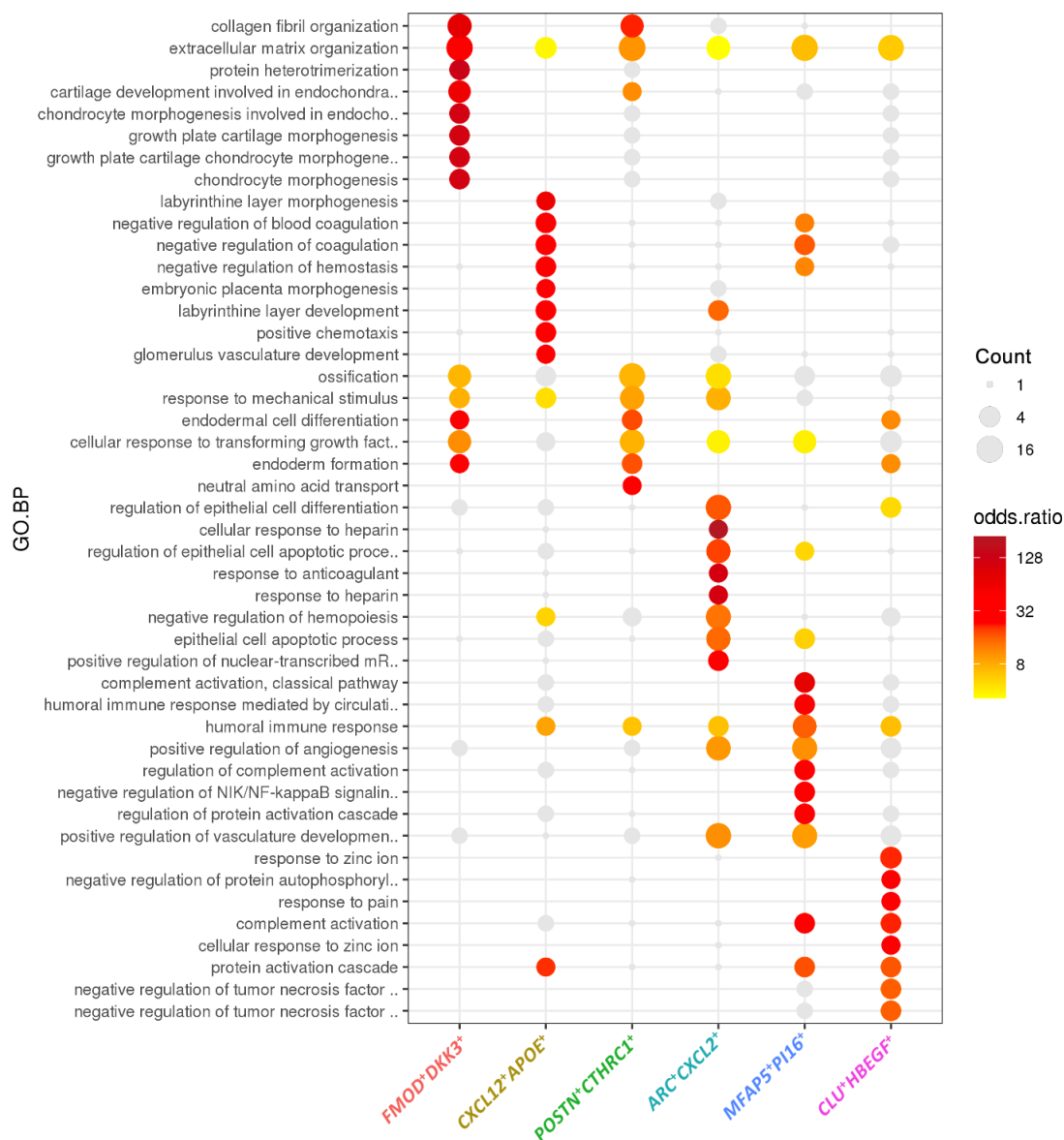


Figure 3.11: **GO** biological process pathway analysis of fibroblast subsets
Over-representation analysis reveals the main enriched **GO** biological process (BP) pathways. The dot size is proportional to the number of genes in the **GO** category that were found as cluster markers (Count). The intensity of the (red) colour represents the odds ratio.

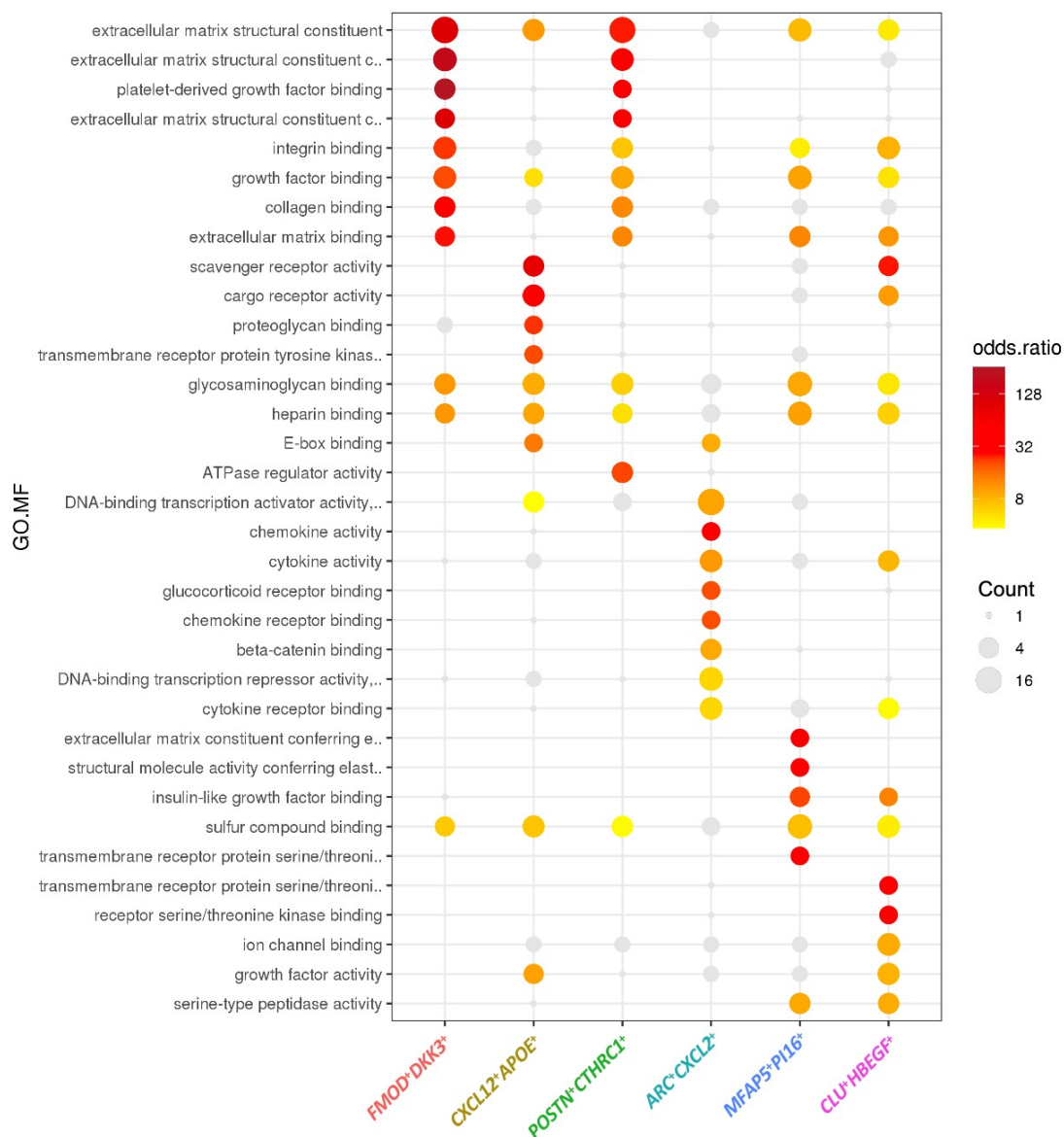


Figure 3.12: **GO** molecular function pathway analysis of fibroblast subsets
Over-representation analysis reveals the main enriched **GO** molecular function (MF) pathways. The dot size is proportional to the number of genes in the **GO** category that were found as cluster markers (Count). The intensity of the (red) colour represents the odds ratio.

3.4.7 Ligand-receptor interaction analysis: macrophages > fibroblasts

The fibroblast cell atlas was interrogated for *IL1R1* expression to identify subsets that express the **IL**-1 receptor gene. *IL1R1* expression was high across all fibroblast subsets (Figure 3.13). Therefore, all fibroblast clusters were included for ligand-receptor interaction analysis.

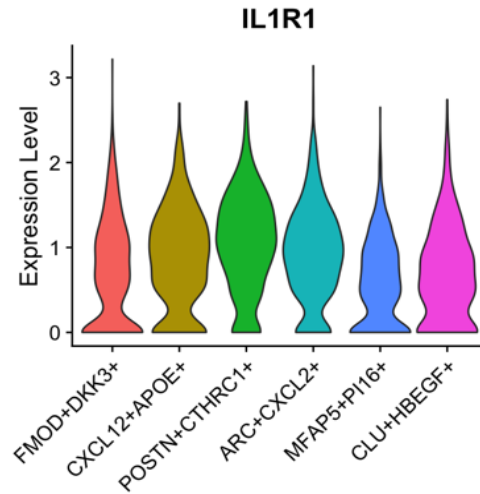


Figure 3.13: *IL1R1* expression by fibroblast populations.

Violin plot showing *IL1R1* expression across six distinct fibroblast populations in the rotator interval of the **GH** joint capsule.

The **scRNAseq** data of the rotator interval of the **GH** joint capsule was interrogated for ligand-receptor interactions between myeloid cells and fibroblasts. Ligand activity analysis revealed 20 potential ligands expressed by myeloid subsets (Figure 3.14A-C). Most of these ligands were mainly expressed by the *CD48*⁺*IL1A*⁺ macrophages, including *IL1B*, *PTGS2*, *IL1A*, and *TGFB1* (Figure 3.14A). There was also a high expression of *ANXA1*, *GAS6* (by *MERTK*⁺*LYVE1*^{high} macrophages), *CXCL16* (by *CD48*⁺*CD1C*⁺ dendritic cells), and *MMP9* (by *MERTK*⁺*LYVE1*^{low}

macrophages) (Figure 3.14A). The predicted ligands were ranked according to their ligand activity (Figure 3.14B). The expression of *IL1A*, *IL1B*, *MMP9*, and *PTGS2* was increased in frozen shoulder relative to comparator *CD48⁺IL1A⁺* macrophages (Figure 3.14C). Ligands expressed by the *CD48⁺IL1A⁺* macrophages (*IL1B*, *PTGS2*, *IL1A*, *TGFB1*) are likely to affect a variety of predicted target genes in the fibroblast clusters, including matrix associated genes (*TIMP3*, *COL1A1*, *COL1A2*, *COL3A1*, *COL5A1*, *FN1*, *TIMP1*, *ITGA6*), chemokine genes (*CXCL2*, *CXCL1*, *CXCL8*), and inflammatory genes (*TNFRSF11B*, *IL6*, *TNC*, *S100A10*, *NKFB1*, *IL33*) (Figure 3.14D).

Following ligand activity analysis, a ligand-receptor analysis was performed by acquiring the receptors of the top predicted ligands (Figure 3.15). Focusing on the ligands from the *CD48⁺IL1A⁺* macrophages, the top predicted ligand-receptor pairs were *TGFB1-TGFBR1*, *TGFB1-TGFBR2*, *IL1B-IL1R1*, *IL1B-IL1RAP*, and *IL1A-IL1R1* (Figure 3.15). Other ligand-receptor pairs (myeloid cells > fibroblasts) with a high prior interaction potential were *GAS6-AXL*, *EFNB1-EPHB3*, and *ITGAM-ICAM1*.

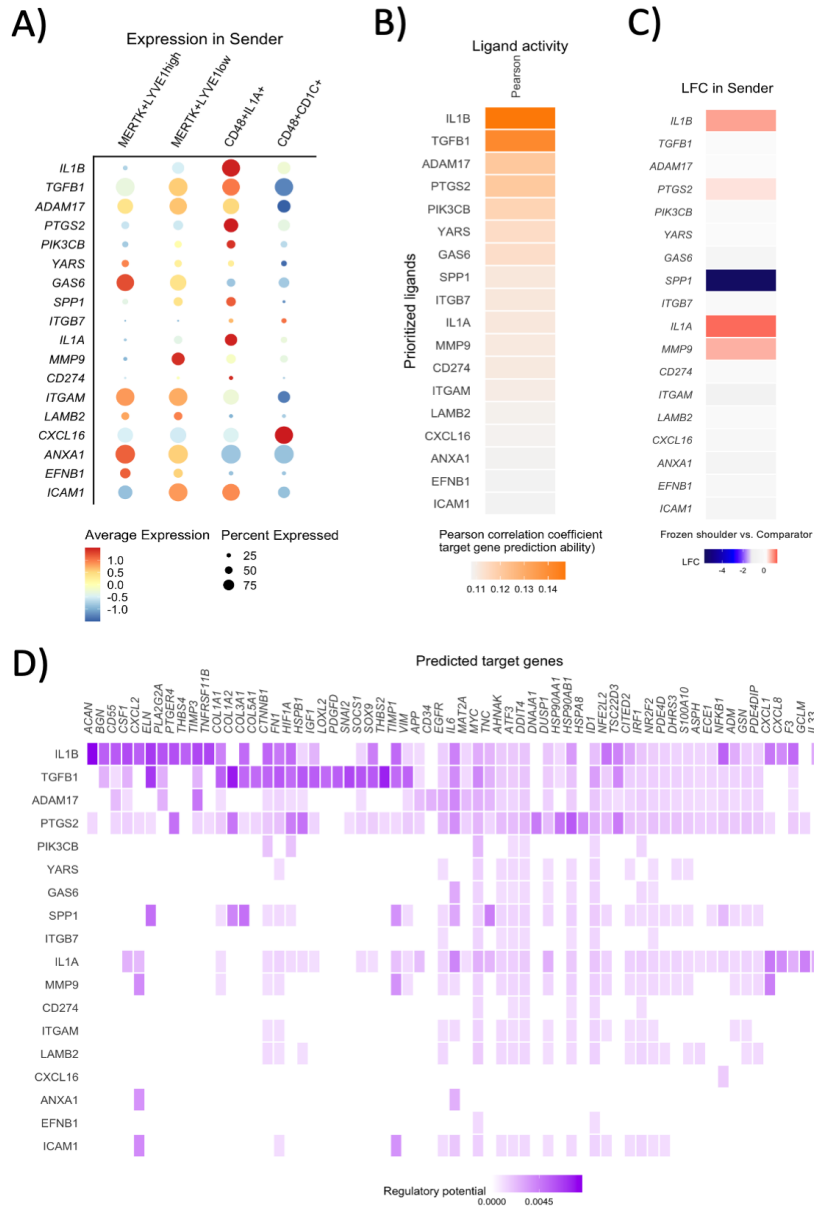


Figure 3.14: NicheNet ligand activity analysis of the myeloid cell clusters.

A) Dot plot showing the expression of the top 20 ligands of the myeloid clusters based on the presence of their target genes in the fibroblasts. The size of the dots represents the percentage of cells, and the colour represents the average expression level. B) Heatmap ranking the top 20 ligands based on ligand activity using the Pearson's correlation coefficient. C) Heatmap of the top 20 ligands and their log fold changes (LFC) based on expression in frozen shoulder relative to comparator cells. D) Heatmap showing the top 100 predicted target genes in fibroblasts based on the top 20 ligands of the myeloid clusters. The intensity of the (purple) colour represents the NicheNet regulatory potential score.

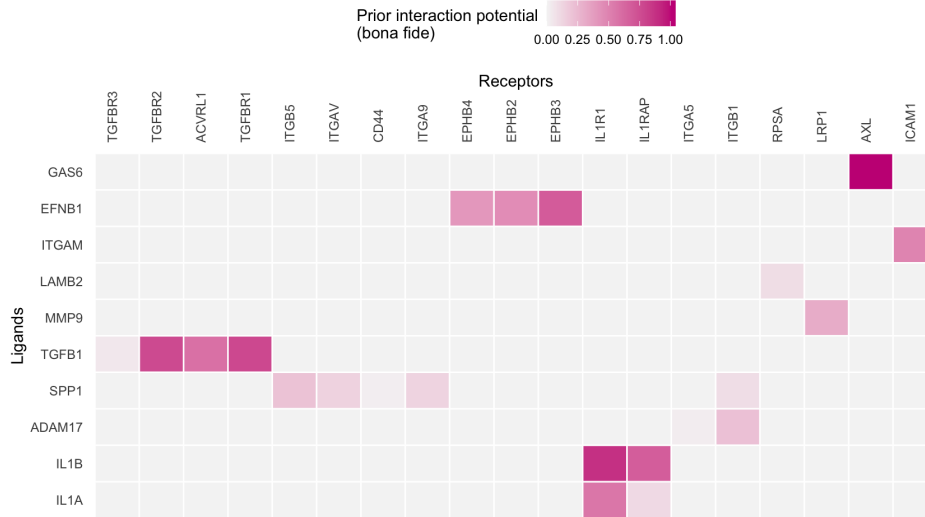


Figure 3.15: NicheNet ligand-receptor analysis of top predicted myeloid ligands and fibroblast receptors.

Heatmap showing the top predicted ligands and their cognate receptors. The intensity of the (dark pink) colour represents the NicheNet prior interaction potential based on the expression of the ligands in the myeloid clusters and the receptors in the fibroblast clusters.

3.4.8 Cell types communicating via the IL-1-axis

To identify the distinct cell types communicating via the IL-1-axis, NATMI analysis was performed on fibroblast- and myeloid cell populations (Figure 3.16). As expected, it is predicted that specific interactions between $CD48^+IL1A^+$ macrophages and all fibroblast clusters take place via the IL-1-axis. $CD48^+IL1A^+$ macrophages and the fibroblast clusters are most likely to interact via $IL1B-IL1R1$, followed by $IL1A-IL1R1$ and $IL1B-IL1RAP$. In addition, $CD48^+IL1A^+$ macrophages are also predicted to communicate with other myeloid cell clusters via most notably $IL1B-IL1R1$, $IL1B-IL1RAP$, and $IL1A$ - or $IL1B-IL1R2$ (Figure 3.16).

NATMI analysis – IL-1-AXIS

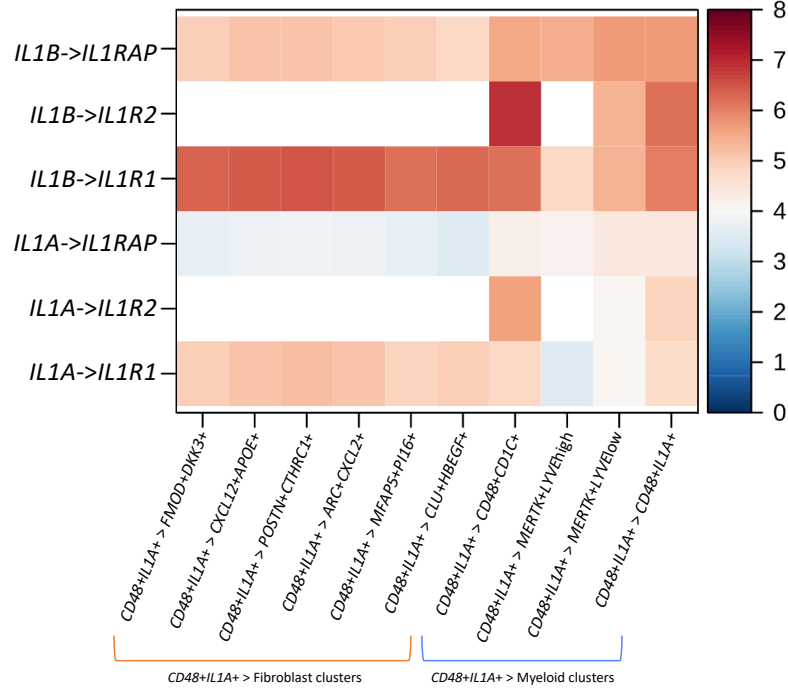


Figure 3.16: **NATMI** analysis of **IL-1-axis** across myeloid cell and fibroblast clusters. Heatmap showing myeloid cells and fibroblast clusters (bottom) predicted to communicate via the **IL-1-axis** ligand-receptor pairs (left). Sender cells = $CD48^+IL1A^+$. Receiver cells = fibroblast subsets and other myeloid cell subsets. The colour indicates overall expression of a ligand-receptor pair (red = high expression; blue = low expression).

3.4.9 Ligand-receptor interaction analysis: fibroblasts > macrophages

Ligand activity analysis revealed 20 potential ligands expressed by fibroblasts (Figure 3.17A-C). Ligands with the highest expression were mainly expressed by the CLU^+HBEGF^+ fibroblast subset, including *CSF1*, *VEGFC*, *TGFB1*, *COL4A1*, and *INHBA* (Figure 3.17A). Other ligands with a high expression included *GAS6*, *HAS2*, *BMP2*, *HSPG2*, and *CADM3* (Figure 3.17A). The predicted ligands were ranked

according to their ligand activity (Figure 3.17B). *CSF1* and *COL4A1* were increased in frozen shoulder relative to comparator fibroblasts (Figure 3.17C). *CSF1* expression was increased in frozen shoulder *CXCL12⁺APOE⁺*, *ARC⁺CXCL2⁺*, *MFAP5⁺PI16⁺*, and *CLU⁺HBEGF⁺* fibroblast subsets (Figure 3.17C). *COL4A1* was mainly increased in frozen shoulder *CXCL12⁺APOE⁺*, *POSTN⁺CTHRC1⁺*, *ARC⁺CXCL2⁺*, and *CLU⁺HBEGF⁺* fibroblast subsets (Figure 3.17C). A variety of predicted target genes were identified in the myeloid cell clusters, including inflammatory and/or pro-fibrotic genes (*ADAM9*, *IGF1*, *FN1*, *SPP1*, *SERPINE1*, *NFKB1*), but also some anti-inflammatory genes (*EGR2*, *ITGAM*, *EGR1*) (Figure 3.17D). Finally, ligand-receptor analysis revealed the top predicted ligand-receptor pairs (fibroblasts > myeloid cells), including *CSF1-CSF1R*, *GAS6-AXL*, *GAS6-MERTK*, and *VEGFC-NRP2* (Figure 3.18).

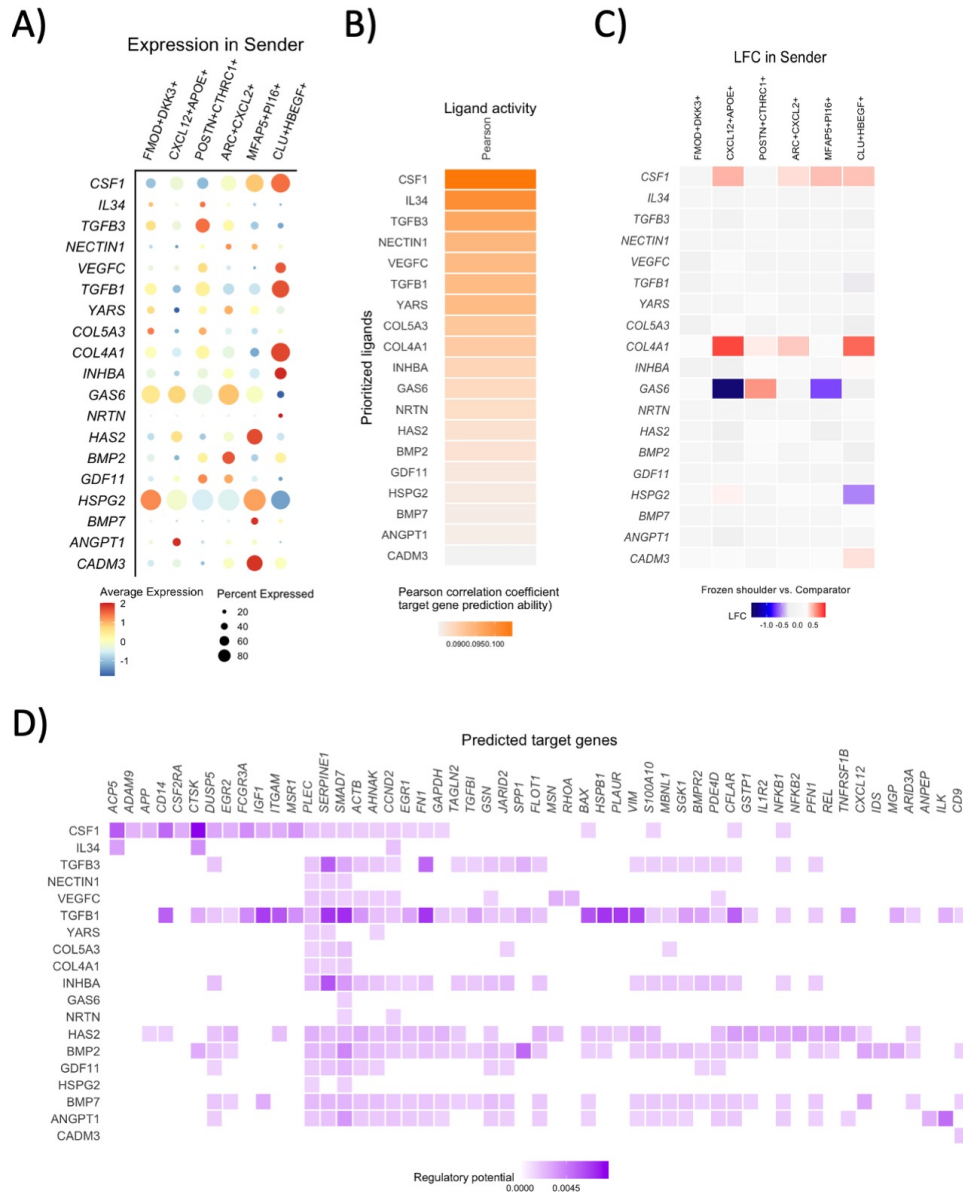


Figure 3.17: NicheNet ligand activity analysis of the fibroblast clusters.

A) Dot plot showing the expression of the top 20 ligands of the fibroblast clusters based on the presence of their target genes in the myeloid cell clusters. The size of the dots represents the percentage of cells, and the colour represents the average expression level. B) Heatmap ranking the top 20 ligands based on ligand activity using the Pearson's correlation coefficient. C) Heatmap of the top 20 ligands and their log fold changes (LFC) based on expression in frozen shoulder relative to comparator cells). D) Heatmap showing the top 100 predicted target genes in myeloid cell clusters based on the top 20 ligands of the fibroblast clusters. The intensity of the (purple) colour represents the NicheNet regulatory potential score.

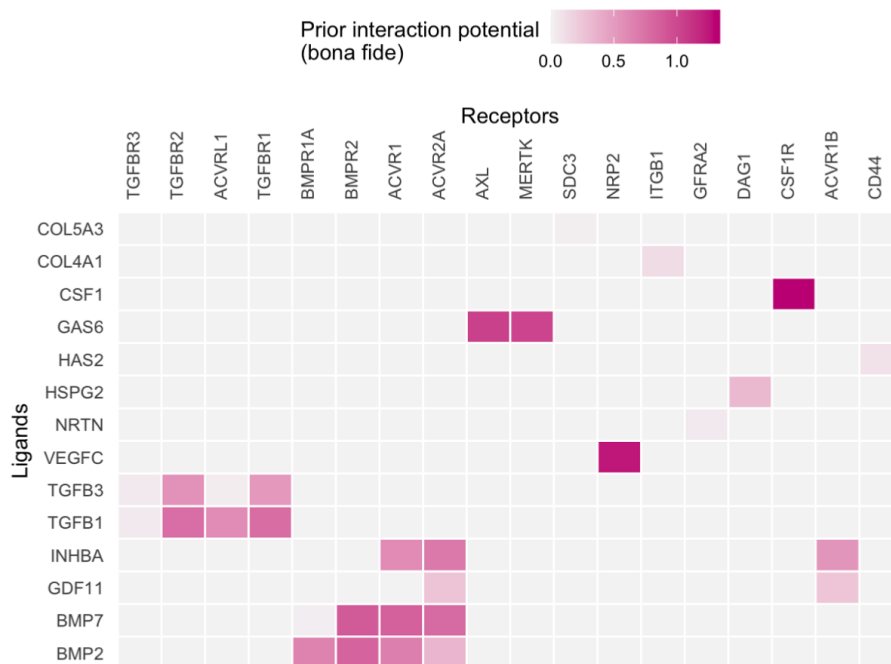


Figure 3.18: NicheNet ligand-receptor analysis of top predicted fibroblast ligands and myeloid cell receptors.

Heatmap showing the top predicted ligands and their cognate receptors. The intensity of the (dark pink) colour represents the NicheNet prior interaction potential based on the expression of the ligands in the fibroblast clusters and the receptors in the myeloid cell clusters.

3.5 Discussion

This chapter had three objectives. The first objective was to identify the micro-anatomical niche in which the cells expressing inflammatory mediators reside. Inflammatory mediators and their cognate receptors onto GH joint capsular tissues from frozen shoulder and comparator patient groups. The second objective was to confirm the protein expression of important inflammatory mediators and respective cognate receptors. Finally, the third objective was to identify cellular interactions between the $CD48^+IL1A^+$ macrophage and fibroblast populations by interrogating the scRNAseq atlas of the rotator interval of the GH joint capsule.

3.5.1 Rotator interval shows increased cellularity and vascularity in frozen shoulder

This chapter investigated the microscopic anatomy of the rotator interval of the GH joint capsule in frozen shoulder and comparator tissue samples. Histology revealed a thin lining layer and a sub-lining area, with significantly increased cellularity in frozen shoulder relative to comparator tissue samples. Findings of previous studies on the anatomy of the GH joint capsule are somewhat ambiguous, but most studies report an increase in cellularity and vascularity in frozen shoulder samples as seen in this chapter [23, 74, 138, 139, 152, 165, 178–181, 202, 224, 225]. Cole et al. (2001) studied foetal and adult cadaveric (non-diseased) rotator interval capsules which showed poorly organised collagen fibres with mostly fibroblast-like cells, which is in line with the histology of comparator samples in this chapter [18]. Interestingly, Kilian et al. (2007) reported a decreased numbers of fibroblast-like cells in frozen shoulder samples relative to control samples [136]. One explanation for

this difference is that the frozen shoulder samples used in this chapter are from patients with advanced-stage disease (duration of symptoms >1 year), whereas Kilian et al. (2007) used earlier-stage disease samples (stage II as per Neviaser (1987)) [123, 136]. It also might be due to their control group consisting of rotator interval samples from GH instability patients only, whereas this chapter included samples from both GH instability and OA patients undergoing elective surgery [136]. While some studies clearly state that the synovium and/or capsule are being investigated, the distinction between the two is not always clear. As any synovial joint, the GH joint is encapsulated by a fibrous joint capsule, which is merged and reinforced with surrounding ligaments. On the inside, the GH joint capsule is lined with synovium. Thus, when taking biopsies of the rotator interval, one might collect both capsule and synovium. Nevertheless, the synovium in frozen shoulder samples also shows increased cellularity and vascularity relative to control tissues [139, 150, 151, 179, 181]. These findings in frozen shoulder are very similar to RA, where the lining layer expands in disease [226].

3.5.2 Frozen shoulder patient tissues show increased expression of inflammatory mediators

To confirm the protein expression of inflammatory mediators and their cognate receptors expressed by the $CD48^+ IL1A^+$ macrophages, capsular tissues were stained with several key markers. Immunohistochemistry revealed a significant increase of CD68, CD48, IL-1 α , IL-1R, and TLR-4 expression in frozen shoulder relative to comparator capsular tissues. IL-1 β expression also seems to be increased in frozen shoulder capsular tissues; however, semi-quantitative image analysis was not performed. S100A8 and S100A9 expression was similar between frozen shoulder and

comparator capsular tissues.

Strong CD68 positive staining is well documented in frozen shoulder literature, and it is suggestive of an increase in macrophages in frozen shoulder [23, 74, 138, 151]. This is the first study to show increased CD48 expression in frozen shoulder relative to comparator capsule, which further supports an increase of $CD48^+IL1A^+$ macrophages (and $CD48^+CD1C^+$ dendritic cells) in frozen shoulder (Chapter 2; section 2.4.5). CD48 expression has been known to increase under inflammatory conditions, particularly on peripheral blood mononuclear cells (e.g., monocytes and lymphocytes) [227, 228]. IL-1 α and IL-1 β expression is also increased in frozen shoulder capsular tissues, which is in line with *IL1A* and *IL1B* mRNA expression (Chapter 2; section 2.4.5), but also existing literature [134, 138, 149]. *IL1A* mRNA expression was found to be increased in capsular tissues and the subacromial bursa of frozen shoulder patients, however this is the first study to identify increased IL-1 α immuno-positive staining in frozen shoulder [134, 149]. IL-1 β protein and mRNA expression were also previously confirmed in frozen shoulder capsular tissues [134, 138, 149]. IL-1 α and IL-1 β are well-studied inflammatory mediators that induce the production of cytokines and chemokines, as well as the infiltration of immune cells via the IL-1 receptor [229]. IL-1 α is a classic alarmin (or DAMP) as its precursor pIL-1 α is present and active in cells from healthy tissues, where it acts as a transcription factor regulating gene expression [230]. In contrast, IL-1 β is only expressed under disease conditions [229]. Both IL-1 α and IL-1 β are believed to be important mediators of inflammation and fibrosis as they are implicated in the pathophysiology of cardiovascular disease, cancer, systemic sclerosis, and RA [231–234]. Moreover, several therapeutics targeting the IL-1-axis have shown promising results in RA

[232, 235]. Increased expression of these inflammatory mediators in advanced-stage samples of frozen shoulder therefore suggest that they are involved in sustaining inflammatory fibrotic processes in frozen shoulder. The cellular biology of the IL-1-axis will be investigated and discussed in more detail in Chapter 4.

Interestingly, S100A8 and S100A9 immuno-positive expression did not differ between frozen shoulder and comparator capsular tissues. So far, only one study has investigated the role of these alarmins in frozen shoulder [152]. Cher et al. (2018) reported an increased expression (immuno-positive staining) of alarmins S100A8, S100A9, IL-33, and HMGB1 in frozen shoulder relative to control tissues [152]. The authors used earlier stage frozen shoulder capsular samples compared to the advanced-stage samples used in this chapter, which could explain the differences in findings [152]. Furthermore, their control group consisted of samples from GH instability patients only, whereas this chapter included samples from both GH instability and OA patients undergoing elective surgery [152]. S100A8 and S100A9 are considered alarmins as they are expressed in physiological conditions, where they regulate a variety of cellular processes [236, 237]. Under disease conditions, myeloid cells release S100A8 and S100A9 into the extracellular environment [238]. Unlike IL-1 α , S100A8 and S100A9 are highly susceptible to oxidation [239]. Depending on their redox status, S100A8 and S100A9 can act as both inflammatory mediators (fully reduced state) or have an anti-inflammatory, protective role (fully oxidised state) [238, 239]. The redox status of the S100 proteins cannot be identified with immunohistochemistry, which adds another layer of complexity to interpreting their role in inflammation. As both mRNA and protein expression of S100A8 and S100A9 did not differ between conditions in this chapter, it is less likely that these molecules

are key players in sustaining inflammation in advanced-stage frozen shoulder.

Fully reduced isoforms of S100A8 and S100A9 (as well as the partially oxidised, disulphide **HMGB1**) can act on **PRRs** (**TLR-2**, **TLR-4**, receptor for advanced glycation endproducts (**RAGE**)), promoting inflammation via the myeloid differentiation primary response gene 88 (**MyD88**)/**NF- κ B** pathway [240, 241]. While **TLR-4** showed significantly increased immuno-positive staining in frozen shoulder relative to comparator capsular tissues, there was little to no **TLR-2** expression in both conditions. This is the first study to confirm immuno-positive staining of **TLR-4** in frozen shoulder and comparator capsular tissues. Yano et al. (2020) did report increased gene expression (**RT-PCR**) of *RAGE*, *HMGB1*, *TLR2*, *TLR4*, and *NKFB* in frozen shoulder tissue samples of the coracohumeral ligament when compared to rotator cuff tear samples [193]. Looking at the cell atlas of the rotator interval, *TLR2* (**mRNA**) expression is mostly expressed by myeloid cells (Chapter 2; Figure 2.8A). However, this did not translate to immuno-positive staining in frozen shoulder capsular tissue sections. As **RAGE** (*AGER*) showed little to no **mRNA** expression, immunohistochemistry was not performed for this receptor (Chapter 2; Figure 2.8A). While increased **TLR-4** expression seems consistent, differences in **TLR-2** and **RAGE** expression in this chapter and in the study by Yano et al. (2020) could be explained by different frozen shoulder stages, different comparator groups and different methods (i.e., comparing gene expression and immunohistochemistry) [193].

HMGB1 and **IL-33** immuno-positive expression were also increased in frozen shoulder relative to comparator capsular samples. This finding is in line with the

findings from previous studies investigating alarmin expression in frozen shoulder [152, 193]. Like the S100 proteins, HMGB1 and IL-33 are alarmins which are susceptible to oxidation and therefore have dual functions, including the ability to act as a cytokine [45, 240]. HMGB1 is expressed by all cell types, whereas IL-33 is mainly expressed by the *PECAM1*⁺ *VWF*⁺ endothelial cells (Chapter 2; Figure 2.8A). As this chapter focuses on inflammatory mediators and alarmins expressed by *CD48*⁺ *IL1A*⁺ macrophages, the possible role of these alarmins in frozen shoulder will not be further discussed here.

3.5.3 Inflammatory mediators and cognate receptors localised to the lining layer of frozen shoulder capsular tissues

To further assess some of the key marker genes of the *CD48*⁺ *IL1A*⁺ macrophages, immunofluorescence was performed to confirm protein expression. Co-localisation analysis revealed that ligands S100A8, IL-1 α , TLR-4, and IL-1R were highly expressed in frozen shoulder capsular tissues, predominantly by the cells localised to the lining layer. The sub-lining area showed CD68⁺ macrophages expressing COX-2, S100A8 and IL-1RA, however there was also a substantial area of CD68⁺ macrophages not expressing these markers. It is likely that these are some of the MerTK⁺ macrophages. In addition, there was little to no co-localisation of cells expressing IL-1 β and CD163, suggesting that IL-1 β is not expressed by cells expressing CD163 (i.e., MerTK⁺ macrophages).

These results support that inflammatory *CD48*⁺ *IL1A*⁺ macrophages are present in frozen shoulder capsular tissues, mainly localised to the lining layer. It also shows

that cells expressing these inflammatory ligands and their cognate receptors are in proximity of each other, making cellular interaction likely. While this is the first study to confirm the co-localisation of these inflammatory mediators in frozen shoulder, previous studies have reported the presence of IL-1 α , IL-1R, IL-1RA, TLR-4, S100A8, and S100A9 in the synovial lining layer of RA [70, 212–216]. Furthermore, Alivernini et al. (2017) reported increased CD68 immuno-positive staining in the lining layer of synovial tissues from RA patients with high/moderate disease compared to RA patients with low disease activity and RA patients in remission [226]. This suggests an increase of macrophages in the lining layer of synovial tissues from RA patients with high/moderate disease [226]. Taken together, it is well established that patients with active RA show an increased number of inflammatory synovial tissue macrophages in the lining layer [70, 212–215, 226]. The lining layer of frozen shoulder capsular tissues seems to behave in a similar way as the synovial lining layer in the synovium of patients with RA.

Fibroblast activation markers CD90 and PDPN were both expressed in frozen shoulder capsular tissues. CD90 was mainly expressed by perivascular cells in the sub-lining. This is in line with a study from Wei et al. (2020), where CD90⁺ cells were identified as perivascular and sub-lining fibroblasts in synovial tissues from patients with RA [242]. Fibroblast activation markers have also been previously identified in frozen shoulder capsular tissues [165, 166]. Akbar et al. (2019) confirmed increased immuno-positive staining of CD248, CD146, VCAM, and PDPN in frozen shoulder capsular tissues [165]. While also mentioning CD90 staining in their methods, it was not reported whether this was present and/or increased in frozen shoulder capsular tissues [165]. Dakin et al. (2019) reported the presence of PDPN, CD106, FAP and

CD90 in frozen shoulder capsular tissues and rotator cuff tendon tears [166]. Interestingly, *CD90 mRNA* expression was reduced in frozen shoulder capsular tissues compared to (chronic) shoulder rotator cuff tendon tears [166]. The authors propose that CD90 represents a key pathogenic marker regulating persistent inflammation, which is increased in chronic rotator cuff tendon tears and RA, whereas it is reduced in frozen shoulder, a self-limiting inflammatory (fibrotic) disease [24, 166]. While this chapter did not include a CD90 semi-quantitative image analysis and/or comparator capsular tissue stained with CD90, its limited expression in advanced-stage frozen shoulder samples concur with findings from the study by Dakin et al. (2019) [166].

Contrary to protein expression (immunohistochemistry), spatial transcriptomics using RNAscope ISH with probes targeting *IL1A* and *IL1B* revealed little mRNA transcripts (puncta) of *IL1A* and *IL1B* in capsular tissue sections of frozen shoulder and comparator patient groups. Looking at the expression of *IL1A* and *IL1B* in the single cell atlas of the rotator interval of the GH joint capsule however, one would have expected to see an abundance of *IL1A* and *IL1B* transcripts in the capsular tissues. Limitations and other possible reasons for this discrepancy are discussed in the limitation section (section 3.5.6).

3.5.4 *COL1A1*⁺*PDGFRA*⁺*FAP*⁺ fibroblast atlas reveals six distinct fibroblast subsets

Frozen shoulder capsular tissues showed significantly increased *IL1A*, *IL1B*, and *IL1R1 mRNA* expression by *CD48*⁺*IL1A*⁺ macrophages (Chapter 2; section 2.4.5). This chapter revealed increased IL-1 α , IL-1 β and IL-1R immuno-positive staining in frozen shoulder capsular tissue. These findings suggest that the IL-1-axis might

be one of the key drivers of inflammation and fibrosis in advanced-stage frozen shoulder. Therefore, the single cell atlas of the rotator interval was interrogated to identify other cell types expressing the *IL1R1* receptor gene. Interestingly, *IL1R1* was predominantly expressed by fibroblasts and endothelial cells. This suggests a potential predicted interaction between *CD48⁺IL1A⁺* macrophages expressing **IL**-1 ligands and fibroblasts expressing **IL**-1R. For this reason, the fibroblasts were computationally isolated and reanalysed to identify sub-populations. Clustering of the fibroblasts (*COL1A1⁺PDGFRA⁺FAP⁺*) revealed six distinct subsets, including five *THY1⁺* clusters and one *THY1^{low}* cluster.

There were two matrix associated *THY1⁺* fibroblast populations (*FMOD⁺DKK3⁺* and *POSTN⁺CTHRC1⁺*), which are likely to be involved remodelling the extracellular matrix. *CXCL12⁺APOE⁺* and *ARC⁺CXCL2⁺* fibroblast populations were enriched for chemoattractant genes. *ARC⁺CXCL2⁺* pathway analyses also revealed enrichment for chemokine and cytokine activity, suggesting that this fibroblast population also has immune-interacting abilities. The *MFAP5⁺PI16⁺* fibroblast population was enriched for biological processes related to the humoral immune response and vascular associated biological processes, suggesting that this is an immune- and vascular-interacting fibroblast population. Finally, the *THY^{low}CLU⁺HBEGF⁺* fibroblasts expressed known (synovial) lining markers and was enriched for biological processes related to complement activation.

The fibroblast populations in the rotator interval of the **GH** joint capsule show similarities with fibroblast populations previously identified in **RA** [51, 70, 242, 243]. Croft et al. (2019) identified sub-lining *THY⁺* clusters and one lining *THY⁻* cluster

in inflamed mouse synovium as well as human RA synovium [51]. The authors identified a human THY^{-} lining layer fibroblast subset expressing *HBEGF*, *CD55*, and *CLIC5* which shows similar gene expression as the $THY^{low}CLU^{+}HBEGF^{+}$ fibroblasts identified in this chapter [51]. Wei et al. (2020) reported a THY^{+} sub-lining fibroblast cluster and a $PRG4^{+}$ lining layer fibroblast cluster [242]. A recent study by Aliverni et al. (2020) identified five fibroblast clusters in synovium from patients with RA [70]. The authors identified a lining fibroblast cluster expressing similar genes as the $THY^{low}CLU^{+}HBEGF^{+}$ fibroblasts identified in this chapter, including *MMP3*, *PRG4*, *HBEGF*, and *CLIC5* [70]. They also reported a HLA^{+} sub-lining cluster expressing *CXCL12*, *APOE*, *PTGDS*, *CCL2* [70]. This cluster shows striking similarities with the $CXCL12^{+}APOE^{+}$ fibroblast cluster (and to a lesser extent the $ARC^{+}CXCL2^{+}$ cluster) identified in this chapter [70]. Their $THY1^{+}$ sub-lining layer fibroblast cluster expressed *COMP*, *CILP*, and collagen genes, which is somewhat similar to the $FMOD^{+}DKK3^{+}$ cluster described this chapter [70]. The $MFAP5^{+}PI16^{+}$ fibroblast cluster identified in this chapter shows similar gene expression as the $THY1^{+}CD34^{+}$ sub-lining cluster identified by Aliverni et al. (2020), as it expresses *MFAP5*, *PI16*, and *FBN1* [70]. Finally, the $THY1^{+}CXCL14^{+}$ sub-lining cluster identified by Aliverni et al. (2020) shows high expression of *MYOC*, *IGFBP7*, and *CFD* which is similar to the expression profile of the $FMOD^{+}DKK3^{+}$ fibroblasts identified in this chapter [70]. Akin to the myeloid cell clusters described in Chapter 2, there is also great similarity between the fibroblast clusters identified in RA and frozen shoulder [243].

3.5.5 Macrophage – fibroblast cellular cross talk in frozen shoulder

First, interactions between $CD48^+IL1A^+$ macrophages (sender cells) and fibroblasts (receiver cells) were assessed. As *IL1R1* expression was high across all fibroblast subsets, all fibroblasts were all included in the ligand-receptor interaction analysis. Ligand activity analysis revealed several important ligands expressed by the $CD48^+IL1A^+$ macrophages, including *IL1B*, *PTGS2*, *IL1A*, and *TGFB1*. Of these, *IL1A* and *IL1B* showed increased expression in frozen shoulder relative to comparator cells. The $CD48^+IL1A^+$ macrophage ligands are predicted to affect a variety of target genes in the fibroblast clusters, including matrix associated genes, chemokine genes, and pro-inflammatory genes. This suggests a possible role for $CD48^+IL1A^+$ macrophage ligands driving inflammation and fibrosis in advanced-stage frozen shoulder. Ligand-receptor interaction analysis ($CD48^+IL1A^+$ macrophages > fibroblasts) predicted several important ligand-receptor pairs, including *TGFB1-TGFBR1*, *TGFB1-TGFBR2*, *IL1B-IL1R1*, *IL1B-IL1RAP*, and *IL1A-IL1R1*. Both **TGF**- β and **IL**-1 are well-studied mediators of inflammation and fibrosis in a variety of diseases [198, 199, 244]. In addition to the fibroblast clusters, $CD48^+IL1A^+$ macrophages are also predicted to interact with other myeloid clusters via the **IL**-1-axis. These results suggest that $CD48^+IL1A^+$ macrophages are stimulating both fibroblasts as well as myeloid cells (e.g., macrophages and dendritic cells) to promote inflammation via the **IL**-1-axis.

To assess macrophage-fibroblast cross talk, ligands of fibroblast clusters affecting myeloid cell clusters were also assessed. Ligand activity analysis revealed several po-

tentially important ligands expressed by different fibroblast clusters, including *CSF1*, *IL34*, *TGFB3*, *TGFB1*, *COL4A1*, and *GAS6*. While most of these markers are pro-inflammatory and/or pro-fibrotic mediators, *GAS6* and *CSF1* have been described as having both pro-inflammatory and anti-inflammatory properties [245–248]. Only *CSF1* and *COL4A1* expression was increased in frozen shoulder relative to comparator fibroblasts, which suggests a role for these markers in frozen shoulder. Hu et al. (2020) reported an anti-inflammatory role of colony-stimulating factor (**CSF**)-1 in neuroinflammation, as activation of its cognate receptor (**CSF**-1R) downregulated **IL**-1 β and **TNF**- α expression [245]. In contrast, Garcia et al. (2016) suggested an inflammatory role of **CSF**-1 as inhibition of **CSF**-1R ameliorated inflammation of **RA** synovial tissues [249]. Furthermore, *in vitro* studies have shown an upregulation of **CSF**-1 after **IL**-1 β stimulation [250, 251]. One could therefore hypothesise that expression of *CSF1* by capsular fibroblasts could be the result of *CD48*⁺*IL1A*⁺ macrophage derived **IL**-1 β stimulation. Predicted target genes expressed by myeloid cell clusters revealed a variety pro-inflammatory and/or pro-fibrotic genes, including *ADAM9*, *IGF1*, *FN1*, *SPP1*, *SERPINE1*, and *NFKB1*. Interestingly, it also showed some predicted anti-inflammatory target genes, including *EGR2*, *ITGAM*, and *EGR1* [252–254]. The identification of anti-inflammatory target genes supports the idea that frozen shoulder tissues used in this chapter derived from patients that were on a resolving trajectory (advanced-stage disease). Finally, top predicted ligand-receptor pairs (fibroblasts > myeloid cells) were *CSF1-CSF1R*, *GAS6-AXL*, *GAS6-MERTK*, *VEGFC-NRP2*, and *TGFB1-TGFBR1/2*. However, as only *CSF1* was increased in frozen shoulder fibroblasts, it is likely that mainly the *CSF1-CSF1R* pathway is active in the frozen shoulder capsular tissues used in this study.

Taken together, this data suggests that interactions between macrophages and fibroblasts are implicated in sustaining inflammation and fibrosis in advanced-stage frozen shoulder capsular tissues. In frozen shoulder, $CD48^+IL1A^+$ macrophages release IL-1 α and IL-1 β , which both act on the IL-1R receptors expressed by fibroblasts (Figure 3.19). Once IL-1R is activated, it is likely that fibroblasts will release chemokines and other inflammatory mediators, further promoting inflammation and fibrosis. Some of these will interact with macrophages (e.g., via the *CSF1-CSF1R* pathway) (Figure 3.19). In addition, there is likely also some autocrine, juxtacrine, and paracrine signalling of IL-1 α and IL-1 β by $CD48^+IL1A^+$ macrophages, as suggested by pathway analyses in Chapter 2, section 2.4.4. Upon release, IL-1 α and IL-1 β act on IL-1R expressed on the secreting cell and/or neighbouring ($CD48^+IL1A^+$) macrophages (Figure 3.19), after which further induces the release of inflammatory mediators (like IL-1 α and IL-1 β).

The next chapter (Chapter 4) will focus on further investigating the role of IL-1 α and IL-1 β in frozen shoulder. The effect of IL-1 α /IL-1 β on fibroblasts and intra-cellular signalling pathways will also be discussed in Chapter 4.

Predicted macrophage-fibroblast cross talk in frozen shoulder

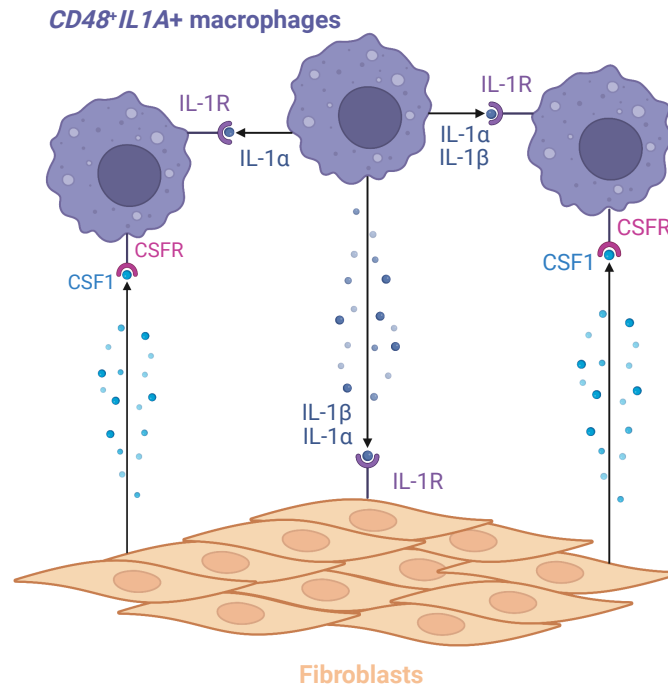


Figure 3.19: Predicted macrophage-fibroblast cross talk in frozen shoulder.

Schematic overview of predicted macrophage-fibroblast cross talk in frozen shoulder. *CD48⁺IL1A⁺* macrophages secrete *IL-1α* and *IL-1β* which act on *IL-1R* expressed by fibroblasts and surrounding macrophages. In turn, fibroblast populations release *CSF-1* which acts on its cognate receptor *CSF-R* on macrophages. Created with [BioRender.com](https://www.biorender.com).

3.5.6 Limitations

This chapter has several limitations. First, there are clinical limitations and challenges to take into consideration as previously described in Chapter 2, section 2.5.5. Second, the capsular tissue from frozen shoulder and comparator patient groups was collected from the rotator cuff interval only. Therefore, histological analysis (and *scRNAseq* analysis) does not represent the entire GH joint, but rather the rotator interval. There is some discussion in literature about the main pathological

site of frozen shoulder, however many studies have suggested that the pathology is predominantly localised to the capsule of the rotator cuff interval, including the coracohumeral ligament that resides within [23, 137, 255–258].

Uitvlugt et al. (1993) found the (intra-articular) synovitis to be localised to the area of the rotator interval in frozen shoulder patients with at least 6 months of complaints [255]. Uhthoff and Boileau (2007) investigated frozen shoulder capsular and synovium samples taken from different biopsy sites, including the posterosuperior capsule, the rotator interval, the coracohumeral ligament, the axillary fold, and the inferior capsule [139]. The patients had at least 12 months of symptoms [139]. Histologically, there was little difference between the synovium and the GH joint capsule across biopsy sites: all showed increased cellularity and vascularity, apart from the inferior capsule [139]. Interestingly, the contracture of the GH joint capsule (immuno-positive vimentin staining) was localised to the anterior capsule (including the axillary fold and the inferior capsule), but not the posterosuperior capsule [139]. Hettrich et al. (2016) collected frozen shoulder tissue samples from both the posterior and the anterior GH joint capsule, however, performed no comparisons between these sample types [179]. Hagiwara et al. (2018) compared protein expression levels of the rotator interval, the middle GH ligament, and the anterior-inferior GH ligament with mass spectrometry [259]. The authors found significant differences between the upper parts of the GH joint capsule and the anterior-inferior GH ligament [259]. As most studies suggest that the rotator interval is the main pathological site, using only the rotator interval capsule for histological analyses is acceptable. In the future, it might be worth comparing samples from different biopsy sites (and different disease stages) by means of histology and scRNAseq.

Another limitation in this chapter is the use of immunohistochemistry for quantification of secreted cytokines in tissue sections of GH joint capsule. While immunohistochemistry allows for detection of receptors and cytokine-producing cells in tissue, there is a limitation for detection and quantification of secreted proteins, especially alarmins. Alarmins are already present in non-diseased cells and undergo post-translational modifications upon stimulation [240]. Therefore, immuno-positive staining (and mRNA expression) might reflect a combination of baseline expression levels and secreted levels of alarmins in tissue sections. Measuring secreted cytokines and alarmins could therefore require different methods, which will be addressed in Chapter 4. As IL-1 α and IL-1 β are regulated post-translationally, there might be some differences in expression between mRNA and protein levels. Yet, both mRNA expression (scRNAseq) and immuno-positive staining for IL-1 α , IL-1 β and IL-1R were increased in frozen shoulder relative to comparator capsular tissues.

Technical challenges and limitations of scRNAseq have been extensively discussed in Chapter 2, section 2.5.5. There are however some additional limitations to consider when performing a ligand-receptor analysis. As mentioned before, mRNA does not always accurately reflect protein levels and therefore results should be interpreted with caution. Furthermore, inferring ligand-receptor interactions from transcriptomics relies mainly on literature-curated lists of interacting proteins. While this curated list is steadily growing, it still does not cover all possible ligand-receptor interactions. Another important limitation is that increased mRNA expression (or protein expression for that matter) of ligands and receptors do not equate to cellular communication, increasing the risk of detecting false positive and negative predicted interactions. Ligands and receptors need to be localised in the correct cellular compartment

and **scRNAseq** data does not inform the spatial location of cells. Communicating cells are expected to be in proximity of each other. For this reason, immunofluorescence was used to both validate cluster markers at a protein level and to confirm their location. This revealed that cells expressing important inflammatory ligands and their cognate receptors are localised close to each other, making cellular interaction more likely. Finally, spatial transcriptomics (in this case RNAscope **ISH**) was used to confirm spatial proximity of cells expressing *IL1A* and *IL1B*. While there was some evidence of *IL1A* and *IL1B* **mRNA** transcripts in capsular tissue sections from frozen shoulder and comparator patient groups, it was less than expected when compared to the **mRNA** expression seen in the **scRNAseq** data and/or the immunostaining. This might be due to some technical challenges. The preparation steps may have degraded **mRNA** transcripts, warranting further optimisation of the RNAscope protocol for **GH** capsular tissue sections. In addition, most capsular tissue sections used were sectioned several months before performing RNAscope **ISH**, possibly allowing for **mRNA** degradation. Future work using newer capsular tissue sections may address this issue. Other methods of spatial transcriptomics may also be explored in future experiments, like for example 10X Visium, which allows for the detection of the whole transcriptome across tissue sections with high sensitivity and specificity.

Ligand-receptor interaction analysis alone is not enough to assess cellular interactions and it is important to stress that these results are merely predictions. Therefore, ligand-receptor interactions that are inferred computationally need validating, including the confirmation of protein expression, confirmation of spatial proximity, and testing of the ligand-receptor interaction using *in vitro* and/or *in vivo* experiments. While some of these issues have been addressed in this chapter, Chapter 4

will further validate the IL-1/IL-1R interaction using patient derived cells. Future tools and algorithms analysing cellular interactions need to generate new scoring systems, which can also account for spatial proximity and the integration of additional omics technologies like proteomics (e.g., with mass spectrometry). This will help identify additional cellular interactions and/or ligand-receptor interactions that current mRNA-based methods are missing.

3.6 Conclusion

This chapter successfully localised the $CD48^+IL1A^+$ macrophage population to both the lining and sub-lining regions of the rotator interval of the GH joint capsule. Immunohistochemistry confirmed the protein expression of important macrophage markers and inflammatory mediators, including CD68, CD48, IL-1 α , IL-1 β , S100A8, S100A9, IL-1R, TLR-4, COX-2, and IL-1RA. There was increased expression of CD68, CD48, IL-1 α , IL-1 β , IL-1R, and TLR-4 in frozen shoulder relative to comparator capsular tissues. Cells expressing IL-1 α , IL-1 β , and IL-1R were in proximity of each other, making cellular interactions likely. ScRNAseq revealed six distinct fibroblast populations in the rotator interval of the GH joint capsule, all of which highly expressed *IL1R1*, suggesting a potential macrophage-fibroblast interaction via the IL-1-axis. Ligand-receptor interaction analysis predicted several important IL-1 ligand-receptor pairs between macrophages and fibroblasts, including *IL1A-IL1R1* and *IL1B-IL1R1*. In turn, fibroblasts are predicted to communicate with myeloid cell populations via the *CSF1-CSF1R* pathway, suggesting that macrophage-fibroblast cross talk is contributes to the pathophysiology of frozen shoulder.

This chapter builds on the knowledge of Chapter 2 and further supports that $CD48^+IL1A^+$ macrophages are involved in sustaining inflammatory processes in advanced-stage frozen shoulder. $CD48^+IL1A^+$ macrophages are likely stimulating both fibroblasts as well as macrophages via the IL-1-axis, promoting ongoing inflammation and fibrosis.

Key findings Chapter 3

- $CD48^+IL1A^+$ macrophages are localised to both the lining and sub-lining regions of the rotator interval of the GH joint capsule.
- Protein expression of important macrophage markers and inflammatory mediators are present in GH joint capsular tissues, including CD68, CD48, IL-1 α , IL-1 β , S100A8, S100A9, IL-1R, TLR-4, COX-2, and IL-1RA.
- The expression of inflammatory mediators is increased in frozen shoulder relative to comparator tissues, including interleukin family members IL-1 α , IL-1 β , and IL-1R.
- The $COL1A1^+PDGFRA^+FAP^+$ fibroblast population of the rotator interval of the GH joint capsule consists of six distinct fibroblast populations, all of which highly express *IL1R1*.
- Ligand-receptor interaction analysis predicts several important IL-1 ligand-receptor pairs between $CD48^+IL1A^+$ macrophages and fibroblast populations, including *IL1A-IL1R1* and *IL1B-IL1R1*.
- Fibroblast populations are predicted to communicate with myeloid cell populations via the *CSF1-CSF1R* pathway.

Investigating the IL-1-axis in frozen
shoulder using an *in vitro* model of
patient-derived cells

4.1 Introduction

The previous chapters identify the IL-1-axis as a candidate pathway that could sustain inflammation and drive fibrosis in advanced-stage frozen shoulder. IL-1 α and IL-1 β are well-studied inflammatory mediators, both of which share a common receptor (IL-1R) [199, 229]. IL-1 α and IL-1 β are secreted by mainly myeloid cells upon cellular stress, after which they activate the IL-1R downstream signalling pathways (i.e., the NF- κ B and the mitogen-activated protein kinase (MAPK) pathways), resulting in an increased expression of inflammatory mediators, including additional IL-1 proteins [199, 229, 260].

A natural IL-1R antagonist (IL-1RA) is also released under inflammatory conditions and is thought to be mainly anti-inflammatory [199, 229]. IL-1RA competes with IL-1 α and IL-1 β for the IL-1R to prevent the autoinflammatory feedback loop of IL-1 proteins [199, 229]. This positive autoinflammatory feedback loop has been a therapeutic target in a variety of inflammatory diseases, including RA and gout [199, 232, 235, 261]. Therapies targeting the IL-1-axis prevent IL-1R activation by either blocking the IL-1R or by neutralising IL-1 α and IL-1 β [199, 232, 235, 261].

In frozen shoulder, IL-1 α and IL-1 β were mainly expressed by CD48⁺IL1A⁺ macrophages, whereas their cognate receptor IL-1R was mainly expressed on fibroblasts (Chapters 2 and 3). To investigate whether the ongoing inflammation in advanced stage frozen shoulder is IL-1 driven, this chapter seeks to investigate the effects of IL-1 in frozen shoulder using patient-derived cells *in vitro*. This could inform a rationale for targeting the IL-1-axis in advanced stage frozen shoulder.

4.2 Objectives and hypotheses

This chapter has two objectives. The first objective is to determine if tissue digests from comparator and frozen shoulder capsular tissues show any differences in the levels of inflammatory mediators they secrete. This will be accomplished by using the Meso Scale Discovery (MSD) platform to measure the concentration of inflammatory mediators in supernatants of freshly digested capsular tissues from both comparator and frozen shoulder patient groups. I hypothesise that the freshly digested capsular tissues from frozen shoulder patients secrete higher levels of pro-inflammatory mediators relative to digested capsular tissues from comparator patient groups.

The second objective is to investigate the effects of recombinant IL-1 proteins on patient-derived cells. Fibroblasts isolated from frozen shoulder capsular tissues will be stimulated with recombinant IL-1 α and IL-1 β . In addition, fibroblasts from frozen shoulder capsular tissues will be pre-treated with recombinant IL-1RA (an IL-1R antagonist) prior to stimulation with IL-1. This will help determine if IL-1RA can moderate IL-1 induced cytokine release and expression of inflammatory fibrotic genes in treated fibroblasts. Bulk RNAseq will then inform on the effects of IL-1 on inflammatory and/or fibrotic gene expression profiles in treated fibroblasts. Finally, the supernatants of these cell experiments will also be analysed with the MSD platform to identify how IL-1 stimulation influences the secretion of inflammatory mediators from frozen shoulder fibroblasts. I hypothesise that IL-1 stimulation of fibroblasts derived from frozen shoulder capsular tissues will result in an increase of inflammatory and fibrotic gene expression, as well as an increase of cytokine and chemokine secretion.

4.3 Materials and methods

4.3.1 Ethics statement and tissue collection

Capsular tissues from the rotator interval of the GH joint capsule used in this chapter were collected under the same ethical approval described in Chapter 2, section 2.3.1. Tissue biopsies were collected in line with the protocols described in Chapter 2, section 2.3.2. Capsular tissue samples from patients undergoing surgery for OA (OMB ethics) and from patients undergoing arthroscopic capsular release for frozen shoulder (ICECAP, group C) were used for MSD assays.

4.3.2 MSD multiplex assay to measure levels of secreted inflammatory mediators

To determine differences in secreted inflammatory mediators between frozen shoulder and comparator capsular samples, supernatants of freshly digested capsular tissues were harvested. Upon arrival, a small biopsy sample of freshly acquired capsular tissue was taken for digestion. All samples weighed 0.1g each (n = 2 comparator and n = 2 frozen shoulder). The samples were placed in a 50ml falcon tube and digested in 9ml DMEM-F12 medium with 100 μ l Worthington type 2 collagenase at 4mg/ml for 45 minutes. After digestion, 2ml of supernatant was acquired for analysis and stored at -20°C until use. In addition, supernatants from cell incubations (section 4.3.4) were also collected for analysis and stored at -20°C until use.

Inflammatory mediators and chemokines in supernatants from freshly digested

capsular tissue or cell incubations were quantified using an **MSD** U-PLEX multiplex assay kit (catalogue number K15067L-1, Meso Scale Diagnostics, Rockville, MD USA, Inc.), which included GM-**CSF**, **IL-1 α** , **IL-1 β** , **IL-1RA**, **IL-6**, **IL-10**, **IL-33**, SDF-1 (**CXCL-12**), and **TNF- α** . All reagents used were provided by the manufacturer (Table 4.1).

Table 4.1: Components of the U-PLEX Multiplex Assay kit

Reagents
- Diluent 43
- Diluent 3
- Stop Solution
- MSD GOLD TM Read Buffer B
Biotinylated antibodies
- GM- CSF
- IL-1α
- IL-1β
- IL-1RA
- IL-6
- IL-10
- IL-33
- CXCL-12
- TNF-α
Unique colour-coded Linkers*
- U-PLEX Linker 1
- U-PLEX Linker 2
- U-PLEX Linker 3
- U-PLEX Linker 4
- U-PLEX Linker 5
- U-PLEX Linker 6
- U-PLEX Linker 7
- U-PLEX Linker 8
- U-PLEX Linker 10
Calibrators**
- Calibrator 1 (contains GM- CSF , IL-1β , IL-6 , IL-10 , and TNF-α)
- Calibrator 3 (contains IL-1α)
- Calibrator 4 (contains CXCL-12)
- Calibrator 6 (contains IL-33)
- Calibrator 9 (contains IL-1RA)
MSD U-PLEX 96-well plate

*Linkers have a biotin-binding domain which binds to the biotinylated antibodies, and a domain that binds with the matching spots on the U-PLEX plate. **Calibrators are buffered diluents containing a mixture of recombinant human proteins.

Preparing the MSD U-PLEX Multiplex Assay

Individual U-PLEX Linker-coupled antibody solutions were created by adding each biotinylated antibody (200 μ l) to an assigned Linker (300 μ l) (Figure 4.1). The mix-

ture was vortexed and incubated at room temperature for 30 minutes. Then, 200 μ l of Stop Solution was added, followed by another 30 minutes of incubation. To create a multiplex coating solution, 600 μ l of each Linker-coupled antibody solution was mixed into a single tube (total of 5.4ml). An additional 600 μ l of Stop Solution was added to make up a total of 6ml of multiplex coating solution. To coat the 96-well U-PLEX plate, 50 μ l of multiplex coating solution was added to each well. The plate was sealed and incubated while shaking for 1 hour at room temperature. Continuing, the plate was washed three times with PBS-T using 150 μ l per well. To generate Calibrator Standard 1 (top of the curve), 50 μ l of Calibrators 1, 3, 4, 6, and 9 were mixed in an Eppendorf tube (Table 4.1; Figure 4.2). For Calibrator Standards 2-8, 225 μ l of Diluent 43 was added to each Eppendorf tube, after which Calibrator Standard 1 was diluted according to the manufacturer instructions (Figure 4.2). After preparing the Calibrator Standards, supernatants of comparator and frozen shoulder capsular samples were randomised and diluted ten-fold ($\sim$ 5 μ l per sample) with Diluent 43, creating a total volume of 50 μ l. Finally, before running the assay, the detection antibody solution was prepared by mixing 60 μ l of each detection antibody (540 μ l) and adding Diluent 3 (5,460 μ l) to bring the final volume up to 6ml.



Figure 4.1: Inflammatory mediators and Linkers (U-PLEX Multiplex Assay kit). A U-PLEX 9-Assay plate was used with recorded antibodies and assigned Linkers. Number 6 was not assigned.

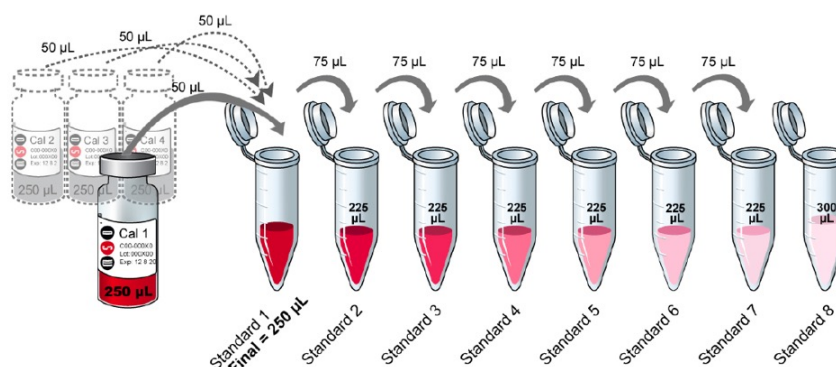


Figure 4.2: Dilution of Calibrator Standards (U-PLEX Multiplex Assay kit).

A dilution scheme for preparing the Calibrator Standards for the U-PLEX Multiplex Assay Kit. Calibrator Standard 8 is made up of 300µl Diluent 43 (zero Calibrator - bottom of the curve). *Image adapted from the manufacturer protocol (MSD U-PLEX multiplex assay kit, catalogue number K15067L-1).*

Running the U-PLEX Multiplex Assay plate

First, 50µl of the prepared Calibrator Standards or (diluted) samples were added to each well, after which the plate was sealed and incubated while shaking at room temperature for 1 hour. Second, the plate was washed three times with **PBS-T** using 150µl per well. Next, 50µl of detection antibody solution was added to each well, after which the plate was sealed and incubated at room temperature for 1 hour again. Finally, the plate was washed three times with **PBS-T** using 150µl per well, and 150µl of **MSD** GOLD Read Buffer B was added to each well for analysis on the **MSD** Sector Imager SQ120 plate reader.

Analysis

Discovery Workbench 4.0 software (Windows 10) was used to analyse the results of the U-PLEX Multiplex assay. A 4-parameter logistic curve fit was used to fit standards. Cytokine and chemokine concentrations were extrapolated from a standard curve. Concentrations below the limit of detection (**LOD**) were recorded as the

lower LOD value. Concentrations above the LOD were assigned as the upper LOD value. The detection ranges and standard curves of each analyte can be found in Appendices C.1 and C.2. Visualisation and statistical analyses were performed with GraphPad Prism (GraphPad Software, version 9.4.0). Due to the small sample, no statistical analysis was performed for differences in concentration between supernatants from freshly digested frozen shoulder ($n = 2$) and comparator ($n = 2$) capsular tissue. The paired T test (parametric) or the Wilcoxon matched pairs signed rank test (non-parametric) was used to detect statistically significant differences in cytokine or chemokine concentrations between different cell experiment conditions. A p value of <0.05 was considered statistically significant.

4.3.3 Cell culture: isolation of patient-derived capsular fibroblasts

Capsular tissues collected from the rotator interval of frozen shoulder patients were cut into small explants (2mm^3) in a tissue culture hood, removing tendon, synovium, and fat where attached. Capsular explants were incubated in a 6-well plate with DMEM-F12 medium containing 50% FCS and 1% penicillin-streptomycin (2ml per well). Medium was replaced twice a week and cells were allowed to grow out the explants over time in a tissue culture incubator at 37°C and 5% CO_2 . After approximately 2 weeks, explants were removed, and cells were cultured in medium containing DMEM-F12 with 10% FCS and 1% penicillin-streptomycin. Cells were allowed to grow until approximately 90% confluence was reached. Finally, the medium was replaced and the cells were scraped. Cells in suspension were centrifuged at 350g for 4 minutes. The supernatant was discarded, and cells were resuspended in 2ml of freezing medium containing FCS with 10% dimethyl sulfoxide (DMSO)

(Sigma Aldrich). Cells suspended in the freezing medium were cryopreserved at a temperature of -80°C until ready for use.

4.3.4 IL-1 stimulation of capsule-derived cells

To investigate the role of the IL-1-axis in the pathophysiology of frozen shoulder, capsular-derived fibroblasts from frozen shoulder patients (n = 3 donors) were stimulated with recombinant human IL-1 α and/or IL-1 β , with and without IL-1RA blockade (Figure 4.3). Recombinant human IL-1 α , IL-1 β , and IL-1RA (R&D Systems, catalogue numbers 200-LA, 201-LB, and 280-RA respectively) were reconstituted in sterile PBS containing 0.1% bovine serum albumin as per manufacturer instructions. A working concentration of 10ng/ml was used for each of the recombinant proteins in starvation medium (DMEM-F12 medium containing 3% FCS and 1% penicillin-streptomycin). Cells were treated with control (starvation) medium (1), recombinant human IL-1 α (2), recombinant human IL-1 β (3), or recombinant human IL-1 α and IL-1 β combined (4) for 48 hours (Figure 4.3). Finally, for the blockade experiment, cells were pre-treated with recombinant human IL-1RA one hour before treating the cells with a combination of recombinant human IL-1 α , IL-1 β , and IL-1RA (5) for 48 hours (Figure 4.3). After 48 hours, supernatants were collected for MSD assay and cells were lysed for RNA extraction. Supernatants were stored at -20°C until use.

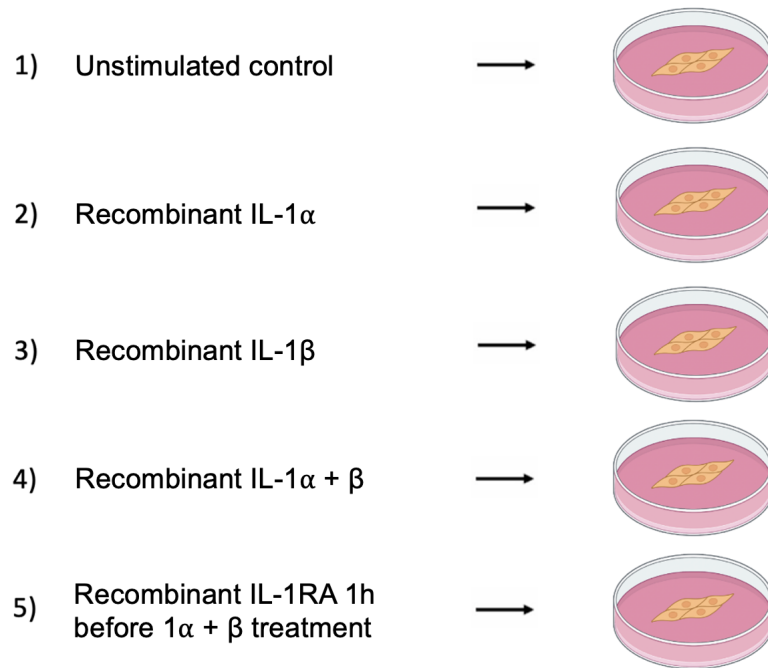


Figure 4.3: **IL-1** experiments on frozen shoulder capsular-derived fibroblasts. Capsular-derived fibroblasts from frozen shoulder patients were either left unstimulated, or treated with **IL-1 α** , with **IL-1 β** , with both **IL-1 α** and **IL-1 β** , or with **IL-1RA** one hour before treatment with **IL-1 α** , **IL-1 β** , and **IL-1RA**. This experiment was run three times with cells from three different frozen shoulder donors.

4.3.5 **RNA** extraction of cell lysates

RNA extraction was performed according to the protocol provided by the RNeasy® Micro Kit by Qiagen (RNeasy Micro Kit, catalogue number 74004, Qiagen). All reagents were provided by the manufacturer. After the **IL-1** experiments were performed, treated cells were harvested and **RNA** was then extracted via several steps as described in the protocol from the manufacturer (Qiagen) (Table 4.2).

Table 4.2: **RNA** extraction steps as per RNeasy Micro Kit protocol (Qiagen)

Step	Description
1	Cells were harvested by direct lysis with 350 μ l Buffer RLT. Lysate was extracted in Eppendorf tubes.
2	350 μ l of 70% ethanol was added to the lysate. The sample was transferred to a RNeasy MinElute spin column (Qiagen) in a 2ml collection tube, after which it was centrifuged for 15 seconds at $\geq 8,000 \times g$. The flow-through was discarded.
3	350 μ l of Buffer RW1 was added to the RNeasy MinElute spin column, after which it was centrifuged again for 15 seconds at $\geq 8,000 \times g$. The flow-through was discarded.
4	DNase I incubation mix was prepared by adding 10 μ l of DNase I stock solution to 70 μ l Buffer RDD. After mixing, 80 μ l of the DNase I incubation mix was added directly to the RNeasy MinElute spin column membrane. This was left for 15 minutes to incubate at room temperature.
5	350 μ l of Buffer RW1 was added to the RNeasy MinElute spin column, and it was centrifuged for 15 seconds at $\geq 8,000 \times g$. After discarding the collection tube, the RNeasy MinElute spin column was placed in a new collection tube.
6	500 μ l of Buffer RPE was added to the spin column, and it was centrifuged for 2 minutes at $\geq 8,000 \times g$. The flow-through was discarded.
7	500 μ l of 80% ethanol was added to the RNeasy MinElute spin column. The columns were centrifuged for 2 minutes at $\geq 8,000 \times g$, after which the collection tube was discarded.
8	The RNeasy spin column was then placed in a new collection tube and with open lids the columns were centrifuged at full speed for 5 minutes to dry the membrane. The collection tube was discarded.
9	The RNeasy MinElute spin column was placed in a new collection tube and 14 μ l of RNase-free water was added directly to the centre of the spin column membrane. Then, the columns were centrifuged for 1 minute at full speed.
10	Finally, the flow-through (RNA) was transferred to 1.5ml Eppendorf tubes.

Samples were stored in -80°C until ready for bulk **RNAseq**. **RNA** quantification was performed with Nanodrop using the NanoPhotometer® NP80 (Implen). A volume of 30 μ l per sample with a concentration of 3.3-33ng/ μ l was deemed sufficient for PolyA library preparation (as per the Oxford Genomics Centre **QC** protocol). The purity of the samples was considered high, with little to no protein contamination (Table 4.4). The samples were transferred into a 96-well plate, after which the

plate was sealed. The plate was placed in a box with dry ice and was sent off to the Oxford Genomics Centre (Dr Angela Lee) for further **RNA** quantification, **RNA** integrity assessment, normalisation, (PolyA) library preparation, and sequencing on an Illumina NovaSeq6000 v1.5. A sequencing depth of 50 to 60 million reads per sample was used.

Table 4.4: **RNA** quantification and integrity (purity) of **IL**-1 experiments.

Sample	Concentration (ng/ μ l)	Volume (μ l)	260/280 ratio
1_unstimulated	993.0	30.0	1.993
1_ IL -1 α	471.0	30.0	2.252
1_ IL -1 β	681.0	30.0	2.009
1_ IL -1 α + β	585.0	30.0	2.053
1_ IL -1RA	648.0	30.0	1.735
2_unstimulated	1521.0	30.0	2.121
2_ IL -1 α	1227.0	30.0	2.128
2_ IL -1 β	1593.0	30.0	2.137
2_ IL -1 α + β	1335.0	30.0	2.234
2_ IL -1RA	1197.0	30.0	2.267
3_unstimulated	1335.0	30.0	2.134
3_ IL -1 α	1644.0	30.0	2.157
3_ IL -1 β	1755.0	30.0	2.123
3_ IL -1 α + β	1875.0	30.0	2.122
3_ IL -1RA	735.0	30.0	2.021

The **IL**-1 experiment was run three times with cells from three different frozen shoulder donors (Sample numbers 1, 2, 3). 260/280 nucleic acid purity ratios between 2.0 and 2.2 are suggestive of high **RNA** purity. Scores <1.9 are suggestive of protein contamination.

4.3.6 Alignment of the bulk **RNAseq** data

The bulk **RNAseq** data was aligned to the **GRCh38** using an ENCODE pipeline for paired-ended and stranded bulk **RNAseq** data: the STAR program was used for mapping of the reads, whereas the RSEM program was used for quantification of transcripts [262, 263]. A count matrix containing raw counts per gene was generated.

The count matrix was then imported in R (version 4.2.0) (<https://www.r-project.org>) and transformed into a DESeq2Dataset object for differential expression analysis with DESeq2, while controlling for batch (e.g., donor) effects in the design [170].

4.3.7 QC and differential expression analysis

Genes with low counts (<10) were removed from the DESeq2Dataset. Sample level QC was performed using PCA and hierarchical clustering (sample-to-sample distances) after variance stabilising transformation of the raw counts using DESeq2 [172]. The limma package was used to remove batch effects when plotting the PC plot (removeBatchEffect) [264]. The likelihood ratio test (LRT) was used to identify DEGs across different conditions using a padj of <0.001 (BH). Clustering was performed using the DEGreport package to identify groups of genes with similar expression profiles across conditions [265]. For pair-wise comparisons between stimulated conditions and vehicle control fibroblasts, the Wald test was used to generate a table of DEGs. Shrinkage of the $\log_2\text{FCs}$ was performed using the ‘apeglm’ method [266]. Significantly DEGs were filtered out using a padj of <0.05 (BH). To visualise the significant DEGs between conditions, volcano plots were generated using padj values <0.05 and $\log_2\text{FCs}$ of ≥ 1.0 in either direction. In addition, dot plots displaying the significantly DEGs plotted by $\log_{10}$ normalised counts were generated.

4.3.8 Pathway analysis

To assess the functionality of the DEGs and genesets that were identified from the bulk RNAseq using DESeq2, geneset enrichment analysis (GSEA) was performed using the GO and the Kyoto Encyclopedia of Genes and Genomes (KEGG) data-

bases in combination with ClusterProfiler in R [174–176, 267]. The most significantly enriched pathways and biological processes were visualised with dot plots displaying the gene count, gene ratio, and **padj** values (**BH** correction).

4.4 Results

4.4.1 Secretion of inflammatory mediators in supernatants of digested GH capsular tissues

Levels of inflammatory mediators were measured in supernatants of collagenase digested capsular tissues ($n = 2$ frozen shoulder and $n = 2$ comparator capsular tissues). Besides the IL-6 concentration in one comparator sample (0.869 pg/ml), the concentrations of the other inflammatory mediators were all below the LOD in supernatant of both frozen shoulder and comparator capsular tissue digest (Figure 4.4). Hence there were no perceivable differences in concentrations of inflammatory mediators between the supernatants of frozen shoulder and comparator capsular tissues (Figure 4.4). The detection ranges and standard curves of each inflammatory mediator can be found in Appendices C.1 and C.2.

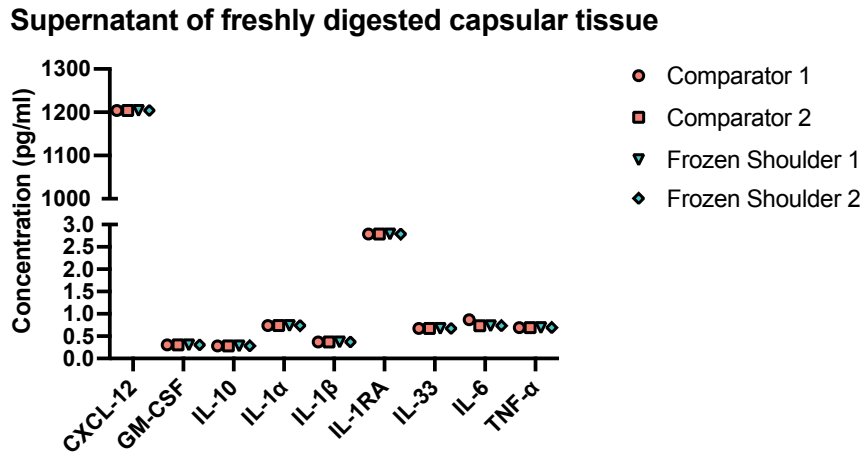


Figure 4.4: Inflammatory mediators secreted by freshly digested capsular tissue. Detection of inflammatory mediators in supernatants of freshly digested capsular tissue (0.1g) from frozen shoulder ($n = 2$) and comparator ($n = 2$) patient groups. Capsular tissues were cut in small pieces and were digested with collagenase for 45 minutes. Supernatants were assayed at a dilution of 1:10. Concentrations below the LOD were assigned the LOD value.

4.4.2 Investigating the effects of IL-1 on patient-derived cells

To investigate the role of IL-1 α and IL-1 β on fibroblasts from frozen shoulder patients, fibroblasts were stimulated with either IL-1 α , IL-1 β , or a combination of IL-1 α and IL-1 β . For the IL-1R blockade experiment, fibroblasts were treated with IL-1RA one hour prior to stimulation with IL-1 α , IL-1 β , and IL-1RA. These conditions were compared to unstimulated fibroblasts (control). Bulk RNAseq analysis was performed to identify differences in gene expression between these conditions. PCA analysis revealed a clear separation between unstimulated fibroblasts and stimulated fibroblasts, whereas the stimulated fibroblasts grouped in proximity of each other (Figure 4.5). Hierarchical clustering showed high overall correlation between the conditions, particularly between stimulated fibroblasts (Figure 4.6).

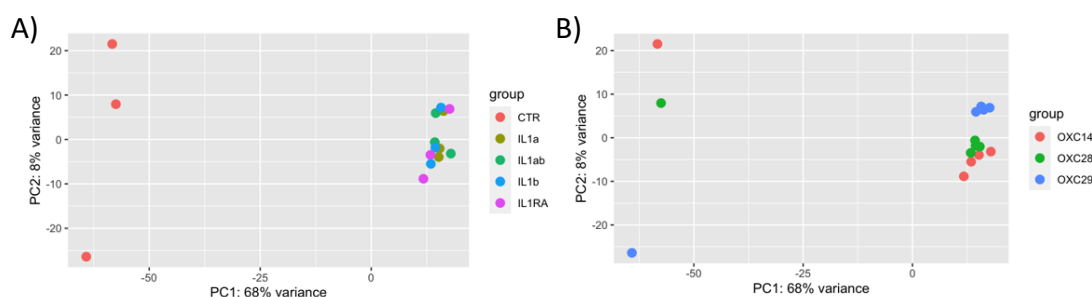


Figure 4.5: QC of the bulk RNAseq data: PCA.

A) Grouping of samples ($n = 15$) by condition after dimensionality reduction by PCA for the first two PCs. CTR, unstimulated fibroblasts (control); IL1a, IL-1 α stimulated fibroblasts; IL1b, IL-1 β stimulated fibroblasts; IL1ab, IL-1 α & IL-1 β stimulated fibroblasts; IL1RA, fibroblasts pre-treated with IL-1RA before IL-1 α , IL-1 β , and IL-1RA stimulation. B) Grouping of samples by donor (OXC14, OXC28, OXC29) after dimensionality reduction by PCA for the first two PCs. The distribution of variance among the PCs is plotted.

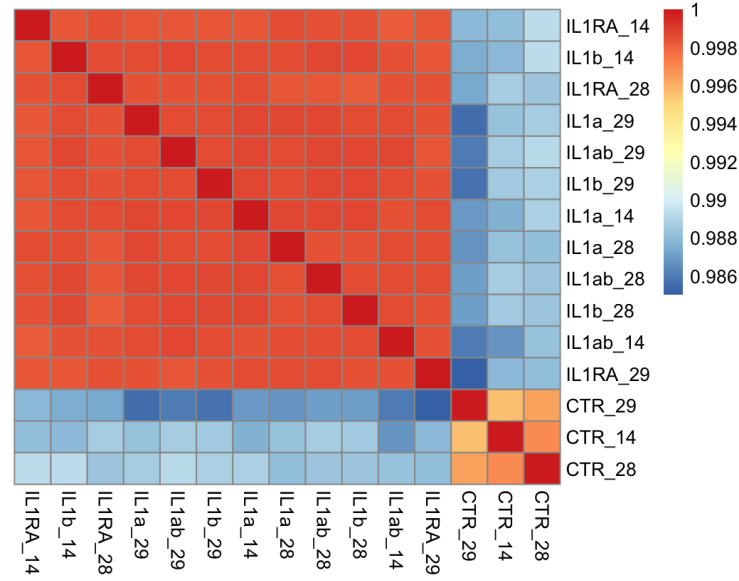


Figure 4.6: QC of the bulk RNAseq data: hierarchical clustering.

Hierarchical clustering heatmap revealing a high overall correlation between the different experiment conditions (>0.985). The different donors are given a number (14, 28, and 29). CTR, unstimulated fibroblasts (control); IL1a, IL-1 α stimulated fibroblasts; IL1b, IL-1 β stimulated fibroblasts; IL1ab, IL-1 α & IL-1 β stimulated fibroblasts; IL1RA, fibroblasts pre-treated with IL-1RA before IL-1 α , IL-1 β , and IL-1RA stimulation.

Differential expression analysis was performed to identify significantly down- and upregulated genes between the different conditions. Clustering of genes revealed eight gene clusters, with each cluster representing a gene expression pattern shared among different DEGs (Figure 4.7). The expression patterns highlighted differences in gene expression between unstimulated and stimulated fibroblasts (Figure 4.7). A heatmap of the top 1,000 DEGs across conditions revealed clear differences in gene expression between unstimulated and stimulated fibroblasts (Figure 4.8). There were little differences in gene expression between the different IL-1 stimulated conditions (Figure 4.8). Top upregulated DEGs in IL-1 stimulated fibroblast conditions included inflammatory and fibrotic genes *CSF3*, *PDE4D*, *WNT5A*, *TNFAIP2*, *NFKBIA*, *TIMP1*, *NFKBIZ*, *CCL2*, *IL6*, *CCL20*, *CSF2*, *CCL8*, *FGF7*, *C1QTNF1*, *CCL5*,

TGFBR1, *TMEM132A*, *CXCL5*, *IGF1*, and *COL3A1* (Figure 4.9). Top upregulated DEGs in unstimulated fibroblasts included *PPL*, *AQP1*, *PLEKHA4*, *ANXA2*, *MYO1D*, *MYL12B*, *CCN3*, and *ASPN* (Figure 4.9).

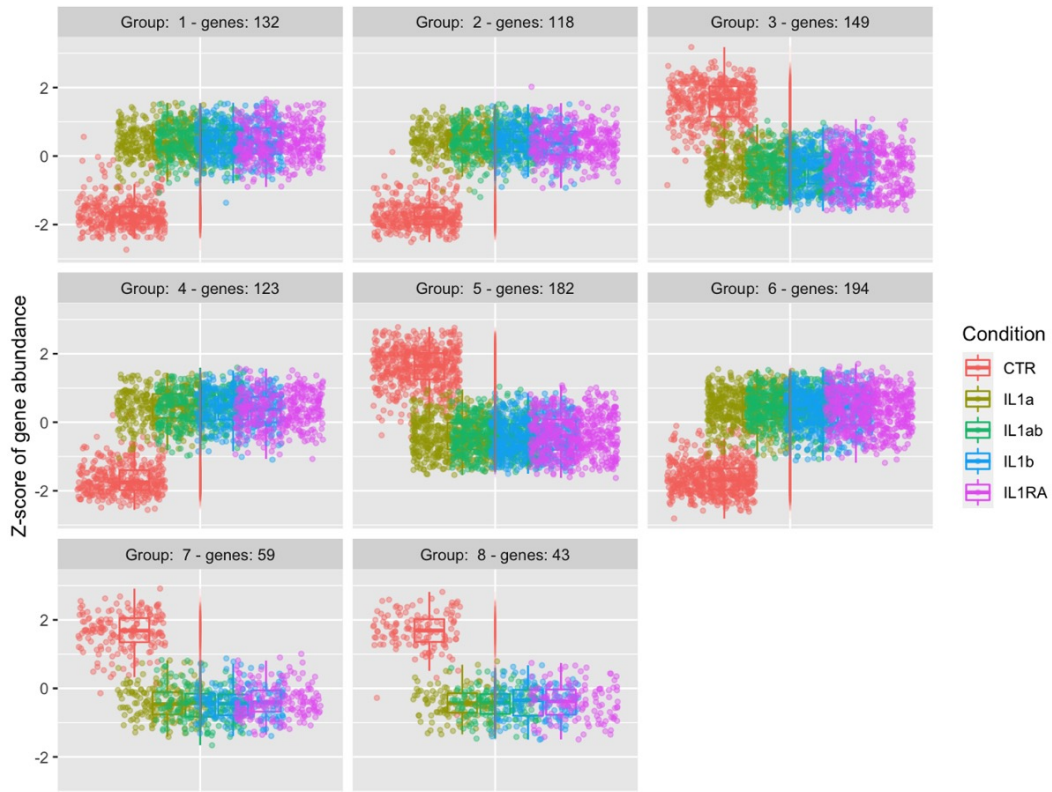


Figure 4.7: Clustered and scaled expression patterns for the top 1,000 DEGs across unstimulated and IL-1 stimulated conditions.

Clustering revealed eight clusters of DEGs with similar gene expression patterns across conditions. Box plots are displaying the mean Z-scores of gene abundance with standard deviation (red line). CTR, unstimulated fibroblasts (control); IL1a, IL-1 α stimulated fibroblasts; IL1b, IL-1 β stimulated fibroblasts; IL1ab, IL-1 α & IL-1 β stimulated fibroblasts; IL1RA, fibroblasts pre-treated with IL-1RA before IL-1 α , IL-1 β , and IL-1RA stimulation.

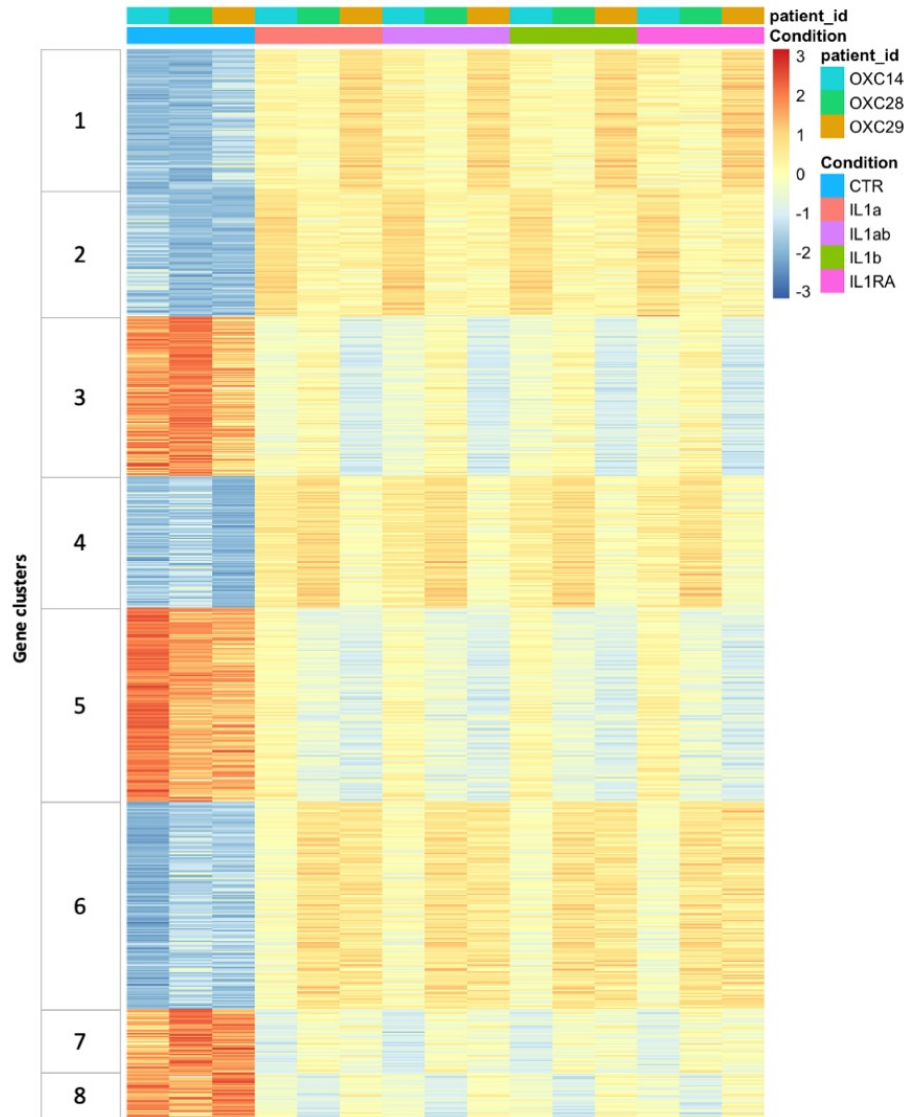


Figure 4.8: Clustering of the top 1,000 **DEGs** across unstimulated and **IL-1** stimulated conditions.

A heatmap of the top 1,000 **DEGs** across different conditions, sorted by gene cluster ($n = 8$). Each cluster shows **DEGs** with similar gene expression patterns across conditions. The different donors are given a number (14, 28, and 29). CTR, unstimulated fibroblasts (control); IL1a, **IL-1 α** stimulated fibroblasts; IL1b, **IL-1 β** stimulated fibroblasts; IL1ab, **IL-1 α** & **IL-1 β** stimulated fibroblasts; IL1RA, fibroblasts pre-treated with **IL-1RA** before **IL-1 α** , **IL-1 β** , and **IL-1RA** stimulation.

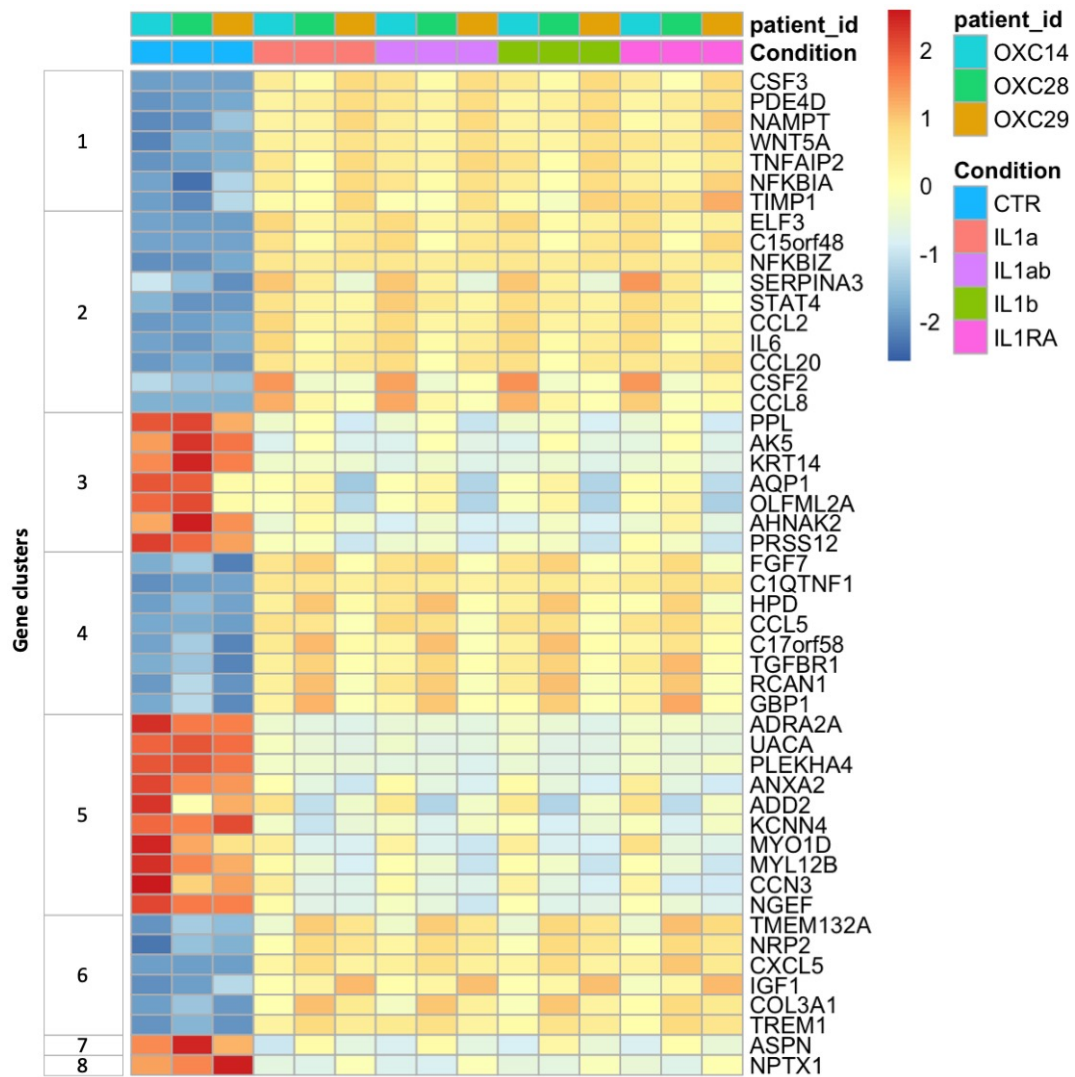


Figure 4.9: Clustering of the top 50 DEGs across unstimulated and IL-1 stimulated conditions.

A heatmap of the top 50 DEGs across different conditions, sorted by gene cluster ($n = 8$). Each cluster shows DEGs with similar gene expression patterns across conditions. The different donors are given a number (14, 28, and 29). CTR, unstimulated fibroblasts (control); IL1a, IL-1 α stimulated fibroblasts; IL1b, IL-1 β stimulated fibroblasts; IL1ab, IL-1 α & IL-1 β stimulated fibroblasts; IL1RA, fibroblasts pre-treated with IL-1RA before IL-1 α , IL-1 β , and IL-1RA stimulation.

4.4.3 Pair-wise comparisons of IL-1 stimulated fibroblast conditions

Pair-wise comparisons of unstimulated (control) and stimulated fibroblasts were performed. Differential expression analysis revealed 3,394 DEGs between IL-1 α stimulated fibroblasts and unstimulated fibroblasts, of which 1,584 were down- and 1,810 up-regulated (Table 4.5; Figure 4.10A). The top 25 significantly DEGs included mostly genes that are upregulated in IL-1 α stimulated fibroblasts, including *NFKBIZ*, *PDE4D*, *FGF7*, *CXCL5*, *TMEM132A*, *CCL5*, *CXCL3*, *IGF1*, *NRP2*, and *IL6* (Figure 4.10B&C). These genes were also upregulated in the other IL-1 stimulated conditions relative to unstimulated fibroblasts (Figure 4.10C). *PPL* was the most significantly downregulated gene in IL-1 α stimulated fibroblasts relative to unstimulated fibroblasts.

Table 4.5: Differential expression analysis: pair-wise comparisons of IL-1 stimulated fibroblasts.

Pair-wise comparison	DEGs	Up-regulated genes	Down-regulated genes
IL-1 α vs. unstimulated (control)	3,394	1,810	1,584
IL-1 β vs. unstimulated (control)	3,319	1,784	1,535
IL-1 α + β vs. unstimulated (control)	3,343	1,805	1,538
IL-1RA* vs. unstimulated (control)	3,202	1,740	1,462
IL-1 α vs. IL-1RA*	2	2	0
IL-1 β vs. IL-1RA*	1	1	0
IL-1 α + β vs. IL-1RA*	0	0	0

Differential expression analysis between two conditions (pair-wise comparisons) using the Wald test with an p_{adj} of 0.05 (BH). A total of 20,869 genes were used for analysis. *Fibroblasts that have been pre-treated with IL-1RA one hour before stimulation with IL-1 α , IL-1 β , and IL-1RA.

Pair-wise comparisons between the other IL-1 stimulated fibroblast groups and unstimulated fibroblasts generated similar results (Table 4.5; Figures 4.11 to 4.13). The top DEGs included mostly upregulated genes of inflammatory mediators, similar to IL-1 α stimulated fibroblasts (Figures 4.11 to 4.13). Interestingly, there were

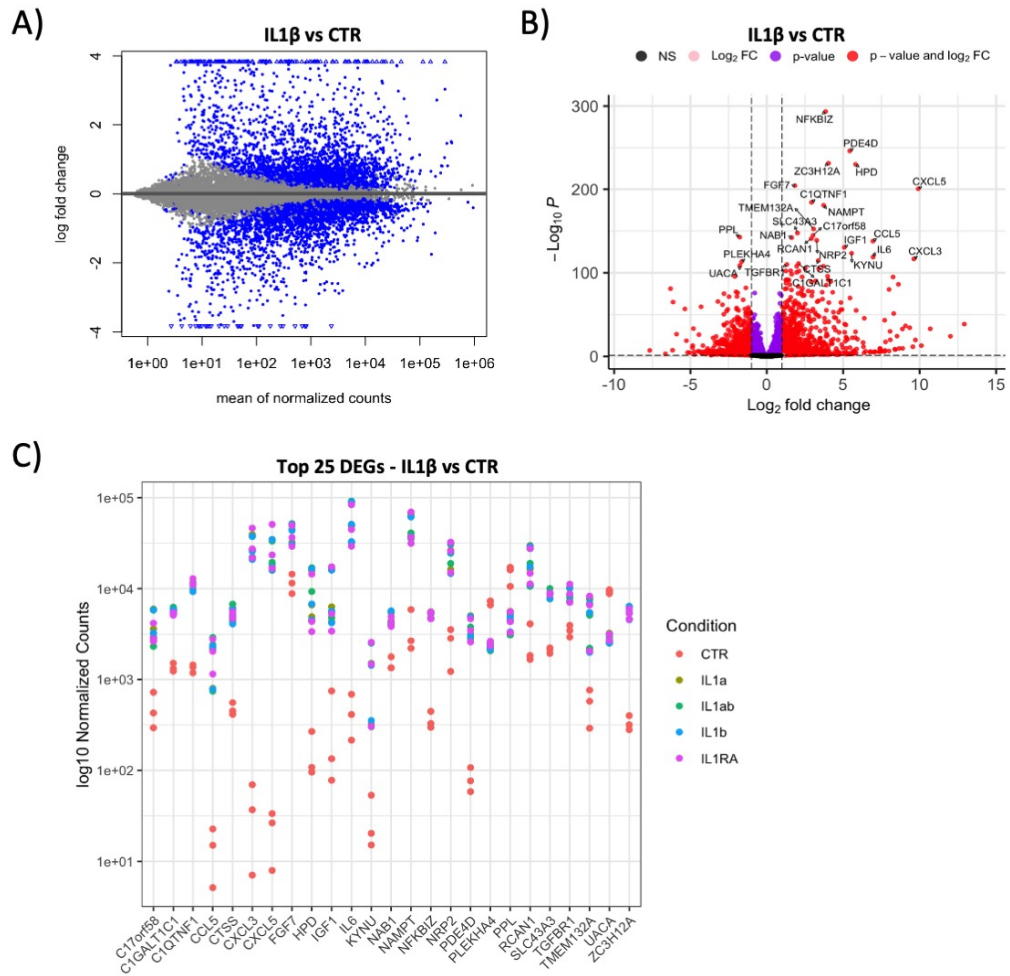


Figure 4.11: Differential expression analysis of **IL-1 β** stimulated fibroblasts relative to unstimulated fibroblasts.

A) MA plot displaying the mean normalised counts versus the shrunk $\log_2$ FCs for all genes. Grey colour displays genes that are not differentially expressed, whereas the blue colour displays **DEGs** in **IL-1 β** stimulated fibroblasts ($n = 3,394$). Points (genes) which fall out of the window are plotted as open triangles. B) Volcano plot displaying the top significantly down- (negative $\log_2$ FC; left) and upregulated (positive $\log_2$ FC; right) genes in **IL-1 β** stimulated fibroblasts relative to control fibroblasts. P_{adj} (BH) set at 0.05 and $\log_2$ FC ≥ 1 in either direction. C) The top 25 significantly **DEGs** in **IL-1 β** stimulated fibroblasts plotted by $\log_{10}$ normalised counts. CTR, unstimulated fibroblasts (control); IL1a, **IL-1 α** stimulated fibroblasts; IL1b, **IL-1 β** stimulated fibroblasts; IL1ab, **IL-1 α** & **IL-1 β** stimulated fibroblasts; IL1RA, fibroblasts pre-treated with **IL-1RA** before **IL-1 α** , **IL-1 β** , and **IL-1RA** stimulation.

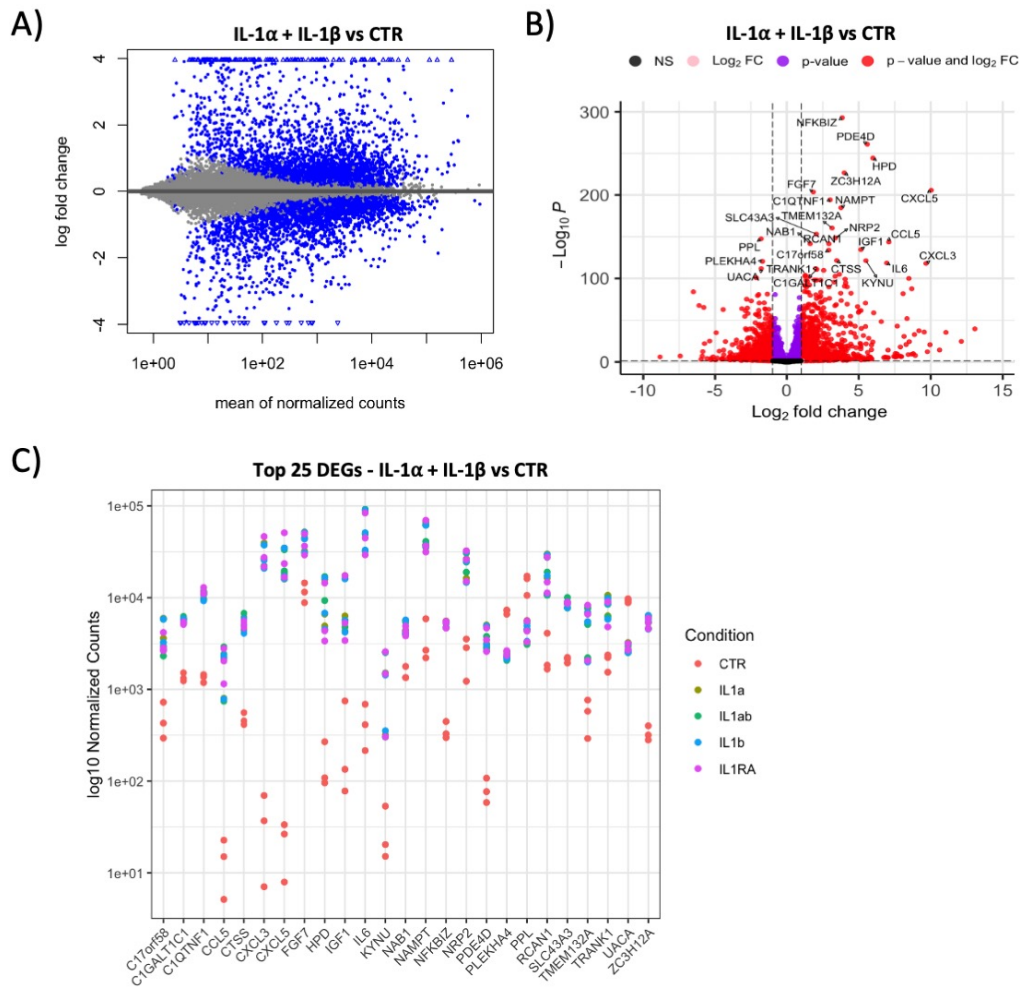


Figure 4.12: Differential expression analysis of **IL-1α** & **IL-1β** stimulated fibroblasts relative to unstimulated fibroblasts.

A) MA plot displaying the mean normalised counts versus the shrunk **log₂FCs** for all genes. Grey colour displays genes that are not differentially expressed, whereas the blue colour displays **DEGs** in **IL-1α** & **IL-1β** stimulated fibroblasts ($n = 3,394$). Points (genes) which fall out of the window are plotted as open triangles. B) Volcano plot displaying the top significantly down- (negative **log₂FC**; left) and upregulated (positive **log₂FC**; right) genes in **IL-1α** & **IL-1β** stimulated fibroblasts relative to control fibroblasts. **P_{adj}** (BH) set at 0.05 and **log₂FC** ≥ 1 in either direction. C) The top 25 significantly **DEGs** in **IL-1α** & **IL-1β** stimulated fibroblasts plotted by log₁₀ normalised counts. CTR, unstimulated fibroblasts (control); IL1a, **IL-1α** stimulated fibroblasts; IL1b, **IL-1β** stimulated fibroblasts; IL1ab, **IL-1α** & **IL-1β** stimulated fibroblasts; IL1RA, fibroblasts pre-treated with **IL-1RA** before **IL-1α**, **IL-1β**, and **IL-1RA** stimulation.

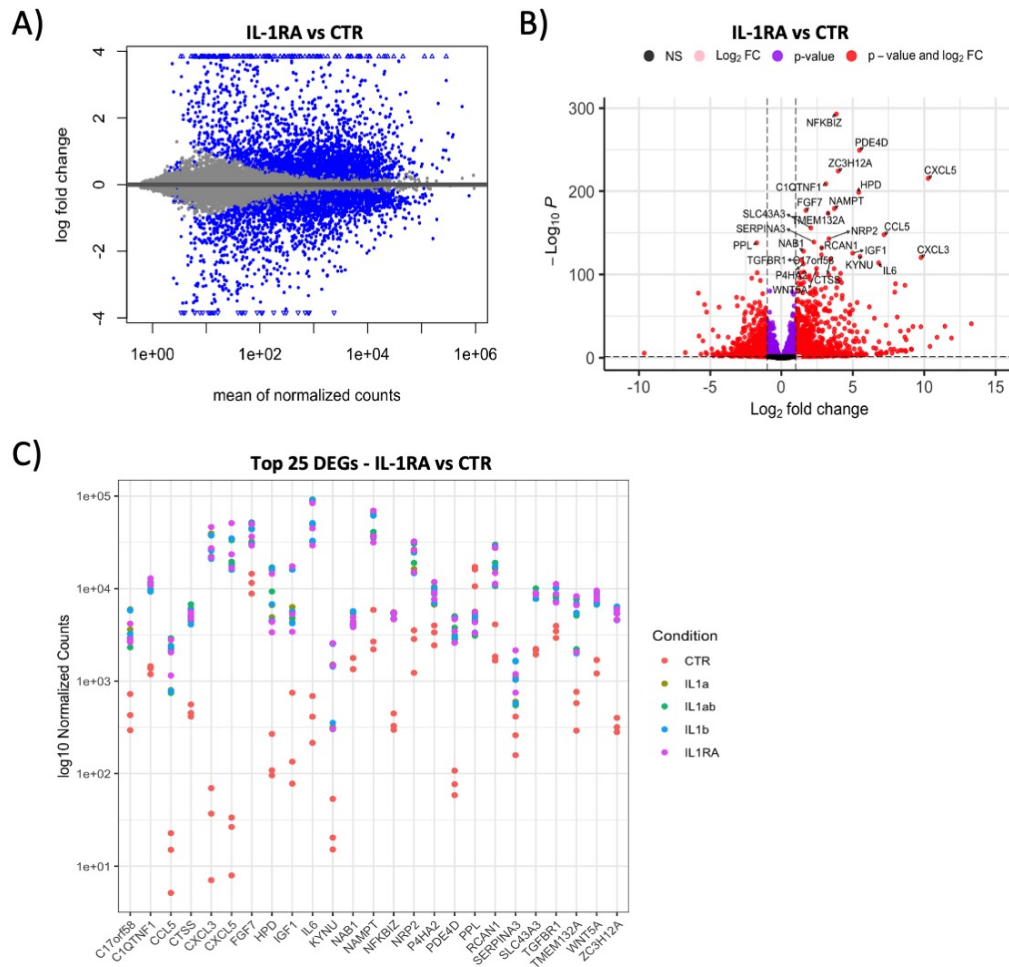


Figure 4.13: Differential expression analysis of **IL-1RA** pre-treated fibroblasts relative to unstimulated fibroblasts.

A) MA plot displaying the mean normalised counts versus the shrunk **log₂FCs** for all genes. Grey colour displays genes that are not differentially expressed, whereas the blue colour displays **DEGs** in **IL-1RA** pre-treated fibroblasts ($n = 3,394$). Points (genes) which fall out of the window are plotted as open triangles. B) Volcano plot displaying the top significantly down- (negative **log₂FC**; left) and upregulated (positive **log₂FC**; right) genes in **IL-1RA** pre-treated fibroblasts relative to control fibroblasts. **Padj** (BH) set at 0.05 and **log₂FC** ≥ 1 in either direction. C) The top 25 significantly **DEGs** in **IL-1RA** pre-treated fibroblasts plotted by log₁₀ normalised counts. CTR, unstimulated fibroblasts (control); IL1a, **IL-1 α** stimulated fibroblasts; IL1b, **IL-1 β** stimulated fibroblasts; IL1ab, **IL-1 α** & **IL-1 β** stimulated fibroblasts; IL1RA, fibroblasts pre-treated with **IL-1RA** before **IL-1 α** , **IL-1 β** , and **IL-1RA** stimulation.

4.4.4 Pathway analyses of IL-1 stimulated fibroblasts from frozen shoulder patients

To further assess the functionality of the different IL-1 stimulated fibroblasts, enrichment analysis was performed using the GO database for biological processes. GSEA of IL-1 α stimulated fibroblasts revealed significant enrichment for the GO biological processes related to the regulation of immune response, response to cytokine, leukocyte activation, cell activation, positive regulation of immune system process and more (Figure 4.14). Suppressed GO terms in IL-1 α stimulated fibroblasts relative to unstimulated fibroblasts included GO biological processes related to cellular processes, including cell cycle processes and DNA replication processes (Figure 4.14). A cnetplot (i.e., a network depicting linkages of genes and biological processes) was created to visualise the upregulated genes that make up the top GO terms, i.e., regulation of immune response and response to cytokine (Figure 4.15). Genes associated with these GO terms included a variety of inflammatory genes, including *CCL* and *CXCL* chemokines, *CSF1*, *CSF2*, *CSF3*, *IL1A*, *IL1B*, *IL33*, *IL6*, *NFKB1*, *ADAM17*, *PTGS2*, *C3*, and many more (Figure 4.15).



Figure 4.14: GO pathway analysis of IL-1 α stimulated fibroblasts.

Pathway analysis of IL-1 α stimulated fibroblasts using the GO biological processes (BP) database. Dot plot displaying the top 15 activated and suppressed genesets in IL-1 α stimulated fibroblasts. The size of the dots represents the number of DEGs associated with a GO term (Count). The colour of the dots represents the adjusted p values (p.adjust) (BH). GeneRatio displays the fraction of DEGs found in a particular geneset.

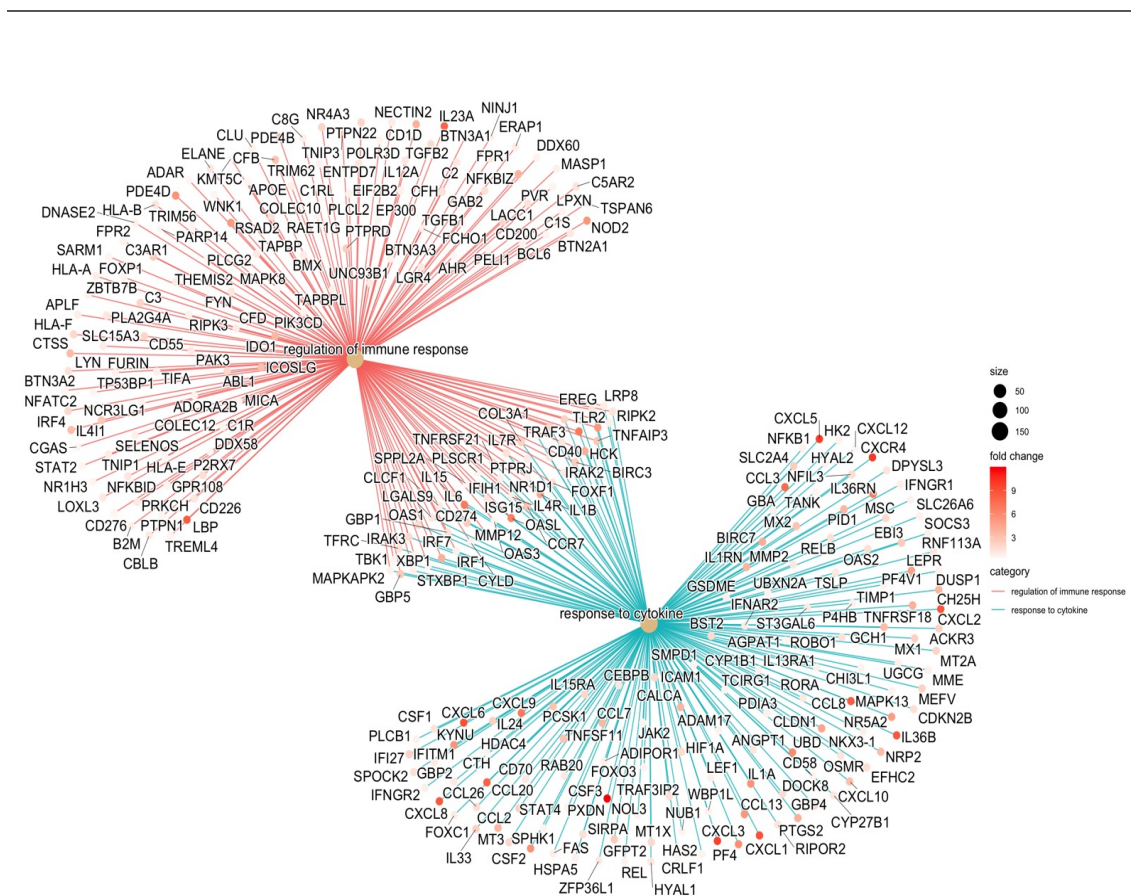


Figure 4.15: Visualisation of enriched GO terms in IL-1 α stimulated fibroblasts. Cnetplot showing genes associated with two enriched GO terms of the biological processes category, including response to cytokine (blue lines) and regulation of immune response (red lines). The size of the dots (GO terms) represents the number of genes associated with a GO term. The colour of the dots (genes) represents the fold change ($\log_2FC$) relative to unstimulated fibroblasts.

There were little to no differences in enriched GO terms between the different IL-1 stimulated conditions, as the same top GO terms were seen across all stimulated conditions (Figures 4.16 to 4.18). A table of the top 100 genesets sorted by condition can be found in Appendix C.4. Additional GSEA using the KEGG database revealed that IL-1 stimulated fibroblasts were enriched for several pro-inflammatory KEGG pathways, including cytokine-cytokine receptor interaction, TNF signalling pathway, RA, NF- κ B signalling pathway, and chemokine signalling pathway (Figure 4.19).



Figure 4.16: GO pathway analysis of IL-1 β stimulated fibroblasts.

Pathway analysis of IL-1 β stimulated fibroblasts using the GO biological processes (BP) database. Dot plot displaying the top 15 activated and suppressed genesets in IL-1 β stimulated fibroblasts. The size of the dots represents the number of DEGs associated with a GO term (Count). The colour of the dots represents the adjusted p values (p.adjust) (BH). GeneRatio displays the fraction of DEGs found in a particular geneset.

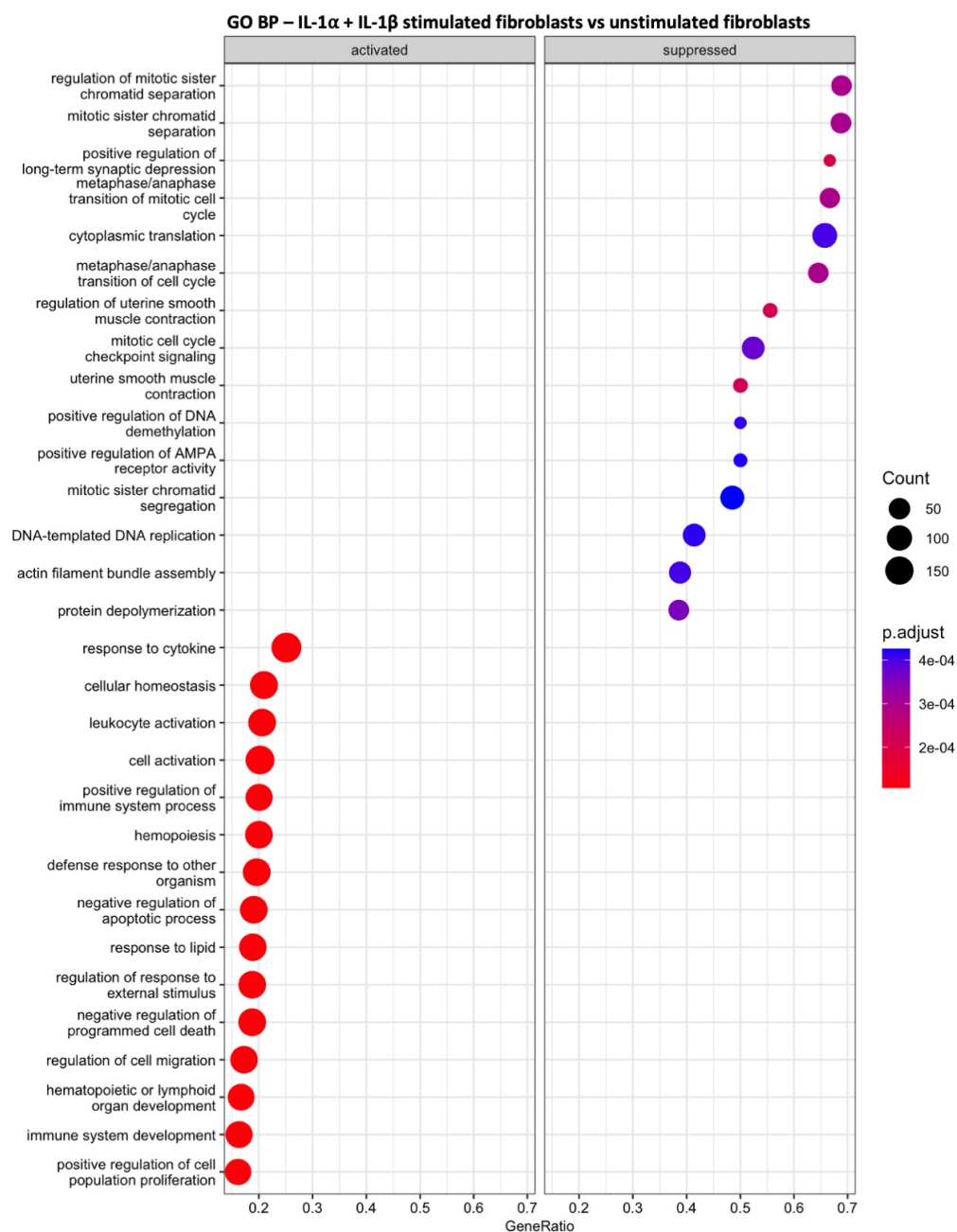


Figure 4.17: GO pathway analysis of IL-1 α + IL-1 β stimulated fibroblasts.

Pathway analysis of IL-1 α and IL-1 β stimulated fibroblasts using the GO biological processes (BP) database. Dot plot displaying the top 15 activated and suppressed genesets in IL-1 α and IL-1 β stimulated fibroblasts. The size of the dots represents the number of DEGs associated with a GO term (Count). The colour of the dots represents the adjusted p values (p.adjust) (BH). GeneRatio displays the fraction of DEGs found in a particular geneset.

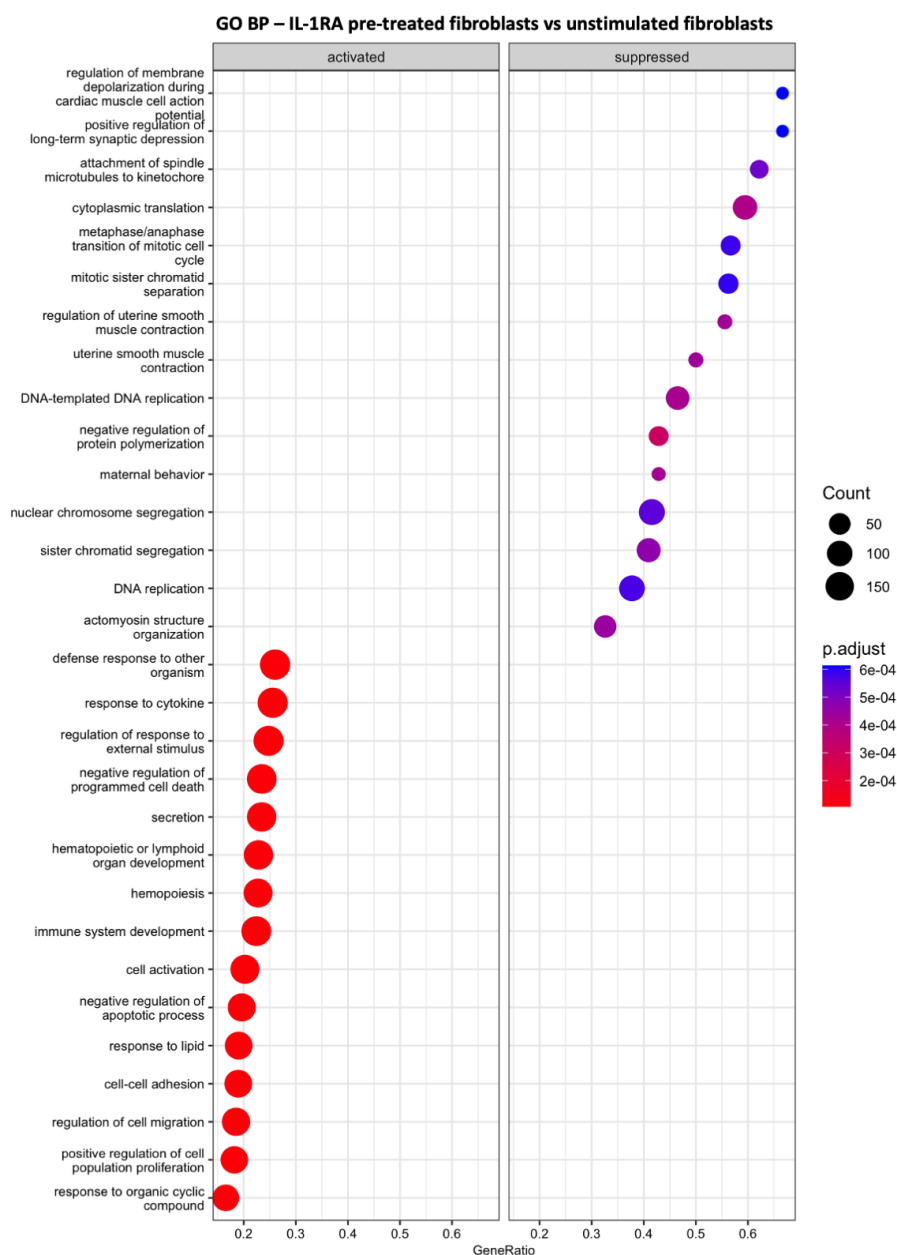


Figure 4.18: **GO** pathway analysis of **IL-1RA** pre-treated fibroblasts. Pathway analysis of fibroblasts pre-treated with **IL-1RA** before **IL-1 α** , **IL-1 β** , and **IL-1RA** stimulation using the **GO** biological processes (BP) database. Dot plot displaying the top 15 activated and suppressed genesets in **IL-1RA** pre-treated fibroblasts. The size of the dots represents the number of **DEGs** associated with a **GO** term (Count). The colour of the dots represents the adjusted p values (p.adjust) (BH). GeneRatio displays the fraction of **DEGs** found in a particular geneset.

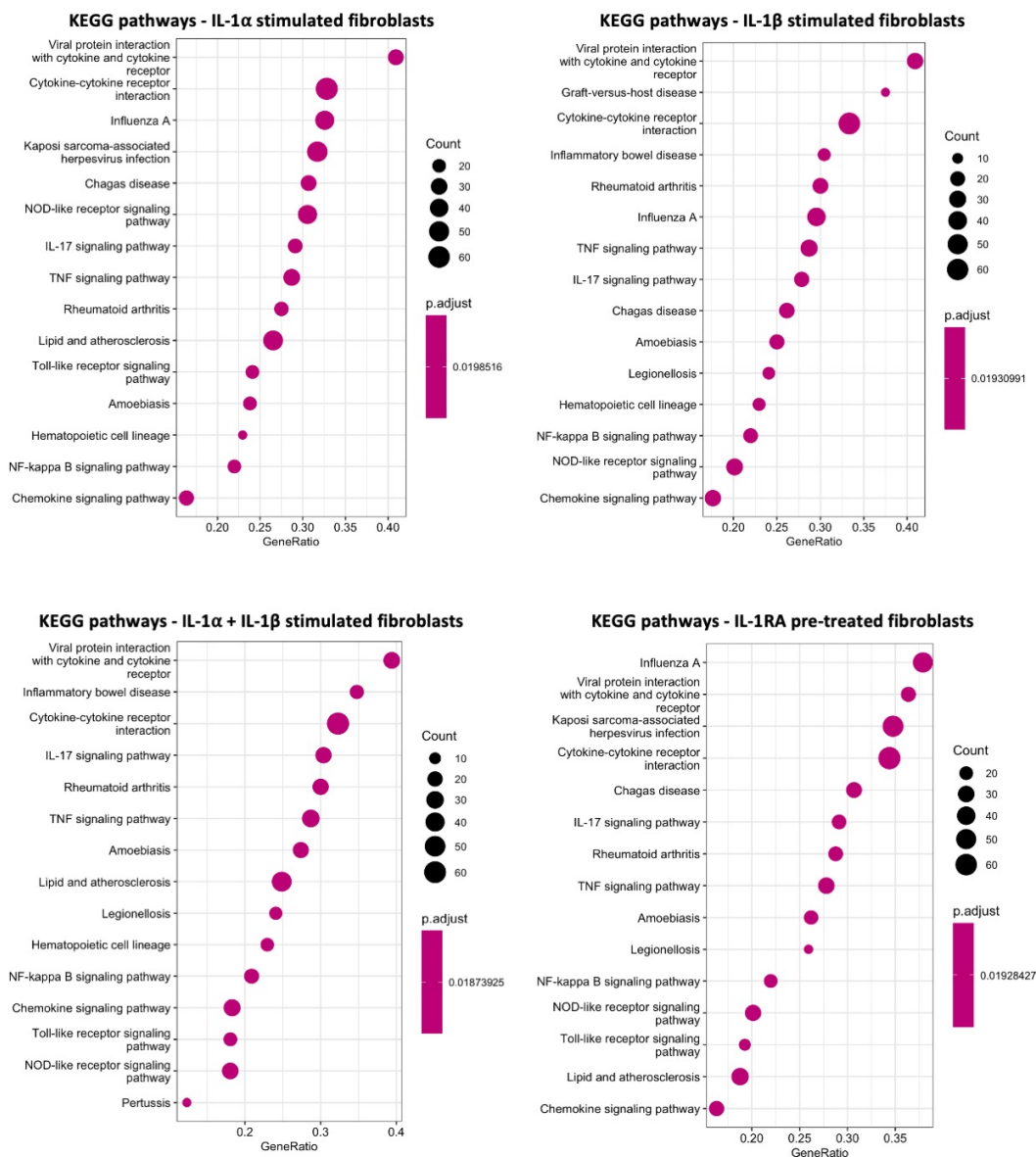


Figure 4.19: KEGG pathway analysis of IL-1 stimulated fibroblasts.

Pathway analysis of fibroblasts stimulated with IL-1 α and/or IL-1 β , and fibroblasts pre-treated with IL-1RA before IL-1 α , IL-1 β , and IL-1RA stimulation using the KEGG database. Dot plots displaying the top 15 enriched KEGG pathways. The size of the dots represents the number of DEGs associated with a KEGG pathway (Count). The colour of the dots represents the adjusted p values (p.adjust) (BH). GeneRatio displays the fraction of DEGs found in a particular geneset.

As expected, pathway analysis of **IL-1** stimulated fibroblasts also revealed several **GO** terms associated with **IL-1** signalling, including response to **IL-1**, **IL-1** production, and **IL-1**-mediated signalling pathway (Figure 4.20). Genes associated with **IL-1** signalling included multiple *CCL* chemokine genes, *IL6*, *CXCL8* (**IL-8**), *IL1RN*, *IRAK2*, *IRAK3*, *MMP2*, *MAPK13*, *NFKB1*, *NOD2*, *IGF1*, *TNFAIP3*, *WNT5A* and more (Figure 4.20).

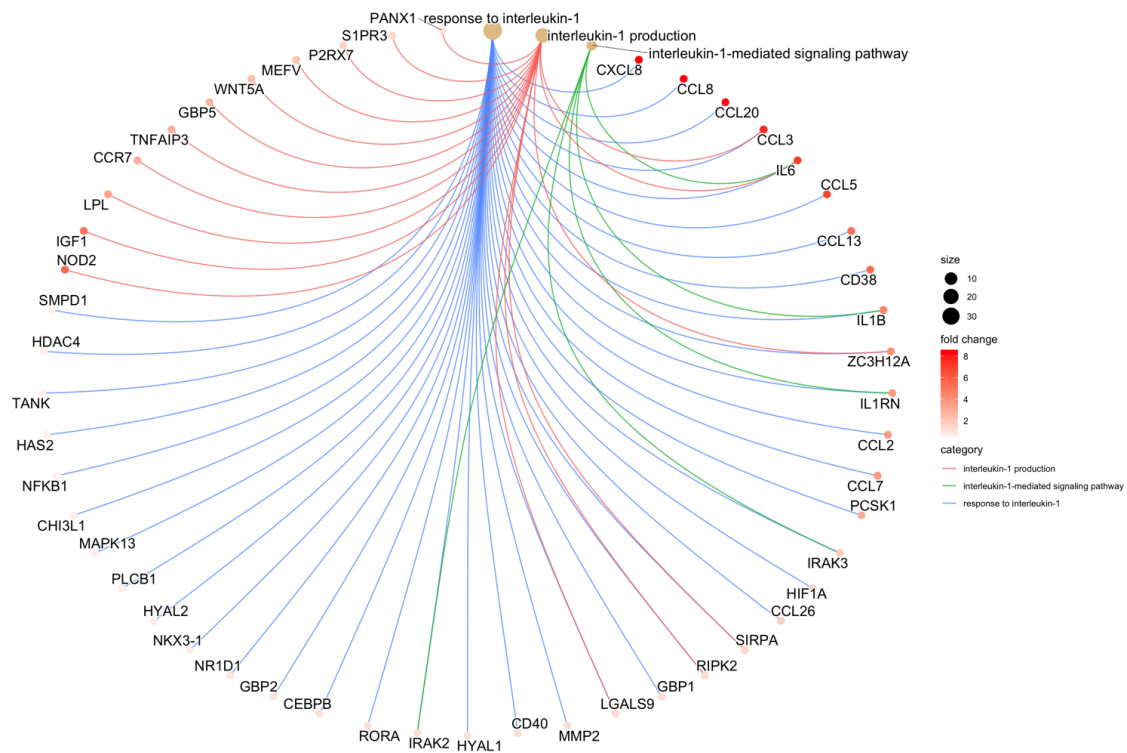


Figure 4.20: Visualisation of **GO** terms associated with **IL-1** signalling in **IL-1** stimulated fibroblasts.

Cnetplot depicting genes associated with three enriched **GO** terms of the biological processes category associated with **IL-1** signalling, including response to **IL-1** (blue lines), **IL-1** production (red lines), and **IL-1**-mediated signalling pathway (green). The size of the dots (**GO** terms) represents the number of genes associated with a **GO** term. The colour of the dots (genes) represents the fold change ($\log_2FC$) relative to unstimulated fibroblasts.

4.4.5 Measuring inflammatory mediators in supernatants of **IL-1** stimulated fibroblasts

After 48 hours of incubation, supernatants of unstimulated (control) and stimulated fibroblasts were collected for analysis of inflammatory mediators. While most concentrations of inflammatory mediators increased after stimulation with **IL-1 α** , **IL-1 β** , and/or **IL-1RA**, only few were statistically significant (Figure 4.21). **IL-6** was significantly increased in supernatants of all stimulated fibroblast conditions (**IL-1 α** , $p = 0.0175$; **IL-1 β** , $p = 0.0082$; **IL-1 α** and **IL-1 β** , $p = 0.0262$; **IL-1RA**, $p = 0.0203$) (Figure 4.21). **CXCL-12** and **IL-1 α** were significantly increased in supernatants of fibroblasts that were stimulated with a combination **IL-1 α** and **IL-1 β** ($p = 0.0045$ and $p = 0.0360$ respectively) (Figure 4.21). **IL-33** was significantly increased in supernatants of fibroblasts stimulated with **IL-1 α** ($p = 0.0285$) (Figure 4.21). Other than **IL-6**, the concentration of all inflammatory mediators in supernatants from unstimulated fibroblasts were below the **LOD** (Figure 4.21).

Looking at the **IL-1** machinery, **IL-1 α** , **IL-1 β** , and **IL-1RA** all increased upon stimulation. Not surprisingly, each of these had a higher concentration if the respective cytokine was included in the stimulation. Yet, treatment with only **IL-1 α** did lead to an increase in **IL-1 β** and vice versa (not significant, $p > 0.05$). **IL-10** and **TNF- α** concentrations were relatively low compared to other inflammatory mediators, with **TNF- α** having most measurements below the **LOD** (Figure 4.21).

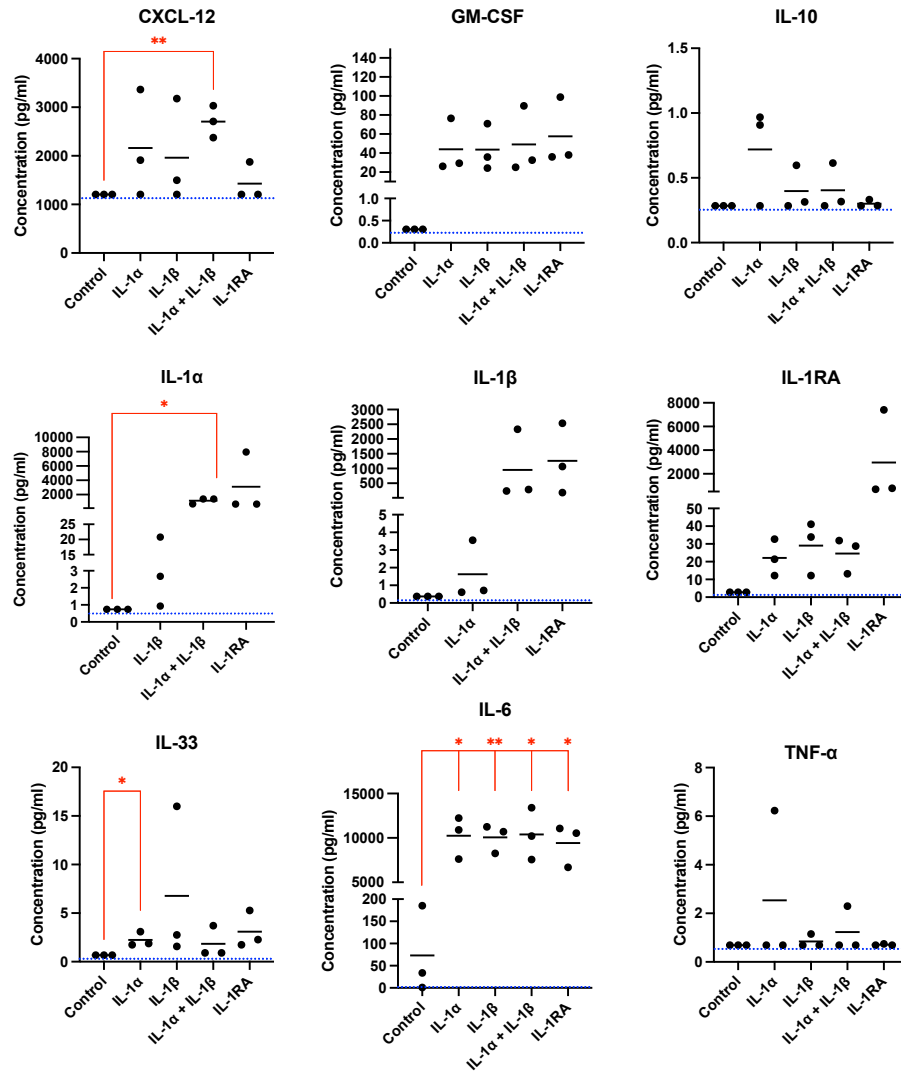


Figure 4.21: Inflammatory mediators secreted by frozen shoulder fibroblasts after stimulation with IL-1.

Scatter plot of inflammatory mediators secreted by frozen shoulder fibroblasts ($n = 3$ frozen shoulder patients). Supernatants taken 48 hours after stimulation with either control medium (unstimulated), IL-1α, IL-1β, IL-1α + IL-1β, or pre-treatment with IL-1RA before IL-1α, IL-1β and IL-1RA stimulation (IL-1RA). Statistical analyses (pair-wise comparisons) are performed relative to unstimulated (control) fibroblasts (red). Black lines represent means. Dots represent replicates. Concentrations below the LOD were assigned the LOD value (blue dotted lines). * $p < 0.05$; ** $p < 0.01$.

4.5 Discussion

This chapter aimed to 1) identify any differences in the secretion of inflammatory mediators by capsular tissues collected from comparator and frozen shoulder donors and 2) investigate the effects of **IL**-1 stimulation on frozen shoulder patient-derived cells. This was accomplished by stimulating fibroblasts isolated from frozen shoulder capsular tissues with recombinant **IL**-1 α and **IL**-1 β proteins.

4.5.1 Secretion of inflammatory mediators by digested frozen shoulder capsular tissues

This chapter investigated the levels of secreted inflammatory mediators in supernatants of digested capsular tissues from frozen shoulder and comparator patient groups. However, the concentrations of all inflammatory mediators were below the **LOD** in supernatants of both frozen shoulder and comparator capsular tissue digest. Therefore, it was not possible to accurately compare the concentration of inflammatory mediators between these two conditions. It is likely that a ten-fold dilution of the supernatant samples resulted in concentrations of inflammatory mediators below the **LOD** of the U-PLEX assay. Other (technical) limitations of this experiment will be discussed in section 4.5.5.

As there are no previous studies that measured the secretion of inflammatory mediators from digested **GH** joint capsular tissues, it is not possible to compare these findings. While there are other studies that investigated the secretion of inflammatory mediators from frozen shoulder patient-derived cells (see section 4.5.2), supernatants from (whole) tissue digests as used in this chapter would likely show

different levels of inflammatory mediators and are therefore not optimal for comparison. This is because inflammatory mediators in supernatants from tissue digests would be secreted by multiple cell types compared to supernatants from isolated fibroblasts.

4.5.2 Frozen shoulder fibroblasts acquire an inflammatory phenotype upon **IL-1** stimulation

To investigate the effects of **IL-1** in frozen shoulder, fibroblasts derived from frozen shoulder capsular tissues were either left unstimulated (control) or were stimulated with **IL-1** α and/or **IL-1** β . Bulk **RNAseq** and differential gene expression analysis revealed that fibroblasts expressed a variety of inflammatory and fibrotic genes across all **IL-1** stimulated conditions, including chemokine associated genes (*CCL2*, *CCL5*, *CCL8*, *CCL20*, *CXCL3*, *CXCL5*), **NF- κ B** pathway genes (*NFKBIA*, *NFKBIZ*), growth factors (*IGF1*, *FGF7*), cytokines (*IL6*, *CSF2*, *CSF3*) and fibrosis related genes (*TGFB1*, *TGFBR1*, *WNT5A*, *COL3A1*, *MMP2*, *TIMP1*). There were no differences between the different **IL-1** stimulated conditions. This suggests that fibroblasts derived from frozen shoulder capsular tissues acquire an inflammatory phenotype upon **IL-1** α and/or **IL-1** β stimulation, which promotes inflammation and fibrosis in (advanced-stage) frozen shoulder. Based on these results, **IL-1** α and **IL-1** β have a similar efficacy and are both potent inducers of inflammation and fibrosis in frozen shoulder fibroblasts. These findings were supported by pathway analyses, which showed enrichment for biological processes related to immune and/or cytokine responses across all **IL-1** stimulated conditions. Unsurprisingly, as both **IL-1** α and **IL-1** β target the same receptor (**IL-1R**). This explains the similar findings between **IL-1** α and **IL-1** β fibroblasts.

Looking back at the different fibroblast populations identified in Chapter 3 (section 3.4.5), several markers seen across IL-1 stimulated fibroblasts were (already) expressed by mainly the chemokine associated fibroblast subsets, including *CXCL12*⁺ *APOE*⁺ and *ARC*⁺ *CXCL2*⁺ fibroblasts. These were mainly *CCL* and *CXCL* chemokine genes, but also *IGF1*, *FGF7*, *NFKBIA*, and some collagen genes (appendix B.9). The expression of these genes are clearly increased upon IL-1 stimulation, compared to non-stimulated fibroblasts from frozen shoulder patients. This is suggestive of an activated phenotype of fibroblasts in frozen shoulder. However, fibroblasts from non-diseased comparator shoulder capsule were not used in this chapter. To fully comprehend the effects of IL-1 on fibroblasts derived from patients with advanced-stage frozen shoulder, future work should also include IL-1 stimulation of fibroblasts from comparator patient groups. This will be discussed in more detail in the limitations section (section 4.5.5).

To date, only one study investigated the effects of IL-1 stimulation in frozen shoulder [165]. Akbar et al. (2019) found an increased mRNA expression of *IL6*, *CXCL8* (IL-8), and *CCL20* in cultured fibroblasts from frozen shoulder patients relative to control fibroblasts from patients with GH instability [165]. The authors found that IL-1 β stimulation resulted in a significant increase of *IL6*, *CXCL8*, and *CCL20* mRNA expression relative to control fibroblasts [165]. Their findings suggest that fibroblasts already have an activated phenotype in frozen shoulder, which is exacerbated upon IL-1 β stimulation [165]. This is further supported by both Akbar et al. (2019) and Dakin et al. (2019), who found an increased expression of fibroblast activation markers (*PDPN*, CD106, CD90, *FAP*, CD248, CD146) in frozen shoulder patient tissues [165, 166].

IL-1 is known to induce cytokine and chemokine release across a variety of inflammatory diseases, including cancer, inflammatory bowel disease, and a variety of joint diseases (e.g., **RA**, **OA**, gout) [268–273]. Similar to the **IL-1** stimulated fibroblasts in this chapter, **IL-1 β** stimulated chondrocytes showed increased **mRNA** expression of a large group of chemokines and cytokines, including *CSF3*, *IL6*, *CCL2*, *CCL5*, *CCL8*, *CCL20*, *CXCL3*, *CXCL5* and more [274]. Chen et al. (2011) showed that fibroblast-like synoviocytes from **RA** patients produced increased levels of **IL-6**, **IL-8**, and **MMP-1** upon **IL-1 β** stimulation [273]. Friedrich et al. (2021) documented that **IL-1 β** induced a big chemokine response (*CXCL1*, *CXCL2*, *CXCL3*, *CXCL5*, *CXCL6*, *CXCL8*), and fibroblast activation (*PDPN*, *FAP*) in stromal cell lines from tissues from patients with inflammatory bowel disease [269]. Thus, the findings of this chapter are in line with existing literature on the effects of **IL-1** [165, 199, 229, 268–277]. Furthermore, a consistent upregulation of **IL-6** is suggesting that this cytokine is likely to have an important biological effect in disease.

For the **IL-1R** blockade experiment, fibroblasts were pre-treated with 10ng/ml **IL-1RA** 1 hour prior to stimulation with **IL-1 α** , **IL-1 β** , and **IL-1RA** for 48 hours. Interestingly, there were no differences between the **IL-1 α** and/or **IL-1 β** stimulated conditions and the blockade experiment (**IL-1RA**). It is therefore likely that the **IL-1Rs** on the fibroblasts were not sufficiently ‘blocked’ with this natural antagonist. Friedrich et al. (2021) pre-treated fibroblasts (Ccd18-Co cell line) with 2mg/ml anakinra (**IL-1RA**) for one hour before stimulating them for three hours with conditioned media produced from tissue digests from patients with inflammatory bowel disease, from ulceration sites with high levels of **IL-1 β** [269]. Anakinra successfully reduced fibroblast activation compared to the vehicle control [269]. Other studies

have used 100 to 500ng/ml of recombinant **IL-1RA** [269, 278]. Therefore, an insufficient concentration of recombinant **IL-1RA** used in this chapter (10ng/ml) is likely to be the reason of the blockade experiment not working. Other limiting factors are discussed below (section 4.5.5).

Top upregulated **DEGs** in unstimulated fibroblasts derived from capsular tissues from frozen shoulder patients included *AQP1*, *PLEKHA4*, *CCN3*, and several cytoskeleton related genes (e.g., *PPL*, *MYO1D*, *MYL12B*). Most of the **DEGs** in unstimulated fibroblasts are commonly expressed in fibroblasts, particularly in fibroblasts from patients with fibrotic disease [278–282]. *AQP1* is upregulated in dermal fibroblasts of patients with systemic sclerosis, another fibrotic condition [278]. *PLEKHA4* is known to enhance Wnt signalling in mammalian cells, an important signalling pathway which is required for **TGF- β** -mediated fibroblast activation and fibrosis [279]. *CCN3* is involved in the proliferation of fibroblasts and the synthesis of **ECM** proteins, thus promoting fibrosis and scar tissue [282]. *PPL* has been identified as an important regulator of lung fibrosis [280, 281]. Hence, frozen shoulder fibroblasts show similarities with other fibroblasts from other fibrotic diseases beyond the joint.

4.5.3 **IL-1 stimulation of frozen shoulder fibroblasts induces secretion of pro-inflammatory mediators**

To investigate whether stimulation of fibroblasts derived from frozen shoulder capsular tissues with **IL-1** proteins induced secretion of inflammatory mediators, the supernatants of **IL-1** stimulated fibroblasts were harvested and analysed. The secretion of inflammatory mediators including **CXCL-12**, **GM-CSF** (*CSF2*), **IL-1RA**, **IL-33**, and **IL-6** was increased upon stimulation with **IL-1 α** and/or **IL-1 β** relative

to unstimulated fibroblasts, whereas the overall concentrations of IL-10 and TNF- α were very low (with some measurements below the lower LOD). Statistical analysis revealed that only the increase of IL-6, IL-33, and CXCL-12 was significant. Due to this experiment being underpowered ($n = 3$ for each condition), it is possible that the increase of other inflammatory mediators would have been significant with additional biological replicates. IL-1 α and IL-1 β levels were also increased; however, elevated levels were inevitable as fibroblasts were stimulated with these inflammatory mediators. Interestingly, stimulation with only IL-1 α did lead to an increase in IL-1 β and vice versa, suggesting that fibroblasts are capable of secreting IL-1 upon cellular stress. As mentioned in section 4.5.2, these findings are in accordance with existing literature on the effects of IL-1 in frozen shoulder fibroblasts, as well as other diseases and tissues [165, 199, 229, 268–277].

Akbar et al. (2019) reported increased secretion of IL-6, IL-8, and CCL-20 fibroblasts derived frozen shoulder capsular tissues relative to control fibroblasts from patients with GH instability, which was also exacerbated upon IL-1 β stimulation [165]. Unstimulated (frozen shoulder) fibroblasts used in this chapter, however, did not secrete high levels of inflammatory mediators as most measurements were below the LOD of the U-PLEX assay. As mentioned in section 4.5.1, this difference might be due to the strong dilution of the supernatants used in this chapter. However, like the findings of Akbar et al. (2019), this chapter also reported an increased secretion of inflammatory mediators upon IL-1 β stimulation [165].

Izadi et al. (2019) found a variety of cytokines and chemokines in the culture supernatant of digested nodular cells from patients with Dupuytren's disease, a

fibrotic condition which is often compared to frozen shoulder [283]. While the authors did not have any control group, the cytokine concentrations (e.g., **TGF- β 1**, **IL-6**, **TNF**, **GM-CSF**, **IL-33**, **IL-1 β**) were higher compared to the supernatants of unstimulated fibroblasts used in this chapter [283]. The difference in cytokine concentrations between their study and this chapter could be a result of different tissue types (e.g., digested nodular cells from patients with Dupuytren's disease and isolated fibroblasts from digested capsular tissues from frozen shoulder patients). The difference in cytokine concentrations might also be explained by Izadi et al. (2019) culturing the digested nodular cells for 24 hours before harvesting the supernatants for analysis, whereas the unstimulated fibroblasts in this chapter were cultured for several weeks [283]. Finally, the supernatants from unstimulated fibroblasts in this chapter may have been diluted too much, hence explaining the relatively low concentrations of secreted inflammatory mediators.

4.5.4 **IL-1** biology and intra-cellular signalling pathways

As discussed above, **IL-1 α** and **IL-1 β** are very potent inflammatory mediators that are involved in a variety of inflammatory diseases and might be involved in sustaining inflammation in advanced-stage frozen shoulder [165, 199, 229, 268–277]. In addition, previous studies have shown that both **IL-1 α** and **IL-1 β** play important roles in fibrosis [284–287]. To understand why **IL-1 α** and **IL-1 β** are such potent inflammatory mediators, their processing, how they are released into the extracellular environment, and the complex **IL-1R** intra-cellular signalling pathways will be discussed briefly below.

IL-1 α processing and release

As IL-1 α exerts both nuclear and extracellular functions in disease and health, it is considered an alarmin [199, 240, 288, 289]. A precursor of IL-1 α (pIL-1 α) can be found in the nuclei of non-hematopoietic cells, where it acts as a transcription factor regulating gene expression [230]. In hematopoietic cells, expression of pIL-1 α only occurs after the cell is activated via PRRs by alarmins or other inflammatory stimuli [288, 289]. The nuclear function and localisation of pIL-1 α is dependent on its N-terminal amino acid residue which contains a nucleotide localisation sequence (IL-1 α contains two cysteine residues, 14 and 47) [290, 291]. Upon cellular activation, the N-terminal domain is cleaved by proteases such as the calcium-activated, membrane-bound, cysteine protease (calpain) (Figure 4.22) [292–294]. This process creates mature IL-1 α , after which it's subsequently secreted into the extracellular environment. Once released from the cell in cases of severe cellular stress, IL-1 α acts as a pro-inflammatory mediator by acting on the IL-1R on surrounding fibroblasts and epithelial cells [276]. IL-1 α also functions as membrane-bound cytokine, where it activates neighbouring cells via IL-1R (juxtacrine signalling) (Figure 4.22) [288, 289]. In addition, IL-1 α is also known for autocrine signalling [244]. Interestingly, in case of apoptosis pIL-1 α is retained by binding to chromatin, whereas necrotic cells leak pIL-1 α into the extracellular environment (thus still being able to promote chemotaxis and inflammation via IL-1R) [295]. At the same time, pIL-1 α is still susceptible to proteolysis by extracellular proteases. While the expression of IL-1 α is related to oxidative stress, extracellular IL-1 α itself is not subject to oxidation like some of the other alarmins (e.g., HMGB1, S100 proteins) [240, 244]. Whilst IL-1 α can be co-secreted with IL-1 β upon inflammasome activation (Figure 4.22), IL-1 α secretion can also be inflammasome independent [296].

IL-1 β processing and release

Unlike IL-1 α , IL-1 β is not considered an alarmin, as the inactive precursor (pIL-1 β) is only expressed by hematopoietic cells after the cell is activated via PRRs [289, 297]. This activates the NF- κ B pathway and induces the synthesis of pIL-1 β (Figure 4.22) [289, 297]. The pIL-1 β cannot act on the IL-1R, hence it needs to be cleaved before becoming active [289, 297]. Upon activation by inflammatory stimuli an inflammasome is assembled. Inflammasomes are cytoplasmic multiprotein complexes, such as the NLR family pyrin domain containing 3 (NLRP3) inflammasome, which activates caspase-1 [289, 297]. Caspase-1 cleaves pIL-1 β (and pIL-18), creating mature IL-1 β (Figure 4.22). There are other proteases which can cleave pIL-1 β via non-canonical inflammasome pathways, however only caspase-1 will be discussed here [289, 297]. Caspase-1 facilitates IL-1 β release by cleaving gasdermin-D (GSDMD), after which it creates pores in the cell membrane (Figure 4.22) [289, 297]. Caspase-1 activation also initiates pyroptosis, resulting in both pIL-1 β and mature IL-1 β being released in the ECM [289, 297]. Once out of the cell, extracellular proteases can cleave pIL-1 β , making it a potent inflammatory mediator [289, 297]. Thus, inflammasome-mediated activation of IL-1 β requires two processes: first, the synthesis of pIL-1 β upon PRRs activation, and second, the activation of the inflammasome (and caspase-1) which leads to the formation of mature IL-1 β (Figure 4.22) [289, 297]. Like IL-1 α , IL-1 β is known to activate IL-1R via autocrine signalling [277]. However, IL-1 β is also able to activate IL-1R via paracrine signalling (systemically) [277].

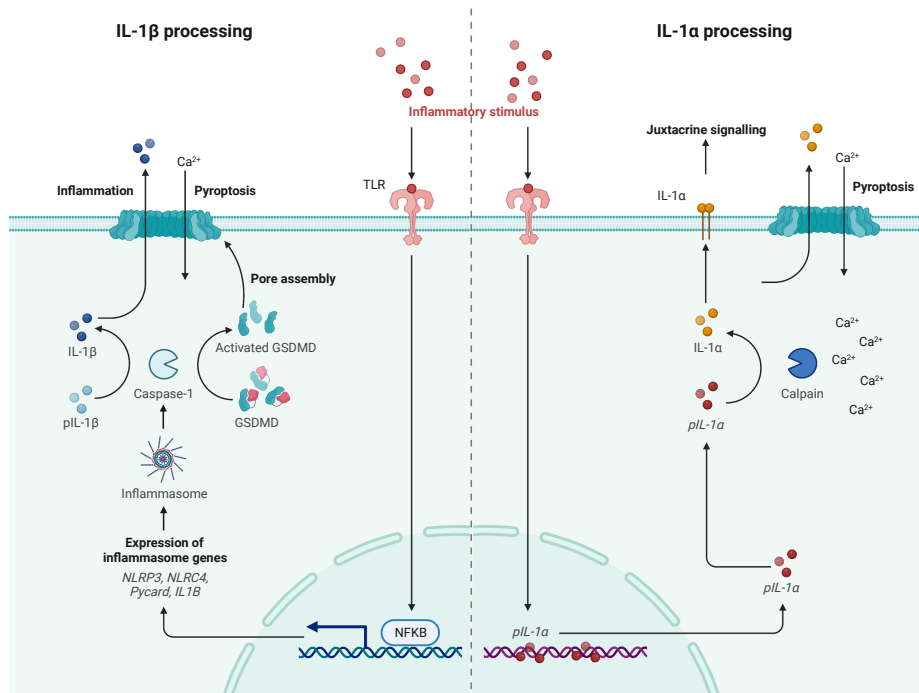


Figure 4.22: **IL-1** processing and activation of the inflammasome.

Schematic overview of the inflammasome activation and **IL-1** β processing (left), and **IL-1** α processing (right). Caspase-1 cleaves precursor **IL-1** β (p**IL-1** β) and **GSDMD**, creating mature **IL-1** β and activated **GSDMD**. **GSDMD** then continues to create pores in the cell membrane and induces pyroptosis which will facilitate **IL-1** β release and subsequently calcium (Ca^{2+}) influx. An increase of calcium influx will facilitate calpain-mediated cleave of precursor **IL-1** α (p**IL-1** α) into mature **IL-1** α . p**IL-1** α will translocate from the nucleus to the cytoplasm upon cellular activation, after which it is cleaved. **IL-1** α is either secreted or act as a membrane-bound juxtacrine signalling cytokine. Created with [BioRender.com](https://www.biorender.com).

IL-1 intra-cellular signalling

First, it must be noted that **IL-1** signalling is highly regulated [229, 277]. **IL-1RA** (a natural antagonist) competes with **IL-1** α and **IL-1** β for the **IL-1R** but does not trigger the downstream signalling [229, 277]. A natural, membrane-bound decoy receptor (**IL-1R2**) can bind to **IL-1** α and **IL-1** β , preventing them from activating the **IL-1R** pathway [229, 277]. In addition, natural soluble forms of **IL-1** receptors (s**IL-1R1**, s**IL-1R2**, s**IL-1RAcP**) are also able to prevent **IL-1** signalling [229, 277]. These natural negative regulators of **IL-1** signalling can limit the effects of **IL-1** [229, 277].

Yet, in (chronic) disease, IL-1 signalling continues to take place [165, 199, 229, 268–277].

Once IL-1R1 is activated however, it recruits the accessory receptor IL-1RAcP and the IL-1 signalling can commence [276]. This IL-1R complex then assembles interleukin-1 receptor-activated protein kinase (IRAK) 4 and MyD88 [277]. Thus, an essential protein complex of IL-1 α /IL-1 β , IL-1R, IL-1RAcP, IRAK4, and MyD88 is formed (Figure 4.23) [277]. Without any of these components, IL-1 signalling cannot take place [298, 299]. Next, autophosphorylation of IRAK4 takes place, after which IRAK4 subsequently phosphorylates IRAK1 and IRAK2 and recruits tumour necrosis factor associated factor (TRAF) 6 (Figure 4.23) [277]. The IRAK1-IRAK2-TRAF6 complex then detaches from the bigger complex and interacts with transforming growth factor- β activated kinase 1 (TAK1) (*MAP3K7*), and the TAK1 binding proteins (TABs) (TAB1 and TAB2) [277]. This newly formed complex (TRAF6-TAK1-TAB1-TAB2) is then transferred to the cytoplasm where TAK1 is phosphorylated and dissociated from this complex [277]. TAK1 then continues to initiate two signalling pathways: NF- κ B pathway and the MAPK pathway (Figure 4.23) [277]. These pathways then will upregulate the expression of inflammatory mediators, including IL-6, TNF- α , and COX-2, further promoting inflammation and fibrosis [277].

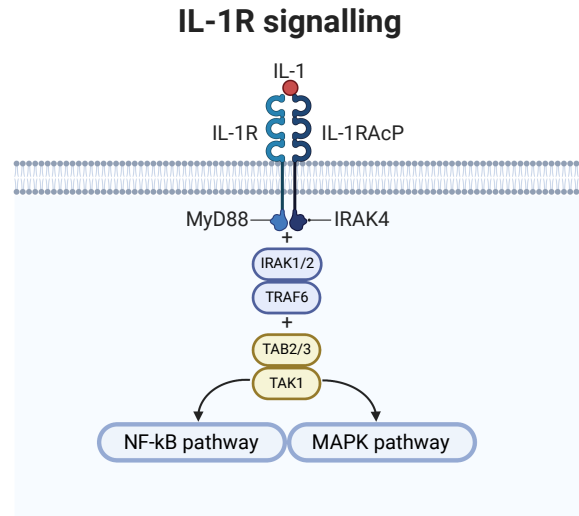


Figure 4.23: **IL-1R** intra-cellular signalling pathways.

A schematic overview of the **IL-1R** signalling pathway. Upon activation of **IL-1R** by **IL-1**, an accessory receptor **IL-1RAcP** is recruited after which intra-cellular signalling proteins **IRAK4** and **MyD88** are assembled. **IRAK4** then initiates autophosphorylation and phosphorylation of **IRAK1** and **IRAK2**, after which **TRAF6** is recruited to the complex. The **IRAK1-IRAK2-TRAF6** complex will then interact with **TAK1**, **TAB2**, and **TAB3**, after which **TAK1** activates the **NF- κ B** pathway and the **MAPK** pathway. Created with BioRender.com.

By releasing pro-inflammatory mediators like **IL-6** and **TNF- α** , inflammation is further promoted and sustained, which eventually results in fibrosis [300, 301]. Differential expression analysis of **IL-1** stimulated fibroblasts in this chapter revealed both inflammatory and fibrotic genes. Gu et al. (2021) identified several key fibrotic genes that were common across a variety of fibrotic diseases, including skin fibrosis and renal fibrosis [302]. Of these genes, *CCL2*, *NOTCH(2)*, *TGFB1*, *MMP2*, and *MMP14* were also highly expressed in **IL-1** stimulated fibroblasts in this chapter, suggesting that **IL-1** also promotes fibrosis in fibroblasts derived from frozen shoulder capsular tissues. While increased *MMP14* expression levels are suggestive of an increase in collagenolysis, a recent study found that a non-synonymous variant (rs1042704) in *MMP14* significantly reduces collagen catabolism and thus promotes fibrogenesis [303]. As mentioned in Chapter 1 (section 1.5.2), this variant in *MMP14* was identi-

fied in **GWAS** of frozen shoulder (and Dupuytren’s disease) [133]. Thus, an increase in *MMP14* expression might be interpreted differently depending on the genotype of patient samples. Genotyping of patient samples might therefore be important in future functional experiments.

Taken together, it is reasonable to think that **IL-1R** signalling is an important driver of inflammation and fibrosis in frozen shoulder. The question is whether **IL-1** cytokines are driving both inflammation and fibrosis in frozen shoulder, i.e., directly influencing both biological processes, or mainly inflammation with secondary fibrosis. Future work using capsular fibroblasts from the **GH** joint capsule could explore this (Chapter 5).

4.5.5 Limitations

There are several limitations that need to be taken into consideration when interpreting the results of this chapter. Clinical limitations and challenges also apply for this chapter; however, these have been discussed in Chapter 2, section 2.5.5 and in Chapter 3, section 3.5.6.

Limitations of measuring secreted inflammatory mediators

The first **MSD** assay had failed due to an error of the **MSD** plate reader. This led to re-freezing the supernatants and thawing them again at a later stage to repeat the **MSD** assay. Although the second run was successful, the inflammatory mediators in the supernatants may have been affected by having undergone two freeze-thaw cycles. The thermal stability of cytokines in supernatant (culture medium) is another limitation. All samples that were used in this chapter were frozen for several

months (<1 year), due to limited capsular tissue samples. Ozbey et al. (2014) found that while IL-6 remains relatively stable, TNF- α degrades quickly and thus the authors advise that TNF- α measurements should be performed in fresh samples [304]. In addition, the authors advise to perform IL-10 measurements in fresh samples or after the first thaw [304]. Future experiments should therefore be done using fresh samples, and if not possible, freeze-thaw cycles should be minimised.

Unfortunately, the concentrations of all inflammatory mediators in supernatants of capsular tissue digests were below the LOD. It is likely that a ten-fold dilution of the supernatant samples resulted in concentrations of inflammatory mediators below the LOD of the U-PLEX assay. For this reason, future experiments should use multiple concentrations to identify the optimal dilution needed. Another issue to take into consideration for future experiments, is that tissues were (mechanically and) enzymatically digested with collagenase at 37°C, which might have induced stress-induced transcriptional changes [305]. This can cause tissues to secrete more inflammatory mediators. In addition, the lower LOD of CXCL-12 was quite high compared to the other inflammatory mediators (1,204 pg/ml; Appendix C.1). The detection range of CXCL-12 (SDF-1 α) as per the specifications on the manufacturer manual should be between 280 and 103,000 pg/ml (U-PLEX Human SDF-1 α Assay). Therefore, it is likely that an error might have been made while preparing and/or diluting the Calibrator Standard for CXCL-12. The concentrations of CXCL-12 in supernatants of IL-1 stimulated fibroblasts were within the detection range, allowing for comparisons between supernatants of IL-1 stimulated and unstimulated fibroblasts.

This chapter was also limited by low sample sizes. For the freshly digested capsular tissues, an $n = 2$ in each group was used. While the concentrations of the inflammatory mediators were below the LOD of the U-PLEX assay, statistical analysis would have not been feasible. For the supernatants of the IL-1 cell experiment, three biological donors were used per condition. While a statistical analysis was performed for these comparisons, this analysis was under-powered. As previously mentioned, it is possible that some of these findings would have been significant with more replicates. Limiting factors and other reasons for limited replicates are extensively discussed in Chapter 2, section 2.5.5.

The IL-1R blockade experiment (pre-treated IL-1RA fibroblasts) was not successful as these fibroblasts were showing similar gene expression profiles as the IL-1 stimulated fibroblasts that were not pre-treated with IL-1RA. While there might be several factors that could have caused this, it is most likely that the working concentration of recombinant IL-1RA was too low (10ng/ml). This became evident when comparing this experiment with other studies (section 4.5.2). For future experiments, multiple concentrations could be used to identify an optimal working concentration before starting the main cell experiment. Future experiments should include time as a factor by harvesting supernatants at several timepoints post-digestion (e.g., 1 hour, 6 hours, 12 hours, 24 hours, 48 hours). This would also inform on the time that is needed for cytokines to be released and/or the time that is needed for the cytokines to be cleared. The same methods could be used for the IL-1 cell experiment.

While there are many advantages to working with the MSD platform over traditional ELISAs, there are still some limitations worth considering. First, like with

ELISA, there are detection thresholds. Several measurements in this chapter were below the **LOD** and therefore the exact concentration could not be retrieved. Fortunately, inflammatory mediators are usually expressed in abundance and almost all measurements of supernatants from **IL-1** stimulated fibroblasts were within the detection range. Another limitation is that only one plate was analysed on the **MSD** platform. When investigating trends in inflammatory mediators like cytokines, it is crucial that the assay is consistent and reproducible. By only running one plate, these quality requirements are not met. Therefore, future experiments should include the analysis of multiple **MSD** plates, enabling the assessment of inter-assay variation and allowing for further validation.

*Bulk **RNAseq** limitations*

Bulk **RNAseq** provides an average gene expression of all the cells in a certain sample, allows for comparisons between conditions, and can identify enriched pathways. However, it does not provide insights into the different cell populations within a sample (tissue heterogeneity). In this case however, the main aim of bulk **RNAseq** analysis was to identify changes in gene expression of fibroblasts post-**IL-1**-stimulation and perform pair-wise comparisons. Second, as with **scRNAseq**, the transcriptome does not necessarily translate to protein expression, particularly when dealing with cytokines. Therefore, supernatants of the **IL-1** cell experiment were also analysed for secreted (protein) levels of inflammatory mediators. Like with **scRNAseq**, artefactual transcriptional stress responses and/or **RNA** degradation as a result of several bulk **RNAseq** preparation steps could have influenced the bulk **RNAseq** analysis. However, this would have happened across all of the investigated conditions, and therefore it is not likely that it would have influenced the comparisons in this chapter.

Using only fibroblasts from advanced-stage frozen shoulder patients was also an important limitation in this chapter. Results should therefore be interpreted with caution. There is clearly an increased expression of important inflammatory and fibrotic genes upon **IL-1** stimulation of frozen shoulder fibroblasts. However, future work should include fibroblasts from comparator patient groups. This would provide more information on how fibroblasts from patients with advanced-stage frozen shoulder respond to **IL-1** compared to fibroblasts from non-diseased comparator patient groups. In addition, one could then also compare the expression profile of unstimulated comparator fibroblasts and unstimulated frozen shoulder fibroblasts. Hypothetically, this would likely show an increased expression of inflammatory and fibrotic genes in frozen shoulder fibroblast. Upon stimulation with **IL-1**, it is likely that frozen shoulder fibroblasts would show a greater inflammatory and fibrotic response relative to comparator fibroblasts. This is similar to the experiments done by Akbar et al. (2019) [165]. Finally, supernatants from these unstimulated and **IL-1** stimulated fibroblasts could then be analysed for levels of secreted inflammatory mediators.

4.6 Conclusion

This chapter found that fibroblasts derived from frozen shoulder capsular tissues acquire an inflammatory fibrotic phenotype upon $\text{IL-1}\alpha$ and/or $\text{IL-1}\beta$ stimulation, which was characterised by an increase in inflammatory and fibrotic gene expression as well as an increased secretion of inflammatory mediators. This included increased expression of chemokine associated genes, $\text{NF-}\kappa\text{B}$ pathway genes, growth factors, cytokines, and fibrosis related genes. Despite its limitations, this chapter builds on the knowledge of Chapters 2 and 3, further supporting a possible role of the IL-1 -axis in maintaining inflammation and fibrosis in advanced stage frozen shoulder. It is likely that $\text{IL-1}\alpha$ and/or $\text{IL-1}\beta$ are secreted from $\text{CD4}^+\text{IL1A}^+$ macrophages and activate fibroblasts via IL-1R to release chemokines and other inflammatory mediators. Therefore, the IL-1 -axis in frozen shoulder should be further investigated *in vitro*, as it could potentially be an important therapeutic target. Future work should investigate whether $\text{IL-1}\alpha$ and/or $\text{IL-1}\beta$ are driving both inflammatory and fibrotic processes simultaneously, or mainly inflammatory processes with secondary fibrosis.

Key findings Chapter 4

- **IL-1 α** and/or **IL-1 β** stimulation exacerbate the inflammatory fibrotic phenotype of fibroblasts derived from frozen shoulder capsular tissues.
- **IL-1 α** and/or **IL-1 β** stimulated fibroblasts from frozen shoulder patients highly express chemokine associated genes (*CCL2*, *CCL5*, *CCL8*, *CCL20*, *CXCL3*, *CXCL5*), **NF- κ B** pathway genes (*NFKBIA*, *NFKBIZ*), growth factors (*IGF1*, *FGF7*), cytokines (*IL6*, *CSF2*, *CSF3*) and fibrosis related genes (*TGFB1*, *TGFBR1*, *WNT5A*, *COL3A1*, *MMP2*, *TIMP1*).
- The secretion of inflammatory mediators (**CXCL-12**, GM-**CSF**, **IL-1RA**, **IL-33**, **IL-6**) by fibroblasts from frozen shoulder patients increases upon stimulation with **IL-1 α** and/or **IL-1 β** relative to unstimulated fibroblasts.
- There are few differences in the expression of inflammatory and fibrotic genes between **IL-1 α** stimulated fibroblasts and **IL-1 β** stimulated fibroblasts, suggesting that both **IL-1 α** and **IL-1 β** are potent drivers of inflammation and possibly fibrosis via the **IL-1R**.

Conclusions and future work

5.1 Summary and conclusion

Efforts have been made to understand the underlying pathophysiology of frozen shoulder since its first description in 1872 [6]. As a result of many histological and other basic studies, there has been growing evidence of the involvement of inflammatory mediators in the pathophysiology of frozen shoulder [134, 135, 138, 149–152]. Therefore, the overarching aim of this thesis was to investigate the role of inflammatory mediators in frozen shoulder.

Firstly, it was important to identify the different cell types expressing inflammatory mediators in the rotator interval of the GH joint capsule, as this had not been established in the literature. ScRNAseq analysis in Chapter 2 revealed that the rotator interval of the GH joint capsule consists of $COL1A1^+PDGFRA^+FAP^+$ fibroblasts, $CD14^+CD68^+$ myeloid cells, $CD2^+CD3^+$ lymphocytes, $PECAM1^+VWF^+$ endothelial cells, and $ACTA2^+NOTCH3^+$ mural cells. $CD14^+CD68^+$ myeloid cells expressed most previously identified inflammatory mediators in frozen shoulder patient tissues. Additional clustering of the $CD14^+CD68^+$ myeloid cells, revealed four myeloid subsets, including $MERTK^+LYVE1^{high}$ macrophages, $MERTK^+LYVE1^{low}$ macrophages, $CD48^+IL1A^+$ macrophages, and $CD48^+CD1C^+$ dendritic cells. Of these, predominantly $CD48^+IL1A^+$ macrophages highly expressed inflammatory mediators and alarmins. In frozen shoulder, $CD48^+IL1A^+$ macrophages showed increased expression of several pro-inflammatory genes, including IL-1 genes ($IL1A$, $IL1B$, $IL1R1$, $IL1RN$). These findings from Chapter 2 suggest that $CD48^+IL1A^+$ macrophages could sustain inflammatory processes in advanced-stage frozen shoulder, particularly via the IL-1-axis.

In Chapter 3, the aim was to identify the micro-anatomical niche in which the $CD48^+IL1A^+$ macrophages reside, to confirm the protein expression of important inflammatory mediators, and to identify potential crosstalk between macrophages and fibroblasts. $CD48^+IL1A^+$ macrophages were localised to both the lining and sub-lining regions of the rotator interval of the GH joint capsule. Immunohistochemistry confirmed the protein expression of the inflammatory mediators identified in Chapter 2, including S100A8, S100A9, IL-1 α , IL-1 β , and IL-1R. Frozen shoulder patient tissues highly expressed IL-1 α , IL-1 β , and IL-1R proteins relative to comparator tissues. Cells expressing IL-1 α and IL-1 β ($CD48^+IL1A^+$ macrophages) and IL-1R (fibroblasts) were in proximity of each other, making cellular interactions between $CD48^+IL1A^+$ macrophages and fibroblasts likely. Clustering of the $COL1A1^+PDGFRA^+FAP^+$ fibroblast population revealed six distinct fibroblast populations, all of which highly expressed *IL1R1*. Ligand-receptor interaction analysis predicted several important IL-1 ligand-receptor pairs between $CD48^+IL1A^+$ macrophages and fibroblast populations, including *IL1A-IL1R1* and *IL1B-IL1R1*. In turn, fibroblast populations were predicted to communicate with myeloid cell populations via the *CSF1-CSF1R* pathway, suggesting that macrophage-fibroblast crosstalk contributes to the pathophysiology of frozen shoulder. Chapter 3 therefore further supports the idea that $CD48^+IL1A^+$ macrophages are involved in sustaining inflammatory processes in advanced-stage frozen shoulder. $CD48^+IL1A^+$ macrophages are likely stimulating fibroblasts (and macrophages) via the IL-1R pathway, promoting ongoing inflammation and fibrosis.

Finally, the aims of Chapter 4 were to determine if tissue digests from comparator and frozen shoulder capsular tissues show any differences in the levels of in-

inflammatory mediators they secrete, and to investigate the effects of IL-1 on patient-derived cells. Unfortunately, there were no detectable differences in the secretion of inflammatory mediators between the tissue digests from comparator and frozen shoulder capsular tissues. However, fibroblasts derived from frozen shoulder capsular tissues acquired an inflammatory phenotype upon IL-1 α and/or IL-1 β stimulation, which was characterised by an increase in inflammatory and fibrotic gene expression, as well as the release of inflammatory mediators. IL-1 stimulated fibroblasts from frozen shoulder patients highly expressed chemokine associated genes (*CCL2*, *CCL5*, *CCL8*, *CCL20*, *CXCL3*, *CXCL5*), NF- κ B pathway genes (*NFKBIA*, *NFKBIZ*), growth factors (*IGF1*, *FGF7*), cytokines (*IL6*, *CSF2*, *CSF3*) and fibrosis related genes (*TGFB1*, *TGFBR1*, *WNT5A*, *COL3A1*, *MMP2*, *TIMP1*). The secretion of inflammatory mediators by IL-1 stimulated fibroblasts from frozen shoulder patients included CXCL-12, GM-CSF, IL-1RA, IL-33, and IL-6. In addition, increased *CSF2* and *CSF3* expression in IL-1 stimulated fibroblasts, as well as increased secretion of GM-CSF (*CSF2*) relative to non-stimulated fibroblasts suggest that IL-1 stimulated fibroblasts release CSF. One could therefore hypothesise that expression of CSF genes by capsular fibroblasts could be the result of CD48⁺IL1A⁺ macrophage derived IL-1 stimulation. In turn, fibroblast derived CSFs could be interacting with myeloid cells, including CD48⁺IL1A⁺ macrophages, further promoting inflammation (as predicted in Chapter 3). Concluding, this final chapter also supports the role of the IL-1-axis in maintaining inflammation in advanced-stage frozen shoulder.

Taken together, it is likely that in (advanced-stage) frozen shoulder, CD48⁺IL1A⁺ macrophages residing in both the lining layer and the sub-lining region of the rotator interval of the GH joint capsule, are secreting IL-1 α and IL-1 β . Both IL-1 α

and **IL-1 β** then activate surrounding macrophages and fibroblasts via **IL-1R** to release chemokines and other inflammatory mediators (Figure 5.1). Fibroblasts could then be interacting with $CD48^+IL1A^+$ macrophages via the release of **CSFs** to further promote inflammation. This data supports the hypothesis that macrophage-fibroblast crosstalk contributes to the underlying pathophysiology of frozen shoulder. Importantly, this thesis provides sufficient data to identify the **IL-1-axis** as a potentially important therapeutic target, which warrants further investigation.

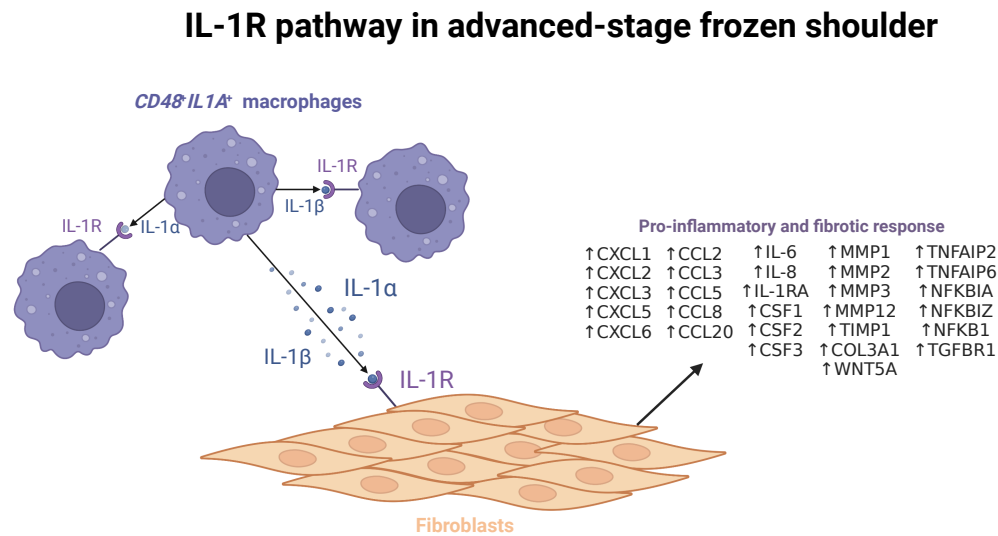


Figure 5.1: **IL-1R** pathway in advanced-stage frozen shoulder.

Schematic overview of **IL-1R** pathway in advanced-stage frozen shoulder. $CD48^+IL1A^+$ macrophages secrete **IL-1 α** and **IL-1 β** which act on **IL-1R** expressed by fibroblasts (and macrophages), which results in the expression and secretion of a variety of pro-inflammatory and fibrotic mediators. These findings support the role of the **IL-1-axis** in maintaining inflammation and fibrosis in advanced-stage frozen shoulder capsular tissues. Created with [BioRender.com](https://www.biorender.com).

5.2 Future work

This thesis adds to existing literature as it highlights a $CD48^+IL1A^+$ macrophage population that expresses a variety of inflammatory mediators that are involved in the underlying pathophysiology of frozen shoulder. This is encouraging as most of the inflammatory mediators that $CD48^+IL1A^+$ macrophages express were previously identified in frozen shoulder. We now know the source of these inflammatory mediators, which suggests a role for $CD48^+IL1A^+$ macrophages in driving the underlying pathophysiology of frozen shoulder. In addition, a potentially important therapeutic target has been identified, i.e., the IL-1R pathway. Below I discuss the next steps and ideas for how the findings from this work inform future research on frozen shoulder.

5.2.1 Exploring the IL-1R pathway in frozen shoulder

Based on the results of this thesis, the next step would be to further explore the IL-1R pathway as a possible therapeutic target in advanced-stage frozen shoulder. Further mechanistic experiments that will examine the effect of blocking IL-1R on IL-1-induced cytokine release and inflammatory/fibrotic gene expression in advanced-stage frozen shoulder is necessary to further establish the IL-1R pathway as a potential therapeutic target. As the IL-1R blocking experiment in Chapter 4 was not successful, limitations and recommendations for this type of experiment have been discussed in Chapter 4, section 4.5.5. Recombinant IL-1RA (anakinra) was used to block the IL-1R and prevent downstream signalling. However, there are many other ways of preventing IL-1R downstream signalling. Instead of blocking the IL-1R, one could also neutralise IL-1 before it activates IL-1R [199, 232, 235, 261]. An anti-IL-1 β

neutralising monoclonal antibody (canakinumab) or a soluble decoy receptor (rilonacept) could also be used to investigate the IL-1R pathway in a frozen shoulder cell culture model [199, 232, 235, 261]. Anakinra, canakinumab, and rilonacept have been tested in clinical trials and have already been fully approved for other inflammatory diseases [199, 232, 235, 261]. Other ways of preventing IL-1R activation are currently being investigated, including an anti-IL-1 α neutralising monoclonal antibody (bermekimab) and a caspase-1 inhibitor (belnacasan) in a variety of inflammatory diseases, including OA and juvenile idiopathic arthritis [306, 307]. Naturally, other steps further down the inflammasome pathway could also be targeted (to prevent IL-1 β release). Thus, there are many ways of investigating the IL-1R pathway in a cell culture model of frozen shoulder (i.e., using patient-derived cells), and one could even investigate the effects of blocking IL-1 α or IL-1 β separately. With each of these experiments, one would have to look for a differential effect on frozen shoulder relative to comparator capsular tissues. If the results of such experiments prove promising, IL-1 blockage could be taken forward to *in vivo* experiments, particularly as some of these agents have already been deemed safe and have been approved for other inflammatory diseases.

There has been some evidence in frozen shoulder literature that already hinted at the IL-1-axis as a potential key driver of inflammation. Several studies have found an upregulation of *IL1A* and *IL1B* mRNA (and protein) expression in tissues from frozen shoulder patients [134, 138, 149]. Interestingly, Bunker et al. (2014) found an association between *Propionibacterium acnes* (*P. acnes*) and frozen shoulder [308]. *P. acnes* has been shown to induce pyroptosis via the NLRP3 inflammasome pathway in intervertebral disc degeneration [309]. As described in Chapter 4, section 4.5.4,

inflammasome-mediated IL-1 β release is also facilitated by pyroptosis [289, 297]. This supports a key role for IL-1 β in frozen shoulder.

Several studies have also investigated IL-1 α or IL-1 β as a possible therapeutic target in fibrotic conditions [234, 284, 285, 287, 310, 311]. Fibroblasts derived from skin lesions from patients with systemic sclerosis show increased expression of IL-1 α and IL-1R compared to normal skin [234]. Blocking IL-1 α expression in fibroblasts derived from skin lesions from patients with systemic sclerosis, reduced IL-6 and pro-collagen expression [234]. In addition, IL-1 has been demonstrated to promote excessive collagen deposition and fibrosis via the PDGF- α pathway [310]. In the absence of IL-1 signalling (*IL1R1*^{-/-} mice), obesity-induced elevations of IL-1 β , TNF- α , Wnt activation, and epithelial proliferation did not occur [311]. Overexpression of IL-1 β in rat lungs led to an acute inflammatory response with severe progressive fibrosis [284]. Bleomycin-induced lung inflammation and fibrosis required inflammasome and IL-1R/MyD88 signalling, suggesting that IL-1 is a key player in the pathophysiology of chronic lung inflammation and fibrosis [285]. Furthermore, *IL1A*^{-/-}*IL1B*^{-/-}*IL1R1*^{-/-} mice do not develop bleomycin-induced pulmonary fibrosis [285, 287]. These findings support IL-1 α and IL-1 β as possible therapeutic targets for fibrotic diseases, including frozen shoulder. However, anti-fibrotic roles for IL-1 β have also been described [312]. Mia et al. (2014) found that IL-1 β without TGF- β 1 had no evident pro-fibrotic on dermal fibroblasts [312]. When given together, IL-1 β decreased the upregulation of TGF- β 1 induced lysyl oxidase (an enzyme involved in cross-linking of collagen), suggesting an anti-fibrotic role for IL-1 β [312]. Nonetheless, it is generally accepted that IL-1 β (and IL-1 α) are potent pro-inflammatory and pro-fibrotic mediators [134, 138, 149, 234, 284, 285, 287, 311].

Future work should investigate whether $\text{IL-1}\alpha$ and $\text{IL-1}\beta$ can drive fibrosis directly without preceding inflammation. Cross-tissue studies are preferable when investigating the effects of $\text{IL-1}\alpha$ and $\text{IL-1}\beta$ on fibroblasts, as fibroblasts from different organs and diseases are likely to respond differently to IL-1 [312]. This is likely due to differences in phenotypical properties (i.e., fibroblast activation) acquired upon different stimuli (e.g., inflammatory stimulus, mechanical stimulus).

5.2.2 Improving three-dimensional (3D) cell culture models of frozen shoulder

Conventional two-dimensional (2D) cell culture methods were used in Chapter 4 to examine the effect of IL-1 on cytokine release and inflammatory/fibrotic gene expression in advanced-stage frozen shoulder. While it's a well-established method, 2D cell cultures are not an accurate representation of human tissue *ex vivo* as these cultures only exist in two dimensions. 3D cell culture models would closely resemble the *in vivo* capsular tissue, recreating the spatial morphology of the GH joint capsule and therefore allowing important cellular crosstalk to take place. This 3D model could be further improved if it resembles the cellular profile of the GH joint capsule as described in Chapter 2. Rather than just using fibroblasts derived from frozen shoulder capsular tissues, one could develop a 3D cell culture model consisting of a combination of fibroblasts, macrophages, and endothelial cells (blood vessels). Such an organoid would consist of several key cell types present in the rotator interval of the GH joint capsule. 3D cultures will also have an ECM, in which cells will form complex structures in contrast to 2D cell cultures monolayers. This could be particularly useful when testing the IL-1R pathway as this is mainly a macrophage-fibroblast interaction in frozen shoulder. This would also provide an extra option to

investigate the IL-1R pathway: one could directly stimulate IL-1R on fibroblasts and macrophages by adding recombinant IL-1 α and IL-1 β to these 3D cell cultures, or you could stimulate the macrophages to release IL-1 α and IL-1 β . A 3D cell culture model would therefore be a better *in vitro* model of frozen shoulder for testing important cellular crosstalk.

5.2.3 Is there a place for animal models of frozen shoulder?

Frozen shoulder is not a naturally occurring disease affecting quadruped animals. This implies that frozen shoulder is a disease that manifests itself exclusively in human (bipedal) shoulders. Interestingly, a case series by Carr et al. (2016) describes adhesive capsulitis in eight dogs [313]. While it is evident there is arthrofibrosis in the shoulders of these dogs, it is unlikely that the disease course is similar to adhesive capsulitis (i.e., frozen shoulder) as seen in human shoulders [313]. The dogs are described as presenting with a history of chronic forelimb lameness; however, the disease course is not further specified and the study group is quite heterogeneous in terms of age, comorbidities, and previous injuries [313]. The different disease stages of frozen shoulder as seen in humans are likely absent in dogs (and difficult to confirm if they were present). Moreover, while they possess similar structures, there is a considerable difference in the anatomy of the quadruped canine and bipedal human shoulders, making comparisons even more difficult.

Several authors have tried to establish an animal model of frozen shoulder to investigate the pathophysiology of frozen shoulder by using a mouse or rat model [314–318]. These models often induce arthrofibrosis of the GH joint, which mimics some of the clinical and histological aspects of the human frozen shoulder [314–318].

However, given the different stages of frozen shoulder, we can assume that the underlying pathophysiology is quite different from these animal models. Furthermore, like with canine shoulders, the macroscopic anatomy of rodent shoulders significantly differ from the human shoulder. In quadrupeds, the forelimb is attached to the trunk via only muscle (i.e., synsarcosis joint), allowing for movement in the anterior and posterior planes only.

Looking at the animal kingdom, humans are most closely related to other apes. An important feature of apes (including humans) that separates them from other primates is a specific scapula and humerus morphology suited to engage in a wide ROM of the GH joint [319]. In comparison to other animals, the shoulder anatomy of apes mostly resembles the human shoulder [319]. Yet, to our knowledge, apes do not develop a frozen shoulder. Thus, while possibly useful as a model for arthrofibrosis in general, it is unlikely that animal models will provide an accurate frozen shoulder disease model. It is therefore recommended that future frozen shoulder research focusses mainly on (human) patient-derived tissues and cells (as described above), and *in vivo* trials in a later stage.

5.2.4 Disease-stage specific analysis of human tissue samples

As discussed in Chapter 2 (section 2.5.5), one of the biggest limitations of basic frozen shoulder research is that it is dependent on acquiring capsular tissues from frozen shoulder patients undergoing a capsular release procedure (as per usual care). This means that, most of the time, researchers are limited to analysing samples collected from advanced-stage frozen shoulder patients (symptoms established for >1 year). Analysing samples from earlier stages of frozen shoulder might provide us with new

insights on potential inflammatory mediators active at an earlier stage of disease. The addition of early-stage frozen shoulder samples to our **scRNAseq** dataset for example, would inform us on the disease trajectory, changes in cell proportions, and changes in regulatory networks over time. Therefore, it would be preferable to also collect capsular tissue samples from patients with early-stage frozen shoulder. The UK FROST trial showed that neither arthroscopic capsular release, physiotherapy, or manipulation under anaesthesia were clinically superior in a multicentre, three-arm, **RCT** [120]. The results of the UK FROST trial have urged clinicians to consider surgical options rather as a last resort if for example conservative management fails [120]. Hence, it is unlikely that we could acquire early-stage frozen shoulder samples via arthroscopic capsular release. Clinicians and researchers are therefore compelled to develop novel, preferably minimally invasive techniques to acquire early-stage frozen shoulder capsular tissues. As a result, the FreeCAP study group (following up from the **ICECAP** study) have set up a new study design to test a minimally invasive technique to acquire capsular tissue samples from the rotator interval of the **GH** joint capsule (<https://www.ndorms.ox.ac.uk/research/research-groups/soft-tissue-joint-disease-dakin-group/clinical-studies/freecap>). The FreeCAP study plans to collect tissue from the rotator interval of the **GH** joint capsule from early-stage frozen shoulder patients undergoing elective ultrasound-guided hydrodilatation. Analysing these tissues will help to identify disease-stage specific therapeutic targets for frozen shoulder patients.

This thesis highlights the **IL-1**-axis as a potential therapeutic target for advanced-stage frozen shoulder. As mentioned before, several studies have found an upregulation of **IL-1 α** and **IL-1 β** expression in tissues from frozen shoulder patients [134, 138,

149]. These patient groups included arguably some earlier-stage frozen shoulder patients, which suggests that the IL-1-axis might also be involved in early-stage frozen shoulder, i.e., throughout the entire disease course [134, 138, 149]. Future work will confirm if this is the case.

5.2.5 Clinical implications

The IL-1R pathway could be a promising therapeutic target for (advanced-stage) frozen shoulder patients, which could be taken forward for *in vivo* trials. As discussed in section 5.2.1, there are many ways one could target the IL-1R pathway [199, 232, 235, 261, 307]. Anakinra for example, which targets IL-1R, could be administered to frozen shoulder patients. Canakinumab (only targeting IL-1 β) and rilonacept (soluble decoy receptor) would also be good candidates. All three of these options have passed phase III clinical trials for other inflammatory diseases like RA and gout [199, 232, 235, 261, 307]. This would significantly speed up the clinical trials on the use for frozen shoulder patients, as they already have been tested on healthy subjects (phase I trials). Once there is sufficient preclinical data to support the IL-1R pathway being one of the key pathways active in maintaining inflammatory processes in (advanced-stage) frozen shoulder, phase II clinical trials could possibly be initiated.

Targeting the IL-1-axis could be effective for advanced-stage frozen shoulder. If future research indicates that the IL-1R pathway is also one of the main active inflammatory pathways of early-stage frozen shoulder, then one could also use treatments targeting the IL-1-axis for early-stage frozen shoulder. While identifying the advanced-stage frozen shoulder patient is quite straight forward, identifying early-stage frozen shoulder can be challenging (see Chapter 1, section 1.3.1). This

is mainly due to pain being the predominant complaint in the early stages (‘freezing phase’), which theoretically could also be the result of other common shoulder diagnoses which manifest in the same age category as frozen shoulder patients (e.g., subacromial pathology).

As frozen shoulder is a localised condition, therapeutic agents targeting the IL-1R pathway would likely be locally delivered injections rather than systemic or oral medication. This would be similar to current intra-articular (or sub-acromial) corticosteroid injections. As it is a condition of the GH joint capsule, it would make sense to go for an intra-articular injection. However, even for corticosteroid injections the literature is not conclusive on this matter [105–109]. There is some data that suggests that intra-articular corticosteroid injections are likely to work faster than sub-acromial injections. However, the overall effectiveness is comparable in the longer term, as injections at both sites do not seem to have an impact on the total disease duration and provide only temporary relief (Chapter 1, section 1.4) [105–109]. Nevertheless, injections are common therapy for shoulder complaints and would be preferable for any therapy targeting the IL-1R pathway. Recently, the Anti-Freeze-F Study ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT05299242) identifier: NCT05299242) has started to recruit frozen shoulder patients for treatment with intra-articular injections of adalimumab (anti-TNF- α monoclonal antibody), after it was found to be effective in treating early-stage Dupuytren’s disease [320]. While advanced-stage frozen shoulder samples seem to have reduced levels of *TNFA* mRNA relative to chronic rotator cuff tendon tear tissues (Dakin et al. 2019), the Anti-Freeze-F study focuses on early-stage frozen shoulder patients [166, 320]. Similar studies could be set up with intra-articular injections targeting the IL-1-axis.

Blocking or dampening inflammatory pathways like the IL-1-axis is one way to tackle the clinical challenge of frozen shoulder. However, as frozen shoulder is ultimately a self-limiting disease, one could also accelerate its resolution, particularly in advanced-stage disease. While this thesis mainly focused on elucidating the role of inflammatory mediators in frozen shoulder, the single cell atlas in Chapter 2 also revealed some macrophages highly expressing genes that resemble the traditional anti-inflammatory M2 macrophages ($MERTK^+LYVE1^{high}$ macrophages). This specific macrophage population was enriched for biological processes related to the regulation of the immune response and is therefore likely to have a role in regulating the ongoing inflammation in advanced-stage frozen shoulder. The $MERTK^+LYVE1^{high}$ macrophages and their role in restraining inflammation were further explored in a study conducted by our group [321]. An interaction of $MERTK^+LYVE1^{high}$ macrophages and matrix associated fibroblasts seems to induce genes and pathways regulating matrix remodelling, possibly moderating the inflammatory phenotype of frozen shoulder capsular fibroblasts [321].

It is undeniable that frozen shoulder is a unique disease which has great importance to research of inflammatory fibrotic diseases in general. Arthrofibrosis in other joints and fibrosis of visceral organs are usually irreversible (e.g., pulmonary fibrosis) [322, 323]. Frozen shoulder therefore presents a unique opportunity to study an example of a chronic inflammatory fibrotic disease that is ultimately self-limiting. This means that it is a tractable disease to therapeutically target. While fully understanding the underlying pathophysiology of frozen shoulder will give us new treatment options for the disease itself, it has also important translational ability to inform on treatments of other chronic inflammatory fibrotic diseases beyond the joint.

5.3 Concluding remarks

This thesis has investigated the role of inflammatory mediators in frozen shoulder. One of the key findings of this thesis is that the IL-1R pathway is likely to be involved in sustaining inflammation in advanced-stage frozen shoulder. Rather than being led by several basic observational studies, this thesis has been led by a comprehensive scRNAseq analysis, which has provided the cellular landscape of one of the main pathological sites of frozen shoulder: the rotator interval of the GH joint capsule. We now understand the main cell types comprising the GH joint capsule, and moreover, this thesis highlighted a specific macrophage-fibroblast interaction that is likely to drive inflammation and fibrosis in advanced-stage frozen shoulder. These findings have been supported by both observational (Chapter 3) and experimental (Chapter 4) data. $CD48^+IL1A^+$ macrophages are the source of many important inflammatory mediators in frozen shoulder, including IL-1 α and IL-1 β , which act on the IL-1R on fibroblast populations. Supported by existing literature, the IL-1R pathway seems to be a key pathway active in advanced-stage frozen shoulder. It must however be noted that, while the IL-1R pathway seems to be biologically important in frozen shoulder, we cannot exclude that other inflammatory mediators may also play a role (i.e., frozen shoulder might not be a mono-cytokine driven disease). Looking at the future, we are merely years removed from fully unravelling the puzzle that is frozen shoulder, and this thesis is a significant contribution towards this goal. It is therefore hoped that this thesis will attune future research to explore the IL-1R pathway and other potentially important pathways in frozen shoulder, and that both clinicians and researchers continue to investigate the cellular and molecular basis underpinning frozen shoulder.

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Appendices

Chapter 2

A.1 RNA expression heatmap of cell clusters identified in the rotator interval capsule

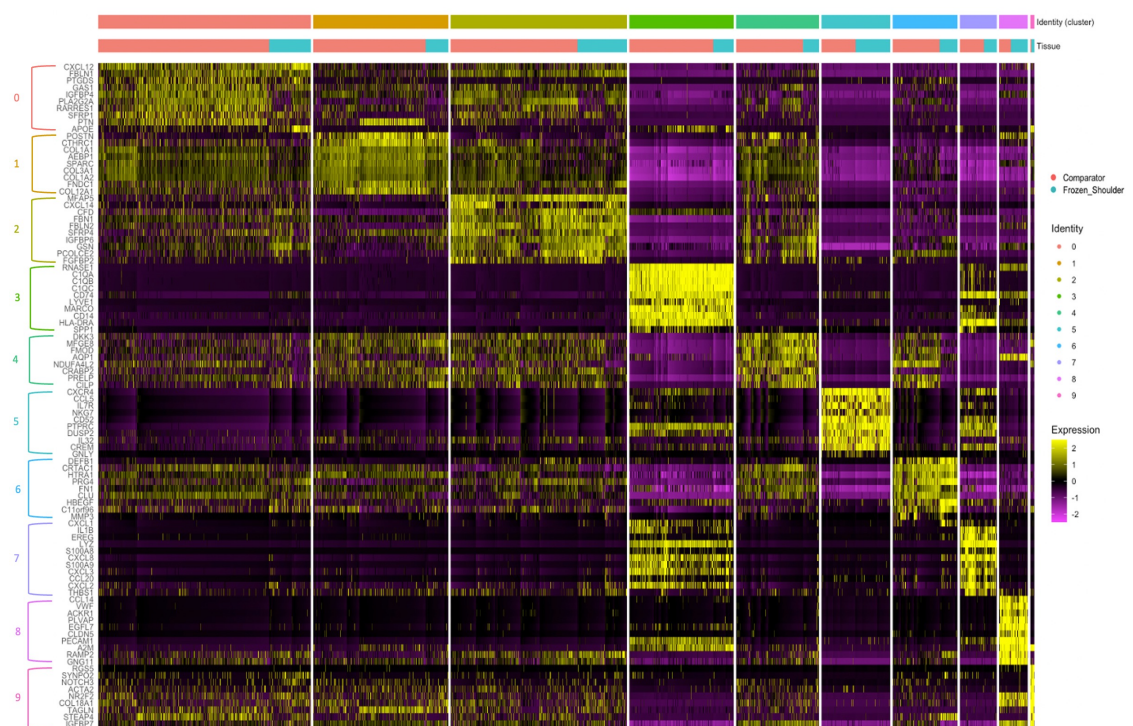


Figure A.1: RNA expression heatmap of cell clusters identified in the rotator interval of the GH joint capsule.

RNA expression heatmap showing the top 10 marker genes (rows) for each of the clusters (coloured columns) in comparator ($n = 6$) and frozen shoulder ($n = 4$) tissue. Yellow indicates increased expression of a particular gene, whereas purple indicates a decrease in expression of a particular gene for each cell.

A.2 Additional QC metrics of the rotator interval single cell atlas

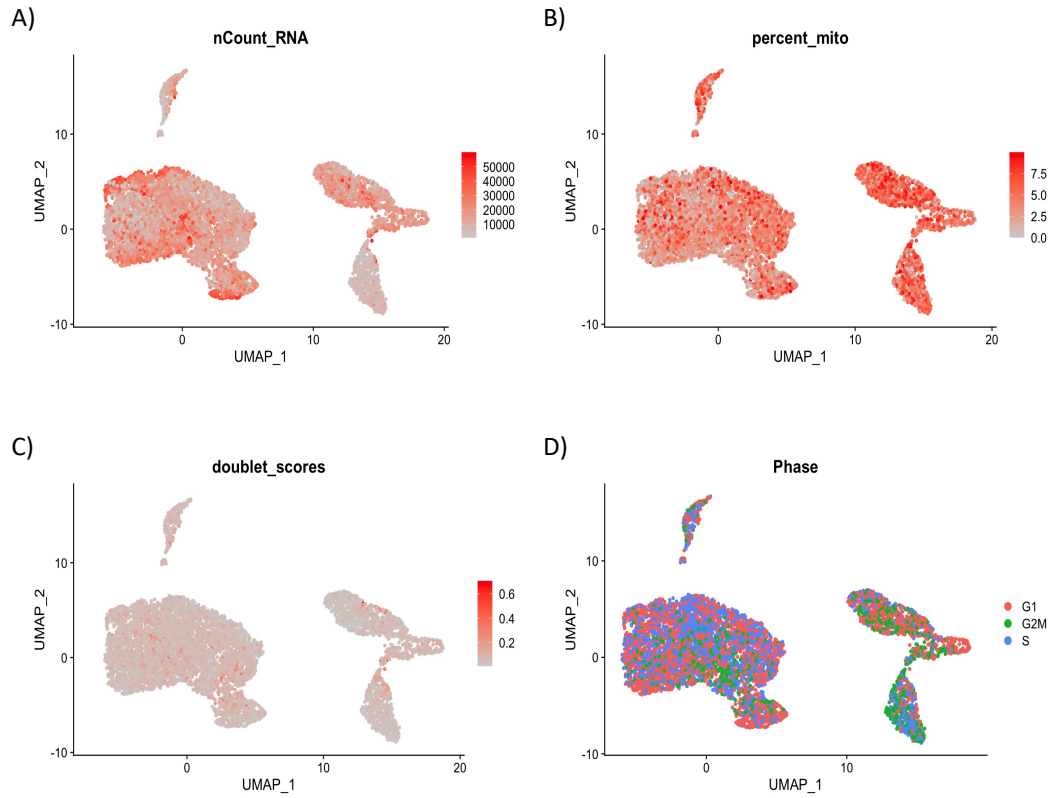


Figure A.2: Additional QC metrics of the rotator interval single cell atlas. Feature plots showing: A) the number of molecules (nCount_RNA), B) the percentage of mitochondrial gene expression, C) the doublet scores (with a score of 1 being a predicted doublet), and D) the cell cycle phase.

A.3 SingleR annotation of the rotator interval cell atlas with the HumanPrimaryCellAtlasData

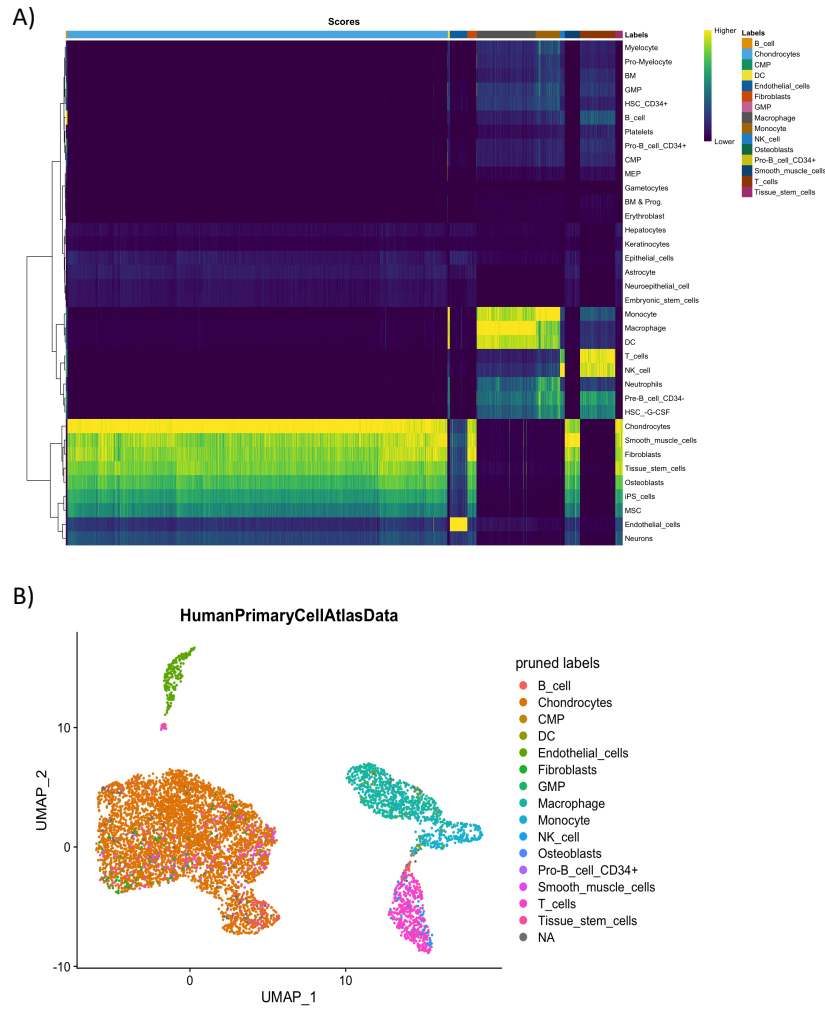


Figure A.3: SingleR annotation of the rotator interval cell atlas with the HumanPrimaryCellAtlasData.

A) singleR predictions ScoreHeatmap. B) pruned singleR predictions UMAP.

A.4 SingleR annotation of the rotator interval cell atlas with the DatabaseImmuneCellExpressionData

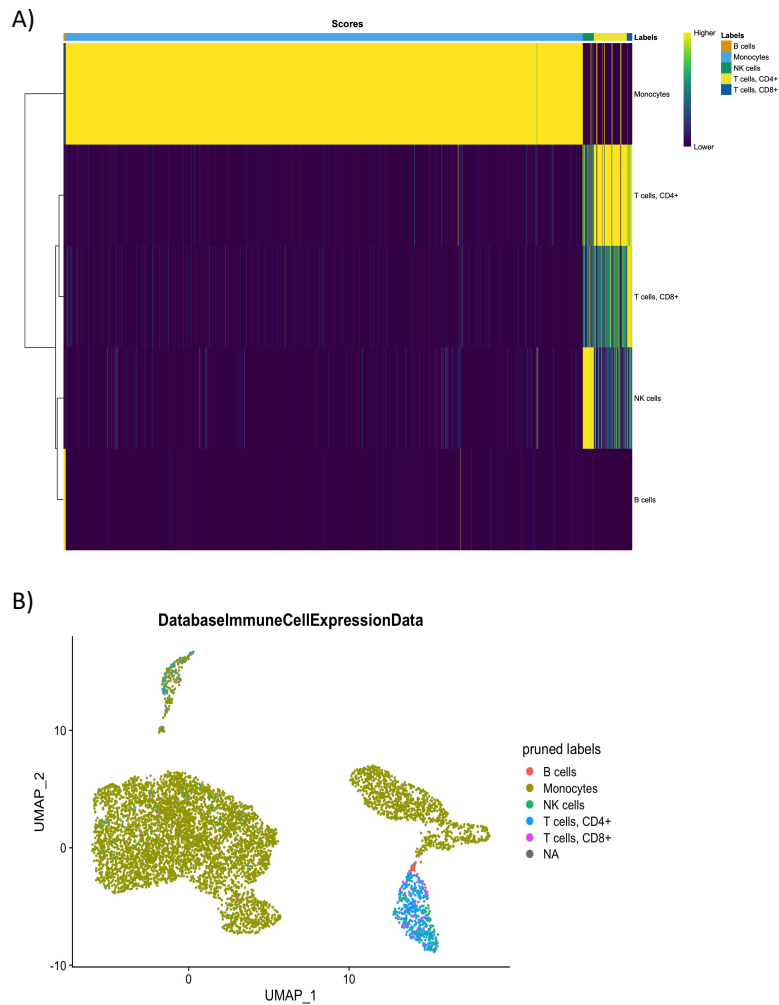


Figure A.4: SingleR annotation of the rotator interval cell atlas with the DatabaseImmuneCellExpressionData.

A) singleR predictions ScoreHeatmap. B) pruned singleR predictions **UMAP**.

A.5 SingleR annotation of the rotator interval cell atlas with the MonacoImmuneData

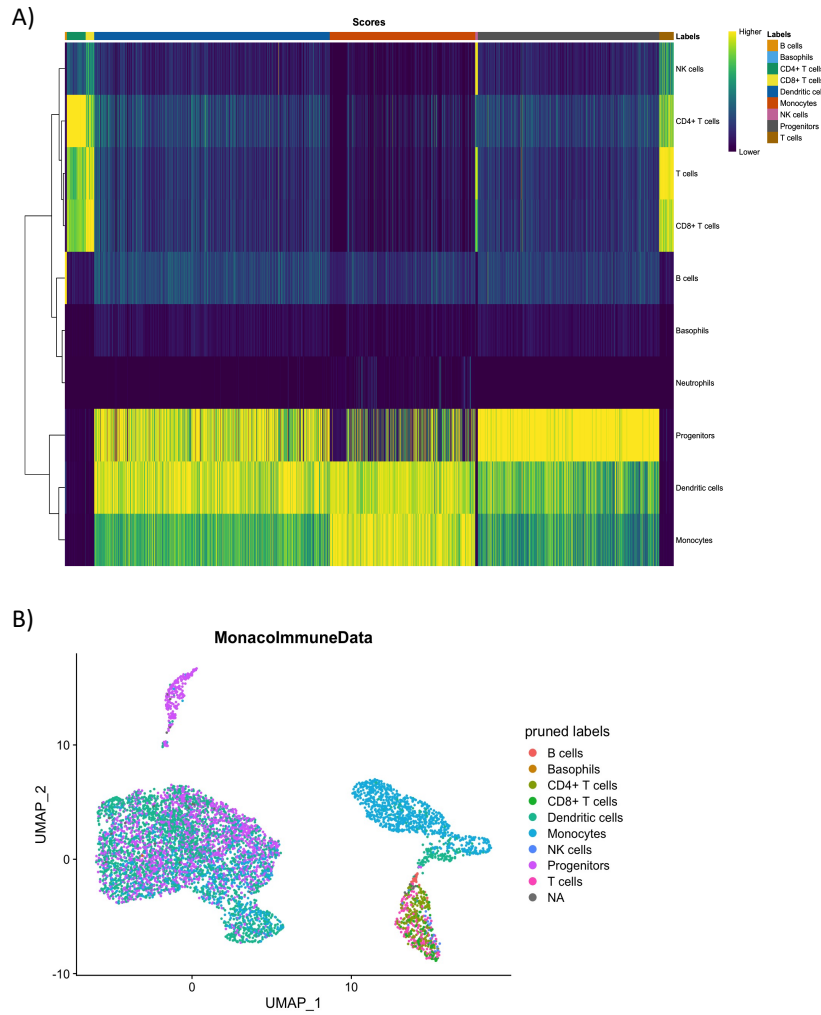


Figure A.5: SingleR annotation of the rotator interval cell atlas with the MonacoImmuneData.

A) singleR predictions ScoreHeatmap. B) pruned singleR predictions **UMAP**.

A.6 UMAPs of the myeloid cell cluster

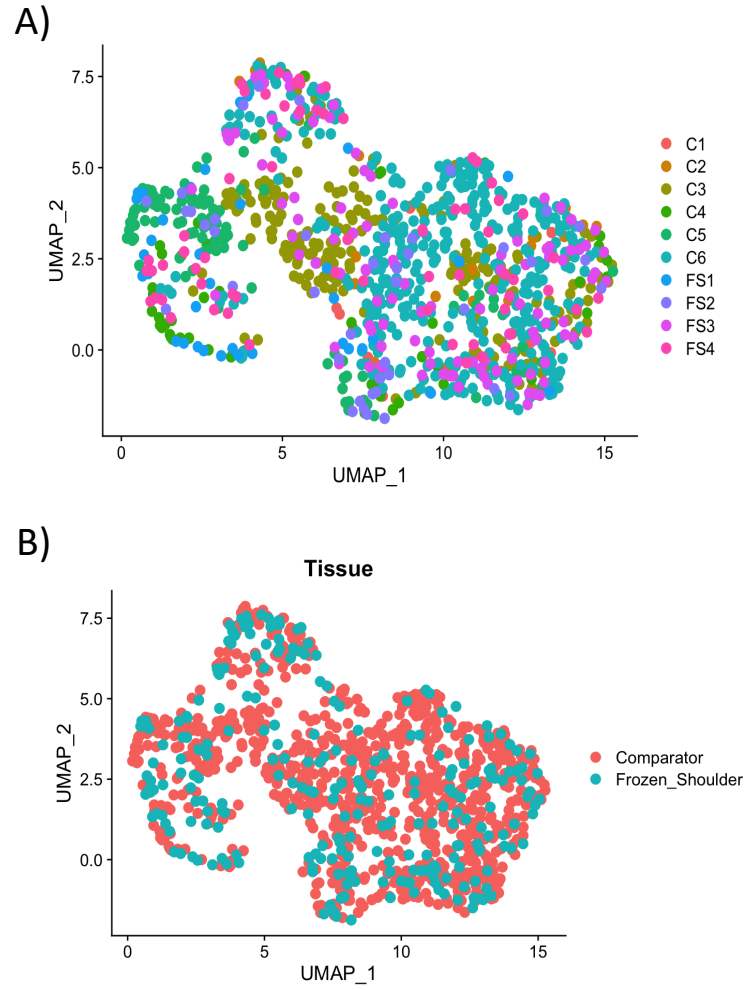


Figure A.6: UMAPs of the $CD14^+CD68^+$ myeloid cell cluster. A) UMAP showing cells from individual donors ($n = 6$ comparator and $n = 4$ frozen shoulder). B) UMAP showing cells from comparator and frozen shoulder samples.

A.7 SingleR annotation of the myeloid cell atlas with the HumanPrimaryCellAtlasData

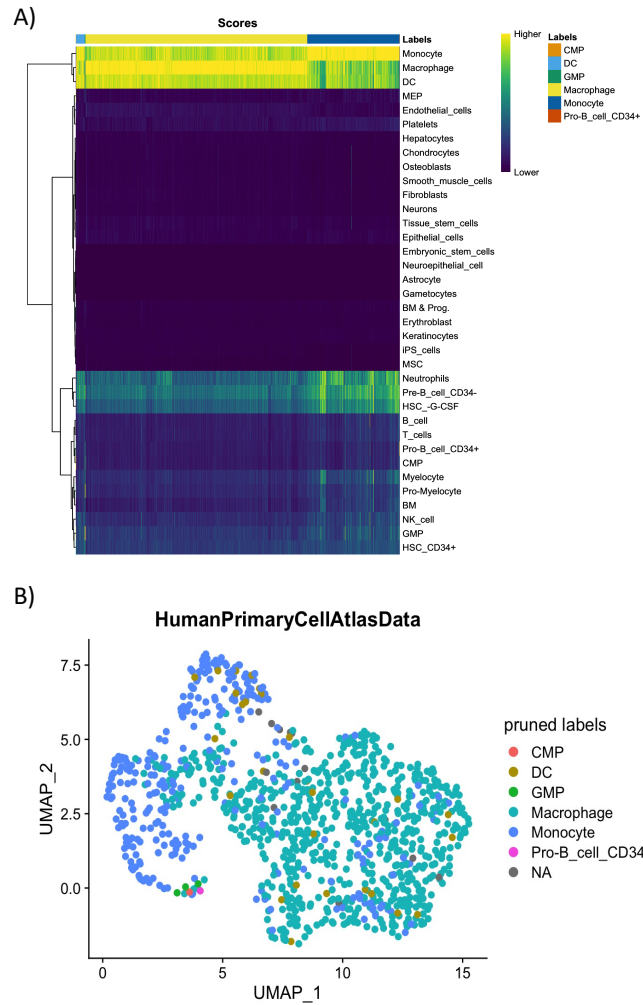


Figure A.7: SingleR annotation of the myeloid cell atlas with the HumanPrimary-CellAtlasData.

A) singleR predictions ScoreHeatmap. B) pruned singleR predictions **UMAP**.

A.8 SingleR annotation of the myeloid cell atlas with the DatabaseImmuneCellExpressionData

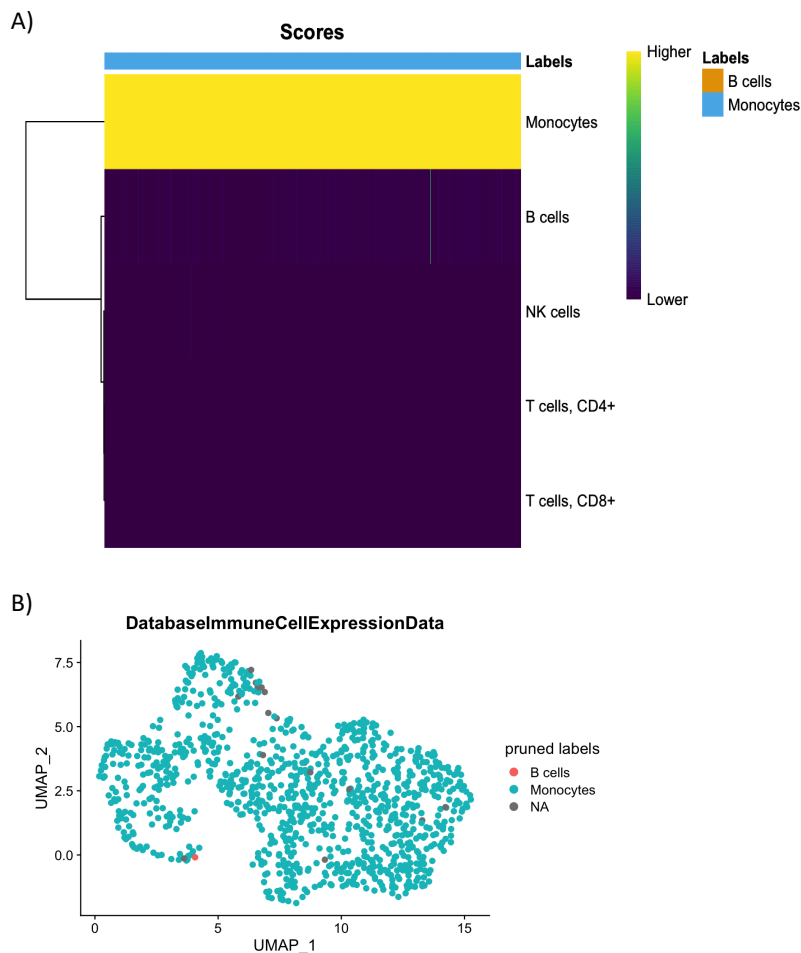


Figure A.8: SingleR annotation of the myeloid cell atlas with the DatabaseImmuneCellExpressionData.

A) singleR predictions ScoreHeatmap. B) pruned singleR predictions **UMAP**.

A.9 SingleR annotation of the myeloid cell atlas with the MonacoImmuneData

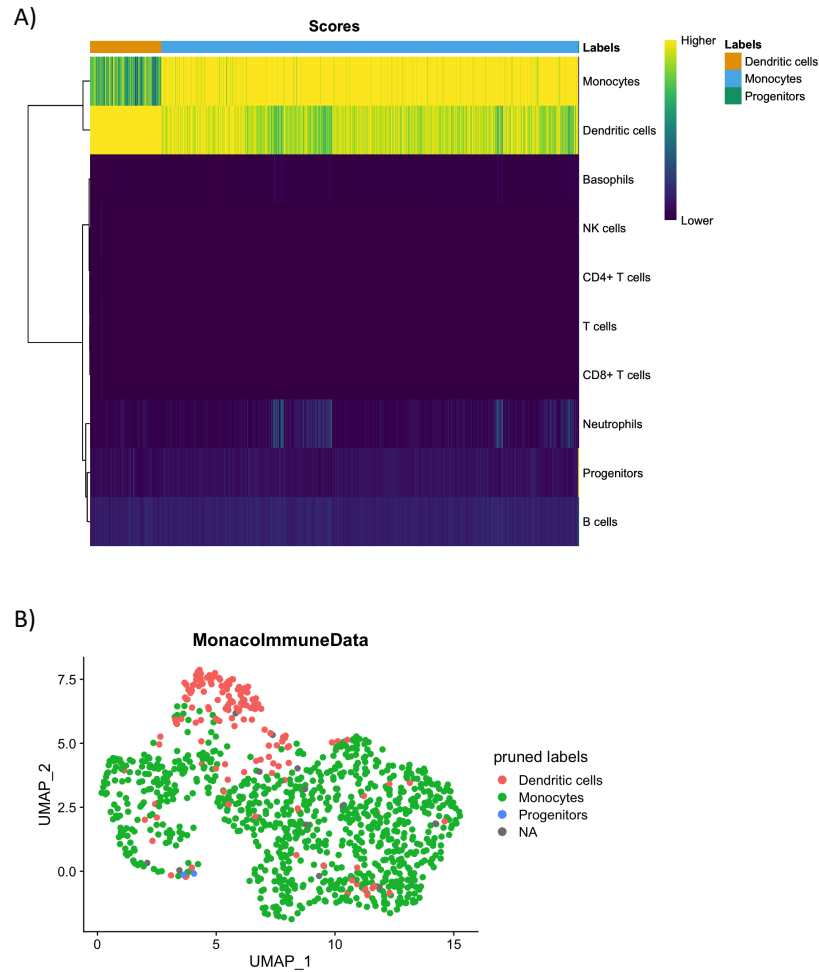


Figure A.9: SingleR annotation of the myeloid cell atlas with the MonacoImmuneData.

A) singleR predictions ScoreHeatmap. B) pruned singleR predictions **UMAP**.

A.10 Cluster markers of myeloid cell subsets

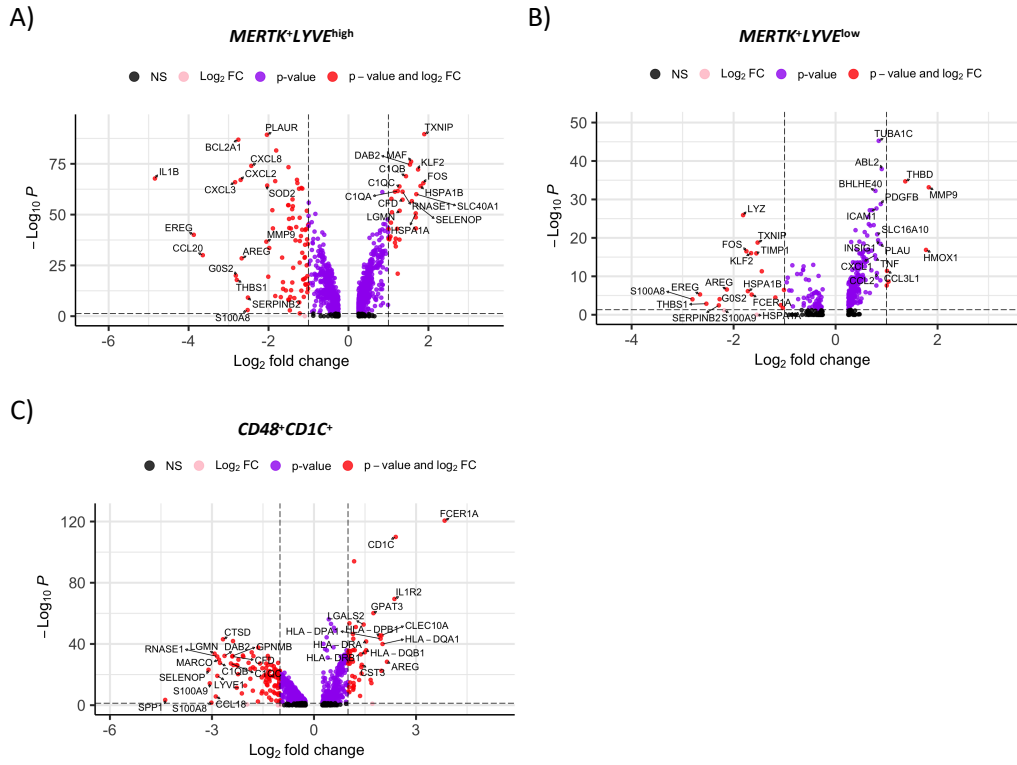


Figure A.10: Cluster markers of $CD14^+CD68^+$ myeloid subsets.

Volcano plots displaying the top negative (negative $\log_2FC$; left) and positive marker genes (positive $\log_2FC$; right) that characterise A) $MERTK^+LYVE1^{high}$ macrophages, B) $MERTK^+LYVE1^{low}$ macrophages, and C) $CD48^+CD1C^+$ dendritic cells.

A.11 GO molecular function pathway analysis of myeloid cell subsets

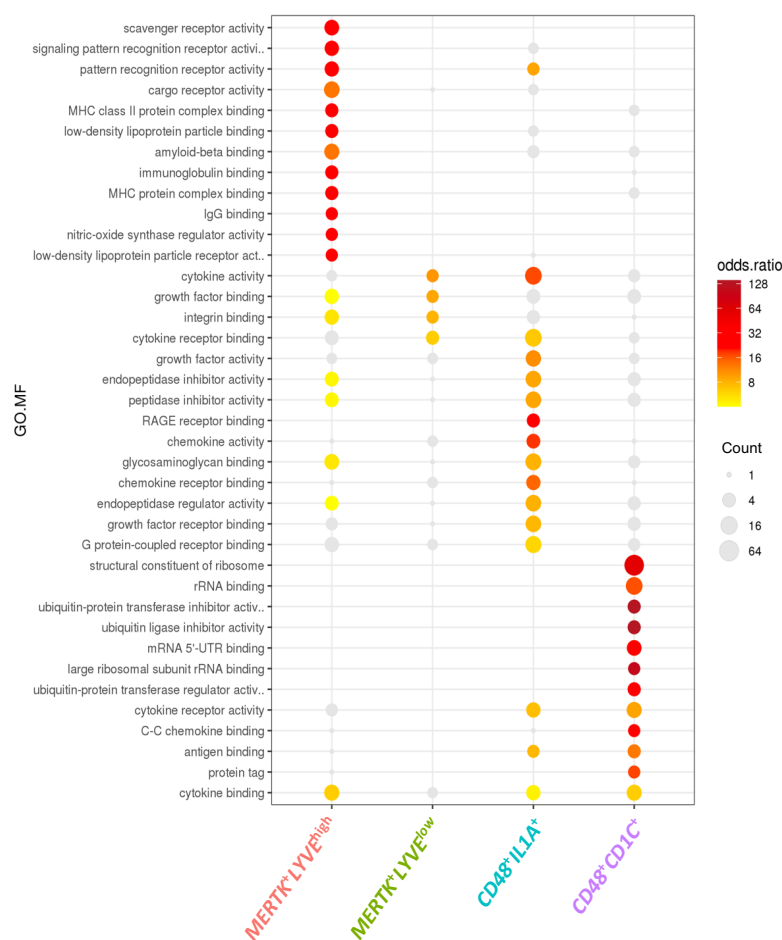


Figure A.11: GO molecular function pathway analysis of $CD14^{+}CD68^{+}$ myeloid subsets.

Over-representation analysis reveals the main enriched GO molecular function (MF) pathways. The dot size is proportional to the number of genes in the GO category that were found as cluster markers (Count). The intensity of the (red) colour represents the odds ratio.

A.12 GO cellular component pathway analysis of myeloid cell subsets

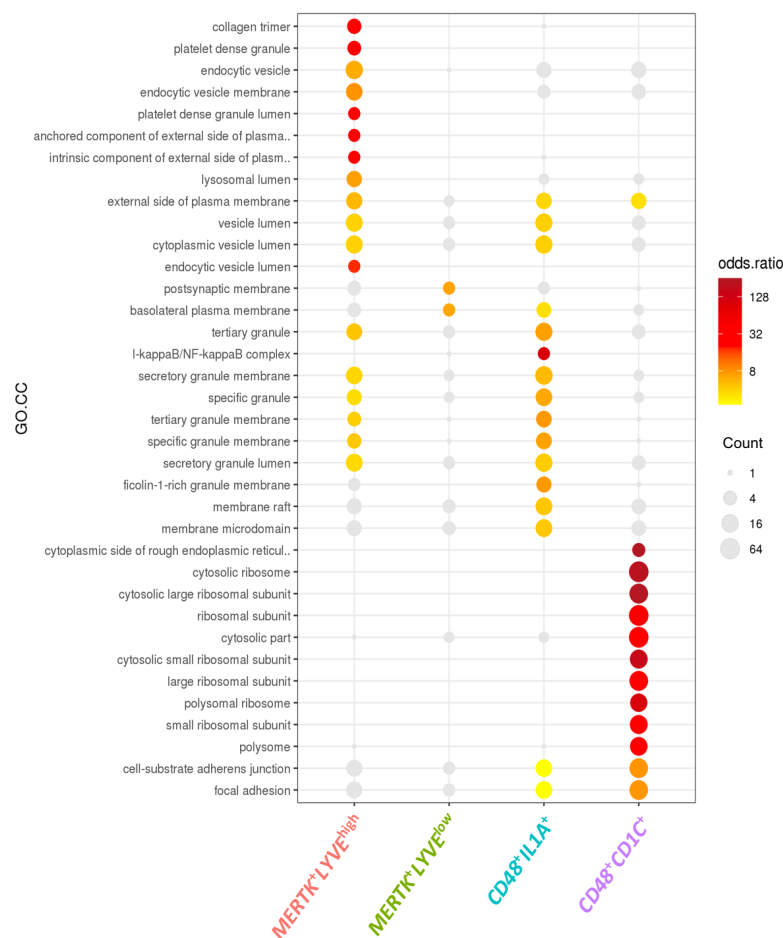


Figure A.12: GO cellular component pathway analysis of $CD14^+CD68^+$ myeloid cell subsets.

Over-representation analysis reveals the main enriched GO cellular component (CC) pathways. The dot size is proportional to the number of genes in the GO category that were found as cluster markers (Count). The intensity of the (red) colour represents the odds ratio.

A.13 MSigDB biocarta pathway analyses of myeloid subsets

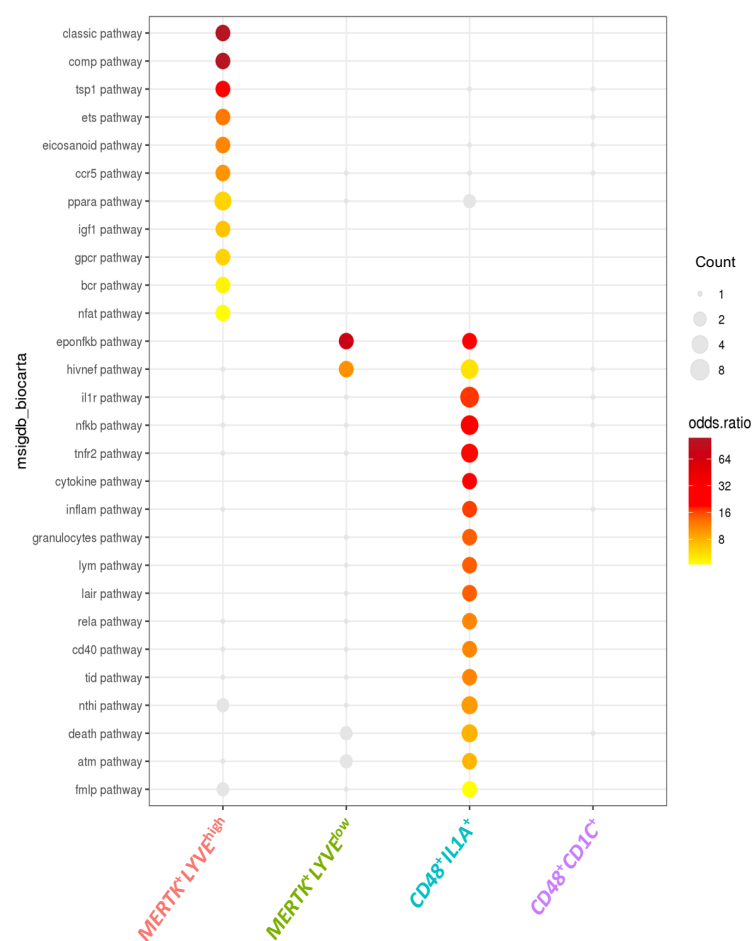


Figure A.13: MSigDB biocarta pathway analyses of $CD14^{+}CD68^{+}$ myeloid subsets. Over-representation analysis reveals the main enriched MSigDB biocarta pathways. The dot size is proportional to the number of genes in the MSigDB category that were found as cluster markers (Count). The intensity of the (red) colour represents the odds ratio.

A.14 MSigDB reactome pathway analyses of myeloid subsets

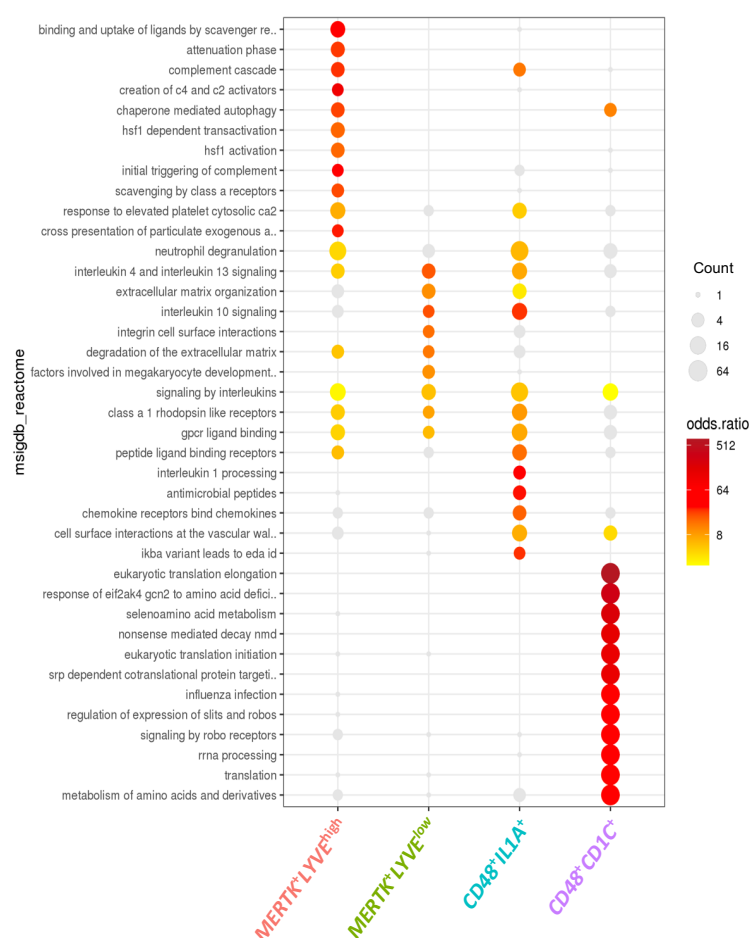


Figure A.14: MSigDB reactome pathway analyses of $CD14^{+}CD68^{+}$ myeloid subsets. Over-representation analysis reveals the main enriched MSigDB reactome pathways. The dot size is proportional to the number of genes in the MSigDB category that were found as cluster markers (Count). The intensity of the (red) colour represents the odds ratio.

A.15 Top 25 downregulated genes in frozen shoulder vs comparator $CD48^+IL1A^+$ macrophages

Table A.1: Top 25 significantly downregulated genes in frozen shoulder vs comparator $CD48^+IL1A^+$ macrophages.

gene	baseMean	log2FC	SE	stat	p value	padj
<i>FN1</i>	573.775121	-5.164008	0.4530321	-11.036674	2.54E-28	1.39E-24
<i>MARCO</i>	97.209991	-3.661258	0.4305224	-8.212072	2.17E-16	5.94E-13
<i>MAMDC2</i>	15.417142	-3.630229	0.5112875	-6.002962	1.94E-09	1.18E-06
<i>COLEC12</i>	21.880262	-3.425206	0.4811943	-6.10514	1.03E-09	7.02E-07
<i>SLCO2B1</i>	17.126129	-3.364681	0.4785482	-5.90101	3.61E-09	1.87E-06
<i>C2</i>	14.50341	-3.228073	0.4891224	-5.581357	2.39E-08	1.00E-05
<i>LILRB5</i>	23.027502	-3.102317	0.4764939	-5.894086	3.77E-09	1.87E-06
<i>LGMN</i>	96.75919	-3.031611	0.4754547	-6.31918	2.63E-10	2.88E-07
<i>ECM1</i>	11.080365	-2.903111	0.4876981	-5.135178	2.82E-07	7.38E-05
<i>NUPR1</i>	7.847602	-2.817674	0.5189926	-4.682966	2.83E-06	5.52E-04
<i>CTSK</i>	12.954952	-2.723046	0.5249873	-4.790874	1.66E-06	3.36E-04
<i>CD276</i>	6.40488	-2.545617	0.5265977	-4.297308	1.73E-05	2.38E-03
<i>MERTK</i>	5.302152	-2.545157	0.5208334	-4.238073	2.25E-05	2.70E-03
<i>DAB2</i>	46.060819	-2.536559	0.4665139	-5.444427	5.20E-08	1.78E-05
<i>OLFML2B</i>	6.429021	-2.533218	0.5201719	-4.351634	1.35E-05	2.18E-03
<i>MAF</i>	18.826329	-2.500076	0.4342963	-5.431452	5.59E-08	1.80E-05
<i>SCD</i>	6.28287	-2.498302	0.5127134	-4.282786	1.85E-05	2.38E-03
<i>PSD3</i>	4.167666	-2.492158	0.5300457	-4.282859	1.85E-05	2.38E-03
<i>SPP1</i>	1055.554973	-2.472476	0.5387078	-7.717613	1.19E-14	2.16E-11
<i>SERPINE1</i>	7.031037	-2.46082	0.5222129	-4.231311	2.32E-05	2.70E-03
<i>ME1</i>	6.882933	-2.457807	0.5102762	-4.310565	1.63E-05	2.38E-03
<i>ACKR3</i>	8.18774	-2.425295	0.506692	-4.295218	1.75E-05	2.38E-03
<i>GPNMB</i>	67.023981	-2.389937	0.4635979	-5.13409	2.84E-07	7.38E-05
<i>EGR1</i>	80.712829	-2.3639	0.5164331	-4.652279	3.28E-06	5.98E-04
<i>KIFC3</i>	5.444769	-2.339047	0.5265923	-4.076985	4.56E-05	5.09E-03

baseMean, mean of normalized counts for all samples; SE, standard error of the log2FC; stat, Wald statistic.

A.16 Top 25 upregulated genes in frozen shoulder vs comparator $CD48^+IL1A^+$ macrophages

Table A.2: Top 25 significantly upregulated genes in frozen shoulder vs comparator $CD48^+IL1A^+$ macrophages.

gene	baseMean	log2FC	SE	stat	p value	padj
<i>GPAT3</i>	34.241247	2.8058669	0.399282	6.946151	3.75E-12	5.13E-09
<i>LAMB3</i>	16.587141	2.1718422	0.5147827	4.235947	2.28E-05	2.70E-03
<i>CHST7</i>	19.500265	2.0754475	0.491247	4.238467	2.25E-05	2.70E-03
<i>PRDM8</i>	44.962447	1.9275201	0.4988898	3.911332	9.18E-05	8.51E-03
<i>TIMP1</i>	816.079265	1.8633577	0.448406	4.166098	3.10E-05	3.53E-03
<i>PLD4</i>	4.145674	1.8523302	0.5257813	3.522131	4.28E-04	2.82E-02
<i>MTRNR2L12</i>	122.954988	1.7466642	0.5208974	3.393241	6.91E-04	3.93E-02
<i>TXNRD1</i>	106.009909	1.682675	0.4604535	3.663298	2.49E-04	1.84E-02
<i>SRXN1</i>	9.509276	1.6215711	0.4554061	3.515438	4.39E-04	2.82E-02
<i>IL1A</i>	217.672395	1.6158864	0.4612761	3.505465	4.56E-04	2.90E-02
<i>ADAM19</i>	11.792299	1.6119909	0.5110265	3.146922	1.65E-03	7.39E-02
<i>HCAR3</i>	22.605784	1.5825508	0.4369275	3.626396	2.87E-04	2.07E-02
<i>GCLM</i>	42.583217	1.5400559	0.4184488	3.688512	2.26E-04	1.69E-02
<i>NSMAF</i>	35.986179	1.506802	0.4014039	3.76285	1.68E-04	1.35E-02
<i>GPR35</i>	9.929532	1.5057826	0.4469382	3.37356	7.42E-04	4.18E-02
<i>PLCXD1</i>	15.033981	1.5049508	0.5285683	2.876719	4.02E-03	1.34E-01
<i>IRF4</i>	8.077654	1.4681569	0.5008543	2.943731	3.24E-03	1.19E-01
<i>EID3</i>	12.464064	1.4590213	0.4992746	2.926605	3.43E-03	1.24E-01
<i>MMP9</i>	132.684497	1.4437439	0.512892	2.834422	4.59E-03	1.45E-01
<i>IL1R1</i>	28.599393	1.4230709	0.5074016	2.818309	4.83E-03	1.48E-01
<i>TNFAIP6</i>	94.293842	1.4159624	0.4890576	2.899449	3.74E-03	1.28E-01
<i>ISG20</i>	22.264422	1.414935	0.486588	2.918209	3.52E-03	1.24E-01
<i>IL1B</i>	4683.19646	1.3667819	0.3960874	3.452149	5.56E-04	3.38E-02
<i>CSF2RA</i>	36.621538	1.3470855	0.3434124	3.927033	8.60E-05	8.11E-03
<i>SMAD3</i>	17.576851	1.3084691	0.4856517	2.736461	6.21E-03	1.70E-01

baseMean, mean of normalized counts for all samples; SE, standard error of the log2FC; stat, Wald statistic.

Chapter 3

B.1 HMGB1, IL-33, and TLR-2 staining in comparator and frozen shoulder capsule

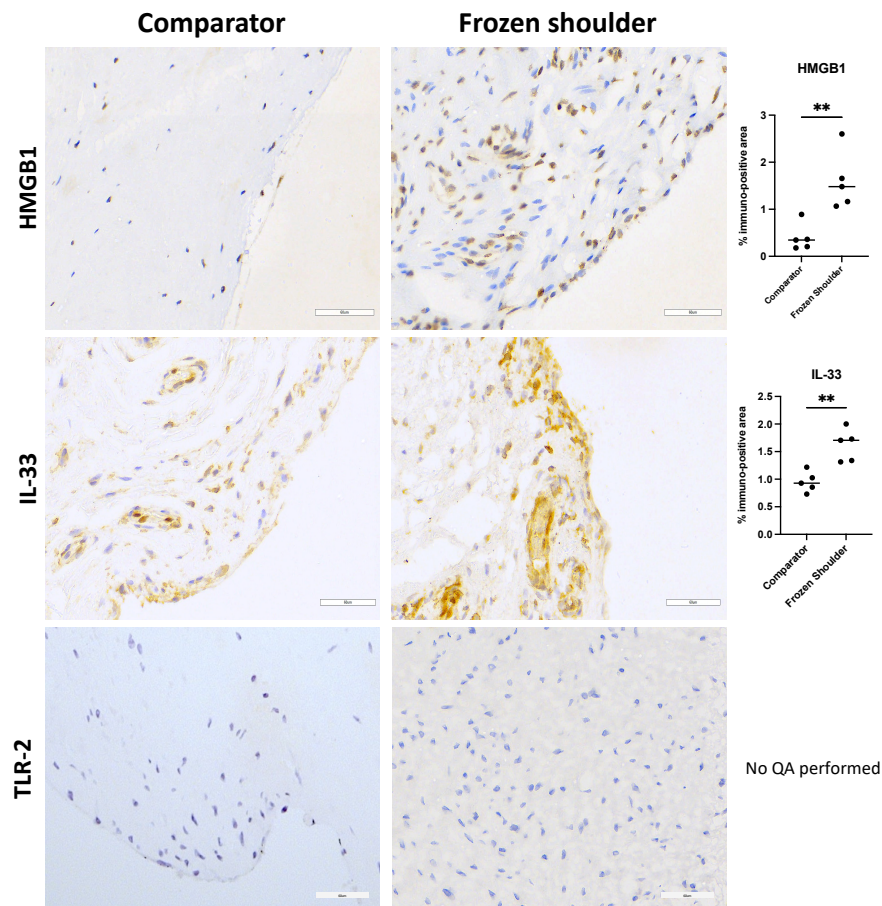


Figure B.1: HMGB1, IL-33, and TLR-2 staining in comparator and frozen shoulder capsule.

Representative images of HMGB1, IL-33, and TLR-2 single staining (= brown) in comparator and frozen shoulder capsular tissue. Haematoxylin was used as nuclear counterstaining (= purple). Scale bar: 60µm (white). The graphs on the right show semi-quantitative image analysis of HMGB1 and IL-33 immunostaining respectively. Every dot represents a unique donor. Statistically significant differences were calculated using the Mann Whitney U test. **p<0.01. Semi-quantitative image analysis (QA) for TLR-2 was not performed.

B.2 Isotype control immunohistochemistry

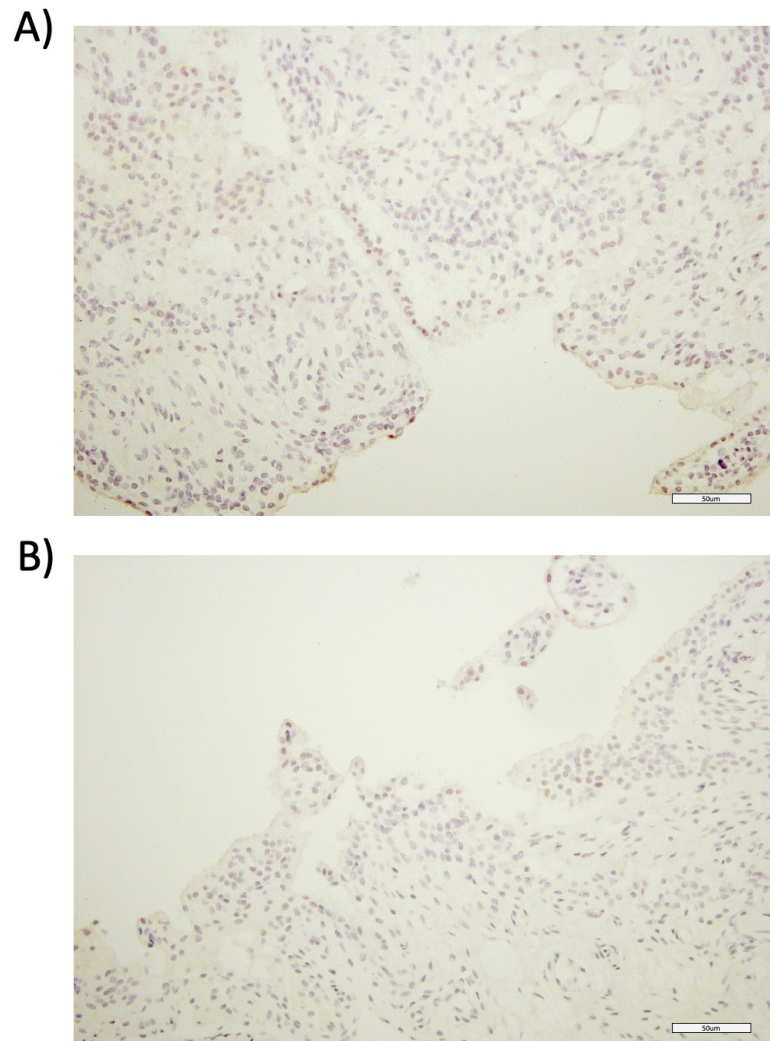


Figure B.2: Isotype control immunohistochemistry.

Immunohistochemistry images of rabbit (A) and mouse (B) isotype control staining in frozen shoulder capsular tissue. Haematoxylin was used as a nuclear counterstain. Scale bar: 50µm (white).

B.3 ISH targeting *IL1A* and *IL1B* in comparator capsular tissues

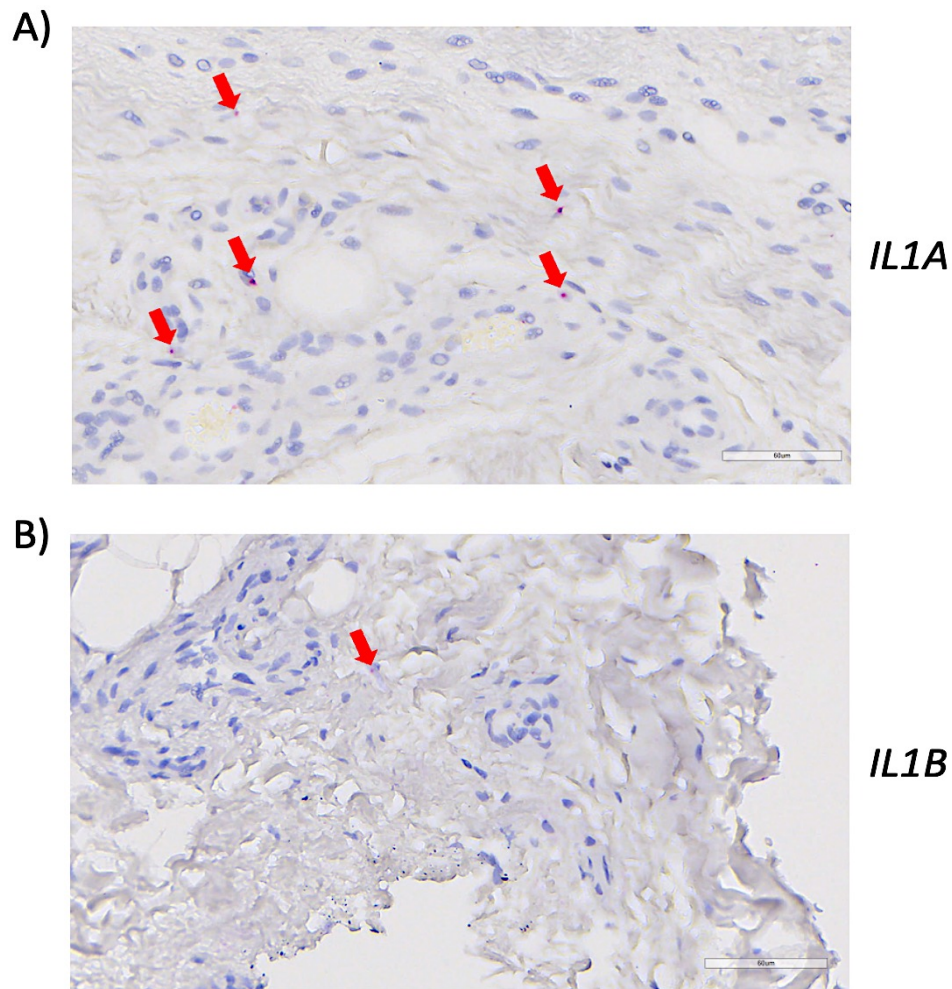


Figure B.3: ISH targeting *IL1A* and *IL1B* in comparator capsular tissues. Representative images of ISH of A) *IL1A* and B) *IL1B* mRNA transcripts in capsular tissue sections of comparator group patients. Red arrows showing RNAscope puncta, which represent single mRNA transcripts. Scale bar: 60µm (white).

B.4 Positive and negative control RNAscope **ISH**

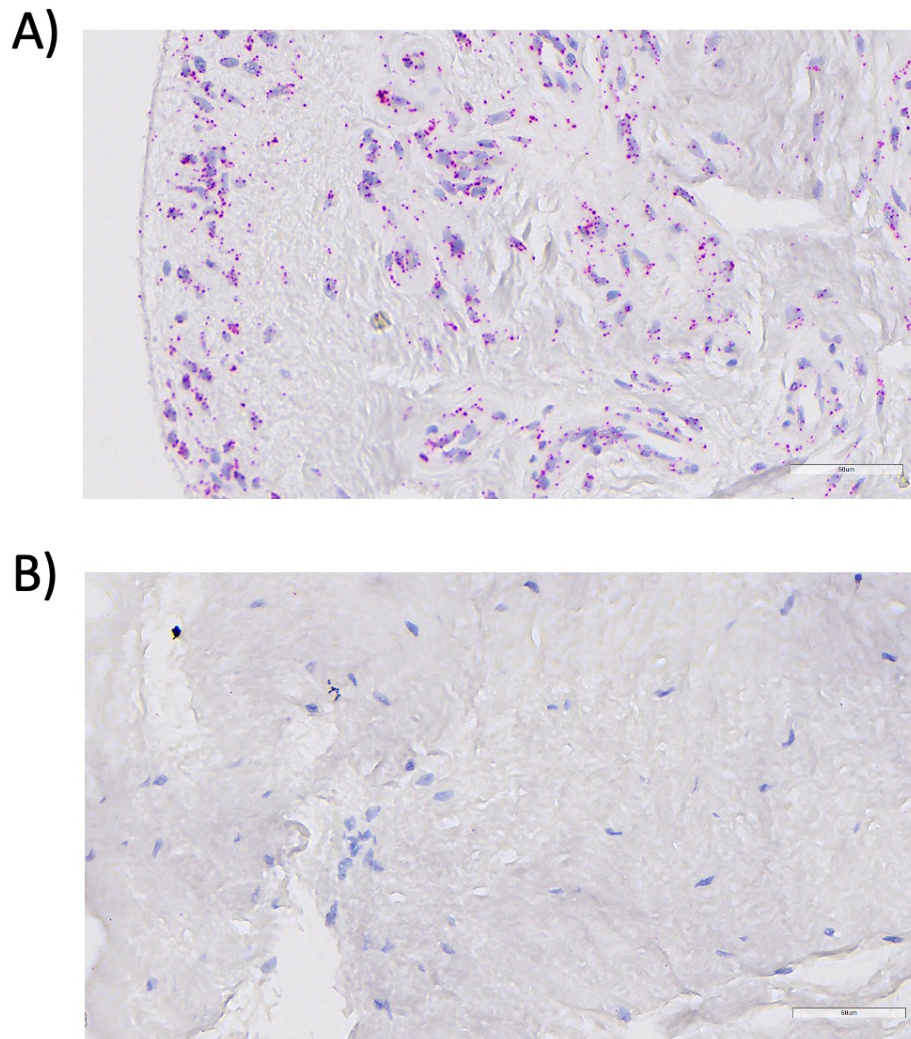


Figure B.4: Positive and negative control RNAscope.

Representative images of **ISH** showing A) positive control with multiple **mRNA** transcripts (red puncta) and B) negative control in capsular tissue sections of comparator patient groups. Scale bar: 60µm (white).

B.5 UMAPs of the fibroblasts of the rotator interval capsule

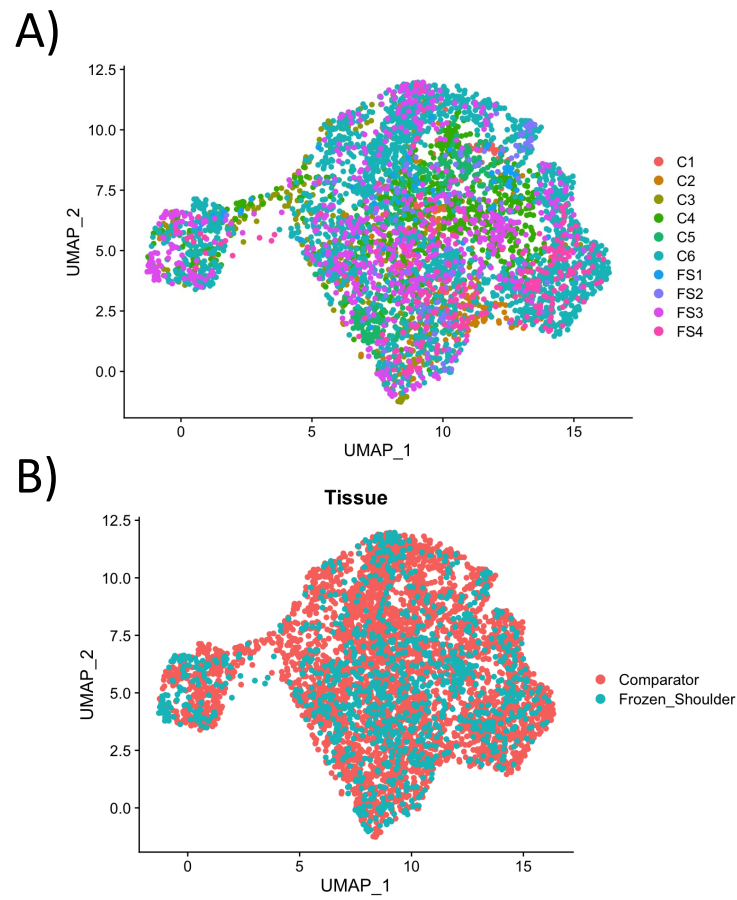


Figure B.5: UMAPs of the $COL1A1^+PDGFRA^+FAP^+$ fibroblasts.

A) UMAP showing cells from individual donors ($n = 6$ comparator and $n = 4$ frozen shoulder). B) UMAP showing cells from comparator and frozen shoulder samples.

B.6 SingleR annotation of the fibroblast atlas with the HumanPrimaryCellAtlasData

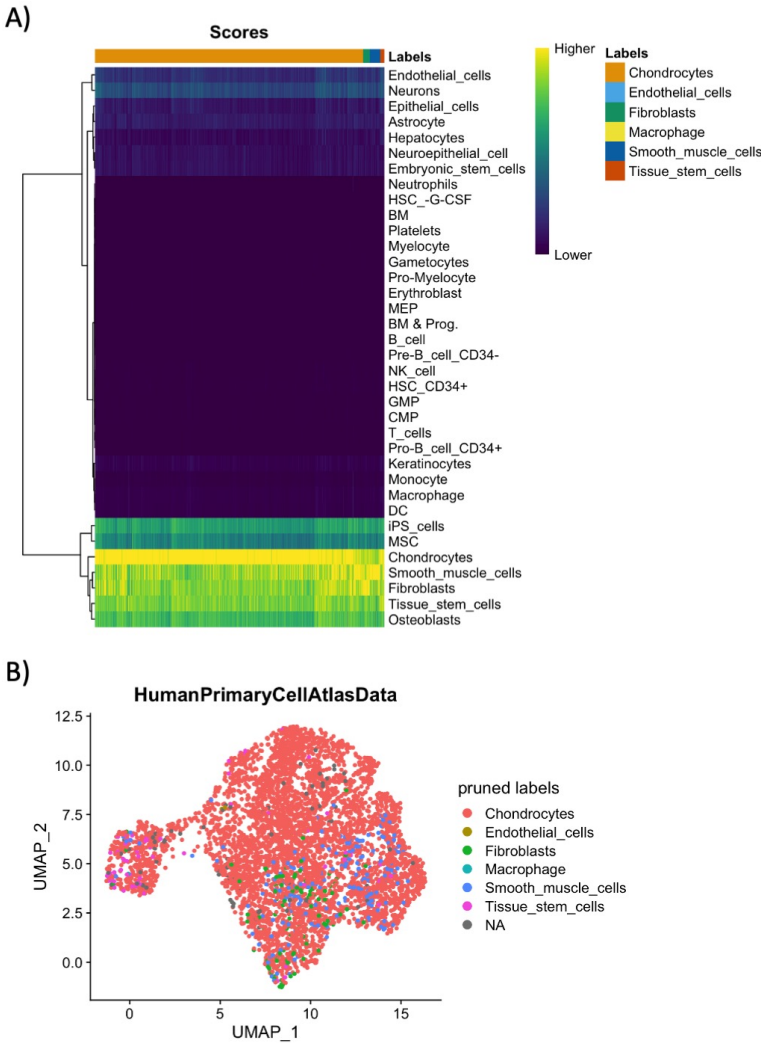


Figure B.6: SingleR annotation of the fibroblast atlas with the HumanPrimaryCellAtlasData.

A) singleR predictions ScoreHeatmap. B) pruned singleR predictions **UMAP**.

B.7 SingleR annotation of the fibroblast atlas with the DatabaseImmuneCellExpressionData

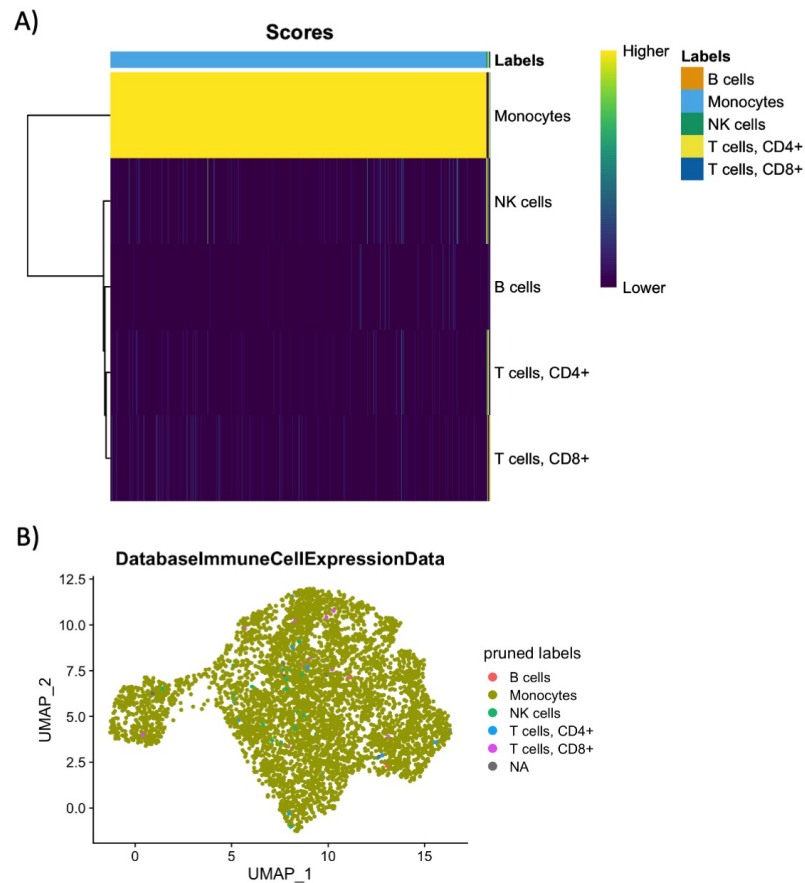


Figure B.7: SingleR annotation of the fibroblast atlas with the DatabaseImmuneCellExpressionData.

A) singleR predictions ScoreHeatmap. B) pruned singleR predictions **UMAP**.

B.8 SingleR annotation of the fibroblast atlas with the MonacoImmuneData

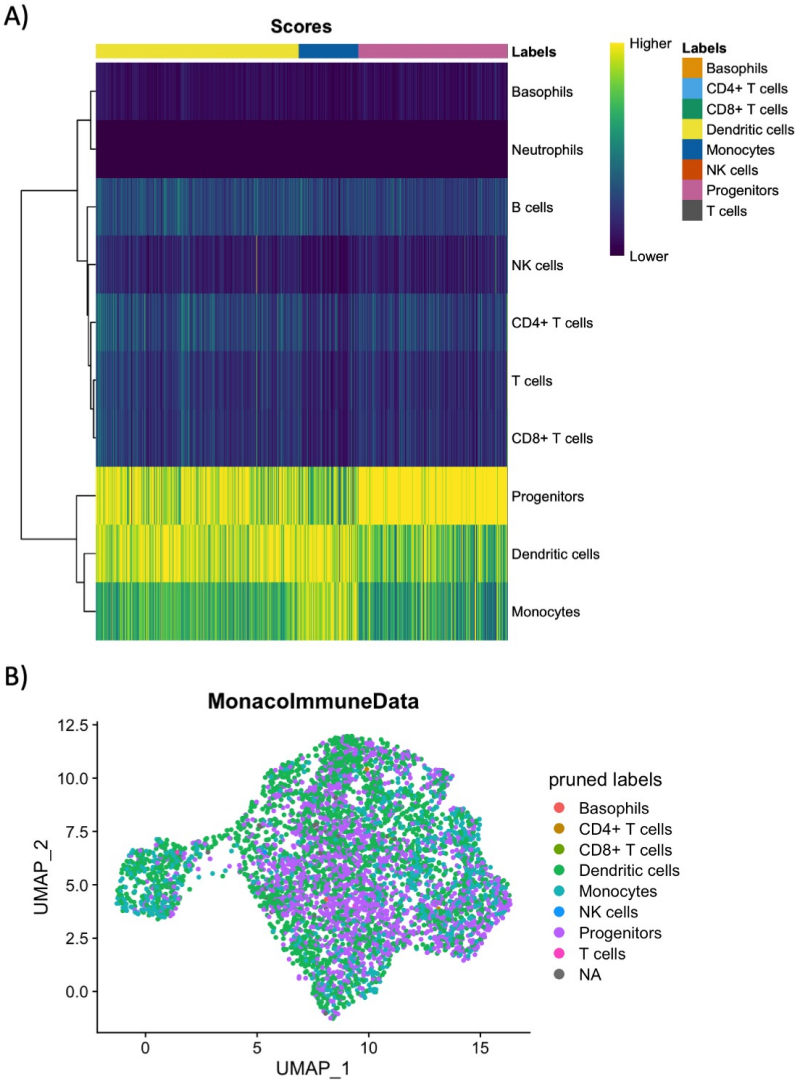


Figure B.8: SingleR annotation of the fibroblast atlas with the MonacoImmuneData. A) singleR predictions ScoreHeatmap. B) pruned singleR predictions **UMAP**.

B.9 Cluster markers of the different fibroblast populations

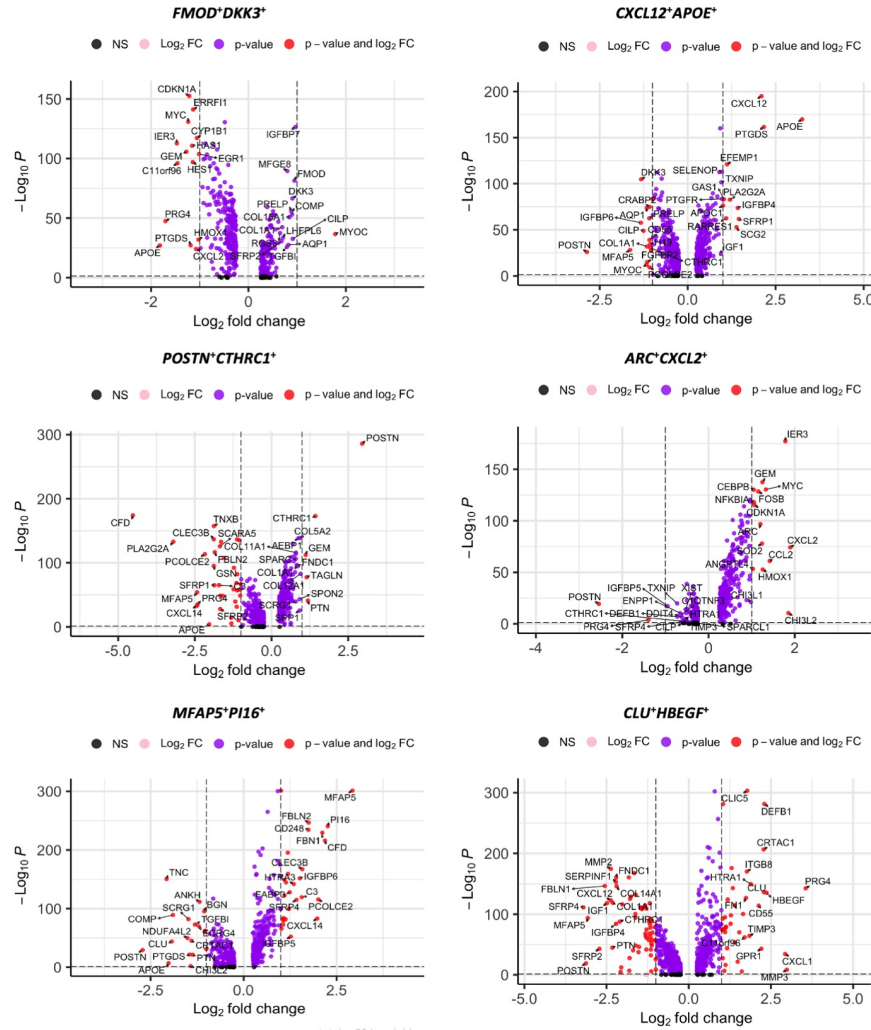


Figure B.9: Cluster markers of the $COL1A1^{+}PDGFRA^{+}FAP^{+}$ fibroblasts. Volcano plots displaying the top negative (negative $\log_2FC$; left) and positive marker genes (positive $\log_2FC$; right) that characterise the $FMOD^{+}DKK3^{+}$, $CXCL12^{+}APOE^{+}$, $POSTN^{+}CTHRC1^{+}$, $ARC^{+}CXCL2^{+}$, $MFAP5^{+}PI16^{+}$ and $CLU^{+}HBEGF^{+}$ fibroblast populations.

Chapter 4

C.1 Detection ranges of the analytes (MSD)

Table C.1: Detection ranges of the different analytes used for MSD.

Analyte	Lower LOD	Upper LOD
CXCL-12	1,204 pg/ml	510,000 pg/ml
GM-CSF	0,303 pg/ml	54,000 pg/ml
IL-10	0,284 pg/ml	19,700 pg/ml
IL-1 α	0,739 pg/ml	29,600 pg/ml
IL-1 β	0,369 pg/ml	22,550 pg/ml
IL-1RA	2,79 pg/ml	22,950 pg/ml
IL-33	0,675 pg/ml	43,000 pg/ml
IL-6	0,735 pg/ml	9,500 pg/ml
TNF- α	0,693 pg/ml	14,500 pg/ml

C.2 Standard plots of the analytes (MSD)

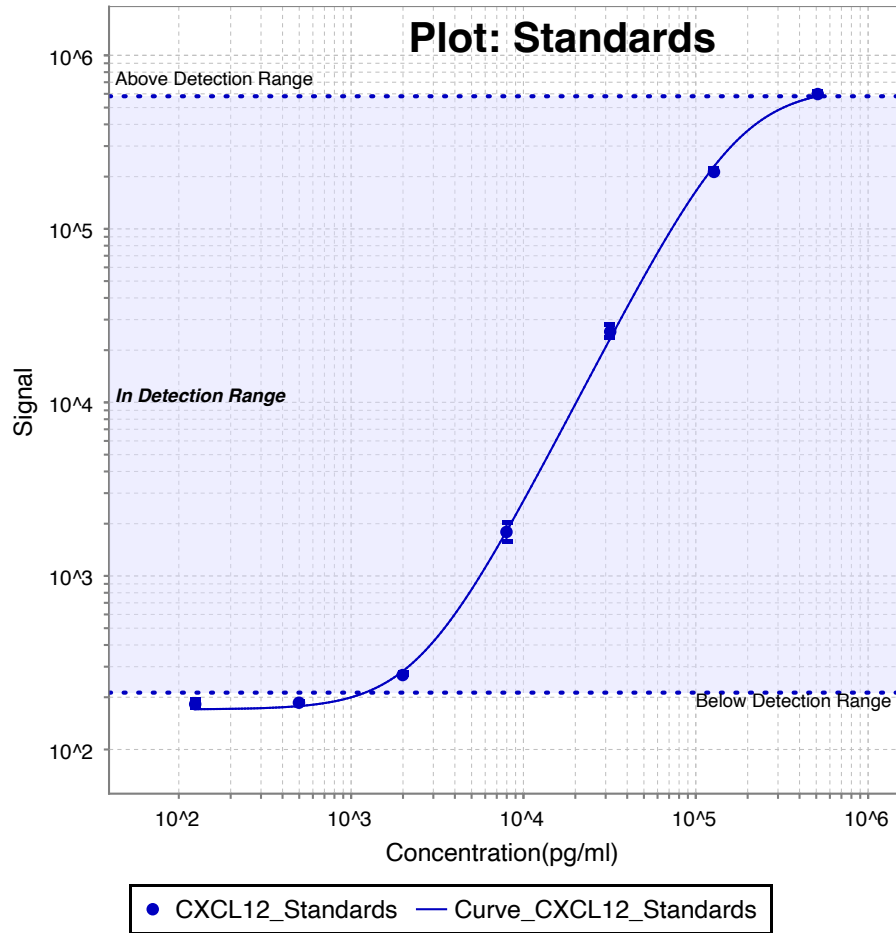


Figure C.1: Standard plot of CXCL-12.

Standard curve of CXCL-12 plotted by the MSD signal (y-axis) and the concentration (x-axis). Blue dots represent the CXCL-12 standards. Blue lines represent the CXCL-12 standard curve. Detection ranges are displayed with blue dotted lines.

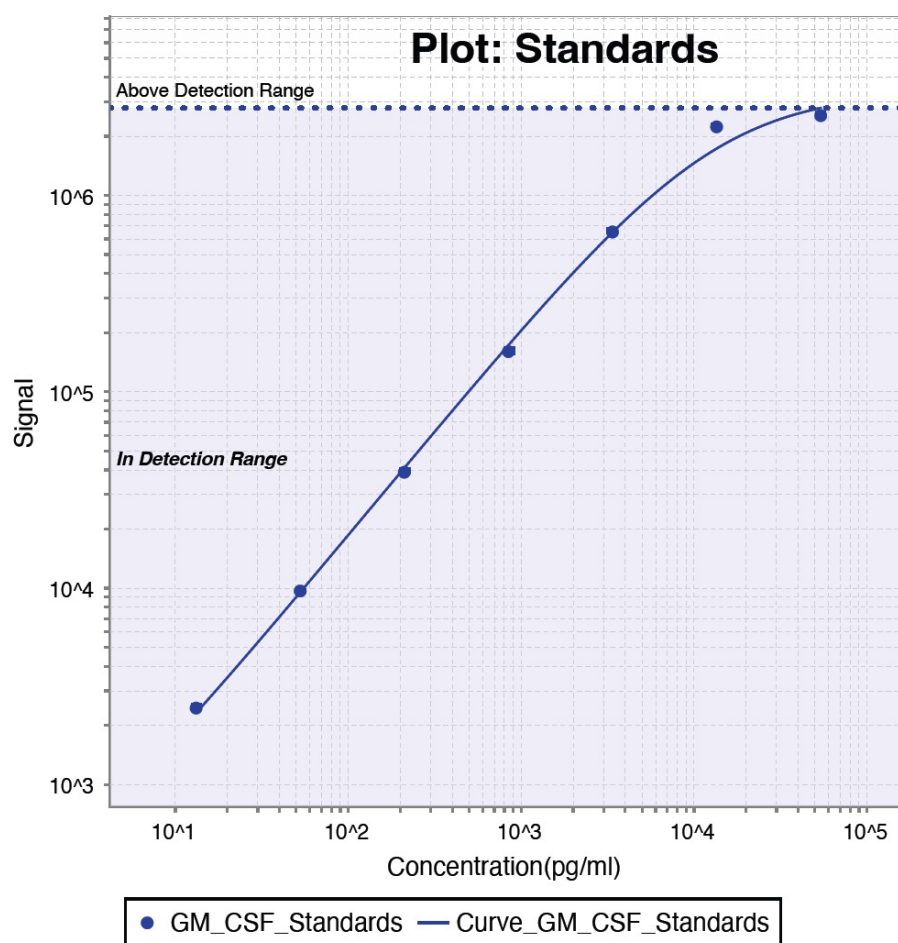


Figure C.2: Standard plot of GM-CSF.

Standard curve of GM-CSF plotted by the MSD signal (y-axis) and the concentration (x-axis). Blue dots represent the GM-CSF standards. Blue lines represent the GM-CSF standard curve. Detection ranges are displayed with blue dotted lines.

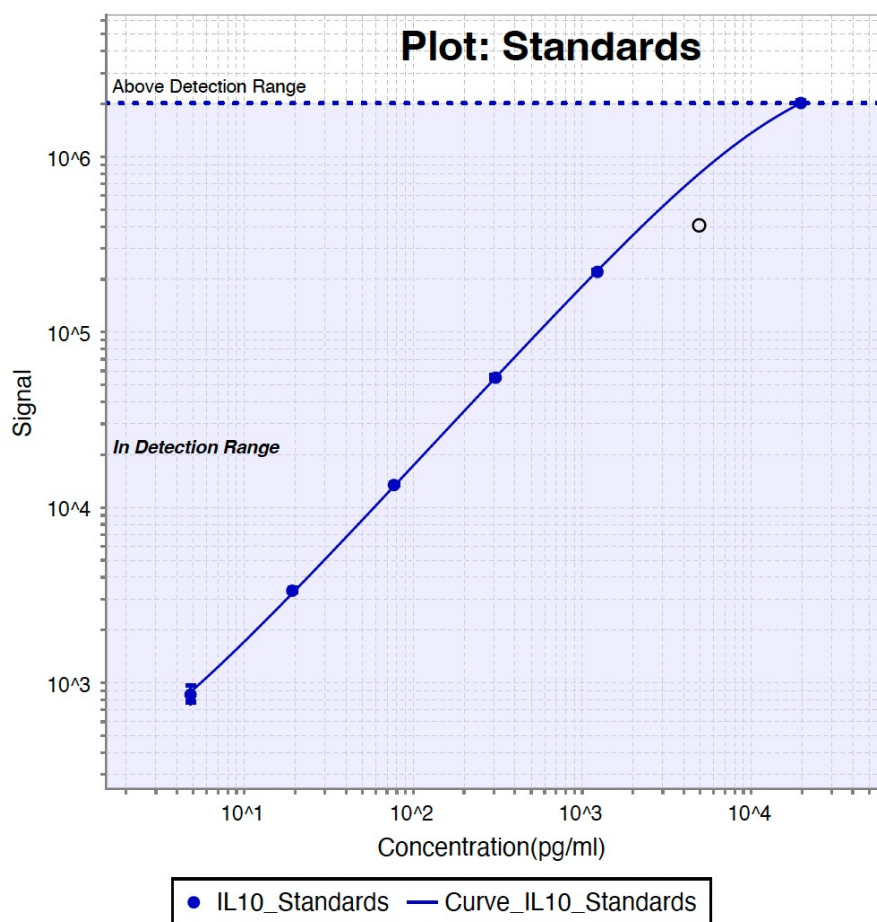


Figure C.3: Standard plot of IL-10.

Standard curve of IL-10 plotted by the MSD signal (y-axis) and the concentration (x-axis). Blue dots represent the IL-10 standards. Blue lines represent the IL-10 standard curve. Detection ranges are displayed with blue dotted lines.

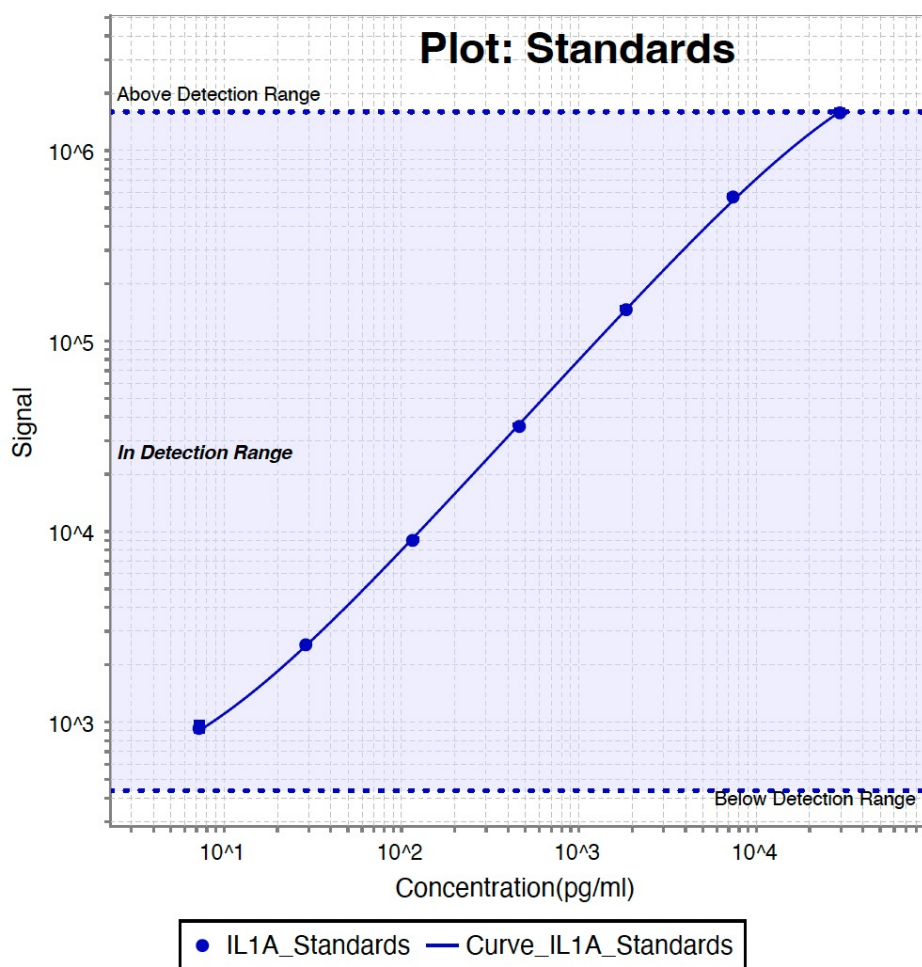


Figure C.4: Standard plot of IL-1 α .

Standard curve of IL-1 α plotted by the MSD signal (y-axis) and the concentration (x-axis). Blue dots represent the IL-1 α standards. Blue lines represent the IL-1 α standard curve. Detection ranges are displayed with blue dotted lines.

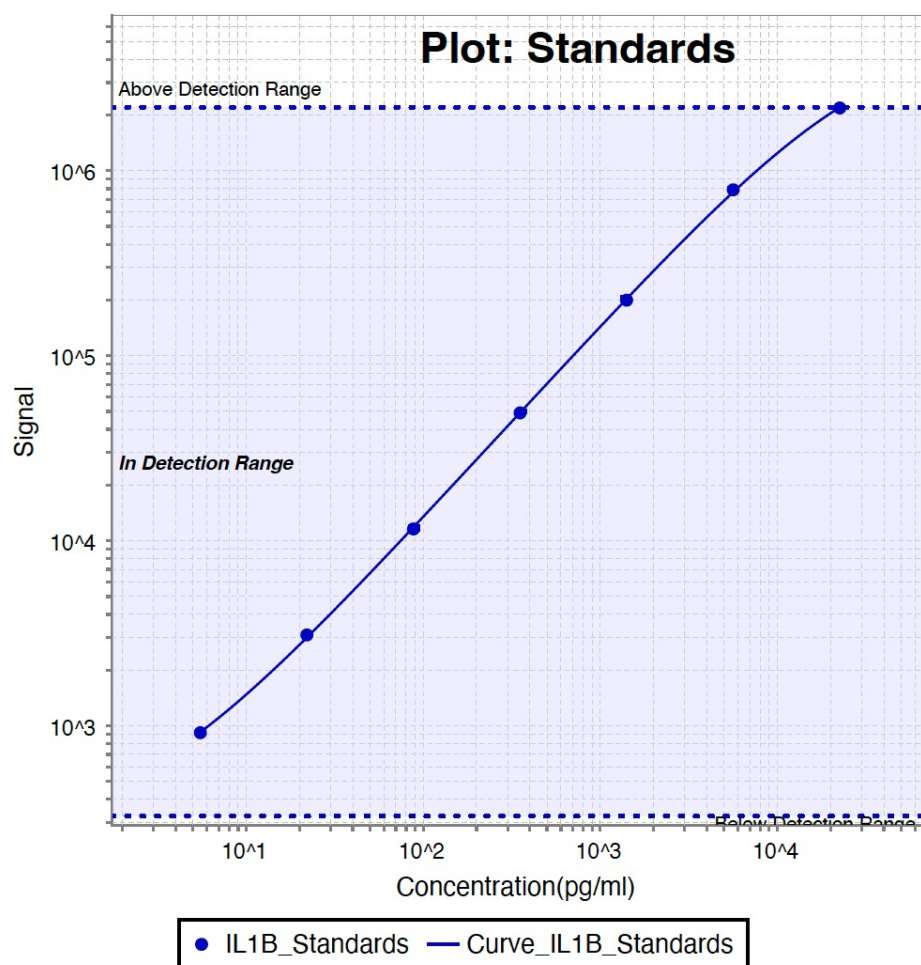


Figure C.5: Standard plot of $\text{IL-1}\beta$.

Standard curve of $\text{IL-1}\beta$ plotted by the MSD signal (y-axis) and the concentration (x-axis). Blue dots represent the $\text{IL-1}\beta$ standards. Blue lines represent the $\text{IL-1}\beta$ standard curve. Detection ranges are displayed with blue dotted lines.

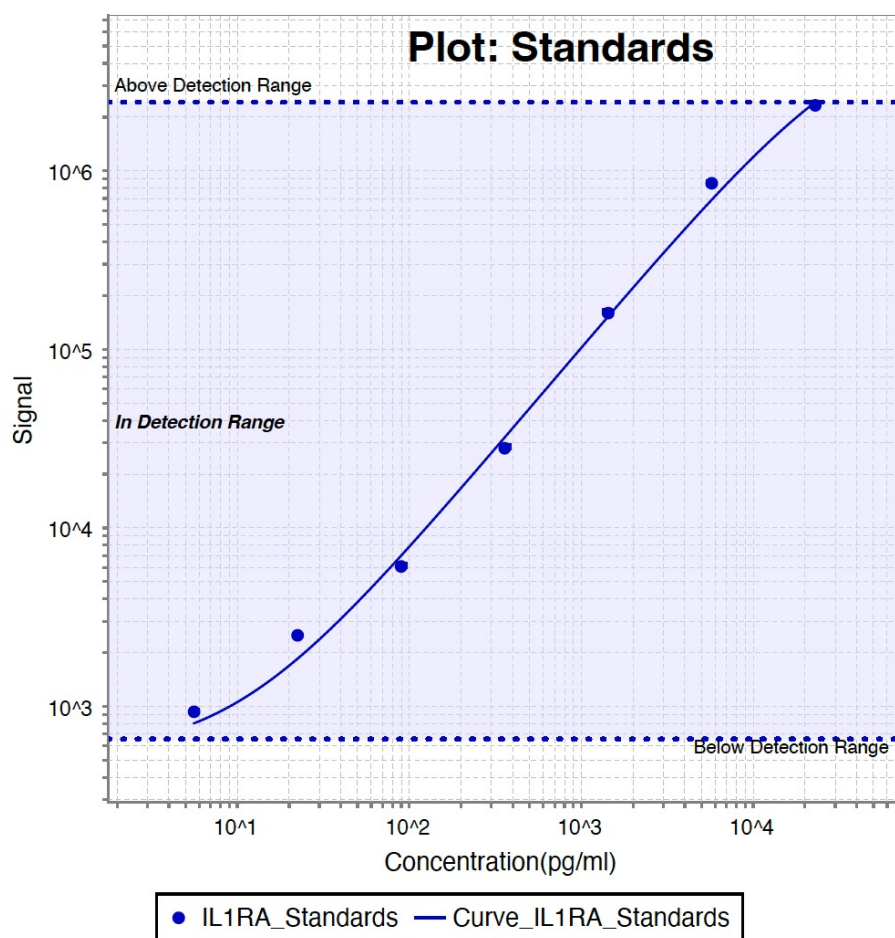


Figure C.6: Standard plot of IL-1RA.

Standard curve of IL-1RA plotted by the MSD signal (y-axis) and the concentration (x-axis). Blue dots represent the IL-1RA standards. Blue lines represent the IL-1RA standard curve. Detection ranges are displayed with blue dotted lines.

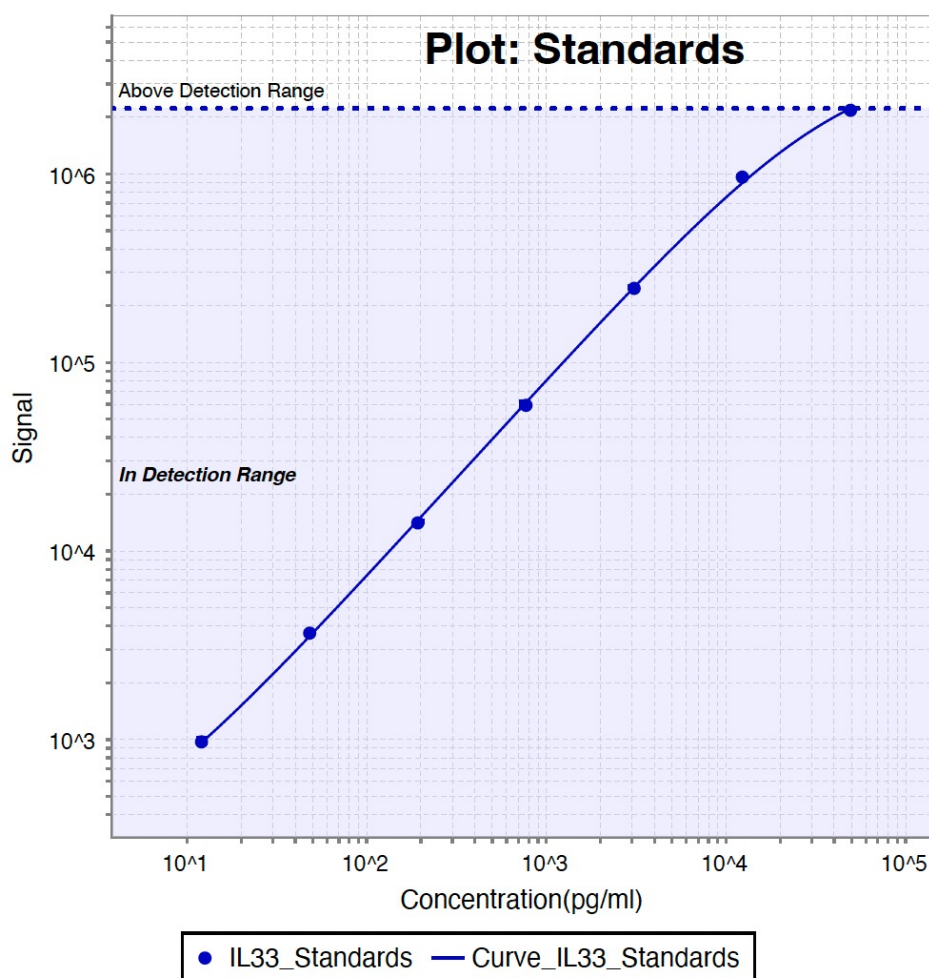


Figure C.7: Standard plot of IL-33.

Standard curve of IL-33 plotted by the MSD signal (y-axis) and the concentration (x-axis). Blue dots represent the IL-33 standards. Blue lines represent the IL-33 standard curve. Detection ranges are displayed with blue dotted lines.

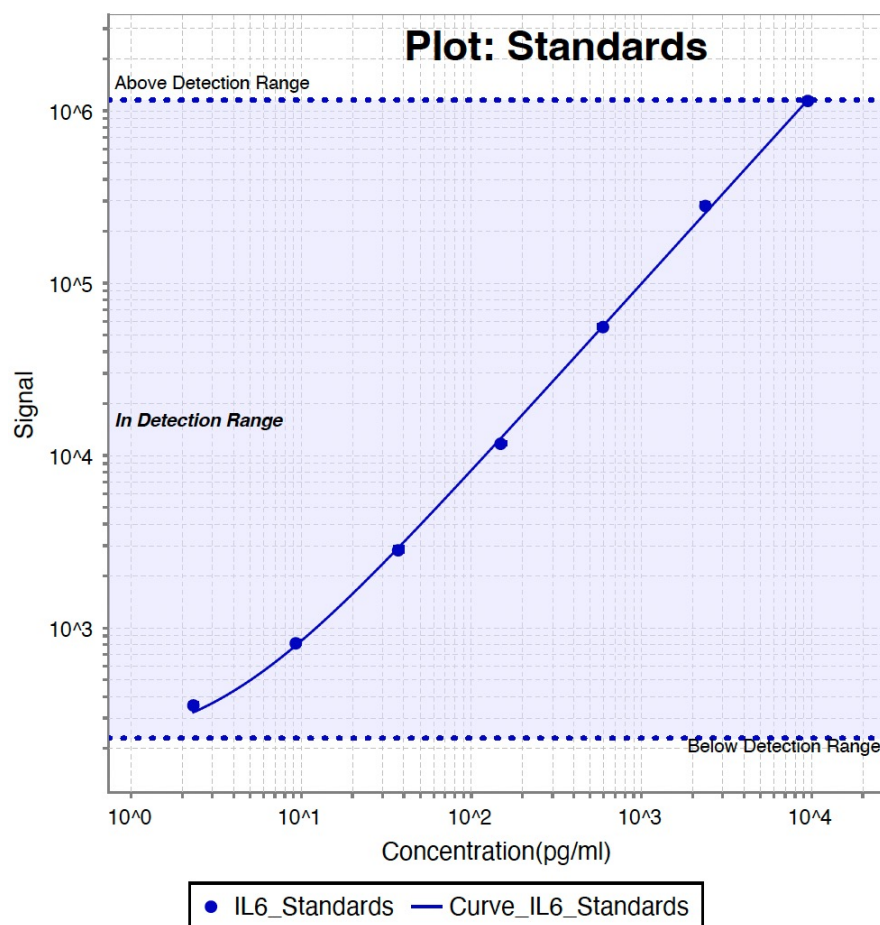


Figure C.8: Standard plot of IL-6.

Standard curve of IL-6 plotted by the MSD signal (y-axis) and the concentration (x-axis). Blue dots represent the IL-6 standards. Blue lines represent the IL-6 standard curve. Detection ranges are displayed with blue dotted lines.

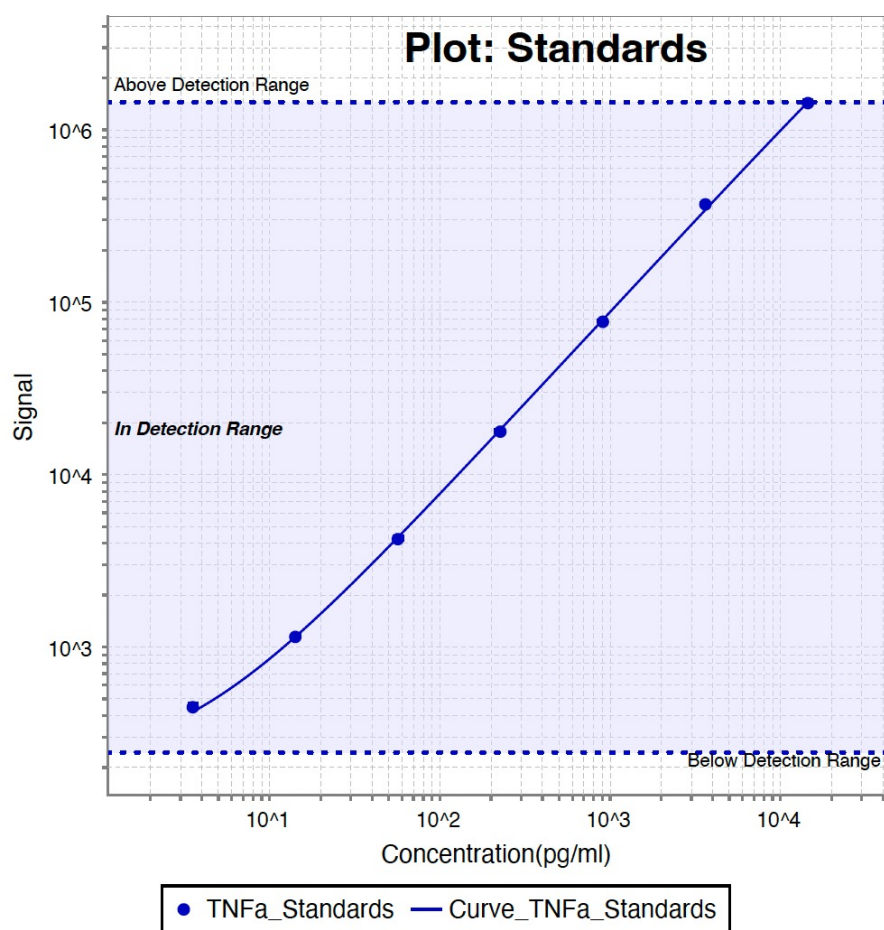


Figure C.9: Standard plot of $\text{TNF-}\alpha$.

Standard curve of $\text{TNF-}\alpha$ plotted by the MSD signal (y-axis) and the concentration (x-axis). Blue dots represent the $\text{TNF-}\alpha$ standards. Blue lines represent the $\text{TNF-}\alpha$ standard curve. Detection ranges are displayed with blue dotted lines.

C.3 Top 50 **DEGs** of stimulated fibroblast conditions (vs unstimulated fibroblasts)

Table C.2: The top 50 **DEGs** of **IL-1 α** stimulated fibroblasts.

Top 50 **DEGs** in **IL-1 α** stimulated fibroblasts vs unstimulated fibroblasts, sorted by **padj**.

gene	baseMean	log2FC	SE	p value	padj
<i>NFKBIZ</i>	4233,313	3,878701	0,104789	2,55E-302	4,71E-298
<i>PDE4D</i>	2941,586	5,492378	0,160813	1,44E-256	1,33E-252
<i>HPD</i>	7240,63	5,805449	0,178464	7,74E-235	4,76E-231
<i>ZC3H12A</i>	4412,059	4,016982	0,123812	3,68E-233	1,70E-229
<i>FGF7</i>	35283,66	1,878601	0,060148	8,89E-216	3,28E-212
<i>CXCL5</i>	19947,4	9,918226	0,326714	2,19E-205	6,73E-202
<i>C1QTNF1</i>	9011,616	3,022667	0,100486	2,49E-200	6,56E-197
<i>NAMPT</i>	37546,91	3,779001	0,129007	2,26E-190	5,21E-187
<i>TMEM132A</i>	4222,663	3,147089	0,115779	2,23E-164	4,57E-161
<i>C17orf58</i>	3085,927	3,100441	0,116985	7,36E-156	1,36E-152
<i>SLC43A3</i>	7339,073	2,003537	0,077068	5,26E-150	8,67E-147
<i>NRP2</i>	20082,82	3,353947	0,128845	5,64E-150	8,67E-147
<i>NAB1</i>	3957,802	1,649354	0,063599	5,28E-149	7,50E-146
<i>RCAN1</i>	15543,26	2,883588	0,114381	1,24E-141	1,63E-138
<i>IGF1</i>	7176,467	5,183815	0,20731	5,76E-140	7,08E-137
<i>CCL5</i>	1502,154	6,900104	0,274197	8,27E-140	9,53E-137
<i>PPL</i>	6408,462	-1,70952	0,068293	1,01E-137	1,10E-134
<i>KYNU</i>	1162,839	5,535951	0,23547	1,68E-126	1,72E-123
<i>CTSS</i>	4249,972	3,428002	0,147091	5,20E-122	5,05E-119
<i>CXCL3</i>	23622,06	9,680307	0,41947	1,02E-121	9,39E-119
<i>IL6</i>	45071,44	6,92323	0,300374	2,08E-121	1,82E-118
<i>TRANK1</i>	6892,448	2,05127	0,087902	3,07E-121	2,58E-118
<i>SLC30A1</i>	7242,093	1,337215	0,057896	1,78E-119	1,43E-116
<i>C1GALT1C1</i>	4799,947	2,081033	0,092118	1,74E-114	1,34E-111
<i>B4GALT1</i>	24465,76	1,612491	0,072111	6,27E-112	4,62E-109
<i>TGFBR1</i>	7647,055	1,310904	0,05885	4,06E-111	2,88E-108
<i>SERPINA3</i>	983,2207	2,009439	0,090806	1,60E-109	1,09E-106
<i>CCL2</i>	37988,35	3,674094	0,167005	1,85E-109	1,22E-106
<i>AK5</i>	688,1611	-2,19041	0,100061	2,46E-107	1,56E-104
<i>UACA</i>	4079,029	-1,7132	0,078549	1,44E-106	8,83E-104
<i>SLC16A4</i>	1840,574	1,64553	0,076864	9,13E-103	5,43E-100
<i>PLXNA4</i>	3368,281	3,342416	0,156994	4,33E-102	2,50E-99
<i>PLEKHA4</i>	3299,534	-1,51731	0,071521	5,45E-101	3,05E-98
<i>LBP</i>	6381,727	8,353487	0,389932	1,76E-100	9,57E-98
<i>PDE4B</i>	2483,752	4,000115	0,189721	3,46E-100	1,82E-97
<i>FNDC3B</i>	16459,74	1,618775	0,076831	8,64E-100	4,43E-97
<i>P4HA2</i>	7750,036	1,421945	0,067488	2,67E-99	1,33E-96
<i>STAT4</i>	637,4869	2,408652	0,114798	6,61E-99	3,21E-96
<i>AKR1B1</i>	21628,09	2,03482	0,097295	2,65E-98	1,25E-95
<i>WNT5A</i>	6652,032	2,311552	0,110836	3,21E-98	1,48E-95
<i>DNAJC1</i>	3059,706	1,270741	0,060948	3,15E-97	1,43E-94
<i>MFSD2A</i>	467,9459	3,208955	0,155736	4,19E-96	1,84E-93
<i>NAMPTP1</i>	453,9358	4,039565	0,196437	2,17E-95	9,30E-93
<i>GBP1</i>	1824,665	1,274393	0,062	9,84E-95	4,13E-92
<i>CLDND1</i>	4343,884	1,285851	0,062701	2,03E-94	8,33E-92
<i>TFPI2</i>	21596,26	4,185017	0,207258	5,93E-93	2,38E-90
<i>ANXA2</i>	49093,86	-1,41898	0,070213	3,53E-92	1,38E-89
<i>PON2</i>	3726,614	1,27296	0,06333	8,68E-91	3,33E-88
<i>CFH</i>	7543,491	1,353454	0,067534	1,76E-90	6,62E-88
<i>CHST11</i>	3133,739	2,405476	0,120223	2,28E-90	8,41E-88

baseMean, mean of normalized counts for all samples; SE, standard error of the **log2FC**; **padj**, **BH** adjusted p values.

Table C.3: The top 50 **DEGs** of **IL-1 β** stimulated fibroblasts.

Top 50 **DEGs** in **IL-1 β** stimulated fibroblasts vs unstimulated fibroblasts, sorted by **padj**.

gene	baseMean	log2FC	SE	p value	padj
<i>NFKBIZ</i>	4233,313	3,854263	0,104808	1,75E-298	3,22E-294
<i>PDE4D</i>	2941,586	5,430238	0,160823	8,84E-251	8,15E-247
<i>ZC3H12A</i>	4412,059	4,040623	0,123775	8,05E-236	4,95E-232
<i>HPD</i>	7240,63	5,811717	0,178782	2,05E-234	9,45E-231
<i>FGF7</i>	35283,66	1,847595	0,060149	7,92E-209	2,92E-205
<i>CXCL5</i>	19947,4	9,906437	0,326865	6,59E-205	2,03E-201
<i>C1QTNF1</i>	9011,616	2,931069	0,100504	1,74E-188	4,59E-185
<i>NAMPT</i>	37546,91	3,724439	0,129066	5,45E-185	1,26E-181
<i>TMEM132A</i>	4222,663	3,070241	0,115816	1,59E-156	3,26E-153
<i>SLC43A3</i>	7339,073	2,014415	0,077057	1,22E-151	2,24E-148
<i>C17orf58</i>	3085,927	3,027939	0,117008	9,30E-149	1,56E-145
<i>PPL</i>	6408,462	-1,7647	0,068315	1,08E-146	1,66E-143
<i>NAB1</i>	3957,802	1,632929	0,063608	5,01E-146	7,11E-143
<i>RCAN1</i>	15543,26	2,915607	0,114397	9,10E-145	1,2E-141
<i>NRP2</i>	20082,82	3,270271	0,128775	9,21E-143	1,13E-139
<i>CCL5</i>	1502,154	6,946284	0,273963	1,05E-141	1,2E-138
<i>IGF1</i>	7176,467	5,0757	0,207452	2,79E-134	3,03E-131
<i>KYNU</i>	1162,839	5,552111	0,235815	3,37E-127	3,46E-124
<i>IL6</i>	45071,44	6,957301	0,300279	1,41E-122	1,37E-119
<i>CXCL3</i>	23622,06	9,623149	0,419622	2,55E-120	2,35E-117
<i>CTSS</i>	4249,972	3,376244	0,147218	2,34E-118	2,06E-115
<i>PLEKHA4</i>	3299,534	-1,63981	0,071683	4,94E-117	4,14E-114
<i>C1GALT1C1</i>	4799,947	2,082921	0,092091	9,45E-115	7,57E-112
<i>TGFB1</i>	7647,055	1,324327	0,058818	1,51E-113	1,16E-110
<i>UACA</i>	4079,029	-1,76064	0,07859	2,55E-112	1,88E-109
<i>TRANK1</i>	6892,448	1,964791	0,087919	2,00E-111	1,42E-108
<i>CCL2</i>	37988,35	3,706299	0,166879	2,43E-111	1,66E-108
<i>STAT4</i>	637,4869	2,523843	0,114377	4,35E-109	2,86E-106
<i>MFS2A</i>	467,9459	3,400963	0,155106	1,97E-108	1,25E-105
<i>B4GALT1</i>	24465,76	1,567819	0,072112	5,27E-106	3,24E-103
<i>SLC30A1</i>	7242,093	1,257515	0,057939	9,65E-106	5,74E-103
<i>AKR1B1</i>	21628,09	2,110843	0,097262	1,18E-105	6,81E-103
<i>SERPINA3</i>	983,2207	1,957471	0,090824	5,53E-104	3,09E-101
<i>SLC16A4</i>	1840,574	1,636427	0,076837	1,09E-101	5,90E-99
<i>PDE4B</i>	2483,752	3,983011	0,189591	2,49E-99	1,31E-96
<i>AK5</i>	688,1611	-2,08355	0,099142	4,03E-99	2,07E-96
<i>WNT5A</i>	6652,032	2,314754	0,110854	1,77E-98	8,82E-96
<i>LBP</i>	6381,727	8,262842	0,390294	2,27E-98	1,10E-95
<i>P4HA2</i>	7750,036	1,390969	0,067502	3,99E-95	1,89E-92
<i>CLDND1</i>	4343,884	1,290329	0,062689	4,16E-95	1,92E-92
<i>NAMPT1</i>	453,9358	4,026249	0,195595	6,00E-95	2,70E-92
<i>DNAJC1</i>	3059,706	1,254074	0,060956	9,70E-95	4,26E-92
<i>TGIF1</i>	3099,307	1,454665	0,070852	1,47E-94	6,30E-92
<i>PLXNA4</i>	3368,281	3,197228	0,157055	1,28E-93	5,36E-91
<i>TFPI2</i>	21596,26	4,185145	0,207734	9,16E-93	3,75E-90
<i>MEIS1</i>	2644,972	2,914446	0,144198	1,44E-92	5,78E-90
<i>PON2</i>	3726,614	1,273719	0,06332	5,67E-91	2,23E-88
<i>CXCL2</i>	11035,65	8,613387	0,439498	1,68E-89	6,47E-87
<i>FNDC3B</i>	16459,74	1,527146	0,076856	4,51E-89	1,69E-86
<i>LIF</i>	7484,508	3,900306	0,197713	1,19E-88	4,37E-86
<i>CD55</i>	8825,105	2,058920	0,104942	6,43E-87	2,33E-84

baseMean, mean of normalized counts for all samples; SE, standard error of the log2FC; padj, BH adjusted p values.

Table C.4: The top 50 DEGs of IL-1 α + β stimulated fibroblasts.

Top 50 DEGs in IL-1 α + β stimulated fibroblasts vs unstimulated fibroblasts, sorted by **padj**.

gene	baseMean	log2FC	SE	p value	padj
<i>NFKBIZ</i>	4233,313	3,84749	0,1048	5,73E-298	1,08E-293
<i>PDE4D</i>	2941,586	5,59138	0,1611	9,51E-266	8,96E-262
<i>HPD</i>	7240,63	5,98404	0,1791	5,89E-249	3,70E-245
<i>ZC3H12A</i>	4412,059	3,99832	0,1237	3,19E-231	1,50E-227
<i>CXCL5</i>	19947,4	10,0326	0,3274	4,29E-210	1,62E-206
<i>FGF7</i>	35283,66	1,84325	0,0601	6,54E-208	2,05E-204
<i>C1QTNF1</i>	9011,616	3,00667	0,1005	2,45E-198	6,60E-195
<i>NAMPT</i>	37546,91	3,76287	0,1292	8,70E-189	2,05E-185
<i>TMEM132A</i>	4222,663	3,14623	0,1156	1,77E-164	3,72E-161
<i>SLC43A3</i>	7339,073	2,04946	0,077	3,66E-157	6,90E-154
<i>NRP2</i>	20082,82	3,37121	0,1289	1,79E-152	3,07E-149
<i>PPL</i>	6408,462	-1,7907	0,0683	1,89E-151	2,98E-148
<i>CCL5</i>	1502,154	7,08334	0,2735	1,71E-147	2,48E-144
<i>NAB1</i>	3957,802	1,62714	0,0635	2,01E-145	2,71E-142
<i>RCAN1</i>	15543,26	2,92096	0,1145	2,55E-145	3,21E-142
<i>IGF1</i>	7176,467	5,1496	0,2077	2,36E-138	2,78E-135
<i>C17orf58</i>	3085,927	2,91142	0,117	1,03E-137	1,14E-134
<i>CTSS</i>	4249,972	3,47139	0,1471	3,89E-125	4,07E-122
<i>KYNU</i>	1162,839	5,49999	0,2353	6,21E-125	6,16E-122
<i>PLEKHA4</i>	3299,534	-1,6894	0,0716	4,31E-124	4,06E-121
<i>IL6</i>	45071,44	6,94447	0,3005	3,87E-122	3,47E-119
<i>CXCL3</i>	23622,06	9,67392	0,4198	1,45E-121	1,24E-118
<i>TRANK1</i>	6892,448	2,00027	0,0879	1,46E-115	1,19E-112
<i>UACA</i>	4079,029	-1,7839	0,0785	1,84E-115	1,44E-112
<i>C1GALT1C1</i>	4799,947	2,07811	0,0921	2,26E-114	1,70E-111
<i>STAT4</i>	637,4869	2,56396	0,114	3,42E-113	2,48E-110
<i>CCL2</i>	37988,35	3,65653	0,1669	1,92E-108	1,34E-105
<i>TGFBR1</i>	7647,055	1,28726	0,0588	3,18E-107	2,14E-104
<i>SLC30A1</i>	7242,093	1,25975	0,0579	2,60E-106	1,69E-103
<i>MFSD2A</i>	467,9459	3,36051	0,155	4,17E-106	2,62E-103
<i>B4GALT1</i>	24465,76	1,56577	0,0721	1,24E-105	7,53E-103
<i>AK5</i>	688,1611	-2,1307	0,0988	2,77E-104	1,63E-101
<i>LBP</i>	6381,727	8,48305	0,3935	2,06E-103	1,18E-100
<i>PDE4B</i>	2483,752	4,05299	0,1896	1,21E-102	6,69E-100
<i>SERPINA3</i>	983,2207	1,93115	0,0907	1,44E-101	7,75E-99
<i>WNT5A</i>	6652,032	2,34949	0,1109	2,37E-101	1,24E-98
<i>AKR1B1</i>	21628,09	2,06196	0,0972	2,46E-101	1,25E-98
<i>PON2</i>	3726,614	1,34051	0,0632	5,38E-101	2,67E-98
<i>P4HA2</i>	7750,036	1,42558	0,0674	5,59E-100	2,70E-97
<i>PLXNA4</i>	3368,281	3,2869	0,1575	6,47E-99	3,05E-96
<i>NAMPTP1</i>	453,9358	4,08833	0,1955	5,55E-98	2,55E-95
<i>SLC16A4</i>	1840,574	1,58422	0,0768	1,48E-95	6,64E-93
<i>DNAJC1</i>	3059,706	1,25086	0,0608	7,21E-95	3,16E-92
<i>CLDND1</i>	4343,884	1,28964	0,0626	8,78E-95	3,76E-92
<i>LIF</i>	7484,508	4,01823	0,1974	5,74E-94	2,41E-91
<i>TFPI2</i>	21596,26	4,19563	0,2079	3,44E-93	1,41E-90
<i>MEIS1</i>	2644,972	2,91955	0,1439	4,93E-93	1,98E-90
<i>CFH</i>	7543,491	1,3703	0,0675	7,54E-93	2,96E-90
<i>CXCL2</i>	11035,65	8,68228	0,4408	6,71E-91	2,58E-88
<i>GBP1</i>	1824,665	1,22166	0,0619	1,20E-87	4,52E-85
<i>CD55</i>	8825,105	2,058920	0,104942	6,43E-87	2,33E-84

baseMean, mean of normalized counts for all samples; SE, standard error of the log2FC; padj, BH adjusted p values.

Table C.5: The top 50 **DEGs** of **IL-1RA** pre-treated fibroblasts.

Top 50 **DEGs** in **IL-1RA** pre-treated fibroblasts vs unstimulated fibroblasts, sorted by **padj**.

gene	baseMean	log2FC	SE	p value	padj.
<i>NFKBIZ</i>	4233,313	3,850431	0,104821	6,54E-298	1,21E-293
<i>PDE4D</i>	2941,586	5,463814	0,160888	4,52E-254	4,17E-250
<i>ZC3H12A</i>	4412,059	3,976103	0,123702	7,00E-229	4,31E-225
<i>CXCL5</i>	19947,4	10,27013	0,326176	5,21E-220	2,40E-216
<i>C1QTNF1</i>	9011,616	3,120073	0,100403	2,31E-213	8,53E-210
<i>HPD</i>	7240,63	5,414371	0,179103	7,93E-203	2,44E-199
<i>NAMPT</i>	37546,91	3,705703	0,12886	3,68E-183	9,68E-180
<i>FGF7</i>	35283,66	1,718559	0,06017	5,81E-181	1,34E-177
<i>TMEM132A</i>	4222,663	3,272253	0,115743	1,51E-177	3,10E-174
<i>SLC43A3</i>	7339,073	2,063664	0,077001	7,15E-160	1,32E-156
<i>CCL5</i>	1502,154	7,183576	0,274442	1,00E-151	1,68E-148
<i>NRP2</i>	20082,82	3,314385	0,128721	9,49E-147	1,46E-143
<i>SERPINA3</i>	983,2207	2,282669	0,090053	8,27E-143	1,17E-139
<i>PPL</i>	6408,462	-1,73494	0,06832	7,96E-142	1,05E-138
<i>RCAN1</i>	15543,26	2,822887	0,114343	7,77E-136	9,56E-133
<i>NAB1</i>	3957,802	1,550628	0,063697	1,02E-131	1,18E-128
<i>IGF1</i>	7176,467	4,98523	0,206838	2,76E-129	2,99E-126
<i>C17orf58</i>	3085,927	2,800769	0,117422	2,55E-127	2,61E-124
<i>KYNU</i>	1162,839	5,507232	0,235345	3,33E-125	3,24E-122
<i>CXCL3</i>	23622,06	9,77108	0,41879	5,91E-124	5,45E-121
<i>CTSS</i>	4249,972	3,422747	0,146822	1,11E-121	9,78E-119
<i>TGFBR1</i>	7647,055	1,368512	0,058815	4,54E-121	3,80E-118
<i>IL6</i>	45071,44	6,806812	0,300604	1,87E-117	1,50E-114
<i>P4HA2</i>	7750,036	1,539695	0,067435	2,96E-116	2,28E-113
<i>WNT5A</i>	6652,032	2,457488	0,110829	1,02E-110	7,55E-108
<i>CCL2</i>	37988,35	3,692037	0,166874	1,66E-110	1,18E-107
<i>B4GALT1</i>	24465,76	1,570479	0,072075	1,63E-106	1,11E-103
<i>UACA</i>	4079,029	-1,70445	0,078528	1,43E-105	9,44E-103
<i>DNAJC1</i>	3059,706	1,321307	0,060867	1,87E-105	1,19E-102
<i>SERPINA1</i>	580,2208	4,144007	0,193221	9,29E-105	5,71E-102
<i>STAT4</i>	637,4869	2,473135	0,114544	1,60E-104	9,53E-102
<i>MFSD2A</i>	467,9459	3,317258	0,155258	6,20E-103	3,57E-100
<i>C1GALT1C1</i>	4799,947	1,959437	0,092134	7,19E-102	4,02E-99
<i>TRANK1</i>	6892,448	1,862093	0,087987	2,58E-100	1,40E-97
<i>GBP1</i>	1824,665	1,304035	0,061917	2,56E-99	1,35E-96
<i>AK5</i>	688,1611	-2,08368	0,099322	6,25E-99	3,20E-96
<i>LIF</i>	7484,508	4,086912	0,198619	3,53E-97	1,76E-94
<i>AKR1B1</i>	21628,09	2,020771	0,09733	4,02E-97	1,95E-94
<i>PDE4B</i>	2483,752	3,929198	0,189549	9,43E-97	4,46E-94
<i>PLEKHA4</i>	3299,534	-1,47864	0,071472	4,14E-96	1,91E-93
<i>FNDC3B</i>	16459,74	1,570447	0,076908	7,44E-94	3,35E-91
<i>TFPI2</i>	21596,26	4,205546	0,207549	1,40E-93	6,15E-91
<i>SLC30A1</i>	7242,093	1,177294	0,058	9,86E-93	4,23E-90
<i>LBP</i>	6381,727	7,991567	0,387964	3,59E-92	1,50E-89
<i>CFH</i>	7543,491	1,364194	0,067534	8,48E-92	3,47E-89
<i>TGIF1</i>	3099,307	1,430997	0,070861	1,55E-91	6,22E-89
<i>CXCL2</i>	11035,65	8,652998	0,436896	2,74E-90	1,07E-87
<i>NAMPTP1</i>	453,9358	3,870803	0,196596	2,08E-87	7,98E-85
<i>PLXNA4</i>	3368,281	3,084604	0,157102	2,62E-87	9,85E-85
<i>COL3A1</i>	563362,9	2,342207	0,120164	6,59E-86	2,43E-83

baseMean, mean of normalized counts for all samples; SE, standard error of the **log2FC**; **padj**, **BH** adjusted p values.

C.4 **GO** Biological Processes pathway analysis of **IL-1** stimulated fibroblasts

Table C.6: Top 50 **GO** Biological Processes genesets of each **IL-1** stimulated fibroblast condition.

Table of the top genesets sorted by Condition and **padj**.

Condition	GO ID	Description	GeneRatio	p value	padj	Count.
IL-1 α	GO:0045229	external encapsulating structure organization	119/2748	3.98E-07	1.72E-02	119
IL-1 α	GO:0030198	extracellular matrix organization	118/2748	6.30E-06	1.72E-02	118
IL-1 α	GO:0043062	extracellular structure organization	118/2748	9.12E-06	1.72E-02	118
IL-1 α	GO:0003013	circulatory system process	170/2748	4.93E-05	6.99E-02	170
IL-1 α	GO:0001525	angiogenesis	170/2748	8.27E-06	9.38E-02	170
IL-1 α	GO:0009617	response to bacterium	164/2748	4.52E-04	4.27E-01	164
IL-1 α	GO:0006935	chemotaxis	166/2748	6.05E-02	4.90E+02	166
IL-1 α	GO:0042330	taxis	166/2748	9.28E-01	6.58E+02	166
IL-1 α	GO:0008015	blood circulation	139/2748	1.19E+00	7.50E+02	139
IL-1 α	GO:0032496	response to lipopolysaccharide	104/2748	1.49E+00	8.43E+02	104
IL-1 α	GO:0002237	response to molecule of bacterial origin	106/2748	1.20E+01	6.20E+03	106
IL-1 α	GO:0003018	vascular process in circulatory system	87/2748	3.06E+01	1.44E+04	87
IL-1 α	GO:0051272	positive regulation of cellular component movement	166/2748	4.38E+00	1.91E+04	166
IL-1 α	GO:0040017	positive regulation of locomotion	165/2748	9.17E+01	3.54E+04	165
IL-1 α	GO:0006036	cell chemotaxis	93/2748	9.38E+01	3.54E+04	93
IL-1 α	GO:2000147	positive regulation of cell motility	162/2748	1.02E+02	3.63E+04	162
IL-1 α	GO:0030335	positive regulation of cell migration	157/2748	2.81E+02	9.37E+04	157
IL-1 α	GO:0043410	positive regulation of MAPK cascade	134/2748	7.36E+02	2.32E+05	134
IL-1 α	GO:0044057	regulation of system process	138/2748	1.31E+03	3.90E+05	138
IL-1 α	GO:0050900	leukocyte migration	108/2748	1.64E+03	4.64E+05	108
IL-1 α	GO:0050865	regulation of cell activation	154/2748	2.04E+03	5.50E+05	154
IL-1 α	GO:0045785	positive regulation of cell adhesion	127/2748	2.80E+03	7.23E+05	127
IL-1 α	GO:0001818	negative regulation of cytokine production	84/2748	4.30E+03	1.06E+06	84
IL-1 α	GO:0071222	cellular response to lipopolysaccharide	69/2748	6.30E+03	1.49E+06	69
IL-1 α	GO:0001503	ossification	124/2748	1.54E+04	3.50E+06	124
IL-1 α	GO:0009611	response to wounding	145/2748	1.80E+04	3.93E+06	145
IL-1 α	GO:0071219	cellular response to molecule of bacterial origin	70/2748	2.02E+04	4.23E+06	70
IL-1 α	GO:0071396	cellular response to lipid	153/2748	2.34E+04	4.74E+06	153
IL-1 α	GO:0001819	positive regulation of cytokine production	122/2748	3.16E+03	6.19E+06	122
IL-1 α	GO:0097529	myeloid leukocyte migration	69/2748	4.23E+04	7.99E+06	69
IL-1 α	GO:1903522	regulation of blood circulation	73/2748	4.54E+04	8.31E+06	73
IL-1 α	GO:0071216	cellular response to biotic stimulus	76/2748	5.32E+03	9.26E+06	76
IL-1 α	GO:0003012	muscle system process	115/2748	5.39E+03	9.26E+06	115
IL-1 α	GO:0001448	connective tissue development	86/2748	6.03E+04	1.00E+07	86
IL-1 α	GO:0001501	skeletal system development	148/2748	6.19E+03	1.00E+07	148
IL-1 α	GO:0030595	leukocyte chemotaxis	70/2748	6.77E+04	1.07E+07	70
IL-1 α	GO:0010035	response to inorganic substance	142/2748	6.98E+04	1.07E+07	142
IL-1 α	GO:0006936	muscle contraction	94/2748	9.43E+04	1.39E+07	94
IL-1 α	GO:0050867	positive regulation of cell activation	101/2748	9.54E+04	1.39E+07	101
IL-1 α	GO:0006959	neutrophil chemotaxis	38/2748	1.54E+05	2.19E+07	38
IL-1 α	GO:0043269	regulation of ion transport	153/2748	1.66E+05	2.29E+07	153
IL-1 α	GO:0002694	regulation of leukocyte activation	138/2748	1.87E+05	2.52E+07	138
IL-1 α	GO:0006959	humoral immune response	55/2748	2.14E+05	2.82E+07	55
IL-1 α	GO:0045765	regulation of angiogenesis	85/2748	3.03E+05	3.90E+07	85
IL-1 α	GO:1901342	regulation of vasculature development	86/2748	3.28E+05	4.13E+07	86
IL-1 α	GO:0050673	epithelial cell proliferation	113/2748	3.55E+05	4.37E+07	113
IL-1 α	GO:0055065	metal ion homeostasis	151/2748	4.23E+05	5.10E+07	151
IL-1 α	GO:0042692	muscle cell differentiation	107/2748	4.32E+05	5.10E+07	107
IL-1 α	GO:1990868	response to chemokine	34/2748	6.01E+05	6.81E+06	34
IL-1 α	GO:1990869	cellular response to chemokine	34/2748	6.01E+05	6.81E+06	34
IL-1 β	GO:0045229	external encapsulating structure organization	116/2684	2.01E-05	6.51E-03	116
IL-1 β	GO:0030198	extracellular matrix organization	115/2684	3.23E-05	6.51E-03	115
IL-1 β	GO:0001525	angiogenesis	168/2684	4.52E-05	6.51E-03	168
IL-1 β	GO:0043062	extracellular structure organization	115/2684	4.61E-05	6.51E-03	115
IL-1 β	GO:0003013	circulatory system process	167/2684	6.92E-05	7.82E-02	167
IL-1 β	GO:0009617	response to bacterium	161/2684	6.81E-04	6.42E-01	161
IL-1 β	GO:0051272	positive regulation of cellular component movement	173/2684	1.86E-02	1.50E+01	173
IL-1 β	GO:2000147	positive regulation of cell motility	169/2684	4.71E-02	3.33E+01	169
IL-1 β	GO:0040017	positive regulation of locomotion	171/2684	9.77E-02	6.14E+00	171
IL-1 β	GO:0030335	positive regulation of cell migration	163/2684	3.25E-01	1.84E+02	163
IL-1 β	GO:0008015	blood circulation	138/2684	4.07E-01	2.09E+02	138
IL-1 β	GO:0006935	chemotaxis	163/2684	7.64E-01	3.60E+02	163
IL-1 β	GO:0042330	taxis	163/2684	1.16E+00	5.06E+02	163
IL-1 β	GO:0032496	response to lipopolysaccharide	102/2684	2.43E+00	9.80E+02	102

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Table C.6: Top 50 **GO** Biological Processes genesets of each **IL-1** stimulated fibroblast condition. (Continued)

Condition	GO ID	Description	GeneRatio	p value	padj	Count.
IL-1 β	GO:0002237	response to molecule of bacterial origin	105/2684	6,61E+00	2,49E+03	105
IL-1 β	GO:0043269	regulation of ion transport	162/2684	5,06E+01	1,79E+04	162
IL-1 β	GO:0003018	vascular process in circulatory system	85/2684	6,89E+01	2,29E+04	85
IL-1 β	GO:0009611	response to wounding	149/2684	1,57E+02	4,94E+04	149
IL-1 β	GO:0060326	cell chemotaxis	90/2684	4,83E+02	1,44E+05	90
IL-1 β	GO:0050865	regulation of cell activation	153/2684	6,31E+01	1,78E+05	153
IL-1 β	GO:0045785	positive regulation of cell adhesion	126/2684	1,18E+03	3,17E+04	126
IL-1 β	GO:0006936	muscle contraction	97/2684	1,74E+03	4,46E+05	97
IL-1 β	GO:0050900	leukocyte migration	106/2684	2,11E+03	5,18E+05	106
IL-1 β	GO:0001503	ossification	124/2684	3,11E+03	7,31E+05	124
IL-1 β	GO:0044057	regulation of system process	134/2684	4,75E+02	1,07E+06	134
IL-1 β	GO:0003012	muscle system process	116/2684	5,44E+03	1,18E+06	116
IL-1 β	GO:0071396	cellular response to lipid	152/2684	7,60E+03	1,59E+06	152
IL-1 β	GO:0006959	humoral immune response	57/2684	7,92E+03	1,60E+06	57
IL-1 β	GO:0042060	wound healing	116/2684	1,54E+03	2,99E+06	116
IL-1 β	GO:0030593	neutrophil chemotaxis	39/2684	1,66E+04	3,13E+06	39
IL-1 β	GO:0043410	positive regulation of MAPK cascade	127/2684	2,84E+04	5,18E+06	127
IL-1 β	GO:0001819	positive regulation of cytokine production	120/2684	3,08E+04	5,43E+06	120
IL-1 β	GO:1903522	regulation of blood circulation	72/2684	4,14E+03	7,09E+06	72
IL-1 β	GO:0010035	response to inorganic substance	140/2684	5,13E+04	8,52E+06	140
IL-1 β	GO:0071222	cellular response to lipopolysaccharide	66/2684	5,53E+04	8,85E+06	66
IL-1 β	GO:0050867	positive regulation of cell activation	100/2684	5,64E+04	8,85E+06	100
IL-1 β	GO:0030595	leukocyte chemotaxis	69/2684	6,49E+04	9,65E+06	69
IL-1 β	GO:0033002	muscle cell proliferation	69/2684	6,49E+04	9,65E+06	69
IL-1 β	GO:0001501	skeletal system development	145/2684	8,22E+03	1,19E+07	145
IL-1 β	GO:0050673	epithelial cell proliferation	113/2684	8,57E+04	1,21E+07	113
IL-1 β	GO:0006873	cellular ion homeostasis	152/2684	1,05E+05	1,44E+06	152
IL-1 β	GO:0097529	myeloid leukocyte migration	67/2684	1,17E+05	1,57E+07	67
IL-1 β	GO:0002694	regulation of leukocyte activation	136/2684	1,43E+05	1,88E+07	136
IL-1 β	GO:0055065	metal ion homeostasis	150/2684	1,47E+05	1,89E+07	150
IL-1 β	GO:0001818	negative regulation of cytokine production	79/2684	1,52E+04	1,91E+07	79
IL-1 β	GO:0071219	cellular response to molecule of bacterial origin	67/2684	1,56E+05	1,92E+07	67
IL-1 β	GO:0006875	cellular metal ion homeostasis	135/2684	1,70E+04	2,05E+06	135
IL-1 β	GO:0071621	granulocyte chemotaxis	43/2684	1,74E+05	2,05E+07	43
IL-1 β	GO:0042692	muscle cell differentiation	106/2684	2,38E+05	2,74E+07	106
IL-1 β	GO:0061448	connective tissue development	83/2684	2,64E+05	2,98E+07	83
IL-1 α + β	GO:0045229	external encapsulating structure organization	120/2689	1,82E-07	8,10E-04	120
IL-1 α + β	GO:0030198	extracellular matrix organization	119/2689	2,93E-07	8,10E-04	119
IL-1 α + β	GO:0043062	extracellular structure organization	119/2689	4,29E-07	8,10E-04	119
IL-1 α + β	GO:0009617	response to bacterium	164/2689	4,99E-05	6,26E-02	164
IL-1 α + β	GO:0001525	angiogenesis	168/2689	5,53E-05	6,26E-02	168
IL-1 α + β	GO:0003013	circulatory system process	166/2689	2,14E-04	2,02E-01	166
IL-1 α + β	GO:0006935	chemotaxis	166/2689	7,73E-02	6,25E+01	166
IL-1 α + β	GO:0042330	taxis	166/2689	1,20E-01	8,48E+01	166
IL-1 α + β	GO:0051272	positive regulation of cellular component movement	169/2689	5,94E-01	3,74E+02	169
IL-1 α + β	GO:2000147	positive regulation of cell motility	166/2689	6,65E-01	3,76E+02	166
IL-1 α + β	GO:0008015	blood circulation	137/2689	1,17E+00	6,03E+02	137
IL-1 α + β	GO:0040017	positive regulation of locomotion	168/2689	1,32E-01	6,20E+02	168
IL-1 α + β	GO:0030335	positive regulation of cell migration	161/2689	1,98E+00	8,60E+02	161
IL-1 α + β	GO:0032496	response to lipopolysaccharide	102/2689	2,76E+00	1,12E+03	102
IL-1 α + β	GO:0002237	response to molecule of bacterial origin	105/2689	7,53E+00	2,84E+03	105
IL-1 α + β	GO:0043269	regulation of ion transport	162/2689	5,98E+01	2,12E+04	162
IL-1 α + β	GO:0003018	vascular process in circulatory system	85/2689	7,70E+01	2,56E+04	85
IL-1 α + β	GO:0009611	response to wounding	148/2689	3,92E+02	1,23E+05	148
IL-1 α + β	GO:0060326	cell chemotaxis	90/2689	5,40E+02	1,57E+05	90
IL-1 α + β	GO:0044057	regulation of system process	137/2689	5,55E+02	1,57E+05	137
IL-1 α + β	GO:0006959	humoral immune response	59/2689	6,91E+02	1,86E+05	59
IL-1 α + β	GO:0045785	positive regulation of cell adhesion	125/2689	2,98E+03	7,67E+05	125
IL-1 α + β	GO:0001503	ossification	124/2689	3,55E+03	8,63E+05	124
IL-1 α + β	GO:0001818	negative regulation of cytokine production	83/2689	3,66E+03	8,63E+05	83
IL-1 α + β	GO:0050900	leukocyte migration	105/2689	5,64E+03	1,28E+06	105
IL-1 α + β	GO:0050865	regulation of cell activation	150/2689	6,24E+03	1,36E+06	150
IL-1 α + β	GO:0071222	cellular response to lipopolysaccharide	68/2689	6,95E+02	1,46E+05	68

Continued on next page

Table C.6: Top 50 **GO** Biological Processes genesets of each **IL-1** stimulated fibroblast condition. (Continued)

Condition	GO ID	Description	GeneRatio	p value	padj	Count.
IL-1 α + β	GO:0006936	muscle contraction	95/2689	1,19E+04	2,41E+06	95
IL-1 α + β	GO:0097529	myeloid leukocyte migration	69/2689	1,58E+04	3,08E+06	69
IL-1 α + β	GO:0042060	wound healing	116/2689	1,74E+04	3,28E+06	116
IL-1 α + β	GO:0071219	cellular response to molecule of bacterial origin	69/2689	2,14E+03	3,92E+06	69
IL-1 α + β	GO:0001501	skeletal system development	147/2689	2,47E+04	4,38E+06	147
IL-1 α + β	GO:0003012	muscle system process	114/2689	3,01E+04	5,16E+06	114
IL-1 α + β	GO:1903522	regulation of blood circulation	72/2689	4,53E+04	7,54E+06	72
IL-1 α + β	GO:0006873	cellular ion homeostasis	153/2689	6,35E+04	1,03E+06	153
IL-1 α + β	GO:0071396	cellular response to lipid	148/2689	1,27E+05	1,99E+07	148
IL-1 α + β	GO:0071216	cellular response to biotic stimulus	74/2689	1,33E+05	2,03E+07	74
IL-1 α + β	GO:0006875	cellular metal ion homeostasis	135/2689	1,94E+05	2,82E+07	135
IL-1 α + β	GO:0033002	muscle cell proliferation	68/2689	1,94E+05	2,82E+07	68
IL-1 α + β	GO:0030003	cellular cation homeostasis	149/2689	2,00E+05	2,83E+07	149
IL-1 α + β	GO:0045765	regulation of angiogenesis	84/2689	2,45E+05	3,39E+07	84
IL-1 α + β	GO:1901342	regulation of vasculature development	85/2689	2,62E+05	3,53E+07	85
IL-1 α + β	GO:0043410	positive regulation of MAPK cascade	124/2689	2,75E+05	3,59E+07	124
IL-1 α + β	GO:0042063	gliogenesis	86/2689	2,79E+05	3,59E+07	86
IL-1 α + β	GO:0055065	metal ion homeostasis	149/2689	3,18E+05	4,00E+06	149
IL-1 α + β	GO:0030593	neutrophil chemotaxis	37/2689	3,53E+05	4,34E+07	37
IL-1 α + β	GO:0034765	regulation of ion transmembrane transport	114/2689	3,68E+04	4,43E+07	114
IL-1 α + β	GO:0060485	mesenchyme development	89/2689	4,12E+05	4,85E+07	89
IL-1 α + β	GO:0030595	leukocyte chemotaxis	67/2689	5,20E+05	5,99E+07	67
IL-1 α + β	GO:0048871	multicellular organismal homeostasis	132/2689	5,29E+05	5,99E+07	132
IL-1RA	GO:0045229	external encapsulating structure organization	117/2599	4,05E-07	1,83E-03	117
IL-1RA	GO:0030198	extracellular matrix organization	116/2599	6,69E-07	1,83E-03	116
IL-1RA	GO:0043062	extracellular structure organization	116/2599	9,68E-07	1,83E-03	116
IL-1RA	GO:0009617	response to bacterium	162/2599	1,02E-05	1,44E-02	162
IL-1RA	GO:0001525	angiogenesis	164/2599	6,95E-05	7,89E-02	164
IL-1RA	GO:0006935	chemotaxis	166/2599	2,75E-03	2,60E+00	166
IL-1RA	GO:0042330	taxis	166/2599	4,33E-03	3,51E+00	166
IL-1RA	GO:0032496	response to lipopolysaccharide	103/2599	9,14E-02	6,48E+01	103
IL-1RA	GO:0003013	circulatory system process	155/2599	1,42E-02	8,93E+01	155
IL-1RA	GO:0002237	response to molecule of bacterial origin	106/2599	2,55E-01	1,44E+02	106
IL-1RA	GO:0060326	cell chemotaxis	95/2599	3,26E-01	1,68E+02	95
IL-1RA	GO:0001818	negative regulation of cytokine production	89/2599	8,13E-01	3,84E+02	89
IL-1RA	GO:0045785	positive regulation of cell adhesion	131/2599	1,48E+00	6,46E+02	131
IL-1RA	GO:0001503	ossification	130/2599	1,79E+00	7,26E+02	130
IL-1RA	GO:0040017	positive regulation of locomotion	163/2599	2,92E+00	1,03E+03	163
IL-1RA	GO:0051272	positive regulation of cellular component movement	163/2599	2,92E+00	1,03E+03	163
IL-1RA	GO:2000147	positive regulation of cell motility	160/2599	3,53E+00	1,18E+03	160
IL-1RA	GO:0050865	regulation of cell activation	155/2599	9,36E+00	2,95E+03	155
IL-1RA	GO:0030335	positive regulation of cell migration	155/2599	1,14E+01	3,41E+03	155
IL-1RA	GO:0006959	humoral immune response	60/2599	4,04E+01	1,15E+04	60
IL-1RA	GO:0033002	muscle cell proliferation	74/2599	5,51E+01	1,49E+04	74
IL-1RA	GO:0050900	leukocyte migration	107/2599	1,08E+01	2,79E+04	107
IL-1RA	GO:0071222	cellular response to lipopolysaccharide	70/2599	1,36E+02	3,35E+04	70
IL-1RA	GO:0009611	response to wounding	145/2599	2,54E+01	6,01E+04	145
IL-1RA	GO:0001819	positive regulation of cytokine production	123/2599	3,19E+02	7,24E+04	123
IL-1RA	GO:0071219	cellular response to molecule of bacterial origin	71/2599	4,66E+02	1,02E+05	71
IL-1RA	GO:0071396	cellular response to lipid	152/2599	6,04E+02	1,23E+04	152
IL-1RA	GO:0050867	positive regulation of cell activation	103/2599	6,08E+02	1,23E+04	103
IL-1RA	GO:0003018	vascular process in circulatory system	81/2599	8,23E+02	1,58E+05	81
IL-1RA	GO:0022407	regulation of cell-cell adhesion	118/2599	8,35E+02	1,58E+05	118
IL-1RA	GO:0008015	blood circulation	126/2599	9,48E+02	1,73E+05	126
IL-1RA	GO:0097529	myeloid leukocyte migration	70/2599	1,05E+02	1,86E+05	70
IL-1RA	GO:0030595	leukocyte chemotaxis	71/2599	1,72E+03	2,95E+05	71
IL-1RA	GO:0001501	skeletal system development	147/2599	1,88E+03	3,14E+05	147
IL-1RA	GO:0043269	regulation of ion transport	153/2599	2,41E+03	3,90E+05	153
IL-1RA	GO:0071216	cellular response to biotic stimulus	76/2599	3,45E+03	5,44E+05	76
IL-1RA	GO:0002694	regulation of leukocyte activation	138/2599	3,60E+03	5,52E+04	138
IL-1RA	GO:0022409	positive regulation of cell-cell adhesion	84/2599	5,83E+03	8,71E+05	84
IL-1RA	GO:0030593	neutrophil chemotaxis	39/2599	6,07E+03	8,83E+05	39

Continued on next page

Table C.6: Top 50 **GO** Biological Processes genesets of each **IL-1** stimulated fibroblast condition. (Continued)

Condition	GO ID	Description	GeneRatio	p value	padj	Count.
IL-1RA	GO:0050673	epithelial cell proliferation	113/2599	1,16E+04	1,64E+06	113
IL-1RA	GO:0071706	tumor necrosis factor superfamily cytokine production	57/2599	1,34E+04	1,81E+06	57
IL-1RA	GO:1903555	regulation of tumor necrosis factor superfamily cytokine production	57/2599	1,34E+04	1,81E+06	57
IL-1RA	GO:0043410	positive regulation of MAPK cascade	125/2599	1,44E+04	1,89E+06	125
IL-1RA	GO:0071621	granulocyte chemotaxis	44/2599	1,52E+04	1,89E+06	44
IL-1RA	GO:0032640	tumor necrosis factor production	56/2599	1,53E+04	1,89E+06	56
IL-1RA	GO:0032680	regulation of tumor necrosis factor production	56/2599	1,53E+04	1,89E+06	56
IL-1RA	GO:0032635	interleukin-6 production	52/2599	2,53E+04	2,99E+06	52
IL-1RA	GO:0032675	regulation of interleukin-6 production	52/2599	2,53E+04	2,99E+06	52
IL-1RA	GO:1990868	response to chemokine	35/2599	2,66E+04	3,02E+06	35
IL-1RA	GO:1990869	cellular response to chemokine	35/2599	2,66E+04	3,02E+06	35

The **GO** ID is a unique seven-digit Gene Ontology (**GO**) identifier. Significance was set at an adjusted p-value (**padj**) of <0.05 (**BH** correction). The Count column represents the number of **DEGs** associated with a **GO** term. **IL-1 α** , **IL-1 α** stimulated fibroblasts; **IL-1 β** , **IL-1 β** stimulated fibroblasts; **IL-1 α + β** , **IL-1 α** & **IL-1 β** stimulated fibroblasts; **IL-1RA**, fibroblasts pre-treated with **IL-1RA** before **IL-1 α** , **IL-1 β** , and **IL-1RA** stimulation.