Understanding the underlying immune mechanisms of Dupuytren's Disease

Kristyn Alexaundria Carter

MSc

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

College of Medicine, Veterinary, and Life Sciences
February 2022

Abstract

Background:

Dupuytren's disease, also called Dupuytren's contracture, is a fibroproliferative condition in which the palmar tissue becomes inflamed and, as the disease progresses, reduced mobility of the hand and affected fingers occurs. Dupuytren's disease consists of two microenvironments: cellular nodules and a fibrous cord. Clinical presentation of the disease is a claw like configuration of the hand with the fingers curling inward. Treatment options vary from enzymatic digestion of nodules, appearing as hardened tissue, to fasciectomy of the affected tissue. Unfortunately, treatment is not always successful as some cases of Dupuytren's recur during the individual's lifetime.

As a model of tissue fibrosis, Dupuytren's is characterised by chronic inflammation and excess matrix protein production. This, coupled with the recurring nature of the disease, suggests the immune system could play a role in mediating persistent, chronic inflammation of the affected tissue. The work encompassed in this thesis aims to understand the immune mechanisms at play and highlight specific cell to cell interactions which could unveil potential therapeutic targets.

Key results:

Preliminary bulk RNA sequencing results describe a heightened presence of myofibroblast and fibrosis related genes in Dupuytren's microenvironments compared to control, carpal tunnel tissue. Cellular characterisation of Dupuytren's tissue led to the identification of activated fibroblasts and myofibroblasts as well as increased matrix protein production. An activated stromal cell phenotype was seen in Dupuytren's tissue and this phenotype, coupled with fibroblast behaviour, was enhanced in response to *in vitro* mechanical damage and/or stimulation with inflammatory or immune cell related cytokines.

Outlining the immune cell landscape of Dupuytren's tissue, via RNA sequencing and flow cytometry, pointed to macrophage and T lymphocytes as the leading cells within the tissue. Visualization of the immune cell populations via immunohistochemistry described not only the presence of dendritic cells and macrophages but also various T cell subsets. Bulk sequencing further supported the presence of T cells by describing T cell related genes within Dupuytren's tissue. T cell activation marker, CD27, and its sole ligand, CD70, were expressed in the tissue and confirmed by immunohistochemistry to be expressed by fibroblasts. Their presence highlights the possibility of stromal-immune interaction between

T cells and fibroblasts. Stimulation of Dupuytren's fibroblasts with CD27 enhanced gene and protein expression of stromal, inflammatory, matrix, and angiogenic related markers; suggesting a role of CD27-CD70 axis in perpetuating Dupuytren's disease.

Conclusion:

This body of work describes an exaggerated stromal activation profile in Dupuytren's disease tissue as well as described enhancement of this stromal activation profile in response to *in* vitro cytokine stimulation. Characterization of immune cell landscape of Dupuytren's tissue featured stromal-immune crosstalk between T cells and fibroblasts and pointed to a signalling axis that could aid in understanding the etiology of Dupuytren's disease.

Table of Contents

List of tables

List of figures

Preface

This body of work was performed from 2018-2022 and it focuses on how the immune system could provide insight on how soft tissue disease develops in response to the immune systems response to damage and/or injury. My research focused on Dupuytren's disease and my passion, which propelled the project forward, was driven by my goal to improve soft tissue treatment via immunological studies. Given the disease's ability to recur post treatment, an improved understanding of the biology behind the disease's etiology and its inflammatory nature would allow for exposure of potential therapeutic targets. The undertaking of this work allowed me to shadow orthopaedic surgeons performing fasciectomies on patients affected by Dupuytren's disease. Shadowing Professor Millar's surgical colleagues not only allowed me to gain firsthand surgical experience but also enabled me to engage with people whose lives are affected by Dupuytren's disease. This exposure enabled me gain perspective on my research and remain focused on the greater goal of improving the affected communities' quality of life. Unfortunately, these shadowing opportunities did not continue once the Covid-19 pandemic hit. The greater duration of my 3-year PhD was impacted by the global pandemic however, the pandemic challenged me to be a better researcher, scientist, and lab member.

Acknowledgements

Thank you to my family and friends for their support during my time in Glasgow. I could not have survived without your compassion and understanding while I settled down in a new country, endured two graduate programs, and braved the pandemic. I could not have leaped so bravely without knowing I had all your love and support.

To my partner, there are no words to describe how much I have leaned on you during my PhD, all while enduring lockdown and the pandemic. Thank you for 'talking me off the ledge' when my cells gave me trouble and for helping me overcome writers block during my thesis write up. I would not have been able to finish this work without your help and the endless supply of memes and animal videos.

Professor Iain McInnes not only are you a pillar in the science community, globally and within Glasgow Uni, you are an incredible role model to me. Despite your numerous responsibilities you consistently found the time to mentor me and provide feedback on my career aspirations and research. You have shown me that one can balance leading an entire research institute while still making meaningful connections with students.

To the Tendon Biology Group (TBG), thank you for not only training me but for answering my litany of questions and troubleshooting my endless mistakes.

Akbar, there is no way I could thank you for all the countless times you helped me during my times of ENDLESS crisis. Thank you thank you thank you; I was so lucky to have you guiding me through my research mistakes during my PhD and masters. This body work would not have been produced without you.

Crowe thank you for your everyday wisdom and calm nature. You helped me during my many tissues culture issues, and you have a seemingly endless wealth of knowledge about everything prism related.

Susan (Suzie Q) you were the best morning work partner I could have asked for and our midday dance breaks got me through the despair that is wet lab research.

Donna, thank you for always helping me with my qPCR issues and helping me gain perspective when my experiments do not work.

Caroline, thank you for being my pre-covid bench buddy. I miss our joint ELISA ventures and your funny stories. Your career as an orthopaedic surgeon-scientist inspires me to continue towards becoming the same, endless thank yous.

To the students I taught during my PhD- Ellen, Sophie, Hussain, Meg, and Anna-thank you for showing me the value of science communication and giving me the opportunity to teach you all practical lab skills. I hope moving forward, you all continue to pursue research and that you time under my tutelage was useful.

To my mentors, thank you for the guidance and wisdom you equipped me with over the years. Your feedback and support not only guided my choices but helped me see what options the future could hold.

Professor Rob Nibbs, thank you for mentoring me not only during my PhD but also during my masters. Without your guidance, I would still be struggling to learn how to read and author papers and still trying to crawl my way to Zurich! Haha!

Dr. Georgia Perona-Wright, thank you for your guidance during my masters and PhD. Having you as an adviser helped me make the right career choices and your stories about your monkey reminded me that life goes on beyond the lab. I am looking forward to our next coffee date with Rob!

Professor Helen Minnis, you and your family have been a saving grace for me during my time at Glasgow; especially when I was not able to visit my home country. Having you as a mentor, one of two tenured Black women at Glasgow Uni, reminded me the importance of achieving my academic goals and, even more so, the importance of serving my community and supporting Black women in STEM.

To my MiSTEAM board, your contribution to this work is immeasurable and I am so proud of our organisation and what we have accomplished towards improving the experience of Glasgow Uni members.

To those who led me to the University of Glasgow: Harriet Washington, Dr. Catherine Manegold, Prof. Lucas Wilson, and Prof. Karen Hollis my thanks extend well beyond these words.

Professor Hollis, without you giving me my first research experience in undergrad, I would have never discovered my research interests and passion. Thank you for advising me and having me over for dinners with your family. You made my time at Mount Holyoke an incredible experience!

Dr. Manegold, without you allowing me to conduct an independent study in African American studies, I would not have come across Medical Apartheid and learned of Dr. James McCune Smith. Nor would I have learned how race is/was used as a key element in history and medicine.

Professor Wilson, thank you for supporting my studies on literature surrounding around the image of the Black superwoman in America. Without this freedom of exploration, and of myself, I would not have felt secure enough to leave America and accept my offer to pursue graduate studies in Scotland.

Harriet Washington, your novel Medical Apartheid has impacted me in more ways than I can describe. As we have discussed, the story of Dr. McCune Smith alone has guided my life towards pursuing studies at the University of Glasgow. Further, your honest presentation of the evidence highlighting how Black Americans have historically, and currently been exploited for medical exploration has further motivated me to becoming the best surgeon scientist I can be. I look forward to our future coffee dates and ongoing zoom chats!

Professor Neal Millar there truly are no words to express my gratitude to you. Without your lecture given to my master's cohort, I would have never known how to combine orthopaedic surgery and research. You are the first orthopaedic surgeon-scientist I have ever met, and I am forever grateful for the opportunity you gave me to first, pursue my masters research in your lab, and second, join your lab as a PhD student. Under your instruction, I not only

learned about soft tissue immunology but also was given the opportunity to shadow you and your colleagues while you performed orthopaedic surgeries. Without this surgical shadowing experience, I would not have networked with surgical residents and orthopaedic surgeons, nor would I have been able to receive career and surgical advice. I hope when I complete my medical school education and surgical training, that I will be able to provide the opportunities and exposure you have given to me to future surgeons and scientists. Here's to you Professor Millar, thank you for everything!

Dedication

Firstly, I am dedicating this body of work to the legacy of Dr. James McCune Smith. University of Glasgow School of Medicine Class of 1837. Without his accomplishments paving the way for me, I would not have pursued my graduate education at the University of Glasgow, nor would I be the first Black woman to complete the University of Glasgow's PhD of Immunology program.

Secondly, I dedicate my years of research to all the Black girls and women who, like me, were told 1) they will never become scientists as science is a white man's world and 2) they do not belong. I hope my journey inspires them to do what they are told they could not and that they take the wise words of Dr. Mae C. Jemison to heart,

"…never be limited by other people's limited imaginations."

Authors declaration

The work described in this thesis represents original work which has been generated through my own efforts and does not consist of work forming part of a thesis to be submitted elsewhere. Furthermore, no data has been given to me by anybody else to be submitted as part of my thesis. Where practical support has been provided by others appropriate acknowledgements have been made.

.....

Kristyn A. Carter

Abbreviations

AP1-

APC – antigen presenting cell

α-SMA – alpha smooth muscle actin

BMP-1 – bone morphogenetic protein 1

CAF-cancer associated fibroblasts

CCL2 – C-C motif ligand 2

CCL20 – C-C motif ligand 20

CCN3 – cysteine-rich protein 61, connective tissue growth factor, and nephroblastoma overexpressed gene 3

CTLA – cytotoxic t-lymphocyte associated antigen

CXCL1 – CXC motif chemokine ligand 1

DAMPs – damage associated molecular patterns

ECM – extracellular matrix

EGF – endothelial growth factor

ELISA – enzyme-linked immunosorbent assay

EMT – endothelial mesenchymal transition

FACS – fluorescence activated cell sorting

FAP – fibroblast activation protein

FGF – fibroblast growth factor

FMT-fibroblast to myofibroblast transition

GMCSF – granulocyte macrophage colony stimulating factor

ICAM-intercellular adhesion molecule

Ig-immunoglobulin

IL-1 – Interleukin 1

IL-1β - Interleukin 1 beta

IL-6 – Interleukin 6

IL-8 – Interleukin 8

IL-13 – Interleukin 13

IL-13Ra1-Interleukin 13 receptor alpha 1

IL-17-Interleukin 17

IL-33-Interleukin 33

Ig- Immunoglobulin

ILC – innate lymphoid cells

IPF - idiopathic pulmonary fibrosis

IFN-γ – Interferon gamma

JAK - janus kinase

JNK – c-Jun N-terminal kinase

LOX - lipoxygenase

LPS - lipopolysaccharide

MACS – magnetic activated cell sorting

MAPK – mitogen activated protein kinase

MCAM – melanoma cell adhesion molecule

MCP- metacarpophalangeal

MCSF – macrophage colony stimulating factor

MMP – matrix metalloproteinase

MSC – mesenchymal stromal cells

NFAT-nuclear factor of activated T cells

NFκB – nuclear factor kappa B

PAMPs – pathogen associated molecule patterns

PBMC – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PDGF – platelet derived growth factor

PDPN – podoplanin

PDL-population doubling level

PDPN- podoplanin

PGE2 – prostaglandin E2

PFD – pirfenidone

PGE2-prostaglandin E_2

PI3K-phosphoinositide 3-kinase

PIP – proximal interphalangeal

qPCR – quantitative polymerase chain reaction

RA – rheumatoid arthritis

ROR – RAR related orphan receptor

RORα – RAR related orphan receptor alpha

RORγ – RAR related orphan receptor gamma

RORγt – RAR related orphan receptor gamma

RPM -rate per minute

RPMI – roswell park memorial institute

ScRNA-seq-

SMAD – SMA genes, drosophila MAD

ST2 – suppressor of tumorigenicity 2

STAT-signal transducer and activator of transcription

STWS-scott's tap water solution

TBS – tris buffered saline

TBST – tris buffered saline tween 20

TCR – T cell receptor

TGF-β – Transforming growth factor beta

TIMP - tissue inhibitor of matrix metalloproteinase

TNF-tumour necrosis factor

TNF-α – tumour necrosis factor alpha

TNFR1 – tumour necrosis factor receptor 1

TNFR2 – tumour necrosis factor receptor 2

TNFSF – tumour necrosis factor super family

TRAF – tumour necrosis factor receptor associated factor

UK – United Kingdom

USA – United States of America

VCAM – vascular cell adhesion molecule

VEGF – Vascular endothelial growth factor

Wnt – Wingless/integrated

Chapter 1: Introduction

1.1 Mesenchymal stromal cells

Dupuytren's disease, also called palmar fibromatosis, is clinically defined as a deformity of the hand that develops over time, causing the fingers to curl inward and reducing functionality of the hand, according to the Mayo Clinic[176]. While Dupuytren's disease is associated with Garrod's pads, also called knuckle pads, people who present with Garrod's do not always suffer from Dupuytren's disease as well; according to British Dupuytren's Society UK[261]. Current literature surrounding the disease describes two cellularly distinct microenvironments: cellular nodules and a fibrous cord; the latter appearing in later stages of the disease. Credited with the development of the disease's fibrous cord, fibroblasts have been highlighted as drivers of the disease and led to the characterisation of the disease as a model of tissue fibrosis[75,231,277].

Frequently described in cancer and fibrotic tissue disease models, stromal compartment activation has been described as a contributor of inflammation and coupled with fibroblast behavior, contributes to the etiology of the disease[24,41,60,196]. During homeostatic tissue repair, the stromal compartment becomes activated in response to factors produced by the surrounding environment. When healing is resolved and the environmental cues dampen, the stromal compartment returns to a resting, non-activated state. During cancer and fibrotic disease, the stromal compartment is persistently activated and thus contributes factors which perpetuate inflammation and fibrosis of the tissue[24,41,60,196]. Understanding whether the stromal compartment is activated in Dupuytren's disease could aid improved understanding of the disease.

The tissue stroma consists of fibroblasts, stromal cells, pericytes, endothelial and epithelial cells, and tissue resident immune cells. The stromal compartment is responsible for maintaining tissue architecture via its secretion of extracellular matrix (ECM) components and expression of cytokines and growth factors aimed at maintaining cell populations within the tissue environment[83,196].

1.1.1 Stromal cell differentiation, structure, and function

Mesenchymal stromal cells (MSCs), also called mesenchymal stem cells are spindle shaped cells that possess the ability to differentiation into various cells[109,130,137,221,243]. The phrase 'mesenchymal stem cell' was popularised by Arnold Caplan in response to MSCs giving rise to bone and cartilage[43,44]. Published works following Dr. Caplan's paper describe the ability of the MSC to give rise to cell lineages including tendon, ligament, and adipose tissue[48,284]; making them incredibly useful in tissue regenerative works[109]. Stem cells are defined as cells which give rise to cells with specialized function. MSCs are described adult stem cells and there is an ongoing debate as to whether the word 'stem' is appropriate, given their limited differentiation and self-renewal capabilities[109,120,137]. Therefore, for the duration of this thesis the acronym MSCs will be used to define mesenchymal stromal cells due to their differentiability and presence in soft tissue.

Mesenchymal stromal cells are a heterogenous class of cells which play a significant role during tissue development, immune response, and injury. They are capable of differentiating into fibroblasts, endothelial cells, pericytes, or epithelial cells[76,86,137,213,236]. These cells populate the area called the 'stroma' which is comprised of a subendothelial membrane and underneath, lies the connective tissue[213]. Within the stroma is not only a rich tapestry of MSCs but also is home to resident immune cells. MSCs aid in tissue organisation and define the architecture of connective tissue via their production of ECM components and cytokines[76,103,122,213].

MSCs have been increasingly studied as they are thought to be complicit in the development of tissue fibrosis[224,243,268]. It has been suggested that activated stromal cells produce ECM components and participate in recruitment of immune cells and secretion of inflammatory factors. Stromal activation has been studied in rheumatoid arthritis and replicated in tendinopathy and frozen shoulder. These works demonstrate that MSC activation markers are present in synovial tissue, in arthritis, and in pathogenic tendon tissue biopsies from patients suffering from tendinopathy and frozen shoulder[2,51,59,185]. These works describe the following markers to indicate activation of stromal cells are podoplanin (PDPN), also referred to as gp38; CD90, also called Thy-1; and CD248, also called endosialin[2,51,59].

1.1.2 Differentiated cells of the stromal compartment

The stroma is comprised of various cell types which function as structural cells and these connective tissue cells play a role in orchestrating the immune response[196]. These cell types include fibroblasts, endothelial cells, pericytes, endothelial and epithelial cells[83,196]. Resident immune cells are present within the stromal compartment and their crosstalk with differentiated stromal cells have been implicated in mediating the tissue healing response[83,196].

Stromal fibroblasts

The fibroblast is the most abundant cell type within the tissue stroma[83]. They have been described as instrumental in immune cell behaviour, and they define the tissue structure[236]. Described in cancer studies, stromal fibroblasts are responsible for the synthesis and remodelling of the ECM within the tissue[24,127,196]. Their involvement in tissue healing, is described at various stages.

Fibroblast-to-myofibroblast transition (FMT) is broken into two stages: pre-myofibroblast phenotype and fully transitioned myofibroblast phenotype. During the first stage, fibroblasts become activated in a semi myofibroblast phenotype, and the second stage is a fully myofibroblastic phenotype[58]. Fibroblasts transition from a resting state, described as fibrocyte, towards an activated FAP^+ phenotype and finally, differentiating into α-SMA^+ producing myofibroblasts (Figure 1.1)[58,160,235].

Persistent fibroblast activation occurs when fibroblasts fail to resolve their inflammatory response or when they plateau in their inflammatory phenotype expression, causing tissue healing to progress into fibrosis[196,236]. The accumulation of myofibroblasts leads to the development of hypertrophic scarring, excessive ECM production, and refraction of the microvasculature[196].

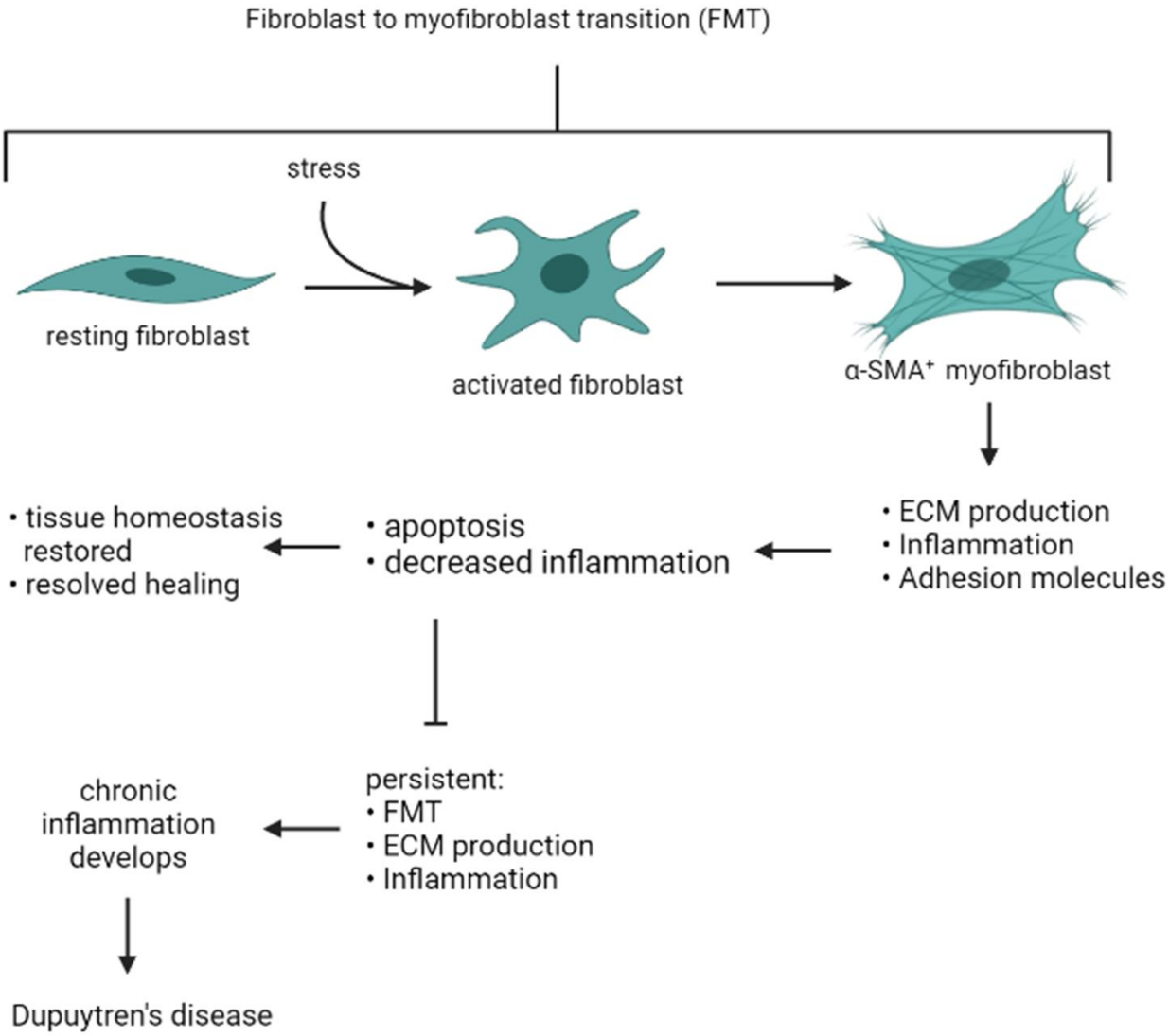


Figure 1.1 Fibroblast to myofibroblast transition is key towards developing Dupuytren's disease.
Quiescent fibroblasts are activated in response to environmental or physical stress, which can be defined as damage, injury, and/or strain. Activated fibroblasts transition into myofibroblasts, identified by their production of a-SMA when stress continues to act upon fibroblasts. Myofibroblasts produce ECM proteins and interact with surrounding cells, contributing to inflammation and increased expression of adhesion molecules. Upon successful tissue healing, myofibroblasts undergo apoptosis and inflammation is decreases, ultimately resulting in restored tissue homeostasis. When apoptosis does not occur, inflammation persists along with constant FMT and maintained ECM protein production. This consistently activated, inflammatory state of the tissue leads to the development of chronic inflammation and manifests clinically as the Dupuytren's disease.

Stromal endothelial cells

During tissue repair, angiogenesis, the formation of blood vessels, is required to support vascular development during tissue healing[83]. Endothelial cells are responsible for regulating the vascular network within the tissue and orchestrating the movement of cells from blood into the tissue[83]. During inflammation, endothelial cells increase blood flow to the site of injury and account for warming of the area and production of swelling[221]. Resting endothelial cells suppress expression of intercellular adhesion molecule 1(ICAM-1) and vascular cell adhesion molecule (VCAM) and during inflammation they express ICAM-1, VCAM, and vascular endothelial growth factor (VEGF) which aids in angiogenesis and encourages vascular leakage to allow an influx of cells to the site of injury[83,111,221].

Published works describe that increased endothelial and angiogenic markers contribute to the development of chronic inflammation and fibrosis via immune cell interacion[124]. Endothelial cells in the tumour microenvironment interact with lymphocytes via TNF-α and VEGF. Stimulation of endothelial cells with VEGF lead to activation of the NFκB pathway and upregulation of adhesion molecules (ICAM-1 and VCAM). Blocking of VEGF signalling increased $CD3^+$ lymphocytes infiltrating into the tumour suggesting that endothelial cell signalling is related to immune cell infiltrate in the tumour microenvironment[111]. An arthritic study demonstrated that suppression of VEGF in animal models inhibit blood vessel formation in inflamed tissue and disease severity was reduced[83,199]. These works highlight the importance of understanding how endothelial cells can mediate inflammation and fibrosis through blood vessel properties and function.

Stromal pericytes

Stromal pericytes wrap around blood vessel walls and regulate permeability of the blood vessel walls via their interaction with endothelial cells[132]. Their interaction with endothelial cells controls the junctions and when they are malfunctioning, vascular leaking occurs, and immune cells can infiltrate from the blood stream[132]. Pericytes can differentiate into myofibroblasts, osteoblasts, and smooth muscle cells. In murine bleomycin induced fibrosis models, they describe pericyte differentiation into α-SMA producing myofibroblasts[113,132]. Pericyte-endothelial cell interactions involve production of soluble factors, matrix protein expression, and vascular development. This, combined with their

ability to in contribute to immune cell influx, describe a potential role for pericytes in stromal activation and chronic inflammation[113,132,230].

Stromal epithelial cells

Stromal epithelial cells are barrier cells involved in the epithelial-mesenchymal transition (EMT) in which epithelial cells undergo biochemical changes to become a mesenchymal cell phenotype[127]. These cells are associated with enhanced migratory capacity, invasiveness, and increased ECM protein production[127]. In kidney fibrosis models, EMT has been suggested as a source of myofibroblasts, however this lineage did not prove true in lung fibrosis models[132]. Transgenic lineage studies provide conflicting evidence regarding an epithelial origin of myofibroblasts however, this does not negate that EMT may occur in human tissues[132]. Future studies would explore how EMT contributes to the fibrotic phenotype seen in Dupuytren's disease.

Resident immune cells

Within the stromal compartment, it has been suggested that tissue resident macrophages and mast cells are activated thereby secreting factors which aid in immune cell recruitment, differentiation, and fibroblast activation[185]. Macrophage polarization reveals a spectrum of macrophages, including an M1 phenotype (classically activated) and an M2 phenotype (alternatively activated) which differ in their cytokine expression and cell interaction[208,299].

It has been suggested that within the stromal compartment, macrophages demonstrate an M2 like phenotype in that they participate in tissue homeostasis[185]. However, in models of tissue fibrosis, macrophages present the M1 phenotype and are responsible for the pro-inflammatory response that develops into tissue fibrosis[185]. Activation of tissue resident macrophages induces production of tumour necrosis factor (TNF), interleukin 1 beta (IL-1β), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and prostaglandin E2 (PGE2)- which contribute to the activation and differentiation of fibroblasts via paracrine signalling[59]. Additionally, mast cells via their secreted factors affect collagen synthesis and matrix metalloproteinase (MMPs) production by fibroblasts[185].

Stromal-immune crosstalk has been implicated in various diseases as a contributing factor to the disease. In cancer, stromal fibroblast, and immune cell crosstalk to mediate cytokine and chemokine production and contributing to chronic inflammation[9,172]. Within the lung, stromal and immune crosstalk regulate injury repair within the tissue[293]. These studies, among others, highlight fibroblasts and immune cells as drivers of the inflammatory response and tissue remodelling.

1.2 Fibrosis

1.2.1 Chronic inflammation is a feature of fibrosis

Chronic inflammation is defined as an immune response in which immune cells are persistently infiltrating the site of repair which triggers excessive ECM component production by fibroblasts[295]. During the initial stages of tissue repair, leukocytes and monocytes are recruited from the peripheral blood and they differentiate at tissue sites and become activated in response to damage associated molecular patterns (DAMPs), pathogen associated molecular patterns (PAMPs), resident immune cells, and stromal cells[37,181]. This inflammatory response is resolved when immune cell recruitment is outweighed by immune cell emigration and apoptosis leading to resolved tissue healing[213]. However, when immune cells and fibroblasts remain activated, inflammation is not resolved, and an inflamed tissue microenvironment is created[213]. Chronic inflammation and fibrosis are encouraged when the balance between MMP production and tissue inhibitor of matrix metalloproteinase (TIMP) regulation is disrupted, leading to the build-up of matrix proteins[295]. This creates disaggregated tissue matrix, aberrant healing, and clinical manifestation ranging from hypertrophic scarring to organ failure.

1.2.2 ECM becomes aberrant during fibrosis

Fibrosis is driven by an imbalance between MMPs and TIMPs where tissue is not cleared from the site of injury and ECM components are continually produced[236]. Fibroblasts play a role in fibrosis via MMPs, inflammatory cytokine/chemokine production, and indirectly, via immune cell crosstalk[196]. Fibroblasts are responsible for secreting ECM components and maintenance of the matrix, as well as secretion of structural and adhesion proteins[83,132]. They produce collagen type I and III, fibronectin, matrix degrading proteins MMPs and, their inhibitors, TIMPs[132,185,213]. It has been reported in Dupuytren's disease that

α-SMA producing myofibroblasts are a hallmark of the disease which leads to increased collagen type III:I ratio and matrix protein expression[117,152,276]. Excess matrix protein expression coupled with the imbalance between MMPs and TIMPs contributes to the dysregulated matrix seen in Dupuytren's[240].

1.2.3 Extracellular matrix proteins

The ECM is a noncellular 3D network of two types of macromolecules, proteoglycans and fibrous proteins: including collagen, proteoglycans, elastin, fibronectin, and various other glycoproteins[86]. It acts as a physical scaffold that regulates cell behaviour including growth, migration, differentiation, and homeostasis[86].

ECM remodelling occurs during physiological conditions and as part of the disease processes modulates the structure and properties of ECMs[86]. In tumours, alterations in the ECM lead to the formation of a fibrotic stroma accompanied by increased stiffness, excess deposition of ECM components, and altered enzyme activity. LOX activity is enhanced in response to abnormal remodelling, and it promotes cross-linking of collagen fibres with ECM components resulting in stiffened matrix and clinically presenting as reduced mobility and hardened tissue[86].

Fibrosis, from the Latin fibre meaning filament or thread, is defined as the hardening, excess accumulation, and/or scarring of tissue[287,295]. It is a pathological feature of chronic inflammatory diseases and is identified by the accumulation of excess ECM proteins[301]. The accumulation of fibrous connective tissue in and around inflamed, damaged tissue leads to permanent scarring, organ malfunction, and in extreme cases death[301]. Several matrix proteins have been studied within the confines of the fibrotic disease, including collagen, fibronectin, periostin, and tenascin C.

Collagens

Collagens are ubiquitous proteins that maintain the structural integrity of connective tissue[32]. Within tissue, they resist shear, tensile, and pressure forces as seen in tendons, bone,

and cartilage[32]. Collagen types are synthesized mainly by cells of the extracellular matrix, namely fibroblasts and myofibroblasts[32]. As seen in liver fibrosis, excessive collagen deposition distorts normal tissue architecture causing functional challenges and ultimately, organ death[32,301].

Type 1 collagen is present throughout the body, except in cartilage, and is the predominant collagen within soft tissue. It is also a major component of mature scar tissue and the principal collagen in the fascia and tendons. Type III collagen dominates within the walls of the blood vessels and co-polymerizes, with type 1 collagen[32]. Described in fibrosis, tendon and heart disease, a high collagen I to collagen III ratio coincides with fibrotic development and organ misfunction[91,193,250,286]. The difference in the physical properties of type 1 & 3 collagen types could define the difference between erratic and resolved matrix repair.

Fibronectin

Fibronectin is a matrix protein that provides structural support and is involved in cell adhesion, migration, growth, and differentiation[86,198,212,240]. Described in cardiovascular disease and tumour studies, fibronectin was shown to be produced by fibroblasts and myofibroblasts. Knockdown murine studies demonstrate the absence of fibronectin leads to resolved tissue healing and no fibrotic development[289]. Additionally, studies describe that fibronectin deficient mice present changes in the tissue architecture coupled with reduced apoptosis[191,307].

Periostin

Periostin, also known as osteoblast-specific factor 2, binds to ECM components including Collagen type 1 and fibronectin. It transmits signals from the ECM to cells via integrin signalling that effect cell adhesion, proliferation, migration, and angiogenesis[13]. Periostin has demonstrated its role as a scaffold for assembly of ECM proteins (type I collagen, fibronectin, tenascin-C) and accessory proteins (BMP-1 and CCN3)[144,198].

Periostin, together with tenascin-C, forms a mesh-like structure with fibronectin upon which type I collagen can cross-link forming the ECM[144]. Secreted in connective tissue during wound healing, periostin is defined as a non-structural ECM component and modulates stabilization of the ECM[198]. Knockdown studies have shown that without periostin collagenous-based tissues were disrupted, demonstrating structural defects, in response to mechanical loading[198].

Tenascin C

Similar to fibronectin, tenascin-C is weakly expressed in the ECM vasculature but upon injury and vascular damage, its expression is upregulated[273]. The tenascin family, consisting of tenascin C, R, W, X, and Y, is not found in normal, uninflamed adult tissue. Tenascin C is expressed during would healing and in tumours and is associated with promoting angiogenesis in disease, including diabetes and inflammatory bowel disease[32,203,273].

Tenascin C is expressed during the inflammatory remodelling process and expression of TGF-b, IL-4, and/or IL-13 stimulation on fibroblasts triggers expression of tenascin-C, periostin, and fibronectin[144]. Interestingly, murine knockdown studies describe that without tenascin-C, tumours develop, and mammary gland show no change with or without the presence of tenascin-C[209]. Wound healing studies have described that its role is crucial for tissue homeostasis in that it is needed for fibronectin expression[209]. These and other published studies, describe tenascin C does not have a consistent effect in disease models. However, within the confines of fibrosis, it is consistently described as crucial for mediating tissue repair and matrix remodelling[182,183].

Matrix metalloproteinases

MMPs and their inhibitors TIMPs, are a family of enzymes which act as regulators of tissue homeostasis via their clearing of matrix proteins[86,279]. A balance between MMPs and TIMPs has been described as a determining factor in ECM turnover[279] and when this balance is disturbed, excess matrix proteins are present within the tissue and chronic inflammation and fibrosis soon follows.

MMPs are produced by several cells (Figure 1.2) and their expression leads to a stiffened tissue matrix. The combination of tissue stiffening leads to mechanically weaker tissue and reduced elasticity, in concert, these changes promote a ‘tissue healing complex’ fibroblast activation and differentiation coupled with immune cell infiltrate are promoted laying the foundation for chronic inflammation and, eventually fibrosis[86].

Group	*MMP*	*Cells source*	*Substrates*
Collagenases	MMP-1	Proliferating and migrating keratinocytes fibroblasts	Collagens I, II, III, V, VII, X, XI; gelatines
	MMP-8	Neutrophils	Collagens I, II, III, VII, VIII, X Gelatines
	MMP-13	Fibroblasts	Collagens I, II, III, IV, IX, X, XIV
		Migrating keratinocytes	
Gelatinases	MMP-2	Fibroblasts Keratinocytes Endothelial cells Macrophages	Gelatines Collagens III, IV, V, VII, X, XI, XIV
	MMP-9	Keratinocytes Neutrophils Macrophages Endothelial cells	Gelatines Collagens I, IV, V
Stromelysins	MMP-3	Basal proliferating keratinocytes	Collagens I, III, IV, V, IX, X
		Fibroblasts	Gelatines
	MMP-10	Migrating keratinocyte	Collagens I, III, IV, V, IX, X
		Fibroblasts	Gelatines
Metalloelastase	MMP-12	Macrophages	Collagen IV; gelatines
Membrane type MMP	MMP-14 (MT1-MMP)	Migrating keratinocytes	Collagen I, II, III Gelatines
Other MMPs	MMP-19	Keratinocyte Fibroblast Endothelial cells	Gelatines Collagen IV

MMPs, matrix metalloproteinases.

Figure 1.2 Matrix Metalloproteinases (MMPs) act as regulators of ECM proteins and are produced by various cell types. A hallmark of tissue fibrosis is an excess of ECM proteins as their production leads to excess fibrous tissue, as seen in Dupuytren's disease. This figure, adapted from Xue and Jackson, 2015, describes various MMPs that are produced by fibroblasts and the matrix proteins they regulate. MMP activity could lead to the development of fibrotic Dupuytren's disease tissue.

1.3 Immune cells contribute to fibrosis

1.3.1 Macrophages in musculoskeletal disease

Macrophages have been described as critical regulators of fibrosis in that they are either tissue resident or infiltrating and differentiating from monocytes[301]. They function as fibrotic regulators via their production of soluble factors and their interaction with various stromal and immune cells. Macrophage populations exert unique effects during the phases of fibrosis, and they drive the pathogenesis of fibrosis[301]. Recruited and resident macrophage populations proliferate and undergo phenotypic and functional changes in response to growth factors and cytokines released from the surrounding tissue microenvironment[299]. Macrophages differentiate into an M1 or M2 phenotype however the relationship between the subtypes is spectral and not polar opposites.

Macrophage subsets

M1, classically activated macrophages

M0 macrophages differentiation into M1-like macrophages in response to TLR ligands, macrophage-colony stimulating factor (MCSF), LPS, TNF-α and IFN-γ[301,310]. Monocytes which differentiation into M1 macrophages are described as classically activated pro-inflammatory macrophages. These macrophages mediate destruction of ECM either directly or indirectly via MMPs release[209].

M2, alternatively activated macrophages

M0 macrophages differentiation into M2-like macrophages when exposed to GM-CSF, IL-4 and IL-13 and are involved in wound healing, tissue remodelling and fibrosis[271,301]. M2 macrophages are referred to as anti-inflammatory M2 macrophages. M2 macrophages support cell proliferation and stabilize deposited ECM components and synthesis of new ECM molecules[289].

M2 macrophages have been implicated in the development of T_H2 effector response and suppression of M1 macrophage response[301]. Work done by Pesce *et al.* (2009) have suggested that M2 macrophages compete with T_H2 cells for factors (L-arginine) which regulate cell growth and collagen synthesis[216,301]. M2 macrophages have also been described

as inducers of regulatory T cells and as exploiters of mechanisms which inhibit ECM synthesis by myofibroblasts[301].

Is the M1/M2 paradigm still true?

Historically, macrophages have been characterized as either M1 or M2[289,299,301]. Current studies describe that macrophage polarization is not one or the other but instead differentiation is defined as a spectrum. Macrophage polarization was formulated by mirroring the T_H1/T_H2 polarization seen in T cells however in vivo, macrophages have been shown to adopt various functional phenotypes, beyond the simplified M1, M2 classification[1,46,116,208]. As the knowledge of macrophage subsets continues to go, studies are using innovative technology, like RNA sequencing, to aid in better understanding phenotypes.

Single cell RNA sequencing (scRNA-seq) of atherosclerosis macrophages revealed several specific clusters of macrophages which are not present in in vitro models[66]. Bleomycin-induced murine fibrosis studies demonstrate that scRNA-seq of lung tissue houses a profibrotic macrophage subpopulation which is localized to the site of hypertrophic scarring[10]. Human idiopathic pulmonary fibrosis (IPF) tissue scRNA-seq confirmed a pro-fibrotic macrophage subpopulation which has been suggested to be a contributing factor in the development of fibrosis[1,17]. The use of ScRNA-seq allows for the ability to build a library of gene expression profiles and potential cellular and biochemical targets in various disease models[1,192,266]. Given the growing literature surrounding the development of macrophage subpopulations, the M1 versus M2 paradigm remains in question as macrophage polarization does not appear to be as black and white, as previous studies suggest. Future studies will continue to investigate and identify macrophage differentiation towards the understanding of macrophage plasticity within the context of fibrotic disease.

Macrophages in fibrosis

Macrophages are the main source for several types of MMPs, namely 1,7,8,9, and 12 which aids in maintain the ECM structure and they also produce their suppressors, TIMPs and a balance between MMPs and TIMPs is crucial for tissue homeostasis[271,289].

Macrophages are a leading producer of TGF-β1 which has been shown to be a major pro-fibrotic agent[271]. In addition to TGF-β1, they also produce IL-4, IL-10, IL-13, TNF-α, and IL-1 which have been shown to activate fibroblasts and thus contribute to matrix protein production[271]. Activated macrophages, in addition to producing pro-fibrotic cytokines, recruit myofibroblasts and exacerbate immune cell infiltration to the site of tissue injury which allows for disaggregated tissue repair, excess matrix deposition, and, finally, fibrosis[271].

Macrophage differentiation, migration, and activation is controlled by IFN-γ producing T cells suggesting a role for the reciprocal relationship between T cells and macrophages in the etiology of chronic inflammation and fibrosis[271]. Additionally, $CD4^+$ T cells generate M2 macrophages via their production of IL-4 and IL-13. Well described within the confines of fibrosis, IL-13 has been described as crucial in lung and liver fibrosis models and cytokines produced by macrophages also function as drivers[295,298]. Macrophage and T cell interactions are not completely understood however, a reciprocal relationship of activation has been described and, even further, crosstalk between these immune cells and stromal fibroblasts have been implicated in the transition towards fibrosis.

Similarly, studies have shown that co-culture systems of macrophages and fibroblasts uncover a reciprocal enhancement of fibrosis as a result of chemokine and cytokine production, suggesting a crucial role for stromal-immune cell interaction in driving fibrosis. Activated macrophages produce TNF-α, TGF-β family members, and platelet derived growth factor (PDGF) which stimulate activation, proliferation, and differentiation of fibroblasts and thus inducing overproduction of ECM proteins[295,298]. The interactions between fibroblasts and immune cells highlight the importance of stromal-immune crosstalk in the etiology of fibrosis.

1.3.2 T cells in musculoskeletal disease

T lymphocytes originate in the bone marrow and migrate to the thymus to mature and undergo selection to produce CD4 or CD8 single positive cells[141,146]. These peripheral T cells consist of various subsets which can respond to memory T cells, regulatory T cells, or

new antigens[146]. The immune response has been described as beginning when a naïve T cell encounters an antigen, or an APC, causing downstream effects like IL-2 production, activation, and differentiation[146].

T cell subsets have varying lifespans. Activated effector cells are short lived and memory T cells can participate in maintaining long term immunity and display a protective response[146]. In addition to varying lifespans, CD4 and CD8 T cell subsets have diverse functions as T helper cells, regulatory T cells, or as killer T cells. T cell subsets such as T_H1, T_H2, T_H17, T_{reg}, and γλ T cells have been described in the context of various diseases and the method in which they contribute to the diseases are under investigation[188]. Inflammation is characterized by an infiltrate of mononuclear cells, including but limited to macrophages, T cells, and eosinophils[298]. Lymphocytes are mobilized and stimulated to produce factors which activate macrophages and these activated macrophages, in turn, produce factors which further stimulate lymphocytes[298].

T cell subsets

T_H1

Th1 cells are characterized by their production of IFN-γ[298]. Naïve $CD4^+$ T cells differentiate into IFN-γ producing T_H1 cells in response to IL-12, described as being secreted by various cells including dendritic cells and macrophages. IFN-γ has been described as an anti-fibrotic due to its ability to suppress collagen synthesis by fibroblasts, which promotes fibrosis[246]. However, IFN-γ has also been shown to increase expression of MMPs, namely 2, 7, 9, and 13 demonstrating a key role of IFN-γ in altering ECM remodelling. T_H1 cells also induce TGF-β expression by myofibroblasts via Smad signalling, again contributing to the development of fibrosis[27,246]. Describing IFN-γ as a double-edged sword, demonstrating both pro- and anti-fibrotic behaviour.

Signal transducer and activator of transcription 4 (STAT4) is critical for IL-12 signalling and commitment to T_H1 differentiation[282]. STAT4 is described as critical for amplifying IFN-γ production but not its initial expression. The defined mechanism is unknown, but this highlights a possible role for STAT4 in mediating T_H1 differentiation[282].

Macrophage activation was first described as a T_H1, IFN-γ mediated process. However, recent works describe macrophage polarization as dependent upon exposure to T_H1 or T_H2 cytokines[298].

T_H2

T_H2 T cell are characterized by their signature cytokines IL-4, IL-5, and IL-13[246,298]. IL-13 has been described as an essential cytokine for the development of fibrosis in that its disruption leads to a lack of developing fibrosis in mouse models[246]. GATA-3, a transcription factor, dictates T_H2 differentiation via regulation of T_H2 cytokine production as it binds to the locus containing IL-4, IL-5, and IL-13 genes[282].

STAT6, activated by IL-4 stimulation, has been historically described as the major signal transducer in IL-4 mediated T_H2 differentiation[282]. Mechanistically, STAT6 promotes Th2 differentiation via its induction of transcription factor GATA-3-as show in in vitro studies[282]. IL-13 and IL-4 share functional activities due to their sharing the IL-4 receptor a-chain and STAT6 signalling pathway. Given that IL-13 is produced in greater concentrations in vivo than IL-4 and IL-5, it has been suggested that IL-13 may play a greater role in tissue remodelling[298]. Studies exploring the role of IL-13 in fibrosis, describe that IL-13 knockdown led to reduced development of fibrosis[228,298].

T_H17

T_H17 T cells are identified by their ability to produce IL-17 family members have been shown to be critical in the inflammatory response in soft tissue disease. T_H17 differentiation is promoted via activation of STAT3 and IL-23R by IL-6 and TGF-β[165,314].

T_H17 transcription factor retinoic acid-related orphan receptor alpha ROR-α and gamma t (RORγt) are critical in promoting T_H17 differentiation. ROR are key transcription factors in T_H17 differentiation. RORγt is upregulated in response to IL-23 and its expression is correlated with IL-17 believed to be via STAT signalling[282]. Within naïve CD4 T cells,

RORγt induces T_H17 differentiation as knockdown studies show that without RORγt, $CD4^+$ T cells did not produce IL-17[282].

IL-17 is produced by T_H17 T cells and γλ T cells, natural killer cells, and innate lymphoid cells. IL-17 production relies on a storm of cytokines, mirroring what occurs in vivo, in vitro studies have described cytokines in this milieu include IL-1β, IL-6, IL-23, and TGF-β[314].

SMAD 4 has been reported as a regulator of T_H17 function and is linked to wound healing and fibrosis, suggesting a synergistic role for TGF-β and T_H17 T cells in regulating the inflammatory response[314]. IL-17A promotes fibroblast proliferation and production of fibrotic factors[246] and most tissue models in which it is studied, it mediates powerful effects on stromal cells[261]; namely in production of inflammatory cytokines and recruitment of immune cells[261].

$T_{regulatory}$ cells

Regulatory T cells (T_{reg}) are pivotal in modulating self-tolerance and immune homeostasis. T_{reg} cells are identified by their ability to produce IL-10 and TGFβ[238]. Within the confines of cell-to-cell communication, IL-10 produced by T_{reg} cells are described as promoting M2 polarization of macrophages[147] and TGFβ is crucial in mediating fibrosis via its interaction with fibroblasts[27]. The downstream effect of T_{reg} action is complex and further divided into regulatory T cell subpopulation 1, Tr1; described as regulating $CD4^+$ T_H1 and T_H2 differentiation[238].

Within the confines of fibrosis, various subpopulations have been characterized under the umbrella of regulatory T cells. CD25, a widely expressed T_{reg} marker, has been identified in human pulmonary fibrosis studies where it is associated with disease severity[140]. Mouse pulmonary studies describe the use of an anti-CD25 antibody leads to reduced fibrotic development[314].

γλ T cells

γλ T cells are characterised by a T cell receptor γ and λ chain and they can instigate or resolve fibrosis depending on cytokines present and the disease model being used[314]. γλ T cells can produce a variety of cytokines, chemokines, and growth factors. They play a key role in regulating the immune response and influencing the behaviour of neighbouring immune cells[246].

In bleomycin induced fibrosis, deletion of γλ T cells results in severe fibrosis and γλ T cells function as the predominant source of IL-17[314]. In a bleomycin-induced pulmonary fibrosis model, T cell receptor delta (TCRλ) deficient mice were injected with γλ T cells from wild type mice yielding a weaker IL-17 signature, as described by reduced $CD4^+$ $IL\text{-}17^+$ cells. Suggesting γλ T cells are needed for T_H17 differentiation and could play a regulatory role in fibrosis via its interaction with T_H17 T cells[314,246].

Furthering their suggestion that γλ T cells modulate T_H17 behaviour, they demonstrate that IFN-γ, a pro-fibrotic cytokine, inhibits IL-17 behaviour by introducing an anti- IFN-γ neutralizing antibody to TCRλ deficient $CD4^+$ T cells which led to a significant increase in $CD4^+$ $IL\text{-}17^+$ T cells differentiation[246]. Confirming that not only do γλ T cells interact with immune cells, $CD4^+$ T cells in this study, and modulate a fibrotic response via IFN-λ production.

1.3.3 T cell activation

T cell activation is instrumented via its co-stimulatory receptors for T cell priming, CD27 and CD28. CD27 is a TNF receptor (TNFR) family member with various mechanisms of action[256]. It is constitutively expressed on naïve and central memory T cells and its expression is lost with differentiation into effector cells[275].

CD28 is a member of the immunoglobulin (Ig) family and serves primarily as a signal amplifier of the T cell receptor[256]. CD28 is expressed on all naïve T cells and maybe lost with differentiation and has shown to decrease with age, this disappearance is accelerated in

susceptible individuals[275]. CD27 activity is induced by CD70, which is found on activated T cells, B cells, and dendritic cells, and CD28 binds to its ligands CD80 or CD86, which are expressed on APCs[275]. CD27 and/or CD28 deficient mice studies describe that both CD27 and CD28 promote clonal expansion of activated T cells via different mechanisms. CD27 acts by counteracting apoptosis via increasing anti-apoptotic molecule, Bcl-xl, and decreasing sensitivity to FasL-induced apoptosis. CD28 affects expression of cell cycle regulatory proteins and activates T cells via the cell cycle[275].

CD27-CD70

CD27 is a member of the TNF receptor family (TNFR) which shares features with CD28, and it is expressed on naïve and central memory T cells[55,95,256,275,280]. CD27 is expressed on lymphocytes and its expression is increased after T cell activation[274]. CD27 belongs to the TRAF-interacting subgroup of the TNF ligand super family (TNFSF), it interacts with TRAF2, 3 and 5[280]. Via TRAF2 and 5, a signalling cascade is activated resulting in the activation of transcription factors of the NFκB family and MAP kinase[280]. Via this pathway, CD27 enhances cellular proliferation and induces anti-apoptotic proteins b cell lymphoma-2 (Bcl-2)[280]. CD27 activity is induced via engagement with CD70, also called CD27 ligand. CD70 is found on activated T cells, B cells and dendritic cells, to name a few[95,274]. CD70 is a member of the TNFSF, and this family can be sub-grouped into three categories: death receptors, TNF receptor associated factors, and decoy receptors[280].

CD70 expression is tightly controlled and occurs strictly after activation. CD27 signalling can lower the threshold of the TCR response to low affinity antigens, promotes differentiation, and enhances proliferation and survival. CD27 signalling is TNF receptor associated factor (TRAF) dependent and primarily targets the NFκB and c-Jun amino terminal kinase/p38 pathways[55]. The importance of the CD27-CD70 pathways has been reflected in animal studies. CD70 blockade in collagen-induced arthritis mouse models describe that CD70 blockade before and after the onset of the disease leads to improved clinical presentation and reduced collagen production[95,151]. In vitro blockade studies show that without CD27, the response of T cell proliferation and cytokine secretion were inhibited and that CD27 deficiency allows for T cell division, but proliferation is poor following activation[55]. In tumours, the CD27/CD70 axis could have pro-tumoral immune suppressive

effects given CD70 has been described as overexpressed in tumour tissue and associated with worsening prognosis[256]. These works suggest a key role for understanding the mechanism of CD27 and CD70 in T cell behaviour within disease models[55,256,280]. Within the confines of musculoskeletal disease, an influx of immune cells is instrumental in mediating tissue repair. Understanding the molecular mechanisms in play during immune cell activation and differentiation could provide crucial understanding towards developing therapeutic targets.

CD28-CD80/86

CD28 is a member of the immunoglobulin family and acts largely as a signal amplifier of the T cell receptor[275]. CD28 is a member of subfamily of co-stimulatory molecules and is expressed by 80% of human CD4+ T cells and 80% of CD8 T cells[81,95]. While CD28's function is not completely understood it has been identified on other cell lineages including bone marrow stromal cells, plasma cells, neutrophils, and eosinophils[81]. It has been suggested that CD28 expression is inversely related to age however TNF can function as a contributing factor in its downregulation. This suggests a cytokine driven mechanism for CD28 expression[95].

The CD28-CD80 pathway is a costimulatory pathway in which CD80/CD86 expression is induced on antigen presenting cells upon stimulation. The interaction between CD28-CD80/86 interaction can be inhibited by cytotoxic T-lymphocyte antigen (CTLA)-4 immunoglobin (Ig)[95]. CTLA4 is expressed after stimulation and limits T cell responses by outcompeting CD28 by binding to CD80 and CD86 with greater affinity[95]. CD80 expression is described as being induced in the immune response prior to CD86. Expression of both CD80 and 86 is dependent on various activation stimuli but the exact mechanism is not known[95]. CD28 acts as a signal for transcription factors like NFκB, nuclear factor of activated t cells (NFAT), and activator protein-1 (AP1). CD28 functions by lowering the TCR threshold and allowing for 'easier' T cell clonal expansion and differentiation[95].

1.4 Dupuytren's disease

1.4.1 History of Dupuytren's disease

Dupuytren's disease is a fibroproliferative condition in which the palmar tissue of the hand becomes chronically inflamed, as a result of unresolved tissue repair, resulting in a fixed flexion of the affected, typically 4th and 5th fingers. Dupuytren's disease was first described by Felix Platter in 1680, Henry Cline in 1808, and Sir Astley Cooper in 1818 before Guillaume Dupuytren's provided an eponym for the disease after his lecture in 1831[84,102,265]. In folklore, Dupuytren's disease has been described as a 'Viking's disease' due to the disease's prevalence in Scandinavia and the spread of the disease in countries colonized by the Vikings[84,105,265]. It has also been described as the papal 'hand of benediction'-inspired by the 'hand of Sabazios' and depicted by an early pope who had the disease- or the curse of the bagpipe craftsmanship of the MacCrimmon clan-a prominent clan of bagpipers who developed the disease and could no longer play[105,265].

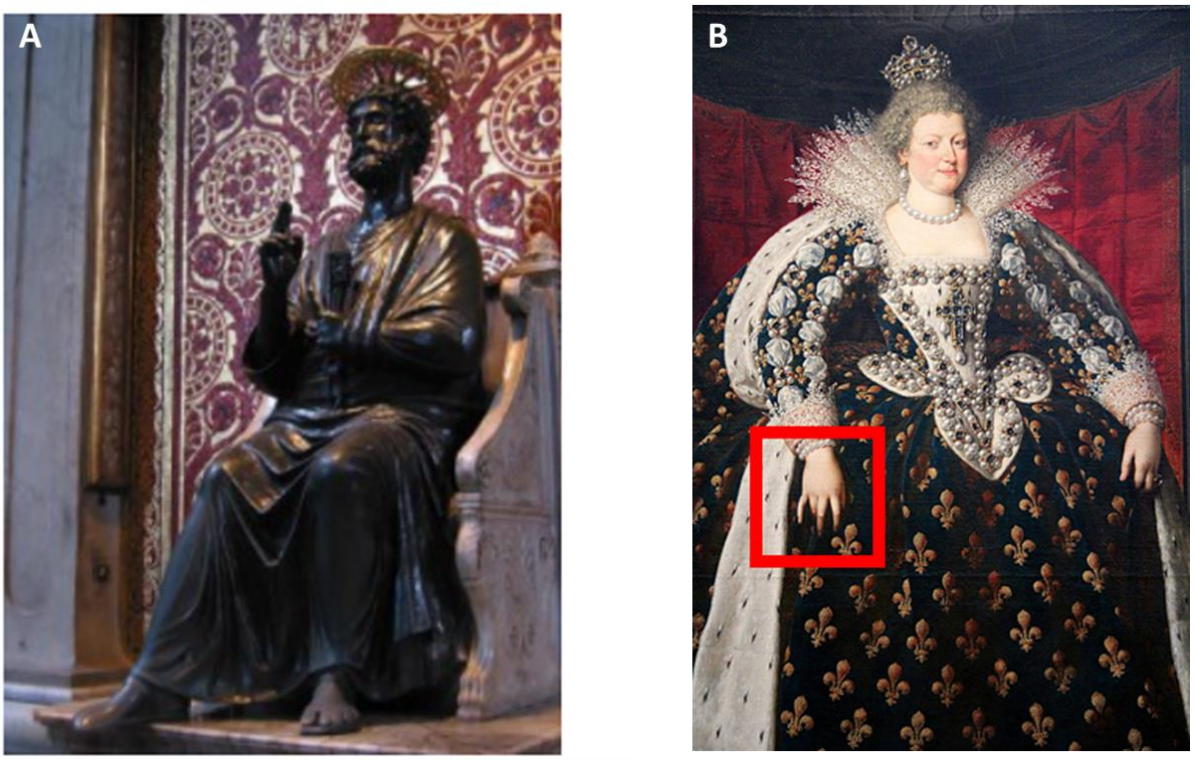


Figure 1.3 Dupuytren's depictions throughout history.
Shown here St. Peter is affected by Dupuytren's disease, noted by the inward curling of his fourth and fifth finger (A). Marie de Medici is also depicted with a contracted fourth finger (B). Image courtesy of Dupuytrens-society.org and cherrychapman.com, respectively. While the name Dupuytren's disease was not given to the contracted finger disease until 1831, depictions of the disease exist throughout history.

1.4.2 Clinical presentation

Dupuytren's disease tissue has two microenvironments: nodules and cord[29,102,218,265]. In early Dupuytren's disease, small tissue masses, called nodules, are palpable on the palm. The nodules develop on the pretendinous cords causing the underlying fascia to thicken in the palm and continue towards the fingers[170]. These nodules develop into a thick, fibrous cord which adheres to the skin, running parallel to the tendons[29,218]. This cord causes a fixed contracture of the metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joint[218].

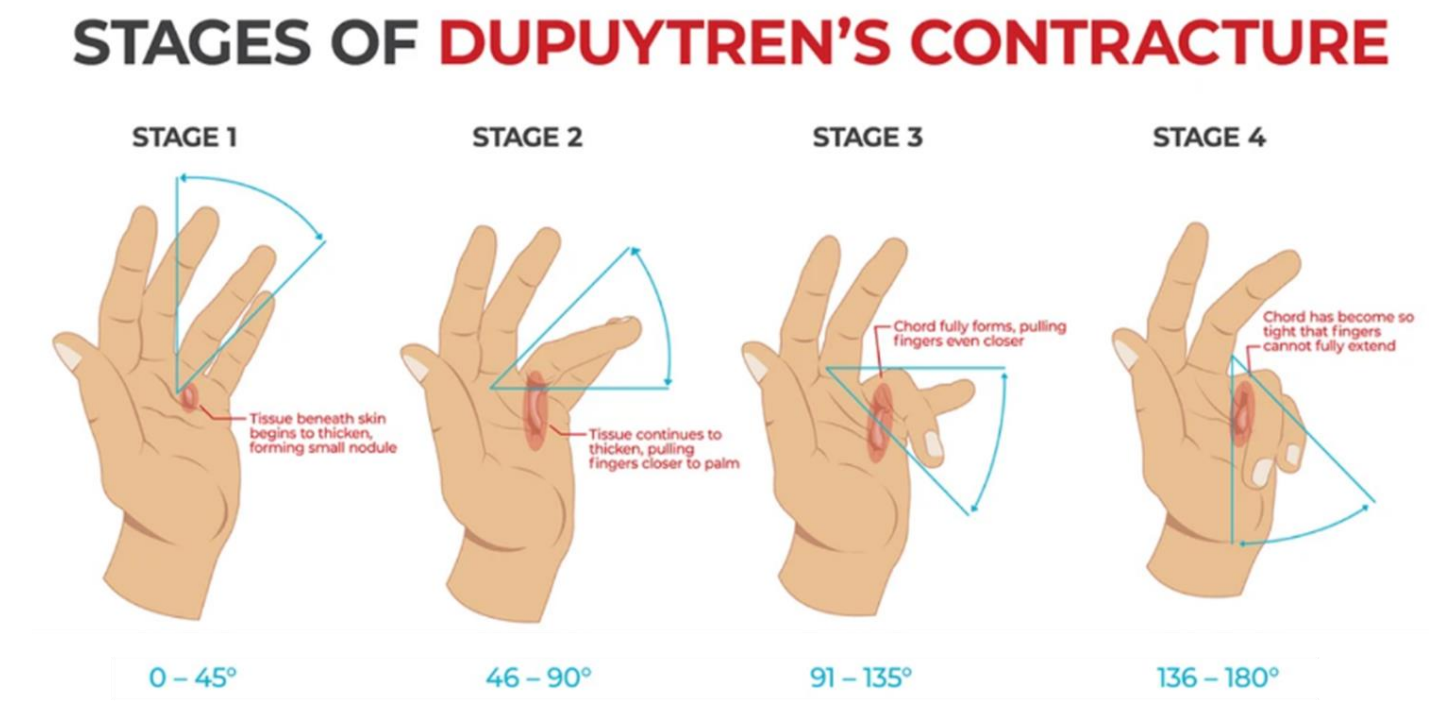


Figure 1.4 Progression of Dupuytren's disease, classified by stage.
Clinical characterization of Dupuytren's disease aids in determining which treatment option would be best, as treatment options vary for each stage of the disease. In addition to feeling the size of the raised skin, the degree of inward contraction is an effective and more concrete method of characterising the disease. Shown here is an image adapted from BraceAbility.

1.4.3 Epidemiology

Dupuytren's cases are frequently reported in western countries, like the United States, England, and Scotland. The disease affects between 2-42% of the population[265] and disproportionately affects white men. The range of percent occurrence is credited to underreporting, as seen in the lower range, and communities disproportionately affected by the disease, indicated by the higher end of the range. Studies have shown that Dupuytren's is reported in Taiwan and Japan as frequently as they are in northern Europe[96,218,248] -however Taiwanese and Japanese cases are not as severe, presenting 'early' stage Dupuytren's disease as noted by the absence of the fibrous cord[218,248]. Icelandic studies have reported 40% of Dupuytren's cases in males over 70 and in the United Kingdom (U.K.), the prevalence is around 4% and rises to 20% in populations of people over 65 years of age[218,248]. However, a United States (U.S.) study reported 4-6% prevalence in the general population[71]; supporting the notion that people over 60 are more likely to develop the disease.

These studies have reported that within the populations studied men, particularly white men, are more susceptible to developing Dupuytren's disease. The link between white men and the disease has been supported by their European heritage, in addition to the possibility of a diathesis, defined as the tendency to suffer from a particular medical condition. However, this notion of Dupuytren's primarily affecting white men is often questioned; it is well understood that across racial groups and genders, white men are more likely to have access to quality care and are taken more seriously when sharing their health challenges with healthcare professionals. Further, it has been suggested that Dupuytren's can be inherited in an autosomal dominant pattern-perhaps in association with risk factors linked to Dupuytren's disease[29,84,105,150,218,240]. These risk factors could explain why Dupuytren's disease is seen in other racial populations with lower prevalence[71,189,287,309].

1.4.4 Risk factors

While Dupuytren's disease has been divided into stages of progression, the cause of the persistent activation and differentiation of fibroblasts has yet to be explained. However, a series of lifestyle factors have been suggested as contributors to the development of the disease. The most discussed are performance of manually laborious tasks[69,105,174], diabetes[102,105,96,69,201,45], smoking[45,96] and alcohol use[96,102,174]. Vibration tasks is the collective

term used to describe actions that lead to the development of microtears or injury to the hand's soft tissue. The performance of vibration tasks can lead to microtears in the fascia that become chronically inflamed, leading to the development of Dupuytren's disease[105, 174].

Musculoskeletal disorders accompany diabetes due to microangiopathy, defined as thickening and weakening of the blood vessels causing leakage, and increased collagen type I production suggesting Dupuytren's could be a complication of diabetes, via an unknown mechanism. Interestingly, it has been suggested that diabetic patients' middle and fourth fingers are affected by Dupuytren's disease instead of the fourth and fifth fingers, as seen in non-diabetic patients[105]. Similarly, diabetes has also been associated with Peyronie's disease, again credited to vascular leakage (as a complication of diabetes) leads to dysregulated tissue homeostasis[92]. Smoking has also been linked to the development of Dupuytren's due to changes in the microvascular of the hand, creating complications in wound healing[105,248]. Alcoholism has also been suggested as a factor of Dupuytren's however, the reasoning has yet to be explained[96,171,174].

Dupuytren's disease belongs to a class of diseases called superficial fibromatosis. Diseases in this class include Dupuytren's disease (palmar fibromatosis), Garrod's pads (palmar fibromatosis), Ledderhose disease (plantar fibromatosis), and Peyronie's disease (penile fibromatosis). Garrod's pads, also called Garrod's nodes or knuckle pads are firm nodules formed on the extensor aspects of the proximal interphalangeal or metacarpophalangeal joints[29,96,177,265,310]. While defined as palmar fibromatosis, like Dupuytren's disease, Garrod's pads are localised to the knuckles prompting the discourse that they should be called knuckle fibromatosis, instead of palmar.

Ledderhose disease, also called plantar fibromatosis, is described as Dupuytren's of the foot; nodules appear along the bottom of the foot causing the toes to contract and painfully curl forward[5,62]. Peyronie's disease affects the penile fascia causing deformation, erectile dysfunction, and pain[96,225,289,310]. The association between these diseases and Dupuytren's disease is credited to cases presenting with both Dupuytren's and another type

of fibromatosis. Studies have also suggested that presenting with Dupuytren's disease could predispose an individual towards developing other types of fibromatosis[202,259,308].

1.4.5 Pathophysiology

Dupuytren's disease has been described in three stages: proliferative, involutional, and residual (Table 1.1). During the proliferative stage, activated fibroblasts, called myofibroblasts, continuously produce α-SMA leading to the formation cellular nodules[29,218]. It has been suggested that transforming growth factor beta (TGFβ) and periostin play a role in the persistent activation and differentiation of fibroblasts into myofibroblasts[27,218]. In addition to α-SMA producing myofibroblasts and excess collagen production, nodule tissue contains narrowed micro vessels and increased type 3:1 collagen ratio, suggesting hypoxia plays a role in the development of the disease[102]. The involutional stage is characterised by the presence of α-SMA^{+} nodules, periostin, and increased expression of collagen type III[218]. The increase in collagen type III expression leads to the development of the fibrous cord in the later stages of the disease. Seen in the residual stage of Dupuytren's, the cord's dense, collagenous matrix contains cellular nodules and is accompanied by flexion contracture of MCP and PIP joints and reduced collagen type III expression[29,102,218].

Table 1.1 Further classification of Dupuytren's disease

As more studies focus on understanding Dupuytren's disease, classification of the stages of disease continues to change. Here is a tabulated assessment of Dupuytren's disease where instead of being characterized into four stages by degree of inward finger curling, the disease is characterized into three stages. This three-stage classification also considers the changes in cell behaviour and decreased mobility. The information in this table was sourced from Walthall e*t al.* 2021[281].

Stages of Dupuytren's	Characterization
Proliferative	• Stage 1 & 2 • phase has a characteristically high concentration of immature myofibroblasts, and fibroblasts arranged in a whorled pattern • mild case, no contraction or mild MCP joint contracture (<30˚)
Involution	• Stage 3 • fibroblasts become aligned in the longitudinal axis of the hand following lines of tension • mild functional challenges, joint contracture (30-60˚)
Residual	• Stage 4 • relatively acellular collagen-rich chords remain causing contracture deformity • severe contracture (>60˚) of MCP joint and PIP joint (>30˚)

1.5 Dupuytren's as a model of fibrosis

The cellular mechanisms of tissue healing are complex and not fully understood. When tissue healing fails to resolve, the tissue becomes chronically inflamed[295,301]. Chronic inflammation is defined as persistent and unresolved inflammation has been described as a contributing factor to erratic healing and in the progression of fibrotic tissue[295]. Tissue fibrosis is characterized by excessive ECM protein production causing permanent scarring and, in severe cases, organ failure[295,301]. Dupuytren's disease is a model of tissue fibrosis as persistent activation and differentiation of fibroblasts and immune cells leads to the maintenance of the inflamed tissue state, presenting histologically as excessive cellular tissue (Figure 1.5)[194,204]. Published works describe Dupuytren's by its activation of the stromal compartment, activation of resident and infiltrating immune cells and production of pro-fibrotic and pro-inflammatory mediators[3, 117,152,276]. Thus, the elucidation of immune mechanisms involved in a local, fibroproliferative disease that is readily accessible for tissue biopsy could provide useful insights in other fibrosis diseases where obtaining a biopsy is more difficult, like in lung and heart fibrosis.

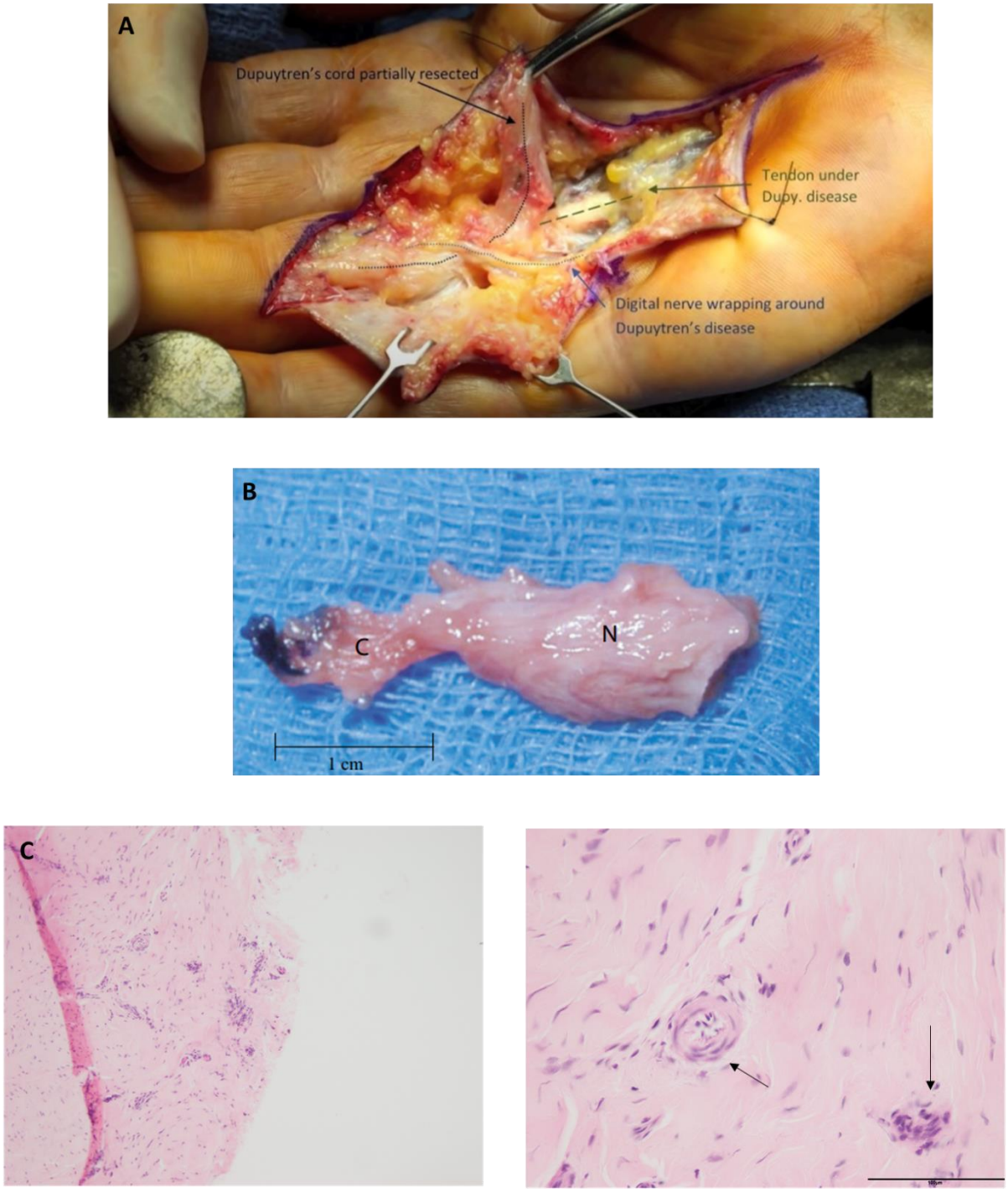


Figure 1.5 Dupuytren's disease fasciectomy and tissue biopsy.
Dupuytren's patients undergo fasciectomy where the affected tissue is removed. Depicted here is an image of a Dupuytren's hand where the fibrous cord is being removed from the affected tendon and nerve (A). Image courtesy of Morgan Town Plastic Surgery, Dr. McClellan. The removed tissue can be visually classified as a cluster of nodules, N or a single, fibrous cord, C (B). The image is adapted from Bisson et al.2016. Haematoxylin and eosin stained Dupuytren's tissue depicts the cellular nature of the removed tissue and a cellular nodule is visible as shown by the arrows (C).

1.5.1 Extracellular matrix makeup of Dupuytren's

Chronic inflammation is defined as an immune response in which immune cells persistently infiltrate the site of repair and can trigger excessive ECM component accumulation[295]. During the preliminary stages of tissue repair, leukocytes and monocytes are recruited from the blood to differentiate and activate in response to factors released by cells at the site of damage[37]. This inflammatory response is resolved when immune cell recruitment is 'outweighed' by immune cell emigration and apoptosis[213]. However, when these immune cells remain, in combination with permanently activated fibroblasts, inflammation is not resolved, and an inflamed tissue microenvironment is created[213].

Matrix proteins in Dupuytren's disease has been characterized by studies describing an increase in myofibroblast expression in response to inflammatory cytokines[117,152,276]. Further Dupuytren's cord composition has been described as primarily made up of collagen type III, presenting as a fibrous cord, raised on the palm[96,133]. Collagen turnover and ECM remodelling is regulated by MMPs and their inhibitors TIMPs[295]. Published works describe that MMP-2 and MMP-14 are key in collagen contractility in Dupuytren's disease[291]. Chronic inflammation and fibrosis are encouraged when the balance between MMP production and TIMP regulation is disrupted, leading to the build-up of matrix proteins, and stiffening of the tissue which manifests clinically as tensed palm tissue and flexed fingers-identifying traits of Dupuytren's disease[295].

1.5.2 Immune cells infiltrate Dupuytren's stroma

When tissue damage has occurred, the cells of the stromal compartment demonstrate an activated phenotype eliciting release of factors which act upon resident and infiltrating immune cells. The immune cell hierarchy of Dupuytren's nodule tissue has been described as macrophages, T cells, mast cells, B cells, and neutrophils[117]. In response to the inflammatory nature of the site of tissue damage, infiltrating immune cells secrete factors that promote further recruitment of immune cells from the blood stream and maintain survival of immune cells, preventing resolution of tissue repair and promoting fibrosis.

Macrophages

Macrophages perform various roles within the tissue where they identify invaders and phagocytosis them and they regulate fibroblast behaviour via their production of TGF-β. In liver fibrosis studies, macrophage depletion led to altered disease pattern[77,299]. Monocytes are recruited from the blood stream in response to CCL2 (monocyte chemoattractant protein-1, MCP-1) produced at the site of injury[301]. The subpopulations of macrophages are described as M1 or M2 and their differentiation depends on surface markers and proteins expressed at the site of injury[289]. Suggested differentiation pathways indicate an M1 phenotype is the result of monocyte exposure to MCSF, bacterial lipopolysaccharide (LPS), TNF-α, or IFN-γ and M2 from GM-CSF, IL-4, or IL-13[289,295,301]. Activated, differentiated macrophages produce pro-inflammatory and pro-fibrotic cytokines which activate and differentiate fibroblasts and drive overproduction of ECM components[289,299]. Additionally, the factors produced continuously recruit and maintain immune cell populations[289].

Mast cells

Mast cells are activated in response to IFN-γ and produce IL-13 which has a role in driving fibrosis via STAT1/IL-13Rα1 pathway[3]. This, in combination with blockade studies, reveal that the absence of fibrosis is associated with decreased IL-4, deemed a pro-fibrotic mediator, and IL-13 expression suggesting that these T_H2 cytokines have a specific role in developing fibrosis[295]. IL-4 has been described as a pro-fibrotic cytokine due to its presence in fibrotic lung fluid[295]. IL-13 has recently been described as a driving factor in Dupuytren's disease[3]. Interestingly, when IL-4 and IL-13 were hindered separately, IL-13 was identified as the dominant cytokine in developing fibrosis[295]-suggesting an instrumental role in activated, IL-13 producing mast cells.

T cells

Infiltrating T cells from the blood stream, particularly $CD4^+$ T cells, have been implicated in the development of fibrosis via their production of inflammatory cytokines[185,301]. The immune cell landscape of Dupuytren's has been described as not only having a clear T cell population but also that T cells are one of the leading immune cells found in nodule tissue[117].

Lymphocyte subsets that have been implicated in having a role in fibrosis are T_H1, T_H2, T_H17 and gamma delta (γδ) T cells[314]. IL-12 has been described as a driver of naïve $CD4^+$ T cells toward the IFN-γ producing T cells which inhibit fibroblast production of collagen thus inhibiting fibrosis[314]. T_H2 subset of T cells are characterised by production of IL-4, IL-5, and IL-13. IL-13 cultured Dupuytren's fibroblast showed an increase in matrix protein expression thus driving the fibrotic response in Dupuytren's[117]. The T_H17 response is characterized by production of IL-17 which activates the STAT3 pathway promoting collagen type 1 production[314].

1.5.3 Cytokines and growth factors in Dupuytren's

Cytokines and growth factors contribute to the development of Dupuytren's disease by maintaining the chronically inflamed nature of the tissue and maintenance of persistently activated myofibroblasts and immune cells. It has been reported that the most prevalent soluble factors in Dupuytren's nodule tissue are IL-6, TGF-β, and TNF-α[117,276]. Studies have shown that culturing Dupuytren's fibroblasts with pro-fibrotic and pro-inflammatory cytokines enhances the elements associated with tissue fibrosis suggesting the possibility of manipulating their pathway of action to reduce or hinder development of fibrosis[3,25,117,276].

TGFβ isoforms

Members of the TGF-β superfamily are secreted by several cell types and implicated in various cell functions: regulating tissue homeostasis and repair, immune and inflammatory response, extracellular matrix deposition, cell differentiation and growth[27]. The three isoforms of TGF-β (TGF-B1, TGF-β2, and TGF-β3) are encoded by three different genes but signal via the same cell surface receptor and have similar targets[27]. TGF-β1 has been described as a key factor leading to the transition from tissue being chronically inflamed to becoming fibrotic; as well as being instrumental in regulating the fibroblast phenotype and function[27,29,218]. TGF-β1 expression in Dupuytren's disease has been described as being highly expressed in the tissue's matrix and by fibroblasts and myofibroblasts within the nodule[25]. It has also been suggested TGF-β1 is the driver of the recurrent nature of the disease as fibroblasts stimulated with TGF-β1 showed a 3-fold increase in α-SMA expression[28] and an increase in collagen type[25,287]. Increased α-SMA is coupled with

increased expression of matrix proteins, contributing to increased contractility of the matrix, a key feature of Dupuytren's[55,267].

IL-1 family

IL-33 is an IL-1 homologue and an alarmin which is released after injury to alert 'alarm' the immune system of tissue damage[185]. It has been described as a key regulator of immune cells as its main receptor, ST2, is expressed by T_H2 T cells, regulatory T cells (T_{reg}), and innate lymphoid cells (ILCs). When expressed, IL-33 induces $ST2^+$ T_{reg} cell proliferation and expression of epidermal growth factor (EGF) which enhances immune regulatory functions and supports tissue repair[159].

Detected in rheumatoid arthritis (RA) synovium, IL-33 correlates with disease severity in mouse models when administration of IL-33 at disease onset worsened the condition and when IL-33 was blocked, via ST2 deletion, RA severity decreased. It has been suggested that the effect of IL-33 is mediated via pro-inflammatory cytokines, namely IFN-γ, TNF-α, IL-17, mast cell degranulation, mobilization of neutrophils into the joints, type 2 cytokines produced by activated T_H2 T cells[159].

In tendinopathy, IL-33 induced changes in collagen and cytokine production via NFκB inhibition. It was suggested that IL-33 acts via a canonical IL-1 receptor signalling pathway, in vitro, and in an in vivo patellar tendon injury model, injection of IL-33 increased collagen type III expression and reduced tendon strength demonstrating the impact of IL-33 on tendon function. In $ST2^{-/-}$ mice, administration of IL-33 showed no effect suggesting IL-33 acts in an ST2 dependent manner[159,185].

Stromal cells in Dupuytren's nodules were described as the main source of IL-33 and both IL-33 and ST2 are present in nodule tissue-the later expressed by mast cells and macrophages[117]. Using neutralizing antibodies to TNFR2 and IL-33, reduced α-SMA, Collagen I, IL-33, and ST2 expression was seen coupled with reduced contraction of the matrix, suggesting that myofibroblasts are involved as they are key for tissue contraction[117].

Suggesting an instrumental role for IL-33/ST2 in perpetuating the fibrotic state of Dupuytren's tissue and tense palmar tissue.

IL-6

IL-6 is a pro-inflammatory cytokine that is a major regulator of the acute inflammatory response and is well studied in fibrotic diseases 40. IL-6 has been shown in Dupuytren's nodules[25] and secreted in its soluble form[117,125] demonstrating the inflammatory nature of the tissue. It has been suggested that the source of IL-6 are the infiltrating immune cells which in turn amplify the immune response[73]. Also suggested as a source of IL-6 are activated fibroblasts, behaving in an autocrine fashion to promote the fibrotic phenotype[132]. In examining the inflammatory response, IL-1β and TNF-α treatment of fibroblasts increase IL-6 secretion and elevating the inflammatory response[301].

IL-13

IL-13 is a T_H2 response cytokine hypothesized to induce fibrosis in a TGF-β dependent manner as it has been shown to be dominant in the development of fibrosis[31,301]. IL-13 is produced by mast cells and T lymphocytes and aid in TGF-β production and activation[3,132]. It has been shown that stimulation of Dupuytren's fibroblasts with IL-13 drives fibrosis via increased myofibroblast differentiation and expression of matrix proteins at the mRNA level[3]. IL-13 signalling occurs via IL-13Rα1 receptor and it was found that blocking IL-13Rα1 decreased matrix protein expression and cell proliferation[3].

IFN-γ

IFN-γ has been implicated in the development of Dupuytren's disease, it has been suggested that IFN-γ targets the pro-fibrotic activity of TGF-β1 and inhibits fibrosis[31,136,301]. However, recent studies show IFN-γ is produced by T cells and its stimulation, in combination with TGF-β1, of fibroblasts increases IL-13 expression from mast cells and driving fibrosis in Dupuytren's tissue[3,218,301].

TNF-α

TNF-α is an inflammatory cytokine that has been described as an essential regulator of the myofibroblast differentiation and instrumental in the development of fibrosis and matrix protein expression[117,276,301]. TNF-α has been shown to be prevalent in Dupuytren's nodules and involved in fibroblast differentiation into myofibroblasts[25,117]. When cultured with TNF, Dupuytren's fibroblasts expressed higher collagen type I, α-SMA, and TNFR1 & 2[117,276]. Published works describe that TNF-α blocking could be a potentially successful treatment as it could reduce contractility of myofibroblasts[126,276].

1.6 Therapies targeting Dupuytren's disease

1.6.1 Current treatments

Treatment for Dupuytren's disease is aimed at palliative care rather than curative[37]. Treatment options depend on the stage of the disease presented and whether surgical or non-surgical treatment would be most successful. Non-surgical treatment options include physical therapy, splints, steroids, radiotherapy, percutaneous fasciotomy, and collagenase injections[29,96]. Non-surgical methods are used when a patient presents early stages of Dupuytren's. The aim of non-surgical treatment is to increase mobility of the fingers and to relax the hardened palmar tissue.

Injection of collagenase clostridium histolyticum, an enzyme, is a non-surgical treatment that aims to digest the collagen that makes up cord tissue[63,96,150]. Collagenase is injected into patient's who present Dupuytren's contracture which is characterized by raised nodules on the palm and affected fingers[71,96]. The following day, mobility of the hand is increased via finger manipulation causing forced extension of the finger followed by placing the hand in a splint to maintaining relaxed finger and palm[63,96]. Besides being non-surgical, this treatment method is performed on an outpatient basis and despite needing multiple treatment session, is initially successful and convenient for patients.

Surgical treatments include local, radical, or limited fasciectomy where limited affected tissue is removed, extensive affected and healthy tissue is removed, and disease tissue is removed, respectively[29]. Dermofasciectomy and skin grafting is also a treatment

option where the overlying tissue is removed, and a skin graft is used to prevent the recurrence of the disease. The goal of surgical treatment is to remove the affected tissue, as well as tissue that could become fibrotic after recovery, to allow the patient to regain use of their hand(s) and relieve palmar tension[29,96]. Surgical treatment is initially successful however, some patients return for treatment after presenting stages of Dupuytren's during their lifetime. Demonstrating the need for further understanding of the disease so treatment options can prevent resurgence of the disease.

1.6.2 Cytokine targeted therapy in Dupuytren's disease

IL-6

Triamcinolone acetonide is a corticosteroid commonly used to treat Dupuytren's disease in the pilot stages. Its administration has been shown to decrease IL-6 expression and fibroblast proliferation and, lastly, VEGF-dependent angiogenesis via STAT[125]. In Dupuytren's disease, corticosteroids improve consistency of nodules and flattens palm tissue. However, in the later stages of the disease, treatment options must be more vigorous (i.e., enzymatic digestion) to disrupt the fibrous tissue.

IL-17

IL-17, also called IL-17A, is a cytokine produced by T_H17 T cells and Secukinumab is an FDA approved anti-IL-17 agent. The effects of Secukinumab have been well demonstrated in mouse and human studies[72,128,179]. A murine lung study showed that bleomycin induced fibrosis was coupled with an IL-17 signature suggesting that a crucial role for the cytokine in fibrotic tissue development[72]. Further mouse studies in skin describe administration of Secukinumab in a bleomycin induced fibrosis model led to amelioration of fibrosis, reduced dermal thickness, and reduced collagen 3 expression[128]. A human psoriatic arthritis study revealed that inhibition of IL-17A, via Secukinumab administration, successfully improved associated symptoms and subjects' quality of life compared to those who received placebo[179]. These and other published works illustrate the targeting of IL-17 as successful in improving the diseased state of inflamed tissue. They go on to label IL-17 related research as a promising avenue of exploration towards developing therapeutics and suggest further human testing is crucial to improve the lives of those affected by fibrotic disease.

TNF-α

Anti-TNF blockade agents currently in circulation for treatment of musculoskeletal diseases are adalimumab, certolizumab, infliximab, etanercept, and golimumab[161]. Adalimumab, Certolizumab, Etanercept, and Golimumab have been shown to decrease contraction of Dupuytren's fibroblasts in *in vitro* studies conducted by Verjee *et al.* They suggest administration of these anti-TNF therapies act upon fibroblasts via Wnt signalling pathways[278]. These blockade therapies are focused on treating early Dupuytren's via nodule injections towards digesting the hardened palmar tissue. Adalimumab injection has been shown to reducing nodule size and hardness in Dupuytren's disease[195,210]. Certolizumab, Etanercept, and Golimumab have been successful in improving patient outcome (i.e., joint mobility, pain management) in patients with RA[90,100,215].

Secondary to these anti-TNF therapies, biosimilar monoclonal antibodies have been developed, like generic drugs, to provide alternative treatment options to patients. A biosimilar is a medicine parallel in structure, pharmacokinetics and function to a Food and Drug Administration (FDA) approved medication but is more accessible and cost effective[166,220]. Infliximab has been developed towards treating rheumatoid arthritis and biosimilars to infliximab, called Remicade and Remsima, are currently being sold in Europe as an effective alternative[39,190]. Future work will assess Infliximab, and its biosimilars, towards altering fibroblast behaviour in Dupuytren's disease.

TGF-β1

TGF-β1, an isoform of TGFβ, is well described in fibroblast biology as an inducer of activation and play a leading role in the pathophysiology of Dupuytren's disease. The use of pirfenidone (PFD) has been shown to inhibit production and activity of TGF-β1 in various pre-clinical and in vitro models in organ fibrosis models[319]. The effect of PFD on canonical (Smad) signalling pathway was examined in Dupuytren's disease, as it has been shown to act via this pathway in other fibrotic models, however this was not seen in Dupuytren's[319].

JAK

Tofacitinib is an inhibitor of janus kinase (JAK) which are vital for STAT1 and STAT6 signalling[3]. Tofacitinib inhibited STAT1 signalling in Dupuytren's disease and that IL-13 production by mast cells was also significantly decreased[3]. Demonstrating the importance of the JAK/STAT pathway and the successful inhibition of STAT signalling.

1.6.3 Signalling pathways in Dupuytren's

Wnt Signalling

Wingless/integrated (Wnt) signalling was first described within the confines of drosophila genetic studies and the name Wnt describes the fusion of the name of the drosophila gene *wingless* and the vertebrate homolog, *integrated* or *int-1*[138]. The Wnt gene family consists of structural genes that encode for glycoproteins and the family is comprised of two signalling pathways: canonical, or Wnt/β-catenin dependent, and non-canonical, or β-catenin dependent[75,138].

TNF canonical signalling was described in Dupuytren's disease by Verjee *et al.* 2013 where they demonstrate that myofibroblast phenotype differentiation is partly mediated by Wnt/β-catenin pathway and that Dupuytren's myofibroblasts and fibroblasts express Wnt/β-catenin related genes[276]. The authors go on to describe that the neutralization of TNF decreases expression of TNFR2 and preventing the development of fibrosis via reduced α-SMA and Collagen type 1 expression[276].

TGF-β1, a pro-fibrotic growth factor, has also been described as acting via both canonical and noncanonical pathways in Dupuytren's disease. Canonically, TGF-β1 binds to its receptors, activating SMAD2/3 dependent signalling leading to the translocation of SMAD 2/3 and 4 to the nucleus and promoting expression of TGF-β1 related genes[80,270,319]. Noncanonically, TGF-β1 induces various branches of MAP kinase (MAPK) including Rho-like GTPase and phosphatidylinositol-3-kinase (PI3K)/AKT pathways independent of SMAD signalling[319]. The role of p38 and Akt phosphorylation has been described as a driver ECM production and proliferation and differentiation of myofibroblasts[229]. The authors go on to describe the effect of Pirfenidone administration as inhibitory of TGF-β1 induced activation

and differentiation of fibroblasts, ECM production, canonical signalling-via attenuation of phosphorylation of SMAD 2/3-and noncanonical signalling via decreased phosphorylation of PI3K/Akt signalling proteins[319].

JAK/STAT pathway

JAK-STAT pathway has been described as a key cytokine signalling pathway[251]. Cytokines binds to their cell-surface receptor resulting in receptor dimerization and activation of JAK tyrosine kinases. These tyrosine residues function as docking site for STATs leading to STAT phosphorylation and translocation to the nucleus where they activate gene transcription[251]. Of the seven mammalian STATs (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6), STAT1 has been described in Dupuytren's disease[3]. Akbar *et al.* (2020) describe T cell produced IFN-γ as an inducer of IL-13 secretion by mast cells, a well described pro-fibrotic cytokine, which leads to upregulation of IL-13Rα1 on fibroblasts-increasing their reactivity to IL-13[3]. The authors use tofacitinib to target STAT1 signalling as it has been shown to be a pan JAK inhibitor and reduces signalling of IFN-γ and Il-13 in vitro. Akbar *et al.* (2020) describes the administration of tofacitinib inhibits an increase expression of IL-13Rα1 from control fibroblasts and IL-13 secretion from mast cells[3]. They also describe that tofacitinib treatment inhibited IL-13-induced STAT6 phosphorylation in Dupuytren's myofibroblasts manifesting downstream as decreased matricellular protein expression and cell proliferation[3].

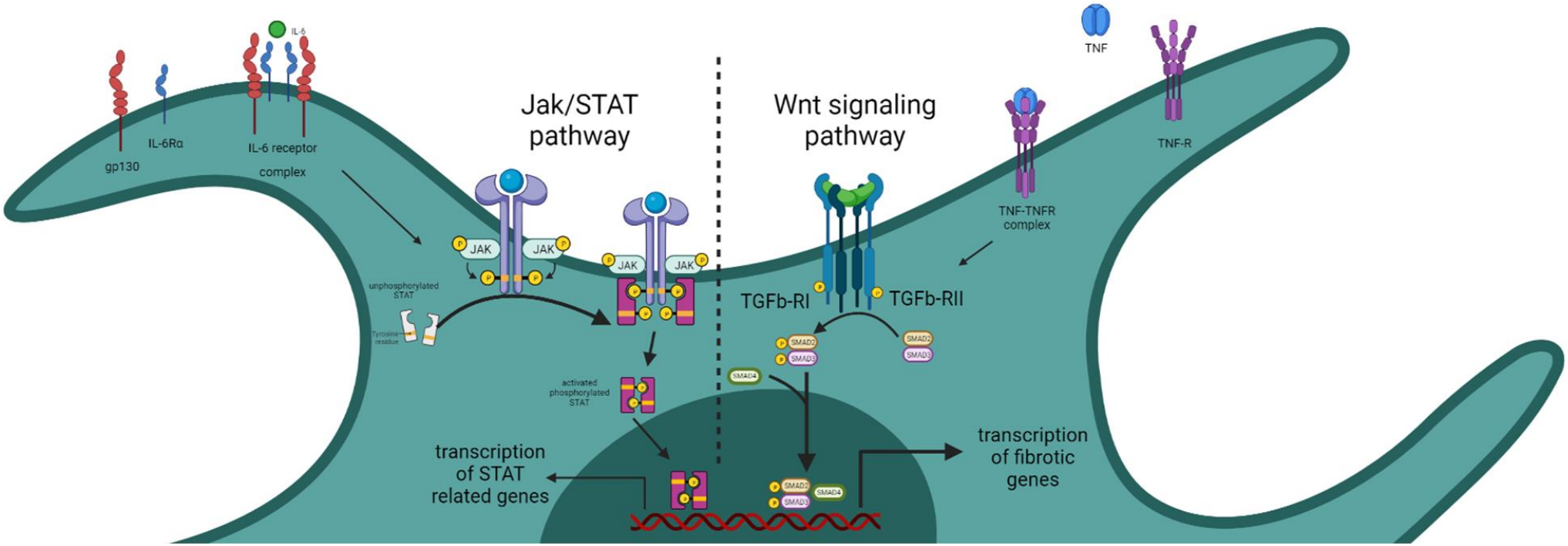


Figure 1.6 Signaling cascades in Dupuytren's fibroblasts which could contribute to the etiology of the disease.
Shown here is a fibroblast with its membrane complexes which lead to gene transcription in the nucleus. Depicted are two heavily published mechanisms that are suggested as contributing to FMT and chronic inflammation. Jak/STAT and Wnt signaling have been shown to be involved in Dupuytren's disease via Jak and TGFβ receptors, respectively. IL-6 has been shown to be involved in Jak/STAT activation and the figure depicts that IL-6 binding to its receptors (IL-6Rα and gp130) leads to Jak/STAT. Similarly, TNF binding to its receptor, TNFR, leads to Wnt signalling activation. Created with BioRender.com.

1.7 Aims

Recent works on Dupuytren's disease describe a role for immune cells in the disease etiology via their interaction with fibroblasts. This immune cell-fibroblast crosstalk could highlight crucial interactions that promote inflammation and excess ECM proteins. Given it is not well understood exactly how the disease begins or how it recurs, acknowledging the role the immune system could play would allow for potential answers to the questions remaining.

This body of work focuses on stromal activation as an element of the disease as published studies describe that stromal cell characterization is associated with tissue disease. We suggest that stromal cell behavior, in combination with immune cell interactions, could emphasize interactions worth pursuing towards creating a fuller understanding of the disease and the factors which impact the tissue stroma.

The aims of this work focus on:

1. Defining stromal cell behaviour within Dupuytren's tissue
2. Characterization of Dupuytren's microenvironments via bulk RNA sequencing
3. Describing the immune cell landscape of Dupuytren's tissue
4. Interrogating stromal-immune cell crosstalk as a contributing factor of Dupuytren's etiology

Chapter 2: Materials and methods

2.1 Buffers and medias

Complete Roswell Park memorial institute (cRPMI)-500mL RPMI 1640 supplemented media supplemented with 10% heat inactivated foetal bovine serum (FBS), 1% L-glutamine (200mM, Thermo fisher scientific), 1% penicillin-streptomycin (10,000 U/mL, Thermo fisher scientific)

MACS buffer 1x PBS supplemented with 2% (w/v) BSA and 2nM EDTA

FACS buffer 1x PBS supplemented with 1% FBS

ELISA assay buffer 1x PBS supplemented with 0.5% (w/v) BSA

ELISA wash buffer 500mL of 10x PBS diluted in 4.5L distilled and 2.5mL of Tween 20

ELISA stop solution 2N sulphuric acid

TBS Tween (TBST) 100mL of 10X TBS buffer (pH=7.6) was diluted in 900mL of water and 0.05mL of Tween 20

2.2 Ethical approval of human tissue collection

Procedures and protocols were approved by the Ethics committee under Central Network, Southeast Health (HREC/96/55, HREC/14/130) and West of Scotland REC (REC14/WS/1035). Tissue was obtained from patients undergoing Dupuytren's fasciectomy or carpal tunnel decompression. Tissue used for experiments was residual tissue not used at the time of surgery under the NHS Greater Glasgow Residual Tissue consent procedure.

2.3 Patient demographic

Tissue samples were obtained from patients undergoing Dupuytren's fasciectomy or Carpal Tunnel decompression. Samples were obtained from individuals who identified as male or female. The median age range for males undergoing Dupuytren's fasciectomy or Carpal tunnel decompression were 55 and 50, respectively. The median age for females undergoing

Dupuytren's fasciectomy or Carpal tunnel decompression were 51 for both. Details about patient's lifestyle and morbidities that could contribute to their presentation of Dupuytren's disease and Carpal tunnel disease (i.e., diabetes, smoking, family history) were not able to be obtained and shared with me as this information could violate the Data Protection Act (DPA) 2018.

2.4 Cell culture

All cell culture experiments were performed in a laminar flow hood. Cell cultures were maintained in T25 or T75 cell culture flask in a humidified incubator at 37℃, 5% CO_2, 95% O_2. The incubator was maintained with Chemgene lab disinfectant diluted in autoclaved water.

2.4.1 Culture of primary human fibroblasts

Dupuytren's and carpal tunnel fibroblasts were explanted from tissue of patients undergoing fasciectomy or compression, respectively. Tissue was dices into small pieces (~2mm) using a sterile blade and placed into a T25 tissue culture flask containing 7mL of complete RPMI. Flasks were incubated for one week, if Dupuytren's tissue, and up to 4 weeks, if carpal tunnel tissue. The cells were allowed to adhere and proliferate. No more than 4 weeks after the specimen's arrival date, or when flasks had reached 90% confluency, and were washed with 1 mL of PBS and 1-2mL of trypsin EDTA was added to the flask. The flask was allowed to rest in the incubator (~5 minutes) until the cells unadhered from the bottom of the flask. Once all cells had detached, fresh, warmed complete media was added in a 1:4 ratio to deactivate trypsin. The cells were spun down in a conical 50mL falcon tube and the pellet was resuspended in 10mL of fresh media and the volume was distributed between sterile T25 flasks and an aliquot was frozen in bambanker (GC lymphotec Inc), in a cryovial, and stored at -80℃. Cells were not used past passage 4 so as to limit population doubling level (PDL) and senescence as cell behaviour and phenotype changes as cells continue to replicate in vitro.

2.4.2 Culture of primary human T cells

2.4.2.1. Isolation of peripheral blood mononuclear cells (PBMCs)

Blood combs were obtained via NHS England. PBMCs were separated from whole blood samples by diluting blood 1:3 in sterile PBS in a 50mL Falcon centrifuge tube. The 40mL of diluted blood was aliquoted into 15mL Falcon centrifuge tubes and 3mL of Ficoll histopaque was added gently to create a top layer. The 15mL Falcon tubes were centrifuged at 400g for 20 minutes without the brake. The layer of PBMCs (white layer) was collected slowly using a Pasteur pipette and transferred to a sterile 50mL Falcon centrifuge tube. The PBMCs were washed twice with sterile PBS and centrifuged at 400g for 5 minutes, with the brake, to create a cell pellet.

2.4.2.2. Isolation of $CD3^+$ T cells from peripheral blood mononuclear cells

Cells were separated using the manual Miltenyi MS column and magnet according to manufacturer's instructions. To prepare cells for separation, the cells were washed in cold PBS and centrifuged at 400g for 5 min and the pellet was resuspended in MACS buffer. The live cells were counted using a haemocytometer and the desired number of cells were obtained, centrifuged, and resuspended in 20µL of $CD3^+$ beads (Miltenyi Biotec), per 10^7 cells, and 80µL of cold MACS buffer, per 10^7 cells. The cells were then placed on ice to allow the beads to bind to the cells. Cells were magnetically separated using medium sized separation columns and the positive and negative fraction was collected for experiments. Purity check of the positive fraction was performed via comparison to the negative fraction and unbound PBMCs.

2.4.3 Stimulation of fibroblasts

Fibroblasts from 2^{nd} passage onward were plated in a sterile 24 well tissue cultures at 2.5 x 10^4 well and allowed 48 hours to adhere in 500µL of RPMI. After the 48-hour rest period, stimulated media in a volume of 600µL of complete RPMI containing the given concentration of each agent (Table 2.1) for, at most, 24 hours at 37℃. Following stimulation, supernatants were transferred into sterile Eppendorf's and cells were lysed using lysis buffer (1:10 dilution of 2-mercaptoethanol in RNase free water); supernatants and lysed cell material were stored at -80℃ for long term storage.

Table 2.1 Agent concentrations used to stimulate Dupuytren's fibroblast stimulation. The concentrations used mimicked those in published works by Akbar *et al.* 2020[3] and Verjee *et al.* 2013[276]. Those with a single asterisk (*) were determined a dose dependent experiment shown later in this body of work. Those with double asterisks (**) were determined by manufacturer dose recommendations.

Agent	Concentration	Manufacturer
IFN-g	10 ng/mL	Biolegend
IL-13	1 ng/mL	Biolegend
TNF-a	0.1 ng/mL	Biolegend
TGF-b	2 ng/mL	Biolegend
CD27*	5 ng/mL	R&D systems
CD70**	5 ng/mL	R&D systems
CD3**	2 ug/mL	Biolegend
CD28**	2 ug/mL	Biolegend

2.4.4 Stimulation of T cells

Isolated $CD3^+$ T cells from PBMCs were seeded in 12 well tissue culture plate at 2.5×10^5 per well. Respective wells were coated with CD3 diluted in sterile PBS and the plate was left overnight at 4℃. The CD3 Ab dilution was removed, the wells were washed with PBS and the T cell suspension was added with 600µL of stimulated media.

Table 2.2 Agent concentrations used in T cell stimulation. The concentrations used were based on manufacturer instructions.

Agent	Concentration	Manufacturer
CD70	5 ng/mL	R & D systems
CD3	2 ug/mL	Biolegend
CD28	2 ug/mL	Biolegend

2.4.5 Scratch assay

Carpal tunnel and Dupuytren's fibroblasts were seeded at 2.5 x10^4 in a 24-well tissue culture plate and allowed to adhere for 48 hours, undisturbed. Medium was removed from cells and scratch in a 'Z formation' was performed using sterile p1000 pipet tip. The wells were washed once with PBS to remove debris and unadhered cells and 600µL of fresh media was added. Cells were incubated and harvested 4 hours or 24-hours after the initial scratch.

2.5 Fluorescence activated cell sorting (FACS)

2.5.1 enzymatic digestion of tissue

Under sterile conditions, carpal tunnel or Dupuytren's disease tissue was cut into small pieces using a sterile petri dish and sterile blade. The pieces were placed in a 50 mL Falcon centrifuge tube containing 10mL of serum free RPMI and 0.125 mg/mL of Liberase (Sigma Aldrich). Enzymatic digestion was performed by incubating tubes at 37℃ for 30 minutes for carpal tunnel tissue and up to 2 hours for Dupuytren's tissue on a MACSMix tube rotator. After digestion, complete RPMI was added to prevent further digestion and the solution was passed through 70µm cell strainer to separate cell suspension from tissue pieces. The flow through, containing media and disaggregated cells, was centrifuged at 400g for 5 minutes. Cells were washed with PBS and spun for 400g for 5 minutes and the cell staining procedure was performed.

2.5.2 Staining of cell surface proteins

Cells obtained from enzymatic digestion were added to round bottom polystyrene FACS tubes and another PBS wash was performed with a spin at 400g for 5 minutes. Excess PBS was poured off and tubes were gently vortexed. 1µL of fixable viability dye was diluted in 1000µL of PBS and 10µL of the dilution was added to each tube and stained on ice, in the dark for 15 minutes. Following the viability stain, 2mL of FACS buffer was added to each tube and cells were centrifuged for 5 minutes at 400g. Excess buffer was gently poured off and the appropriate quantity and concentration of each antibody was added to each tube. Cells were stained on ice for 30 minutes, in the dark. If flow cytometry was not immediately performed, 250µL of cell fix was added to each tube containing cells, covered in aluminium foil, and stored at 4℃ in the dark. FACS analysis was performed using BD LSRII flow cytometer and the data was analysed via FlowJo software.

Table 2.3 Antibodies used for flow cytometry. The following antibodies were used in flow cytometry experiments and their isotypes were chosen based off of manufacturer recommendations.

Target protein	Conjugate	Clone	Isotype	Supplier
CD3	V450, pacific blue	UCHT1	Mouse IgG1, κ	Biolegend
CD4	FITC PE/Cy5	RPA-T4	Mouse IgG1, κ	BD pharmigen Biolegend
CD8	PE BV605	HIT8a SKI	Mouse IgG1, κ	BD pharmigen Biolegend
CD11c	BV510	3.9	Mouse IgG1, κ	Biolegend
CD14	V500	M5E2	Mouse IgG2a, k	BD Horizon
CD17A	PE	BL168	Mouse IgG1, k	Biolegend
CD19	APC FITC	HIB19 4G7	Mouse IgG1, κ	Biolegend
CD25	AF700	M-A251	Mouse IgG1, κ	BD pharmigen
CD27	PerCP	O323	Mouse IgG1, κ	Biolegend
CD45	AF700 PerCP	2D1	Mouse IgG1, κ	Biolegend
CD56	FITC	HCD56	Mouse IgG1, κ	Biolegend
CD64	BV605	10.1	Mouse IgG1, κ	Biolegend
CD69	APC	FN50	Mouse IgG1, κ	Biolegend
CD117	PE	104D2	Mouse IgG1, κ	Biolegend
γλ T cell	PeCy7	B1	Mouse IgG1, κ	Biolegend
HLA-DR	PeCy7	1243	Mouse IgG1, κ	Biolegend
IFN-γ	FITC	MAb11	Mouse IgG1, κ	Biolegend
TNF-α	AF700	MAb11	Mouse IgG1, κ	Biolegend

Table 2.4 Antibodies used for T cell purity assessment. T cell populations were verified against monocyte and B cell markers to ensure the cells were only $CD3^+$.

Target protein	Conjugate	Clone	Excluding cell type	Supplier
CD19	APC	HIB19	B cells	Biolegend
CD14	V500	M5E2	Monocytes	BD Horizons
CD3	Pacific blue	UCHT1	T cells	Biolegend

2.5.3 CD3 purity check

Flow cytometry was performed to validate the purity of MACS positive fraction for $CD3^+$ T cells from PBMCs. Isolation controls include pure PBMCs and negative fraction from the $CD3^+$ isolation.

All cells and control cells were washed with PBS and stained using a viability dye as previously stated. Cells were then washed with FACS buffer and spun at 400g for 5 minutes before being stained for the full antibody panel for 30 minutes on ice. After the stain incubation, cells were washed with FACS buffer and spun at 400g for 5 minutes. Cell fix was added to tubes if flow cytometry was not being performed on the day of preparation, as previously described.

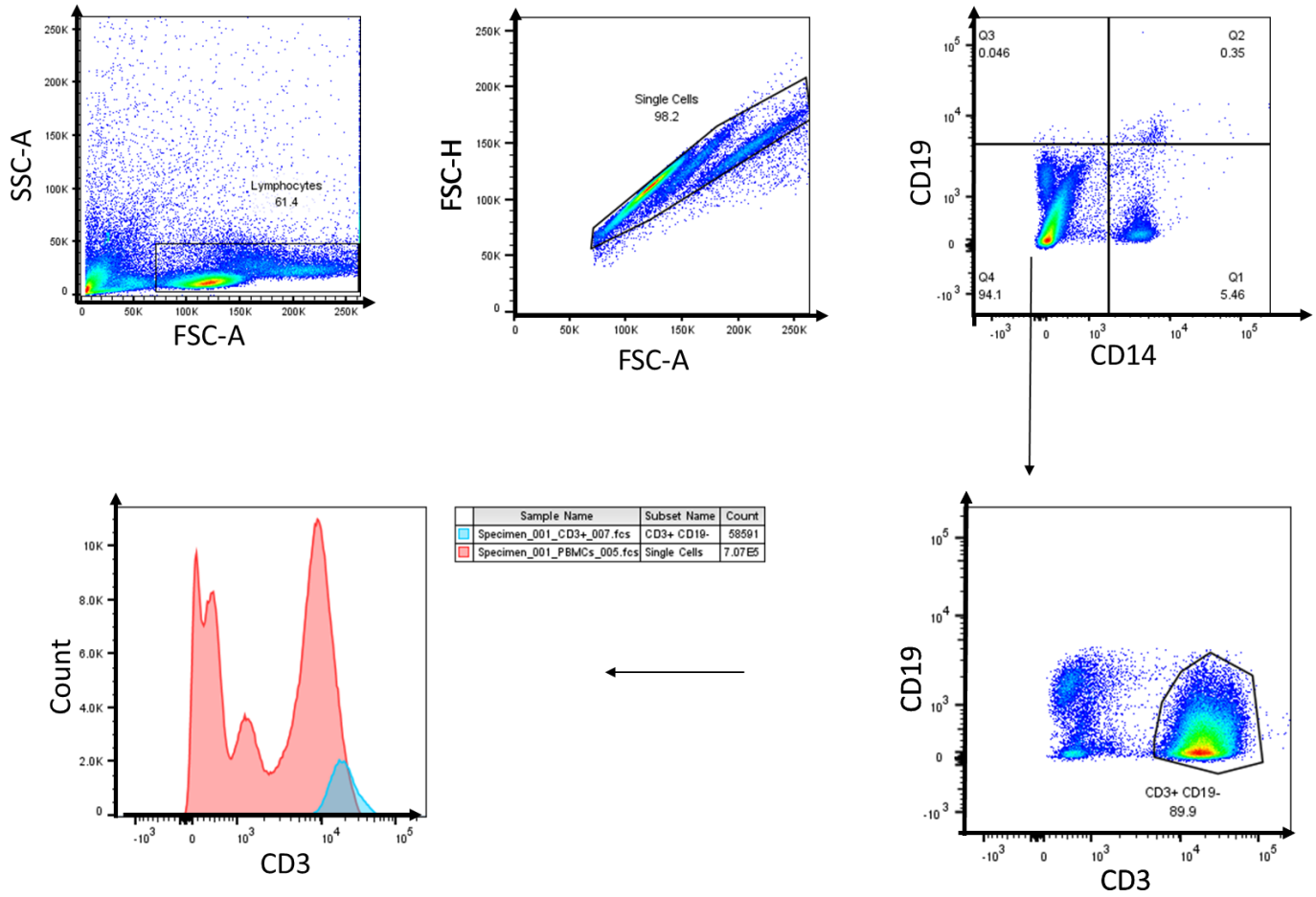


Figure 2.1 CD3 purity check against PBMCs. PBMCs were isolated from whole blood and CD3 beads were used to extract $CD3^+$ T cells. The positive fraction was split into unstained and stained aliquots. Each stained tube contained the full antibody panel, minus one to perform flow minus one (FMO) to determine cell populations. The unstained and stained controls were compared against a PBMC control.

2.6 Digestion of Dupuytren's and Carpal tunnel biopsies for flow cytometry

Dupuytren's tissue biopsies were cut into small pieces (~2mm) and digested in serum-free RPMI and Liberase for up to two hours. Cell suspension was filtered through a 70µm strainer and washed with complete RPMI and centrifuged at 400g for 5 minutes; this wash cycle was completed twice. After a 24-hour rest period in a 12-well plate, fibroblasts were stimulated with PMA-ionomycin and brefeldin and allowed to stimulate for 4-hours. Cells were collected in FACS tubes, and the wells were washed with cold PBS and added to the FACS tube. Tubes were spun and washed with PBS and stained.

2.7 Quantitative polymerase chain reaction (qPCR)

2.7.1 Total RNA extraction from cells

Total RNA isolation and purification was performed using PureLink RNA kit (Thermo Fisher Scientific). The RNA extraction procedure was performed using RNase free water and filter tips and each centrifugation step was performed at room temperature at 12,000g.

Adherent fibroblasts were lysed using 300µL of lysis buffer (dilution of 1% 2-mercaptoethanol diluted in lysis buffer) immediately after supernatant was harvested and incubated for no more than 5 minutes at room temperature. Lysed material was added to fresh Eppendorf's and stored at -20℃.

One volume of 100% ethanol was added to the lysed material and gently pipetted to disperse the precipitate. Samples were transferred to the PureLink spin column and centrifuged according to the manufacturer's protocol. Flow through was discarded and 700µL of wash buffer 1 was added and the tubes were spun. Flow through was discarded and 500µL of wash buffer 2 was added to the column and spun, this step was performed twice. The spin column was placed into a new collection tube and centrifuged for 2 minutes to dry the membrane of the spin column. 25-30µL of RNase free water was added to the membrane and the column was incubated for 1 minute before being spun and the bound RNA was eluted from the membrane into a fresh Eppendorf tube. RNA samples were stored on ice or frozen at -20℃ or -80℃ for long term storage.

2.7.2 Total RNA extraction from human tissue

Carpal tunnel and Dupuytren's samples were submerged in RNAlater (Thermo Fisher Scientific) and stored at 4℃ or -20℃ for long term storage.

Samples were thawed at room temperature and cut into small pieces (~2mm) using a sterile blade in a petri dish. In 2mL round bottom Eppendorf's, ball bearings were added along with tissue pieces and enough lysis buffer to cover the tissue sample. The TissueLyser LT was used to disaggregate tissue via high-speed shaking. 10µL of Proteinase K was diluted in 590µL of RNase free water and 600µL of diluted proteinase K was added to the disaggregated solution. The tubes were incubated at 55℃ for 10 minutes and spun at 12,000g for 3 min and the supernatant was collected in a fresh Eppendorf. Samples underwent the RNA extraction protocol according to the manufacturer's instruction.

2.7.3 Quantifying concentration of nucleic acids

The concentration and purity of RNA extracted from cells and tissue were evaluated via a Nanodrop spectrophotometer. RNase free water was used as a blank and 1µL of sample was added to measure the absorbance at 260nm and 280nm. An A260/A280 ratio was quantified to determine the purity of the sample and A260/A230 assessed protein quality.

2.7.4 cDNA synthesis

cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit. RNA samples were diluted to 10ng/µL using RNase free water and 10µL of pure RNA in a 200µL PCR tube with High-capacity cDNA master mix. Tubes were vortexed and centrifuged before being placed in the thermocycler.

Table 2.5 PCR mastermix recipe. The recipe was designed by the manufacturer, Thermo Fisher Scientific.

Component	Volume x reaction (µL)
10X RT buffer	2
10X RT random primers	2
dNTP (100nM)	0.8
Multiscribe reverse transcriptase	1
RNase free water	4.2
Total	10 µL

2.7.5 SYBR Green PCR mRNA quantification

qPCR experiments were performed using PowerUp SYBR green Master mix (Thermo Fisher) according to the manufacturer's instructions. Upon completion of reverse transcription of the cDNA template, samples were diluted 1 in 5 for a final volume of 100µL. 1µL of cDNA template was added to 9µL of PowerUp SYBR master mix in duplicate wells, in a 96-well qPCR plate (Table 2.7). Plates were sealed with optical adhesive film and centrifuged at 400g for 2 minutes. The StepOnePlus real time qPCR system (Applied Biosystems) was used to measure mRNA expression and a control of nuclease free water was used for each primer. The cycling parameters and melt curve analysis described the presence of a single amplified product.

Table 2.6 qPCR master mix recipe. This was taken from the manufacturer's protocol.

Reagent	Volume x reaction (µL)
PowerUP SYBR green	5
Forward primer	0.1
Reverse primer	0.1
Nuclease free water	3.8
cDNA template	1
Total	10 µL

Table 2.7 qPCR primers designed using NCBI Blast primer design tool.

Target	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
ACTA2	CCTCCCTTGAGAAGAGTTACG	GACTCCATCCCGATGAAGGAT
CCL20	GTCTTGGATACACAGACCGTATT	GTGTGAAAGATGATAGCATTGATGT
CD27	TCACTCTGCTCACTCTGTCT	GATCACTTGAGTCCAGGAAGTG
CD34	AGCAGGCTGATGCTGATG	TGCTGGAAATTTCTGTTCTGTTG
CD70	CAACCTCACTGGGACACTTT	TAATCAGCAGCAGTGGTCAG
CD90	CTAACAGTCTTGCAGGTCTCC	ACTGCTGGTATTCTCATGGC
CD146	CGGCACGGCAAGTGAAC	GCATTCAACACCTGTCTCCAAC
CD248	CCCAAATCCCAAGGGAAGAT	CTGTGCTCGGCAAGACC
Collagen type I	CAATGCTGCCCTTTCTGCTCCTTT	CACTTGGGTGTTTGAGCATTGCCT
Collagen type III	GTCAAGTCTGGAGTAGCAGTAG	GAACCAGGATGACCAGATGT
CXCL1	ACTGAACTGCGCTGCCA	TTCCGCCCATTCTTGAGTGT
FAP	ACGGATATACCAGTTATTGCCTATT	GGATTCGCTCCAGCCTTT
Fibronectin	CAAGGCTGGATGATGGTAGATT	TCCTGATCGTTGCATCTATTTCT
GAPDH	GAAGGACTCATGACCACAGT	GTAGAGGCAGGGATGATGTT
ICAM-1	AGCTTCTCCTGFTCTGCAA	GGGCCATACAGGACACGAA
IL-6	CACTCACCTCTTCAGAACGAA	GCTGCTTTCACACATGTTACTC
IL-8	GTGCATAAAGACATACTCCAAACC	GCTTTACAATAATTTCTGTGTTGGC
IL-17A	AGGCCATAGTGAAGGCAGGAA	ATTCCAAGGTGAGGTGGATCG
IL-17RA	GATTCACCCTCGAAACCTGA	ATGCTGGCGTCTGTCTG
IL-17B	TGTCACGGATGAAACCGTATG	TTCTCTGGGCCAGCTCT
IL-17RB	GTCGCTCGTGCTGCTAAG	CAGTTTCAGAGCCACATTGAAC
IL-17C	CCACCATGACGCTCCTC	TCAGCCGAGTAGCAGTGT
IL-17RC	CGTGCATCTGGTTCTGAATG	GCGGTCCAGTCAGGTTT
IL-17D	TGGGCCTACAGAATCTCCTA	GACGGTGGGCATGTAGAC
IL-17RD	GGGCTGTACAACATCACCTT	GGCAAGCATACTGGCTGA
IL-17E	CCCTGGAGATATGAGGCTT	TGCACTGACCTGGTACAT
IL-17RE	CACCTGCTCAGAGGAAGC	TTTAGAGCGAAGTCCCTTGTG
IL-17F	CCCAGCCATGGTCAAGTA	TCAAGCTTCATACTACCTCCTG
Podoplanin	CTTGACAACTCTGGTGGCA	GCGCTTGGACTTTGTTCTTG
Periostin	TTGAGACGCTGGAAGGAAAT	AGATCCGTGAAGGTGGTTTG
ROR-γ	TTTGAGGAACACAGGCATCC	ACCAGGTGCTCTATCTCTGT
Tenascin C	GTGCCAGGAGACCGTACCAC	CTTTGGCTGGGTTGCTTGAC
VCAM-1	GCAAGTCTACATATCACCCAAGA	TAGACCCTCGCTGGAACA

2.7.6 Analysis of qPCR data

A housekeeping gene, or endogenous control, was compared to each sample alongside the target gene. GAPDH was used for tissue and 18S was used for cells as they are expressed in all cell types and their expression in consistent during the duration of the experiment.

mRNA relative expression ($2^{-\Delta Ct}$)

The expression of the target gene is represented as mRNA relative expression, $2^{-\Delta Ct}$. This was calculated by subtracting the target gene Ct from the housekeeping Ct to give ΔCt. The final value was expressed as $2^{-\Delta Ct}$ and described as mRNA expression relative to the housekeeping gene.

Fold change expression ($2^{-\Delta\Delta Ct}$)

Fold change expression represents gene expression relative to the experimental control. ΔCt was calculated by subtracting the target gene Ct from the housekeeping. The experimental ΔCt is subtracted from the experimental control ΔCt to give ΔΔCt. The ΔΔCt is expressed as $2^{-\Delta\Delta Ct}$ indicating fold change relative to the experimental control.

2.8 Enzyme linked immunosorbent assay (ELISA)

ELISA was performed to determine the concentration of cytokines present in cell culture supernatants. All ELISAs were performed according to manufacturer instructions and using 96-well half well plates and so all volumes were halved. Supernatant was diluted up to 1:50 in assay buffer to ensure concentrations were within detection range of the ELISA kit. ELISA readings were normalised to standards made according to manufacturer's instructions using assay buffer and experimental blank, which was assay buffer alone.

Plates were coated using coating antibody diluted in PBS and incubated at either room temperature or at 4℃ overnight. The following day, plates were washed with ELISA wash buffer, tapped dry, and blocked with 150µL of assay buffer for at least an hour.

According to Thermo Fisher ELISA kit protocols, 50µL of standards and samples were added to the plate and the plate shook continuously for 2 hours at room temperature. Plates were washed five times with wash buffer, tapped dry, and 50µL of streptavidin-HRP was added; plates were incubated with continual shaking for 30 minutes.

Using R&D kits, following manufacturer instructions, 50µL of sample and standards were added and plates were left at room temperature for 2 hours without shaking. Following three washes of wash buffer, detection antibody diluted in assay buffer was added and the plate was incubated again at 2 hours. Three washes were performed followed by 30 minutes of incubation with streptavidin-HRP diluted in assay buffer.

After final washes were completed and plates were tapped dry, 50µL of TMB chromagen substrate was added at room temperature until desired color change was seen. The reaction was stopped by adding 50µL of 2N sulphuric acid and the plate was read at 450 nm on a Dynex technology MRX plate reader.

Table 2.8 ELISA kit detection ranges described by the manufacturer's protocol.

Target protein	Detection range (pg/mL)	Manufacturer
IL-6	31.25-2000	ThermoFisher
Collagen type 1	31.25-2000	R&D systems
CD27	125-8000	R&D systems
IFN-g	31.25-2000	ThermoFisher
IL-8	12.5-800	ThermoFisher

2.9 Immunohistochemistry of paraffin embedded sections

To prepare samples for paraffin embedding, they were completely submerged in formalin for at least 24-hours at room temperature and then transferred to ethanol to begin dehydration. Tissue, embedded in paraffin wax, was sectioned into 5µm sections and 2 sections were adhered to microscope slides and left undisturbed for at least one hour to allow the sections to adhere to the microscope slide.

Slides ready for staining were heated at 65℃ for at least 30 minutes to allow the paraffin wax to melt and the slides underwent further dewaxing via xylene and dehydrated via ethanol. Slides were washed in TBST, and endogenous peroxidase was used to block nonspecific staining for 10 minutes at room temperature via Bloxall (Vector laboratories). Antigen retrieval was performed by diluting antigen retrieval concentrate (TCS Bioscience) in water and samples were microwaved for up to 20 minutes at 30% power. Following a cooling stage, slides were washed in TBST, and water and nonspecific blocking was performed using 5% horse serum for 30 minutes. The blocking buffer was tapped off and the antibody and isotype were diluted in 5% horse serum, 95% TBST and incubated on tissue sections overnight at 4℃.

The following day, slides were warmed to room temperature, washed in TBST, and incubated with secondary antibody, diluted in TBST, for 30 minutes. Slides were washed in TBST, and the stain was developed using DAB (Vector laboratories) at room temperature until signal was visible. Sections were washed in TBST and running water before being dipped in haematoxylin and returned to running water. Slides were incubated in Scott's tap water solution (STWS) for 30 seconds and left in running water to wash off unbound STWS before beginning rehydration. Slides were mounted using DPX and coverslip.

2.9.1 Bonar scoring

Bonar scoring is a scoring system used to quantify and evaluate staining in Dupuytren's and carpal tunnel tissue. Five random high-power images (400X) were taken, and each field was given a score of 0, 1, 2, or 3 based on visual assessment by two independent assessors. A

score of 0 correlates to no positive staining, 1 indicates less than 10% staining, 2 describes 10-20% positive staining, and 3 indicates more than 20% positive staining.

2.9.2 Semi-quantitative analysis

Of the five high power field images taken that were used for Bonar scoring, 3 were chosen to analyse semi-quantitatively. Each positive and negatively stained cell was counted by eye and a percent positive value was calculated. This was done by dividing the number of positive cells by the total number of cells in the high-power field. This quotient was multiplied by 100 to yield a percent positive value for the image. An average of the three images was calculated for the donor of the tissue section in question. This was performed by two independent assessors.

2.10 Bulk RNA sequencing

High quality total RNA was used to build Illumina mRNA sequencing libraries using NEBNext Ultra II directional RNA library prep kit for Illumina. Manufacturer's protocol was followed, and sample purity and quantification were performed via Qubit. cDNA libraries were made via Chromium Controller and Chromium Single-Cell 3/ v2 reagent kit (10X genomics). Libraries were pooled and they were sequenced on a HiSeq 4000 (Illumina) to a depth of 30,000 reads per cell and the alignment of reads to the genome and generation of gene counts per cell was determined via Cell Ranger Software (10X genomics). Dr. Moeed Akbar assessed the quality of samples and poor-quality cells were removed based on the number of genes expressed (>200), of unique molecular identifiers, and percentage of mitochondrial reads mapped (>5%) prior to performing analysis.

2.11 Single cell sequencing analysis

Using a publicly available data set, GSE173252, single cell RNA from Dupuytren's and Carpal tunnel tissues were evaluated. Seurat Package from the Satija Lab, for R software, was used for analysis. Immune cells were computationally isolated for analysis. Principal component analysis and high-quality cells were clustered based on a graph-based routine implemented within Seurat R package. CellChat and CellphoneDB packages for R were used to identify specific immune-stromal cell interactions, using clustered immune cells, and previously published clustered stromal cells[74].

2.12 Statistics

The results presented in this study are represented as mean ± SEM. Comparisons between groups were evaluated using the Kruskal-Wallis one way analysis of variance and Dunn's multiple comparison was used as a post hoc test. Further, student's t-test was used and samples with a p value less than 0.05 were considered significant. Throughout this thesis, statistical tests are described within the figure legends.

Chapter 3: Characterizing the stromal activation phenotype in Dupuytren's disease

3.1 Introduction

Dupuytren's disease is a fibroproliferative condition in which the palmar tissue becomes chronically inflamed resulting in a permanent contracture of the affected fingers, eventually developing into cellular nodules and a fibrous cord. The etiology of the disease has been described as unresolved healing of microtrauma the palmar tissue leading to persistent stromal cell activation and excess matrix protein production[96,218].

The stromal compartment consists of stromal cells which, upon activation, produce cytokines and chemokines which elicit an inflammatory response and recruit immune cells from the bloodstream[83]. The diverse population of the stromal compartment has been well described in tumours as consisting of fibroblasts, mesenchymal stromal cells, endothelial cells, pericytes, adipocytes, and immune cells[41,83].

Fibroblasts are the most abundant cell within the tissue stroma and their activation is well documented in several soft tissue diseases like rheumatoid arthritis[59], tendinopathy[59], frozen shoulder[2], and Dupuytren's disease[153,276]. It has been described that stromal fibroblast fail to switch off their inflammatory programme and they continually produce factors-chemokines, cytokines, ECM proteins-which contribute to the inflammatory nature of the aforementioned diseases[59]. A growing list of cell surface markers indicate stromal cell/stromal fibroblast activation include, but are not limited to, FAP[59], CD90[59], CD248[2,59], and PDPN[2,59]. Given that persistent stromal activation causes stromal cells to maintain their inflammatory phenotype, producing pro-inflammatory mediators and matrix proteins, we suggest stromal activation could be instrumental in understanding the etiology of Dupuytren's disease.

The stromal compartment houses various cell types, including fibroblasts, endothelial cells, and stromal cells[41,313]. CD90 has been identified in Dupuytren's fascia and described within cancer as a marker for myofibroblasts and dispersed throughout the tumour stroma[121,129]. CD248 is associated with stromal cell proliferation and migration and fibrosis[56,260]. CD90, CD248, and podoplanin have also been described as characterizing markers for stromal fibroblasts and pericytes[260].

Podoplanin has been described and seen to be upregulated in various diseases, but the exact role of the marker has yet to be elucidated[14]. In cancer studies, PDPN expression promoted metastasis and its knockdown decreases tumour migration and growth[14]. These stromal markers have been shown to be upregulated in chronic inflammatory diseases like rheumatoid arthritis[56], tendinopathy[59], and frozen shoulder[2] podoplanin is expressed on fibroblasts and macrophages[38,56, 59].

Cytokines are secreted proteins which have specific effects on various cell types, and they play a role in cell-to-cell communication[23,313]. IFN-γ, described as being produced by T cells in Dupuytren's, has been implicated in fibrotic pathways via its IFN-γ's ability to induce mast cells to produce IL-13[3]. IL-13 is a cytokine described as a core mediator of fibrosis and has been shown to induce proliferation of myofibroblasts and ECM proteins by Dupuytren's fibroblasts[3].

TGF-β has a well described role in inducing the myofibroblast phenotype[276]. TNF-α has also been described as myofibroblast phenotype inducer and a driver of matrix protein expression in Dupuytren's fibroblasts[276]. Based on growing literature surrounding the role of stromal activation in chronic musculoskeletal diseases, this chapter will investigate a potential activated stromal compartment phenotype in Dupuytren's tissue and describe the response of the stromal compartment after stimulation with previously mentioned cytokines[153,276].

3.2 Aims:

1. Define stromal compartment activation within Dupuytren's disease

2. Assess the effect of cytokine stimulation and *in vitro* mechanical damage of Dupuytren's fibroblasts on inflammatory, matrix and stromal expression

3.3 Results

3.3.1 Fibrotic genes are expressed in Bulk RNA sequenced Dupuytren's tissue

Bulk RNA sequencing of Dupuytren's tissue primarily demonstrated upregulated expression of fibrosis associated genes compared to control, carpal tunnel tissue (Figure 3.1). While the Covid-19 pandemic led to a decrease in human sample availability, which manifests here as not significantly distinct data, this data describes a select number of fibrotic genes, expressed in Dupuytren's tissue compared to control; particularly those associated with stromal activation.

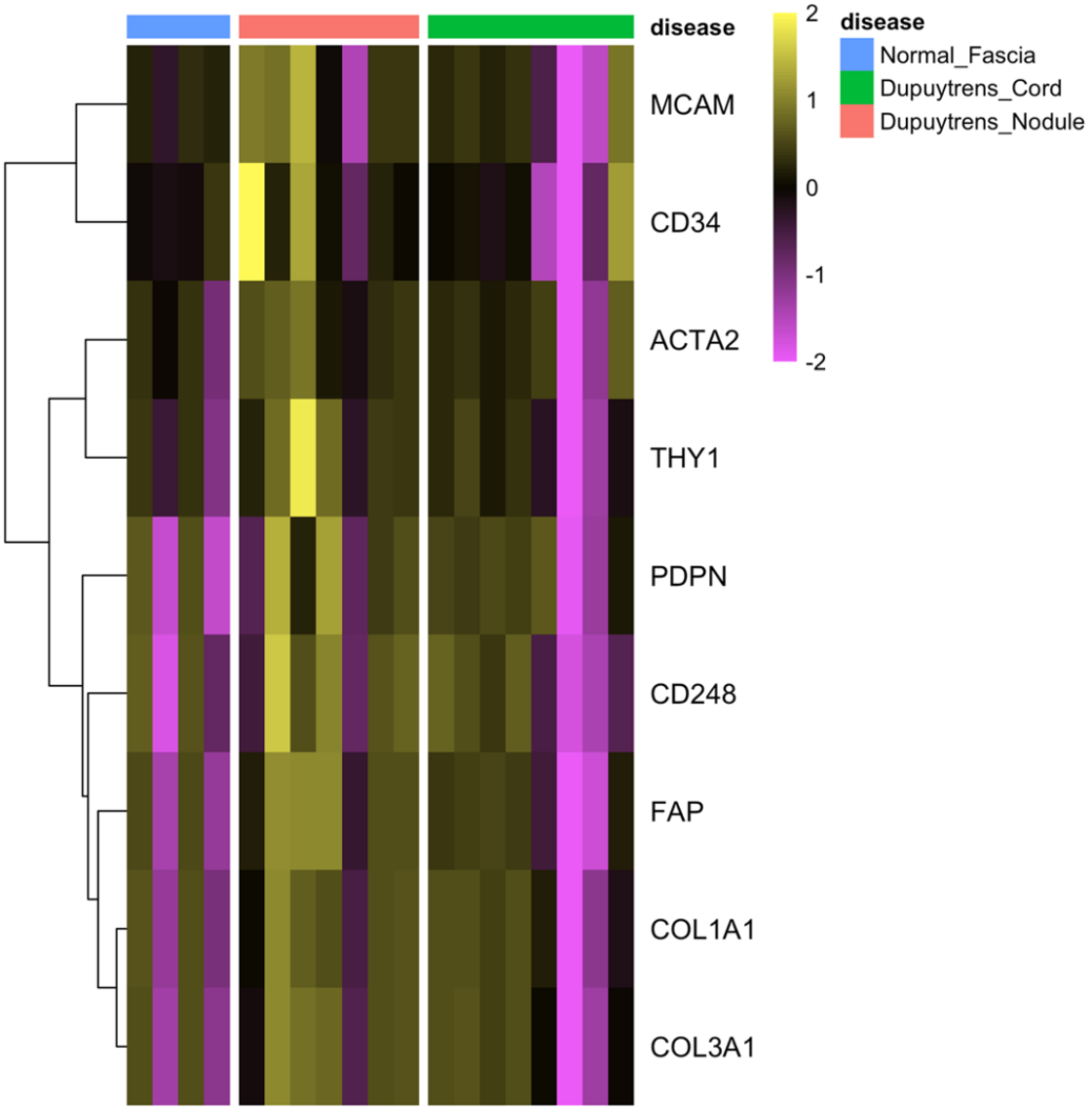


Figure 3.1 Bulk sequencing of Dupuytren's microenvironments and carpal tunnel tissue highlights prevalence of fibrotic genes.

Selected fibrotic genes were analysed by bulk RNA sequencing of Dupuytren's nodules (n=7) and cords (n=8) and carpal tunnel (control, n=4) tissue. The colour intensity represents row scaled (z-score) transcript expression, with purple representing low expression and yellow representing high.

3.3.2 Characterising stromal compartment activation in Dupuytren's disease

Activated fibroblasts are recognized by their expression of FAP and differentiated fibroblasts, called myofibroblasts, are identified by α-SMA. In each case, activated and differentiated fibroblasts produce factors that mediate the tissue healing response. α-SMA and FAP mRNA level expression in Dupuytren's disease were not significantly different compared to control tissue and within the samples examined, existed a range in expression for each marker (Figure 3.2A). Histological analysis (Figure 3.2B) depicts a significant increase in α-SMA staining in Dupuytren's tissue ($p<0.05$) via Bonar scoring and semi-quantitative analysis (% positive stained) compared to control tissue. FAP evaluation via Bonar scoring and semi-quantitative analysis showed little to no expression compared to control, carpal tunnel tissue (Figure 3.2B, C). Semi-quantitative analysis suggested that α-SMA (67.8%) was expressed more so in Dupuytren's compared to FAP (4.1%), describing fibroblasts as presenting an α-SMA^{+} myofibroblast phenotype within tissue biopsies.

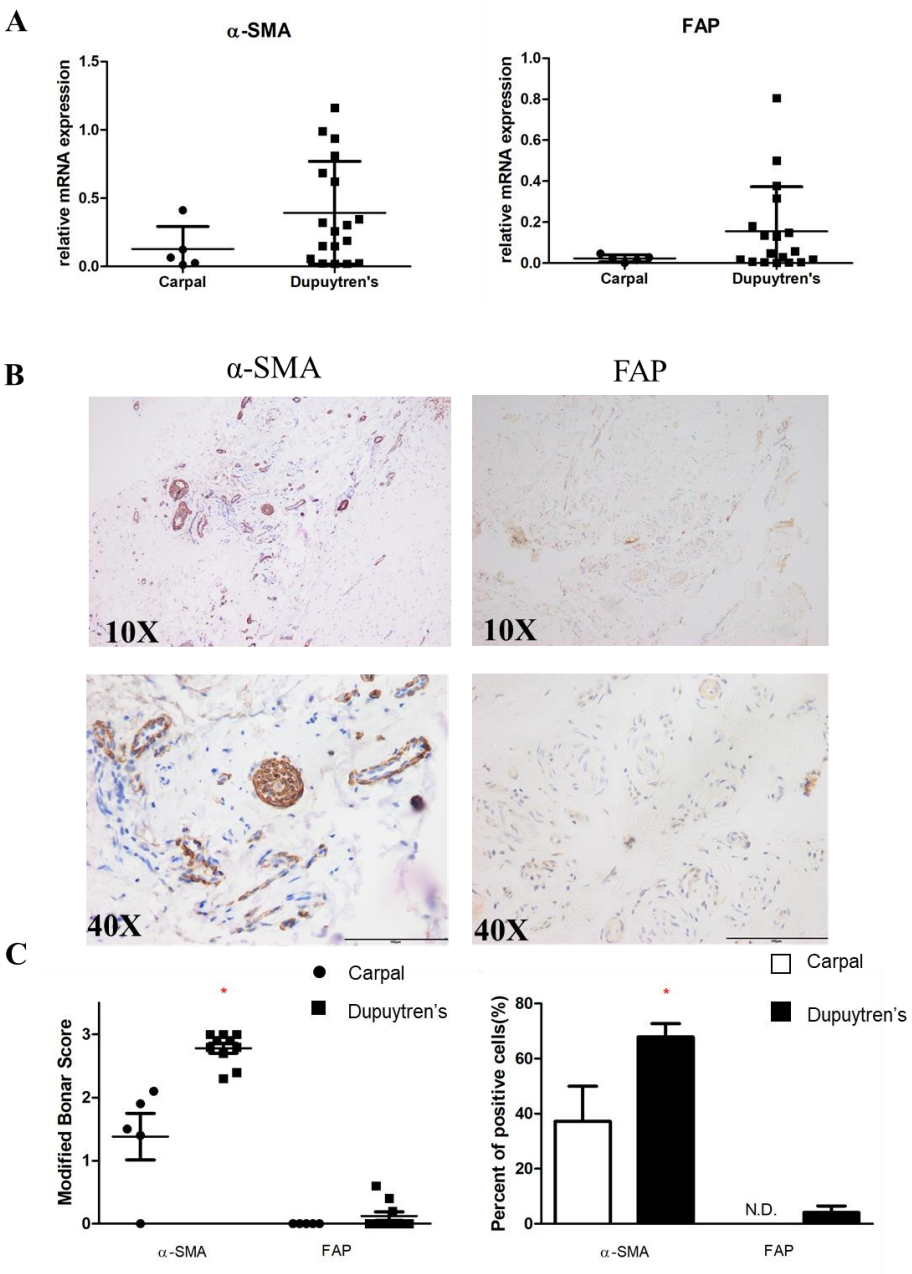


Figure 3.2 Dupuytren's fibroblasts express FAP and α-SMA.
(A) Relative mRNA expression ($2^{-\Delta Ct}$) of α-SMA and FAP in carpal tunnel (n=5) and Dupuytren's disease (n=10) tissue. Data is represented at mean ± SEM relative to GAPDH as the housekeeping gene (mean of duplicate analysis). (B) Immunohistochemistry of α-SMA and FAP in Dupuytren's disease at 10x and 40x magnification. (C) Graphical representation of Bonar scoring and percent of positive cells in carpal tunnel (n=5) and Dupuytren's disease (n=10) tissue biopsies for expression of α-SMA and FAP; mean ± SEM shown. Modified Bonar scoring system describes the mean score per donor based on five random high-powered fields. 0 indicates no staining, 1 indicates les than 10% of cells are stained positive, 2 indicates between 10-20% of cells are stained positive, and 3 indicates over 20% of cells are stained positive. T-test $p<0.05$ * $p< 0.01$ **

Stromal endothelial cells were identified by CD34 and CD146 (MCAM) in Dupuytren's disease. mRNA level expression was not significantly increased for either marker, compared to carpal tunnel (Figure 3.3A). Positive staining of both markers was seen throughout Dupuytren's tissue (Figure 3.3B) and modified Bonar scoring described Dupuytren's expression of CD34 and CD146 as significantly increased compared to carpal tunnel ($p<0.01$). Further analysis, via semi-quantitative evaluation, showed that CD34 (21.7%) was not expressed significantly higher in Dupuytren's tissue but CD146 (15.7%) was significantly expressed higher than carpal tunnel, CD34 (12.8%) and CD146 (5.8%) ($p<0.01$) (Figure 3.3C).

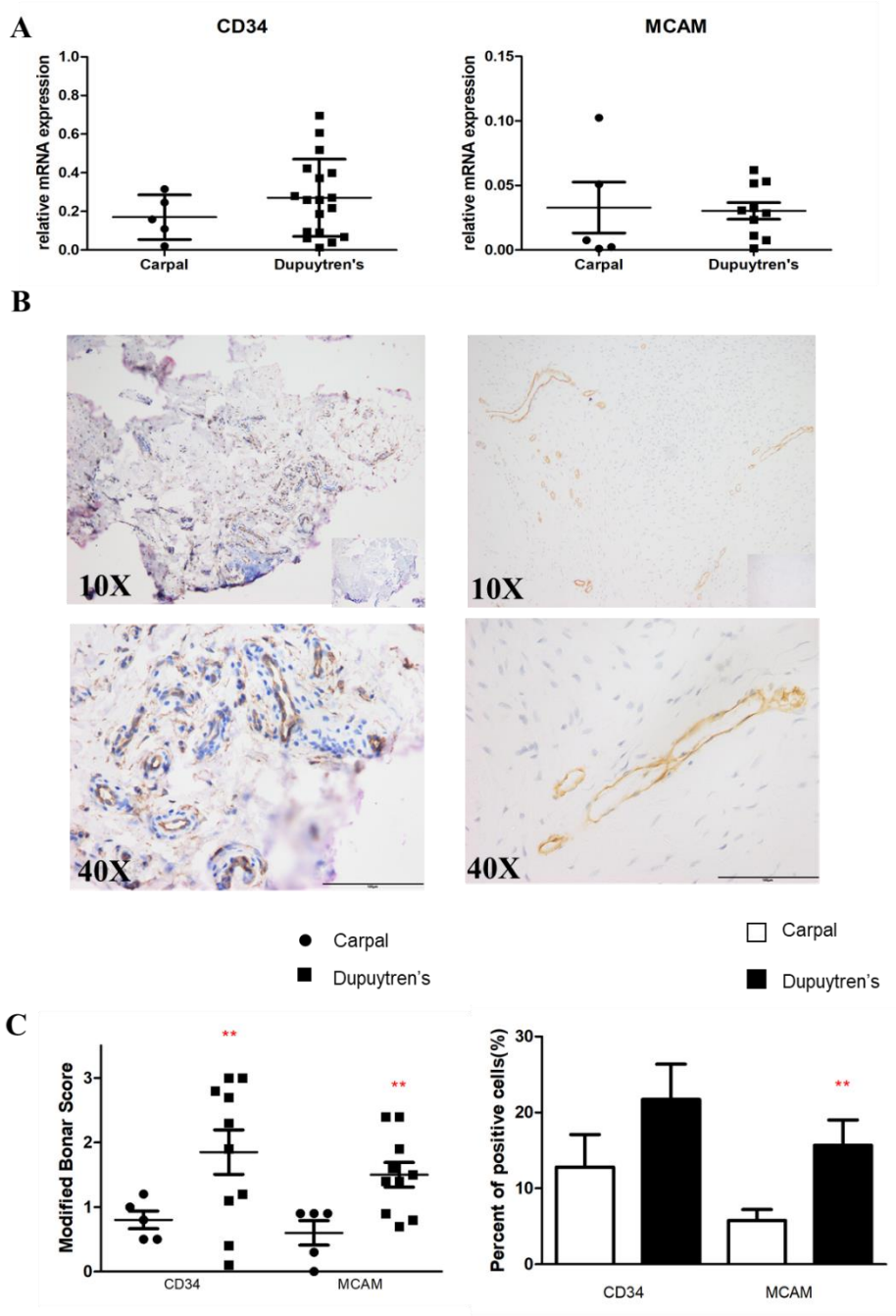


Figure 3.3 Endothelial cells are present in Dupuytren's disease.
(A) Relative mRNA expression ($2^{-\Delta Ct}$) of CD34 and CD146 in carpal tunnel (n=5) and Dupuytren's disease (n=10) tissue. Data is represented at mean ± SEM relative to GAPDH as the housekeeping gene (mean of duplicate analysis). (B) Immunohistochemistry of CD34 and CD146 in Dupuytren's disease at 10x and 40x magnification. (C) Graphical representation of Bonar scoring and percent of positive cells in carpal tunnel (n=5) and Dupuytren's disease (n=10) tissue biopsies for expression of CD34 and CD146; mean ± SEM shown. Modified Bonar scoring system describes the mean score per donor based on five random high-powered fields. 0 indicates no staining, 1 indicates les than 10% of cells are stained positive, 2 indicates between 10-20% of cells are stained positive, and 3 indicates over 20% of cells are stained positive. T-test $p<0.05$ * $p< 0.01$ **

Stromal fibroblast activation markers CD90, CD248, and podoplanin were evaluated in Dupuytren's disease. mRNA level expression of the aforementioned markers was increasingly expressed in Dupuytren's biopsies, only podoplanin was significantly higher compared to control biopsies ($p<0.01$) (Figure 3.4A). Via immunohistochemistry evaluation, these markers were seen throughout Dupuytren's tissue (Figure 3.3B) and Bonar scoring evaluation revealed that compared to carpal tunnel, CD90 ($p<0.05$) and CD248 ($p<0.01$) in Dupuytren's was significantly increased. Semi-quantitative analysis showed that expression of the CD90 (16%), CD248 (19%), and PDPN (31%) were significantly upregulated ($p<0.01$) in Dupuytren's disease compared to carpal tunnel expression of CD90 (4.2%), CD248 (3.8%), and PDPN (19.7%) (Figure 3.4C).

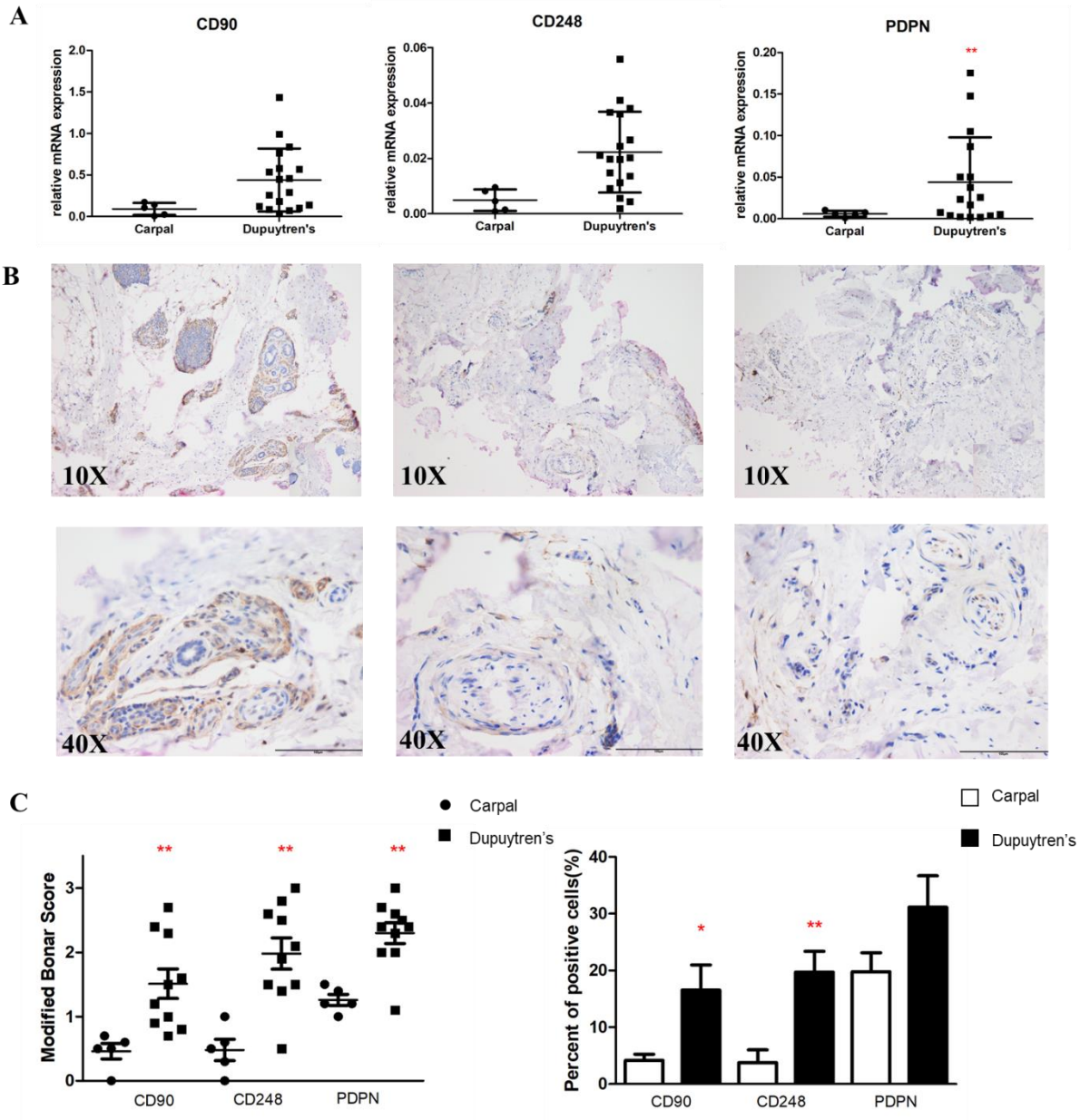


Figure 3.3 Mesenchymal stromal cells are present in Dupuytren's tissue.
(A) Relative mRNA expression ($2^{-\Delta Ct}$) of CD90, CD248, and podoplanin in carpal tunnel (n=5) and Dupuytren's disease (n=10) tissue. Data is represented at mean ± SEM relative to GAPDH as the housekeeping gene (mean of duplicate analysis). (B) Immunohistochemistry of CD90, CD248, and podoplanin in Dupuytren's disease at 10x and 40x magnification. (C) Graphical representation of Bonar scoring and percent of positive cells in carpal tunnel (n=5) and Dupuytren's disease (n=10) tissue biopsies for expression of CD90, CD248, and podoplanin; mean ± SEM shown. Modified Bonar scoring system describes the mean score per donor based on five random high-powered fields. 0 indicates no staining, 1 indicates les than 10% of cells are stained positive, 2 indicates between 10-20% of cells are stained positive, and 3 indicates over 20% of cells are stained positive. T-test $p<0.05$ * $p< 0.01$ **

3.3.3 Mechanical damage alters Dupuytren's fibroblast expression

Previous studies have identified *in vitro* mechanical damage, also called a scratch assay or mechanical injury model, as a method used to study a cell's response to microtrauma[121]. We explored whether mechanical damage could induce an α-SMA^{+} myofibroblast phenotype coupled with an inflammatory response, described by IL-6.

We observed an incremental increase in α-SMA mRNA expression by Dupuytren's fibroblasts after damage was induced (Figure 3.5A). IL-6 mRNA expression by Dupuytren's fibroblasts increased at 4-hours post scratch and expression resided at 24-hours (Figure 3.5B). IL-6 secretion by Dupuytren's fibroblasts increased at all time points, relative to control and regardless of cells being damaged; Carpal tunnel fibroblasts saw the largest increase 24-hours after damage was induced (Figure 3.5C).

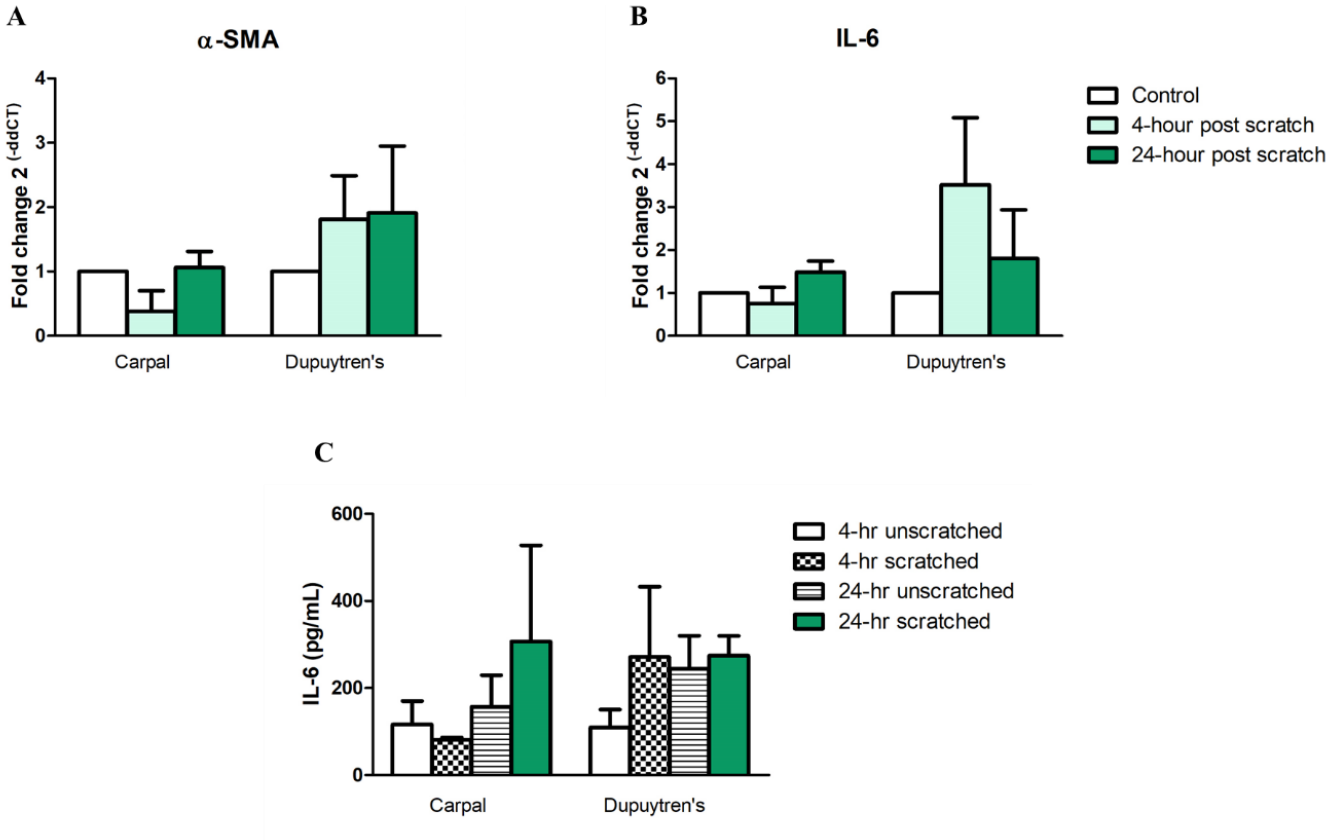


Figure 3.5 Myofibroblast and inflammatory expression following mechanical damage. α-SMA (A) and IL-6 (B) mRNA expression and IL-6 secretion (C) by Carpal tunnel fibroblasts and Dupuytren's fibroblasts 4 and 24 hours after scratch. Data represents mean ± SEM for carpal tunnel and Dupuytren's fibroblasts (n=6). Relative fold change is normalised to control (unstimulated) samples. * P<0.05 versus control (Kruskal Wallis test).

Matrix protein expression by fibroblasts occurs upon activation and differentiation into myofibroblasts. These activated myofibroblasts produce excess matrix proteins which contribute to the fibrotic and chronic inflammatory nature of Dupuytren's disease. Collagen type I and type III are key proteins of the ECM, and both increased in expression 4-hours after damage and reduced their mRNA level expression 24-hours after damage (Figure 3.6). Secretion levels of Collagen type I did not mirror the same results seen at the mRNA level, instead 24-hour unscratched and 24-hour scratched were higher than their 4-hour unscratched control (Figure 3.6). Periostin and fibronectin are well documented matrix proteins that aid in tissue matrix organisation[2, 30]. Both increased mRNA level expression in response to mechanical damage 4-hours after scratch by Dupuytren's fibroblasts.

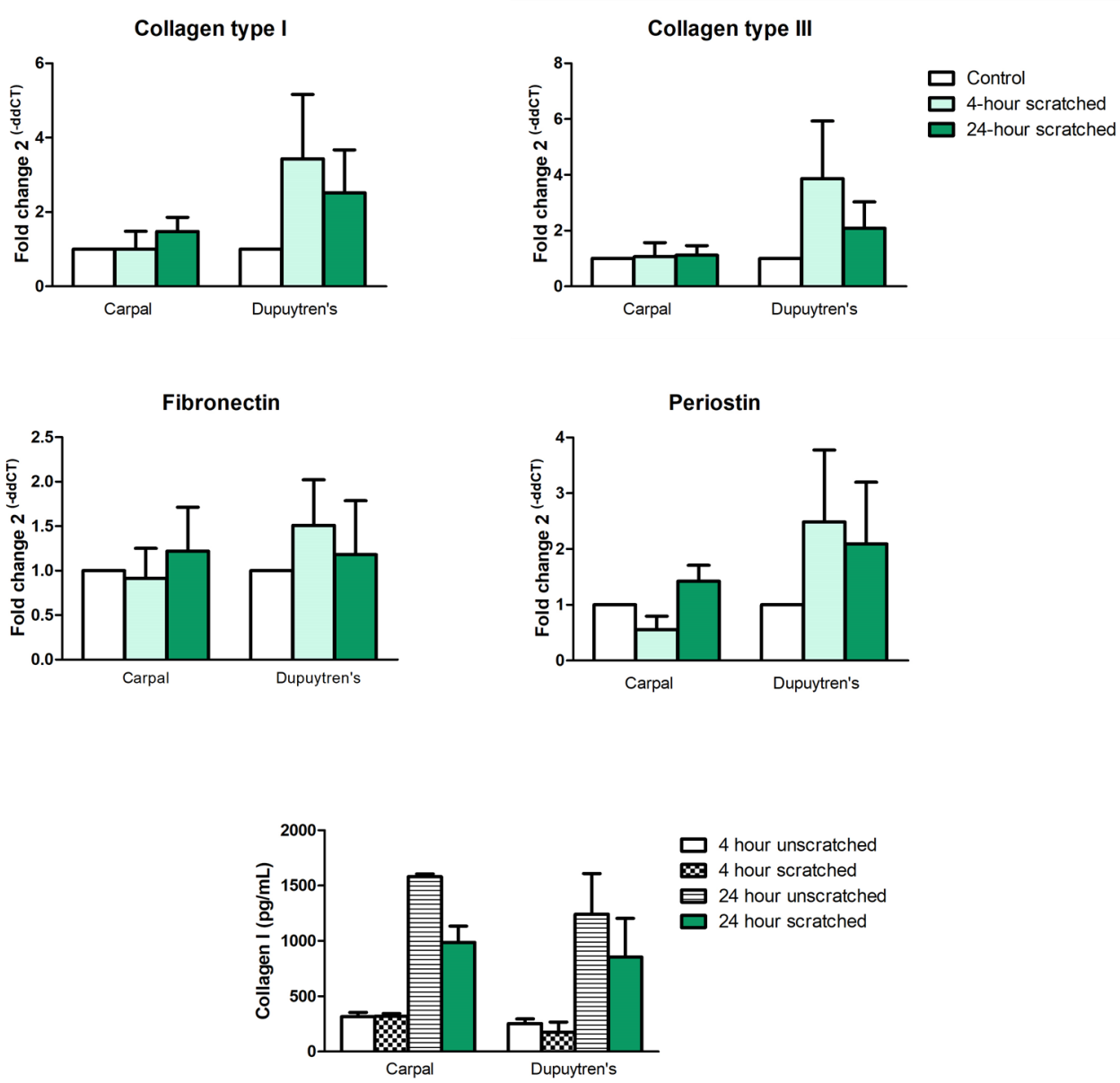


Figure 3.6 Matrix protein expression in response to mechanical damage.
4 and 24 hours post mechanical damage on matrix expression by Carpal tunnel and Dupuytren's fibroblasts. Data represents mean ± SEM for carpal tunnel and Dupuytren's fibroblasts (n=6). Relative fold change is normalised to control (unstimulated) samples. * $P<0.05$ versus control (Kruskal Wallis test).

Stromal compartment activation occurs in response to damage and aids in persistent chronic inflammation[15]. This suggests a stromal activation phenotype can be induced in vitro by damaging fibroblasts. This was seen in all markers assessed. CD90 expression increased the most at 4-hours post scratch and reduced at 24-hours. CD248 expression by Dupuytren's fibroblasts was highest 4-hours post scratch. Podoplanin expression by Dupuytren's fibroblasts increased at both 4 and 24-hours post scratch, but highest change was seen at 4-hours (Figure 3.7).

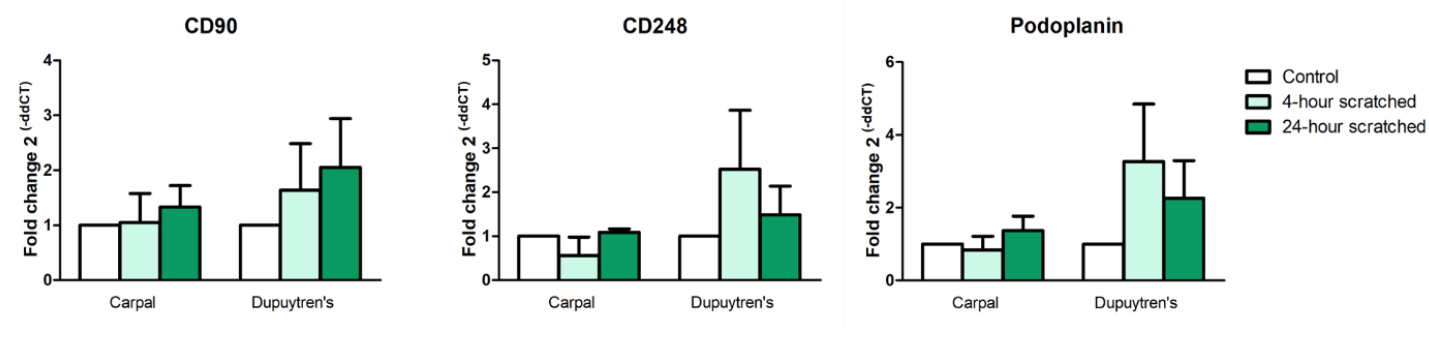


Figure 3.7 Mesenchymal stromal cell marker expression following mechanical damage. Mechanical damage on stromal surface marker expression by Carpal tunnel and Dupuytren's fibroblasts 4 and 24 hours after scratch. Data represents mean ± SEM for carpal tunnel and Dupuytren's fibroblasts (n=6). Relative fold change is normalised to control (unstimulated) samples. * P<0.05 versus control (Kruskal Wallis test).

3.3.4 Dupuytren's fibroblasts respond to stimulation with cytokines

Previous studies have shown that fibroblast behaviour can be manipulated via stimulation with cytokines produced by immune cells or in response to environmental cues[14,16]. We sought to examine the effect of cytokines, produced by immune cells and fibroblasts, on myofibroblast differentiation and inflammatory response. IL-13 on Dupuytren's fibroblasts further enhanced α-SMA production (Figure 3.8B) and TGF-β on control fibroblasts induced a myofibroblast phenotype (Figure 3.8A). This phenotype was consistent in IL-6 expression by carpal tunnel fibroblasts with IL-13 driving the mRNA level expression (Figure 3.8C) and TGF-β driving secretion level expression (Figure 3.8E). IL-6 mRNA expression by Dupuytren's fibroblasts in response to IFN-γ (Figure 3.8D) and secretion expression was driven by TGF-β (Figure 3.8F).

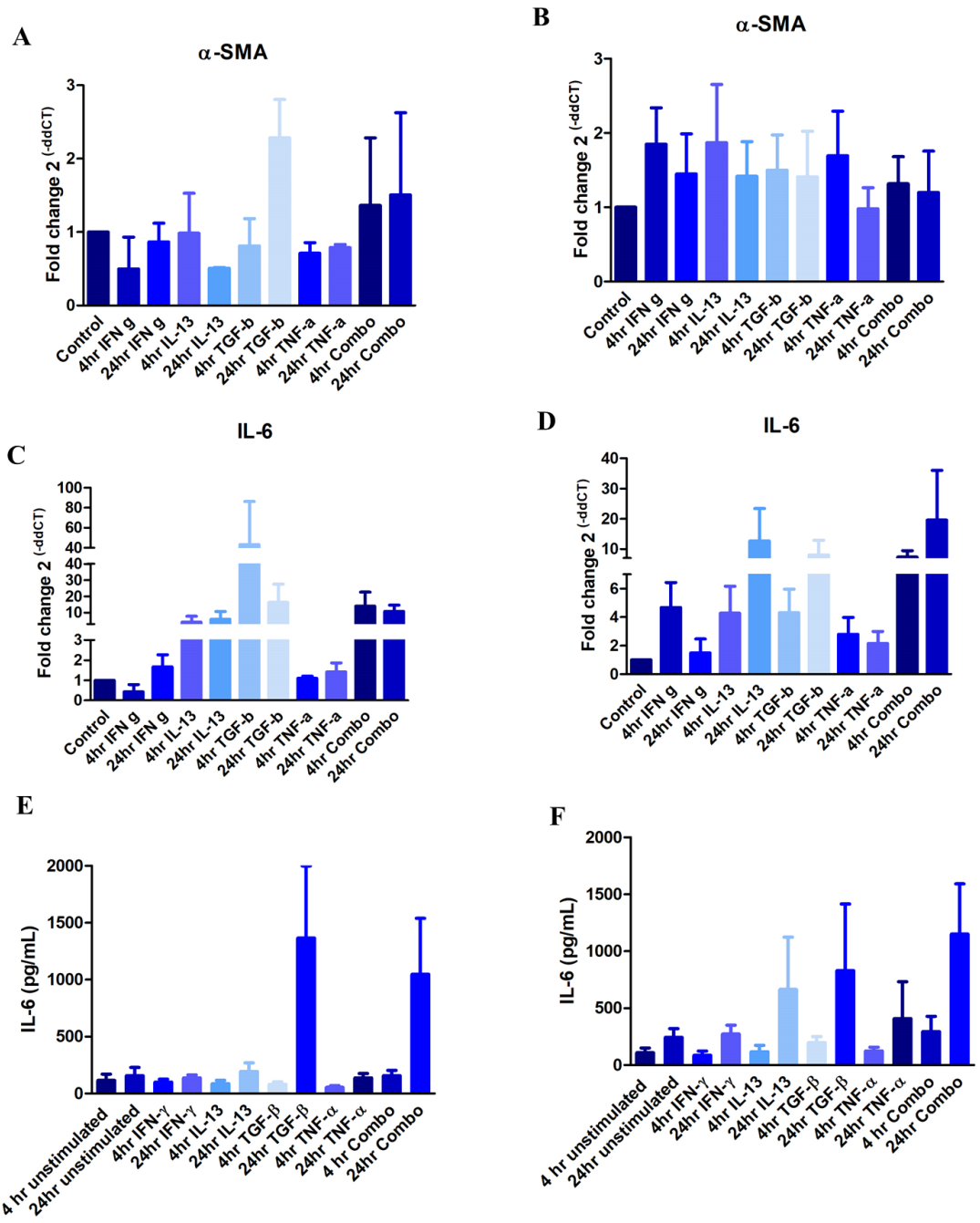


Figure 3.8 Cytokine stimulation on Dupuytren's fibroblasts.
Fibroblasts were stimulated with IFN-γ (10 ng/mL), IL-13 (1 ng/mL), TGF-β (2 ng/mL), TNF-α (0.1 ng/mL) and a combination of all 4 cytokines at 25% of the individual concentration. α-SMA expression in Carpal (A) and Dupuytren's (B), IL-6 mRNA level expression in Carpal (C) and Dupuytren's (D) and secretion level expression of Carpal tunnel fibroblasts (E) and Dupuytren's fibroblasts (F) were evaluated. Data represents mean ± SEM for carpal tunnel (n=3) and Dupuytren's fibroblasts (n=6). Relative fold change is normalised to control (unstimulated) samples. * $P<0.05$ versus control (Kruskal Wallis test).

Given cytokine ability to drive a myofibroblast phenotype, we sought to examine if α-SMA myofibroblast differentiation is accompanied by matrix protein expression. Carpal tunnel fibroblasts mRNA matrix protein expression was driven by 24-hour stimulation with TGF-β (Figure 3.9A). Dupuytren's fibroblasts mRNA level matrix protein expression was enhanced in response to 4-hour stimulation with TNF-α (collagen type I and periostin) and IFN-γ (collagen type III and fibronectin) (Figure 3.9B). Secretion level of collagen type 1 by carpal tunnel (Figure 3.9C) and Dupuytren's fibroblasts (Figure 3.9D) increased in response to 24-hour TGF-β stimulation.

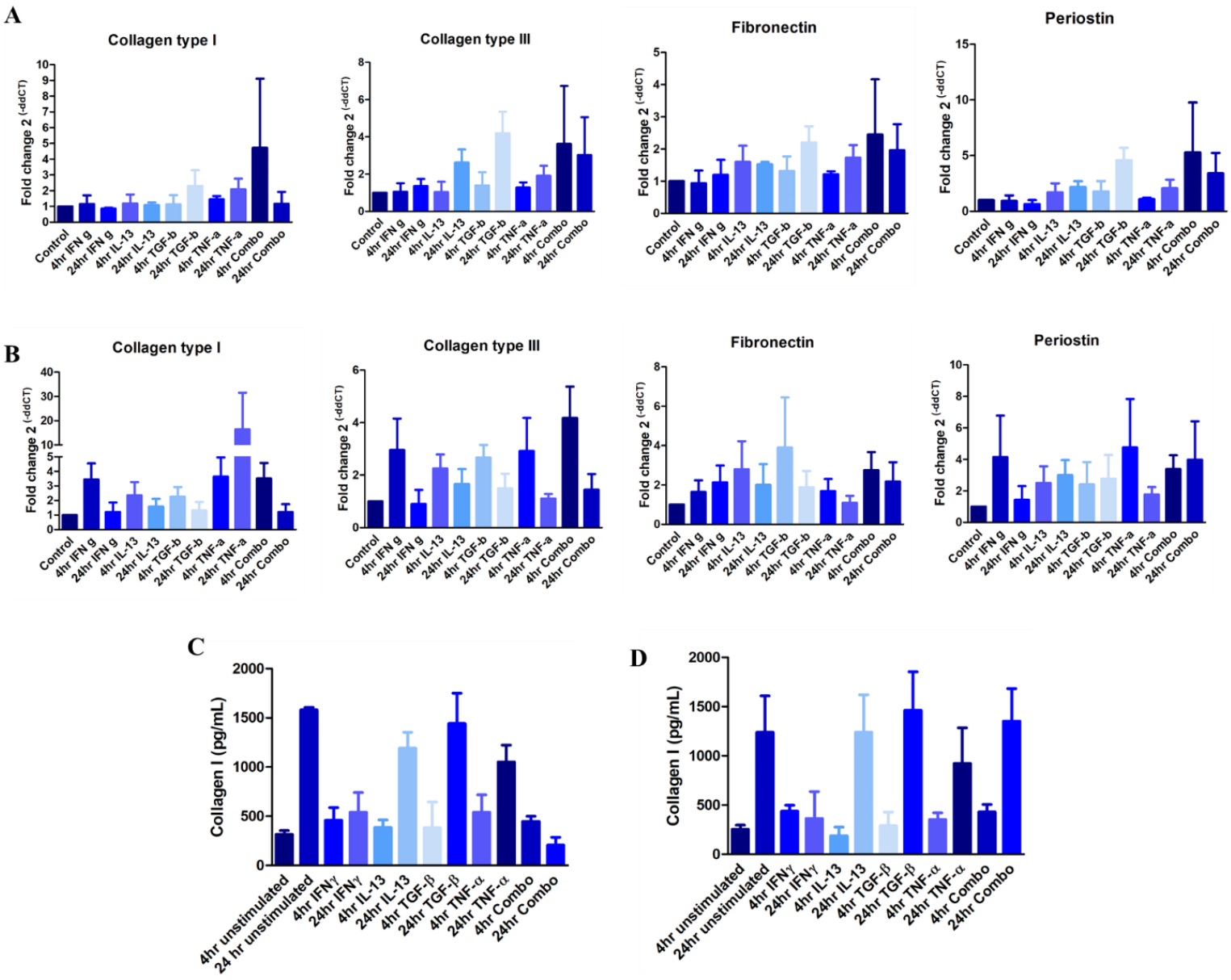


Figure 3.9 Matrix protein expression after cytokine stimulation of Dupuytren's fibroblasts.
Fibroblasts were stimulated with IFN-γ (10 ng/mL), IL-13 (1 ng/mL), TGF-β (2 ng/mL), TNF-α (0.1 ng/mL) and a combination of all 4 cytokines at 25% of their individual concentration. Effect of cytokine stimulation on matrix protein production by Carpal tunnel (A, C) and Dupuytren's fibroblasts (B, D) was evaluated. Data represents mean ± SEM for carpal tunnel (n=3) and Dupuytren's fibroblasts (n=6). Relative fold change is normalised to control (unstimulated) samples. * $P<0.05$ versus control (Kruskal Wallis test).

Carpal tunnel fibroblasts increased mRNA expression of stromal activation marker CD90, CD248, and PDPN after 4-hours of stimulation with a cytokine storm of all four cytokines (Figure 3.10A). Dupuytren's fibroblasts increased mRNA level expression of CD90 and CD248 4-hours after stimulation with TNF-α and podoplanin after 4-hours of stimulation with IFN-γ (Figure 3.10B).

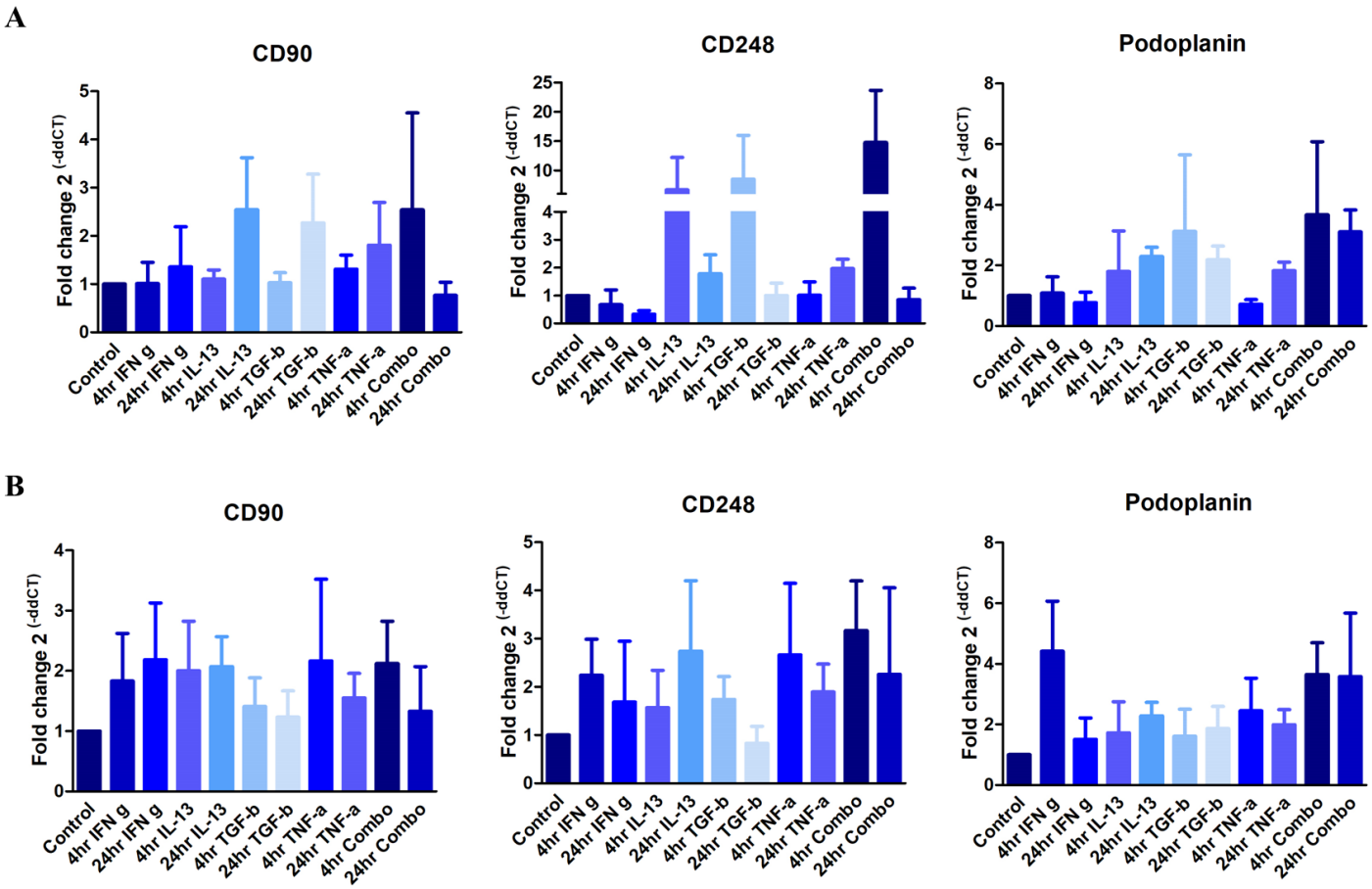


Figure 3.10 Mesenchymal stromal marker expression after cytokine stimulation of Dupuytren's fibroblasts.

Fibroblasts were stimulated with IFN-γ (10 ng/mL), IL-13 (1 ng/mL), TGF-β (2 ng/mL), TNF-α (0.1 ng/mL) and a combination of all 4 cytokines at 25% of their individual concentration. Effect of cytokine stimulation on stromal activation markers by Carpal tunnel (A) and Dupuytren's fibroblasts (B) were evaluated. Data represents mean ± SEM for carpal tunnel (n=3) and Dupuytren's fibroblasts (n=6). Relative fold change is normalised to control (unstimulated) samples. * $P<0.05$ versus control (Kruskal Wallis test).

3.3.5 Mechanical damage and cytokine stimulation alter stromal fibroblast behaviour *in vitro*

Having demonstrated the individual effect of mechanical damage and cytokine stimulation on fibroblast behaviour, we sought to explore whether the combination of both would further drive their inflammatory response. We describe that carpal tunnel fibroblasts differentiation into myofibroblasts 24-hours post damage and stimulation with TGF-β (Figure 3.11A) and Dupuytren's 4-hours after IL-13 stimulation and damage (Figure 3.11B). Accompanying this differentiation is an increase in inflammatory signature, increased mRNA IL-6 expression, by Carpal tunnel fibroblasts in response to stimulation with TGF-β at 4 and 24 hours (Figure 3.11C). Dupuytren's fibroblasts, after being stimulated with IFN-γ and 4-hours post damage, increases IL-6 expression at the mRNA level and Carpal tunnel fibroblasts increased their IL-6 mRNA expression 4 hours post damage and stimulation with TGFβ and all four cytokines combined (Figure 3.11D). Secretion of IL-6 by Carpal tunnel fibroblasts increased 24-hours after stimulation with TGF-β and damage (Figure 3.11E). Dupuytren's fibroblasts increased IL-6 secretion 24-hours after stimulation with all cytokines and damage (Figure 3.11F).

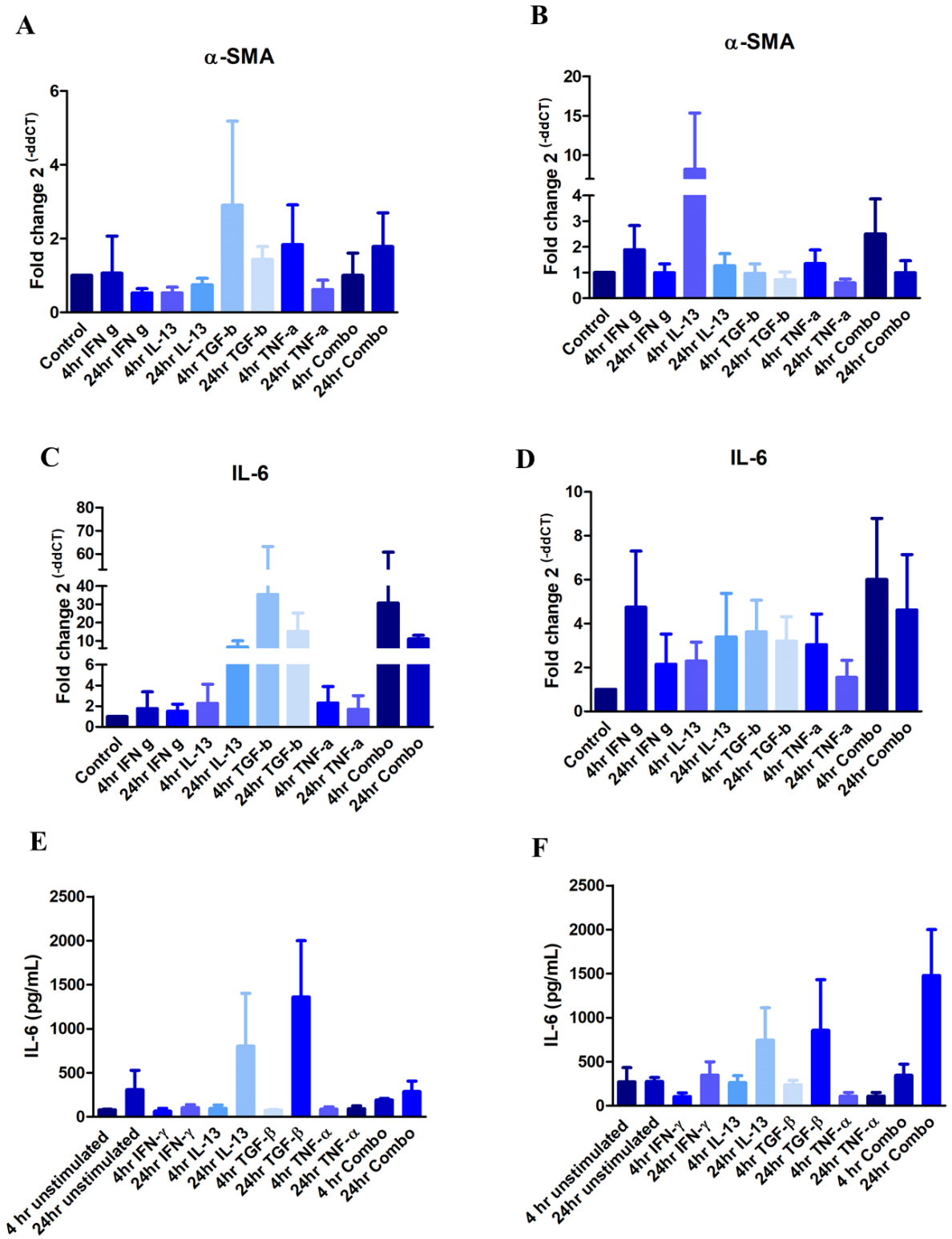


Figure 3.11 Dupuytren's fibroblast expression of myofibroblast and inflammation markers after mechanical damage and cytokine stimulation.

Fibroblasts were stimulated with IFN-γ (10 ng/mL), IL-13 (1 ng/mL), TGF-β (2 ng/mL), TNF-α (0.1 ng/mL) and a combination of all 4 cytokines at 25% of their individual concentration. α-SMA production by carpal tunnel (A) and Dupuytren's (B) fibroblasts, IL-6 mRNA level expression by Carpal tunnel (C) and Dupuytren's (D) fibroblasts and IL-6 secretion by Carpal tunnel (E) and Dupuytren's fibroblasts (F) were evaluated 4 and 24 hours post scratch. Data represents mean ± SEM for carpal tunnel (n=3) and Dupuytren's fibroblasts (n=6). Relative fold change is normalised to control (unstimulated) samples. * $P<0.05$ versus control (Kruskal Wallis test).

mRNA matrix protein expression by carpal tunnel fibroblasts was most notably seen at 4-hours post scratch and TGF-β stimulation (Figure 3.12A) and by Dupuytren's fibroblasts at 4-hours post scratch and IFN-γ stimulation (Figure 3.12B). Secretion level expression of collagen type I was increased by carpal tunnel fibroblasts after 24-hour post scratch and stimulation with TNF-α (Figure 3.12C) and by Dupuytren's fibroblasts 24-hour post scratch and IL-13 stimulation (Figure 3.12D).

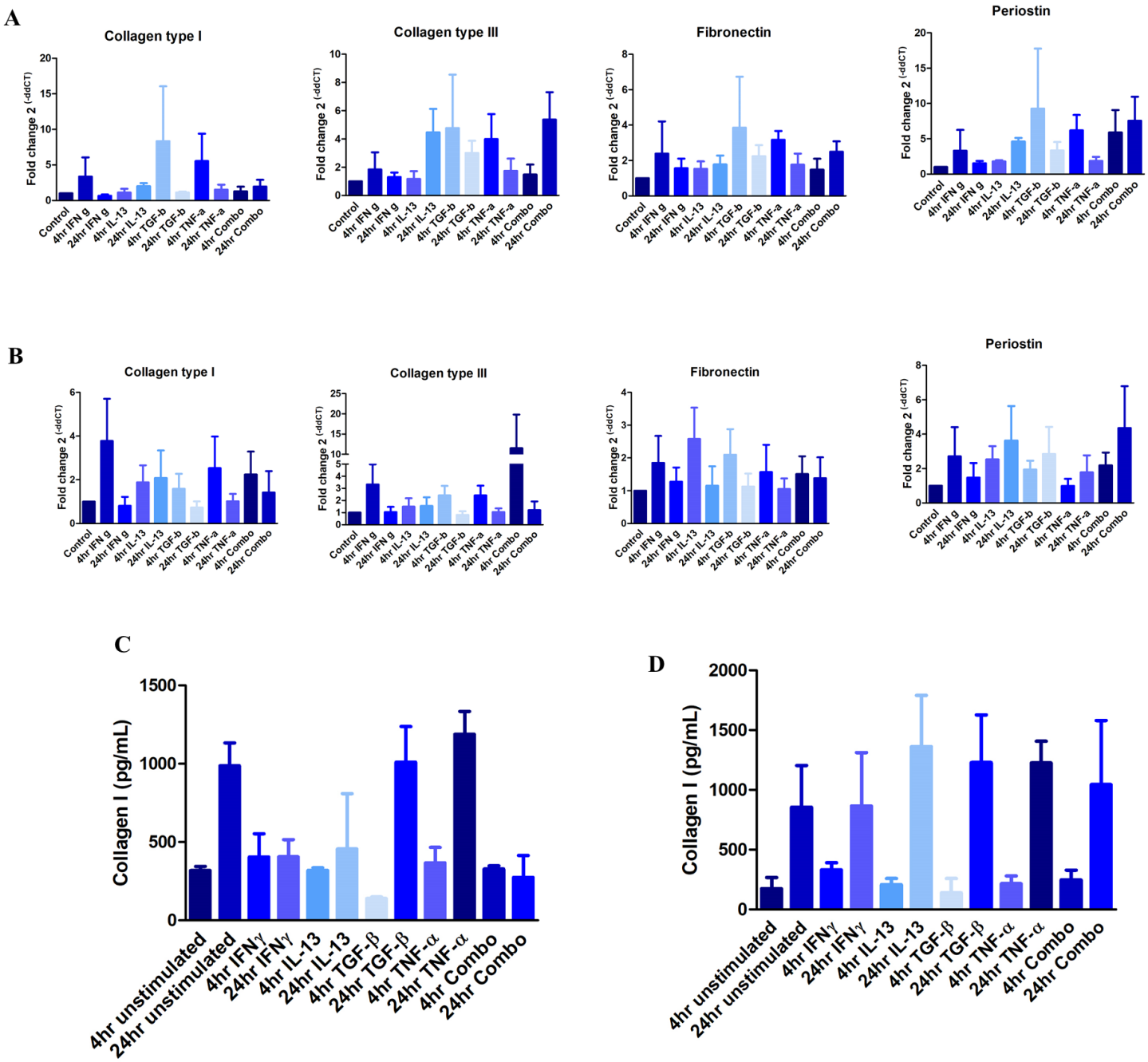


Figure 3.12 Matrix protein expression following mechanical damage and cytokine stimulation of Dupuytren's fibroblasts.

Fibroblasts were stimulated with IFN-γ (10 ng/mL), IL-13 (1 ng/mL), TGF-β (2 ng/mL), TNF-α (0.1 ng/mL) and a combination of all 4 cytokines at 25% of their individual concentration. Matrix expression by Carpal tunnel (A, C) and Dupuytren's (B, D) fibroblasts were evaluated 4 and 24 hours post scratch. Data represents mean ± SEM for carpal tunnel (n=3) and Dupuytren's fibroblasts (n=6). Relative fold change is normalised to control (unstimulated) samples. * $P<0.05$ versus control (Kruskal Wallis test).

Stromal activation marker expression by carpal tunnel fibroblasts increased 4-hours post scratch and stimulation with TGF-β (Figure 3.13A) and Dupuytren's fibroblasts increased 4-hour post scratch and stimulation with IFN-γ (Figure 3.13B).

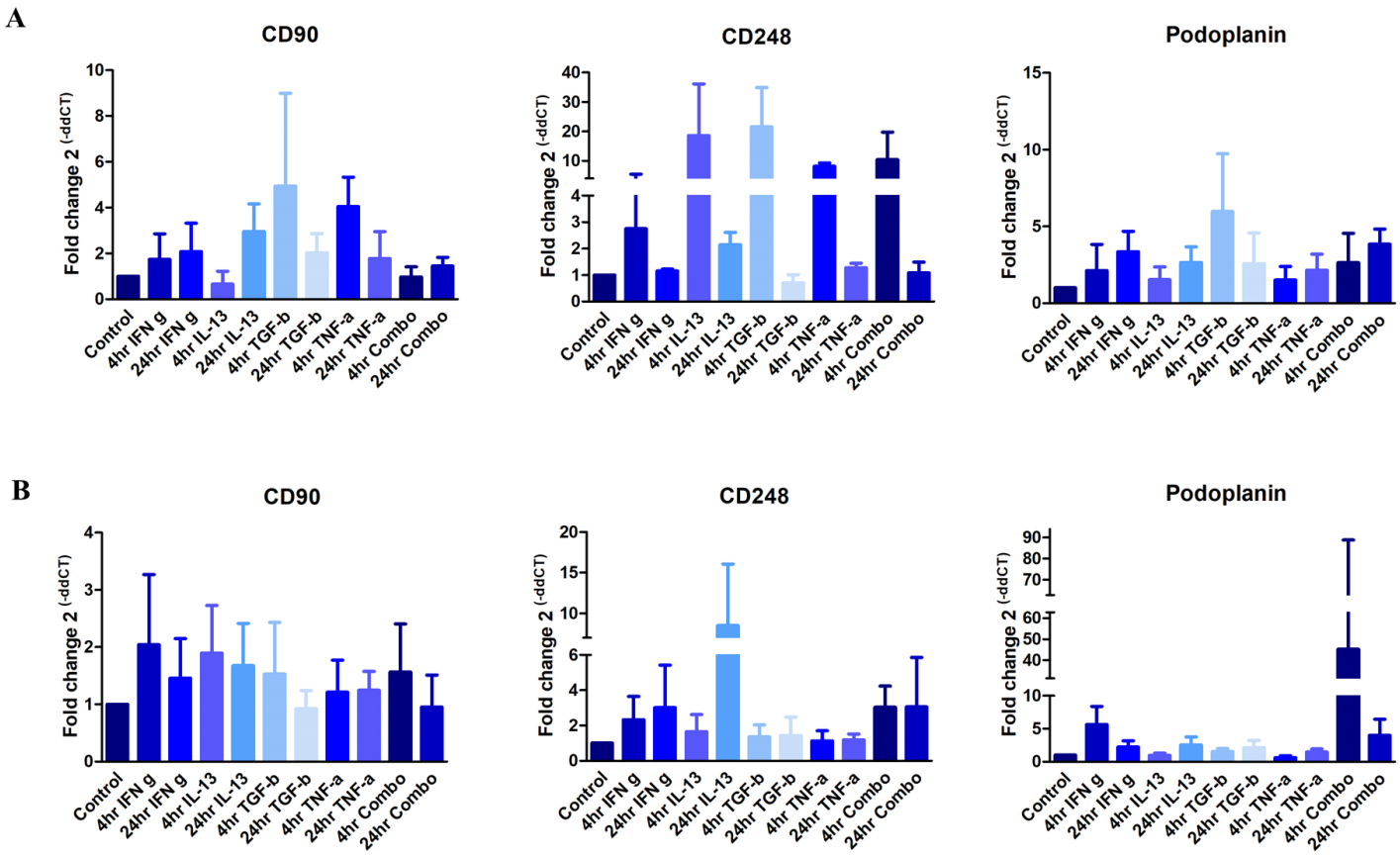


Figure 3.13 Mesenchymal stromal cell marker expression in response to mechanical damage and cytokine stimulation by Dupuytren's fibroblasts.
Fibroblasts were stimulated with IFN-γ (10 ng/mL), IL-13 (1 ng/mL), TGF-β (2 ng/mL), TNF-α (0.1 ng/mL) and a combination of all 4 cytokines at 25% of their individual concentration. Matrix expression by Carpal tunnel (A) and Dupuytren's (B) fibroblasts were evaluated 4- and 24-hours post scratch. Data represents mean ± SEM for carpal tunnel (n=3) and Dupuytren's fibroblasts (n=6). Relative fold change is normalised to control (unstimulated) samples. * P<0.05 versus control (Kruskal Wallis test).

3.4 Discussion and conclusion

This chapter describes the presence of MSCs, stromal fibroblasts, and stromal endothelial cells within Dupuytren's disease tissue. We further demonstrate that immune cell related cytokines act upon these cells allowing them to increase their 'pro-fibrotic phenotype'- defined as increased stromal, matrix, and inflammation related markers. We also describe that *in vitro* mechanical damage of Dupuytren's fibroblasts, in combination with cytokine stimulation, highlights a potential role for stromal-immune cell crosstalk via paracrine signalling.

Stromal fibroblasts have been described as the most abundant cell in the tissue stroma and its role as a contributor to fibrosis is well described in published works[59,132]. We describe these stromal fibroblasts are not only present in the tissue but are also activated, as described by FAP expression; fibroblasts are also differentiated into a-SMA^{+} myofibroblasts in Dupuytren's tissue. Myofibroblast differentiation is associated with higher contractility of the affected tissue, a clinical element of Dupuytren's disease[276-278]. Tissue fibrosis is characterised by persistently chronically inflamed tissue accompanied by a heightened immune cell presence and excess matrix protein production[47,168,214]. Within the confines of Dupuytren's disease, palmar tissue is inflamed and hardened causing a fixed conflexion of the affected fingers. Previous studies have described persistent stromal fibroblast activation as a hallmark feature of Dupuytren's and their differentiation into α-SMA producing myofibroblasts, leading to production of factors which prevent resolved tissue healing[2,51,59,76,153].

In addition to fibroblasts and myofibroblasts, we also describe endothelial and MSC's present in the tissue. Stromal endothelial cell localisation around the blood vessels suggests angiogenesis occurs in Dupuytren's tissue and could aid in vascular leaking, allowing immune cells to infiltrate the palmar tissue[104,221]. Future work would explore the changes in adhesion molecules to clarify whether there are changes in adhesion leading to increased, or decreased, vessel permeability. MSCs activation surface markers describe activated stromal cells present in the tissue. Published works describe that activation of stromal cells could contribute to soft tissue inflammation and aid in perpetuating chronic inflammation[2,51,59,76,154].

Understanding how cells respond to damage could aid in understanding how disease primed cells can exasperate the fibrotic response seen in Dupuytren's disease. We used an *in* vitro scratch to mimic the damaged environment of Dupuytren's disease and Carpal tunnel tissues and analyse how the cell monolayer responds to this damage. Time point analysis described an incremental increase in differentiation of Dupuytren's fibroblasts into myofibroblasts, and matrix protein and inflammatory marker expression, in response to damage. Additionally, Dupuytren's fibroblasts increased expression of MSC activation markers. Carpal tunnel fibroblasts did not increase their damage phenotype-FMT, inflammation, and matrix protein expression- post scratch. We suggest these cells do not respond as dramatically as Dupuytren's fibroblasts because Carpal tunnel is a less inflammatory soft tissue disease and thus, the Carpal tunnel fibroblasts are not as 'disease primed' as Dupuytren's disease fibroblasts.

Chronic inflammation and fibrosis are hallmarks of various soft tissue disease[2,38,117,288]. Within the confines of this disease, persistent stromal activation and excess matrix protein production are said to be the drivers of fibrosis. Published works have well described how individual or a combination of cytokines can influence fibroblast behaviour and these works dissect how molecular pathways can play a role[2,117,289]. Modelling the chronically inflamed *in vivo* environment of Dupuytren's disease, we stimulated cells with recombinant cytokines, in addition to inducing damage, to assess cell behavior and further understand which factors function as drivers of the disease.

Verjee et al. describes TGFβ indiscriminately activates fibroblasts and myofibroblast differentiation[276]. Our data describe that when carpal tunnel fibroblasts were stimulated with TGF-β1, they increased their expression of matrix, stromal and inflammatory markers, with and without the induction of mechanical damage. Similarly, Dupuytren's fibroblast differentiation into myofibroblasts, indicated by α-SMA, and inflammation marker, IL-6, in response to TGF-β1 stimulation. IL-6 expression is associated with increased contractility of myofibroblasts which, clinically speaking, contributes to the tightened palm tissue[40]. Additionally, matrix protein expression was increased by TGFβ. Verjee *et al.* describe that TGF-β1 activates and differentiates fibroblasts regardless of whether the fibroblasts were disease primed and that TGF-β1 acts on fibroblasts via canonical Wnt signalling[257,276];

Supporting literature describe that Yes-associated protein 1 (YAP1), via its interaction with TGF-β1, could contribute the stiffness seen in Dupuytren's disease by promoting matrix protein expression[219].

Stromal-immune crosstalk has been implicated in several disease models and we suggest that immune cells interact with fibroblasts to mediate the fibrotic response seen in Dupuytren's. Dupuytren's fibroblasts stimulated with TNF-α increased matrix expression. Verjee *et al.* described macrophages are a primary source of TNF-α within Dupuytren's tissue[276]. Increased matrix expression by fibroblasts in response to TNF-α supports the idea that macrophages and fibroblasts interact via cytokine signalling. Recent works describe that TNF-α and TGF-β1 could interact with fibroblasts via Wnt/β-catenin signalling[200,276].

Akbar *et al.* has described a role for STAT1 signalling, via IL-13Rα1, as a driver of fibrosis in Dupuytren's disease[276]. They describe that IFN-γ, produced by T cells, promotes myofibroblast differentiation and promotes mast cell production of IL-13; IL-13 promotes matrix protein expression by myofibroblasts[4]. The present study demonstrates that IFN-γ aggregated matrix and inflammatory marker expression by Dupuytren's fibroblasts. Stimulation with IL-13 increased myofibroblast differentiation and IL-6 expression in by Dupuytren's fibroblasts, with and without cells being damaged. Activated MSC marker expression by Dupuytren's fibroblast increased when stimulated by IFN-γ and IL-13.

We suggest that cytokines could behave in concert to aid in inducing fibrosis, as opposed to a linear relationship between one cytokine acting upon fibroblasts. The present study evaluated the combined effect of IFN-γ, IL-13, TNF-α, and TGF-β-defined as a cytokine storm-upon Dupuytren's and Carpal tunnel fibroblasts. Carpal tunnel fibroblasts stimulated with the cytokine storm increased the inflammatory, matrix, and stromal marker expression. We presume Carpal tunnel fibroblasts require more stimulation, via the storm instead of single cytokine stimulation, because they do not have a disease phenotype. Similarly, Dupuytren's fibroblasts respond in an increasingly diseased manner-increased inflammatory, matrix, and stromal marker expression, mimicking the behaviour of the tissue stroma *in vivo*. Given that Dupuytren's and Carpal tunnel fibroblasts respond dramatically to cytokine storm

stimulation, we suggest that paracrine signalling pathways engage in the etiology of fibrosis. Future work should aim to elucidate which cells and cytokines participate in the mechanisms which contribute to Dupuytren's disease, and on a larger scale tissue fibrosis.

This work highlights the role of paracrine signalling in exacerbating the pro-fibrotic phenotype of Dupuytren's fibroblasts. Specifically, we highlight the roles of immune cell derived TNF-α, IL-13 and IFN-γ in driving the stromal and matrix response by Dupuytren's disease and Carpal tunnel fibroblasts, suggesting a role for immune cell and fibroblast crosstalk in perpetuating tissue fibrosis. Additionally, future work studying cytokine stimulation of fibroblasts and paracrine signalling in fibrotic tissue stroma should increase the number of donors to create statistical significance between conditions and sequencing data. One of the greatest impacts of the Covid-19 pandemic on my research is the lack of tissue samples. Given that hospitals turned their attention towards treating those who are seriously ill, elective surgeries, like Dupuytren's fasciectomy and Carpal tunnel decompression ceased. Human sample acquisition is the foundation of my work and throughout this thesis, the lack of patient samples unfortunately rendered my data nonsignificant. Hopefully, with vaccination numbers increasing, surgeons will be able to return to performing elective surgeries and samples will resume being collected so this work can be continued towards producing significant data for publication.

Chapter 4: Exploring the role of T cell-fibroblast interaction as a contributing factor to the inflammatory nature of Dupuytren's disease

4.1 Introduction

Well-described within the confines of tumours, the influx of immune cells from the bloodstream and activation of resident immune cells acts as defining factors of the inflammatory response. The composition of the influx can serve as biomarkers for predicting the healing response[316]. Tumour infiltrating lymphocytes and macrophages have been shown to be major players in the immune response in that their activation and differentiation is associated with favourable outcomes and their role as regulators between the immune system and tumours, respectively[316].

Infiltrating immune cells have been studied in soft tissue disease models which describe the remodelling process being heavily influenced by immune cells, resident, and infiltrating, as well as the factors they produce[3,8,37,115,117,155,175,276,316]. Upon arrival at the site of inflammation, immune cells differentiate in response to factors secreted by surrounding cells. Activated immune cells produce elements which encourage stromal cells to activate and differentiate leading to production of inflammation related markers and matrix proteins. Upon completing tissue repair, a 'loss of survival signals' (i.e., produced by activated fibroblasts and stromal cells) causes immune cells to cease production of their inflammatory mediators, undergo apoptosis, and drain into the lymphatic system[236]. When tissue healing is not resolved, the recruitment of immune cells persists, stromal cells continue to be activated and together drive tissue healing towards chronicity and fibrosis[236,316].

The immune cell landscape in fibrotic tissue has been described as instrumental in perpetuating the tissue disease state[110,155]. In the context of Dupuytren's disease, previous studies have defined a milieu of immune cells which include, but are not limited to, TNF-α producing macrophages in response to IFN-γ and IL-13 producing T cells, and IL-13 producing mast cells[3,117,175,276]. While the precise mechanism of immune cell involvement in Dupuytren's disease is not fully understood, current literature support that immune cells contribute to the diseased nature of the tissue and support immune cell interaction as a promising avenue of research. Published works describe that sequencing Dupuytren's tissue can guide research questions towards interrogating specific niches that could contribute to the

etiology of the disease[74,232,260,281]. Stromal-immune cell interactions are spotlighted for the development of therapeutic targets.

Fibroblasts are incredibly versatile connective tissue cells, and within the tissue lie a heterogenous population of fibroblasts. This diversity in phenotype and function could shed light on the mechanism by which fibroblasts interact with various immune cells[37]. Elegantly described by Akbar et al. 2020 IFN-γ producing T cells in Dupuytren's disease promote IL-13 production from mast cells which upregulate fibroblast differentiation into myofibroblasts via STAT signalling[3]. Izadi et al. 2019 defines the role of IL-33 producing stromal cells in inducing TNF-α production by M2 macrophages and mast cells which then interact with Dupuytren's fibroblasts leading to differentiated α-SMA$^+$ myofibroblasts[117]. These works demonstrate how an understanding of the immune cell landscape can lead to the development of therapeutic interventions.

Highlighted within the confines of cancer[20,101,288], rheumatoid arthritis[131,156,178], and fibrosis[35,247,314], T cell and fibroblast crosstalk has been suggested as a mediator of the inflammatory nature of the disease. CD27 is a member of the TNF receptor family and alongside its costimulatory molecule, CD28, mediates T cell activation[95]. CD27 is expressed by naïve and memory T cells and interacts with its sole ligand, CD70 which is expressed on activated T cells, B cells and dendritic cells[95,119]. CD70 expression is induced via toll like receptor (TLR) signalling and CD27 occurs via NFκB signalling[99]. CD70 expression is tightly controlled and upon binding with CD27, a signalling cascade involving TRAF2 and 5 leads to T cell activation[164].

CD70 is also a member of the TNF family but its signalling cascade is not well understood[119]. Identified in colorectal cancer studies as an immune checkpoint molecule, CD70 was not found on tumour cells but strongly expressed on cancer associated fibroblasts (CAF)[119]. These works describe that CD70$^+$ CAFs could be used as a predictor for cancer patients given their role in cancer cell migration and T_{reg} accumulation. CD70 overexpression is described on malignant cells, fibroblasts, and stromal cells and its interaction with CD27 allows for induction of T cell apoptosis, T cell exhaustion and suppression of regulatory T

cells[119]. Deletion studies describe that knockdown or blocking of CD70 on Tregs or CD27 on T cells significantly enhanced T_{reg} suppression and it was suggested that CD27/CD70 interaction are key for understanding how T cells regulate inflammation[11]. Further, these works describe $CD70^{-/-}$ T cells are unable to upregulate immune checkpoint molecules like PD-1 and CTLA-4 thus inhibiting caspase mediated apoptosis[205]. This CD27/CD70 activation axis as an apoptosis inhibitor could aid in explaining how immune cells remain active in the tissue stroma and provide rationale for the development of chronic inflammation; given that without apoptosis to inactivate immune cells, inflammatory factors are continually produced.

4.2 Aims

These publications support the theory that fibroblast-T cell interaction could be important towards understanding the underlying immune mechanisms of Dupuytren's disease. In this chapter we aim to:

1. Delineate the distinct biology of both Dupuytren's microenvironments via RNA bulk sequencing
2. Define the immune cell landscape of Dupuytren's disease tissue
3. Interrogate T cell-fibroblast interaction within Dupuytren's disease

4.3 Results

4.3.1 Immune cells are present within Dupuytren's tissue

Histological representation of immune cells in Dupuytren's disease tissue describe CD3, a common marker for the T cell lineage, present in Dupuytren's disease significantly more so than in control, carpal tunnel tissue via modified Bonar scoring ($p<0.01$) and percent positive ($p<0.001$). Representative images show that CD3 is expressed in the cellular nodules of Dupuytren's tissue (Figure 4.1).

CD11c has been described as a dendritic cell marker and while it was selectively expressed within Dupuytren's disease, compared to carpal tunnel it was not expressed significantly higher (Figure 4.1). CD11c expression was not localised to cellular nodules but its expression was sporadically seen in more dispersed cellular areas of the tissue.

CD68 is a macrophage marker and is also expressed by cells of the monocyte lineage. Histological representation shows it is clearly expressed throughout Dupuytren's tissue and unlike T cells, that it is not localized to the nodules. CD68 expression in Dupuytren's was more significantly expressed than in carpal tunnel as determined evaluated by modified Bonar scoring ($p<0.001$) and percent positive evaluation ($p<0.01$) (Figure 4.1).

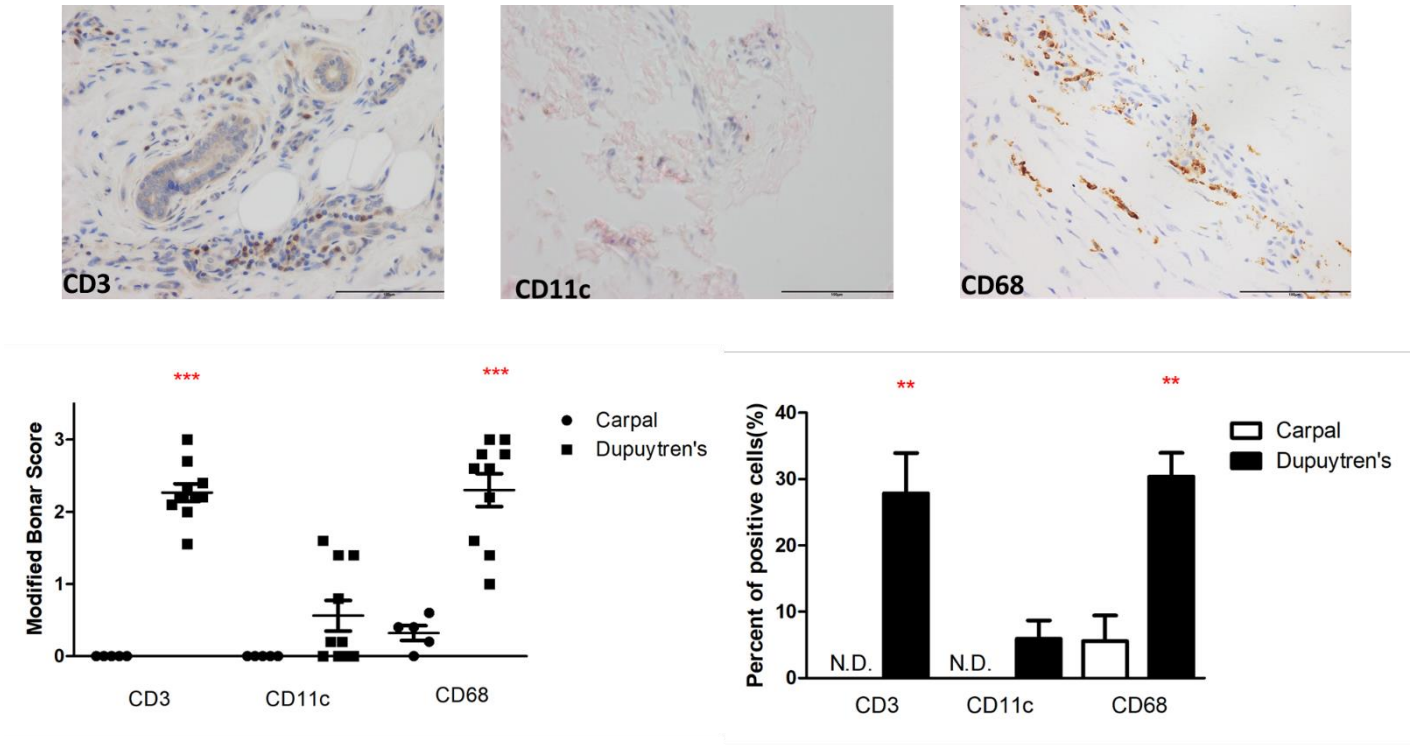


Figure 4.1 Immune cells are dispersed throughout Dupuytren's tissue. (A) Immunohistochemistry of CD3, CD11c, and CD68 in Dupuytren's disease at 40x magnification. (B) Graphical representation of Bonar scoring and percent of positive cells in carpal tunnel (n=5) and Dupuytren's disease (n=10) tissue biopsies for expression of CD3, CD11c and CD68, mean ± SEM shown. Modified Bonar scoring system describes the mean score per donor based on five random high-powered fields. 0 indicates no staining, 1 indicates les than 10% of cells are stained positive, 2 indicates between 10-20% of cells are stained positive, and 3 indicates over 20% of cells are stained positive. T-test $p< 0.01$ ** $p<0.001$***

Histological representation demonstrates the presence of leading immune cells highlighted by sequencing of Dupuytren's tissue. However, we explored a wider panel of markers to encompass those highlighted in sequencing data. Fresh Dupuytren's tissue was digested and evaluated via flow cytometry for general immune cell markers. Our work describes the presence of lymphocytes, macrophages, mast cells, natural killer cells and dendritic cells. Further we show hierarchy of immune cells present and further supporting sequencing results we show that the leading immune cell populations were T cells (Figure 4.2). Carpal tunnel tissue was not cellular enough to provide flow cytometry and immunohistochemistry staining of immune cell populations.

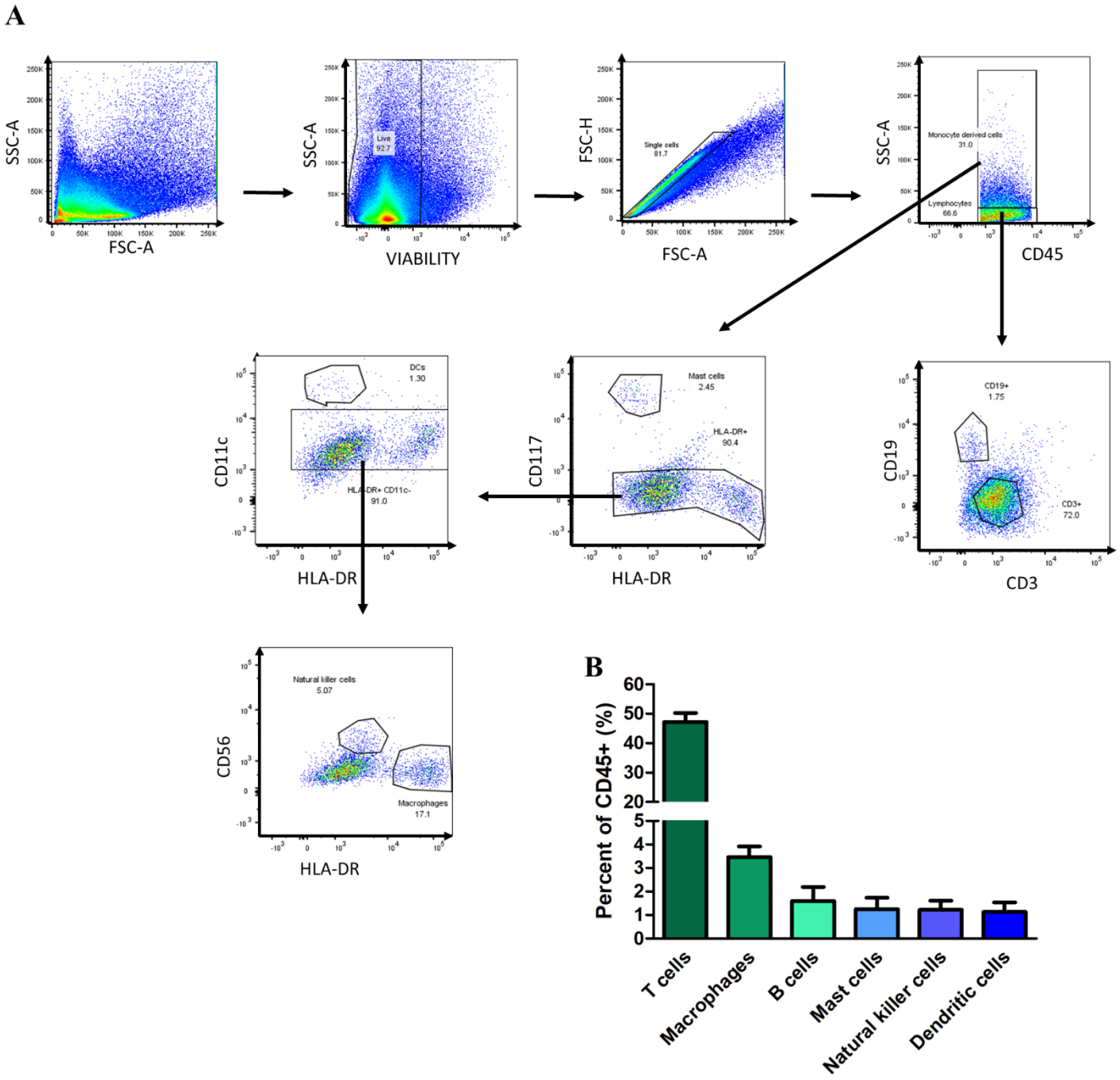


Figure 4.2 Freshly digested Dupuytren's tissue describes abundance within immune cell landscape.
(A) Flow cytometry gating strategy of fresh Dupuytren's tissue. (B) Immune cell populations represented as percent of CD45+ cells from freshly digested Dupuytren's tissue (n=3). Data represented as mean ± SEM shown.

Following my initial findings of significant immune cell influx in Dupuytren's tissue I planned to undertake scRNA-seq to help better define the subpopulations of immune cells present in disease. Unfortunately, I was unable to complete this experiment myself due to the onset of COVID restrictions, lack of sample availability and the lack of laboratory time due to the pandemic.

However, I was able to access a publicly available dataset via Dobie *et al.* (2022)[167] and therefore was able to conduct a limited single cell analysis to help and better define immune cell subpopulation in Dupuytren's disease. This work was further completed with the help of Dr. Akbar. This was based on n=5 Dupuytren's tissue and n=3 control fascial tissue obtained prior to the pandemic and tissue draught that followed. UMAP visualisation of both tissue microenvironments describe myeloid and T-lymphoid cells as leading immune cells in Dupuytren's disease (Figure 4.3, Figure 4.4). Further elucidation demonstrated that Dupuytren's disease myeloid and T lymphocytes make up the majority of cells seen. Quantitative analysis describes percent evaluation of immune cell populations within the tissue (Figure 4.3C).

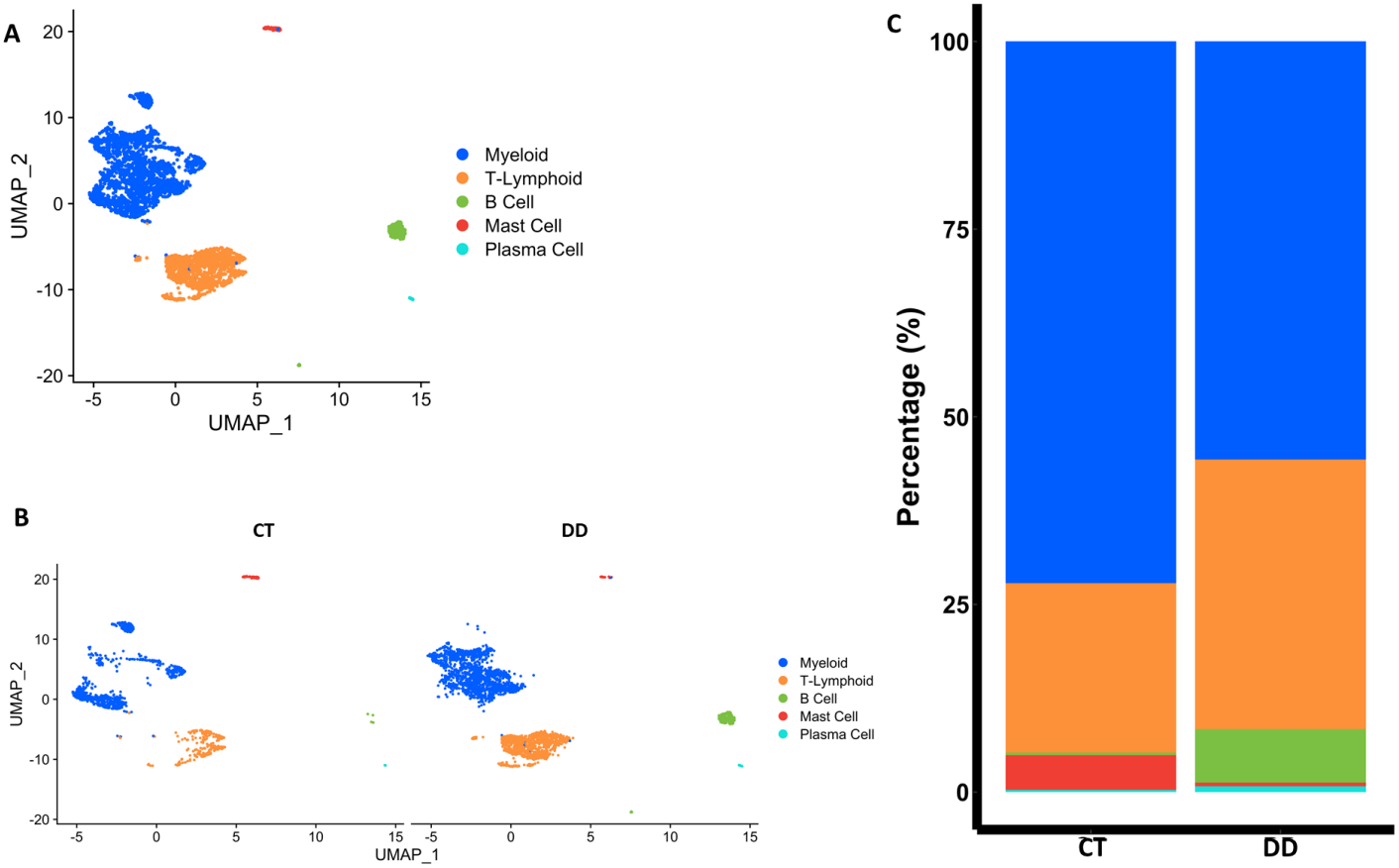


Figure 4.3 Immune cell landscape of Dupuytren's and carpal tunnel tissue. Carpal tunnel (n=3) and Dupuytren's tissue(n=3) underwent single cell RNA sequencing (A,B). The immune cell landscape was quantified in percentage of immune cells in the overall population (B). Quantitative analysis of immune cell populations (n=3,150 total immune cells sequenced) further supports the notion of a greater T lymphocyte population in Dupuytren's tissue relative to carpal tunnel (C).

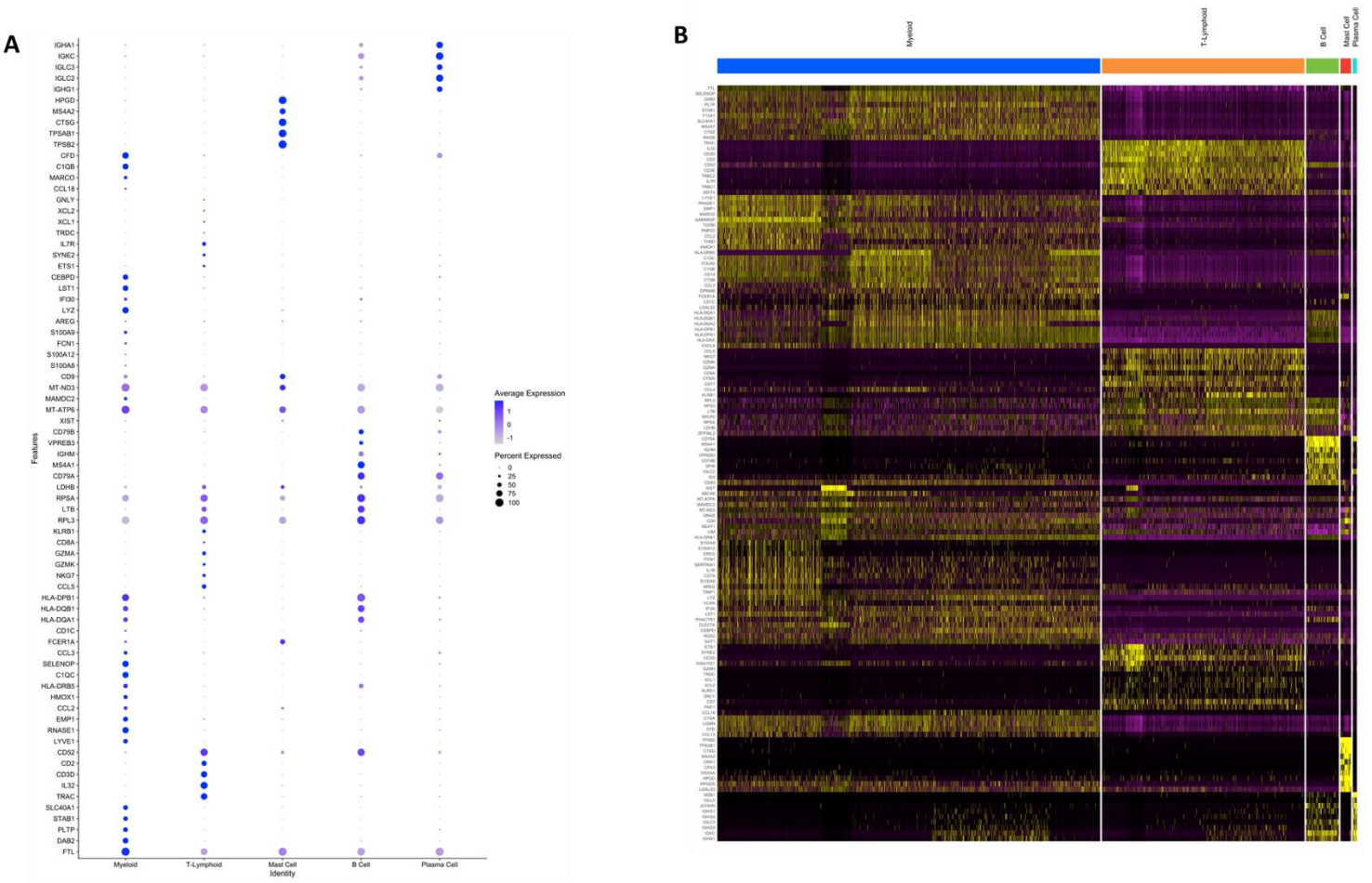


Figure 4.4 Clusters of immune cell related genes from bulk sequenced Dupuytren's tissue. Dupuytren's tissue (n=3) underwent single cell sequencing and immune cell related genes are described as percentage expressed (A) and heat map expression relative to the group mean (B) describe the leading populations as myeloid and T lymphocyte.

4.3.2 T cell subtypes are present within Dupuytren's disease

To better understand the role of T cells in Dupuytren's disease, we visualised CD4 (Figure 4.5A) and CD8 (Figure 4.5B) populations via immunohistochemistry. Interestingly, CD4 was expressed in cellular nodules and the tissue stroma whereas CD8 was expressed strictly in the cellular nodule of Dupuytren's tissue. Immunofluorescent visualization showed $CD3^+$ $CD4^+$ T cells throughout the tissue (Figure 4.5C); $CD3^+$ $CD8^+$ is localized to cellular nodules (Figure 4.5D).

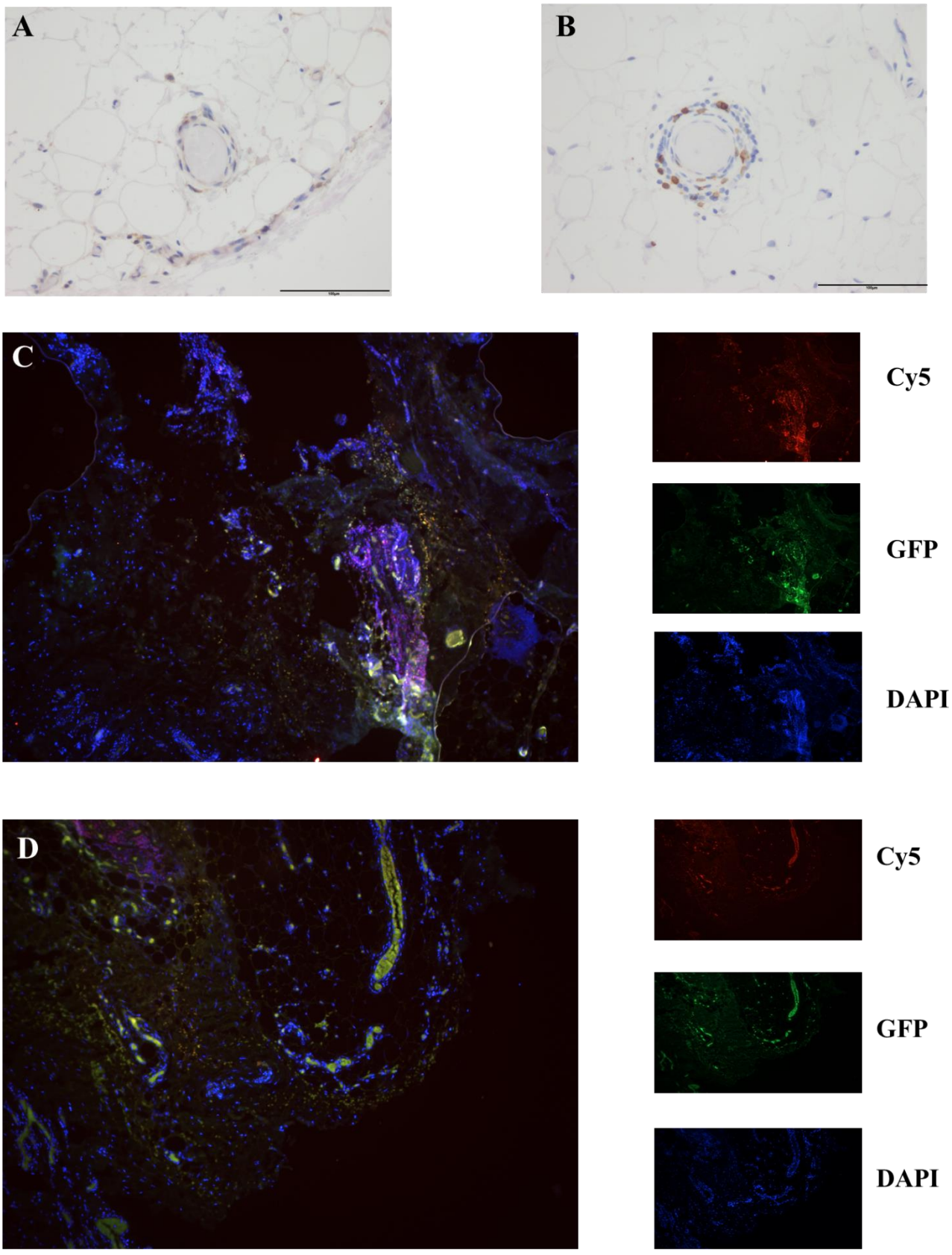


Figure 4.5 CD4 and CD8 T cells are present within Dupuytren's tissue.
Representative images of CD4 (A) and CD8 (B) T cells within Dupuytren's tissue (40X). Immunofluorescent staining of CD3 and CD4 (C) and CD3 and CD8 (D) in Dupuytren's tissue.

T helper 1 (T_H1) cells are members of the $CD4^+$ effector lineage and the T_H1 response is characterized by their secretion of IFN-γ, as previously described in this body of work. T helper 2 (T_H2) cells engage in the type 2 immune response and as previously described, produce IL-13 and are dependent upon transcription factors, like STAT, to elicit their effects. To further confirm the presence of these subtypes in Dupuytren's, we performed double immunofluorescent staining to visualize $CD3^+$ $CD4^+$ (Figure 4.5C) and $CD3^+$ $CD8^+$ (Figure 4.5D) subpopulations within the tissue stroma. This work describes the presence of T_H1 and T_H2 populations as described by CD4 and CD8, respectively.

T_H17 biology is implicated in several inflammatory diseases where matrix destruction is a predominant feature. I therefore evaluated the T_H17 T cell subtype expression of RAR-related orphan receptor gamma (RORγ), a transcription factor associated with IL-17. While mRNA level its expression was not notable (Figure 4.6A), representative images, show RORγ is dispersed throughout Dupuytren's tissue stroma (Figure 4.6B, C). Expression was confirmed quantitatively and semi-qualitatively via Bonar scoring ($p<0.01$) and percent positive staining ($p<0.01$) (Figure 4.6C &D).

Additionally, I investigated IL-17A signature in Dupuytren's tissue (Figure 4.7). Taken together, the presence of IL-17A supports a key role of T cells in the pathology of Dupuytren's disease. I planned to pursue further in vitro work on the effect of rhIL-17A on inflammatory related markers and matrix proteins in addition to experiments with Secukinumab, an IL-17A neutralising antibody. However, given the time constraints and lack of tissue samples available, I was unable to complete this.

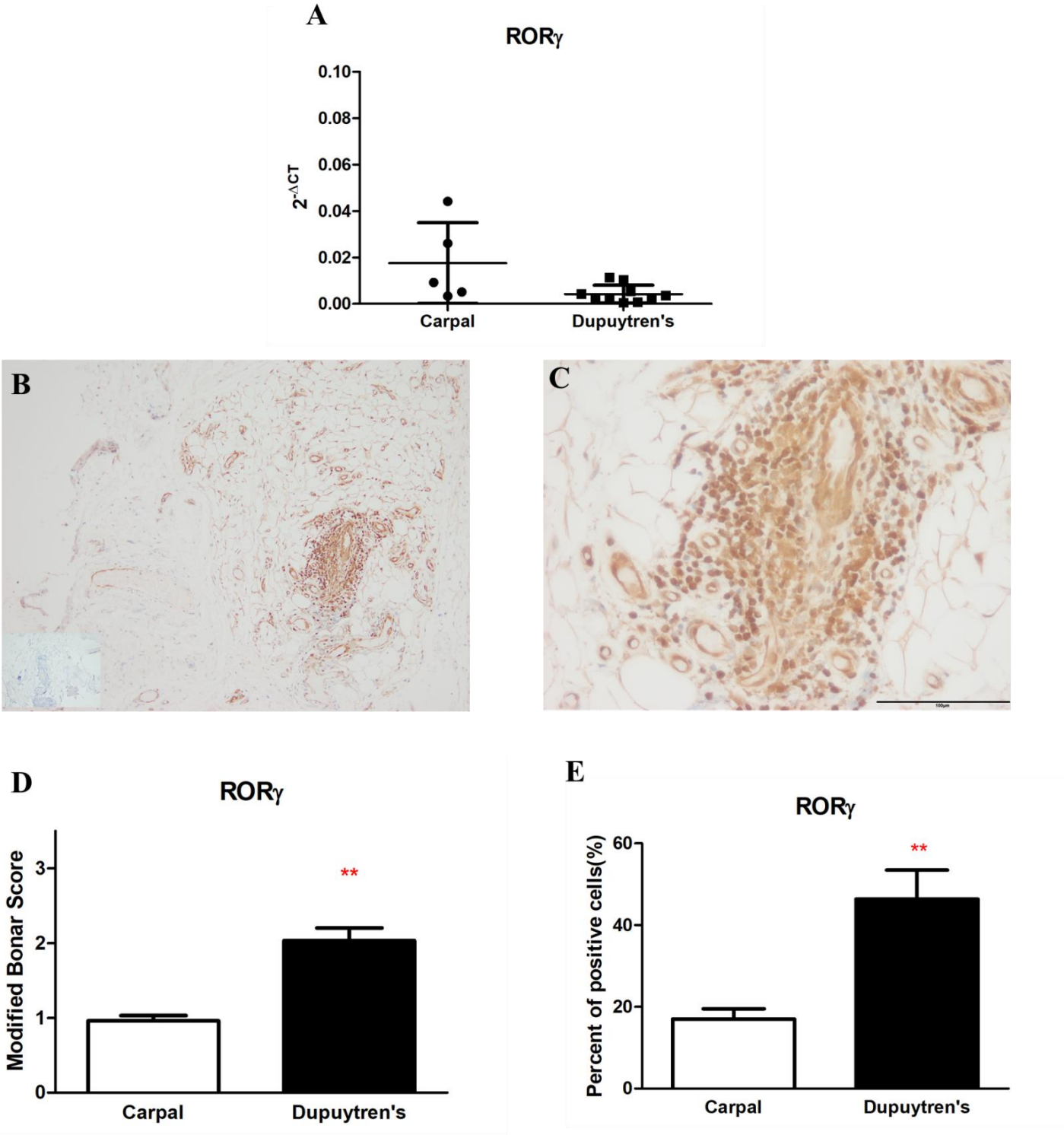


Figure 4.6 RORγ is expressed by immune cells and fibroblasts throughout Dupuytren's disease tissue.
(A) Relative mRNA expression of RORγ ($2^{-\Delta Ct}$) in carpal tunnel and Dupuytren's disease tissue. (B) Representative images of RORγ at 10X and 40X (C). (D) Graphical representation of modified bonar scoring and percent of positive cells (E). T-test $p<0.05$ * $p< 0.01$ **

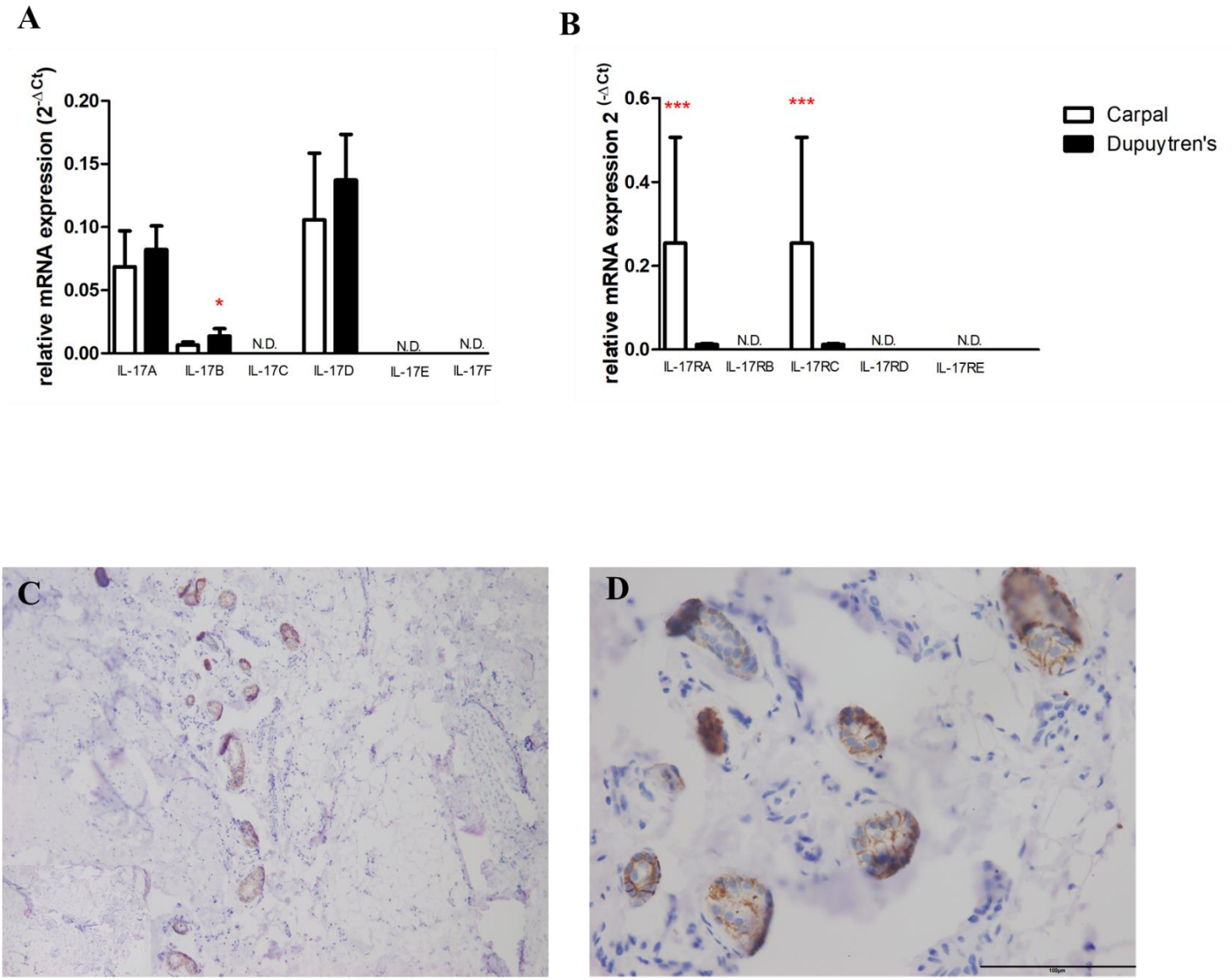


Figure 4.7 IL-17 isoforms within Dupuytren's disease tissue.
Relative mRNA level ($2^{-\Delta Ct}$) of IL-17A isoforms (A) and receptors (B) in carpal tunnel (n=5) and Dupuytren's tissue (n=10). Data is represented as mean ± SEM relative to GAPDH as the housekeeping gene (mean of duplicate analysis). Representative images of IL-17A immunohistochemistry 10x (C) and 40X (D). T-test *p<0.05 ***p<0.001

4.3.3 T cells are activated in Dupuytren's disease

Despite the limitations of being unable to conduct scRNA-seq on Dupuytren's nodules and cords, the samples used here were obtained earlier in my studies. Following the gradual release of easing restrictions, I was able to conduct library preparations to send the samples for bulk RNA sequencing of Dupuytren's microenvironments. This indicated that T cell related genes are expressed in Dupuytren's tissue (Figure 4.8). Further evaluation of the data highlight that T cells are present, CD27, and activated, CD69, CD70, within Dupuytren's tissue (Figure 4.8-4.10).

This data also describes a cellular difference between nodule and cord tissue. Dupuytren's nodules are highly cellular structures and within the sequencing data, they express T cell related genes higher than cord tissue. This, perhaps, is due to the fibrous nature of cord tissue as it is less cellular than nodules and therefore would not produce a high T cell marker signature.

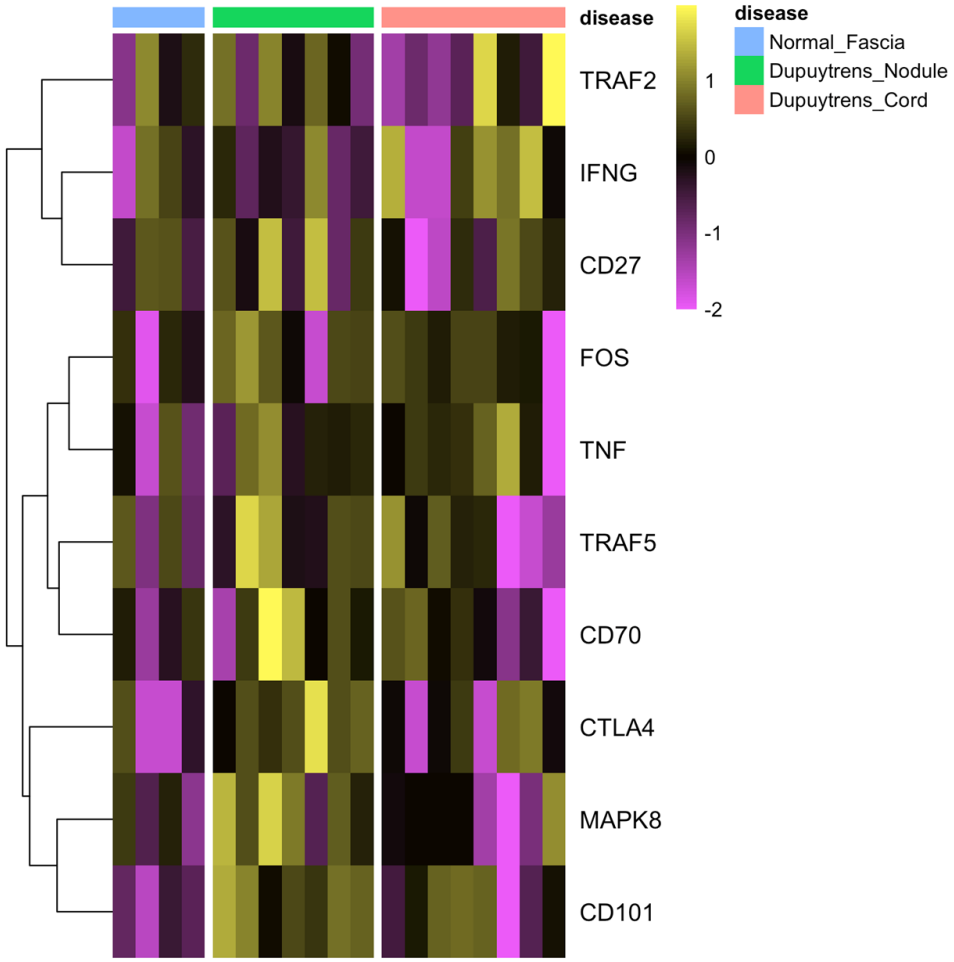


Figure 4.8 T cell related gene expression in Dupuytren's and control tissue. Normal fascia tissue (n=4), Dupuytren's nodules (n=7) and cords (n=8) underwent bulk RNA sequencing. The colour intensity represents row scaled (z-score) transcript expression, with purple as low and yellow as high expression.

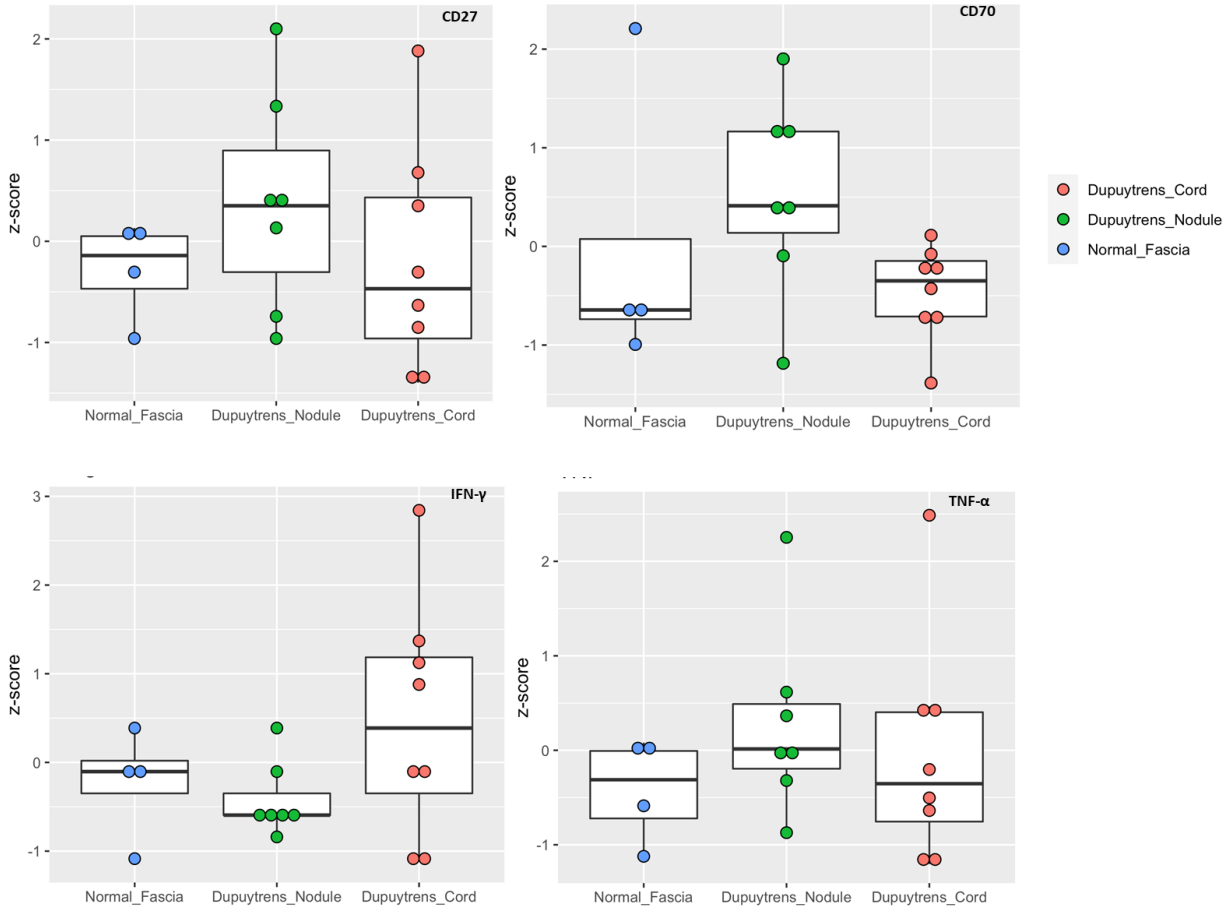


Figure 4.9 Z-score representation of T cell activation related genes sourced from bulk sequenced Dupuytren's microenvironments and carpal tunnel tissue. Carpal tunnel (n=4), Dupuytren's nodules (n=7) and cords (n=8) z-scores from the heatmap demonstrate T cell activation genes are present and allocate a score ranging from 3 to -1 describing how many standard deviations the tissue is from the mean.

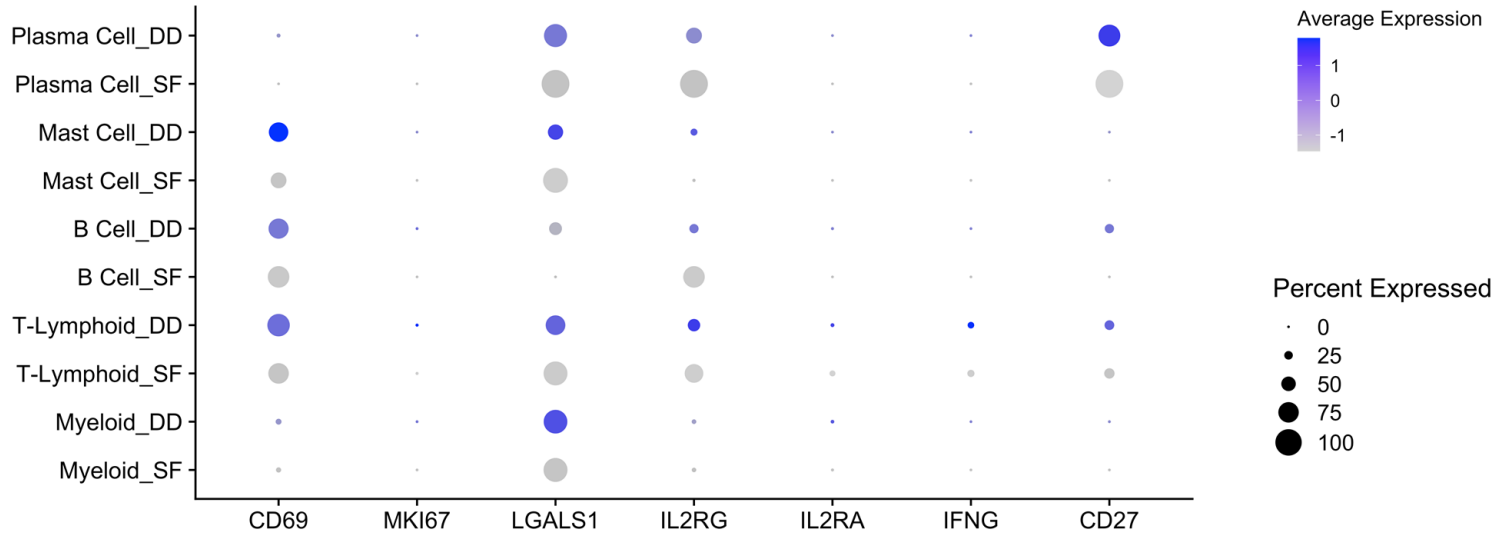


Figure 4.10 T cell activation marker expression in Dupuytren's and control tissue. Expression of selected genes associated with T cell activation in Dupuytren's tissue (DD) or control tissue (SF) from single cell RNA data. Size indicates percent expressed and color depicts deviation from the average expression, blue indicates higher gene expression than mean and grey describes less expression.

mRNA evaluation describes the presence of CD27 and CD70 is expressed more so in Dupuytren's compared to carpal tunnel (Figure 4.11A). Histological evaluation shows that T cells are present and activated ($CD3^+$ $CD27^+$) in Dupuytren's tissue alongside $CD70^+$ fibroblasts (Figure 4.11B). T cell associated markers are expressed more significantly in Dupuytren's disease compared to carpal tunnel tissue (Figure 4.11 C, D).

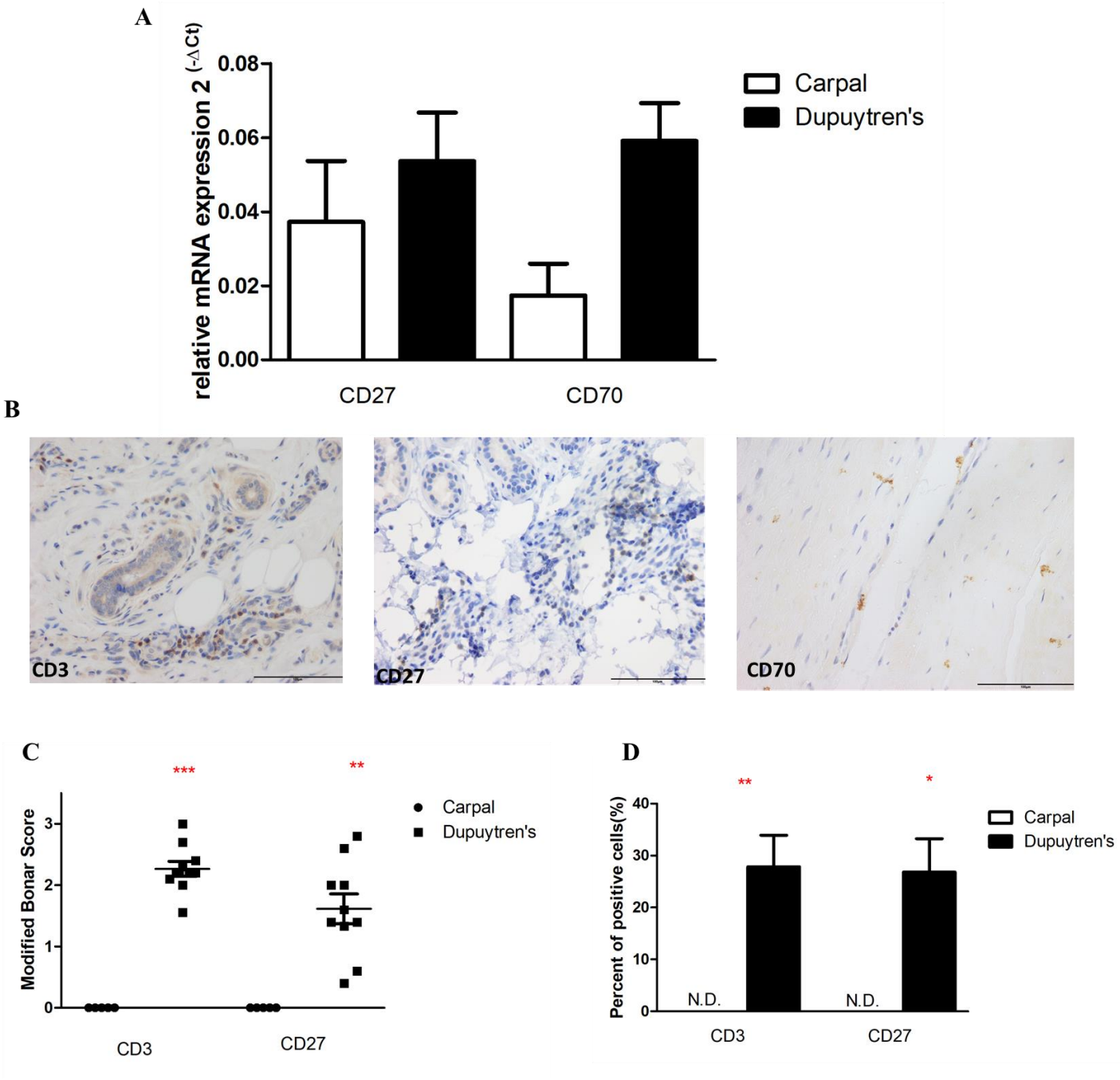


Figure 4.11 T cell activation markers in Dupuytren's disease.
(A) Relative mRNA expression of CD27 and CD70 in Dupuytren's disease and carpal tunnel tissue. (B) Representative immunohistochemistry images (40X) of CD3, CD27, and CD70. (C) Graphical representation of modified bonar score and percent positive staining of carpal tunnel (n=5) and Dupuytren's disease (n=10), mean ± SEM shown. Modified Bonar scoring system describes the mean score per donor based on five random high-powered fields. 0 indicates no staining, 1 indicates les than 10% of cells are stained positive, 2 indicates between 10-20% of cells are stained positive, and 3 indicates over 20% of cells are stained positive. T-test $p<0.05$* $p< 0.01$ ** $p<0.001$***

.

We additionally conducted cell to cell interaction analysis between stromal and immune cells from the single cell dataset using the programme CellChat and CellPhoneDB. Highlighting stromal-immune cell interaction within Dupuytren's tissue (Figure 4.9) and further interrogation points to the strength of T cell-fibroblast/myofibroblast interactions (Figure 4.10-4.14).

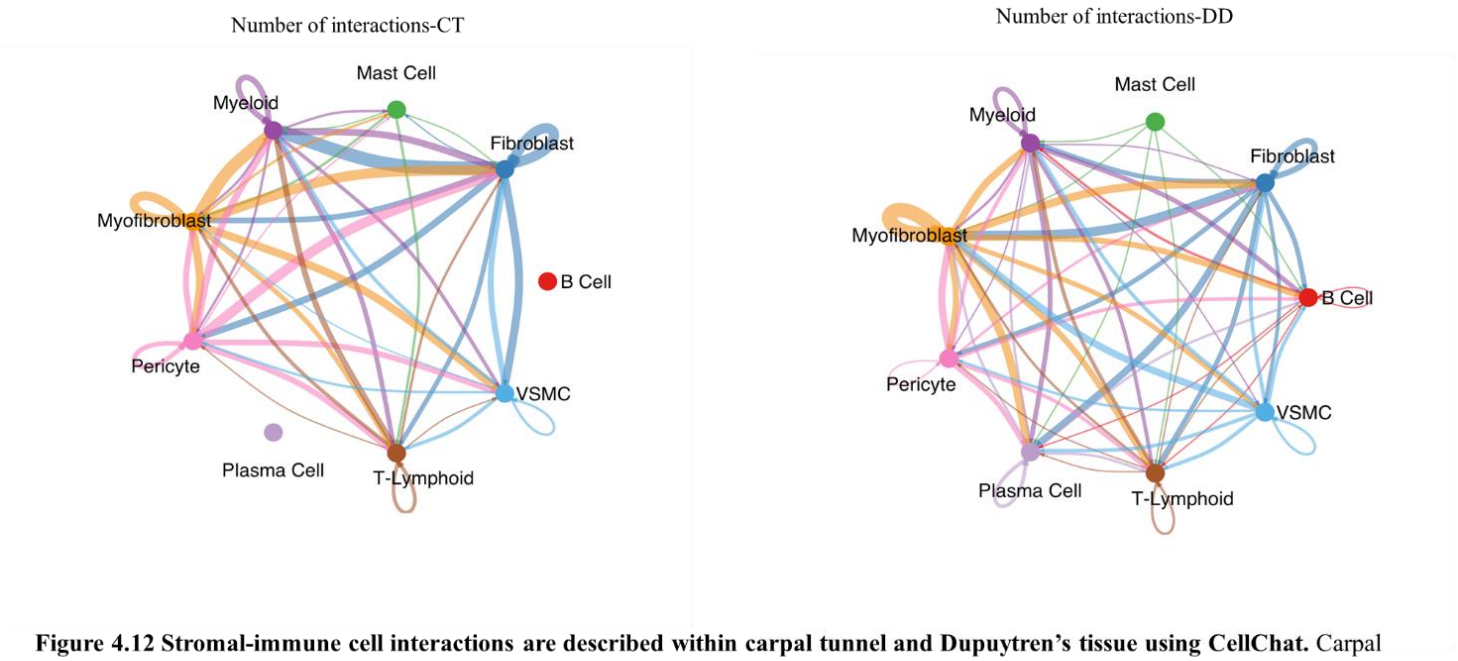
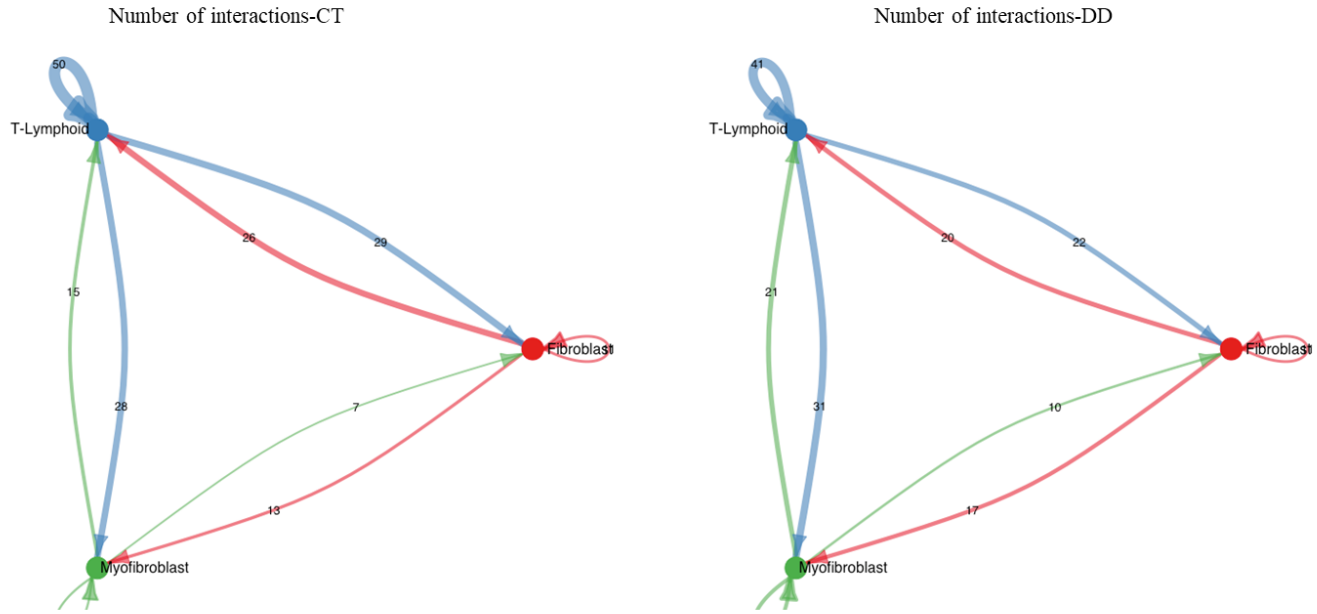


Figure 4.12 Stromal-immune cell interactions are described within carpal tunnel and Dupuytren's tissue using CellChat. Carpal tunnel (n=3) and Dupuytren's nodules (n=3) cell interactions were depicted by line and strength of interaction is depicted by line thickness. Color of line is used to describe different cell populations.

A



B

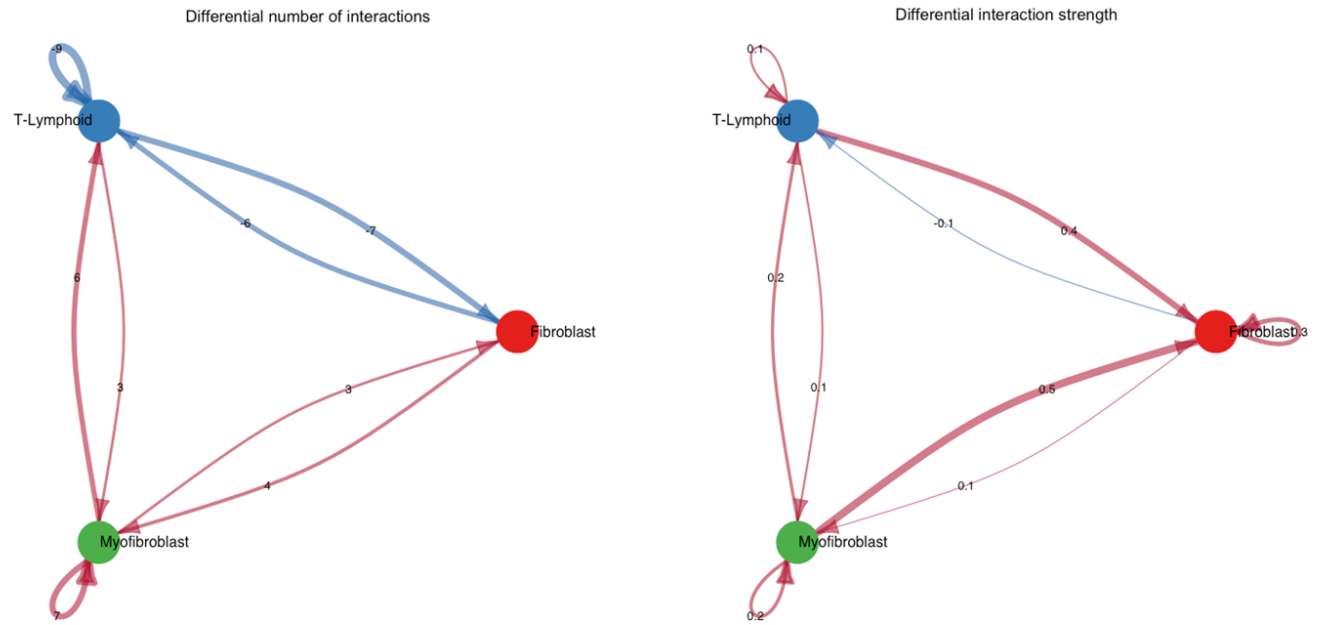


Figure 4.13 Quantification of T cell-fibroblast interaction in carpal tunnel and Dupuytren's tissue via CellChat.

Carpal tunnel (n=3) and Dupuytren's nodules (n=3) cell interactions were described quantitatively with the number of interactions between fibroblasts, myofibroblasts, and T-lymphoids (A). The interaction was further defined by line thickness where thicker lines equates to stronger interactions between cells (B).

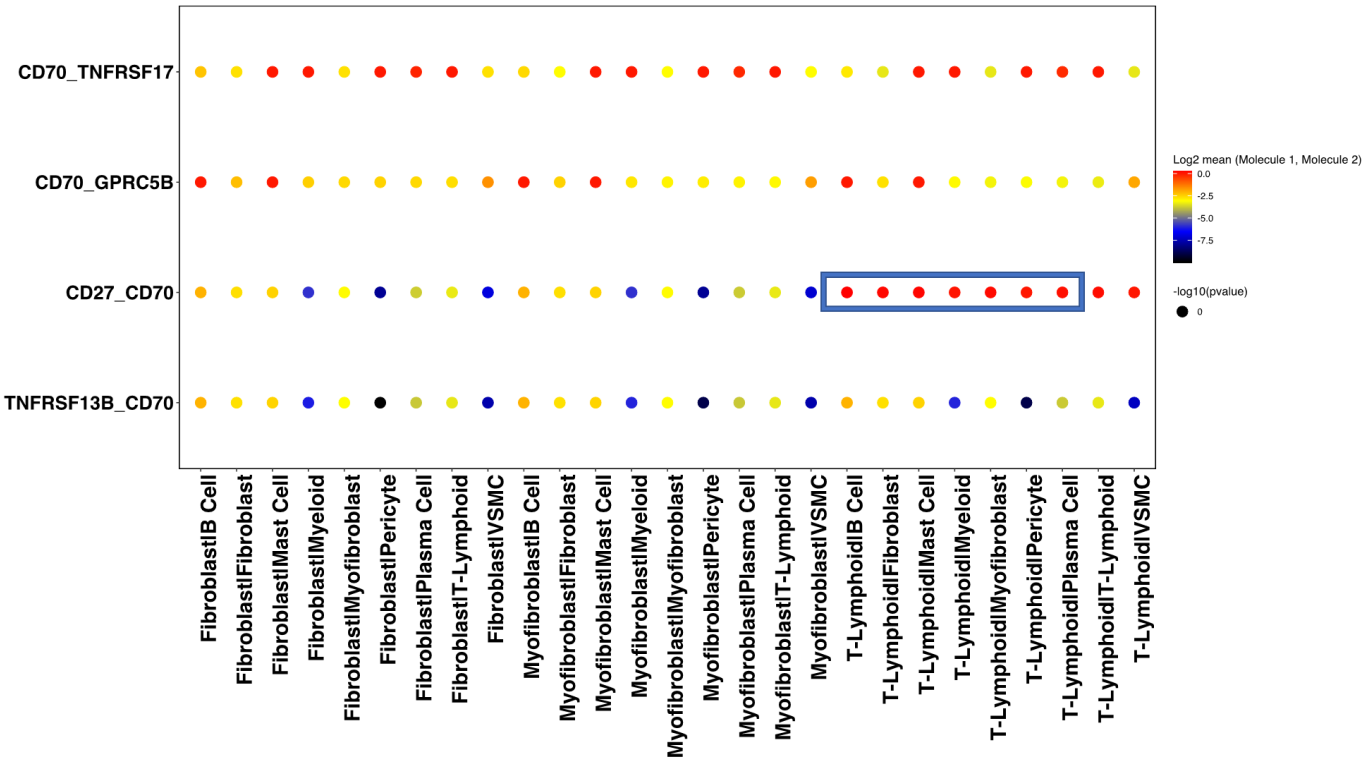


Figure 4.14 T cell-myofibroblast interaction and activation markers assessed in carpal tunnel and Dupuytren's tissue and visualised via CellPhoneDB. Carpal tunnel (n=3) and Dupuytren's nodules (n=3) cell interactions were depicted by color describing the deviation from the mean and further highlighting the importance of T cell-myofibroblast interaction (blue box).

4.3.4 Stimulation of Dupuytren's fibroblasts with CD27 and/or TNF-α mimics T cell, fibroblast interaction *in vivo*

CD27 is a member of the TNF superfamily (TNFSF) and a costimulatory molecule for T cell activation that interacts with CD70. We show that CD70 was expressed by Dupuytren's fibroblasts and suggest an aggravated response of matrix protein expression post stimulation with CD27 (Figure 4.15).

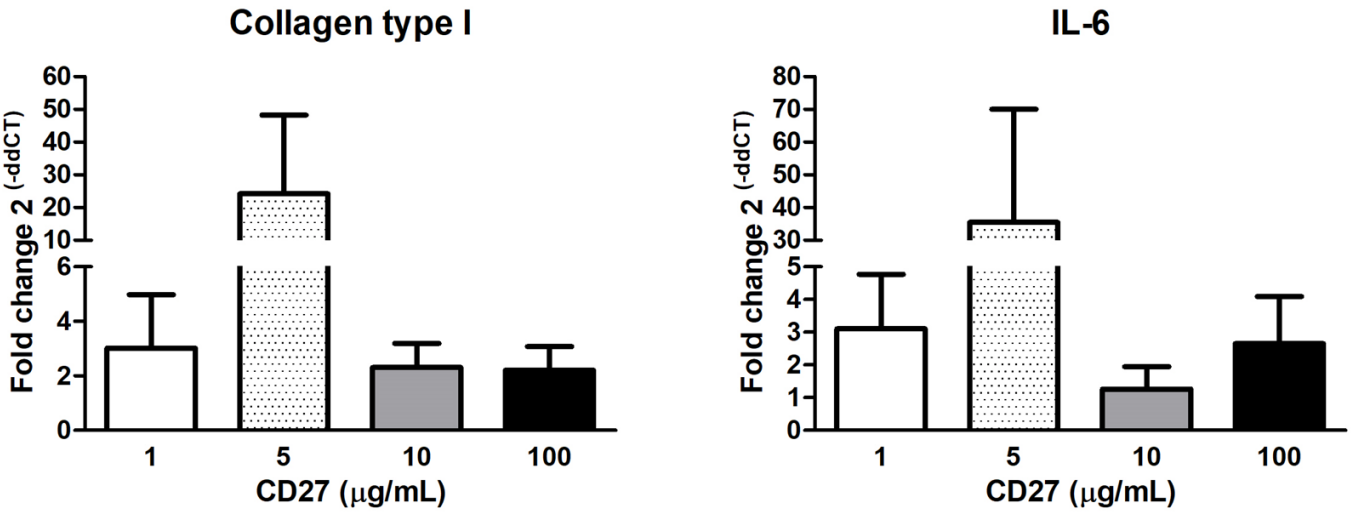


Figure 4.15 Effect of CD27 administration on Dupuytren's fibroblasts.
Dupuytren's fibroblasts (n=5) were stimulated for 24 hours with CD27 in a dose dependent manner. Fold change is normalised to control (unstimulated) samples. $p<0.05$* versus control Kruskal Wallis test

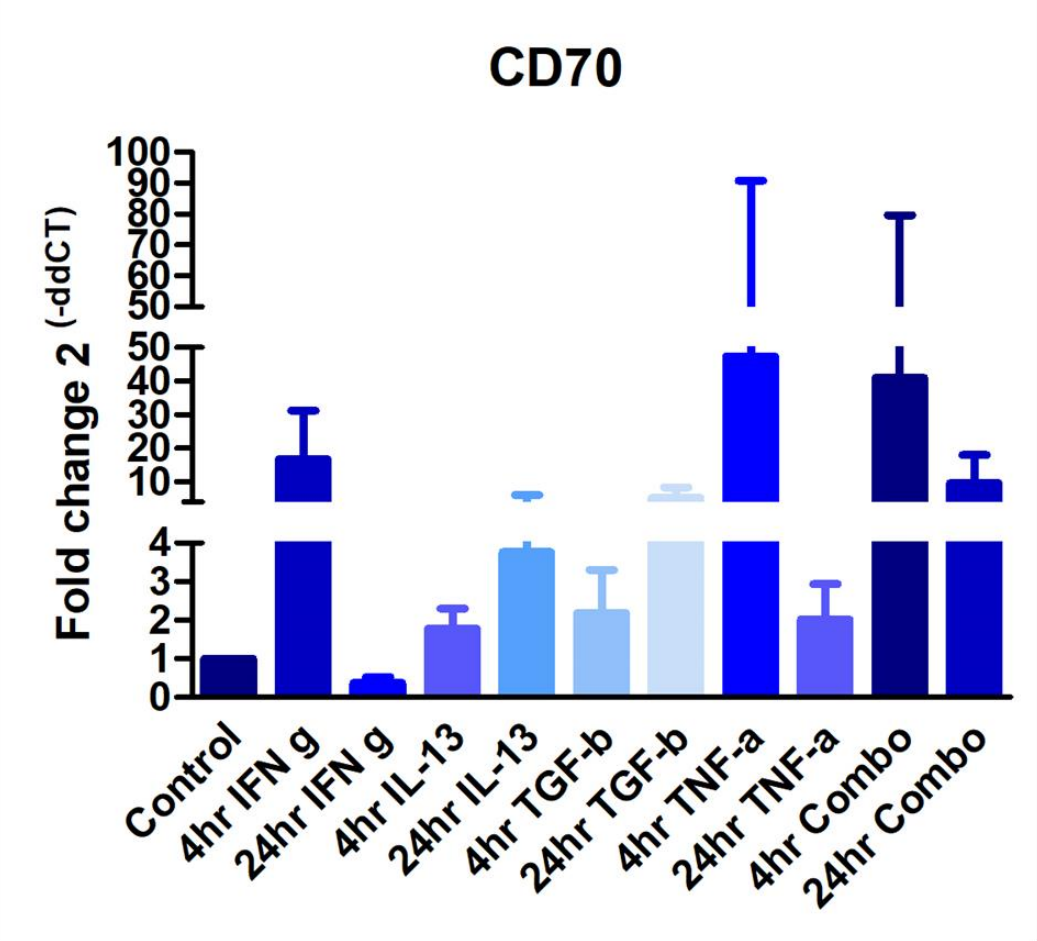


Figure 4.16 CD70 expression by Dupuytren's fibroblasts in response to cytokine stimulation.

Dupuytren's fibroblasts (n=5) were stimulated for 4 or 24 hours with IFN-γ (10 ng/mL), IL-13 (1 ng/mL), TGF-β (2 ng/mL), TNF-α (0.1 ng/mL) and a combination of all 4 cytokines at 25% of their individual concentration. $p<0.05$ * versus control Kruskal Wallis test

CD27 is a member of the TNF family, we sought to explore how CD70 expression changes under cytokine stimulation. We describe that CD70 expression by Dupuytren's fibroblasts increases in response to TNF-α (Figure 4.16).

Furthering our understanding of CD27 and CD70 interaction, we examined the effect of stimulating fibroblasts with CD27 and/or TNF-α. We found that matrix (Figure 4.17), inflammatory (Figure 4.18), angiogenesis (Figure 4.19), and stromal activation (Figure 4.20) marker expression increased in response to CD27 and TNF-α singularly and in concert.

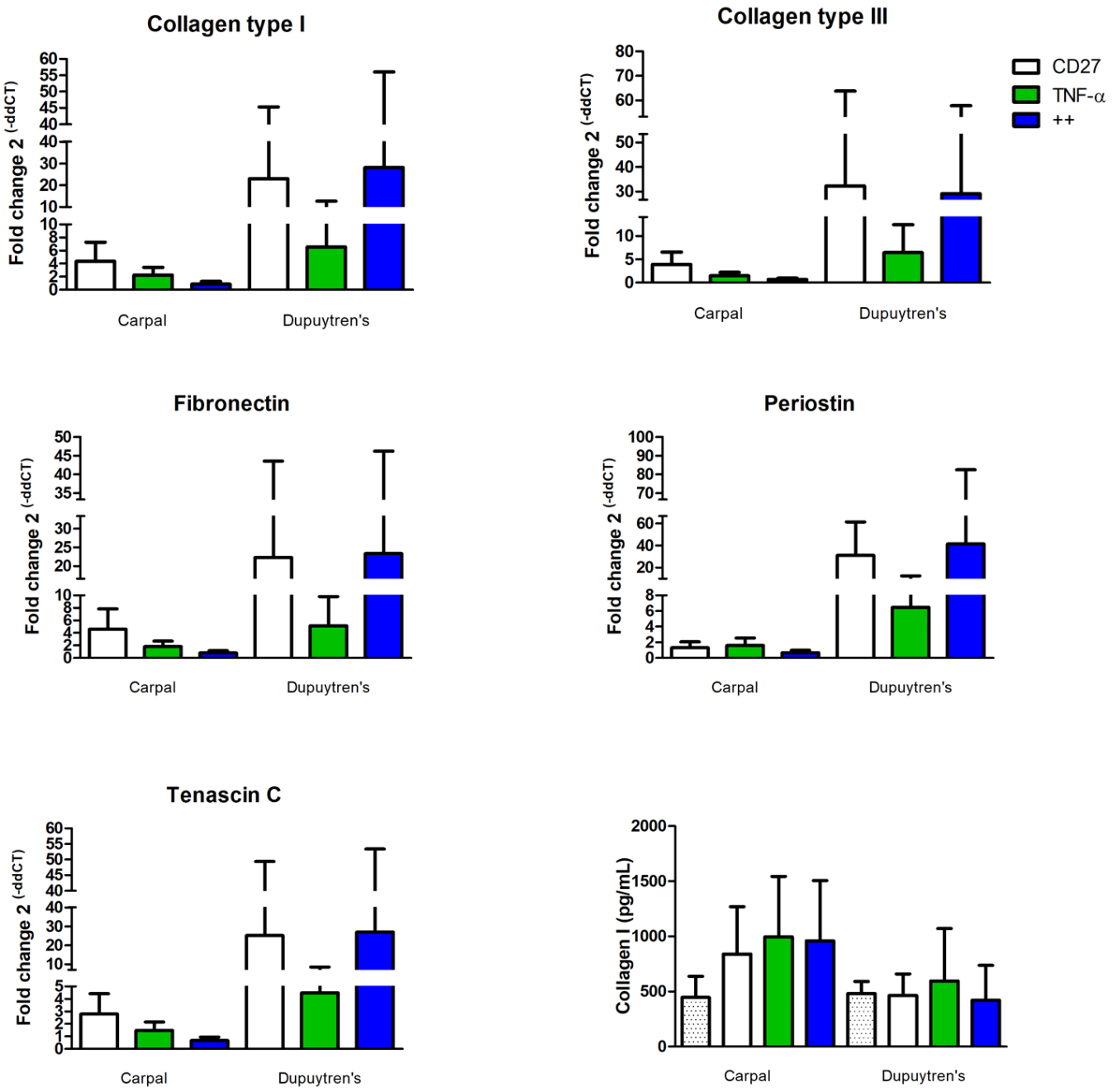


Figure 4.17 Matrix protein expression by Dupuytren's fibroblasts following CD27 and/or TNF-α stimulation.

Carpal tunnel and Dupuytren's fibroblasts (n=3) were stimulated for 24 hours with CD27 and/or TNF-α. Fold change is normalised to control (unstimulated) samples. $p<0.05$* versus control Kruskal Wallis test

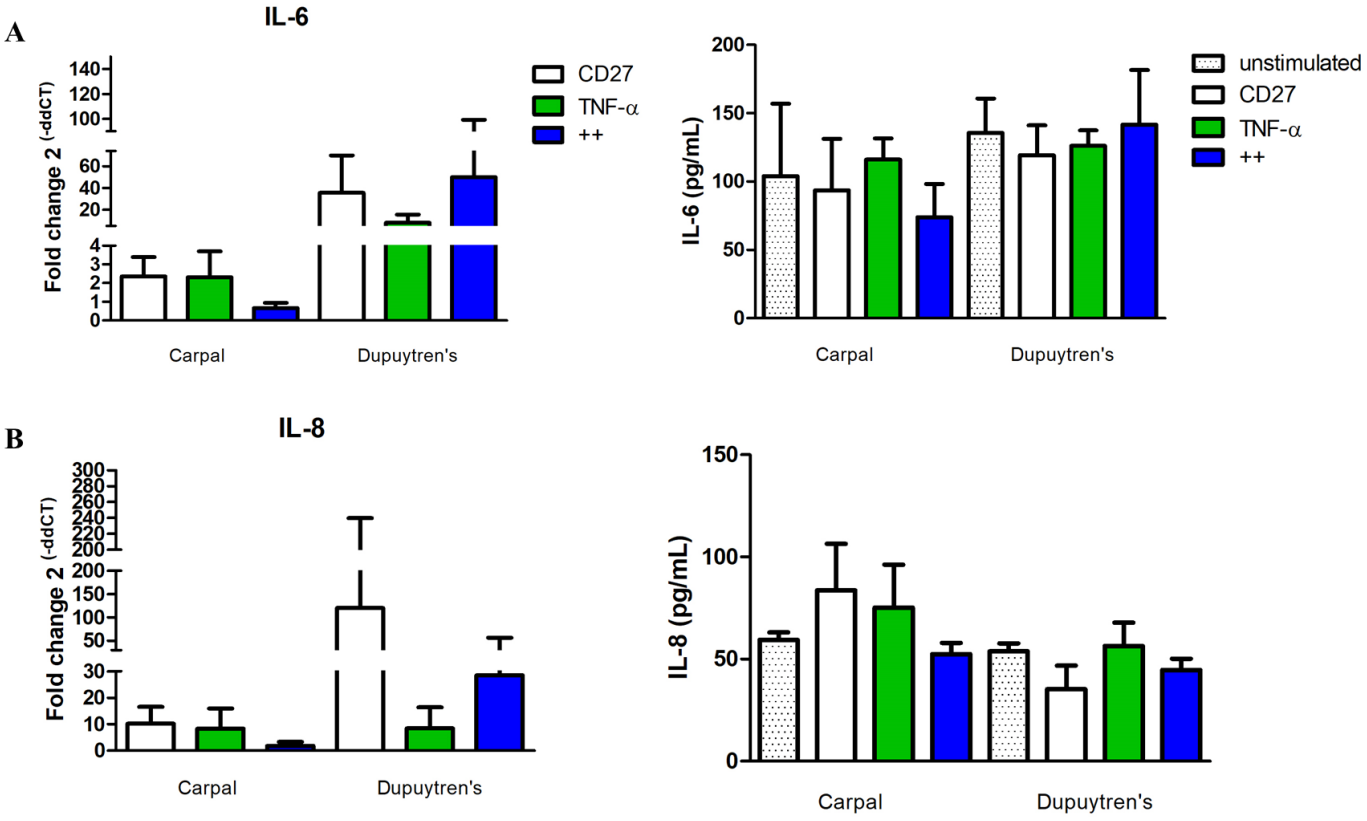


Figure 4.18 Inflammatory marker expression by Dupuytren's fibroblasts after cytokine stimulation. Carpal tunnel and Dupuytren's fibroblasts (n=3) were stimulated for 24 hours with CD27 and/or TNF-α. Fold change is normalised to control (unstimulated) samples. IL-6 (A) and IL-8 (B) secretion by fibroblasts was evaluated by ELISA. $p<0.05$* versus control Kruskal Wallis test

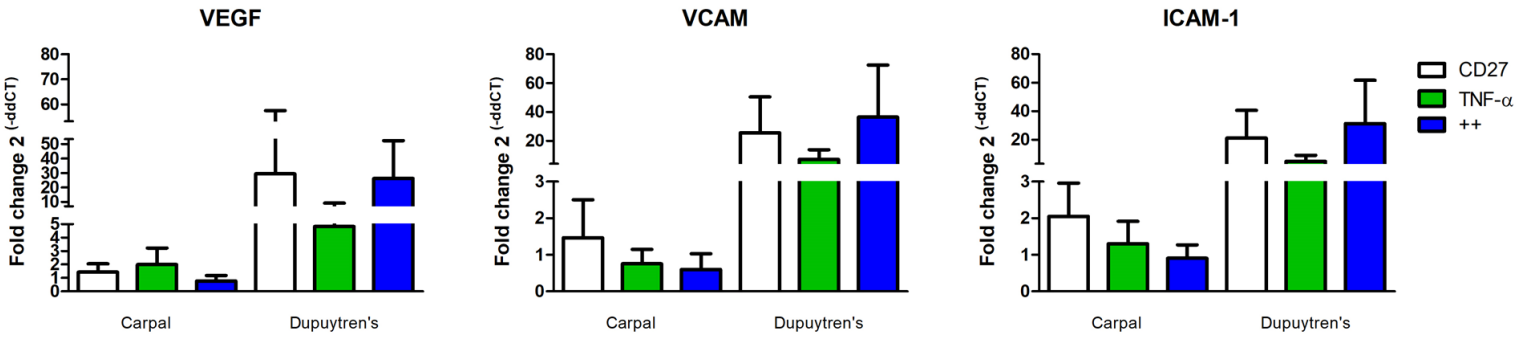


Figure 4.19 Angiogenesis marker expression by Dupuytren's fibroblasts after cytokine stimulation.
Carpal tunnel and Dupuytren's fibroblasts (n=3) were stimulated for 24 hours with CD27 and/or TNF-α. Fold change is normalised to control (unstimulated) samples. $p<0.05$* versus control Kruskal Wallis test

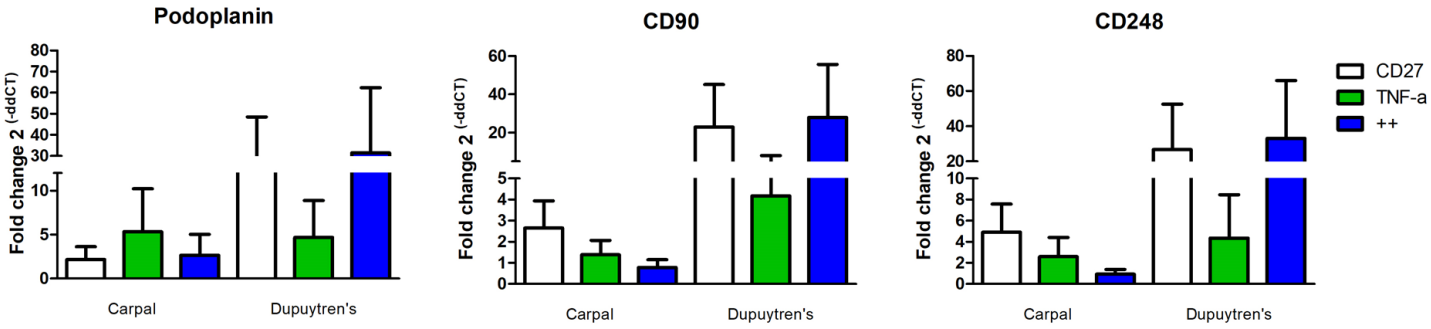


Figure 4.20 Mesenchymal stromal marker expression by Dupuytren's fibroblasts after stimulation with cytokines.

Carpal tunnel and Dupuytren's fibroblasts (n=3) were stimulated for 24 hours with CD27 and/or TNF-α. Fold change is normalised to control (unstimulated) samples. $p<0.05$* versus control Kruskal Wallis test

4.3.5 Freshly isolated T cells are not activated in response to paracrine factors

We previously describe, within this body of work, the effect of recombinant CD27 on fibroblasts and we sought to explore if T cells were similarly affected by CD70 and its costimulatory molecule CD28. CD69 appears on the surface of T cells and is involved in T cell subset differentiation and signalling between T cells and APCs[52]. We expect, given the co-stimulatory nature of CD27 and CD28 on T cell activation, the addition of CD28 would increase CD69 and, perhaps, CD27.

We found that T cells isolated from fresh blood did not alter their expression of CD27 or CD69, upon stimulated with CD28, CD70 or Dupuytren's cultured supernatant, representing soluble CD70 (Figure 4.21, 4.22). However, CD3+CD70 stimulation reduced CD27 expression by fresh T cells, suggesting a negative feedback loop within the stroma. Secretion levels of CD27 and IFN-γ did not change in response to stimulation, however compared to stimulation with CD3, T cells stimulated with CD3 and CD70 expressed CD27 significantly more ($p<0.05$). This work was impacted by the Covid-19 pandemic as blood combs were no longer available and while this data is interesting, it is difficult to draw conclusions as this work needs to be redone to verify this data and evaluate for statistical significance.

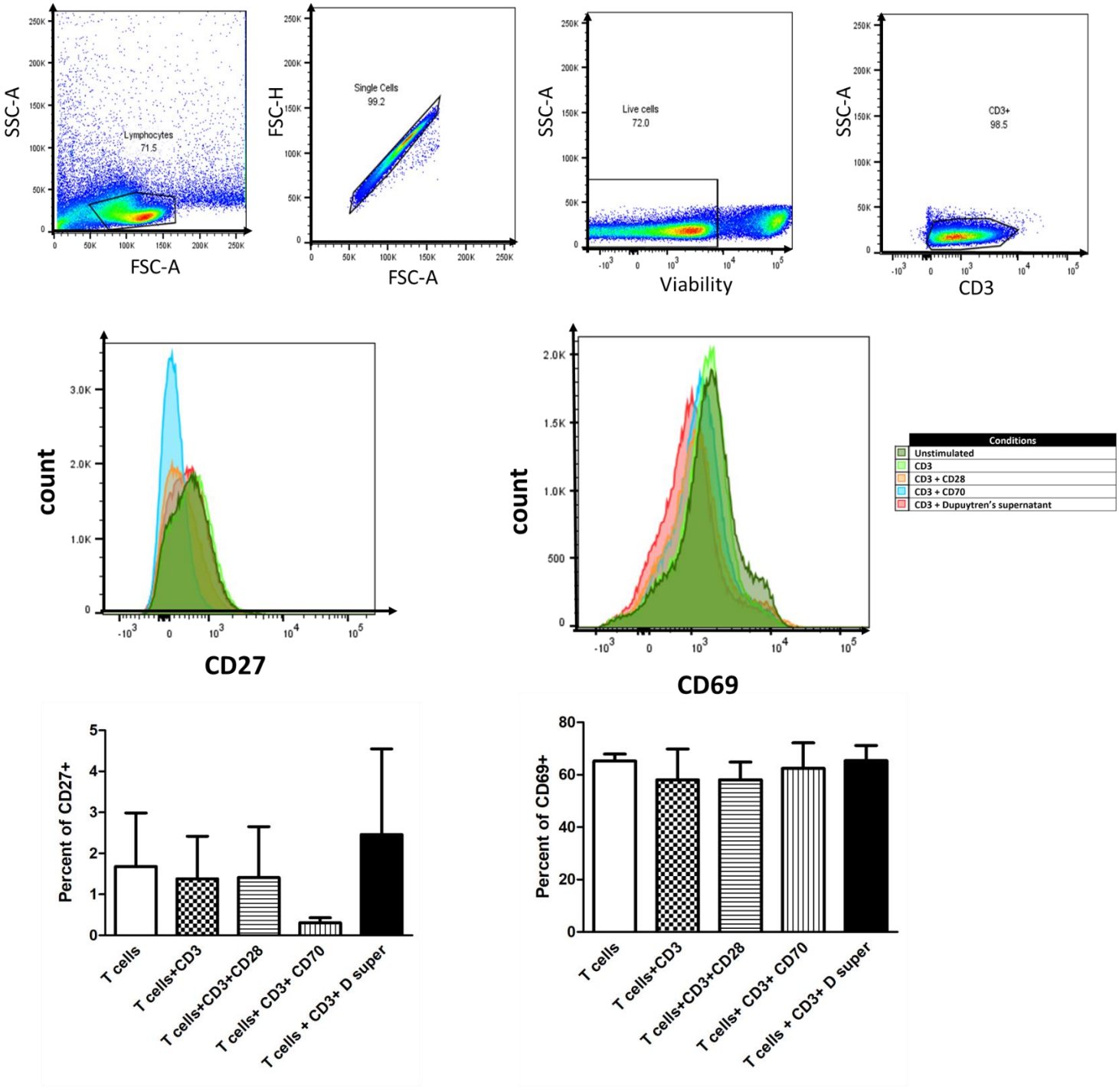


Figure 4.21 Freshly isolated T cells were stimulated with recombinant protein CD28 or CD70 or Dupuytren's supernatant. CD3+ T cells were stimulated with CD28, CD70, or Dupuytren's supernatant and T cell activation was evaluated by flow cytometry. . * $p<0.05$ Kruskal Wallis test

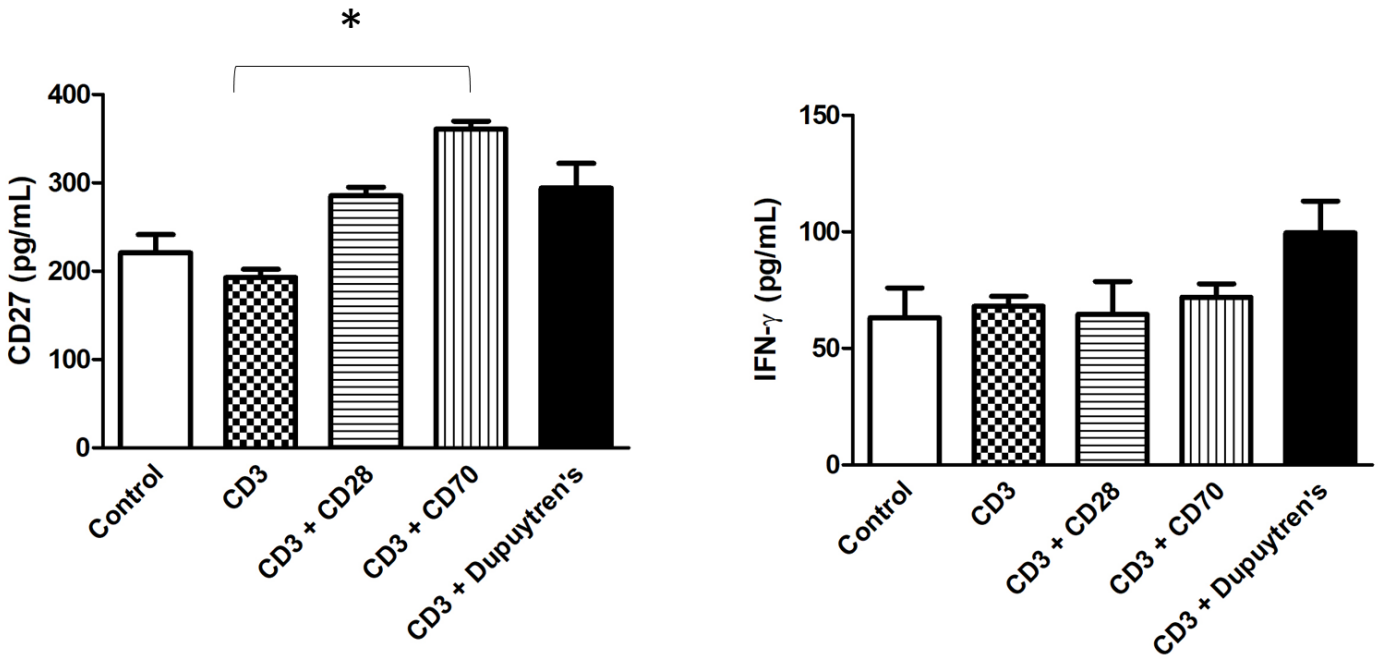


Figure 4.22 Secretion levels of T cell activation markers is unaltered by stimulation.
CD3+ T cells were stimulated with CD28, CD70, or Dupuytren's supernatant and T cell activation marker secretion was evaluated via ELISA. * $p<0.05$ Kruskal Wallis test

4.4 Discussion and conclusion

Dupuytren's disease is classified by two microenvironments: cellular nodules, characteristic of early disease, and a fibrous cord, which develops in later stages; giving rise to inwardly contracted fingers[29,84,102,105,265,310]. Given the differences in tissue composition[270,283], further analysis of the distinct nature of each environment could lead to a more complete understanding of the disease from early to late Dupuytren's[133,270]. The use of RNA sequencing has allowed for the advancement of cell interaction in various fibrotic diseases and allowed for the interrogation of specific cellular populations[41,65,74,233,283]. Current literature describes the presence of immune cells and fibroblasts as the primary cells driving the inflammatory, fibrotic nature of Dupuytren's. Well-described in cancer and arthritis, immune cells produce factors which drive the inflammatory response allowing them to be used as indicators of chronic inflammation[97,115].

Single cell RNA sequencing of Dupuytren's tissue describes myeloid and T lymphocytes, as the leading immune cells in Dupuytren's tissue. We visualized dendritic cells, macrophages and T cells via immunohistochemistry and Bonar scoring describes these population significantly more prevalent in Dupuytren's tissue compared to carpal tunnel. Further examination of the tissue reveals the immune cell landscape of Dupuytren's tissue is more cellular than carpal tunnel and hierarchical representation, via flow cytometry, describes T cells as the predominant cell in digested, fresh tissue. These results complement published works describing the importance of T cell-fibroblast interaction in cancer[21,127,314], renal fibrosis[21,314], and cardiac fibrosis[35,271,314].

Continuing the interrogation of T cells in Dupuytren's, analysis of sequenced Dupuytren's tissue reveal T cell related genes are prevalent within the tissue microenvironments. Further characterized of the T cell profile unveils clear T_H1 ($CD3^+$ $CD4^+$), T_H2 ($CD3^+$ $CD8^+$), and T_H17 ($ROR\gamma^+$) populations. Further interrogation of T_H17 behaviour highlight promotional role of IL-17A in promoting fibroblast behaviour. Published literature describe IL-17A in disease as a driving factor of inflammation and matrix protein expression[296,311].

T cell dysfunction is described as partial, or complete, loss of effector function and/or proliferative ability via inhibition of immune checkpoint pathways[101,232,288]. CAFs produce various immune checkpoint ligands, including PD-L1 and PD-L2, which interact with T cell receptors, promoting activation and differentiation. These ligand-receptor interactions have been demonstrated in vitro and are described as the activating and inhibiting effects of fibroblasts co-cultured with T cells[101,232,288]. Immunohistochemistry staining of Dupuytren's tissue define CD27 expression by T cells and CD70 on fibroblasts. Suggesting the importance of CD27-CD70 in mediating T cell inflammatory response[30,68,157,275].

CD70 is a transmembrane protein and the sole ligand for CD27, a transmembrane protein and member of the TNFR[148]. Studies also describe the presence of $CD70^+$ fibroblasts and how they engage in crosstalk with Tregs to promote their survival[119,163,172]. Our work describes that $CD70^+$ fibroblasts are present, and their tissue repair response is heightened after stimulation with CD27.

Within the tissue stroma, cells do not interact in a linear fashion as their cytokines interact with various cell types to mediate inflammation. Our sequencing data, and published works[3,117,276], describe that stromal-immune interactions occur in Dupuytren's. We show that Dupuytren's fibroblasts increase their expression of CD70 in response to various cytokines but most notably by TNF-α. TNF-α expression in Dupuytren's disease has been credited to macrophages[276] and coupled with the myeloid signature in the sequencing results, we suggest the possibility of a tri-cell interaction of macrophages enhancing CD70 expression by fibroblasts.

Within the tissue stroma, fibroblasts have been shown to produce soluble factors which influence angiogenesis and immune cell recruitment as well as directly affect T cell behaviour in cancer[21,127,314] and enhance fibroblast behaviour[314] and ECM component expression[35]. Furthering our exploration of a potential tri-cell interaction, we stimulated

fibroblasts with CD27, TNF-α, or both. CD27 mediates matrix production by fibroblasts in a dose dependent manner, which was further enhanced by the addition of TNF-α. CD27/TNF-α stimulation demonstrated an increase in inflammatory cytokine production, angiogenesis, and stromal activation related markers, and matrix protein expression further highlighting the effect of T cells on fibroblast behaviour.

Understanding the signalling cascade between T cells and fibroblasts can reveal potential targets. TRAFs have been described as signal transducers for the TNFR family[149,285,302]. Previous works describe that CD27 activates the NFκB and SAPK/JNK pathways via TRAF2 and TRAF5 and knockdown studies demonstrate that inhibition of TRAF2 and TRAF5 prevents CD27 signalling[6,285]. In murine arthritic studies, blockade of the CD27-CD70 axis via anti-CD70 antibody prevented development of collagen induced arthritis[99,206]. Similarly, anti-TNF therapy prevented the effect of CD27-CD70 axis in arthritic models[234,294]. TRAF2 is expressed by various cell types (including macrophages, fibroblasts, and T cells) and has been shown to be crucial in mediating inflammation[6,285].

TRAF2 activates the NFκB pathway, and it activates MAPKs: JNK, ERK 1 &2, and p38[149,234,285,302]. In cardiovascular murine models, TRAF2 overexpression led to fibrosis and ventricular dysfunction[206,234]. TRAF5, a similar homolog to TRAF2, plays a role in the inflammatory response instrumented by $CD4^+$ T_H2 and T_H17 T cells-among other cell types like fibroblasts and macrophages- and elicits activation of NFκB1 and NFκB2 pathways[34,149]. In atherosclerosis models, TRAF5 knockdown led to the development of accelerated atherosclerosis coupled with an influx of immune cells. In cardiac fibrosis models, TRAF5 deficiency led to the development of fibrosis suggesting that TRAF5 plays anti-fibrotic and anti-inflammatory role[149,303]. Our sequencing data describes the presence of not only TRAF2 but also TRAF5 in both Dupuytren's nodules and cord tissue. Future work could explore whether TRAF2 and TRAF5 act like MMPs/TIMPs where an imbalance leads to the development of disease and explore their role in CD27 signalling.

Studies examining the signalling pathways of CD70 in murine B cells describe the administration of anti-CD70 antibodies dampens downstream PI3K and MEK pathway targets PKB and ERK, respectively[118]. Manipulation of the PI3K and MEK pathways is common in cancer studies, and these have not been studied in soft tissue disease[87]. We suggest that manipulation of their downstream targets will shed light on the signalling cascade of CD70 and this, in combination, with TRAF2/TRAF5, could help further understand T cell inflammation in Dupuytren's disease.

Given that Dupuytren's fibroblasts responds dramatically in response CD27 stimulation, we suggested that CD70 could provide a similarly robust signal on $CD3^+$ T cells. Stimulation of freshly isolated T cells with CD3, CD3 & CD28, CD28 recombinant CD70 or CD3+ soluble CD70, via Dupuytren's supernatant, did not elicit the expected effect. Compared to unstimulated T cells, the addition of CD70 or Dupuytren's supernatant produced no significant changes in CD69 expression; also suggesting that the CD69 signature seen in sequenced Dupuytren's disease could stem from mast cells and lymphocytes. Given that we saw a strong mast cell population expressing CD69 by sequenced tissue, could support the notion that CD69 in Dupuytren's comes from mast cells.

Published works describe that CD70 production is tightly controlled[11,145] and given our findings that paracrine signalling did not result in an increase in CD27 expression prompts the suggestion that CD27-CD70 interaction could require direct cell to cell communication. Future works would assess T cell and fibroblast behaviour in a direct co-culture system. This interaction could also allow for exploration of TRAF2/5 signalling by T cells and its effect on CD70 expression. As well as the possibility of a negative feedback loop between CD27/CD70 expression.

Chapter 5: Discussion and future directions

5.1 Summary of key findings

Dupuytren's disease a fibroproliferative condition defined by chronic inflammation and excess matrix protein expression. Treatment can involve removal of the affected tissue but unfortunately, in some cases, the disease reappears within the patients' lifetime. Accounting for the disease's recurring nature, we suggest the immune system plays a role in not only perpetuating chronic inflammation but also the tissue memory associated with its resurgence.

Our findings in the present study describe that the stromal compartment, identified by the presence of mesenchymal stromal fibroblasts, stromal cells, and endothelial cells, is activated within the tissue. The 'activated phenotype' is further exacerbated in response to mechanical damage and/or stimulation by cytokines circulating within the tissue, creating an in vitro environment which mimic the *in vivo* tissue environment.

scRNA sequencing of Dupuytren's tissue microenvironments revealed that myeloid and T lymphocytes were the leading cell populations seen. Further analysis revealed strong fibroblast/myofibroblast-T lymphocyte interactions and $CD70^{+}$ T cell activation was explored. T cells expressed activation markers and their interaction with fibroblasts was mediated via CD27-CD70 interaction. Fibroblast stimulation with recombinant CD27 enhancing fibroblast production of inflammatory, matrix, angiogenic, and stromal related markers. Highlighting T cell-fibroblast crosstalk as a contributing factor in mediating Dupuytren's fibrotic signature.

5.2 Stromal compartment activation contributes to the fibrotic nature of Dupuytren's

The stromal compartment consists of mesenchymal stromal cells which differentiate in response to environmental factors. Mesenchymal stromal cells have been identified in Dupuytren's disease and these populations have been described as functionally distinct and representing various states of activation[19,153]. Studies that perform single cell analysis to

describe the landscape of the tissue microenvironments and, within the stromal compartment, describe the presence of myofibroblasts, endothelial cells, and immune cells[19,153].

Sequencing of Dupuytren's tissue revealed a myofibroblast population and matrix protein expression. Visualisation of the tissue demonstrated stromal fibroblasts were characterized as not only activated (FAP^+) but also differentiated into α-SMA^+ myofibroblasts. Published works describe that fibroblast populations in disease are heterogeneous in their expression of FAP and α-SMA and this contributes to the developing of fibrosis[154,207]. Future studies will further describe whether fibroblast and myofibroblast populations are heterogenous in regard to their expression of FAP and α-SMA within nodules and cord tissues.

We also characterized stromal endothelial cells dispersed within the tissue and localized around the blood vessels. Stromal cells in the tissue and their associated activation markers: CD90, CD248, and podoplanin are present in Dupuytren's and, described in other disease models, play a role in the development of fibrosis.

Studies describe the use of an *in vitro* scratch assay as a means to study cell behaviour in response to damage[158,237]. We used this model to explore whether Dupuytren's fibroblasts respond in an increasingly damaged manner post-scratch. Our data supports the finding that in vitro scratch increases the inflammatory nature of Dupuytren's fibroblasts. This mechanical damage model also showed changes in matrix protein and stromal activation expression. Within the stromal compartment, stromal cells interact with resident and infiltrating immune cells to mediate the inflammatory response[21,196]. Mimicking the paracrine signalling seen in the tissue between immune and stromal cells, we stimulated Dupuytren's fibroblasts with IFN-γ, IL-13, TNF-α, and TGF-β1 and performed a scratch assay.

Immune cells have been shown to mediate the inflammatory response and tissue repair[47,110]. Published works describe crosstalk between fibroblasts and macrophages via TNF-α promote myofibroblast differentiation and matrix protein expression[276]. TNF-a signalling in Dupuytren's disease is described as being mediated, in part, to Wnt/β-catenin signalling. TGF-β stimulation of Dupuytren's fibroblasts acts via Smad signalling. Akbar *et al.* 2020 describe that mast cells interact with myofibroblasts via IL-13[3]. Additionally, T cells produce IFN-γ to differentiate fibroblasts into matrix protein producing myofibroblasts. They conclude mast cells, T cells and fibroblasts/myofibroblasts communicate via STAT signalling. Our results describe the cytokine storm assessed, or individual cytokines, acts upon fibroblasts to increase inflammation, matrix, and stromal markers, with and without the mechanical damage model. The changes we see can be credited to specific stromal-immune cell crosstalk and further explained via signalling cascades from which therapeutic targets can be developed.

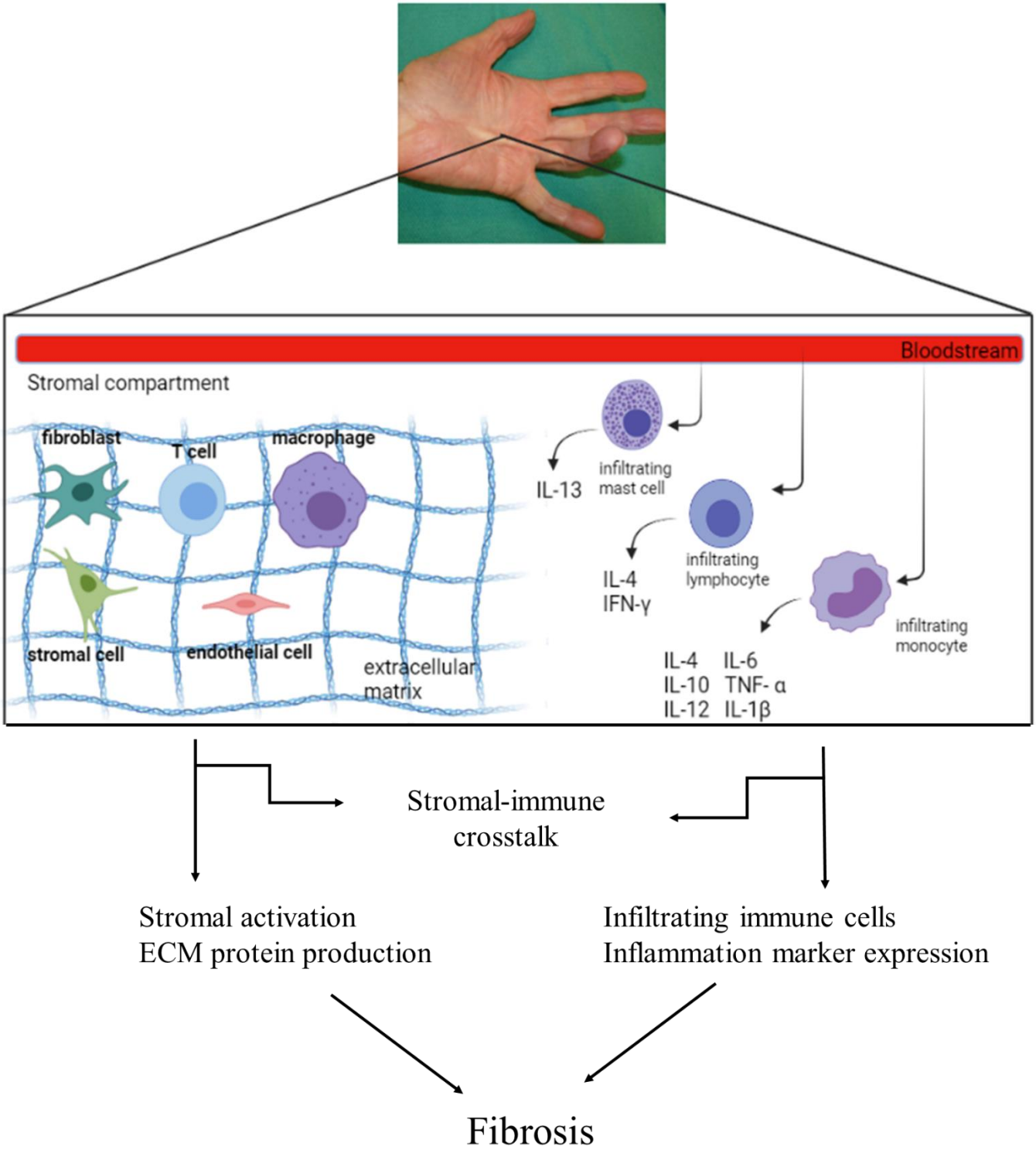


Figure 5.1 Illustration of stromal compartment activation and immune cell infiltrate lying the foundation for fibrosis. When damaged, palmar tissue does not heal in a resolved manner, the stromal compartment is persistently activated and consistently producing matrix proteins and immune cells continue to infiltrate and produce inflammatory mediators. This steady production of matrix proteins and inflammatory mediators leads to chronically inflamed tissue and tissue fibrosis. Figure made using Biorender.

5.3 Immune cell hierarchy in Dupuytren's disease highlights potential stromal-immune crosstalk

Published studies describe fibrosis as a complication of tissue healing, driven by fibroblasts and immune cells[110]. Immune cells infiltrate from the blood stream and differentiate in response to environmental factors. Once activated, innate and adaptive immune cells produce cytokines, chemokines, and growth factors which modulate signalling cascades resulting in chronic inflammation and fibrosis[110]. Immune cells are complex in their behaviour and a descriptive understanding of the immune cell landscape of Dupuytren's microenvironment could aid in the development of targeted therapies.

Using RNA sequencing, we were able to uncover a variety of immune cell populations and decipher which cells were interacting within the tissue environment and which capacity. Results describe strong myeloid and T lymphocyte populations and visualisation via immunohistochemistry and flow cytometry depict that not only are T cells the leading immune cell but Th1, Th2, and Th17 subsets are present in the tissue.

Literature describing T cell interaction in soft tissue diseases like tendinopathy, arthritis and frozen shoulder describe the role of T_H17 immune cells in disease[4,89,184]. Interrogating Th17 behaviour in Dupuytren's disease, pointed to IL-17 isoforms and receptors present in the tissue coupled with the presence of one of its transcription factors, RORγ. Future work exploring the role of IL-17A on Dupuytren's fibroblasts will examine the mechanism of interaction and whether Secukinumab can reduce the inflammatory signature.

5.4 T cell-fibroblast interaction is a contributing factor in the inflamed nature of Dupuytren's

Fibrosis disease models describe T cells interact with fibroblasts promoting myofibroblast differentiation and matrix production. This also promotes T cell related cytokine production which further exacerbate the immune response[314]. Classically published works describe the

presence of T cells in Dupuytren's disease[19,98,180]. Akbar *et al.* (2020) describe that Dupuytren's fibroblasts and T cells interact leading to differentiation into myofibroblasts and increased matrix protein[3]. The present study supports that not only is this stromal-immune cell interaction present in the tissue, but T cells are the leading immune cell in the population in fresh Dupuytren's tissue. Within the tissue, we characterize T cells as activated via the presence of CD27 on T cells and CD70 on fibroblasts.

CD27 is a member of the TNF receptor (TNFR) family and, in humans, in strictly expressed by cells of the lymphoid lineage[275]. CD70 has been described as being expressed on T cells, but the present study describes CD70 expression only on fibroblasts. CD27 interacts with TNFR associated factors 2 and 5 (TRAF2, TRAF5) mediating activation of NFκB signalling causing enhanced cell survival and cytokine production[285,302].

Dupuytren's fibroblasts stimulated with CD27 increased matrix and inflammatory marker expression and stimulation with macrophage produced[276] TNF-α increased CD70 expression. We suspect this finding could be due the large myeloid population seen within the tissue and that CD27 is a member of the TNF receptor family[107,223,254]. Examining the effect of stimulation with recombinant CD27 and/or TNF-α demonstrated an increase in matrix, stromal and inflammation related markers. Further purporting stromal-immune crosstalk between T cells and fibroblasts mediating the stromal activation and the inflammatory signature seen in Dupuytren's disease. Interestingly, freshly isolated T cells did not increase their expression of CD27 in response to stimulation with co-stimulatory molecule CD28, recombinant CD70 or soluble CD70, via Dupuytren's cultured media. Future works would explore whether CD27 agonism could be achieved via direct Dupuytren's fibroblast/T cell interaction.

T cells have been reported to play a role in revascularization of damaged tissue. Fibrotic literature describes angiogenesis as a contributing factor to disease progression in liver and pulmonary fibrosis[79,124,235]. Additionally, murine studies describe CD70 knockout

results in impaired vascular development[252]. We examined common angiogenesis related marker expression in response to stimulation and found that Dupuytren's fibroblasts increase all angiogenic markers examined. Future studies could further examine the role of angiogenesis in the development of Dupuytren's disease and determine if within the mechanism of action there are potential therapeutic targets.

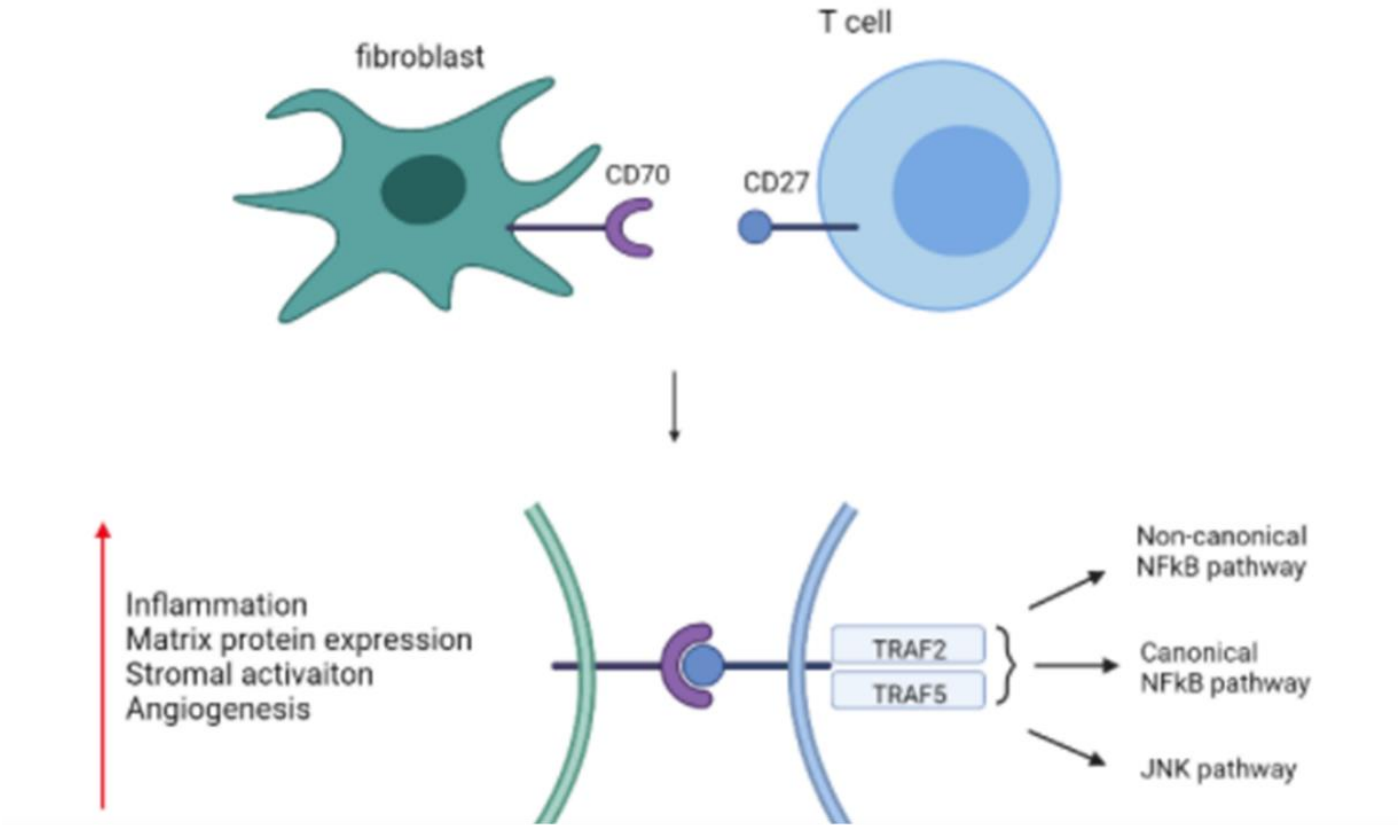


Figure 5.2 Schemata of CD27-CD70 T cell activation fibroblasts axis.
CD27 is expressed on T cells, and it binds to CD70 on fibroblasts during T cell activation. This CD27-CD70 axis leads to increased inflammation, matrix, stromal, and angiogenesis related markers by Dupuytren's fibroblasts. Published works describe that TRAF2/5 on T cells are involved in T cell stimulation via signaling pathways including non-canonical NFκB pathway, canonical NFκB pathway, and JNK pathway.

5.5 Conclusion and future directions

We hypothesized that stromal and immune cells contribute to the fibrotic nature of Dupuytren's disease. We describe that an activated stromal cell phenotype is present in Dupuytren's disease and, in accordance with published works, could be an element that drives chronic inflammation of the tissue. We also examine the immune cell landscape of Dupuytren's tissue towards understanding what stromal-immune cell interactions could be involved.

Current works describe immune cells as contributing element of Dupuytren's disease, namely macrophages, T cells, and mast cells[3,57,129,211,278]. This shift in attention towards immune cells provides a niche for this thesis as it focuses on T cell-fibroblast crosstalk, a potential feedback loop in T cell activation, and a mechanism towards therapeutic intervention as a means of understanding Dupuytren's disease etiology. While myeloid and T lymphocytes were highlighted as the main immune cells in the tissue microenvironments, T lymphocyte and fibroblast interaction has been shown to be key in understanding the immune mechanisms of the disease. Future analysis of T cell behaviour will interrogate the mechanism of IL-17 on fibroblasts and assess Secukinumab as a potential fibrotic inhibitor.

Lastly, the CD27-CD70 axis should be further examined via direct cell to cell interaction between T cells and Dupuytren's fibroblasts. A major challenge with studying human disease *in vitro* is the inability to mimic the 3D tissue stroma. However, recent advancements in biomaterials describe the ability to mimic a free floating, 3D environment using collagen matrices or hydrogels to study cell interaction and behavior. The examination of a tri-culture system of macrophages, T cells, and fibroblasts could provide insight into the stromal-immune cell interactions within Dupuytren's tissue, given the fibroblast response to stimulation with T cell produced CD27 and macrophage produced TNF-α. Lastly, the use of TNF-α neutralizing antibodies would allow for the exploration of anti-TNF therapy as a treatment option for Dupuytren's disease.

Chapter 6: Glossary

Adaptive immune response: this process is conducted by lymphocytes (B and T cells) which react against a foreign antigen presented on the surface of antigen presented cells (Alberts et al, 2002).

Adipocyte: a fat cell that makes up adipose tissue, that stores energy as fat within the human body (church,2012)

Angiogenesis: the process of developing blood vessels

Antibody: blood protein which binds to antigen

Antigen: foreign substance which binds to antibody

Antigen presenting cells: immune cells which engulf the foreign substance and present a 'piece' of the substance to the adaptive immune cells

Arthritis: inflammation of the joints causing reduced mobility and stiffness that can worsen as one gets older

Chemokine: a group of small proteins that attract white blood cells to the site of infection and they play a role in cell migration

Clusters of differentiation: identified by the acronym CD, this designation refers to the proteins found on the surface of the cell followed by a unique number which allows for universal identification

Cytokine: proteins secreted by cells that are involved in cell-to-cell communication, cell activation and differentiation

Degranulation: the process of mast cells reacting to wounded tissue and producing mediators which lead to further inflammation

Fibroproliferative: defined as progressive fibrosis in diseased tissue

Frozen Shoulder: also called adhesive capsulitis and characterized by stiffness and pain in shoulder joints

Granulocyte: a class of immune cells with granules containing enzymes; they are visually distinct from other cells given their ‘grainy’ look. Cells within this class consist of neutrophiles, eosinophils, and basophils.

Housekeeping gene: used in qPCR to create a baseline, or internal control, of genetic expression by cells

Innate immune response: the first line of defence in the immune system, consisting of leukocytes and phagocytic cells that interact with invading pathogens and communicate with adaptive immune cells to elicit a specific immune response (Alberts, 2002; innate immunity)

Interferon: abbreviated as IFN and activate the innate immune response by interacting with cells

Interleukin: a group of proteins which mediate cell to cell correspondence

Isotype: used in histology and flow cytometry as a negative control to confirm staining of tissue and cells

Lipoxygenase enzyme

Lymphocyte: a group of B and T cells

Mesenchymal stromal cell: adherent, spindle shaped cells that can be isolated from bone marrow, adipose and connective tissue

Monocyte: play a role in the inflammatory response and they differentiate into macrophages and dendritic cells based on paracrine factors

Paracrine signalling: a form of cell signalling between nearby cells

Pathophysiology: physiological processes that are associated with disease and/or injury

Pericyte: cells in the walls of capillaries

Phagocytosis: the ingestion of material by phagocytic cells like macrophages

Polymerase Chain Reaction: lab technique of amplifying DNA segments

Quantitative polymerase chain reaction: RNA is transcribed into complementary DNA (cDNA) by the enzyme, reverse transcriptase

Stromal compartment: ‘compartment’ of cells within the connective tissue which mediate support and function of the organ

Tendinopathy: overuse injury of tendons manifesting as inflammation and pain

Transmembrane protein: protein that spans the cell membrane and allows for transport of substances across the membrane

Chapter 7: References

1. Adams, T. S. et al. (2020) ‘Single-cell RNA-seq reveals ectopic and aberrant lung-resident cell populations in idiopathic pulmonary fibrosis’, Science Advances. American Association for the Advancement of Science, 6(28), p. 1983.
2. Akbar, M. et al. (2019) ‘Fibroblast activation and inflammation in frozen shoulder’, PLoS ONE. Public Library of Science, 14(4).
3. Akbar, M. *et al.* (2020) Attenuation of Dupuytren’s fibrosis via targeting of the STAT1 modulated IL-13Rα1 response, *Sci. Adv*. Sci. Adv., 6 (28).
4. Akbar, M. et al. (2021) ‘Translational targeting of inflammation and fibrosis in frozen shoulder: Molecular dissection of the T cell/IL-17A axis’, Proceedings of the National Academy of Sciences. National Academy of Sciences, 118(39).
5. Akdag, O. et al. (2016) ‘Dupuytren-Like Contracture of the Foot: Ledderhose Disease’, *The Surgery Journal*. 2(03), pp. e102–e104.
6. Akiba, H. et al. (1998) ‘CD27, a Member of the Tumor Necrosis Factor Receptor Superfamily, Activates NF-κB and Stress-activated Protein Kinase/c-Jun N-terminal Kinase via TRAF2, TRAF5, and NF-κB-inducing Kinase’, Journal of Biological Chemistry. Elsevier, 273(21), pp. 13353–13358.
7. Alivernini, S., et al. (2020). Distinct synovial tissue macrophage subsets regulate inflammation and remission in rheumatoid arthritis. Nature medicine, 26(8), 1295–1306.
8. Amatya, N., Garg, A. V. and Gaffen, S. L. (2017) ‘IL-17 Signaling: The Yin and the Yang’, Trends in immunology 38(5), p. 310.
9. An, Y. et al. (2020) ‘Crosstalk between cancer-associated fibroblasts and immune cells in cancer’, Journal of Cellular and Molecular Medicine. pp. 13–24.
10. Aran, D. et al. (2019) ‘Reference-based analysis of lung single-cell sequencing reveals a transitional profibrotic macrophage’, Nature immunology. 20(2), p. 163.
11. Arroyo Hornero, R. et al. (2020) ‘CD70 expression determines the therapeutic efficacy of expanded human regulatory T cells’, Communications Biology. Nature Research, 3(1), pp. 1–17.
12. Ascensión, A. M., Araúzo-Bravo, M. J., & Izeta, A. (2022). The need to reassess single-cell RNA sequencing datasets: the importance of biological sample processing. *F1000Research*, *10*, 767.
13. Ashley, S. L. et al. (2017) ‘Periostin regulates fibrocyte function to promote myofibroblast differentiation and lung fibrosis’, Mucosal Immunology. Nature Publishing Group, 10(2), pp. 341–351.
14. Astarita, J. L., Acton, S. E. and Turley, S. J. (2012) ‘Podoplanin: Emerging functions in development, the immune system, and cancer’, *Frontiers in Immunology*. Frontiers Media SA.
15. Augello A, De Bari C. (2010) ‘The regulation of differentiation in mesenchymal stem cells.’ Human Gene Therapy. 21(10):1226-38.
16. Avery, D., et al. (2018). ‘Extracellular matrix directs phenotypic heterogeneity of activated fibroblasts.’ Matrix Biology, 67, 90–106.
17. Ayaub, E., et al. (2021). Single Cell RNA-seq and Mass Cytometry Reveals a Novel and a Targetable Population of Macrophages in Idiopathic Pulmonary Fibrosis. BioRxiv, Published Jan 5, 2021
18. Baeri, Alberto, et al. (2022) A role for metformin in the treatment of Dupuytren disease?, *Biomedicine & Pharmacotherapy*, Volume 150
19. Baird, K. S. et al. (1993) ‘T-cell-mediated response in Dupuytren’s disease’. Lancet, 341(8861), pp. 1622–1623.

20. Baker, A. T. et al. (2021) ‘Cancer-Associated Fibroblasts and T Cells: From Mechanisms to Outcomes’, Journal of immunology. J Immunol, 206(2), pp. 310–320.
21. Barnas, J. L. et al. (2010) ‘T cells and stromal fibroblasts in human tumor microenvironments represent potential therapeutic targets’, in Cancer Microenvironment. Springer, pp. 29–47.
22. Barron, L. and Wynn, T. A. (2011) ‘Fibrosis is regulated by Th2 and Th17 responses and by dynamic interactions between fibroblasts and macrophages’, American Journal of Physiology - Gastrointestinal and Liver Physiology. American Physiological Society, 300(5), p. G723.
23. Behm, B. et al. (2012) ‘Cytokines, chemokines and growth factors in wound healing’, Journal of the European Academy of Dermatology and Venereology, 26(7), pp. 812–820.
24. Bhowmick, N. A., Neilson, E. G. and Moses, H. L. (2004) ‘Stromal fibroblasts in cancer initiation and progression’, Nature. 432(7015), p. 332.
25. Bianchi, E. et al. (2015) ‘Involvement of pro-inflammatory cytokines and growth factors in the pathogenesis of Dupuytren’s contracture: A novel target for a possible future therapeutic strategy?’, Clinical Science. Portland Press Ltd, 129(8), pp. 711–720.
26. Bianco, P. (2014) ‘“Mesenchymal” Stem Cells’, Annu. Rev. Cell Dev. Biol, 30, pp. 677–704.
27. Biernacka, A., Dobaczewski, M. and Frangogiannis, N. G. (2011) ‘TGF-β signaling in fibrosis’, Growth Factors. NIH Public Access, 29(5), pp. 196–202.
28. Bisson MA, et al. (2003) The different characteristics of Dupuytren's disease fibroblasts derived from either nodule or cord: expression of alpha-smooth muscle actin and the response to stimulation by TGF-beta1. J Hand Surg Br. 28(4):351-6.
29. Black, E. M. and Blazar, P. E. (2011) ‘Dupuytren disease: An evolving understanding of an age-old disease’, Journal of the American Academy of Orthopaedic Surgeons, 19(12), pp. 746–757.
30. Borst, J., Hendriks, J. and Xiao, Y. (2005) ‘CD27 and CD70 in T cell and B cell activation’, Current opinion in immunology. Curr Opin Immunol, 17(3), pp. 275–281.
31. Borthwick, L. A., Wynn, T. A. and Fisher, A. J. (2013) ‘Cytokine mediated tissue fibrosis’, Biochimica et Biophysica Acta - Molecular Basis of Disease. NIH Public Access, pp. 1049–1060.
32. Bosman, F. T. and Stamenkovic, I. (2003) ‘Functional structure and composition of the extracellular matrix’, The Journal of Pathology. John Wiley & Sons, Ltd, 200(4), pp. 423–428.
33. Boutet, M. A., et al. (2021). Novel insights into macrophage diversity in rheumatoid arthritis synovium. *Autoimmunity reviews*, *20*(3), 102758.
34. Bradley, J. R. and Pober, J. S. (2001) ‘Tumor necrosis factor receptor-associated factors (TRAFs)’, Oncogene 2001 20:44. Nature Publishing Group, 20(44), pp. 6482–6491.
35. Bradshaw, A. D. and DeLeon-Pennell, K. Y. (2020) ‘T-cell regulation of fibroblasts and cardiac fibrosis’, Matrix biology. journal of the International Society for Matrix Biology. Matrix Biol, 91–92, pp. 167–175.
36. Brembilla, N. C., Senra, L. and Boehncke, W.-H. (2018) ‘The IL-17 Family of Cytokines in Psoriasis: IL-17A and Beyond’, Frontiers in Immunology. Frontiers, p. 1682.
37. Buckley, C. D. et al. (2001) ‘Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation’, Trends in Immunology. Elsevier Current Trends, 22(4), pp. 199–204.

38. Buckley, C. D. et al. (2004) ‘Defining a role for fibroblasts in the persistence of chronic inflammatory joint disease’, in Annals of the Rheumatic Diseases. BMJ Publishing Group, pp. 92–95.
39. Buer, L. C. T. et al. (2017) ‘Switching from Remicade® to Remsima® is well Tolerated and Feasible: A Prospective, Open-label Study’, Journal of Crohn’s and Colitis. Oxford Academic, 11(3), pp. 297–304.
40. Bujak, M. *et al.* (2015) ‘Inflammatory gene expression upon TGF-β1-Induced p38 activation in Primary Dupuytren’s Disease fibroblasts’, *Frontiers in Molecular Biosciences*. Frontiers Media S.A., 2(DEC), p. 68.
41. Bussard, K. M. et al. (2016) ‘Tumor-associated stromal cells as key contributors to the tumor microenvironment’, Breast Cancer Research. BioMed Central Ltd., pp. 1–11.
42. Capone, A. and Volpe, E. (2020) ‘Transcriptional Regulators of T Helper 17 Cell Differentiation in Health and Autoimmune Diseases’, Frontiers in Immunology. Frontiers, 0, p. 348.
43. Caplan, A. I. (1991) ‘Mesenchymal stem cells’, *Journal of Orthopaedic Research*. John Wiley & Sons, Ltd, 9(5), pp. 641–650.
44. Caplan, A. I. (2017) ‘Mesenchymal Stem Cells: Time to Change the Name!’, *Stem cells translational medicine.* Stem Cells Transl Med, 6(6), pp. 1445–1451.
45. Chammas, M. et al. (1995) ‘Dupuytren’s disease, carpal tunnel syndrome, trigger finger, and diabetes mellitus’, Journal of Hand Surgery, 20(1), pp. 109–114.
46. Chávez-Galán, L. et al. (2015) ‘Much more than M1 and M2 macrophages, there are also CD169+ and TCR+ macrophages’, Frontiers in Immunology. Frontiers Media. 6, p. 263.
47. Chen, L. et al. (2018) ‘Inflammatory responses and inflammation-associated diseases in organs’, Oncotarget. Impact Journals, LLC, 9(6), p. 7204.
48. Chen, Q. et al. (2016) ‘Fate decision of mesenchymal stem cells: adipocytes or osteoblasts?’, *Cell Death & Differentiation* 2016 23:7. Nature Publishing Group, 23(7), pp. 1128–1139.
49. Chen, S., et al. (2022). miR-491-5p inhibits Emilin 1 to promote fibroblasts proliferation and fibrosis in gluteal muscle contracture via TGF-β1/Smad2 pathway. *Physiological research*.
50. Cheng, L., et al. (2021). New Insights From Single-Cell Sequencing Data: Synovial Fibroblasts and Synovial Macrophages in Rheumatoid Arthritis. *Frontiers in immunology*, *12*, 709178.
51. Choi, I. Y. et al. (2017) ‘Stromal cell markers are differentially expressed in the synovial tissue of patients with early arthritis’, PLoS ONE. Public Library of Science, 12(8).
52. Cibrián, D. and Sánchez-Madrid, F. (2017) ‘CD69: from activation marker to metabolic gatekeeper’, European journal of immunology. Europe PMC Funders, 47(6), p. 946.
53. Costa, A. et al. (2018) ‘Fibroblast Heterogeneity and Immunosuppressive Environment in Human Breast Cancer’. Cancer Cell, 33, pp. 463-479.e10.
54. Costa, A., et al. (2018). Fibroblast Heterogeneity and Immunosuppressive Environment in Human Breast Cancer. *Cancer cell*, *33*(3), 463–479.e10.
55. Croft, M. (2003) ‘Co-stimulatory members of the TNFR family: Keys to effective T-cell immunity?’, Nature Reviews Immunology. European Association for Cardio-Thoracic Surgery, pp. 609–620.
56. Crowe, L. A. N. et al. (2021) ‘Stromal “activation” markers do not confer pathogenic activity in tendinopathy’, Translational Sports Medicine. Wiley, 4(2), pp. 268–279.

57. Correa-Gallegos, D., Jiang, D. and Rinkevich, Y. (2021) ‘Fibroblasts as confederates of the immune system’, *Immunological Reviews*. John Wiley & Sons, Ltd, 302(1), pp. 147–162.
58. D’Urso, M. and Kurniawan, N. A. (2020) ‘Mechanical and Physical Regulation of Fibroblast–Myofibroblast Transition: From Cellular Mechanoresponse to Tissue Pathology’, Frontiers in Bioengineering and Biotechnology. Frontiers Media S.A., 8, p. 1459.
59. Dakin, S. G. et al. (2017) ‘Persistent stromal fibroblast activation is present in chronic tendinopathy’, Arthritis Research and Therapy. BioMed Central Ltd., 19(1).
60. Dakin, S. G. et al. (2018) ‘Pathogenic stromal cells as therapeutic targets in joint inflammation’, Nature Reviews Rheumatology. Springer US, 14(12), pp. 714–726.
61. Darby, I. A. et al. (2014) ‘Fibroblasts and myofibroblasts in wound healing’, Clinical, Cosmetic and Investigational Dermatology. Dove Medical Press Ltd., pp. 301–311.
62. de Souza, D. F. et al. (2010) ‘Ledderhose Disease: An Unusual Presentation’, The Journal of Clinical and Aesthetic Dermatology. *Matrix Medical Communications,* 3(9), p. 45.
63. Degreef, I. (2016) ‘Collagenase Treatment in Dupuytren Contractures: A Review of the Current State Versus Future Needs’, Rheumatology and Therapy. Springer Science and Business Media LLC, 3(1), pp. 43–51.
64. Deng, C. C. et al. (2021) ‘Single-cell RNA-seq reveals fibroblast heterogeneity and increased mesenchymal fibroblasts in human fibrotic skin diseases’, Nature Communications 2021 12:1. Nature Publishing Group, 12(1), pp. 1–16.
65. Deng, C. et al. (2021). Single-cell RNA-seq reveals fibroblast heterogeneity and increased mesenchymal fibroblasts in human fibrotic skin diseases. *Nature communications*, *12*(1), 3709.
66. Deng, H. et al. (2020) ‘New Classification of Macrophages in Plaques: a Revolution’, Current Atherosclerosis Reports. Springer, 22(8), pp. 1–9.
67. Denkler, K. A., Park, K. M., & Alser, O. (2022). Treatment Options for Dupuytren's Disease: Tips and Tricks. *Plastic and reconstructive surgery. Global open*, *10*(1), e4046.
68. Denoeud, J. and Moser, M. (2011) ‘Role of CD27/CD70 pathway of activation in immunity and tolerance’, Journal of leukocyte biology. J Leukoc Biol, 89(2), pp. 195–203.
69. Descatha, A. et al. (2011) ‘Should we consider Dupuytren’s contracture as work-related? A review and meta-analysis of an old debate,’ BMC Musculoskeletal Disorders. BioMed Central, p. 96.
70. de Souza, D. F. *et al.* (2010) ‘Ledderhose Disease: An Unusual Presentation’, *The Journal of Clinical and Aesthetic Dermatology*. Matrix Medical Communications, 3(9), p. 45.
71. DiBenedetti, D. B. et al. (2011) ‘Prevalence, incidence, and treatments of Dupuytren’s disease in the United States: Results from a population-based study’, Hand. SAGE Publications, 6(2), pp. 149–158.
72. Ding, W. et al. (2015) ‘Interleukin-17A promotes the formation of inflammation in the lung tissues of rats with pulmonary fibrosis’, Experimental and Therapeutic Medicine. Spandidos Publications, 10(2), pp. 491–497.
73. Distler, J. H. W. et al. (2019) ‘Shared and distinct mechanisms of fibrosis’, Nature Reviews Rheumatology. 15, 705–730.
74. Dobie, R. et al. (2022) ‘Deciphering Mesenchymal Drivers of Human Dupuytren’s Disease at Single-Cell Level’, Journal of Investigative Dermatology. Elsevier, 142(1), pp. 114-123.e8.

75. Dolmans, G. H. et al. (2011) ‘Wnt Signaling and Dupuytren’s Disease’, N Engl J Med, 365, pp. 307–324.
76. Douglas M.R. et al. 2002. Why does inflammation persist: a dominant role for the stromal microenvironment? Expert Rev Mol Med. Dec 9;4(25):1-18.
77. Duffield, J. S., et al. (2005). ‘Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair.’ Journal of Clinical Investigation, 115(1), 56.
78. Dullea, A., et al. (2022). Whole-Genome Sequencing Identifies Novel Heterozygous Mutation in ALMS1 in Three Men With Both Peyronie's and Dupuytren's Disease. *Urology*, S0090-4295(22)00207-2.
79. Elpek, G. Ö. (2014) ‘Cellular and molecular mechanisms in the pathogenesis of liver fibrosis: An update’, World Journal of Gastroenterology. 20(23), p. 7260.
80. Enzo, M. V. *et al.* (2015) ‘The Wnt/β-catenin pathway in human fibrotic-like diseases and its eligibility as a therapeutic target’, *Molecular and Cellular Therapies 2015 3:1*. BioMed Central, 3(1), pp. 1–13.
81. Esensten, J. H. et al. (2016) ‘CD28 Costimulation: From Mechanism to Therapy’, Immunity. Cell Press, pp. 973–988.
82. Filer, A. et al. (2007) ‘Targeting Stromal Cells in Chronic Inflammation’, Discovery medicine. Europe PMC Funders, 7(37), p. 20.
83. Filer, A., Pitzalis, C. and Buckley, C. D. (2011) ‘Targeting the stromal microenvironment in chronic inflammation’, 6(4), pp. 393–400.
84. Flatt, A. E. (2001) ‘The Vikings and Baron Dupuytren’s Disease’, Baylor University Medical Center Proceedings. Informa UK Limited, 14(4), pp. 378–384.
85. Fletcher, A. L., & Turley, S. J. (2021). Who am I? (re-)Defining fibroblast identity and immunological function in the age of bioinformatics. *Immunological reviews*, *302*(1), 5–9.
86. Frantz, C., Stewart, K. M. and Weaver, V. M. (2010) ‘The extracellular matrix at a glance’, Journal of Cell Science. The Company of Biologists, 123(24), pp. 4195–4200.
87. Fruman, D. A. et al. (2017) ‘The PI3K pathway in human disease’, Cell. NIH Public Access, 170(4), p. 605.
88. Fujie, H. et al. (2012) ‘A Distinct Regulatory Role of Th17 Cytokines IL-17A and IL-17F in Chemokine Secretion from Lung Microvascular Endothelial Cells’, Inflammation 2012 35:3. Springer, 35(3), pp. 1119–1131.
89. Gaffen, S. L. (2009) ‘The role of interleukin-17 in the pathogenesis of rheumatoid arthritis’, Current Rheumatology Reports. NIH Public Access, 11(5), pp. 365–370.
90. Garcia, M. V., Rosa, J. and Soriano, E. R. (2011) ‘Patient Considerations in the Management of Rheumatoid Arthritis: Role of Once-A-Month Golimumab Injection’. SAGE Publications Sage UK: London, England, 3, pp. 415–423.
91. Garcia-Melchor, E. et al. (2021) ‘Novel self-amplificatory loop between T cells and tenocytes as a driver of chronicity in tendon disease’, Ann Rheum Dis, 0, pp. 1–11.
92. Gelbard, M. K. and Rosenbloom, J. (2020) ‘Fibroproliferative disorders and diabetes: Understanding the pathophysiologic relationship between Peyronie’s disease, Dupuytren disease and diabetes’, *Endocrinology, diabetes & metabolism*. Endocrinol Diabetes Metab, 4(2).
93. Gonga-Cavé, B. C., Pena Diaz, A. M. and O’Gorman, D. B. (2021) ‘Biomimetic analyses of interactions between macrophages and palmar fascia myofibroblasts derived from Dupuytren’s disease reveal distinct inflammatory cytokine responses’, *Wound Repair and Regeneration*. John Wiley & Sons, Ltd, 29(4), pp. 627–636.

94. Göransson, et al (2021). Hand function 5 years after treatment with collagenase Clostridium histolyticum injection for Dupuytren's disease. *Journal of Hand Surgery: European Volume*, *46*(9), 985–994.
95. Goronzy, J. J. and Weyand, C. M. (2008) 'T-cell co-stimulatory pathways in autoimmunity', Arthritis Research and Therapy. BioMed Central, pp. 1–10.
96. Grazina, R. et al. (2018) 'Dupuytren's disease: Where do we stand?'. British Editorial Society of Bone and Joint Surgery, 4(2), pp. 63–69.
97. Grivennikov, S. I., Greten, F. R. and Karin, M. (2010) 'Immunity, Inflammation, and Cancer', Cell. Cell Press, 140(6), pp. 883–899.
98. Gudmundsson, K. G. et al. (1998) 'T-and B-lymphocyte subsets in patients with Dupuytren's disease. Correlations with disease severity,' Journal of hand surgery, 23(6), pp. 724–727.
99. Han, B. K., Olsen, N. J. and Bottaro, A. (2016) 'The CD27–CD70 pathway and pathogenesis of autoimmune disease', Seminars in Arthritis and Rheumatism. W.B. Saunders, 45(4), pp. 496–501.
100. Haraoui, B. and Bykerk, V. (2007) 'Etanercept in the treatment of rheumatoid arthritis', Therapeutics and Clinical Risk Management. Dove Press, 3(1), p. 99.
101. Harryvan, T. J. et al. (2019) 'Targeting of the Cancer-Associated Fibroblast—T-Cell Axis in Solid Malignancies', Journal of Clinical Medicine 2019, Vol. 8, Page 1989. Multidisciplinary Digital Publishing Institute, 8(11), p. 1989.
102. Hart, M. G. (2005) 'Clinical associations of Dupuytren's disease', Postgrad Med J, 81, pp. 425–428.
103. Haworth, O., et al. (2005). Stromal cells as new therapeutic targets in rheumatoid arthritis. Therapy, 2(1), 121–129.
104. Herbert, S. P. and Stainier, D. Y. R. (2011) 'Molecular control of endothelial cell behaviour during blood vessel morphogenesis', *Nature Reviews Molecular Cell Biology 2011 12:9*. Nature Publishing Group, 12(9), pp. 551–564.
105. Hindocha, S., McGrouther, D. A., & Bayat, A. (2009). 'Epidemiological evaluation of dupuytren's disease incidence and prevalence rates in relation to etiology.'Hand Vol. 4, Issue 3, pp. 256–269.
106. Hinz, B. et al. (2001). Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. American Journal of Pathology, 159(3), 1009–1020.
107. Hiroyasu Yamamoto, M., & Kishimoto, T. (1998). 'Functional Region of CD27 Factors in Its Signaling and Identification of Involvement of TNF Receptor-Associated B Activation in CD27 Signaling: κ NF'. J Immunol References, 4753, 4753–4759.
108. Honma, M., et al. (2012). 'Podoplanin expression in wound and hyperproliferative psoriatic epidermis: Regulation by TGF-β and STAT-3 activating cytokines, IFN-γ, IL-6, and IL-22.' *Journal of Dermatological Science*, *65*(2), 134–140.
109. Horwitz, E. M., Andreef, M., & Frassoni, F. (2006). 'Mesenchymal Stromal Cells.' Current Opinion in Hematology, 13(6), 419.
110. Huang, E., et al. (2020). 'The Roles of Immune Cells in the Pathogenesis of Fibrosis.' International Journal of Molecular Sciences, 21(15), 1–27.
111. Huang, H., et al. (2015) 'VEGF suppresses T-lymphocyte infiltration in the tumor microenvironment through inhibition of NF-κB-induced endothelial activation.' *The FASEB Journal*, *29*(1), 227–238.
112. Hueber, W., et al. (2012).' Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial.' *Gut, 61*(12), 1693.

113. Hung, C., et al. (2013) ‘Role of Lung Pericytes and Resident Fibroblasts in the Pathogenesis of Pulmonary Fibrosis.’ *American Journal of Respiratory and Critical Care Medicine* 188(7), 820.
114. Hurst, L. C., et al (2009). Injectable collagenase clostridium histolyticum for Dupuytren's contracture. *The New England journal of medicine, 361*(10), 968–979.
115. Ino, Y., et al. (2013) ‘Immune cell infiltration as an indicator of the immune microenvironment of pancreatic cancer.’ British Journal of Cancer 2013 108:4, 108(4), 914–923.
116. Italiani, P., & Boraschi, D. (2014) ‘From monocytes to M1/M2 macrophages: Phenotypical vs. functional differentiation’. Frontiers in Immunology p.514
117. Izadi, D., et al. (2019) ‘Identification of TNFR2 and IL-33 as therapeutic targets in localized fibrosis.’ In Sci. Adv vol. 5 p12.
118. J van Oers Heemskerk, M. H., et al. (2021) ‘Activation and IgG Production Signaling through CD70 Regulates B Cell.’ *J Immunol References, 173,* 3901–3908.
119. Jacobs, J., et al. (2018) ‘Unveiling a CD70-positive subset of cancer-associated fibroblasts marked by pro-migratory activity and thriving regulatory T cell accumulation.’ Oncoimmunology, 7(7).
120. Jaishankar, A. *et al.* (2009) ‘Human embryonic and mesenchymal stem cells express different nuclear proteomes’, *Stem cells and development*. Stem Cells Dev, 18(5), pp. 793–801.
121. Jiang, D., & Rinkevich, Y. (2018) ‘Defining skin fibroblastic cell types beyond CD90.’ Frontiers in Cell and Developmental Biology Vol. 6, p. 133.
122. Jiang, W., & Xu, J. (2020) ‘Immune modulation by mesenchymal stem cells.’ *Cell Proliferation, 53*(1), e12712.
123. Jin, R., et al. (2022). Effect of nanoparticle-mediated delivery of SFRP4 siRNA for treating Dupuytren disease. *Gene therapy*, 10.1038/s41434-022-00330-9. Advance online publication.
124. Johnson, A., & DiPietro, L. A. (2013) ‘Apoptosis and angiogenesis: an evolving mechanism for fibrosis.’ The FASEB Journal, 27(10), 3893.
125. Johnson, B. Z., et al. (2020) ‘The Role of IL-6 in Skin Fibrosis and Cutaneous Wound Healing.’ *Biomedicines,* 8(5), 101.
126. Kalliolias, G. D. and Ivashkiv, L. B. (2016) ‘TNF biology, pathogenic mechanisms and emerging therapeutic strategies’, *Nature reviews. Rheumatology*. NIH Public Access, 12(1), p. 49.
127. Kalluri, R. (2016). ‘The biology and function of fibroblasts in cancer.’ Nature Reviews Cancer 16(9), 582–598.
128. Karatas, A., et al. (2021) ‘Secukinumab and metformin ameliorate dermal fibrosis by decreasing tissue interleukin-17 levels in bleomycin-induced dermal fibrosis.’ International Journal of Rheumatic Diseases, 24(6), 795–802.
129. Karkampouna, S., et al. (2016) ‘Connective Tissue Degeneration: Mechanisms of Palmar Fascia Degeneration (Dupuytren’s Disease).’ Current Molecular Biology Reports, 2(3), 133–140.
130. Keating, A. (2012). ‘Mesenchymal Stromal Cells: New Directions.’ *Cell Stem Cell*, *10*(6), 709–716.
131. Kehlen, A., et al. (2002). ‘Expression, modulation and signalling of IL-17 receptor in fibroblast-like synoviocytes of patients with rheumatoid arthritis.’ *Clinical and Experimental Immunology*, *127*(3), 539–546.
132. Kendall, R. T. and Feghali-Bostwick, C. A. (2014) ‘Fibroblasts in fibrosis: Novel roles and mediators’, Frontiers in Pharmacology. Frontiers Research Foundation.

133. Ketchum, L. D. (2014) ‘The Rationale for Treating the Nodule in Dupuytren’s Disease’, Plastic and Reconstructive Surgery Global Open. Wolters Kluwer Health, 2(12).
134. Kim, S. S. et al. (2019) ‘Fibronectin synthesis, but not α-smooth muscle expression, is regulated by periostin in gingival healing through FAK/JNK signaling’, Scientific Reports. *Nature Publishing Group*, 9(1), pp. 1–17.
135. Kirk, T., Ahmed, A., & Rognoni, E. (2021). Fibroblast Memory in Development, Homeostasis and Disease. *Cells*, *10*(11), 2840.
136. Kloen, P. (1999). New insights in the development of Dupuytren’s contracture: A review. *British Journal of Plastic Surgery*, 52(8), 629–635.
137. Kolf, C. M., Cho, E., & Tuan, R. S. (2007). Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: Regulation of niche, self-renewal and differentiation. *Arthritis Research and Therapy*, 9(1), 1–10.
138. Komiya, Y., & Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis*, *4*(2), 68.
139. Kondrup, F., Gaudreault, N., & Venne, G. (2022). The deep fascia and its role in chronic pain and pathological conditions: A review. *Clinical anatomy (New York, N.Y.)*, 10.1002/ca.23882. Advance online publication.
140. Kotsianidis, I. *et al.* (2012) ‘Global Impairment of CD4+CD25+FOXP3+ Regulatory T Cells in Idiopathic Pulmonary Fibrosis’. *American Thoracic Society*, 179(12), pp. 1121–1130.
141. Krammer, P. H., Arnold, R. and Lavrik, I. N. (2007) ‘Life and death in peripheral T cells’, *Nature Reviews Immunology 2007 7:7*. Nature Publishing Group, 7(7), pp. 532–542.
142. Krueger, J. G. *et al.* (2019) ‘IL-17A inhibition by secukinumab induces early clinical, histopathologic, and molecular resolution of psoriasis’, *Journal of Allergy and Clinical Immunology*. Mosby, 144(3), pp. 750–763.
143. Kuboi, T., et al (2021). Collagenase *Clostridium histolyticum* Injection Therapy Improves Health-related Quality of Life in Patients with Dupuytren's Disease. *Progress in rehabilitation medicine*, *6*, 20210023.
144. Kudo, A., & Kii, I. (2018). Periostin function in communication with extracellular matrices. *Journal of Cell Communication and Signaling* (Vol. 12, Issue 1, pp. 301–308).
145. Kuka, M. *et al.* (2013) ‘CD70 is downregulated by interaction with CD27’, *Journal of immunology (Baltimore, Md. : 1950)*. NIH Public Access, 191(5), p. 2282.
146. Kumar, B. V., Connors, T., & Farber, D. L. (2018). Human T cell development, localization, and function throughout life. *Immunity*, 48(2), 202.
147. Kwiecień, I. et al. (2019) ‘CD163 and CCR7 as markers for macrophage polarization in lung cancer microenvironment’, *Central European Journal of Immunology*. Termedia Publishing House Ltd., 44(4), pp. 395–402.
148. Jin, L . *et al.* (2018) ‘CD70, a novel target of CAR T-cell therapy for gliomas.’, *Neuro-oncology*. Oxford University Press, 20(1), pp. 55–65.
149. Lalani, A. I. *et al.* (2018) ‘TRAF molecules in inflammation and inflammatory diseases’, *Current pharmacology reports*. NIH Public Access, 4(1), p. 64.
150. Lanting, R. *et al.* (2014) ‘A systematic review and meta-analysis on the prevalence of Dupuytren’s disease in the general population of western countries’, *Plastic and Reconstructive Surgery*, 133(3), pp. 593–603.
151. Law, C.-L. *et al.* (2021) ‘Anti-CD70 Antibody Ameliorates Joint Blocking of CD27-CD70 Pathway by’, *J Immunol References*, 183, pp. 3770–3777.

152. Layton, T. and Nanchahal, J. (2019) 'Recent advances in the understanding of Dupuytren's disease', *F1000Research*. F1000 Research Ltd.
153. Layton, T. B. *et al.* (2020) 'Cellular census of human fibrosis defines functionally distinct stromal cell types and states', *Nature Communications*. Nature Research, 11(1).
154. Layton, T. B. *et al.* (2020) 'Single cell force profiling of human myofibroblasts reveals a biophysical spectrum of cell states', *Biology Open*. Company of Biologists Ltd, 9(3).
155. Lech, M. and Anders, H. J. (2013) 'Macrophages and fibrosis: How resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*. Elsevier, 1832(7), pp. 989–997.
156. Lee, W.-W. *et al.* (2007) 'Unchecked CD70 Expression on T Cells Lowers Threshold for T Cell Activation in Rheumatoid Arthritis', *The Journal of Immunology*. The American Association of Immunologists, 179(4), pp. 2609–2615.
157. Lens, S. M. A. *et al.* (1998) 'Control of lymphocyte function through CD27-CD70 interactions', *Seminars in Immunology*. Academic Press, 10(6), pp. 491–499.
158. Liang, C.-C., Park, A. Y., & Guan, J.-L. (2007). In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols 2007 2:2*, *2*(2), 329–333.
159. Liew, F. Y., Girard, J. P. and Turnquist, H. R. (2016) 'Interleukin-33 in health and disease', *Nature Reviews Immunology*. Nature Publishing Group, pp. 676–689.
160. Lindner, T. *et al.* (2019) 'Targeting of activated fibroblasts for imaging and therapy', *EJNMMI Radiopharmacy and Chemistry*. Springer Science and Business Media LLC, 4(1), pp. 1–15.
161. Lis, K., Kuzawińska, O. and Bałkowiec-Iskra, E. (2014) 'Tumor necrosis factor inhibitors – state of knowledge', *Archives of Medical Science : AMS*. Termedia Publishing, 10(6), p. 1175.
162. Liu, T. *et al.* (2017) 'NF-κB signaling in inflammation', *Signal Transduction and Targeted Therapy*. Springer Nature, p. 17023.
163. Liu, T. *et al.* (2019) 'Cancer-associated fibroblasts: An emerging target of anti-cancer immunotherapy', *Journal of Hematology and Oncology*. BioMed Central Ltd., 12(1), pp. 1–15.
164. Liu, W. *et al.* (2021) 'Structural delineation and phase-dependent activation of the costimulatory CD27:CD70 complex', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology Inc., 297(4), p. 101102.
165. Lorè, N. I., Bragonzi, A. and Cigana, C. (2016) 'The IL-17A/IL-17RA axis in pulmonary defence and immunopathology', *Cytokine and Growth Factor Reviews*. Elsevier Ltd, pp. 19–27.
166. Lu, X. Q. *et al.* (2021) 'Efficacy and Safety of Adalimumab Biosimilars: Current Critical Clinical Data in Rheumatoid Arthritis', *Frontiers in Immunology*. Frontiers Media SA, 12, p. 638444.
167. Luo, J. *et al.* (2022) 'Sustained AWT1 expression by Dupuytren's disease myofibroblasts promotes a proinflammatory milieu', *Journal of Cell Communication and Signaling 2022*. Springer, pp. 1–14.
168. Luster, A. D., Alon, R. and von Andrian, U. H. (2005) 'Immune cell migration in inflammation: present and future therapeutic targets', *Nature Immunology 2005 6:12*. Nature Publishing Group, 6(12), pp. 1182–1190.
169. MacFadyen, J. R. *et al.* (2005) 'Endosialin (TEM1, CD248) is a marker of stromal fibroblasts and is not selectively expressed on tumour endothelium', *FEBS Letters*. Elsevier, 579(12), pp. 2569–2575.

170. Mandel, D. R. and Demarco, P. J. (2014) ‘Overview of the pathogenesis, diagnosis and treatment of Dupuytren’s disease’, *International Journal of Clinical Rheumatology*. Future Medicine Ltd., 9(2), pp. 217–225.
171. Mansur, H. G., Oliveira, E. R. de and Gonçalves, C. B. (2018) ‘Epidemiological analysis of patients with Dupuytren’s disease’, *Revista Brasileira de Ortopedia (English Edition)*, 53(1), pp. 10–14.
172. Mao, X. *et al.* (2021) ‘Crosstalk between cancer-associated fibroblasts and immune cells in the tumor microenvironment: new findings and future perspectives’, *Molecular Cancer 2021 20:1*. BioMed Central, 20(1), pp. 1–30.
173. Maruhashi, T. *et al.* (2010) ‘Interaction between periostin and BMP-1 promotes proteolytic activation of lysyl oxidase’, *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 285(17), pp. 13294–13303.
174. Mathieu, S. *et al.* (2020) ‘Dupuytren’s Disease and exposure to vibration: Systematic review and Meta-analysis’, *Joint Bone Spine*. Société française de rhumatologie, 87(3), pp. 203–207.
175. Mayerl, C. *et al.* (2016) ‘Characterisation of the inflammatory response in Dupuytren’s disease’, *Journal of plastic surgery and hand surgery*, 50(3), 171–179.
176. mayoclinic.org. 2020. Dupuytren's contracture - Symptoms and causes. Available at: <https://www.mayoclinic.org/diseases-conditions/dupuytrens-contracture/symptoms-causes/syc-20371943> [Accessed 19 May 2022].
177. McCarty, S., Syed, F. and Bayat, A. (2010) ‘Role of the HLA system in the pathogenesis of dupuytren’s disease’, *Hand*. SAGE Publications, 5(3), pp. 241–250.
178. Mcgonagle, D. G. *et al.* (2019) ‘The role of IL-17A in axial spondyloarthritis and psoriatic arthritis: recent advances and controversies’, *Ann Rheum Dis*, 78, pp. 1167–1178.
179. Mease, P. J. *et al.* (2015) ‘Secukinumab Inhibition of Interleukin-17A in Patients with Psoriatic Arthritis’, *N Engl J Med 2015;* 373:1329-1339.
180. Meek, R. M. D., Mclellan, S., & Crossan, J. F. (1999). Dupuytren’s disease: a model for the mechanism of fibrosis and its modulation by steroids. *J Bone Joint Surg [Br]*, 81, 732–740.
181. Mescher, A. L. (2017) ‘Macrophages and fibroblasts during inflammation and tissue repair in models of organ regeneration’, *Regeneration*. Wiley, 4(2), pp. 39–53.
182. Midwood, K. S., & Orend, G. (2009). The role of tenascin-C in tissue injury and tumorigenesis. *Journal of Cell Communication and Signaling*, *3*(3–4), 287.
183. Midwood, K. S., Williams, L. V. and Schwarzbauer, J. E. (2004) ‘Tissue repair and the dynamics of the extracellular matrix’, *International Journal of Biochemistry and Cell Biology*. Elsevier Ltd, pp. 1031–1037.
184. Millar, N. L. et al. (2016) ‘IL-17A mediates inflammatory and tissue remodelling events in early human tendinopathy’, Scientific Reports. Nature Publishing Group, 6(1), pp. 1–11.
185. Millar, N. L., Murrell, G. A. C. and Mcinnes, I. B. (2017) ‘Inflammatory mechanisms in tendinopathy - towards translation’, *Nature Reviews Rheumatology*. Nature Publishing Group, 13(2), pp. 110–122.
186. Milutinovic, Masa (2022) Culturing Dupuytren Fibroblasts in Hypoxic Conditions Presents Different Levels of α-SMA, Collagen Type I and Collagen Modifying Enzymes. Bachelor's Research Project. Rijksuniversiteit Groningen
187. Mimpen, J. Y. *et al.* (2021) ‘Interleukin-17A Causes Osteoarthritis-Like Transcriptional Changes in Human Osteoarthritis-Derived Chondrocytes and Synovial Fibroblasts In Vitro’, *Frontiers in Immunology*. Frontiers, p. 1677.

188. Miossec, P. and Kolls, J. K. (2012) 'Targeting IL-17 and T H 17 cells in chronic inflammation', *Nature Reviews Drug Discovery 2012 11:10*. Nature Publishing Group, 11(10), pp. 763–776.
189. Mitra, A., & Goldstein, R. Y. (1994). Dupuytren's contracture in the black population: a review. *Annals of plastic surgery*, 32(6), 619–622.
190. Monaco, C. et al. (2015) 'Anti-TNF therapy: past, present and future', International Immunology. Oxford University Press, 27(1), p. 55.
191. Moriya, K. et al. (2012) 'Fibronectin is essential for survival but is dispensable for proliferation of hepatocytes in acute liver injury', *Hepatology* (Baltimore, Md.). NIH Public Access, 56(1), p. 311.
192. Mulder, K. *et al.* (2021) 'Cross-tissue single-cell landscape of human monocytes and macrophages in health and disease', *Immunity*. Cell Press, 54(8), pp. 1883-1900.e5.
193. Murrell, G. A., Francis, M. J., & Bromley, L. (1991). The collagen changes of Dupuytren's contracture. *Journal of hand surgery*, 16(3), 263–266.
194. Musumeci, M. *et al.* (2015) 'Dupuytren's disease therapy: targeting the vicious cycle of myofibroblasts?'. Informa Healthcare, 19(12), pp. 1677–1687.
195. Nanchahal, J. *et al.* (2018) 'Anti-Tumour Necrosis Factor Therapy for Dupuytren's Disease: A Randomised Dose Response Proof of Concept Phase 2a Clinical Trial', *EBioMedicine*. Elsevier, 33, p. 282.
196. Naylor, A. J., Filer, A. and Buckley, C. D. (2013) 'The role of stromal cells in the persistence of chronic inflammation', *Clinical & Experimental Immunology*. John Wiley & Sons, Ltd, 171(1), pp. 30–35.
197. Nguyen, H. N. *et al.* (2017) 'Autocrine Loop Involving IL-6 Family Member LIF, LIF Receptor, and STAT4 Drives Sustained Fibroblast Production of Inflammatory Mediators'. *Immunity*, 46(2), 220–232.
198. Nikoloudaki, G. *et al.* (2020) 'Periostin and matrix stiffness combine to regulate myofibroblast differentiation and fibronectin synthesis during palatal healing', *Matrix Biology*. Elsevier B.V., 94, pp. 31–56.
199. Nishida, N. *et al.* (2006) 'Angiogenesis in Cancer', *Vascular Health and Risk Management*. Dove Press, 2(3), p. 213.
200. Niu, K., et al . (2022). Study on the Action Mechanism of Dkk-1, TGF-β1 and TNF-α Expression Levels in Dupuytren's Contracture. Untersuchung der Expressionsniveaus von DKK-1, TGF-β1 und TNF-α bei Dupuytren-Kontraktur. *Organ der Deutschsprachigen Arbeitsgemeinschaft fur Mikrochirurgie der Peripheren Nerven und Gefasse : Organ der V...*, *54*(2), 149–154.
201. Noble, J., Heathcote, J. G., & Cohen, H. (1984). Diabetes mellitus in the aetiology of Dupuytren's disease. *The Journal of bone and joint surgery*. British volume, 66(3), 322–325.
202. Nugteren, H. M. et al. (2011) 'The association between Peyronie's and Dupuytren's disease', *International Journal of Impotence Research*. 23(4), pp. 142–145.
203. O'Connell, J. T. *et al.* (2011) 'VEGF-A and Tenascin-C produced by S100A4 + stromal cells are important for metastatic colonization'. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(38), 16002–16007.
204. O'Gorman, D. B., Vi, L. and Gan, B. S. (2010) 'Molecular mechanisms and treatment strategies for Dupuytren's disease', *Therapeutics and Clinical Risk Management*. Dove Press, 6, p. 383.
205. O'Neill, R. E. *et al.* (2017) 'T cell-derived CD70 delivers an immune checkpoint function in inflammatory T cell responses', *Journal of immunology (Baltimore, Md.: 1950)*. NIH Public Access, 199(10), p. 3700.

206. Oflazoglu, E. *et al.* (2009) ‘Blocking of CD27-CD70 Pathway by Anti-CD70 Antibody Ameliorates Joint Disease in Murine Collagen-Induced Arthritis’, *The Journal of Immunology*. American Association of Immunologists, 183(6), pp. 3770–3777.
207. Öhlund, D., et al. (2017). Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. *The Journal of experimental medicine*, *214*(3), 579–596.
208. Orecchioni, M. *et al.* (2019) ‘Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS–) vs. Alternatively Activated Macrophages’, *Frontiers in Immunology*. Frontiers, 0(MAY), p. 1084.
209. Orend, G. and Chiquet-Ehrismann, R. (2006) ‘Tenascin-C induced signaling in cancer’, *Cancer Letters*. *Elsevier*, 244(2), pp. 143–163.
210. Ox.ac.uk. 2022. Breakthrough in treatment for Dupuytren’s disease | University of Oxford. [online] Available at: <https://www.ox.ac.uk/news/2022-05-03-breakthrough-treatment-dupuytren-s-disease> [Accessed 19 May 2022].
211. Pakshir, P. and Hinz, B. (2018) ‘The big five in fibrosis: Macrophages, myofibroblasts, matrix, mechanics, and miscommunication’, *Matrix biology : journal of the International Society for Matrix Biology*. Matrix Biol, 68–69, pp. 81–93.
212. Pankov, R. and Yamada, K. M. (2002) ‘Fibronectin at a glance’, *Journal of Cell Science*. The Company of Biologists, 115(20), pp. 3861–3863.
213. Patel, R. *et al.* (2014) ‘Stroma: Fertile soil for inflammation’, *Best Practice and Research: Clinical Rheumatology*. Bailliere Tindall Ltd, pp. 565–576.
214. Pahwa, R., Goyal, A. and Jialal, I. (2021) ‘Chronic Inflammation’, *Pathobiology of Human Disease: A Dynamic Encyclopedia of Disease Mechanisms*. StatPearls Publishing, pp. 300–314. org
215. Pelechas, E., Voulgari, P. V. and Drosos, A. A. (2019) ‘Golimumab for Rheumatoid Arthritis’, Journal of Clinical Medicine 2019, Vol. 8, Page 387. Multidisciplinary Digital Publishing Institute, 8(3), p. 387.
216. Pesce, J. T., Ramalingam, T. R., Mentink-Kane, M. M., Wilson, M. S., El Kasmi, K. C., Smith, A. M., Thompson, R. W., Cheever, A. W., Murray, P. J., & Wynn, T. A. (2009). Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. *PLoS pathogens*, *5*(4), e1000371.
217. Phan, R., et al. (2021). The influence of skin tears following collagenase treatment of Dupuytren's disease on treatment outcomes. *The Journal of hand surgery, European volume*, *46*(4), 398–402.
218. Picardo, N. E. and Khan, W. S. (2012) ‘Advances in the understanding of the aetiology of Dupuytren’s disease’, *Surgeon*. Elsevier Ltd, 10(3), pp. 151–158.
219. Piersma, B. *et al.* (2015) ‘YAP1 Is a Driver of Myofibroblast Differentiation in Normal and Diseased Fibroblasts’, *The American journal of pathology*. Am J Pathol, 185(12), pp. 3326–3337.
220. Pfizer.com. 2022. Biologics vs Biosimilars: Understanding the differences | Pfizer. <https://www.pfizer.com/news/articles/biologics_vs_biosimilars_understanding_the_differences> [Accessed 18 May 2022].
221. Pober, J. S. and Sessa, W. C. (2007) ‘Evolving functions of endothelial cells in inflammation’, *Nature Reviews Immunology 2007 7:10*. Nature Publishing Group, 7(10), pp. 803–815.
222. Postlethwaite, A. E., et al. (1987). Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta. *The Journal of experimental medicine*, *165*(1), 251–256.
223. Prasad, K. V. S. *et al.* (1997) ‘CD27, a member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein’, *Proceedings of the*

National Academy of Sciences of the United States of America. National Academy of Sciences, 94(12), pp. 6346–6351.
224. Prockop, D. J. (2016) 'Inflammation, fibrosis, and modulation of the process by mesenchymal stem/stromal cells', *Matrix Biology*. Elsevier, 51, pp. 7–13.
225. Qian, A. *et al.* (2004) 'Comparison of gene expression profiles between Peyronie's disease and Dupuytren's contracture', *Urology*. Urology, 64(2), pp. 399–404.
226. Rabelink, T. J. and Little, M. H. (2013) 'Stromal cells in tissue homeostasis: Balancing regeneration and fibrosis', *Nature Reviews Nephrology*. Nature Publishing Group, 9(12), pp. 747–753.
227. Radhamony, N. G. *et al.* (2022) 'Residual deformity versus recurrence following Dupuytren's palmar fasciectomy-a long term follow-up of 142 cases', *Annals of Medicine and Surgery*. Elsevier, 73, p. 103224.
228. Ramalingam, T. R. *et al.* (2016) 'Enhanced protection from fibrosis and inflammation in the combined absence of IL-13 and IFN-γ', *The Journal of pathology*. NIH Public Access, 239(3), p. 344.
229. Ratkaj, I. *et al.* (2012) 'Microarray Analysis of Dupuytren's Disease Cells: The Profibrogenic Role of the TGF-β Inducible p38 MAPK Pathway', *Cellular Physiology and Biochemistry*. Karger Publishers, 30(4), pp. 927–942.
230. Raza, A., Franklin, M. J. and Dudek, A. Z. (2010) 'Pericytes and vessel maturation during tumor angiogenesis and metastasis', *American Journal of Hematology*. John Wiley & Sons, Ltd, 85(8), pp. 593–598.
231. Rehman, S. *et al.* (2011) 'Dupuytren's: A systems biology disease', *Arthritis Research and Therapy*. BioMed Central, p. 238.
232. Ribas, A. and Wolchok, J. D. (2018) 'Cancer Immunotherapy Using Checkpoint Blockade', *Science (New York, N.Y.)*. NIH Public Access, 359(6382), p. 1350.
233. Riester, S. M. *et al.* (2015) 'RNA sequencing reveals a depletion of collagen targeting microRNAs in Dupuytren's disease', *BMC Medical Genomics*. BioMed Central Ltd., 8(1), pp. 1–14.
234. RO, W. *et al.* (2000) 'Evaluation of TNF-alpha and IL-1 blockade in collagen-induced arthritis and comparison with combined anti-TNF-alpha/anti-CD4 therapy', *Journal of immunology (Baltimore, Md. : 1950)*. J Immunol, 165(12), pp. 7240–7245.
235. Rockey, D. C., Bell, P. D. and Hill, J. A. (2015) 'Fibrosis — A Common Pathway to Organ Injury and Failure', *New England Journal of Medicine*. Massachusetts Medical Society, 372(12), pp. 1138–1149.
236. Rodrigues, M. *et al.* (2019) 'Wound Healing: A Cellular Perspective', *Physiol Rev*, 99, pp. 665–706.
237. Rodriguez-Menocal, L. *et al.* (2012) 'Stimulation of Skin and Wound Fibroblast Migration by Mesenchymal Stem Cells Derived from Normal Donors and Chronic Wound Patients', *STEM CELLS Translational Medicine*. John Wiley & Sons, Ltd, 1(3), pp. 221–229.
238. Roncarolo, M. G. *et al.* (2001) 'Type 1 T regulatory cells', *Immunological Reviews*. John Wiley & Sons, Ltd, 182(1), pp. 68–79.
239. Ruan, Q. *et al.* (2011) 'The Th17 immune response is controlled by the Rel-RORγ-RORγT transcriptional axis', *Journal of Experimental Medicine*. The Rockefeller University Press, 208(11), pp. 2321–2333.
240. Sahai, E. *et al.* (2020) 'A framework for advancing our understanding of cancer-associated fibroblasts', *Nature Reviews Cancer*. Nature Research, pp. 174–186.
241. Sawant, M., et al (2021). A story of fibers and stress: Matrix-embedded signals for fibroblast activation in the skin. *Wound repair and regeneration: official publication of the Wound Healing Society [and] the European Tissue Repair Society*, *29*(4), 515–530.

242. Sayadi, L. R. *et al.* (2019) 'The Molecular Pathogenesis of Dupuytren Disease: Review of the Literature and Suggested New Approaches to Treatment', *Annals of Plastic Surgery*. Lippincott Williams and Wilkins, 83(5), pp. 594–600.
243. Schildberg, F. A. and Donnenberg, V. S. (2018) 'Stromal cells in health and disease', *Cytometry Part A*. John Wiley & Sons, Ltd, 93(9), pp. 871–875.
244. Schonfeldova, B., Zec, K., & Udalova, I. A. (2022). Synovial single-cell heterogeneity, zonation and interactions: a patchwork of effectors in arthritis. *Rheumatology* (Oxford, England), 61(3), 913–925.
245. Schuster, R., et al. (2021). The inflammatory speech of fibroblasts. *Immunological reviews*, *302*(1), 126–146.
246. Segawa, S. *et al.* (2016) 'The regulatory role of interferon-γ producing gamma delta T cells via the suppression of T helper 17 cell activity in bleomycin-induced pulmonary fibrosis', *Clinical and Experimental Immunology*. Wiley-Blackwell, 185(3), p. 348.
247. Selman, M. *et al.* (1990) 'Effect of lung T lymphocytes on fibroblasts in idiopathic pulmonary fibrosis and extrinsic allergic alveolitis', *Thorax*. Thorax, 45(6), pp. 451–455.
248. Shaw, R. B. *et al.* (2007) 'Dupuytren's disease: History, diagnosis, and treatment', *Plastic and Reconstructive Surgery*, 120(3), pp. 44–54.
249. Shchudlo N.A., et al. (2021) Comparative Clinical and Pathomorphological Characteristics of Dupuytren's Contracture in Men and Women. *Traumatology and Orthopedics of Russia*.Vol. 27.P.166-174.
250. Shoemaker, C. T. *et al.* (1984) 'Elevated Ratios of Type I/III Collagen in the Lungs of Chronically Ventilated Neonates with Respiratory Distress', *Pediatric Research 1984 18:11*. Nature Publishing Group, 18(11), pp. 1176–1180.
251. Shuai, K. and Liu, B. (2003) 'Regulation of JAK–STAT signalling in the immune system', *Nature Reviews Immunology 2003 3:11*. Nature Publishing Group, 3(11), pp. 900–911.
252. Simons, K. H. *et al.* (2018) 'The role of CD27-CD70-mediated T cell co-stimulation in vasculogenesis, arteriogenesis and angiogenesis', *International Journal of Cardiology*. Elsevier, 260, pp. 184–190.
253. Smeraglia, F., et al (2016). Collagenase clostridium histolyticum in Dupuytren's contracture: a systematic review. *British medical bulletin*, *118*(1), 149–158.
254. Smith-Garvin, J. E., Koretzky, G. A. and Jordan, M. S. (2009) 'T Cell Activation', *Annual Review of Immunology*. Annual Reviews , 27(1), pp. 591–619.
255. Spees, J. L., Lee, R. H. and Gregory, C. A. (2016) 'Mechanisms of mesenchymal stem/stromal cell function', *Stem Cell Research & Therapy 2016 7:1*. BioMed Central, 7(1), pp. 1–13.
256. Starzer, A. M. and Berghoff, A. S. (2019) 'New emerging targets in cancer immunotherapy: CD27 (TNFRSF7)', *ESMO Open*. Elsevier, 4, p. e000629.
257. Tai, Y. *et al.* (2021) 'Myofibroblasts: Function, Formation, and Scope of Molecular Therapies for Skin Fibrosis', *Biomolecules 2021, Vol. 11, Page 1095*. Multidisciplinary Digital Publishing Institute, 11(8), p. 1095.
258. Tai, Y., et al. (2021). Myofibroblasts: Function, Formation, and Scope of Molecular Therapies for Skin Fibrosis. *Biomolecules*, *11*(8), 1095.
259. Tamborrini, G., Gengenbacher, M. and Bianchi, S. (2012) 'Knuckle pads – a rare finding', *Journal of Ultrasonography*. Polish Ultrasound Society, 12(51), p. 493.
260. Teicher B. A. (2019). CD248: A therapeutic target in cancer and fibrotic diseases. *Oncotarget*, *10*(9), 993–1009.
261. Tesmer, L. A. *et al.* (2008) 'Th17 cells in human disease', *Immunological Reviews*. John Wiley & Sons, Ltd, pp. 87–113.

262. The British Dupuytren's Society | Supporting people with Dupuytren's, Ledderhouse and Peyronie's disease. 2022. Symptoms of Dupuytren's | The British Dupuytren's Society. [online] Available at: <https://dupuytrens-society.org.uk/information/related-conditions/> [Accessed 19 May 2022].
263. To, W. S. and Midwood, K. S. (2011) 'Plasma and cellular fibronectin: Distinct and independent functions during tissue repair', *Fibrogenesis and Tissue Repair*. BioMed Central, pp. 1–17.
264. Tomasek, J. J. *et al.* (2002) 'Myofibroblasts and mechano-regulation of connective tissue remodelling', *Nature Reviews Molecular Cell Biology 2002 3:5*. Nature Publishing Group, 3(5), pp. 349–363.
265. Townley, W. A. *et al.* (2006) 'Dupuytren's contracture unfolded', *British Medical Journal*, pp. 397–400.
266. Travaglini, K. J. *et al.* (2020) 'A molecular cell atlas of the human lung from single-cell RNA sequencing', *Nature 2020 587:7835*. Nature Publishing Group, 587(7835), pp. 619–625.
267. Tripoli, M., Cordova, A. and Moschella, F. (2016) 'Update on the role of molecular factors and fibroblasts in the pathogenesis of Dupuytren's disease', *Journal of Cell Communication and Signaling*. Springer, 10(4), p. 315.
268. Usunier, B. *et al.* (2014) 'Management of Fibrosis: The Mesenchymal Stromal Cells Breakthrough', *Stem Cells International*. Hindawi Limited, 2014.
269. Van Beuge, M. M. *et al.* (2015) 'Wnt pathway in Dupuytren disease: connecting profibrotic signals', *Translational research : the journal of laboratory and clinical medicine*. Transl Res, 166(6), pp. 762-771.e3.
270. van Beuge, M. M. *et al.* (2016) 'Matrix and cell phenotype differences in Dupuytren's disease', *Fibrogenesis and Tissue Repair*. BioMed Central Ltd., 9(1), pp. 1–11.
271. Van Linthout, S., Miteva, K. and Tschöpe, C. (2014) 'Crosstalk between fibroblasts and inflammatory cells', *Cardiovascular Research*. Oxford Academic, 102(2), pp. 258–269.
272. Van Nuffel, M. *et al.* (2022) 'Palmar skin elasticity measured by the Cutometer MPA 580 is decreased in mild Dupuytren's disease compared to healthy controls' *Hand Therapy*. 2022;27(1):14-21.
273. van Obberghen-Schilling, E. *et al.* (2011) 'Fibronectin and tenascin-C: Accomplices in vascular morphogenesis during development and tumor growth', *International Journal of Developmental Biology*, 55(4–5), pp. 511–525.
274. van Oosterwijk, M. F. *et al.* (2007) 'CD27-CD70 interactions sensitise naive CD4+ T cells for IL-12-induced Th1 cell development', *International Immunology*. Oxford Academic, 19(6), pp. 713–718.
275. Ven, K. van de and Borst, J. (2015) 'Targeting the T-cell co-stimulatory CD27/CD70 pathway in cancer immunotherapy: rationale and potential', *http://dx.doi.org/10.2217/imt.15.32*. Future Medicine Ltd London, UK , 7(6), pp. 655–667.
276. Verjee, L. S. *et al.* (2009) 'Myofibroblast Distribution in Dupuytren's Cords: Correlation With Digital Contracture', *The Journal of Hand Surgery*. W.B. Saunders, 34(10), pp. 1785–1794.
277. Verjee, L. S. *et al.* (2010) 'Post-transcriptional regulation of α-smooth muscle actin determines the contractile phenotype of Dupuytren's nodular cells', *Journal of Cellular Physiology*. John Wiley & Sons, Ltd, 224(3), pp. 681–690.
278. Verjee, L. S. *et al.* (2013) 'Unraveling the signaling pathways promoting fibrosis in Dupuytren's disease reveals TNF as a therapeutic target'.

279. Vincenti, M. P. (2001) ‘The Matrix Metalloproteinase (MMP) and Tissue Inhibitor of Metalloproteinase (TIMP) Genes’, *Methods in molecular biology (Clifton, N.J.)*. Humana Press, 151, pp. 121–148.
280. Wajant, H. (2016) ‘Expert Opinion on Therapeutic Targets Therapeutic targeting of CD70 and CD27 Therapeutic targeting of CD70 and CD27’. *Expert opinion on therapeutic targets*, *20*(8), 959–973.
281. Walthall, J., Anand, P. and Rehman, U. H. (2021) ‘Dupuytren Contracture’, *Essentials of Physical Medicine and Rehabilitation: Musculoskeletal Disorders, Pain, and Rehabilitation*. StatPearls Publishing, pp. 154–158.
282. Wan, Y. Y. and Flavell, R. A. (2009) ‘How Diverse—CD4 Effector T Cells and their Functions’, *Journal of Molecular Cell Biology*. Oxford Academic, 1(1), pp. 20–36.
283. Wang, M. L. *et al.* (2021) ‘Circulating inflammatory cytokines alter transcriptional activity within fibrotic tissue of Dupuytren’s disease patients’, *Journal of Orthopaedic Research*. John Wiley and Sons Inc, pp. 1–12.
284. Wang, Y. et al. (2014) ‘Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications’, *Nature Immunology* 2014 15:11. Nature Publishing Group, 15(11), pp. 1009–1016.
285. Watts, T. H. (2005). TNF/TNFR family members in costimulation of T cell responses. *Annu. Rev. Immunol*, 23, 23–68.
286. Wei, S. *et al.* (1999) ‘Left and right ventricular collagen type I/III ratios and remodeling post-myocardial infarction’, *Journal of Cardiac Failure*. Churchill Livingstone, 5(2), pp. 117–126.
287. Weinstein, A. L., Haddock, N. T., & Sharma, S. (2011). Dupuytren's disease in the Hispanic population: a 10-year retrospective review. *Plastic and reconstructive surgery*, *128*(6), 1251–1256.
288. Weyand, C. M. and Goronzy, J. J. (2021) ‘The immunology of rheumatoid arthritis’, *Nature Immunology*. Nature Research, pp. 10–18.
289. Wick, G. *et al.* (2013) ‘The Immunology of Fibrosis’. (2013). *Annual review of immunology*, *31*, 107–135.
290. Wilkins, S. et al. (2021) ‘From Mechanisms to Outcomes Cancer-Associated Fibroblasts and T Cells’. *Journal of immunology (Baltimore, Md. : 1950)*, *206*(2), 310–320.
291. Wilkinson, J. M. *et al.* (2012) ‘MMP-14 and MMP-2 are key metalloproteases in Dupuytren’s disease fibroblast-mediated contraction’, *Biochimica et biophysica acta*. Biochim Biophys Acta, 1822(6), pp. 897–905.
292. Williams, A. R. and Hare, J. M. (2011) ‘Mesenchymal stem cells: Biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease’, *Circulation Research*. Lippincott Williams & Wilkins Hagerstown, MD , 109(8), pp. 923–940.
293. Wong, et al. (2022). Basic science approaches to common hand surgery problems. *The Journal of hand surgery, European volume*, *47*(1), 117–126.
294. Wong, M. *et al.* (2008) ‘TNFα blockade in human diseases: Mechanisms and future directions’, *Clinical immunology (Orlando, Fla.)*. NIH Public Access, 126(2), p. 121.
295. Worrell, J. C. and MacLeod, M. K. L. (2021) ‘Stromal–immune cell crosstalk fundamentally alters the lung microenvironment following tissue insult’, *Immunology*. Wiley-Blackwell, 163(3), p. 239.
296. Wu, L. *et al.* (2014) ‘Cardiac fibroblasts mediate IL-17A–driven inflammatory dilated cardiomyopathy’, *The Journal of Experimental Medicine*. The Rockefeller University Press, 211(7), p. 1449.

297. Wynn T. A. (2008). Cellular and molecular mechanisms of fibrosis. *The Journal of pathology*, *214*(2), 199–210.
298. Wynn, T. A. (2004) 'Fibrotic disease and the TH1/TH2 paradigm', *Nature Reviews Immunology*. Nature Publishing Group, pp. 583–594. doi: 10.1038/nri1412.
299. Wynn, T. A. and Vannella, K. M. (2016) 'Macrophages in Tissue Repair, Regeneration, and Fibrosis', *Immunity*. Cell Press, pp. 450–462.
300. Wynn, T. A., & Barron, L. (2010). Macrophages: master regulators of inflammation and fibrosis. *Seminars in liver disease*, *30*(3), 245–257.
301. Wynn, T. A., & Ramalingam, T. R. (2012). Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nature medicine*, *18*(7), 1028–1040.
302. Xie, P. (2013) 'TRAF molecules in cell signaling and in human diseases', *Journal of Molecular Signaling*. Ubiquity Press, 8, p. 7.
303. Xu, W. *et al.* (2020) 'TRAF5 protects against myocardial ischemia reperfusion injury via AKT signaling', *European Journal of Pharmacology*. Elsevier, 878, p. 173092.
304. Xue, M. and Jackson, C. J. (2015) 'Extracellular Matrix Reorganization During Wound Healing and Its Impact on Abnormal Scarring', *Advances in Wound Care*. Mary Ann Liebert, Inc., 4(3), p. 119.
305. Yamanaka, Y. *et al.* (2018) 'Blocking fibrotic signaling in fibroblasts from patients with carpal tunnel syndrome', *Journal of Cellular Physiology*. Wiley-Liss Inc., pp. 2067–2074.
306. Yang, D. D. et al. (1998) 'Differentiation of CD4+ T Cells to Th1 Cells Requires MAP Kinase JNK2', Immunity. Cell Press, 9(4), pp. 575–585.
307. Yokoi, H., et al (2002). Role of connective tissue growth factor in fibronectin expression and tubulointerstitial fibrosis. *American journal of physiology. Renal physiology*, *282*(5), F933–F942.
308. Yun DH, et al (2020) Ledderhose Disease With Concomitant Presence of Dupuytren Contracture: A Case Report and Review of the Literature. *Plastic Surgery Case Studies.*
309. Zaworski, R. E., & Mann, R. J. (1979). Dupuytren's contractures in a black patient. *Plastic and reconstructive surgery*, *63*(1), 122–124.
310. Zdilla, M. J. (2017) 'The Hand of Sabazios: Evidence of Dupuytren's Disease in Antiquity and the Origin of the Hand of Benediction', *The journal of hand surgery Asian-Pacific volume*, 22(3), pp. 403–410.
311. Zhang, J. *et al.* (2019) 'Profibrotic effect of IL-17A and elevated IL-17RA in idiopathic pulmonary fibrosis and rheumatoid arthritis-associated lung disease support a direct role for IL-17A/IL-17RA in human fibrotic interstitial lung disease', *American Journal of Physiology - Lung Cellular and Molecular Physiology*. American Physiological Society, 316(3), pp. L487–L497.
312. Zhang, J. *et al.* (2020) 'Cancer-associated fibroblasts promote the migration and invasion of gastric cancer cells via activating IL-17a/JAK2/STAT3 signaling', *Annals of Translational Medicine*. AME Publications, 8(14), pp. 877–877.
313. Zhang, J. M. and An, J. (2007) 'Cytokines, inflammation, and pain', *International Anesthesiology Clinics*. NIH Public Access, pp. 27–37.
314. Zhang, M., & Zhang, S. (2020). T Cells in Fibrosis and Fibrotic Diseases. *Frontiers in Immunology*, 11, 1142.
315. Zhang, S., Zhang, Y., & Min, P. (2022). Single-Cell and Bulk Transcriptome Data Integration Reveals Dysfunctional Cell Types and Aberrantly Expressed Genes in Hypertrophic Scar. *Frontiers in genetics*, *12*, 806740.
316. Zhang, S.-C. et al. (2019) 'Clinical Implications of Tumor-Infiltrating Immune Cells in Breast Cancer', Journal of Cancer. Ivyspring International Publisher, 10(24), p. 6175.

317. Zhao, J. *et al.* (2020) ‘The role of interleukin-17 in tumor development and progression’, *Journal of Experimental Medicine*. The Rockefeller University Press, 217(1).
318. Zheng, L. *et al.* (2022) ‘Risk Factors for Dupuytren’s Contracture: A Case-Control Study’, *Computational Intelligence and Neuroscience*. Edited by S. Ahmad, 2022, pp. 1–6.
319. Zhou, C., et al (2016). Anti-fibrotic action of pirfenidone in Dupuytren's disease-derived fibroblasts. *BMC musculoskeletal disorders*, *17*(1), 469.
320. Zimmerman, M., Gottsäter, A., & Dahlin, L. B. (2022). Carpal Tunnel Syndrome and Diabetes-A Comprehensive Review. *Journal of clinical medicine*, *11*(6), 1674.

Bibliography

Born in Michigan to Cassandra and Kevin Carter, Kristyn is the second oldest of their daughters and the first in her family to complete a PhD program. She began her scientific research pursuits at Mount Holyoke College (MHC) where she graduated with a Bachelor of Arts as a Biochemistry major and mathematics minor. During her time at MHC, she pursued several independent studies where she researched neuroscience and animal behaviour under Dr. Karen Hollis and Black studies under Professor Lucas Wilson and Catherine Manegold. During her Black studies research, she uncovered the story of Dr. James McCune-Smith. He was born enslaved and upon being freed, attended the University of Glasgow's School of medicine. In 1837, he graduated at the top of his class and returned to America as the University of Glasgow's first African American physician and, in America, was the first University trained African American physician. Following his footsteps, she travelled to Glasgow to pursue her Masters of Immunology and upon completing her degree went on to pursue her PhD in Immunology. Similar to Dr. McCune Smith, Kristyn will be the first Black graduate of the University of Glasgow PhD in Immunology program.

After completing her PhD, Kristyn returned to America and began her postdoctoral training at Yale University under Professor Valerie Horsley. Applying the work done in her PhD on tissue immunology, Kristyn's postdoctoral studies will focus on how engineered materials can improve diabetic wound healing. Upon completing her postdoctoral training, she plans to matriculate into medical school to pursue her MD, aiming to practice orthopaedic surgery. This will allow her to reach her ultimate career goal of becoming a physician scientist and leading her own tissue biology research group.

Aside from her pursuing her career as physician scientist, Kristyn is also passionate about creating opportunities for those who are historically excluded from the fields of science, engineering, and medicine. During her time at the University of Glasgow, she dedicated her free time to the race equality group towards the aim of improving student and staff life at Glasgow Uni. In parallel, she founded her own organisation Minorities in Science, Technology, Engineering, Arts, and Mathematics (MiSTEAM) where she, and her board, hosted events pointed towards supporting those in STEAM to create a safe space for those intentionally disenfranchised and neglected in their field. As she progresses in her career, she intends to expand her organisation and continue to create equitable opportunities for those without access, as this is the only way to increase visibility in S.T.E.A.M.