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The role of stromal-immune cell interactions in the pathogenesis of tendinopathy

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Abstract

Background: Overuse injuries of the tendon—encompassed by the term 'tendinopathy'—represent a largely underestimated group of musculoskeletal disorders associated with chronic inflammation and dysregulated tissue repair. Tendinopathies account for 30-50% of all sporting injuries and a high proportion of rheumatological and orthopaedic referrals from primary care physicians. Despite historical disagreement between 'inflammation' vs 'degeneration' hypotheses it is now widely accepted that inflammatory mechanisms elicited by persistent mechanical injury at a microscopic level disturb the intricate homeostatic balance that exists between stromal and immune cell compartments within the tendon during the initial stages of disease. The molecular mechanisms that regulate inflammatory pathways in tendinopathy are largely unknown therefore this thesis sought to characterize mechanisms involved in activation of the innate immune system and subsequent development of persistent inflammation and aberrant matrix repair.

Key results: Gene expression analysis of tendon tissue identified the presence of myeloid associated alarmins S100A8 and S100A9 in early tendinopathy. Treatment of primary human tenocytes with exogenous S100A8 & A9 enhanced cytokine and chemokine release; however, no alterations in genes associated with matrix remodelling were observed indicating these alarmins act to exaggerate the inflammatory response in the early stages of disease. Extensive phenotyping of tendon stromal cells by flow cytometry identified the presence of novel subsets of tenocytes that are expanded under chronic inflammatory conditions. Furthermore, enhanced expression of markers associated with stromal cell activation was observed *ex vivo* in late tendinopathic tissue and *in vitro* in response to inflammatory stimuli. I next identified a contact dependent mechanism through which stromal cells influence immune cell phenotype and differentiation in a direct tenocyte-monocyte co-culture model. Finally, expression of stromal activation markers podoplanin and VCAM1 in tenocytes was silenced by siRNA mediated knockdown; however, no discernible alterations in tenocyte behaviour or changes in monocyte phenotype (induced by monocyte-tenocyte co-culture) were observed indicating alternate mechanisms are responsible for these interactions.

Conclusions: This study has identified novel stromal-immune cell crosstalk in tendinopathy and highlighted the significance of these interactions in the development of non-resolving chronic inflammation.

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Author's 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.

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Abbreviations

APC – Antigen presenting cell

SMA - smooth muscle actin

bHLH - Basic helix loop helix

CAD11- Cadherin 11

CAF- Cancer associated fibroblast

CAR - Chimeric antigen receptor

CF – Cystic fibrosis

CLEC – C-type lectin

COMP – Cartilage oligomeric matrix protein

COX – Cyclooxygenase

CRP – C reactive protein

CTLD – C type lectin domain

DAB – 3,3-diaminobenzidine

DAMP – Damage associated molecular pattern

DC – Dendritic cell

DNA – Deoxyribonucleic acid

ECM – Extracellular matrix

ELISA – Enzyme linked immunosorbent assay

ERK – Extracellular signal-regulated kinase

FACS – Fluorescence activated cell sorting

FAP – Fibroblast activation protein

FGF – Fibroblast growth factor

FLS – Fibroblast like synoviocyte

GAG - Glycosaminoglycan

GM-CSF – Granulocyte-macrophage colony-stimulating factor

HLA – Human leukocyte antigen

HMG – High mobility group

HSP – Heat shock protein

IBD – Inflammatory bowel disease

ICAM – Intracellular adhesion molecule

IL - Interleukin

LIF – Leukemia inhibitory factor

LPS – Lipopolysaccaride

LT - Leukotriene

MAPK – Mitogen activated

M-CSF – Macrophage colony-stimulating factor

MCP – Monocyte chemoattractant protein

MDSC – Myeloid-derived suppressor cell

TK – Tyrosine kinase

MHC – Major histocompatibility complex

MIP – Macrophage inflammatory protein

MMP – Matrix metalloproteinase

MPS – Mononuclear phagocyte system

MRP – Myeloid related protein

MS – Multiple sclerosis

MSC – Mesenchymal stem cell

MTJ – Myotendinous junction

NF – Nuclear factor

NK – Natural killer

NO – Nitric oxide

NOS – Nitric oxide synthase

NSAID – Non steroidal anti-inflammatory drug

OA - osteoarthritis

PAMP – Pattern associated molecular pattern

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PDAC – Pancreatic ductal adenocarcinoma

PDGF – Platelet derived growth factor

PG - Prostaglandin

PNS – Peripheral nervous system

PRR – Pattern recognition receptor

PsA – Psoriatic arthritis

RA – Rheumatoid arthritis

RAGE – Receptor for glycation end product

RNA – Ribonucleic acid

ROS – Reactive oxygen species

SCID – Severe combined immunodeficiency

SLE – Systemic lupus erythematosus

SLRP – Small leucine-rich repeat protein

SOCS – Suppressor of cytokine signalling

SPADE – Spanning-tree progression of density normalized events

STAT – Signal transducer and activator of transcription

TBST – Tris buffered saline

TGF – Transforming growth factor

TIMP – Tissue inhibitor of metalloproteinase

TLR – Toll-like receptor

TNF – Tumour necrosis factor

TNFR – Tumour necrosis factor receptor

TNMD - Tenomodulin

tSNE – t-distributed stochastic neighbour embedding

VCAM – Vascular cell adhesion molecule

VEGF – Vascular endothelial growth factor

Chapter 1: Introduction

1.1 Tendon Biology

1.1.1. Tendon structure and function

Tendons are connective tissue structures interposed between muscles and bones. The primary function of a tendon is to enable transduction of mechanical load generated by a muscle to bone thus enable movement at a joint.[1] Tendons are highly organised structures composed mainly of water, type 1 collagen (approximately 70% of its dry weight) and a combination of other matrix proteins. The basic cellular component of the tendon is the 'tenocyte'; a fibroblast like cell that regulates turnover of the extracellular matrix.[2]

The high tensile strength of the tendon is derived from the hierarchical organisation of collagen molecules interspersed with a highly hydrated matrix at each level of the hierarchy.[3] Commonly referred to as ground substance, this is composed largely of water, proteoglycans and glycoproteins.[4] The axial and lateral organisation of collagen molecules ensures strong intermolecular interactions and cross linkage. At the molecular level, insoluble collagen molecules are formed by the cross linking of soluble tropocollagen. Arrangement into microfibrils and subsequent assembly into fibrils make up the macroscopic units of the hierarchy.[5] Tendon structure and composition can vary according to both anatomic location and specific sites within the tendon.[6]

A 'bunch' of collagen fibrils makes up a collagen fibre which is the basic structural unit of a tendon. This is encapsulated by a fine sheath of connective tissue called the endotenon that binds fibres together. A bunch of fibres makes up the subfascicle or primary fibre bundle that in turn aggregates into a group of secondary fascicles. These tertiary bundles make up the whole tendon that is encased by the epitenon.[7] In regions away from joints a loose connective tissue structure called the paratenon surrounds tendons to enable movement beneath the skin. Around joint structures the tendon is contained within a synovial sheath to protect it from bony surfaces that may cause friction.[8]

The point of union between the tendon and adjoining muscle is the myotendinous junction (MTJ). This is the primary site of force transmission and is based on structural relationships between cytoskeletal proteins and the extracellular

matrix.[9] Proximally, the osteotendinous junction or enthesis comprises the tendon bone interface. The enthesis has a unique molecular and cellular composition a gradual transition from tendinous to bone tissue. The enthesis may be classified as fibrous or fibrocartilaginous with the former representing an area where the tendon attaches directly to the bone. The fibrocartilaginous enthesis describes an area where chondrogenesis has occurred through several zones that ultimately results in enthesial tissue indistinct from bone.[10] It has been postulated that the enthesis is in fact a collection of related tissues termed the 'enthesis organ'. This is composed of the fibrocartilages, bursae and fat pad that act synergistically to dissipate stress.[11]

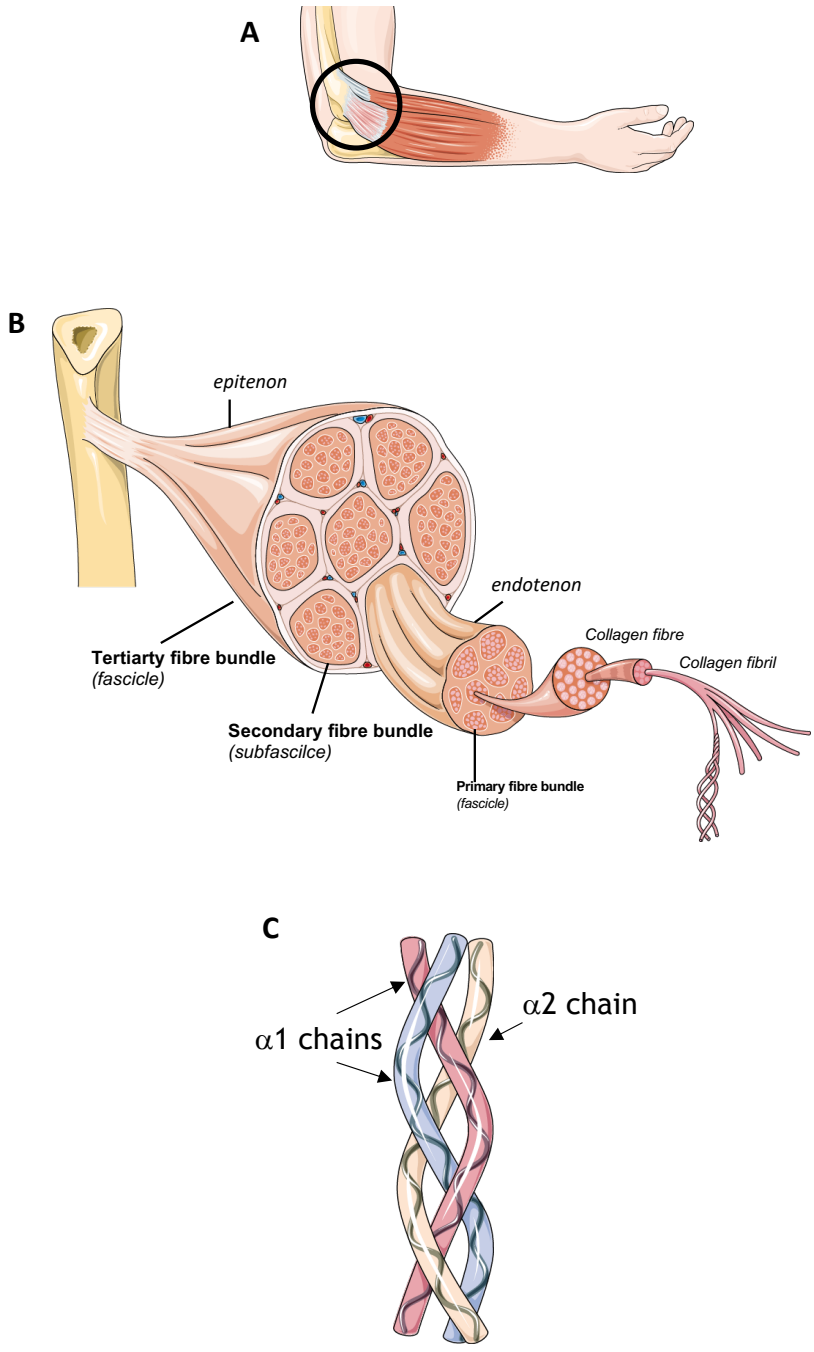


Figure 1.1 Tendon structure
(A) Anatomical location of extensor tendons in the arm
(B) Hierarchical structure of tendon
(C) Structure of collagen triple helix

1.1.2. Extracellular tendon matrix

Collagen

Collagen protein is comprised of a triple helix, most commonly two identical alpha chains (α1) and one structurally similar α2 chain.[12] Collagen I is the primary component of tendon tissue accounting for approximately 70% of its dry weight and 95% of total collagens. The next most abundant is type III collagen accounting for 3% of total tendon dry weight and is mainly found in the endotenon and epitenon.[13] Types II, IV, XI are also present and, in cartilaginous areas, type IX, X and XI have been described.[2]

Ground substance

Ground substance is a highly hydrated gel like substance that confers the viscoelastic properties to a tendon.[14] It is a complex mixture of glycoproteins and proteoglycans and binds tenocytes to collagen fibres. It is an extremely viscous substance that provides structural support to the collagen fibres and also acts as a medium for diffusion of gases and essential nutrients.[15]

Proteoglycans

Proteoglycans- a subclass of glycoproteins- are the most abundant non fibrous proteins in the tendon accounting for between 1 and 5% of its dry weight.[15] Small leucine rich proteoglycans (SLRPs) consist of a protein core covalently attached to one or more glycosaminoglycan side chains (GAGs) and are capable of holding up to fifty times their weight in water.[16] They play an important regulatory role in matrix assembly, cytokine binding and hydration.[17] The most abundant proteoglycan within the tendon matrix is **decorin** which accounts for 80% of total proteoglycans. Decorin shares a common collagen 1 binding site with another SLRP **biglycan** with decorin possessing higher affinity binding.[18] Tendons also express type II SLRPs **fibromodulin**[19] and **lumican**[20] in smaller quantities. **Aggrecan**—the major proteoglycan in articular cartilage—is expressed throughout the tendon and is reported to be most abundant in fibrocartilaginous regions.[21,22]

Glycoproteins

Structurally, glycoproteins are macromolecules that consist of a protein to which a carbohydrate molecule is attached. They differ to proteoglycans in their protein-carbohydrate ratio. A variety of these proteins are expressed within the tendon

including **fibronectin, COMP, tenascin, vitronectin, tenomodulin**.[23] Fibronectin possesses binding sites for cells, collagen and glycosaminoglycans and acts to facilitate cell adhesion and migration. Fibronectin binds fibroblasts to collagens and enables proliferation and phagocytosis thus facilitating wound healing responses.[24] **Tenascin C** is a glycoprotein that regulates cell behaviour and matrix organization during remodelling processes. It is found in regions of compression such as the osteotendinous junction. Its anti-adhesive properties are thought to result from a cellular adaptation to compression that aids in maintaining the fibrocartilage.[25,26]

Elastin

Elastin is the core component of elastic fibres that comprise approximately 1-2% of tendon dry mass.[15] Generally, elastin content is low and limited to sparse distribution within the fascicles. It has recently been postulated that elastin is most abundant in energy storing tendons such as the Achilles and lowly expressed in positional tendons.[27]

Molecule	Structure/ type	Properties
Proteoglycan		
Decorin	SLRP	Binds collagen, affects collagen-fibril formation, binds growth factors
Biglycan	SLRP	Binds collagen, affects collagen-fibril formation, binds growth factors
Fibromodulin	SLRP	Binds collagen, affects collagen-fibril formation, binds growth factors
Lumican	SLRP	Binds collagen, affects collagen-fibril formation
Aggrecan	Hyalectin	Resists compression, expression prominent in fibrocartilage and low in tensional areas of tendon
Versican	Hyalectin	Lubricates boundaries between fibres
Glycoprotein		
Tenascin-C	Branched molecule	Mediates cell-matrix interactions
Fibronectin	Modular protein	Mediates cell-matrix interactions, role in tendon healing
COMP	Branched molecule	Mediates cell-matrix interactions, role in fibril formation
Tenomodulin	Type II transmembrane glycoprotein	Role in collagen fibril maturation
Elastin	Branched network	Forms elastic fibres, provides elastic properties of tissue

Table 1.1 Properties of tendon extracellular matrix components

1.1.3. Tendon cells

Tenocytes and tenoblasts make up 90-95% of the cellular component of tendon tissue. The remaining percentage is accounted for by chondrocytes at insertion sites, synovial cells of the tendon sheath and vascular cells (smooth muscle cells and endothelial cells) of the arterioles in the endotenon and epitenon.[28] Under homeostatic conditions there is also a small compartment of tissue resident immune cells that may be expanded under pathological conditions.[29]

Tenoblasts are spindle shaped immature tendon cells (approximately 20-70μm in length). Young tendon has a high cell-matrix ratio and tenoblasts exhibit numerous cytoplasmic organelle that reflect the high metabolic activity of the developing tissue.[30] As tenoblasts elongate they develop into tenocytes (approximately 80-300μm in diameter). Tenocytes have extremely elongated nuclei that span almost the entire length of the cell. Consequently, the lower nucleus to cytoplasm ratio results in reduced metabolic activity.[1] Mature tendon is relatively acellular with tenocytes interspersed between collagen fibrils accounting for 20% of total tissue volume.[31]

Tenocytes are mechanosensitive cells capable of detecting changes in mechanical load. Deformation of their cell membrane and cytoskeleton due to ECM strain activates membrane bound integrin receptors that signal to the nucleus and alter gene expression.[32] As such, this cellular response to mechanical load is a particularly practical adaptation in the context of tissues exposed to high levels of strain.

1.1.4. Tendon development and tenocyte lineage commitment

Mesenchymal stem cells (MSCs) are the common progenitors for skeletal muscle and tenocyte progenitors and lineage commitment is regulated by a number of environmental variables. During embryogenesis genes expressed in the developing tendon include homeobox genes six1, six2 and their transcriptional co-activators eyes absent homologue 1 (Eya1) and Eya2, tenascin, follistatin, mohawk and scleraxis.[33,34,35] Scleraxis—a basic helix loop helix (bHLH) transcription factor—is the most well characterised regulator of tendon development.[36] It is expressed in tendon progenitors and differentiated cells during embryogenesis and at later

stages of development. At embryonic day (E) 10.5 scleraxis is detected in the developing limb bud in the somitic compartment called the syndetome.[37,38] Conditional knockout of the scleraxis gene in a mouse model of tendon development resulted in significant deficits in tendon differentiation and consequent loss of tendons that transmit force to the limbs, tail and trunk.[37] Tenomodulin (TNMD) is a type II transmembrane protein predominantly expressed in dense connective tissue including tendon and ligament. Tenomodulin is considered a marker of tenocyte differentiation and its expression is positively regulated by scleraxis.[39] In mice deletion of the tenomodulin gene was shown to significantly reduce tenocyte proliferation and alter collagen fibril structure.[40]

1.1.5. Innervation and vasculature

Tendon is a bradytrophic tissue that generally displays a low degree of innervation and is poorly vascularized.[41] This may contribute to its propensity for injury and explain periods of protracted, inferior healing.

Nerve supply

Innervation of the tendon originates in neighbouring muscular, cutaneous and peritendinous nerve trunks. From the myotendinous junction nerve fibres enter the tendon septa and form plexuses in the paratenon.[42] Under normal conditions nerve fibres do not enter the tendon but terminate on the tendon surfaces (paratenon, epitenon and endotenon).[43] Innervating nerves consist of lowly abundant myelinated fast transmitting fibres (Aα and Aβ) with three types on nerves endings responsible for mechanoception. Ruffin corpuscles (Type 1) are pressure and stretching sensors. Similarly, Vater-Pacini corpuscles (Type II) are pressure sensors that react to accelerating and decelerating forces. Golgi tendon organs (Type III) are tension receptors predominantly found at the myotendinous junction and around insertion sites. [44]

Within the tendon sheath there is a greater number of unmyelinated slow transmitting fibres (Aγ, Aδ, B and C fibres). These fibres predominantly function in nociception and mediate deep tissue pain. Autonomic function is conferred by B fibres that exert vasomotor actions on the small arterial and venous structures.[45]

The peripheral nervous system (PNS) is also involved in tendon homeostasis and efferent physiological functions such as cell proliferation, hormone release and immune responses, particularly during periods of stress. This is mediated by classical neurotransmitters including monoamines and acetylcholine as well as neuropeptides such as substance P.[46]

Blood supply

Blood vessels generally enter the tendon through the myotendinous junction, osteotendinous junction and surrounding connective tissue structures including the paratenon. Within the endotenon vessels pass around collagen fibre bundles in a longitudinal manner.[47] The number of vessels and the manner in which they are arranged is highly specific to each tendon. For example, there is a great difference in bloody supply of sheathed and unsheathed tendons. In unsheathed tendons vessels pass through the paratenon at any given point. Conversely, in sheathed tendons around joint capsules blood supply is significantly more organised and well defined.[41]

1.1.6. Mechanobiology of tendon

Tendons possess unique biomechanical properties that facilitate transmission of dynamic mechanical forces. This is predominantly derived from their hierarchical collagen structure and viscoelastic properties.[48] Tissue mechanical adaptation occurs in response to altered load conditions and allows for changes in tendon structure and function. Under physiological conditions loading may be beneficial; however, excessive loading may have detrimental effects and ultimately lead to tendon rupture.[49]

A typical stress strain curve for a tendon consists of four regions. The initial toe region consists of tendon strained up to 2% and represents ‘stretching out’ of crimped tendon fibrils as a result of mechanical loading on the tendon. The ‘crimp pattern’ influences the tendons biomechanical properties and differs according to tendon type.[50] The linear region accounts for strain of up to 4%; this is the point at which collagen fibres lose their crimp pattern. The slope of the line at this point is referred to as Young’s module of tendon which describes the relationship between tensile stress and tensile strain. At strain above 4% (the physiological limit of strain) microscopic tearing occurs resulting in microtear failure. At 8-10%

strain macroscopic tearing occurs that may ultimately result in tendon rupture.[51,48]

Both age and anatomical location of tendons affect their mechanical properties. For example, the Young's modulus of the patellar tendon is 600±266MPa while the elastic modulus of the tibialis anterior is 1200MPa. Similarly, young patellar tendons (29-50 years) have a Young's modulus of 660±266MPa compared with 504±222 in old patellar tendons (64-93 years).[52]

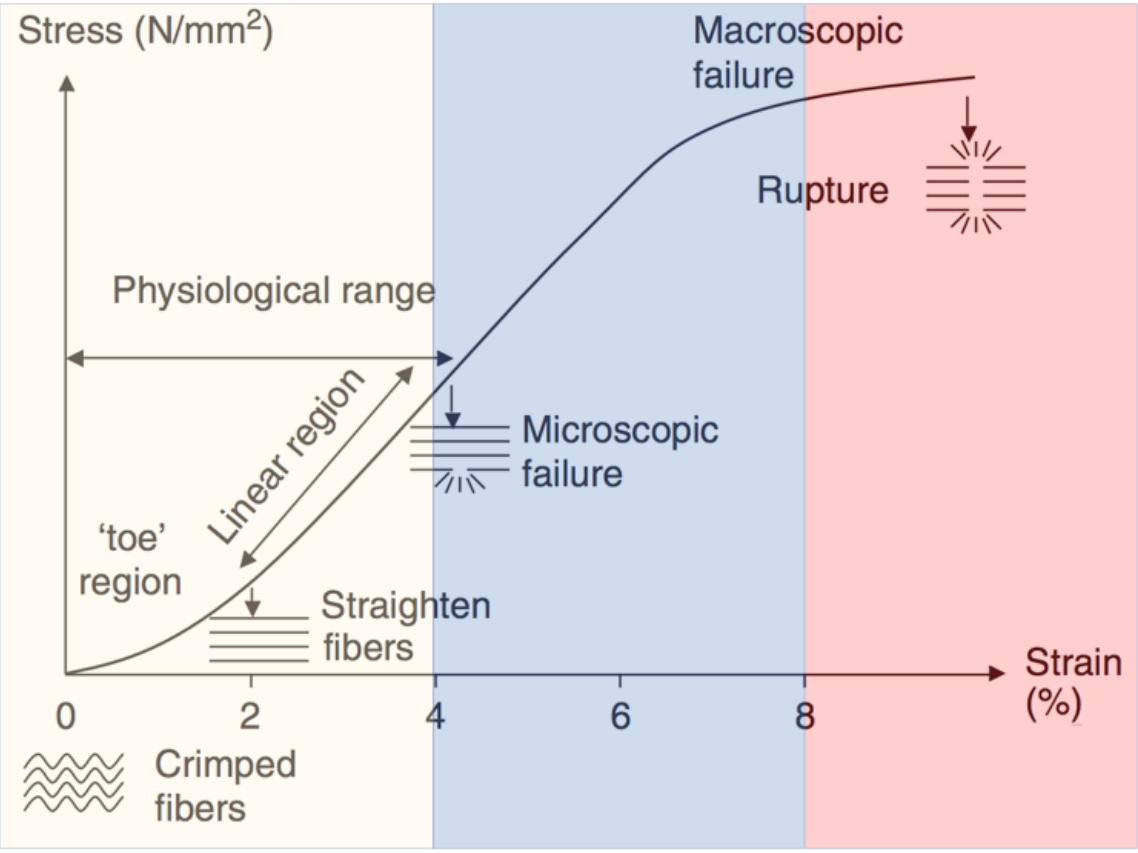


Figure 1.2 Tendon stress-strain curve
0-4% strain represents the physiological range, stretching out of crimped tendon fibrils occurs . Strain above 4% (the physiological limit of strain) microscopic tearing occurs resulting in microtear failure. At 8-10% strain macroscopic tearing occurs that may ultimately result in tendon rupture. Figure adapted from Wang *et al* 2006.

1.1.7. Tendon injury and healing

Tendon injuries can be acute or chronic and arise as result of a combination of both intrinsic and extrinsic factors. Tendon repair occurs over several days in three overlapping stages: inflammation, proliferation and remodelling.[53]

The Inflammatory Phase

In the initial inflammatory phase, rupture of blood vessels results in the immediate formation of a blood clot and release of cytokines, chemokines, growth factors and vasoactive substances that result in local acute inflammation. Inflammatory cells, red blood cells and platelets infiltrate the area using the clot as a preliminary scaffold.[54] Initial cell influx (within hours) is predominately neutrophils. In the following 24 hours monocytes and macrophages predominate and phagocytosis of necrotic debris occurs.[55] Vasoactive substances increase vascular permeability and initiate angiogenesis and the inflammatory milieu generated by the accumulated cells stimulates recruitment and proliferation.[56] This gives rise to the proliferation or repair stage where collagen III synthesis is initiated.[57]

The Proliferative Phase

At approximately two days into the injury response the proliferative or healing phase commences. This phase is characterized by high synthetic activity and recruitment and expansion of the tenocyte population.[58] Tenocytes are recruited to the wound from the paratenon and synovial sheath and proliferate. Similarly, intrinsic tenocytes from the endotenon and epitenon migrate to the site of injury and proliferate. This new pool of tenocytes together with macrophages present in the wound direct synthesis of ECM components, release of growth factors, cell recruitment and neoangiogenesis. The role of macrophages shifts from phagocytic to reparative.[59] Collagen III production peaks as tenocytes lay down a temporary, mechanically inferior, matrix composed of type III collagen and glycosaminoglycans.[53]

The Remodelling Phase

The remodelling phase begins approximately 6 weeks after the initial wound formation. It is characterised by decreased cellularity, reduction of ECM synthesis and organization of the collagen matrix.[58] Between 6-10 weeks tenocyte metabolism remains high and alignment of tenocytes and fibril collagen occurs. Type I collagen synthesis predominates at the stage. Cellularity and synthetic activity gradually decline as the healing tissue changes from cellular to fibrous. After 10 weeks the tissue starts to become scar like and the healing process can take up to one year to resolve.[60] However; the repaired tissue remains inferior and the tendon never fully recuperates the biomechanical properties it possessed prior to injury.[61]

Time (days)	Phase	Process
0	Post injury	Clot formation around the wound
0-1	Inflammatory	Initial release of growth factors and inflammatory mediators, recruitment of neutrophils
1-2	Inflammatory	Influx of monocytes/macrophages, enhanced phagocytosis
2-4	Proliferation	Recruitment and expansion of the tenocyte population
4-7	Reparative	Collagen deposition, revascularization
7-14	Reparative	High synthetic activity and organization of collagen matrix
14-21	Remodelling	Reduced cellular and vascular content, increased type I collagen synthesis
21+	Remodelling	Further organization of the collagen matrix and integration with existing healthy matrix, collagen ratios, water content and cellularity approach normal levels

Table 1.2 Phases of tendon injury and healing

1.2 Tendinopathy

Tendinopathies account for approximately 30% of musculoskeletal consultations in general practice and 30-50% of all sporting injuries.[62,63] It is commonly associated with overuse injury of the tendon and characterised by pain, decline in function and reduced exercise tolerance.[28] Tendon injury affects people of all ages and the prevalence of tendinopathy amongst the general population poses a significant economic burden to public health services. Chronic or acute injuries can occur in any tendon but are particularly prevalent in major tendons with high loading demands including the rotator cuff, Achilles, patellar and forearm extensor tendons.[64,65]

1.2.1 Aetiology and pathophysiology

The aetiology of tendinopathy remains unclear; however, it is becoming increasingly recognised that a multitude of factors are implicated in disease initiation and progression. Chronic disorders are attributed to a combination of extrinsic and intrinsic factors while acute injury is generally associated with one isolated overloading event. Intrinsic factors include sex, age and chronic conditions including type 2 diabetes and obesity.[65] The following section will address theories pertaining to the pathophysiology of tendinopathy. The role of inflammation in tendinopathy will be discussed in greater detail in the subsequent section.

Mechanical stress

Excessive loading is considered the main causative factor in tendon degeneration.[66] Tendons subject to mechanical strain above the physiological limit exhibit inflammation of their sheath and degeneration of the tendon body.[67] Weakening of tendons occurs when fatigue damage is not sufficiently repaired and may ultimately lead to complete rupture. In addition, damage may occur under physiological conditions as a result of repetitive microtrauma and incomplete healing.[66] Mechanical strain is thought to disrupt the hierarchical organisation of collagen structure within the tendon causing vessel disruption and impaired tissue perfusion.

Under physiological conditions mechanical loading of the tendon results in increased collagen synthesis likely regulated by the mechanoresponsive properties

of tenocytes.[68,69] Degradation of collagen proteins occurs simultaneously (due to increases in matrix degrading enzymes such as MMPs) and a net increase in collagen requires a restoration period.[70] It has been postulated that insufficient rest leads to progressive collagen loss and tendinopathy occurs as a result of an imbalance between synthesis and breakdown of matrix proteins.[71,65]

Mechanical stretching of human tenocytes was shown to stimulate production of prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) and an inverse relationship between static load and MMP-1 expression has been observed in rodent tenocytes.[72,73] This indicates that mechanical stress can act directly on the cellular component of the tendon as well as influencing the structure of the extracellular matrix. The precise role of mechanical load in the pathogenesis of tendinopathy remains unclear; however, evidence suggests that a combination of biological factors and structural alterations may weaken the overall architecture of the tendon.

Matrix turnover

Proteolytic activity is required to orchestrate maintenance and repair of tissue. Following injury, proteolysis is required to clear debris from damaged ECM and remodel the injured tissue. MMPs have been implicated in ECM turnover under both physiological and pathological conditions.[6] MMP production in tendon explants in stimulated by cytokines which indicates that inflammation influences proteolytic activity. Tenocytes have been shown to respond to stress by increasing production of MMPs; as alluded to previously, this provides indication that mechanical strain is an important stimulus for ECM remodelling.[74] In a rodent model of acute tendon injury MMPs were identified at different locations and time points following injury. Levels of MMP-9 and MMP-13 peaked between days 7-14, whereas MMP-2, MMP-3 and MMP-14 levels increased following injury and maintained high levels until day 28. The authors propose that MMP-3 and MMP-13 participate in collagen degradation in the inflammatory phase of tendon healing while MMP-2, MMP-3 and MMP-14 predominantly function in the remodelling phase.[75] Increased activity of MMP-1, MMP-2 and MMP-3 has been observed in ruptured supraspinatus tendons that corresponds with collagen turnover and deterioration of the collagen network. This activity is thought to represent a repair function that is associated with degenerative processes caused by repeated

injury or mechanical strain.[76] Furthermore, expression of MMP-13, MMP-3, TIMP-2, TIMP-3 and TIMP-4 mRNA was shown to be altered in both torn and intact tendinopathic rotator cuff tendons.[77,78]

Apoptosis

Several studies have highlighted the presence of increased apoptosis in tendinopathy. Excessive apoptosis of tendon cells has been described in supraspinatus, patellar and tibialis anterior tendons.[79] Further studies have shown that apoptosis is induced by oxidative stress in human tenocytes and is mediated by release of cytochrome C from mitochondria to the cytosol and subsequent activation of caspase-3.[80] A rodent running overuse model demonstrated increased levels of heat shock proteins and apoptotic genes. This was confirmed in human supraspinatus and subscapularis tendons and suggests that heat shock proteins play a role in stress-activated cell death.[81] In addition, IL-18, IL-15 and IL-6 expression has been associated with expression of caspases 3 and 8 and Fas ligand; all of which are genes associated with oxidative stress induced apoptosis.[82]

Oxygen free radicals

Reactive oxygen species (ROS) have traditionally been viewed as inducers of cellular or tissue damage; however, it is now accepted that ROS (alone or in concert with reactive nitrogen species) at lower levels may be physiological and induce processes such as proliferation, differentiation and cellular adaptation.[83]
Several studies have demonstrated a role for nitric oxide (NO) signalling in the pathophysiology of tendinopathy.[84,85] Following tendon injury, NO is produced by all three isoforms of nitric oxide synthase (inducible NOS, endothelial NOS and brain NOS).[86] In a rodent model of Achilles tendon injury iNOS, eNOS and bNOS have been detected sequentially and all three isoforms were detected in tenocytes.[87] Expression of all isoforms has been observed in human rotator cuff biopsy samples.[85] Furthermore, human tenocytes respond to NO by increasing collagen synthesis *in vitro*[88] and microarray analysis has demonstrated increases of type I, III and IV collagen as well as biglycan, decorin, laminin and MMP10.[89]

Vascular changes and hypoxia

Historically, it has been proposed that vascular damage and impaired blood flow due to trauma or aging results in tissue degeneration that precedes acute rupture.[90] Decreased microcirculation and vessel number has been reported in areas of degeneration or rupture in rotator cuff tendons.[91] Furthermore, hypoxic damage and associated apoptosis has been observed in rotator cuff tendons through the progression of pathology and in both partial and full thickness tears.[92] Hypoxic alterations have also been observed in tenocytes forty eight hours after tendon rupture.[93] Conversely, neovascularization and increased blood flow have also been observed in tendinopathic lesions.[94,95] This presents an interesting paradox regarding the implications of vascular changes in tendinopathy as hypoxia is capable of inducing angiogenesis while impaired vascularity may ultimately lead to hypoxia.

Neurological factors

The primary symptom of tendinopathy is pain thus it has been postulated that neurological factors may play a role in the pathogenesis of tendinopathy. Healthy tendon bodies are virtually devoid of nerve fibres and innervation is confined to the paratenon.[96] However, several studies have reported abberations in the distribution and types of nerve fibres present in tendinopathy in addition to increased vascular innervation.[97,98] The neuropeptide substance P (associated with the sensation of pain) has been reported to be increased in patients with rotator cuff tendinopathy.[99] Substance P has also been reported to stimulate neurogenic inflammation in and around the tendon.[100] Substance P has also been shown to induce mast cell degranulation resulting in release of growth factors that modulate fibroblast proliferation, angiogenesis and tissue oedema.[6] In addition, glutamate signalling has been shown to induce a pro-apoptotic response in rat tenocytes *in vitro* which suggests a role for excitotoxic neurotransmitter responses in tendinopathy.[101]

Drug induced tendinopathy

Fluoroquinolones including ciprofloxacin and levofloxacin have been associated with the development of tendinopathy, generally presenting as rupture of the Achilles tendon (accounting for 90% of cases, half of which are bilateral). Tendinopathy occurs in 0.5% to 2% of patients treated with fluroquinolones. Fluoroquinolones inhibit tenocyte metabolism thus reducing cell proliferation and synthesis of ECM components including collagen.[102,103] Furthermore, ciprofloxacin has been shown to induce IL-1β mediated MMP-3 release which may contribute to inflammation and degeneration of tendon tissue.[104]

1.2.2 Histopathology of tendinopathy

Painful tendons show alterations in cellularity (both increased and decreased), decreased matrix organization and increased infiltration of blood vessels.[94] Ruptured tendons display similar degenerative features; however, reduced cellularity is observed and there is little evidence of neovascularization.[105] Healthy tendon appears white in colour and has a firm fibroelastic texture, whereas tendinopathic tendon is grey or brown and thin and fragile.[106] Microscopically, collagen bundles appear disorganised and at the electron microscope level fibres are angulated and vary in diameter and orientation.[107,108] There is also increased ground substance and an increased number of nuclei.[109] Infiltrations of small blood vessels and ingrowths of small nerves are observed in tendinopathic tendon.[110],[107] Furthermore, the presence of lipid vacuoles, degranulated endoplasmic reticulum and enlarged lysosomes are consistent with tissue hypoxia.[105]

Many early histological studies failed to demonstrate the presence of inflammatory cells in human tendon tissue.[107,105,106] However, more recently the presence of several immune cell subsets has be documented in tendinopathy.[111] This will be reviewed in the forthcoming section.

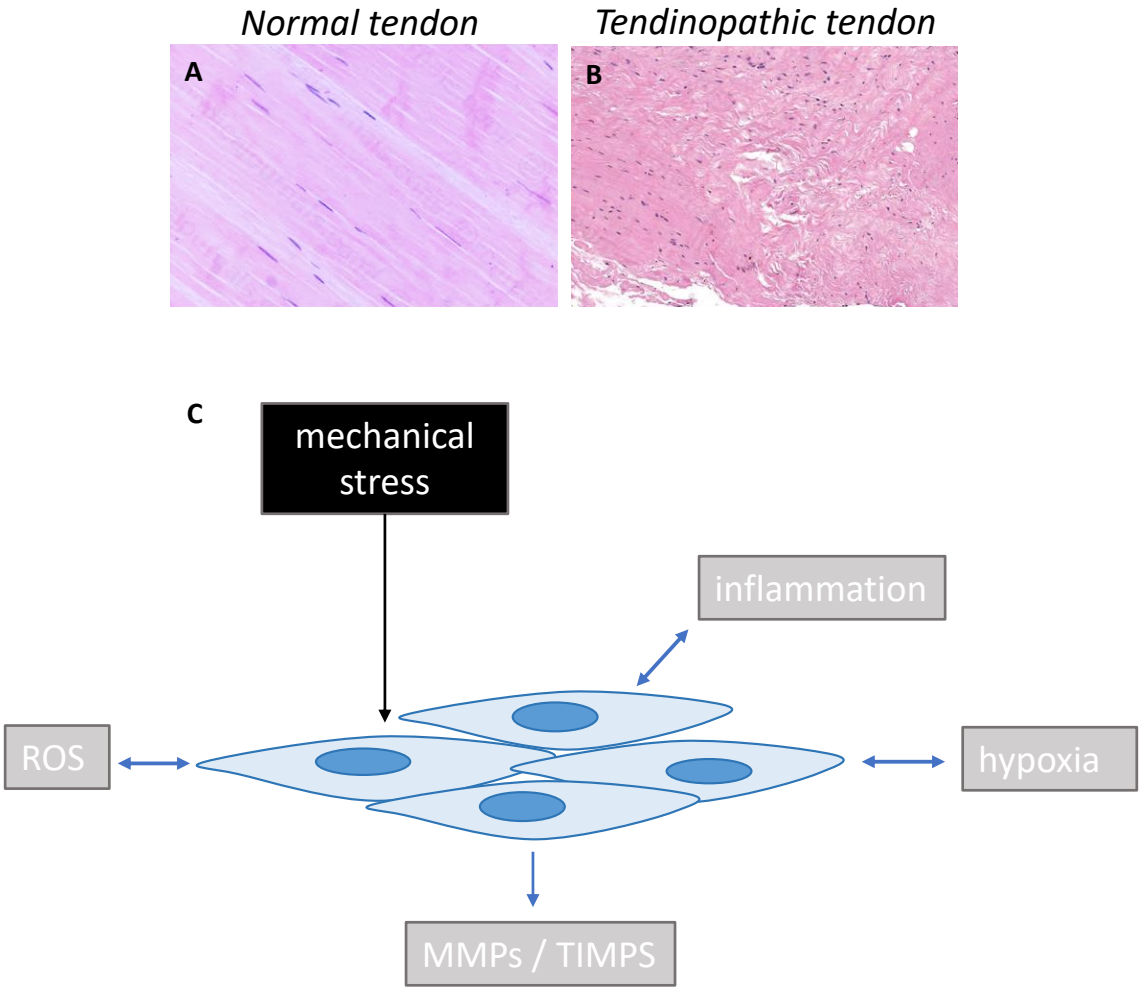


Figure 1.3 Pathophysiology and histopathology of tendinopathy
(A & B) Histological sections illustrate disorganized structure of collagen matrix in tendinopathy **(C)** Aetiological factors in the development of tendinopathy

1.3 Inflammation in tendinopathy

Historically, there has been considerable disagreement with regard to classification and terminology related to tendon disorders. The term 'tendonitis' was traditionally used to describe painful symptoms attributed to inflammation of the tendon while 'tendinosis' refers to damage at a cellular level and is associated with chronic degenerative changes.[112,113] Divergence from the original, widely accepted 'tendonitis' paradigm came from histological observations that showed load bearing regions of tendons were devoid of an inflammatory cell component while studies documenting long term administration of NSAIDs and periodic local corticosteroid injections showed little alleviation of symptoms.[66] Henceforth, theories relating to the 'degenerative' hypothesis became prominent and widely advocated by many research groups and clinicians.[114,115,116,117,118]

However, due to recent advancements in immunohistochemistry and gene expression analysis it is becoming increasingly apparent that a lack of observation of acute inflammatory infiltrate does not exclude a pivotal role for inflammation in the pathogenesis of acute and chronic tendon injury.[119,120] Chronological restraints including late presentation of symptoms in human patients, cases of recurrent injury and the availability of tissue samples for study (the majority of biopsy samples are usually acquired during surgery following acute rupture) may have a bearing on observations of inflammation during different stages of disease. With regard to the resurgence of interest in inflammation in tendinopathy a comparison can be made with the classification of joint conditions where 'arthritis' now takes precedence over 'arthrosis' as infiltration of inflammatory cells and cytokines have been identified as a key contributing factor to pathology, in addition to long term degenerative processes.[121,122,123]

It is now well established that inflammatory mediators are essential in the initiation and maintenance of tendinopathy. Increased inflammatory cell infiltrate (particularly of macrophages, mast cells and T cells) and expression of inflammatory markers has been observed in the early stages of tendinopathy.[111] Several studies have outlined a role for a diverse range of inflammatory mediators including various cytokines and alarmins.[82,124]

1.3.1 Alarmins in tendinopathy

As discussed in the previous section, the main external precipitating factor in the development of tendinopathy is mechanical strain. This can occur beneath the physiological threshold which results in repetitive microtrauma and cumulative injury due to incomplete healing.[125] Alternatively, forces that exceed the elastic modulus of the tendon result in acute traumatic injury. In either case, damage to the tendon matrix occurs resulting in cellular damage or necrosis.[48]

Historically, the nature of host-innate immune response to pathogen invasion has been well documented.[126] Over the last decade, damage associated molecular patters (DAMPs) or alarmins have been identified as an analogue to pathogen associated molecular patterns (PAMPs) and are considered signalling mediators of sterile inflammatory responses. 'Alarmins' are so called as they provide the initial molecular signal that alerts surrounding cells to damage or danger.[127] The 'danger hypothesis' was first described in 1994 and suggested that cells are activated by alarm signals from injured cells such as those exposed to toxins and mechanical damage.[128] It was suggested that alarmins were intracellular factors that become exposed and available for recognition by the immune system when cells lose their compartmentalization during necrosis. Being entirely theoretical the hypothesis attracted a great deal of debate; however, the existence of several 'alarmins' have since been revealed.[129] Upon binding of pattern recognition such as toll like receptors (TLRs) on the cell surface, alarmins induce the expression of cytokines and chemokines to mobilize and recruit immune cells to the site of injury.[130]

S100 proteins

S100 proteins are low molecular weight calcium binding proteins (approximately 10-12kDA) that regulate a variety of intracellular and extracellular processes including cell growth and motility, cell cycle regulation, transcription, differentiation, energy metabolism and inflammation.[131] S100 proteins may function in an intracellular or extracellular manner while some display both properties.[132,133,134] The extracellular S100 proteins are considered 'alarmins' and play a role in immune homeostasis, post-traumatic injury and inflammation. In addition, some have been adopted as surrogate biomarkers in a number of diseases.[135] S100 proteins with extracellular functions include S100B, S100A4, S100A8, S100A9 S100A12, S100A13. S100B exhibits neurotrophic activity, S100A4

is pro-angiogenic, S100A12 participates in the host-parasite response and S100A13 is a component of a secreted complex containing fibroblast growth factor FGF-1. [136] Of the S100 proteins, S100A8 and S100A9 are the most well studied in the context of tissue damage and inflammation.[137]

S100A8 and S100A9—also known as myeloid-related proteins 8 and 9 (MRP8 and MRP9)—are constitutively expressed in monocytes and neutrophils. Human S100A8 and S100A9 are composed of 93 and 113 amino acid residues, respectively and are able to form a stable heterodimer.[138] Under inflammatory conditions they are actively secreted to modulate the inflammatory response through induction of leukocyte recruitment and cytokine secretion.[137] S100A8/S100A9 heterodimer or calprotectin is the most abundant naturally occurring S100 heterodimer and is thought to play a cytokine-like role in inflammation. S100A8 and S100A9 bind to pattern recognition receptors including toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE).[139,140]

S100A8 and S100A9 in inflammation

S100A8 protein is abundant in some immune cells (accounting for 20% of neutrophil cytoplasm) and genetic deletion of S100A8 in mice is lethal.[141] Its expression is induced in macrophages, dendritic cells, epithelial cells and fibroblasts in response to inflammatory stimuli.[142] S100A8 has been implicated in a variety of immunomodulatory roles including myeloid cell differentiation, scavenging of ROS generated by activated neutrophils, and inhibition of transendothelial migration of neutrophils. Studies in the context of inflammation have shown S100A9 inhibits differentiation of macrophages, DCs and accumulation myeloid-derived suppressor cells (MDSCs) in cancer through generation of ROS.[87] Furthermore, S100A9 gene deletion compromises neutrophil responses to chemoattractants and cytoskeletal dynamics thus inhibiting transendothelial cell migration.[143]

S100A8 and S100A9 have also been associated with myeloid cell differentiation toward macrophages and dendritic cells.[144] They are co-expressed in fetal myeloid progenitors where their expression level correlates with commitment to the myeloid lineage.[145] S100A8/A9 heterodimer is thought to amplify pro-inflammatory cytokine production by macrophages via NFκB and p38 MAPK in rheumatoid arthritis.[146] In addition, S100A8/A9 has been implicated in monocyte

and neutrophil transmigration and has been shown to enhance expression of CD11b and adhesion of phagocytes.[107,144]

S100A8 and S100A9 in inflammatory diseases

Expression of S100A8 and S100A9 has been documented in various models of osteoarthritis and rheumatoid arthritis.[147] In mice, S100A8/A9 is upregulated in early but not late phase OA while S100A9 knockout mice display significantly reduced osteophyte size.[148] In antigen induced arthritis articular cartilage destruction is absent in $S100A9^{-/-}$ mice and synovial gene expression of MMP-3, -9 and -13 is low. Furthermore, expression of these genes was elevated in murine macrophages stimulated with S100A8 or S100A8/A9.[149] In human OA patients S100A8/A9 plasma levels are elevated at baseline.[148] S100A8, S100A9 and the heterodimer have all been identified in joint fluid in patients with RA and levels of S100A8/A9 correlate with disease activity. Histologically, S100A8 and S100A9 expression has been identified in synovial lining and sublining macrophages. Furthermore, expression of S100A8/A9 heterodimer is strongly correlated with severity of radiological bone erosion in RA.[150] Taken together, these data suggest that S100A8 and S100A9 play a role in the pathogenesis of musculoskeletal disease.

S100A8 and S100A9 proteins have been proposed as biomarkers of disease activity in several chronic inflammatory pathologies including RA[151], psoriatic arthritis (PsA)[152], inflammatory bowel disease (IBD)[153] and cystic fibrosis (CF).[154] In RA S100A8/A9 was proposed as a biomarker for predicting clinical outcome of treatment. Others have proposed S100A8/A9 levels may be a more accurate predictor of synovial inflammation in RA patients compared to traditional markers such as C-reactive protein (CRP).[155]

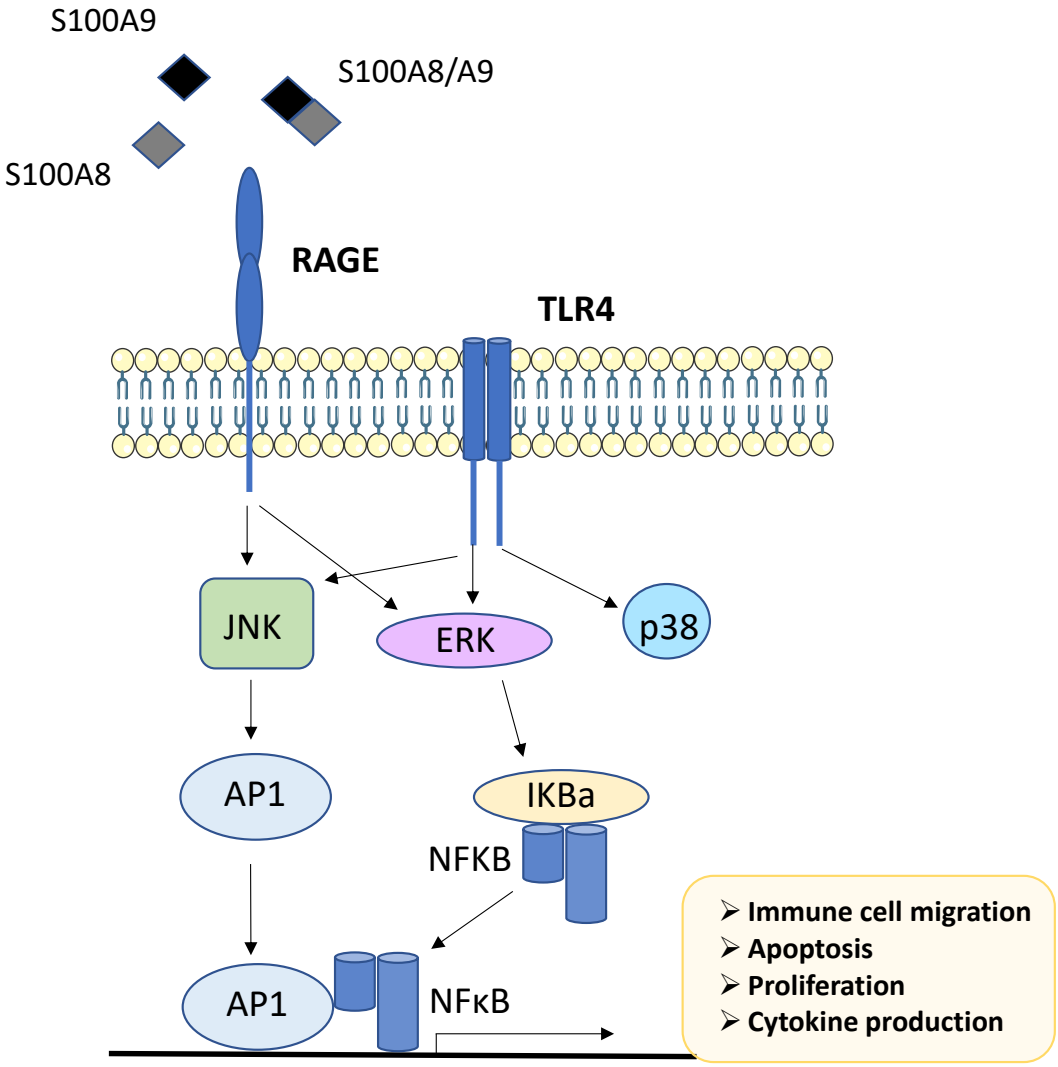


Figure 1.4 Summary of S100A8 & A9 signaling
Figure adapted from Xia *et al* 2018

High Mobility Group Box 1

High mobility group box 1 (HMGB1) is a highly abundant nuclear protein that modulates intracellular and extracellular biological activities. Within the nucleus HMGB1 is involved in determining chromosomal architecture and regulating transcription.[156] In the extracellular space it functions as an alarmin to stimulate the innate immune system, alone or in concert with various cytokines, endogenous or exogenous molecules.[157] HMGB1 is released from cells passively under necrotic conditions or secreted actively during late apoptosis (secondary necrosis).[158] It is also secreted actively by immune cells including monocytes, macrophages and DCs.[159] HMGB1 acts through PRRs including TLR2, TLR4, TLR9 AND RAGE to modulate immune activities including cytokine production, cell proliferation, chemotaxis and differentiation.[160]

Extracellular HMGB1 is thought to contribute to the pathogenesis of numerous chronic inflammatory and auto-immune diseases including RA, systemic lupus erythematosus (SLE), atherosclerosis and pulmonary fibrosis.[161] Several studies have identified the expression of HMGB1 in tendon pathology.[162,163,164] In a human model of tendinopathy HMGB1 was shown to regulate expression of inflammatory mediators and matrix proteins. Furthermore, blocking HMGB1 signalling via TLR4 silencing reversed these inflammatory and matrix changes.[162] Recently, several *in vitro* and *in vivo* studies targeting HMGB1 signalling have provided promising results in the context of inflammation and matrix remodelling.[165]

IL-33

IL-33 is a nuclear cytokine of the IL-1 family and is most well studied in the context of type-II innate immunity and asthma.[166] Biologically active full length IL-33 can be released into the extracellular space following cell damage or mechanical injury thus it is considered a classical cytokine alarmin.[167,166] It is constitutively expressed in normal tissue and can be induced in haematopoietic cells during allergic inflammation and infection.[168] Increased expression of IL-33 has been observed in pathologies with large numbers of necrotic cells including RA and SLE; it is thought to act in a similar manner to HMGB1 as both are released from dying cells and both are nuclear factors.[169,170]

Heat shock proteins

Heat shock proteins are the most abundant soluble intracellular proteins found in all prokaryotes and eukaryotic cells. Although some HSPs are constitutively expressed, expression is generally induced in response to stress including heat shock, inflammation and infections.[171] Functioning as alarmins, they are released into the extracellular space upon necrotic cell death and signal through PRRs such as TLRs to activate innate immunity. HSPs induce secretion of pro-inflammatory cytokines including TNF-α, IL-1β and GM-CSF by macrophages[172], induce iNOS and subsequent NO production by macrophages and DCs[173] and promote secretion of chemokines including monocyte chemoattractant protein (MCP-1) and macrophage inflammatory protein (MIP-2) from T cells.[174] The reported cytokine-like effects of HSPs have been postulated to contribute to the pathogenesis of many autoimmune and chronic inflammatory diseases.[175]

HSP27 and HSP70 expression has been identified in rat and human models of tendinopathy and was found to correlate with expression of apoptotic regulators including caspases 3 and 8 and cFLIP. The authors propose that extracellular heat shock proteins released under conditions of cellular stress are implicated in degenerative processes in tendinopathy.[81]

1.3.2 Cytokines in tendinopathy and chronic inflammation

Cytokines are small secreted proteins (of approximately 30-50KDa) released by cells to facilitate cellular communication. Cytokines are crucial mediators of both acute and chronic inflammation.[176] Dysregulated cytokine production and accumulation is thought to contribute to persistence of inflammation and pathological chronic inflammation.[177] Cytokines can be classified according to their cell of origin or purpose. For example, interleukins are cytokines made by leukocytes that predominantly act on other leukocytes and chemokines are cytokines with chemotactic properties. Cytokines are extremely versatile and may act in an autocrine, paracrine or endocrine manner thereby facilitating a diverse range of cellular functions.[178] Historically, cytokines have been classified according to their 'pro-inflammatory' or 'anti-inflammatory' properties; however, it is now widely accepted that cytokines and their signalling pathways are extremely sophisticated and non-binary in their mode of action.

IL-6

IL-6 was first cloned and studied in 1986 and has since been described as a pleiotropic cytokine that influences antigen specific immune responses and inflammatory responses.[179] It is produced by several types of cell including T cells, B cells, monocytes, fibroblasts and endothelial cells and modulates numerous biological activities.[180] IL-6 is a key mediator of the acute phase response which is characterised by changes of concentration of plasma proteins known as 'acute phase reactants' and several other biochemical and physiological responses.[181] As well as mediating acute inflammatory processes IL-6 is crucial in the switch from acute to chronic inflammation. In acute inflammation the leukocyte infiltrate is primarily neutrophilic; after 24 to 48 hours monocytic cells predominate. Histologically, chronic inflammation is associated with the presence of mononuclear cells including macrophages and lymphocytes.[182,183] The transition from neutrophil to monocyte recruitment is dependent on the specificity of chemokines produced at the inflammatory site. IL-8 and CCL2 (MCP-1) are the predominant cytokines involved in recruitment of neutrophils and monocytes, respectively. IL-8 production by neutrophils occurs in the 24 hours following initiation of the inflammatory cascade resulting in further recruitment and local activation of neutrophils. CCL2 production is delayed but often persists for several days resulting in monocyte recruitment and accumulation at the site inflammation.[184,185] The shift in from neutrophil to monocyte recruitment may also mediated by chemokines secreted by stromal cells or macrophages.[186]

The IL-6-IL-6Rα complex has been shown to activate endothelial cells to secrete IL-8 and CCL2 in addition to the expression of adhesion molecules. IL-6-IL-6Rα complex associates with signal transducing membrane protein gp130 to initiate intracellular signalling cascades in a mechanism termed trans signalling.[187] Gp130 is ubiquitously expressed; however, in humans IL-6Rα expression is limited to leukocytes and hepatocytes. Soluble IL-6Rα has been identified in neutrophil rich inflammatory fluids such as synovial fluid.[188] Several studies have identified a mechanism through which the IL-6-IL-6Rα complex favours leukocyte accumulation and a transition from acute to persistent inflammation through regulation of IL-8 and CCL2 production.[189,190]

The expression of IL-6 and its interactions with transcription activator STAT3 in tendinopathy has been well characterised. IL-6 expression was first identified in tendon and found to be upregulated in tendon pathology.[191] Tendons of surgically injured IL-6 knockout mice show inferior mechanical and structural properties compared with controls indicating IL-6 is implicated in tendon healing.[192] Furthermore, cyclical loading of tendons resulted in increased expression of IL-6 and collagen 1 suggesting a role for IL-6 in tendon adaptation to exercise.[193] Additional mechanical studies showed that tenocytes release IL-6 in response to mechanical loading; however, IL-6 did not exhibit any autocrine function in an *in vitro* model of tenocyte stimulation.[194,195] Cytokines and alarmins including IL-1β, IL-33, HMBG1 and S100A8 & A9 induce IL-6 gene expression and protein release in human tenocytes.[196,197,162,198] Furthermore, damaged tendons show dysregulated IL-6 signalling as a result of decreased expression of IL-6R and upregulation of intracellular signal transducer STAT3.[199] In line with its pleiotropic properties, the data suggest that IL-6 is capable of modulating a number of cellular processes in tendinopathy which may involve pro- or anti-inflammatory actions.

TNF-α

TNF-α is a prototypic pro-inflammatory cytokine that is a product of activated monocytes/macrophages, fibroblasts, mast cells, T cells and NK cells. It is a potent paracrine inducer of other inflammatory cytokines including IL-1, IL-6, IL-8 and GMCSF as well as acting as an autocrine stimulator.[200] It is also known to induce expression of adhesion molecules on fibroblasts.[201] The physiological role of TNF-α has been widely studied in RA where TNF plays a crucial role in disease pathogenesis. Individuals with RA have high levels of TNF-α in the synovial fluid and Inhibiting TNF signalling in RA fibroblast-like synoviocytes (FLS) reduces the production of IL-6, IL-8, IL-1 and GMCSF.[202,203]

Expression of TNF-α and TNFR1 has been identified in inflamed equine tendon and TNFR1 and TNFR2 expression is co-localized on the surface of equine tenocytes. This expression can be upregulated in response to TNF-α stimulation suggesting the existence of an autocrine feedback mechanism.[204,205] In human tendon tissue TNF-α mRNA is increased 11-fold in torn supraspinatus tendon (representative of late pathology) compared with control and increased expression of TNF receptors has been observed in Achilles tendon.[82,206,207] Cultured human tenocytes

stimulated with TNF-α showed reduced type I collagen deposition and increased elastin gene expression. In addition expression of MMP-1, TNF-α, IL-1β, IL-6 and IL-10 was highly upregulated in response to TNF-α stimulation.[195] Culture of tendon explants with exogenous TNF-α resulted in increased expression of TLR2 but not TLR4.[208] Evidence suggests that TNF signalling through both TNFR1 and TNFR2 has an autoregulatory effect on amplifying the inflammatory response in tendinopathy.

IL-1 family cytokines

IL-1 family cytokines are important regulators of innate and adaptive immunity regulating early non-specific defence to pathogen invasion and other insults such as injury or stress. They are unique in their properties as IL-1 cytokines and TLRs share a common cytoplasmic domain called the Toll-IL-1-Receptor (TIR).[209] Signal transduction mechanisms and biological consequences of TLR and IL-1 family ligands are virtually indistinguishable. IL-1 family members include IL-1α, IL-1β, IL-18 and IL-33. IL-1 cytokines are potent inducers of inflammation and amplify the immune response thus their expression must be tightly regulated to avoid tissue damage.[210] Dysregulated activity of these cytokines is associated with autoimmunity and chronic inflammatory diseases including RA, SLE and IBD.[211]

IL-1β is a potent inducer of several inflammatory mediators in human tenocytes including IL-6, IL-8, CCL2, COX-2 and PGE2.[196,212,197] In addition, it has been shown to induce the expression of MMP-1, -3 and -13.[196] Stimulation of tenocytes with IL-1β downregulates expression of type I collagen with a resultant increase in type III collagen indicating this inflammatory stimulus may have a detrimental effect on the integrity of the tendon matrix.[213] IL-8 mRNA expression has also been identified in human tissue samples from patients with rotator cuff tears.[82] More recently, the effect of IL-33 has been investigated in tendinopathy. As discussed previously, IL-33 is released into the extracellular space following tissue damage and acts as an 'alarmin' molecule to activate the innate immune system. Millar *et al* demonstrated significantly increased expression of IL-33 in early human tendinopathy that is lost in end stage disease.[197] Increased expression of type I and type III collagen mRNA and protein levels in tenocytes was also reported in response to stimulation with recombinant human IL-33. This was accompanied by increases in inflammatory mediators IL-6, IL-8 and CCL2. The stimulatory effect

of IL-33 was abrogated by inhibition of NF-κB signalling suggesting canonical IL-1R signalling may be responsible for this effect. Further *in vivo* work demonstrated rhIL-33 increased synthesis of type III collagen and resulted in diminished tendon tensile strength in a murine tendon injury model. In mice that lack IL-33 decoy receptor (ST2), rhIL-33 had no effect on ECM synthesis indicating the action of IL-33 is dependent on ST2 signalling. Furthermore, pharmacological neutralization of endogenous IL-33 mitigated the switch from type I to type III collagen and resulted in improved tendon biomechanical properties.[197]

IL-21 is a proinflammatory cytokine of the IL-1 family and is produced mainly by $CD4^+$ lymphocytes and natural killer T cells (NK). It is known to modulate T cell proliferation, B cell differentiation, and cytotoxic properties of NK cells, as well as the antigen presenting and T-cell activating abilities of dendritic cells.[214] Overexpression is associated with chronic inflammatory diseases including rheumatoid arthritis and inflammatory bowel disease.[215] IL-21R expression has been identified in early tendinopathy and TNF-α and IL-1β induce expression of IL-21R transcript and protein in human tenocytes.[216]

IL-8

IL-8 is a member of the CXC chemokine family and an important modulator of neutrophil accumulation and activation of acute inflammation. IL-8 is produced by a number of cell types including monocytes, endothelial cells and fibroblasts.[217] As described previously IL-8, acting in concert with CCL2 and IL-6, is an important mediator in the transition from acute to chronic inflammation.[218] Several studies have documented the expression of IL-8 in human tenocytes whereby expression is induced by IL-1β, IL-33, IL-17A, HMGB1, S100A8 and S100A9.[197,219,162,198] In addition, IL-8 expression is upregulated in ruptured Achilles tendons.[220]

CCL2

CCL2, otherwise referred to as monocyte chemoattractant protein (MCP-1), is a member of the CC chemokine family and a potent chemotactic factor for monocytes.[221] It is produced by fibroblasts, endothelial cells, smooth muscle cells, epithelial cells and myeloid cells although monocytes/macrophages are thought the main source of CCL2.[222] CCL2 exerts its effects through binding of G protein coupled receptors on the surface of leukocytes targeted for activation and

migration.[223] The CCL2/CCR2 axis plays a critical role in monocyte recruitment in response to infection or insult acting to promote resolution of inflammation by phagocytosis of cellular debris and apoptotic cells. Under homeostatic conditions, CCL2 also promotes emigration monocytes from the bone marrow in to circulation.[224] As discussed with IL-6 and IL-8, CCL2 is involved in the transition from acute to chronic inflammation and its expression is upregulated in human tenocytes in response to various cytokines and alarmin molecules. [197,219,162,198]

CXCL10

CXCL10 (interferon γ inducible protein) is a member of the CXC chemokine family which binds to the CXCR3 receptor to exert its biological effects.[225] It is secreted by a variety of cells including activated neutrophils, leukocytes, monocytes, eosinophils, monocytes, epithelial cells, endothelial cells and stromal cells.[226] It is chemoattractant for monocytes, T cells and NK cells. CXCL10 plays a key role in leukocyte homing to inflamed tissues thus has potential to exacerbate inflammation and cause tissue damage.[227] CXCL10 overexpression has been observed in several inflammatory pathologies and cancer.[228,229,2302]

CCL20

CCL20 (also referred to as macrophage inflammatory protein-3alpha (MIP-3α)) is the only known chemokine to interact with its receptor CCR6. It is widely expressed by immune cells and stromal cells under both homeostatic and inflammatory conditions.[231] The CCL20-CCR6 axis regulates chemotaxis of DCs, T cells and B cells. Diseases associated with CCL20 include RA and certain cancers.[232] CCL20 levels are significantly elevated in the synovial fluid of patients with RA and its expression is upregulated in RA-FLS in response to TNF-α, IL-1 and IL-17.[233]

IL-4/IL-13

IL-4 and IL-13 are related cytokines that play an important role in regulating responses of lymphocytes, myeloid cells and non-hematopoietic cells.[234] IL-4 and IL-13 induce the differentiation of naïve T cells into Th2 cells and is implicated in 'alternative activation' of macrophages.[235] In a murine tendon injury model IL-4$^{-/-}$ mice show disorganised collagen structure and impaired mechanical properties.[192] In addition expression of IL-4Rα, IL-13Rα1 and IL-13Rα2 but not the common gamma receptor chain are expressed in tendon tissue and cultured

tenocytes. Furthermore, tenocytes stimulated with rhIL-4 and IL-13 display increased proliferation rates.[236]

IL-17 family cytokines

The IL-17 family consists of IL-17A-F plays a crucial role is host defence to microbial organisms and the development of inflammatory disease.[237] IL-17A is primarily produced by the Th17 subset of T helper cells and excessive production leads to excessive inflammation and consequent tissue damage.[238] Increased expression of IL-17A and IL-17F has been identified in tendinopathy.[219,199] In early tendinopathy, IL-17A expression was localised to mast cells, macrophages and T cells. Furthermore, tenocytes stimulated with IL-17A exhibit increased production of pro-inflammatory cytokines, altered expression of ECM components including type III collagen and increased expression of several genes associated with regulation of apoptosis.[219]

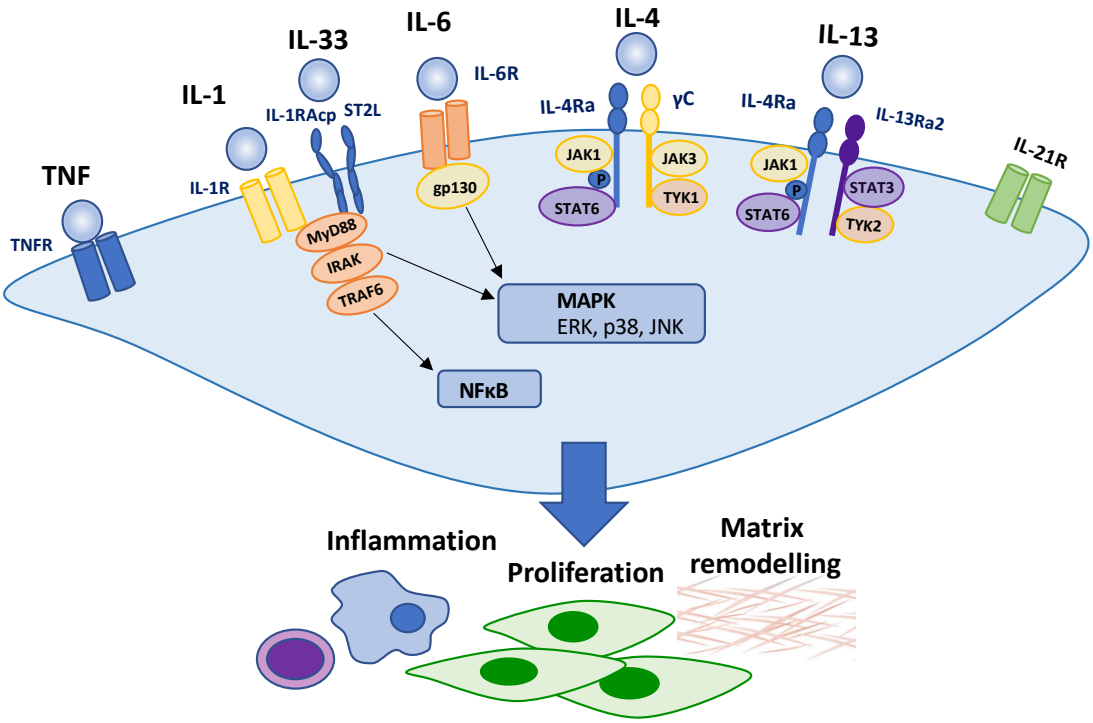


Figure 1.5 Cytokine receptor signaling pathways in tenocytes
Figure adapted from Millar *et al* 2017

1.4 Immune cells in tendinopathy

The concept of cells existing in specific subsets or 'compartments' within the tendon is a theory that has emerged over the past decade following the departure of the 'degenerative hypothesis'. In 2001 Marsolais *et al* published a study documenting the sequential accumulation of neutrophils and monocytes in tendon post injury.[239] Due to the emergence of the 'tendinosis' model it was almost ten years until the concept of immune cells in tendinopathy was revisited. In 2010 a study in human rotator cuff tendon biopsies demonstrated the presence of macrophages in the subintimal layer, mast cells around the vasculature and diffuse distribution of T cells. In particular, mast cells and 'M2' macrophages were observed in biopsies with increased fibroblast cellularity.[111] Subsequent studies of macrophage populations in equine tendons demonstrated the presence of 'M1' and 'M2' macrophages in sub-acute and chronic tendinopathy, respectively.[240] Furthermore, increased numbers and inflammatory activity of mast cells was observed in rodent and human tendinopathy.[241,242] A systematic review detailing evidence of immune cell subsets in human tendinopathy identified four studies highlighting increased numbers of macrophages and three of increased mast cells.[243]

A recent review has proposed the presence of three cellular 'compartments' within the tendon, comprising of the stromal compartment, the immune sensing compartment and the infiltrating compartment. The authors propose the 'immune-sensing' compartment consists of tissue resident macrophages and mast cells while the 'infiltrating compartment' is comprised of neutrophils and circulating monocytes/macrophages recruited to the site of injury.[29] A study detailing infiltration of inflammatory cells in surgically induced tendon injury showed rapid and transient accumulation of neutrophils immediately following injury. This was succeeded by gradual infiltration of macrophages 1-28 days post injury.[239] NK cells and T cells may also be present in this infiltrating compartment.[244] This evidence, together with activation of inflammatory signalling pathways, suggests there is a dynamic immune environment within the tendon both under homeostatic conditions and following injury.

1.4.1 Monocytes and macrophages

Monocytes and macrophages are critical regulators of inflammation and the innate immune response. The 'mononuclear phagocyte system' (MPS) is a population of cells derived from erythromyeloid progenitors in the bone marrow that differentiate to form blood monocytes and circulate in the blood before entering tissues in response to environmental cues.[245] The MPS is divided into three subsets including (monocytes, dendritic cells and macrophages) on the basis of their functional and phenotypical characteristics.[246] Monocytes and monocyte-derived macrophages from the peripheral blood are actively recruited to sites of inflammation to perform phagocytosis and participate in the immune response by secreting cytokines and chemokines. [247] They are also recruited in the steady state to aid in tissue homeostasis.[248]

The original MPS classification was based on the premise that the majority of cell division occurs in the monoblast and pro-monocyte stage and replication of tissue macrophages was a minor mechanism that aided in the maintenance of tissue macrophage populations.[246] More recent studies have refuted this hypothesis based on evidence of a separate lineage of 'tissue resident' macrophages that are embryonic in origin.[249] Tissue macrophages are widely distributed throughout the body and are able to undergo self-renewal *in situ* by proliferation. They are highly specialized to their environment and fulfil tissue-specific and niche-specific functions.[250]

The majority of ontogenetic and phenotyping studies in monocytes/macrophages have been conducted in mice. Monocytes have been divided into subsets based on their expression of the membrane monocytic marker Ly6C and CCR2.[251] 'Classical' or inflammatory monocytes are $Ly6C^{+}$ $CCR2^{+}$ and 'alternative' monocytes are $Ly6C^{low}CCR2^{-}$. In humans, monocytes are characterized based on their expression of CD14 (TLR4 co-receptor) and CD16 (FcγRIII) where 'classical' monocytes are $CD14^{+}CD16^{-}$, intermediate monocytes are $CD14^{+}CD16^{+}$ and 'alternative' monocytes are $CD14^{low}CD16^{+}$.[252] The classification of macrophages is somewhat more complex; however, F4/80 (murine) and CD68 (human) are generally considered pan macrophage markers.[253]

Under normal homeostatic conditions macrophages perform housekeeping functions independently of activating stimuli generated by immune cells. Macrophages recognize both exogenous (PAMPs) and endogenous danger signals generated by necrotic cells (DAMPs) and signal through PRRs such as TLRs.[254] Upon activation macrophages express a wide variety of receptors including scavenger receptors that enhance their activity and capacity for phagocytosis, endocytosis and adhesion.[255,256] Furthermore, integrin receptors play an important role in tissue homeostasis by facilitating the attachment of macrophages to the extracellular matrix and signal transduction from the ECM to the cell.[257]

Macrophages are essential mediators of innate and adaptive immunity and display a remarkable degree of plasticity. Initial observations of phenotypical and functional adaptability were likened to polarization of T helper cell responses that represent two distinct and opposing states of activation. Henceforth, macrophages were described as 'classically activated' (M1) or 'alternatively activated' (M2) mirroring Th1 and Th2 nomenclature.[258] Interactions with T lymphocyte subsets were crucial to defining their phenotype as M1 macrophages are activated as a result of stimulation with IFN-γ or initiation of TLR signalling by LPS while M2 macrophages are activated by IL-4 and IL-13 predominantly released by Th2 cells.[259] 'M1' macrophages have a largely pro-inflammatory cytokine profile acting through the release of IL-1, IL-6 and IL-23 thus promoting a Th1 response. Moreover, classically activated macrophages have strong antimicrobial and tumoricidal properties conferred by release of reactive oxygen and nitrogen species. [260]

As the classification of macrophages as 'M1' and 'M2' represented two distinctly polarized states, nomenclature was expanded to include three subsets of M2 macrophages: 'M2a' activated by IL-4/IL-13 signalling, 'M2b' induced by immunocomplexes and TLR agonists and 'M2c' activated by IL-10 and glucocorticoids.[261] It has since been postulated that the process of macrophage polarization is not confined to distinct subsets but rather it represents a variable and fluid spectrum of activation states dependent upon the tissue microenvironment.[262]

1.4.2 Monocytes and macrophages in wound healing and tendinopathy

Previous studies have identified the presence of macrophages in normal and diseased human tendons.[111,212] Thus far, the question of the origin of these macrophages has not been addressed. Monocyte-derived macrophages are actively recruited to the tissue as part of the acute inflammatory infiltrate in response to injury. Tissue resident macrophages are unique to their microenvironment and critical for maintaining homeostasis within the cell; they also retain the capacity to respond to local cues within the tissue milieu.[250] Taking evidence from literature that documents the contribution of macrophages to inflammation and fibrosis in other chronic inflammatory pathologies, it may be postulated that both foetal tissue macrophages and monocyte-derived macrophages co-exist within the tendon under inflammatory conditions.[263,264,265]

There is evidence to suggest that the 'activation' state of macrophages is fully and rapidly reversible.[266] This potential activation plasticity is relevant to tendinopathy where, in the early stages, inflammation is the major factor contributing to matrix dysregulation. At this stage, it is conceivable that a 'pro-inflammatory' macrophage phenotype dominates and contributes to matrix destruction and alterations in tissue architecture by release of MMPs.[267] In cutaneous wound healing, various studies have proposed a role for 'pro-inflammatory' macrophages during the onset of injury (inflammatory phase) while an 'alternatively activated' or pro-resolving phenotype dominates in the resolution phase.[268,269] These interpretations led to the creation of further 'M2 like' subgroups such as 'wound healing macrophages'.

It has been postulated that the phenotype of macrophages evolves with the stages of wound healing based on spatiotemporal cues within the wound.[262] Pro-wound healing macrophages secrete a variety of factors including platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-1),TGF-ß1,VEGF and IL-10. Secretion of these factors by macrophages is thought to stimulate fibroblast proliferation and synthetic activity.[268] Furthermore, wound-healing macrophages secrete TIMPs to counteract the activity of MMPs and facilitate formation of the extracellular matrix.[267]

Literature from various pathologies attributed to inflammation induced matrix deposition has suggested alternative activation of macrophages is responsible for a failed healing response.[265,270,271] It is evident that dysregulated macrophage function can lead to aberrant tissue repair, perpetuated inflammation and impaired communication between macrophages and stromal cells. If left unchecked this may lead to chronic inflammation and development of pathological fibrosis.

1.4.2 Surface markers associated with a pro-resolving phenotype

Nomenclature surrounding the various activation states of macrophages is complex and widely disputed. As such, the term 'pro-resolving' macrophage will be used hereafter in reference to macrophages displaying characteristics of 'alternative activation'. The following section will briefly describe surface markers associated with a 'pro-resolving' phenotype relevant to this thesis.

CD163

CD163 (haemoglobin scavenger receptor) expression is specific to monocyte-macrophage lineage. It is highly expressed in certain types of macrophages (including bone marrow macrophages, alveolar macrophages and Kupffer cells) and it exhibits modest expression in monocytes.[272] High CD163 expression is characteristic of tissues responding to inflammation and its expression has been documented in chronic inflammatory pathologies including inflammatory arthritis and atherosclerosis.[273,274]

CD206

CD206 (also referred to as mannose receptor or MRC-1) is a transmembrane glycoprotein that belongs to the C-type lectin family. It is predominantly expressed by macrophages but may be expressed by immature DCs and specialized lymphatic and endothelial cells.[275] Functionally, CD206 is active in antigen processing, endocytosis and phagocytosis, playing an important role in the innate immune response.[276] In addition, increased expression of CD206 has been observed in human tendinopathy.[212]

MerTK

The TAM receptors (Tyro3, Axl and Mer) are receptor tyrosine kinases that play an important role in anti-inflammatory feedback mechanisms. The receptors share ligands Gas6 and Protein S (Pros1).[277] MerTK is expressed on macrophages and DCs where ligand binding enhances phagocytic capacity of the cell. It is also thought TAM receptors negatively regulate inflammation by inducing SOCS (suppressor of cytokine signalling) proteins 1 and 3 that inhibit TLR and cytokine signalling.[278] TAM deficient mice develop auto-immunity associated with impaired inhibition of inflammation and dysregulated phagocytosis.[279] MerTK expression is associated chronic inflammatory pathologies including RA, SLE and MS.[280,281,282]

HLA-DR

HLA-DR is an MHC class II cell surface receptor expressed on antigen-presenting cells (macrophages, B cells, DCs). MHC-II expression is upregulated in alveolar macrophages and tumour associated macrophages (associated with an 'alternatively activated' phenotype).[283,284]

1.5 Stromal Biology

Stromal cells are tissue resident cells that support tissue function and homeostasis.[285] Fibroblasts, endothelial cells, pericytes and epithelial cells are stromal cells that modulate a variety of niche specific functions. Fibroblasts are highly specialized to their microenvironment and are essential in the maintenance of tissue architecture.[286] 'Fibroblast' is an umbrella term that encompasses cells within tissues that synthesize components of the extracellular matrix. As discussed in section 1.1.3, tenocytes are fibroblast-like differentiated cells that perform this function within the mature tendon.[31] The composition of ECM products secreted by fibroblasts is highly influenced by the local microenvironment thus cellular heterogeneity is observed between tissues.[287] It has also been postulated that more discreet differences exist between fibroblasts from various anatomical locations within the same tissue.[288]

Historically, immunologists have considered fibroblasts somewhat benign in the co-ordination of immune responses within tissues. Studies largely focused on lymphocyte-myeloid cell interactions and antigen-driven responses in the development of chronic inflammation and autoimmunity.[289] However, more recently it has been acknowledged that molecular danger signals are not antigen specific and the immune environment has since been broadened to include a role for fibroblasts in inflammation.[290] Key questions in the current fibroblast literature focus on identifying factors that trigger persistence of inflammation with the ultimate aim of developing strategies to prevent disease chronicity.

1.5.1 Chronic inflammation

Chronic inflammation is a feature of many diseases of differing aetiology and pathophysiology. Autoimmune diseases associated with chronic inflammation include RA, psoriasis, SLE and IBD.[291] Cancers include gastric cancer (caused by chronic *H.pylori* infection), colorectal cancer (arising as a result of IBD), liver carcinoma due to hepatitis C infection and pancreatic ductal adenocarcinoma (PDAC).[292] In addition, fibrotic disorders associated with excessive ECM production manifest as end organ pathology in the lung, heart, kidneys and liver and fibrotic disorders of connective tissue include scleroderma, adhesive capsulitis (frozen shoulder), Dupuytren's contracture and Peyronie's disease.[293]

Inflammation as a result of tissue insult or infection is generally beneficial as recruitment of inflammatory cells to the site of injury aids in the clearance of cellular debris and pathogens.[294] Chronic inflammation occurs as a result of an imbalance between inflammatory cell recruitment, proliferation and apoptosis. Stromal cells, specifically vascular endothelial cells, play a key role in initiation of the innate immune response.[295] Damage compromises the vessel wall triggering an acute vascular response characterized by increased blood flow, vascular permeability, activation of fibroblasts and endothelial cells and infiltration of polymorphonuclear cells. This is succeeded by the chronic cellular response that consists of increased trafficking of mononuclear cells. Here, the term chronic is used to represent a physiological phenomenon that occurs over weeks and is characterized by phagocytosis, tissue repair by fibroblasts, emigration and apoptosis of leukocytes and ultimately resolution of inflammation.[296]

Identifying the point at which physiological self-limiting inflammation becomes truly chronic or pathological is crucial to understanding the pathogenesis of many inflammatory diseases. Mechanisms regulating chemotaxis of immune cells to sites of inflammation are well characterized; however, mechanisms that govern retention and accumulation of leukocytes are less well defined.[178] Theories relating to selective accumulation of leukocyte subsets have been attributed to endothelial selection at the point of entry mediated by expression of a variety of adhesion molecules.[297] It is now widely accepted that upon entry to the stromal microenvironment, cells are subject to a variety of stimulating factors that promote the expansion of leukocyte pools.[298] Studies have shown that induction of apoptosis and cytokine deprivation are key to removal of lymphocytes during resolution of inflammation.[299] However, other inflammatory cells including macrophages appear to be relatively resistant to deprivation of external cytokines and growth factors.[300]

1.5.2 Stromal activation

The involvement of the stroma in chronic inflammatory pathologies has generated a great deal of interest over the last few years. The idea of fibroblast activation was first proposed in the late 1990s as fibroblasts were found to secrete chemokines (which had recently been identified as novel group of chemotactic cytokines).[301,302] Initial studies focused on the ability of bacterial products such as LPS to induce secretion of chemokines and facilitate recruitment of inflammatory cells. [303] Expression of these mediators by fibroblasts was found to be transient but sufficient enough to induce recruitment of haematopoietic cells capable of perpetuating 'fibroblast activation' through expression of pro-inflammatory cytokines such as TNF-α.[304] CD40 (a member of the TNF receptor superfamily) was deemed a major 'activation antigen' on fibroblasts and was proposed to act to activate fibroblasts through engagement of CD40L on the surface of immune cells. CD40 expression and engagement was found to correlate with expression of adhesion molecules, activation of NFκB pathways and secretion of cytokines and chemokines.[305] These fibroblast-immune cell interactions were considered analogous to interactions between leukocytes and antigen presenting cells (APCs). As fibroblasts are relatively long lived cells, it was quickly acknowledged that this mechanism must be tightly regulated in the steady state to avoid overstimulation of the immune response.[306] These findings were succeeded by a body of work in RA that established fibroblasts derived from the synovium of patients were phenotypically distinct from those in non-inflamed joints.[307,308] Furthermore, a distinctive subset of 'tumour-associated' activated fibroblasts has been identified in several cancers.[309]

As yet, the concept of stromal activation in tendinopathy is relatively unexplored. The RA synovium and tumour micro-environment are highly dynamic areas of exaggerated inflammation. To provide context for this thesis, the following sections will review the concept of 'stromal activation' and fibroblast heterogeneity in RA and cancer.

1.5.3 Synovial fibroblasts

In RA, cells of the synovium were originally classified as type A synoviocytes (macrophage-like synovial cells of myeloid origin expressing CD11b, CD68, CD14, CD163 and MHC-II) and type B synoviocytes (fibroblast-like synoviocytes of mesenchymal origin that express cadherin 11, VCAM-1, ICAM-1 and CD55).[310] Type B synovial cells or synovial fibroblasts are the most abundant cell type within the joint synovium responsible for synthesis of ECM in the synovial fluid. RA-FLS have been described as 'aggressive' and likened to tumour cells due to their expression of oncogenes such as c-Myc and Raf-1 that promote growth and invasion.[311] Furthermore, their aggressive phenotype is associated with tissue destruction through dysregulated activity of MMPs/TIMPs. Synovial fibroblasts express a number of PRRs including TLR1-9 that are involved in recognition of pathogens and products joint damage. Activation of TLRs in synovial fibroblasts has been shown to induce the production of cytokines, chemokines and MMPs.[312] FLS interact with several cell types in the synovium including macrophages, T cells, B cells, endothelial cells and osteoclasts.

Synovial fibroblasts isolated from different joints have different phenotypic characteristics and heterogenous populations of fibroblasts have been observed in the synovium.[313] Differences in FLS phenotype were first noted in fibroblasts derived from the lining and sub-lining layer. Cells were acquired through enzymatic digestion, cultured and stimulated with cytokines (TNF-α, IL-1β and TGF-β). Fibroblasts from the lining layer in RA were shown to express high levels of podoplanin compared to normal synovium while CD248 expression was restricted to sub-lining layer cells. Stimulation with TNF-α or IL-1β resulted in increased expression of podoplanin while TGF-β stimulation induced expression of CD248. Furthermore, in a SCID human-mouse mode RA-FLS induced expression of PDPN and CD248. This study was the first to identify distinct populations of fibroblasts based on their surface marker expression.[313]

This was succeeded by characterization studies in two cohorts of patients with early RA to identify any possible relation of expression of stromal markers and relationship to diagnosis and prognostic outcome. Immunofluorescence was used to detect stromal markers CD55, CD248, FAP and podoplanin in synovial tissue. Expression of CD55, CD248, FAP and podoplanin was observed in synovial tissue of

all early arthritis patients regardless of prognostic outcome and baseline expression of FAP was higher in patients who fulfil criteria for diagnosis of established RA. The authors conclude that significant fibroblast activation occurs in the early stages of RA.[314]

Further studies by the aforementioned authors and others have emerged describing distinct subsets of fibroblasts in RA using next generation sequencing technologies. The first study by Mizgouchi *et al* identified three fibroblast subsets using a combination of bulk transcriptomics on pre-sorted subpopulations and unbiased single cell analysis. Populations identified were defined according to their expression of CD34, CD90 and cadherin 11. It was found that the anatomical localization of fibroblasts differed according to their surface phenotype with $CD34^{-}CD90^{+}$ localized to the perivascular zone in the sublining layer of the synovium and near accumulations of lymphocytes and $CD34^{+}CD90^{+}$ fibroblasts were observed in both superficial lining and deeper sublining layers of the synovium. $CD34^{-}CD90^{-}$ fibroblasts were mostly observed in the lining layer. Cadherin 11 was expressed in the majority of fibroblasts with highest expression in the lining layer and the $CD34^{-}CD90^{+}CAD11^{+}$ subpopulation was found to be expanded threefold in patients with RA compared to OA. The authors proceeded to explore the functional differences between fibroblast subsets and found $CD34^{+}$ fibroblasts have a transcriptomic profile characterized by the expression of IL-6, CXCL12 and CCL2. This effect was replicated *in vitro* with $CD34^{+}$ fibroblasts stimulated with TNF-α found to secrete large amounts of IL-6, CXCL12 and CCL2. Furthermore, this subset demonstrated enhanced recruitment of peripheral blood monocytes in a transwell leukocyte recruitment assay suggesting $CD34^{+}$ fibroblasts may play a role in monocyte recruitment in inflamed synovial tissue.[315] Another study using droplet-based techniques for single-cell transcriptome profiling of synovial tissue identified distinct subsets of $CD55^{+}$ and $CD90^{+}$ fibroblasts that are localized to the lining and sublining layer, respectively.[316]

Deletion of FAP-α^{+} fibroblasts in mice has been shown to suppress inflammation and bone erosion in models of resolving and persistent inflammation.[317] Single cell RNA sequencing identified two additional fibroblasts within the FAP-α^{+} fibroblasts based on their expression of CD90. $FAP\alpha^{+}CD90^{+}$ fibroblasts were deemed 'immune effector fibroblasts' localised to the sublining and $FAP^{+}CD90^{-}$ 'destructive

fibroblasts' were found in the lining layer. Adoptive transfer of FAPα^+CD90$^-$ fibroblasts into the joint resulted in bone and cartilage damage while transfer of FAPα^+CD90$^+$ fibroblasts resulted in more severe arthritis characterised by persistent inflammation with limited bone or cartilage erosion.[317] In addition, subsequent studies integrating single cell transcriptomics and mass cytometry identified four fibroblast subpopulations (based on expression of CD34, HLA-DRA and CD90) displaying differential expression in leukocyte rich RA, leukocyte poor RA and OA. In leukocyte rich RA (representative a highly inflammatory environment) sub-lining specific subsets showed enriched expression of genes associated with regulation of leukocyte migration, regulation of the inflammatory response, NFκB signalling and IL-6 production.[318] Taken together, it is evident that fibroblast populations within that RA synovium are heterogeneous and display different phenotypical and functional characteristics according to their anatomical localization.

1.5.4 Fibroblasts in cancer

Cancer tissue is composed of cancer cells and associated stromal cells. Stromal cells are non-malignant; however, fibroblasts within the tumour microenvironment can acquire an 'activated' phenotype.[292] The tumour microenvironment regulates the influx and retention of inflammatory cells such as macrophages and is also responsible for the development of 'carcinoma associated fibroblasts' (CAFs) characterized by expression of α-smooth muscle actin (α-SMA) and vimentin; features typical of activated myofibroblasts during wound healing responses.[319] The concept of 'fibroblast activation' was first described in the context of solid cancers and expression of fibroblast activation protein (FAP) by fibroblasts is now considered a hallmark feature of activated stroma.[320,321,322] In primary fibroblasts partial silencing of FAP using siRNA resulted in reduction of proliferation and production of collagen I, laminin and fibronectin.[323] Furthermore, FAP$^{-/-}$ mice show reduced vascularization in lung tumours.[324] Conversely, overexpression of FAP in CAFs resulted in changes to the CAF secretome by inducing expression of inflammatory, proliferative and ECM remodelling factors.[325]

FAP has also been shown to promote immunosuppression by CAFs through activation of STAT3-CCL2 signalling that enhances recruitment of myeloid-derived suppressor cells (MDSCs) thereby promoting tumour growth.[326] In addition, accumulation of ROS has been shown to induce activation of CAFs through induction of growth factors including PDGF and TGF-β.[327] Several studies targeting FAP have been conducted including the use of CAR-T cells to recognize FAP-positive cells and allow specific targeting of stromal cells.[328] T cell mediated elimination of FAP^+ CAFs resulted in reduced collagen density allowing for greater uptake of chemotherapeutic drugs.[329] Studies using cancer vaccines specific for FAP have also resulted in tumour regression in the absence of cytotoxic drugs.[330]

More recently, studies have highlighted the significance of fibroblast heterogeneity in PDAC where CAFs are associated with chemotherapy resistance. The authors identified two CAF subtypes designated as myofibroblastic (characterized by increased expression of α-SMA) or inflammatory (associated with increased expression of IL-6 and leukaemia inhibitory factor (LIF)).[331] Subsequently, tumour secreted factors IL-1 and TGF-β were found to promote CAF heterogeneity as IL-1 induces expression of LIF and activation of the JAK/STAT pathway to generate inflammatory CAFs and TGF-β reversed this through downregulation of IL-1R expression and promotion of differentiation to a myofibroblastic phenotype.[332] Furthermore, single cell RNA-seq of cells isolated from human lung tumours identified five subsets of fibroblasts in tumours compared with matched non-malignant lung samples and all subsets of fibroblast were found to have differing collagen and ECM molecule expression profiles.[333]

1.5.5 Candidate markers of stromal activation and fibroblast heterogeneity in tendinopathy

The concepts of stromal activation and fibroblast heterogeneity are inextricably linked and perhaps only defined by the evolution of modern methodologies. Given the nature and diversity of proteins expressed by stromal cells, identifying markers relevant to specific disease pathologies is crucial. This section will summarise the properties of a panel of cell surface proteins deemed relevant to this study.

Name(s)	Properties	References
CD146 melanoma cell adhesion molecule (MCAM)	▫ Cell adhesion molecule involved in cell migration ▫ Regulates inflammatory response in chronic inflammatory diseases ▫ Involved in recruitment of activated T cells to sites of inflammation ▫ Regulates cancer cell adhesion ▫ Marker for synovial membrane angiogenesis in RA	334,335,336,337,
CD90 Thy-1	▫ Promotes adhesion of leukocytes to endothelial cells and fibroblasts ▫ Promotes T cell activation ▫ Modulates fibroblast phenotype in wound healing and fibrosis	338,339,340
CD34	▫ Ligand for L-selectin (CD62L); promotes adhesion of leukocytes to vascular endothelium ▫ Regulates cell proliferation and differentiation ▫ Enhances trafficking and migration of haematopoietic cells ▫ Expression prevalent in solid tumours	341,342,343
VCAM1 (Vascular cell adhesion molecule-1) CD106	▫ Mediates contact between cell surface and stroma ▫ Mediates leukocyte extravasation to sites of tissue inflammation ▫ Influences behavior of inflammatory cytokines	344,345,346
CD10 Neutral endopeptidase	▫ ECM degrading enzyme involved in matrix remodelling ▫ Influences activation and degradation of pro-inflammatory peptides involved in immune system regulation and autoimmune diseases	347,348,349

Table 1.3.1 Candidate stromal cell surface markers in tendinopathy

Name(s)	Properties	References
CD47 Integrin associated protein (IAP)	▫ Regulates the function of monocytes and macrophages ▫ Interacts with the thrombospondin family of extracellular matrix proteins ▫ Interacts with SIRPα to regulate lymphocyte homeostasis, DC activation and cellular translocation ▫ Involved in the induction of apoptosis in fibroblasts	350,351,352,353,354
CD29 Integrin β1 (ITGB1)	▫ Associates with α integrins to form receptors for collagen, laminin, fibronectin, vitronectin and VCAM1 ▫ Links the actin cytoskeleton with the ECM to transmit bidirectional signals ▫ Migration and development of T and B cells ▫ Migration of monocytes, macrophages and DCs	355,356,357,358
FAP Fibroblast activation protein Seprase	▫ Exhibits exopeptidase and endopeptidase activities ▫ Specifically expressed by cells under stress ▫ Marker of carcinoma associated fibroblasts (CAFs) ▫ Involved in pathological fibrosis	359,360,328,325
Podoplanin PDPN	▫ Associates with the ECM to facilitate migration, adhesion and proliferation ▫ Plays a key role in platelet aggregation ▫ Expression is upregulated in areas of wound healing ▫ Expressed on CAFs and involved in invasion and metastasis	361,362,363,364,365

Table 1.3.2 Candidate stromal cell surface markers in tendinopathy

Name(s)	Properties	References
CD248 Endosialin Tumour endothelial marker-1 (TEM-1)	▫ Contains C-type lectin domains (CTLD) which function in cell adhesion and regulation of inflammation ▫ Plays a role in tissue remodelling associated with increased stromal cell proliferation and migration ▫ Expressed on pericytes and fibroblasts during inflammation ▫ Highly expressed in most cancers and on activated fibroblasts in RA	366,367,368,369
Cadherin 11	▫ Regulates collagen and elastin synthesis ▫ Regulates inflammation and cartilage damage in models of RA ▫ Promotes malignant transformation and tumour invasiveness ▫ Expression is increased in fibrotic tissue	370,371,308,372,373
CD81 Target of anti-proliferative antibody-1 (TAPA-1)	▫ Associates with CD4 and CD8 on T cells ▫ Mediates adhesion of T and B cells to stroma ▫ Promotes tumour growth and metastasis by modulating activities of Tregs and MDSCs ▫ Expression is upregulated in RA FLS	374,375,376
CD91 low-density lipoprotein receptor related protein-1 (LRP1)	▫ Involved in receptor mediated endocytosis ▫ Regulates cell growth, migration, apoptosis and inflammation ▫ Regulates macrophage mediated inflammation and ECM synthesis in atherosclerosis ▫ Tumour suppressing properties through clearing of proteases such as MMPs	377,378,379

Table 1.3.3 Candidate stromal cell surface markers in tendinopathy

1.6 Strategies targeting inflammation in musculoskeletal disease

1.6.1 Current treatments for tendinopathy

The pathophysiology of tendinopathy has been a subject of considerable disagreement which has resulted in significant challenges in the management and treatment of tendon disorders. To date, treatments have been largely conservative and surgery is only offered as a last resort. Many proposed treatment options have been largely experimental with some lacking adequate scientific rationale. Current treatments are summarised in Table 1.4.

1.6.2 Targeting cytokines in tendinopathy

With the advent of modern molecular techniques the role of inflammation in tendinopathy is now well established. Over the last decade the use of cytokine targeted therapies has become widespread in other musculoskeletal pathologies, most notably RA.[380] The presence of IL-1 family cytokines (IL-1β and IL-33), TNF, IL-6 and IL-17 has been documented in tendinopathy and all represent potential targets for pharmacological manipulation.

TNF

Anti-TNF therapy is one of the most studied treatments in RA to date due to the central role of TNF in disease pathogenesis.[381] Therapeutic agents (anti-TNF monoclonal antibodies) include infliximab, etanercept, adalimumab, certizumab pegol and golimumab. Inhibition of TNF-α in RA results in reduced IL-6 production, reduced recruitment of immune cell to the synovium and lower chemokine production.[382] TNF blockade also results in fewer macrophages and lymphocytes in the synovium.[383] In a pilot study in tendinopathy, adalimumab was trialled in a group of 10 athletes with symptomatic unilateral tendinopathy for more than six months. Significant improvement in pain at rest was recorded after 7 days and there was trend towards improvement in walking pain at 7 days and 12 weeks post treatment.[384]

Treatment	Mode of action
Rest or modification of activity	Removal of precipitating factors and prevention of injury
Orthotics	Removal of precipitating factors and prevention of injury
Cryotherapy	Reduction of acute inflammation and reduction in cell metabolism
Heat treatment	Stimulation of cellular activity and increased blood flow
Physiotherapy	Stimulation of cellular activity and increased blood flow
Electrical stimulation	Stimulation of cellular activity and increased blood flow, reduction in pain perception
Laser treatment	Possible analgesic effects and unspecified effects on cell activity
Pulsed electromagnetic fields	Possible analgesic effects and unspecified effects on cell activity
Electrocorporeal Shock-wave therapy	Possible stimulatory effects on neovascularization and inhibition of nociception
NSAIDs	Reduction in inflammation through inhibition of prostaglandin synthesis
Pertendinous corticosteroid injection	Inhibition of inflammation
Low dose heparin	Effect on blood flow possibly resulting in improved healing
Glycosaminoglycan polysulfate	Inhibition of inflammation, possible inhibition of MMP activity
Eccentric exercise therapy	Thought to promote restoration of tissue architecture through effects on cell activity and matrix remodelling
Sclerosant injection	Blocks tendon blood flow to target neovascularization and nerve in-growth
Platelet-rich plasma injection	Contains growth factors (including TGF-β and PDGF) thought to promote matrix synthesis and repair
Surgery	Excision of degenerative tissue

Table 1.4 Summary of current treatments for tendinopathy
Adapted from Riley *et al* 2005

IL-1

Agents involved in blockade of IL-1β signalling include Anakinra, rilonacept and canakinumab. Anakinra is an IL-Rα antagonist that is used in the treatment of moderate-severe RA.[385] A pilot study in patients with chronic Achilles tendinopathy administered anakinra by ultrasound guided peritendinous injection. The investigators observed a substantial increase in tendon thickness over time; however, no changes in pain walking or at rest was noted. In addition, no changes in intratendinous blood flow were observed.[384]

IL-6

IL-6 expression in tendinopathy is well documented and several agents targeting IL-6 signalling are currently used as treatment in inflammatory arthritis including tocilizumab, sarilumab, clazakizumab, sirukumab.[386] Toclizumab is a humanised monoclonal antibody against IL-6R that has shown efficacy in treatment of RA, particularly in those patients who have previously not responded to anti-TNF-α therapy.[387,388] As yet, no approved IL-6 targeting biologics have been trialled in human tendinopathy; however, an historic study assessing alterations in ECM as a result of peritendinous infusion of recombinant human IL-6 found stimulation of collagen synthesis in the Achilles tendon.[389]

IL-17

IL-17 has been shown to play a role in inflammation and matrix remodelling in tendinopathy.[390] A recent study has examined the effect of secukinumab (an anti-IL-17 monoclonal antibody) in human tenocytes and in an *in vivo* rat model of tendinopathy. Results showed IL-17A blockade reduces the pro-inflammatory signature in human tenocytes and significantly improved tendon structure and function *in vivo* indicating potential for use in human tendinopathy.[391]

Pilot studies have highlighted the potential of targeting inflammatory signalling in tendinopathy using biologic agents. Thus far these studies have been non-blinded and non-randomized limiting their interpretation. It is evident that biologic agents represent an attractive step forward in the treatment of tendon disorders; however, further quality studies are required to validate these findings.

1.6.3 Targeting signalling pathways in tendinopathy

Recently, studies have identified NFκB as a potential therapeutic target in human tendinopathy.[392] It was first noted that 65% of NFκB-associated genes assayed were dysregulated in clinical samples of early tendinopathy. Most notably, increases in the regulatory serine kinase subunit IKKβ were observed and cre-mediated overexpression of IKKβ resulted in degeneration of mouse rotator cuff tendons with associated increases in pro-inflammatory cytokines and innate immune cells within the joint. In addition, conditional knockout of IKKβ improved outcomes after surgical repair while overexpression resulted in impaired healing. The authors propose that NFκB directed therapy could specifically target the stromal cell compartment while allowing tissue resident and infiltrating immune cells to initiate the healing response.[392]

Several *in vivo* studies have identified a role for MAPK signalling in tenocyte behaviour. Increased extracellular signal regulated kinase (ERK) 1/2 signalling was increased in a rodent model of tendinopathy while inhibition of p38 decreased IL-6 expression in a rodent model of plantaris tendon growth. In addition, reduced expression of ECM and cell proliferation genes was observed.[393,394] *In vitro*, ERK and p38 inhibitors were shown to reduce expression of inflammatory cytokines and chemokines but did not induce tenocyte apoptosis.[82] Several phase II trials using small molecule inhibitors of MAPK signalling have been conducted although all have proved unsuccessful. However, JAK inhibitors including tofacitnib and baricitnib have recently been approved for clinical use in RA which provides rationale for further studies interrogating signalling pathways in tendinopathy.

Recently, regulatory pathways involving microRNAs have been described in human and murine models of tendinopathy.[197] In a study detailing IL-33 mediated alterations in collagen synthesis, reduced expression of miR-29a was observed in human rotator cuff biopsy samples. Functional reduction of miR-29a activity led to the development of tendinopathy and miR-29a was shown to induce expression of type I collagen but not type III in tenocytes. The authors propose that reintroduction of miR-29a to an injured tendon could reverse the switch from type I to type III collagen which is the hallmark feature of pathological matrix remodelling.

Evidence suggests there is clear justification for the translation of immune based therapies used in other chronic inflammatory pathologies to tendinopathy. Targeting pathways and signalling molecules that regulate immune-cell matrix interactions may assist in restoring tendon homeostasis.

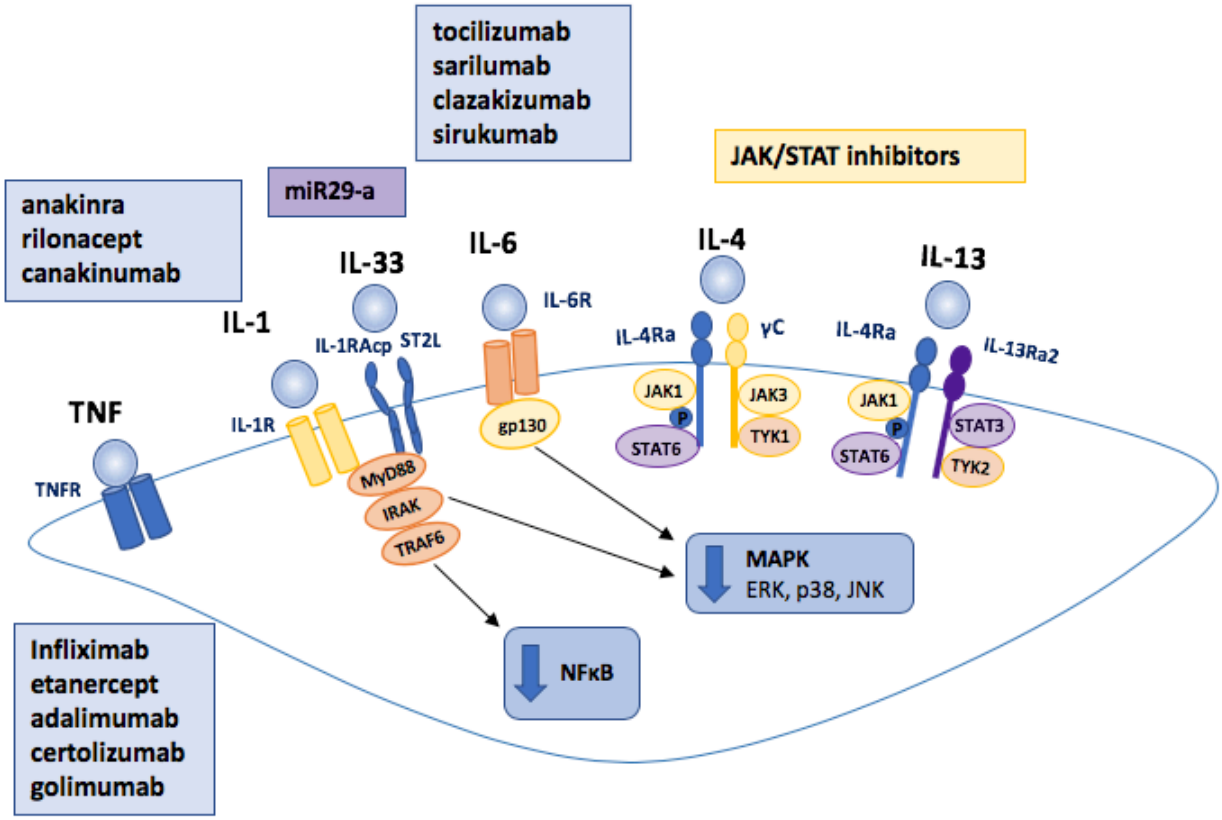


Figure 1.6 Potential therapeutic agents targeting inflammation in tendinopathy

1.7 Aims

It is now well established that inflammation is a key precipitating factor in the development of tendinopathy. Studies investigating immune regulation in tendinopathy have largely focused on cytokine biology and the role of specific immune cell subsets and their interactions with stromal cells remains uncovered. This work seeks to define mechanisms through which immune cell-stromal interactions regulate inflammation in both early and late stage disease.

The aims of this project were:

1. To identify mechanisms through which damage regulates initiation of the innate immune response in tendinopathy
2. Profile stromal cells in healthy and tendinopathic tendon and identify disease relevant characteristics
3. Explore mechanisms through which markers of activated stroma regulate tenocyte behaviour
4. Directly assess the effect of tenocyte-monocyte interactions on immune cell phenotype

Chapter 2: Materials and Methods

2.1 Buffers and Media

Complete Roswell Park Memorial Institute (RPMI) – RPMI 1640 medium supplemented with 10% heat inactivated foetal bovine serum (FBS), 1% L-Glutamine (2nM), 1% Penicillin-Streptomycin (100µg/ml) (all Thermo Fisher Scientific).

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

FACS buffer- 1x PBS supplemented with 1% FBS and 0.09% (w/v) sodium azide

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

ELISA wash buffer- 1 x PBS in distilled water with 0.5% Tween 20 (Sigma Aldrich)

ELISA stop solution- 2N sulphuric acid

TBS Tween (TBST): 10x TBS buffer was made using: 876.6 g NaCl, 121.1 g Tris, 40 ml HCl and adjusted to pH pH 8.0. 10x solution was diluted 1:10 with dH_2O and 0.05% Tween added.

2.2 Ethics and collection of human tendon tissue

All procedures and protocols were approved by the Ethics Committee under approval numbers Central Network, South East Health (HREC/96/55, HREC/14/130) and West of Scotland REC (REC14/WS/1035) with informed consent obtained and carried out in accordance with standard operative procedures. Tissue for explant culture was obtained following patient informed consent to use any residual tendon tissue not utilised at the time of surgery under the NHS Greater Glasgow Residual Tissue consent procedures which is a Pan Glasgow Ethics facility.

Supraspinatus tendon samples were collected from patients with rotator cuff tears undergoing shoulder surgery. Control groups obtained comprised of subscapularis tendon collected from patients undergoing arthroscopic surgery for shoulder stabilisation without rotator cuff tears, no previous shoulder surgery, no radiographic signs of shoulder fracture, or history of RA or OA. The absence of rotator cuff tears was confirmed by arthroscopic examination. A separate control group of hamstring tendons was obtained at the time of routine anterior cruciate ligament reconstruction (utilised as control due to lack of healthy subscapularis tissue). Standardised patient demographics were obtained preoperatively and included the duration of shoulder symptoms experienced by the patient and the number of subacromial steroid injections.

2.3 Cell culture

All cell culture was undertaken in a laminar flow hood. Cultures were maintained in a humidified environment at 37°C, 5% $C0_2$ in a cell culture incubator.

2.3.1 Culture of primary human tenocytes

Normal human tenocytes were explanted from hamstring tendon of patients undergoing anterior cruciate ligament reconstruction surgery. Healthy tendon tissue was cut into small pieces with a sterile blade and placed in a T25 tissue culture flask containing 5ml of complete RPMI. Flasks were incubated without disturbance for two weeks to allow tenocytes to adhere. Following this 1ml of RPMI was added to each flask on a weekly basis to replenish medium. When flasks reached confluency (no more than 4 weeks after the date of arrival of the specimen) the tissue was removed and cells were washed with 5mls of 1x PBS. To lift the cells 1ml of Trypsin EDTA was added and flasks were incubated at 37°C for approximately 5 minutes or until the cells were completely detached. Fresh complete medium containing 10% FBS was then added to inactivate the trypsin and cells were transferred to a fresh, sterile 15ml centrifuge tube. Cells were then reseeded in fresh, sterile T25 flasks.

'Tendinopathic' tenocytes were explanted from supraspinatus tendon obtained from patients undergoing arthroscopic shoulder surgery using the same method as above.

If required for immediate use all cells were maintained in complete RPMI and passaged at 70% confluency up to four times using the above method. If intended for future use, cells were stored in cryovials at -80°C. Following trypsinization and resuspension in complete RPMI cells were centrifuged at 1500rpm for 5 minutes, supernatant was removed and tenocytes were resuspended in 1ml of BAMBANKER (Alpha Laboratories) per 10^6 cells.

2.3.2 Culture of primary human monocytes

2.3.3.1 Separation of peripheral blood mononuclear cells

Buffy coats were obtained from the Scottish National Blood Transfusion Service (SNBTS). To separate PBMCs from whole blood samples the blood was diluted 1:1 with sterile PBS in a 50ml centrifuge tube. In a sterile 15 ml tube 10mls of blood was carefully overlaid on to 4mls of Histopaque-1077 (Sigma Aldrich) and centrifuged at 2100 rpm for 25 minutes with no brake. The PBMC containing interface was carefully collected using a Pasteur pipette and transferred to a 50ml centrifuge tube. PBMCs were washed twice using cold MACS buffer and counted using a 1 in 10 dilution of trypan blue (Sigma).

2.3.3.2 Isolation of $CD14^+$ monocytes from peripheral blood mononuclear cells

Cells were separated using the Miltenyi autoMACS Pro Separator according to manufacturer's instructions. To prepare the cells for separation, once counted, the desired number of PBMCs were resuspended in 80μl of cold MACS buffer per 10^7 cells. Samples were then placed in the chilled autoMACS tube rack where they are automatically labelled with magnetic anti-CD14 conjugated beads (Miltenyi Biotec). Cells were subsequently magnetically separated using the 'PosselD' setting that allows for positive selection of the $CD14^+$ population. Purity check of $CD14^+$ populations will be discussed in section 2.4.3. Generally enrichment of the $CD14^+$ population was > 95% purity.

2.3.4 Stimulation of tenocytes

Tenocytes from the 2nd or 3rd passage were seeded in sterile 12 or 24 well tissue culture plates at a density of $1x10^5$/ml and stimulated in a volume of 500µl complete RPMI containing the given concentration of each stimulating agent (Table 2.1) for 24 hours at 37°C. Following stimulation supernatants were transferred into sterile Eppendorfs (under sterile conditions if intended for further use in tissue culture) and stored at -20°C for short term storage or -80°C for longer term storage.

	Concentration	Manufacturer
S100A8	0.1µg/ml 1µg/ml	Abcam
S100A9	0.1µg/ml 1µg/ml	Abcam
IL-6	100ng/ml	Biolegend
CCL2	100ng/ml	Biolegend
IL-1β	10ng/ml	Biolegend
LPS	1ng/ml	Sigma-Aldrich
M-CSF	25ng/ml	Biolegend
GM-CSF	100ng/ml	Biolegend

Table 2.1 Concentrations of agents used for cell stimulations

2.3.5 Transfection of human tenocytes

In order to knockdown a markers expressed on the tenocyte surface, tenocytes were transfected using Silencer Select predesigned siRNAs (PDPN, assay ID S20884, VCAM1 assay ID S14760, Thermo Fisher Scientific) or a scrambled negative control (miRIDIAN microRNA Mimic Negative Control #1, Horizon). The following transfection protocol using DharmaFECT 3 (Dharmacon) was adapted from the manufacturer's recommended protocol. DharmaFECT 3 and OptiMEM (Thermo Fisher Scientific) were added to an eppendorf, Tube A. 10 μM stock concentration siRNA and OptiMEM were added to a separate eppendorf, Tube B. The contents were mixed well and incubated at room temperature for 5 minutes. The contents of Tube A were added to Tube B and mixed thoroughly. This mixture was then incubated at room temperature for 15 minutes. The medium in which the cells were cultured was replenished with 200μl of fresh medium and 50μl of the transfection mixture was added for a total volume of 250μl in a 24 well plate. The cells were incubated at 37°C in a CO_2 incubator for 48 hours. Cells were then retransfected for a further 24 hours using the same protocol.

Reagent	**Volume per reaction**	**Reagent**	**Volume per reaction**
Tube A		**Tube B**	
OptiMEM	24.5μl	OptiMEM	23.75 μl
DharmaFECT 3	0.5 μl	Mimic or siRNA (10 μM)	1.25 μl
Final Volume	**25.0 μl**	**Final Volume**	**25.0 μl**

Table 2.2 Components of transfection mixture

2.3.6 Scratch assay

Normal tenocytes were seeded at 5×10^4/ml in 12 well culture plates and allowed to adhere for 48 hours. Medium was then replenished and cells were scratched 4 times across the diameter of the plate with a sterile pipette tip. Injured cells were incubated for 24 hours before harvesting of supernatants.

For monocyte experiments using tenocyte scratch supernatants human CD14+ monocytes isolated from buffy coats were seeded in 24 well culture plates at a density of $2.5x10^5$ per well with supernatants (diluted 1 in 2 with complete RPMI) obtained from the tenocyte scratch assay for 24 hours.

2.3.7 Direct co-cultures

Tenocytes were seeded at a density of $1x10^5$/ml in 24 well plates and allowed to adhere for 48 hours. Monocytes were isolated from buffy coats according to the aforementioned procedure, resuspended at $1x10^6$/ml in complete RPMI and the tenocyte medium was replaced by the monocyte cell suspension. Following 48 hours incubation, supernatants were transferred to FACS tubes and centrifuged at 400 x g for 5 mins to allow any remaining suspended monocytes to adhere to the tube. Adherent cells were washed with PBS, detached using 250μl accutase for 10 minutes at 37°C and the mixed cell suspension was then stained for analysis by flow cytometry.

2.3.8 Transwell co-cultures

Tenocytes were seeded at a density of $1x10^5$/ml in 24 well plates and allowed to adhere for 48 hours. Monocytes were isolated from buffy coats according to the aforementioned procedure, resuspended at $1x10^6$/ml in complete RPMI. Tenocyte medium was replaced with 500μl of fresh RPMI and pre-soaked 0.4μM transwell inserts (Corning) were added to the appropriate wells. 250μl of the monocyte cell suspension was then added for a total volume of 750μl. Co-cultures were incubated for 48 hours and supernatants were harvested for ELISA.

2.4 Fluorescence activated cell sorting (FACS)

2.4.1 Enzymatic digestion of tendon tissue

The product of enzymatic digestion of tendon tissue contains a heterogenous cell population that includes tenocytes, immune cells and endothelial cells. To identify these populations cells were stained with antibodies directed against cell surface markers. Under sterile conditions, healthy hamstring or diseased supraspinatus tendon was cut into small pieces in a petri dish containing serum-free, phenol-free RPMI using a sterile blade. Tissue was then transferred to a sterile 20ml universal tube with 10mls of serum-free, phenol-free RPMI containing 0.125mg/ml Liberase (Sigma Aldrich). To allow for optimal enzymatic digestion, tubes were incubated at 37°C for 2 hours on a MACSMix tube rotator (Miltenyi Biotec). The tubes were shaken briefly and 10mls of phenol free RPMI supplemented with 10% heat inactivated FBS was added to inhibit any further digestion. The solution containing the cellular component was passed through a 100μm cell strainer into a sterile 50ml centrifuge tube and any remaining fibrous tissue debris was discarded. Cells were centrifuged at 1500rpm for 5 minutes, media was aspirated and 50mls of FACS buffer was added for a 5 minute wash at 1500rpm. Following aspiration of FACS buffer cells were carried on to the cell surface staining protocol as detailed below.

2.4.2 Staining of cell surface proteins

Cells obtained from cultures or enzymatic digestion of tissue were transferred to a round bottomed polystyrene FACS tube (BD Biosciences), 2mls of PBS was added and tubes were centrifuged at 1500rpm. This step was repeated once more to remove any residual serum. Excess PBS was poured off leaving a residual volume of approximately 100μl with the cells in a pellet to the bottom of the tube. Tubes were then vortexed gently to create a dense cell suspension. Fixable viability dye (eBioscience eFluor506™ or eFluor780™ viability dye, Thermo Fisher Scientific) working solution was made using a 1 in 1000 dilution with PBS. 100μl was added to each tube and cells were stained for 15 minutes at room temperature in the dark.

Following viability staining, 2mls of FACS buffer was added and cells were centrifuged at 1500rpm for 5 minutes at 4°C. Excess buffer was poured off and

the appropriate concentration of antibody was added to each tube. Cells were stained for 15 minutes at room temperature in the dark and washed a further two times with FACS buffer. If analysis was to be performed immediately, 250μl of FACS buffer was added to each tube for acquisition. For next day analysis, cells were resuspended in 250μl of BD Cell Fix (BD Biosciences) and stored at 4°C away from light.

Exclusion channel staining (Table 2.4) was performed when tendon digests were stained for macrophage markers.

FACS analysis was carried out using a BD LSR Fortessa or BD LSRII flow cytometer (BD Biosciences) and data was analysed with FlowJo software.

Target protein	Conjugate	Clone	Isotype	Supplier
Podoplanin	PerCP/Cy5.5	NC-08	Rat IgG2a k	Biolegend
CD29	PE	TS2/16	Mouse IgG1 k	Biolegend
CD10	BV605	H110a	Mouse IgG1 k	Biolegend
CD44	FITC	BT18	Mouse IgG1 k	Biolegend
CD146	Pe/Cy7	PIH12	Mouse IgG1 k	Biolegend
CD34	APC	561	Mouse IgG2a k	Biolegend
CD47	APC/Fire750	CC2C6	Mouse IgG1 k	Biolegend
CD81	Pe/Cy7	5A6	Mouse IgG1 k	Biolegend
CD90	BV421	5E10	Mouse IgG1 k	Biolegend
CD163	PerCP/Cyanine5.5	GH1/61	Mouse IgG1 k	Biolegend
CD206	BV421	15-2	Mouse IgG1 k	Biolegend
HLA-DR	BV785	L243	Mouse IgG2a k	Biolegend
MERTK	PE	590H11G1E3	Mouse IgG1 k	Biolegend
CD64	BV605	10.1	Mouse IgG1 k	Biolegend
CD45	Pe/Cy7	2D1	Mouse IgG1 k	Biolegend
CD90	AF700	5E10	Mouse IgG1 k	Biolegend
VCAM1	PE	STA	Mouse IgG1 k	Biolegend
CD91	FITC	A2MR-α2	Mouse IgG1 k	BD Bioscience

Table 2.3 Antibodies used for flow cytometry

Target protein	Conjugate	Clone	Cell type to exclude	Supplier
CD117	FITC	104D2	mast cells	Biolegend
CD15	FITC	H198	neutrophils	Biolegend
CD19	FITC	H1B19	B cells	Biolegend
CD31	FITC	WM59	endothelial	Biolegend
CD56	FITC	5.1H11	NK cells	Biolegend
CD3	FITC	HIT3a	T cells	Biolegend
CD90	FITC	5E10	tenocytes	Biolegend

Table 2.4 Antibodies used for flow cytometry (exclusion channel)

2.4.3 CD14 purity check

Flow cytometry was used to analyse the efficiency of AutoMACS automated separation of $CD14^+$ cell populations from PBMCs. After the separation was completed, $1x10^6$ cells each were taken from the $CD14^+$ and $CD14^-$ fractions. $1x10^6$ cells from the original PBMC sample were also counted and prepared for FACS analysis. The cells were washed with 1ml FACS buffer followed by centrifugation at 300xg for 5 minutes. Samples were resuspended in 100µl FACS buffer and the appropriate concentration of antibodies. Next, the cells were incubated at 4 °C in the dark for 30 minutes. Samples were washed once more with 1ml FACS buffer followed by centrifugation at 300xg for 5 minutes. The cells were resuspended in 250µl FACS buffer and analysed using the BD LSRII flow cytometer (BD Biosciences).

Under this set of experimental conditions, $CD14^+$ cells generally make up 15-25% of total PBMCs isolated from whole blood and AutoMACS automated sorting isolated $CD14^+$ cell populations with an efficiency of ~95%.

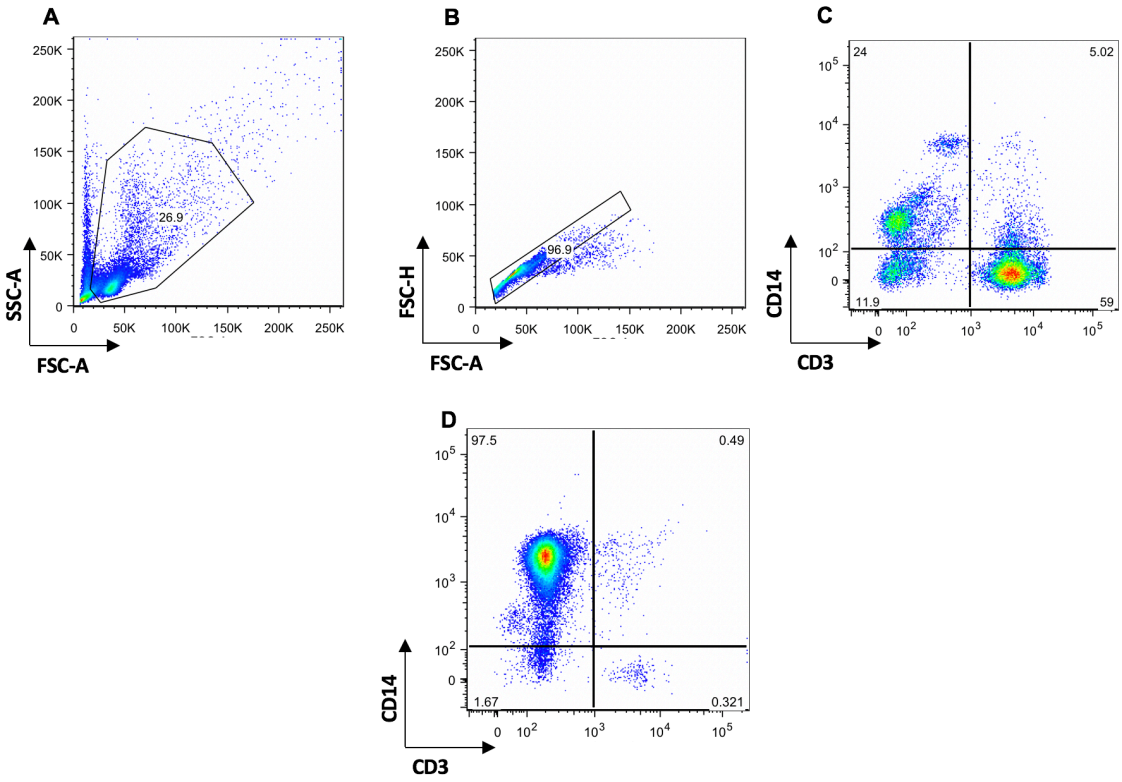


Figure 2.1 Analysis of purity of $CD14^+$ population isolated from PBMCs

(A) Gating strategy for analysis of proportion of $CD14^+$ cells in a PBMC sample. Cells were selected based on size and granularity. **(B)** Doublets were omitted by segregating height and area. **(C)** $CD14^+CD3^-$ cells were identified. **(D)** Analysis of the same $CD14^+CD3^-$ population from the $CD14^+$ selected fraction. Images are representative of one donor.

2.5 Quantitative Polymerase Chain Reaction

2.5.1 Total RNA extraction from cells

Total RNA was isolated and purified from cells using the PureLink RNA Mini Kit (Thermo Fisher Scientific). All RNA extraction procedures were carried out using RNase free, filter pipette tips. Each centrifugation step was performed at 12000xg at room temperature.

Adherent cells (tenocytes and macrophages) were lysed for RNA extraction using 350µl of lysis buffer (provided with the PureLink kit) containing 1% (w/v) 2-mercaptoethanol (Sigma Aldrich). This was added to each well immediately after harvesting of the supernatant and incubated at room temperature for approximately 5 minutes. Samples were transferred into fresh eppendorfs, vortexed thoroughly and used immediately or stored at -20°C.

Nonadherent cells were transferred into fresh eppendorfs and centrifuged at 1500 rpm for 5 minutes to form a pellet. Supernatants were then carefully removed and 350µl of lysis buffer containing 1% (w/v) 2-mercaptoethanol was added and tubes were vortexed thoroughly.

One volume of 70% ethanol was added for each volume of cell lysate and vortexed thoroughly to disperse any visible precipitate. Samples were then transferred to the PureLink spin column and centrifuged for 15 seconds. Flow through was discarded and 700µl of Wash Buffer 1 was added and spun for 15 seconds. This process was repeated twice using 500µl of Wash Buffer 2. The spin column was placed into a new collection tube and centrifuged for 2 minutes to dry the membrane. 30µl of RNase-free water was added directly onto the membrane of the spin column and incubated at room temperature for 1 minute. The column was centrifuged for a further 2 minutes to elute the bound RNA from the membrane into a fresh Eppendorf. The columns were subsequently discarded. Henceforth, all RNA samples were stored on ice or frozen at -20°C or -80°C for long term storage.

2.5.2 Total RNA extraction from human tissue

Normal, diseased subscapularis or diseased supraspinatus tendon samples were immersed in RNA later solution (Thermo Fisher Scientific) and stored overnight at 4°C to allow the solution to penetrate the tissue. Samples were then transferred to -80°C for storage.

Samples were thawed at room temperature and cut into small pieces with a sterile blade in a petri dish containing 700μl of PureLink lysis buffer (supplemented with 1% (w/v) 2-mercaptoethanol). Ball bearings (purchased from Qiagen) were cleaned with RNase Zap (Thermo Fisher Scientific) and placed into round bottomed eppendorfs (4 per sample) alongside the tissue samples and lysis buffer. The Tissue Lyser LT (Qiagen) was used to disrupt and homogenize tissue by high speed shaking. Samples were shaken for 2 minutes, a total of 3 times, with a one minute incubation on ice between each disruption. The supernatant was then carefully removed from the tube into a fresh RNase-free eppendorf and the tissue debris discarded. Samples were centrifuged at 1500rpm for 5 minutes to remove the remaining debris and transferred into new RNase-free eppendorfs. At this stage, the samples are ready undergo RNA extraction and purification using the PureLink kit as detailed previously.

2.5.3 Measuring concentration of nucleic acids

The concentration and purity of RNA isolated from cells or tissue was measured using a Nanodrop NC-100 spectrophotometer (Thermo Scientific). Nuclease-free water was used as a blank. The absorbance of the RNA sample was measured at 260 and 280nM with a background correction of 230nM. The A260/280 ratio is used to assess RNA purity and A260/230 ratio determines the level of protein contamination.

2.5.4 cDNA synthesis

cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to manufacturer's instructions.

RNA samples were diluted to a concentration of 10ng/μl with nuclease-free water and 10μl (a total of 100ng per reaction) of RNA was added to 200μl thin walled PCR tubes (Starlabs) with 2x High Capacity cDNA mastermix (Thermo Fisher Scientific) for a final volume of 20μl (Table 2.5). Tubes were vortexed thoroughly and placed in a thermal cycler (Applied Biosystems) for the following reverse transcription cycle outlined in Table 2.6.

Reagent	Volume per reaction (μl)
10x RT Buffer	2.0
dNTP (100mM)	0.8
10X RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase (50 U/μl)	1.0
Nuclease-free water	4.2
Total	**10.0**

Table 2.5 High Capacity 2x reaction mix

Settings	Step 1	Step 2	Step 3	Step 4
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 minutes	120 minutes	5 minutes	∞

Table 2.6 Cycling parameters for High Capacity cDNA synthesis

2.5.5 SYBR Green PCR mRNA quantitation

All qPCR experiments were performed using PowerUp SYBR Green Master Mix (Thermo Fisher) according to manufacturer's instructions. Following reverse transcription cDNA samples were diluted 1 in 5 for a final volume of 100μl. 1μl of cDNA template was added with 9μl of PowerUp SYBR Green Master Mix (including RNase-free water and forward and reverse primers) in duplicate to 96 well qPCR plates (Table 2.7). Plates were covered with optical adhesive film (both Starlabs) and centrifuged at 300xg for 1 minute. The StepOnePlus Real Time PCR system (Applied Biosystems) was used to measure mRNA expression and a non-template control (using RNase-free water in place of template cDNA) was used for each primer. The cycling parameters used are shown in table and a melt curve was performed after each run to confirm the presence of a single amplified product. qPCR primers used are shown in Table 2.8.

Reagent	Volume per reaction (μl)
PowerUp SYBR® Master Mix	5.0
Forward and Reverse Primers (5nM each primer)	1.0
cDNA template	1.0
Nuclease free water	3.0
Total	**10.0**

Table 2.7 PowerUp SYBR® reaction mix per well

Step	Temperature	Duration	Cycles
Uracil-DNA Glycosylase (UDG) Activation	50 °C	2 minutes	1X
AmpliTaq Fast DNA polymerase, UP activation	95 °C	2 minutes	1X
Denature	95 °C	1 seconds	40X
Anneal/Extend	60 °C	30 seconds	

Table 2.8 Cycling parameters for PowerUp SYBR®

Target	Forward	Reverse
GAPDH	5′-TCGACAGTCAGCCGCATCTTCTTT-3′	5′-ACCAAATCCGTTGACTCCGA CCTT-3’
18S	5’-GTAACCCGTTGAACCCCATT-3’	5’-CCATCCAATCGGTAGTAGCG-3’
Col1α1	5’-CAATGCTGCCCTTTCTGCTCC-3’	5’-CACTTGGGTGTTTGAGCATTG-3’
Col3α1	5’- TATCGAACACGCAAGGCTGTG-3’	5’-CACTTGGGTGTTTGAGCATTG-3’
S100A8	5’-AGACCGAGACCGAGTGTCCTC-3’	5’-CAGCTGCTTGTCTGCATTTG-3’
S100A9	5’-TCAAAGAGCTGGTGCGAAA-3’	5’-CAGCTGCTTGTCTGCATTTG-3’
Tenascin C	5’-CTTTGGCTGGGTTGCTTGAC-3’	5’-GTGCCAGGAGACCGTACCAC-3’
Periostin	5′-TTGAGACGCTGGAAGGAAAT-3′	5′-AGATCCGTGAAGGTGGTTTG-3′
Decorin	5′-CGCCTCATCTGAGGGAGCTT-3′	5′-TACTGGACCGGGTTGCTGAA-3′
PDPN	5’-CTTGACAACTCTGGTGGA-3’	5’-GGGCTTGGACTTGTTCTTG-3’
VCAM1	5’-GCAAGTCTACATATCACCCAAGA-3’	5’-TAGACCCTGGCTGGAACA-3’
CD44	5’-CCTGCAGGTATGGGTTCATAG-3’	5’-GGTGTTGGATGTGAGCATGT-5’
CD90	5’-CTAACAGTCTTGCAGGTCTCC-3’	5’ACTGCTGGTATTCTCATGCC-3’
CD34	5’-AGCAGGCTGATGCTGATG-3’	5’-TGCTGGAAATTTCTGCTCCAGCCTTT-3’
FAP	5’-ACGCATATACCAGTTATTGCCTATT-3’	5’-GGATTCTTAGCTCCAGCCTTT-3’
CD29	5’-GGCCTTGCATTACTGCTGATA-3’	5’-GTGTCCCATTTGGCATTCATTT-3’
CD146	5’-CGGCACGGCAAGTGAAC-3’	5’-GCATTCAACACCTGTCTCCAAC-3’
CD47	5’-TACAGAGACTCAGTCCAACCA-5’	5’-TTAGTACAGCGATTGGATTAACCT-3’
CD10	5’-GGTCTCGGGAATCACATACGTT-3’	5’-AAATCAGCTGCTCGACTGATCC-3’
CD248	5’-CTGTGCTCGGCAAGACC-3’	5’-CCCAAATCCCAAGGGAAGAT-3’
MMP3	5’-ACCCGACCTTACATACAGGATT-3’	5’-GTCACCTCTTCCCAGACTTTC-3’
MMP9	5’-GGGCTTAGATCATTCCTCAGTG-3’	5’-TTCAGGGCGAGGACCATA-3’
TIMP1	5’-GAACTAACCAGACCACCTAT-3’	5’-TTCAGGGCGAGGACCATA-3’
TIMP2	5’-GGCCTTCCTGCAATGAGATA-3’	5’-AGGGCCTGAGAAGCATATAGAG-3’

Table 2.9 Primers used for qPCR

2.5.7 Analysis of qPCR results

For each sample, the target gene was run alongside an endogenous control or 'housekeeping' gene that is required for maintenance of cell function. GAPDH and 18S were used as they are ubiquitously expressed in all cell types and their expression is known to remain constant over the course of the experiment.

$2^{\wedge-\Delta CT}$ quantification

The expression of target genes was expressed as $2^{-\Delta CT}$. This figure was obtained by subtracting the Ct value of the housekeeping gene from the Ct value of the target gene, to give the ΔCt value for each sample. The final value was expressed as $2^{-\Delta CT}$.

$2^{\wedge-\Delta\Delta CT}$ Relative Fold Change quantification

Relative Fold Change Quantification represents the expression values for a given gene as a fold change relative to control samples within a data set. First, the Ct value of the housekeeping gene was subtracted from the Ct value of the target gene, to give the ΔCt for each sample. Next, the ΔCt value of the control sample was subtracted from the experiment sample, to give the ΔΔCt value of the sample. The final value was expressed as $2^{\wedge-\Delta\Delta CT}$ which indicates the fold change between the control and any given experimental condition.

2.6 Illumina BeadChip Array

RNA was extracted from tendon tissue biopsies using the procedure outlined in section 2.5.2 and processed for the BeadChip array using an Illumina TotalPrep RNA Amplification kit (Life Technologies) according to manufacturer's protocol as follows:

1. Reverse transcription to synthesise first strand cDNA
2. Second strand cDNA synthesis
3. cDNA purification
4. In vitro transcription to synthesise cRNA
5. cRNA purification

The hybridization procedure (HumanHT-12_v4_BeadChip, Illumina) was performed according to manufacturer's instructions. GenomeStudio software was used for quality control including background correction. Genome studio was also used to calculate differential gene expression between the two groups ($p<0.05$) and the Euclidian method was used to cluster the differential genes.

2.7 Enzyme-Linked-Immunosorbent Assay (ELISA)

ELISA was used to measure the concentration of cytokines in cell culture supernatants. Commercially available ELISA kits (summarised in table 2.10) were performed according to manufacturer's instructions. All ELISAs were carried out using 96 Well Half Area High Bind Microplates (Corning) therefore all volumes were scaled down accordingly. Cell culture supernatants were used neat, diluted 1 in 2, 1 in 5,1 in 10 or 1 in 20 with ELISA assay buffer to ensure sample O.D did not exceed that of the highest standard. The ELISA is performed with sets of standards (range specific to each kit) prepared by a 1 in 2 serial dilution in ELISA assay buffer or complete RPMI (dependent upon which medium most closely reflects the sample diluent). Assay buffer or RPMI alone was used as a blank.

Plates were coated with capture antibody diluted in PBS, covered and incubated overnight at 4°C. They were washed once with ELISA wash buffer, blotted dry and blocked with 150μl ELISA assay buffer for one hour at room temperature.

For Thermo Fisher Scientific kits, 50μl of samples and standards were then added to the plate with 25μl of detection antibody (diluted in ELISA assay buffer) and incubated with continual shaking for 2 hours at room temperature. Plates were washed 5 times, 50μl of Streptavidin-HRP working solution was added and incubated at room temperature for a further 30 minutes.

For all other kits 50μl of samples and standards were added to the plate and incubated at room temperature for 2 hours with continual shaking at room temperature. Plates were washed 5 times, 50μl of working detection antibody was added and plates were incubated for an hour at room temperature. After five more washes, 50μl of Streptavidin-HRP working solution was added and incubated at room temperature for a further 30 minutes.

Finally, the wash steps were repeated and 50μl of TMB chromagen solution (Thermo Fisher Scientific) was added and incubated at room temperature until the desired blue colour change had been achieved. The reaction was stopped with 50μl of 2N sulphuric acid and the plate was read at 450nm on an MTX TC II microplate reader (Dynex Technologies).

Target protein	Sensitivity	Manufacturer
IL-6	31.25-2000pg/ml	Thermo Fisher Scientific
IL-8	12.5-800pg/ml	Thermo Fisher Scientific
CCL2	31.25-2000pg/ml	Thermo Fisher Scientific
CCL20	2.5-160pg/ml	Biolegend
CXCL10	7.8-500pg/ml	Biolegend
S100A8	31.25-2000pg/ml	R & D Systems
S100A9	31.25-2000pg/ml	R & D Systems
MMP3	1-2000pg/ml	Thermo Fisher Scientific

Table 2.10 ELISA kits

2.8 Proteome profiler array

Cell supernatants were evaluated for the presence and relative amounts of 35 proteases using the Proteome Profiler Human Protease Array Kit (R&D Systems) according to the manufacturer's protocol. For tenocyte experiments equal volumes of supernatants from tenocytes stimulated were pooled from three donors and applied to the respective array membrane. Samples were then mixed with a combination of biotinylated detection antibodies and incubated overnight at 4°C. The membranes were then subject to a series of washes before addition of diluted solution of horseradish peroxidase-conjugated streptavidin at room temperature for 30 minutes. Visualization of protease expression was carried out by chemiluminescence and signal intensity was quantified using an Azure c500 imaging system (Azure Biosystems). Relative optical densities of immunoreactive bands were determined using Image Studio Lite software (Li-Cor Biosciences).

2.9 Immunohistochemistry of paraffin embedded sections

For paraffin embedded sections, tissue was stored in formalin for 24 hours and transferred to 70% ethanol to retard the fixation process. Tissues were paraffin embedded and subsequently cut to thickness of 5µm. A minimum of 2 sections were mounted on one slide to facilitate isotype staining on the same slide in IHC protocols. For back-to-back sections, single sections were mounted on a slide with the next section being used on a different slide having the same anatomical surface in common. Sections for isotypes were added subsequently. Haematoxylin and eosin (H&E) staining was performed on one of the serial sections to allow tissue morphology to be assessed.

Slides containing paraffin embedded sections were heated to 65°C for 35 minutes followed by dewaxing in xylene and rehydration through ethanol to TBS Tween (TBST). Endogenous peroxidase activity was blocked using H_2O_2/methanol (5%/95%). To expose the relevant antigen, sections were then microwaved in 0.5M citrate buffer, pH 6 for 8 min. The sections were blocked for 1 hour at room temperature in 2.5% serum TBST of the species in which the secondary antibody was raised. The relevant primary antibody was applied overnight at 4°C 2.5% serum TBST (antibodies were purchased from Abcam, Abnova and LSBio). The following day the sections were washed with TBST, incubated with relevant secondary antibody for 30 minutes in 5% horse serum containing TBST, washed twice with TBST then incubated with substrate Vector ABC (Vector) for 30 minutes. Sections were washed twice with TBST before developing with 0.6mg/ml 3,3'- diaminobenzidine tetrahydrochloride (DAB) (Sigma) with 0.01% H_2O_2 for up to 5 minutes at room temperature until brown reaction product was apparent. Sections were then washed in water and counterstained using Harris's haematoxylin (BDH Ltd). Finally sections were dehydrated in ethanol, cleared in xylene and finally mounted in DPX mountant (both from BDH Ltd.).

Alternatively, the second day staining protocol was altered by using the ImmPRESS kit (Vector) in which sections were incubated with a species specific polymer for 30 min replacing the secondary antibody. Sections were washed with TBST and then developed with ImmPACT DAB (Vector) for up to 2 minutes. Further staining of the sections was carried out as described above.

2.9.1 Bonar score

The Bonar scoring system is used to classify the histopathological findings of tendinopathy. Sections were stained with H&E and toluidine blue for determination of the degree of tendinopathy as assessed by a modified version of the Bonar score[395] (grade 4 = marked tendinopathy; grade 3 = advanced tendinopathy; grade 2 = moderate degeneration; grade 1 = mild degeneration; grade 0 = normal tendon). This included the presence or absence of oedema and degeneration together with the degree of fibroblast cellularity and chondroid metaplasia.

We applied a scoring system based on previous methods to quantify immunohistochemical staining.[396] Five random high power fields (x400) were evaluated. In each field the number of positive and negatively stained cells were counted and the percentage of positive cells calculated giving the following semi-quantitative grading; Grade 0 = no staining, Grade 1 = <10% cells stained positive, 2 = 10–20% cells stained positive, Grade 3 = >20% cells positive.

2.10 Statistics

Results are reported as mean values ± SEM or median. Comparisons between groups were made with Kruskal-Wallis One Way Analysis of Variance on ranks, Ordinary One-Way ANOVA with Tukey's correction for multiple comparisons, Friedman test with Dunn's correction for multiple comparisons, two-way paired Student's *t* tests or Mann-Whitney *U* test using Graph Pad Prism 5 software. In all analysis $p<0.05$ was considered statistically significant. Details of all statistical methods used and number of biological replicates for each experiment are stated in the figure legend.

2.11 Bioinformatic analysis

2.11.1 Spanning Tree Progression Analysis of Density-normalized events (SPADE)

SPADE organizes cellular populations into hierarchies based on similar phenotypes. It provides a 2D depiction of multiple cell-types in a branched tree structure. A typical SPADE tree is comprised of nodes representing cell clusters that are connected through edges to represent relationships and provide information about the underlying similarity of cell-types.[397] Normal and tendinopathic tissue was digested and stained for a panel of cell surface markers as described previously and data was acquired on a BD Fortessa or LSRII cytometer.

The acquired data was loaded into Cytobank (https://www.cytobank.org), manually gated for singlet cells then subjected to SPADE analysis using the following settings:

- Target number of nodes=200
- Percentage downsampling =10%

The SPADE algorithm performs 4 steps[398]:

i. Density dependent downsampling to equalie the density in the point cloud of cells
ii. Agglomerative clustering to partition the point cloud of cells into cell clusters
iii. Minimum spanning tree construction to link the cell clusters
iv. Up-sampling to map all the cells onto the resulting tree structure

The SPADE tree generated was then analysed for populations of cells expressing surface markers stained for prior to FACS analysis. The tree was manually gated for positive populations by drawing ‘bubbles’ around selected nodes.

2.11.2 viSNE analysis

viSNE is a tool that uses the t-distributed stochastic neighbour embedding (tSNE) algorithm to analyse and display high-dimensional data on a two-dimensional map.[399] As stated previously, normal and tendinopathic tissue was digested and stained for a panel of cell surface markers and data was acquired on a BD flow cytometer. Data files were then compiled using the FCS file concatenation tool on FlowJo software. Tenocytes were manually gated out and data was loaded into Cytobank. viSNE analysis was run with the following settings:

- Equal sampling (equal number of events sampled per FCS file)
- Iterations – 1000
- Perplexity- 30
- Theta- 0.5

An overlay plot coloured for different cell surface proteins was then generated by performing manual gating of positive cell populations.

Chapter 3: Dissecting the role of alarmins S100A8 and S100A9 in tendinopathy

Content of this chapter has been published in the following manuscripts:

Crowe LAN, McLean M, Kitson SM, Melchor EG, Patommel K, Cao HM, Reilly JH, Leach WJ, Rooney BP, Spencer SJ, Mullen M, Chambers M, Murrell GAC, McInnes IB, Akbar M, Millar NL

S100A8 & S100A9: Alarmin mediated inflammation in tendinopathy

Scientific Reports. 2019 Feb 6;9(1):1463.

3.1 Introduction

Tendinopathy is a damage associated pathology whereby repetitive microtrauma elicits an immune response. Mobilization of immune cells within the tendon matrix is triggered by microenvironmental changes that occur in response to injury.[212]

Alarmins, also referred to as damage associated molecular patterns (DAMPS), are endogenous molecules rapidly released into the extracellular milieu following tissue damage.[400] S100A8 and S100A9, also known as myeloid related protein 8 (MRP8) and MRP14, are low molecular weight calcium binding proteins constitutively expressed by cells of myeloid origin.[401] Under pathological conditions they are induced in other cell types in response to environmental triggers. Acting as alarmins they are released passively by necrotic cells or by active secretion from activated immune cells.[136] Extracellular S100A8 and S100A9 bind pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) and receptor for advanced glycation end products (RAGE) to activate the innate immune system and mediate inflammation by influencing monocyte and macrophage behavior.[402]

In a rat model of Achilles tendon injury increased infiltration and accumulation of immune cells including neutrophils and macrophages was observed between 1 and 28 days post injury.[239] Subsequent human studies have identified distinct populations of myeloid monocytes and macrophages in both early and late tendinopathy.[29,212]

Monocytes recruited to areas of damage enter tissue in response to activation of chemokine pathways such as the CCL2/CCR2 axis.[403] In addition to monocytes, the myeloid compartment within the tendon may also include mature tissue macrophages that are programmed to respond to chemotactic factors following injury and assist in the initial inflammatory response. Current evidence suggests that immune cell infiltration and inflammatory mediators play diverse roles in the initiation and maintenance of tissue repair.[404] In the context of tendinopathy an initial inflammatory response promotes beneficial healing;

however, sustained inflammatory conditions may eventually lead to dysregulated matrix remodelling.

Both S100A8 and S100A9 are chemotactic for monocytes and have been implicated in myeloid cell maturation where their expression directly correlates with state of differentiation.[405 ,406] Moreover, they may exert both pro and anti-inflammatory effects by manipulating the cytokine profile of cells through PRR binding.[136] S100A8 and S100A9 are considered biomarkers of disease activity in chronic inflammatory pathologies associated with impaired matrix remodelling such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and cystic fibrosis.[407]

3.2 Aims

Recent investigations have established tendinopathy as an alarmin-mediated pathology[197,162] thus we sought to:

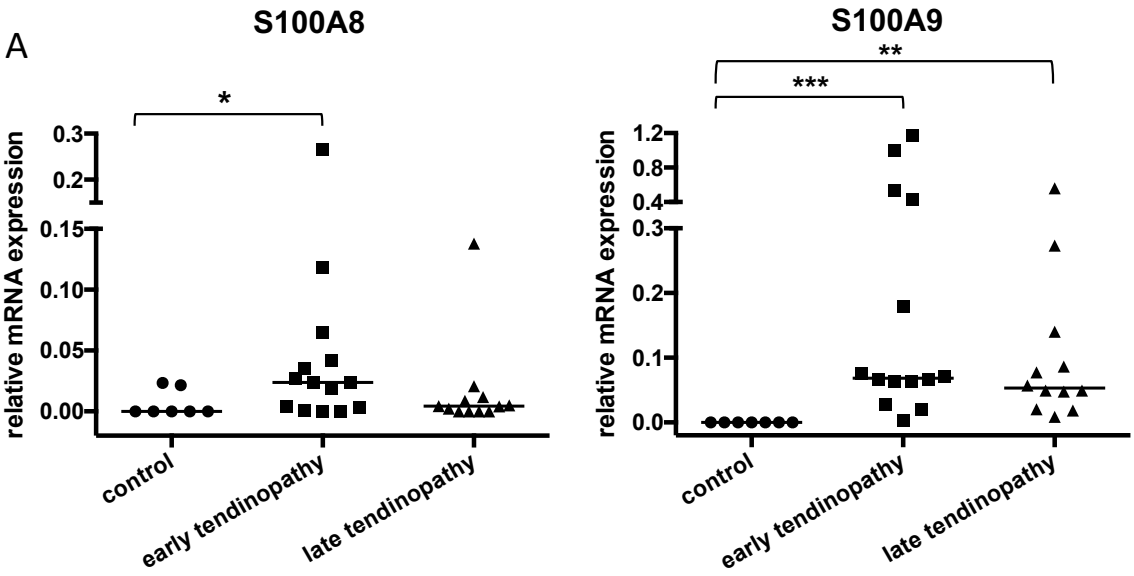
1. Characterise the expression of S100A8 and S100A9 in human tendinopathy

2. Assess their effect on inflammation and matrix production in tenocytes *in vitro*

3.3 Results

3.3.1 Characterising expression of S100A8 and S100A9 in tendinopathy

S100A8 mRNA expression is significantly upregulated in early tendinopathy compared with control ($p<0.05$) (**Fig. 3.1A**). S100A9 expression was more profoundly increased in both intact ($p<0.001$) and torn tendon biopsies ($p<0.01$) compared with control (**Fig. 3.1A**). We observed greatest significant upregulation of S100A8 and S100A9 in early tendinopathy and a relative absence of S100A8 mRNA expression in late tendinopathy suggesting these alarmins are key regulators in the early stage of disease. We noted positive staining of the alarmin molecules S100A8 and S100A9 in the early tendinopathy biopsy sections (**Fig. 3.1B**). The modified Bonar Score showed significantly increased expression of S100A8 in early tendinopathy vs control ($p<0.05$). S100A9 expression was significantly increased in both early and late tendinopathy ($p<0.01$ and $p<0.05$, respectively). In addition, there was a significant difference in S100A9 expression between early and late tendinopathy sections ($p<0.05$) (**Fig. 3.1C**). Semi-quantitative analysis suggested that S100A9 (9% early tendinopathy, 5% late tendinopathy, % of cells stained positive) was more frequently expressed than S100A8 (4% early tendinopathy, 1% late tendinopathy, % of cells stained positive) in tissue biopsies (**Fig. 3.1C**).


A
S100A8
S100A9
relative mRNA expression
0.3
0.2
0.15
0.10
0.05
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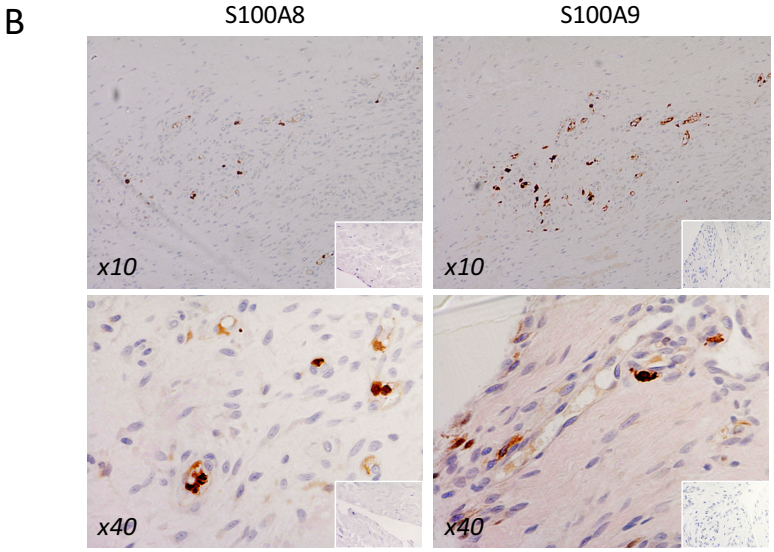
control
early tendinopathy
late tendinopathy


B
S100A8
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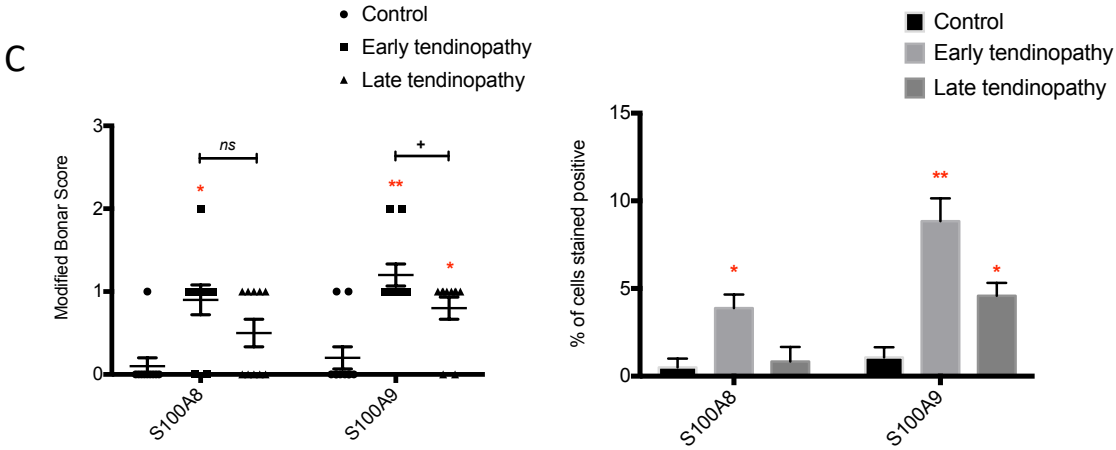
C
Control
Early tendinopathy
Late tendinopathy
Modified Bonar Score
ns
+
% of cells stained positive
S100A8
S100A9

Figure 3.1 S100A8 and S100A9 expression is increased in tendinopathy.
(A) Relative mRNA expression (2^-**Δ**CT) of S100A8 & S100A9 in control (hamstring tendon, n=7), early tendinopathy (intact subscapularis biopsy, n=14) and late tendinopathy (torn supraspinatus tendon, n=12). Data represent mean ± SEM relative to housekeeping gene GAPDH (mean of duplicate analysis). * p<0.05, **p<0.01, ***p<0.001 (Kruskal-Wallis test with Dunn's multiple comparisons) versus control.
(B) Immunostaining of S100A8 and S100A9 in early tendinopathy (subscapularis biopsy) at 10x and 40x magnification.
(C) Graphs illustrate modified Bonar scoring and percentage of cells stained positive for samples of human tendon biopsies for expression of S100A8 and A9 with mean and SEM shown. n=10 for control tendon, n=10 for early and late tendinopathy. Modified Bonar scoring system depicts mean score per sample based on five high-power fields. 0= no staining, 1=<10%, 2=10%–20%, 3=>20% positive staining of cells per high-power field. *p<0.05, **p<0.01 (ANOVA) versus control biopsies. +p<0.05 late versus early tendinopathy.

There were no significant correlations between S100A8 & A9 expression and the mean duration of symptoms, patient age or number of steroid injections (data not shown). Late tendinopathy samples exhibited marked degeneration, mucoid change and frank chondroid metaplasia (grade 4), whereas matched subscapularis tendon biopsies had grade 2–3 changes indicative of early tendinopathy. All control samples were classified as grade 1 consistent with normal fibrotendinous tissue with large distinct collagen fibrils.

Histological analysis showed both S100 proteins appeared not to be localised around the stroma. As S100A8 & A9 are known to be of myeloid origin, sub analysis using back-to-back staining with the macrophage marker CD68 was performed. Back-to-back staining utilises sections of tissue that are almost anatomically identical thus allowing comparison of multiple sections stained for different markers. Staining revealed that S100A9 was localised to macrophage cells in tendinopathic sections (**Fig. 3.2A & B**). To confirm the presence of S100A8 and S100A9 in macrophages, human monocyte-derived macrophages were grown on chamber slides and stained with antibodies directed against S100A8 & A9 (**Fig. 3.2E & F**). We observed positive staining of both S100A8 & A9 which suggests that myeloid cells rather than stromal cells may be the source of these alarmin proteins in tendinopathy.

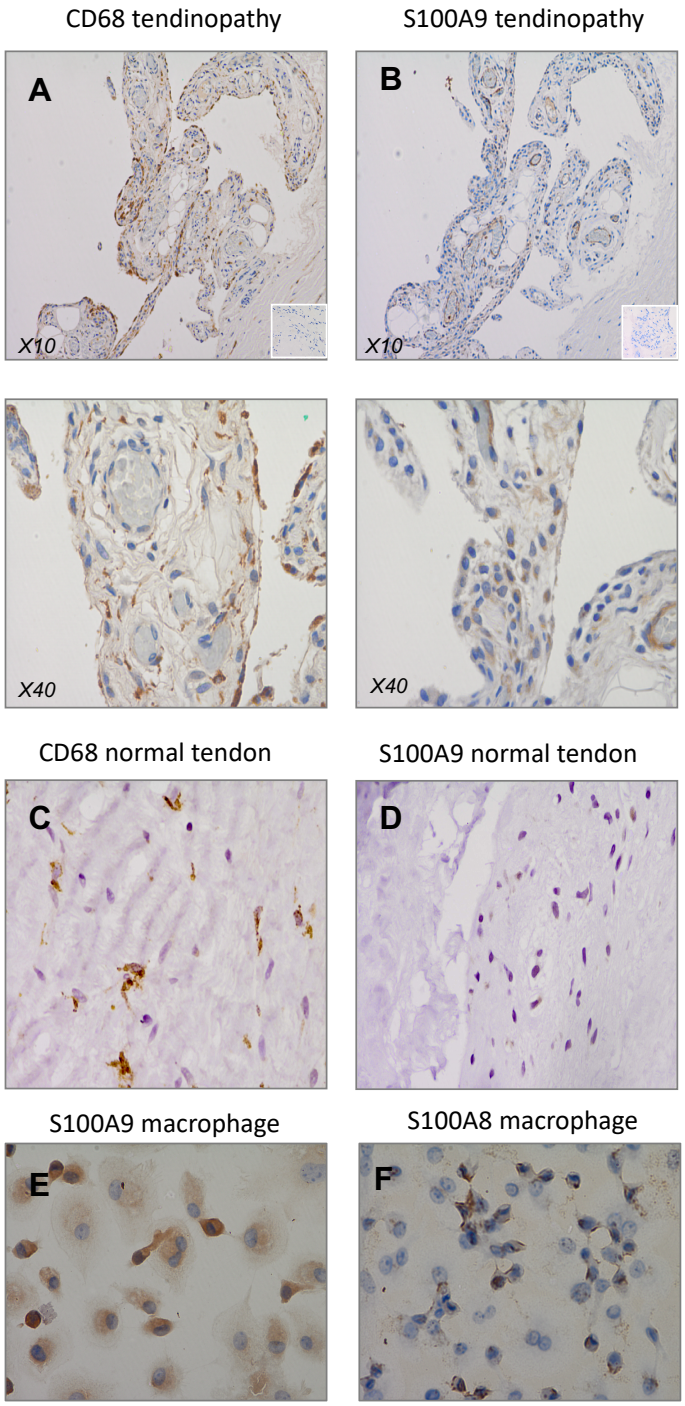

Figure 3.2 S100 expression is localized to immune cell infiltration in tendinopathy
(A) shows positive staining for CD68 (pan macrophage marker) localised to positive staining of S100A9 **(B)**. **(C), (D)** CD68 and S100A9 staining in normal tendon **(E)** Macrophages stained for S100A9 and S100A8 **(F).**

3.3.2 Damage induces release of chemokines and S100 proteins *in vitro*

Previous studies have shown that S100 proteins are released from myeloid cells in response to inflammatory stimuli[408] thus we measured the effect of recombinant LPS, CCL2 and IL-6 on S100 expression in human monocytes. We observed a significant increase in S100A8 (**Fig. 3.3A,** $p<0.05$) and S100A9 (**Fig. 3.3B**) transcript in response to CCL2 and IL-6.

Given that alarmins are released into the extracellular compartment by necrotic cells, we used an *in vitro* model of microtrauma to investigate potential mechanisms of alarmin release in tendinopathy. The scratch assay did not induce direct release S100 protein from tenocytes (data not shown); however, we did observe significantly increased release of CCL2 versus control indicating damage precipitates an inflammatory reaction and potential recruitment of immune cells to the site of injury (**Fig. 3.4A**, $p<0.01$). We did not detect release of other chemokines (including CCL20 or CXCL10) following injury further indicating CCL2 release from tenocytes is the primary mechanism of immune cell mobilization following tendon damage.

As CCL2 is a known chemokine for monocyte recruitment we hypothesised that these cells are recruited post-injury within the tendon. To simulate the stromal environment following injury $CD14^+$ monocytes were incubated with conditioned medium obtained from scratched tenocytes. We measured a significant increase in S100A8 protein release from monocytes by ELISA compared with unscratched control ($p<0.05$) (**Fig 3.4B**). Although not statistically significant, S100A9 release following incubation with tenocyte conditioned medium was also greater than unscratched and comparable to values observed with LPS stimulation (**Fig. 3.4B**). Taken together, these data suggest that tendon damage primes the local microenvironment to induce alarmin release from monocytes.

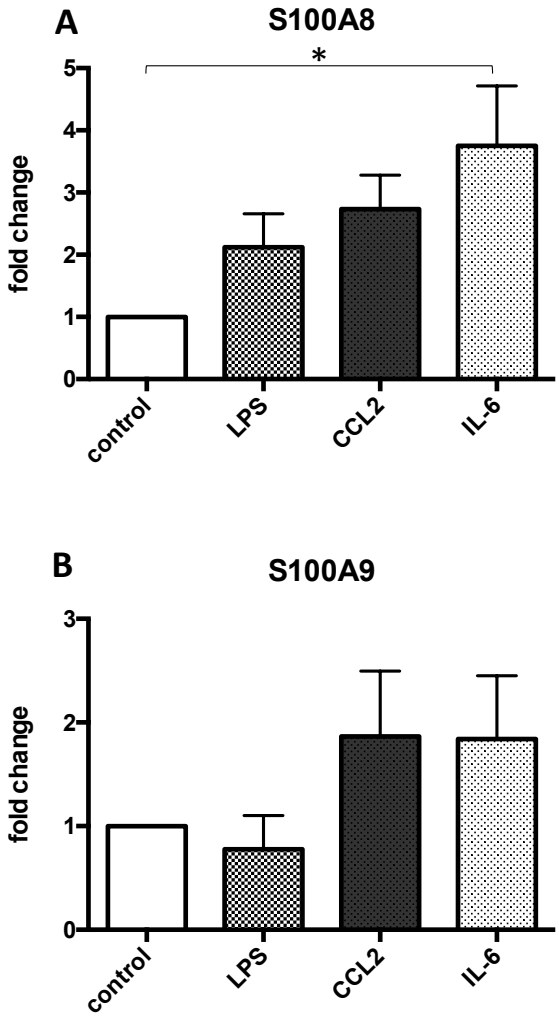

Figure 3.3 Inflammatory stimuli induce expression of S100A8 & A9 in human CD14+ monocytes
(A) (B) Tenocytes stimulated with 1ng/ml LPS , 100ng/ml CCL2 or 100ng/ml IL-6. Data represent mean ± SEM of duplicate samples expressed as relative fold change normalised to control (unstimulated) samples, n= 3. *p<0.05 versus control (Kruskal Wallis test).

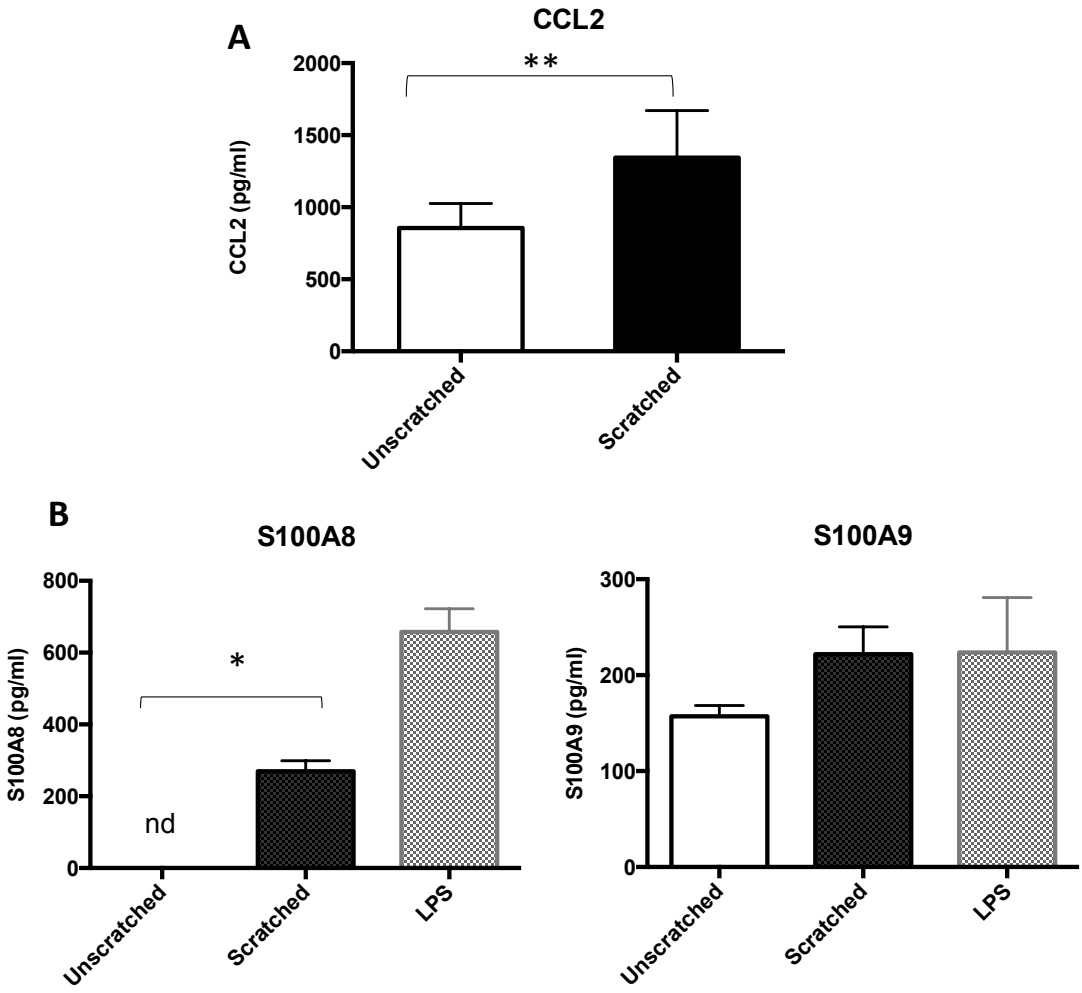

Figure 3.4 Damage induces release of CCL2 from tenocytes.
(A) CCL2 release measured by ELISA in supernatants collected from tenocytes scratched and incubated for 24 hours post injury compared to unscratched control. **(B)** S100A8 and S100A9 concentration in supernatants from $CD14^+$ monocytes (isolated from buffy coats) cultured in conditioned medium from tenocyte scratch assay for 24 hours. 'nd' indicates not detected. All data represent mean ± SEM. n=3 donors *p<0.05, ** p<0.01 versus control **(**paired *t*-test**).**

3.3.3 Measuring the effect of S100A8 and S100A9 on matrix regulation in tendinopathy

Previous investigations have shown that alarmins differentially regulate collagen synthesis and expression of matrix proteins in tendinopathy[197,162] thus we sought to assess the effect of extracellular S100A8 and S100A9 on matrix regulation in primary human tenocytes. Neither S100A8 or S100A9 had any significant effect on Col1a1, Col3a1, Decorin or Tenascin C gene expression following 24 hours stimulation relative to controls (**Fig. 3.5A-D**). Furthermore, no effect was observed on collagen I protein release from tenocytes in response to S100 stimulation (**Fig. 3.5E**).

As we found no significant alterations in genes associated with matrix deposition we performed an array (that allows simultaneous detection of 35 proteases) to further assess the potential effect of S100A8 & A9 on matrix turnover in tenocytes (**Fig. 3.6A**). We observed a marked increase in MMP3 (**Fig 3.6B**) and MMP9 (**Fig. 3.6E**) expression between control vs S100A9 and a modest increase in MMP12 (**Fig. 3.6D**), Cathepsin B (**Fig. 3.6G**) and ADAMTS12 (**Fig. 3.6H**).

This was validated at transcript and protein level where we found upregulation of MMP3 and MMP9 transcript in response to both S100A8 and S100A9 (**Fig. 3.7A & B**) and a significant increase in MMP3 release from tenocytes upon stimulation with S100A9 (**Fig. 3.7E**). We observed no discernible effect of S100A8 or S100A9 on inhibitors of metalloproteinases TIMP1 or TIMP2 (**Fig. 3.7C & D**).

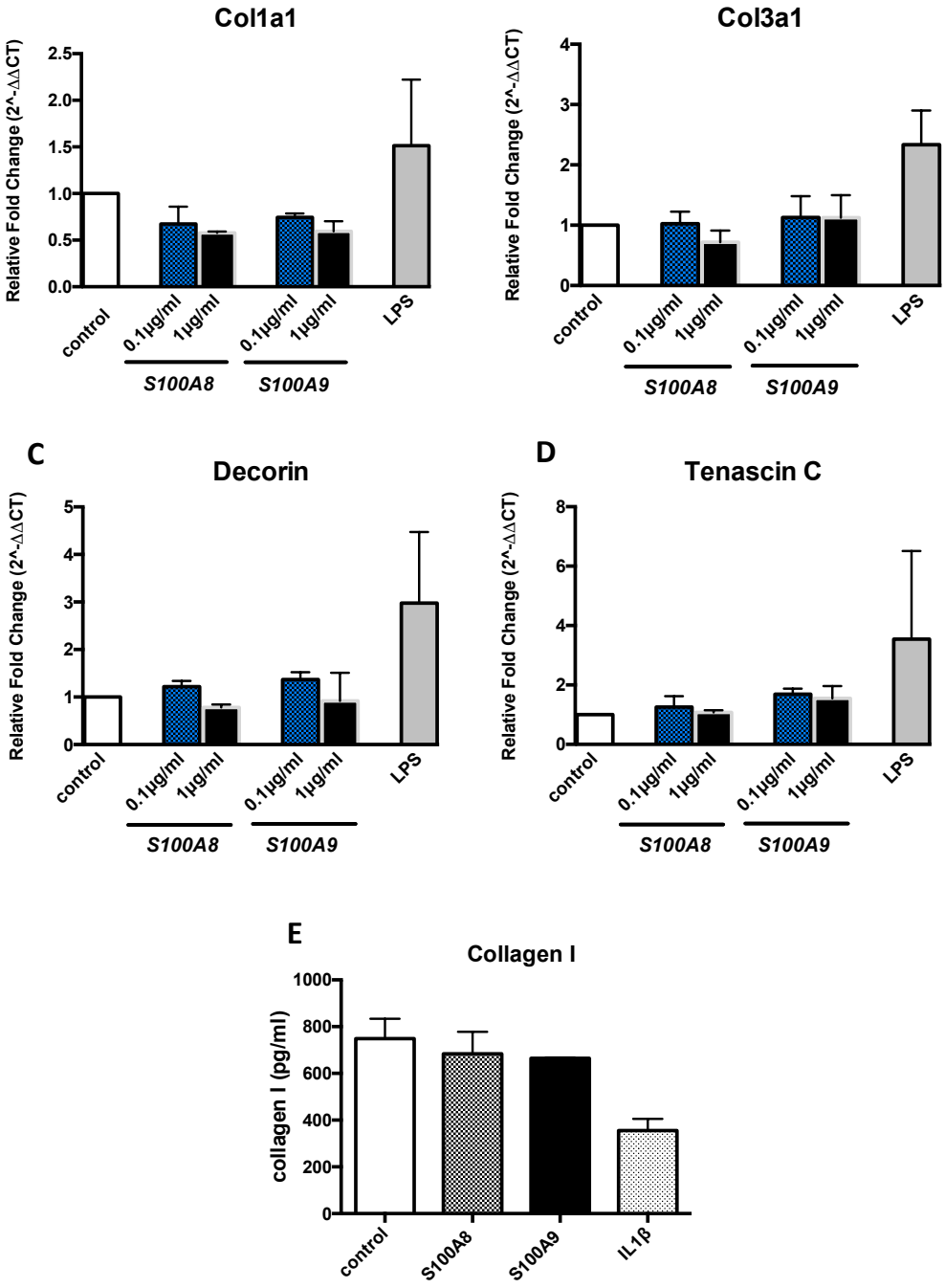

Figure 3.5 S100A8 and S100A9 do not directly alter matrix proteins *in vitro*

(A) Col1a1, **(B)** Col3a1, **(C)** Decorin, **(D)** Tenascin C expression in tenocytes stimulated 0.1 or 1µg/ml recombinant S100A8, 0.1 or 1µg/ml recombinant S100A9 or 1ng/ml LPS for 24 hours was determined by real time PCR. **(E)** Collagen protein release measured by ELISA from tenocytes stimulated with 1µg/ml recombinant S100A9, S100A8 or 1ng/ml LPS. Data represent mean ± SEM of duplicate samples expressed as relative fold change normalised to control (unstimulated) samples, n= 3.

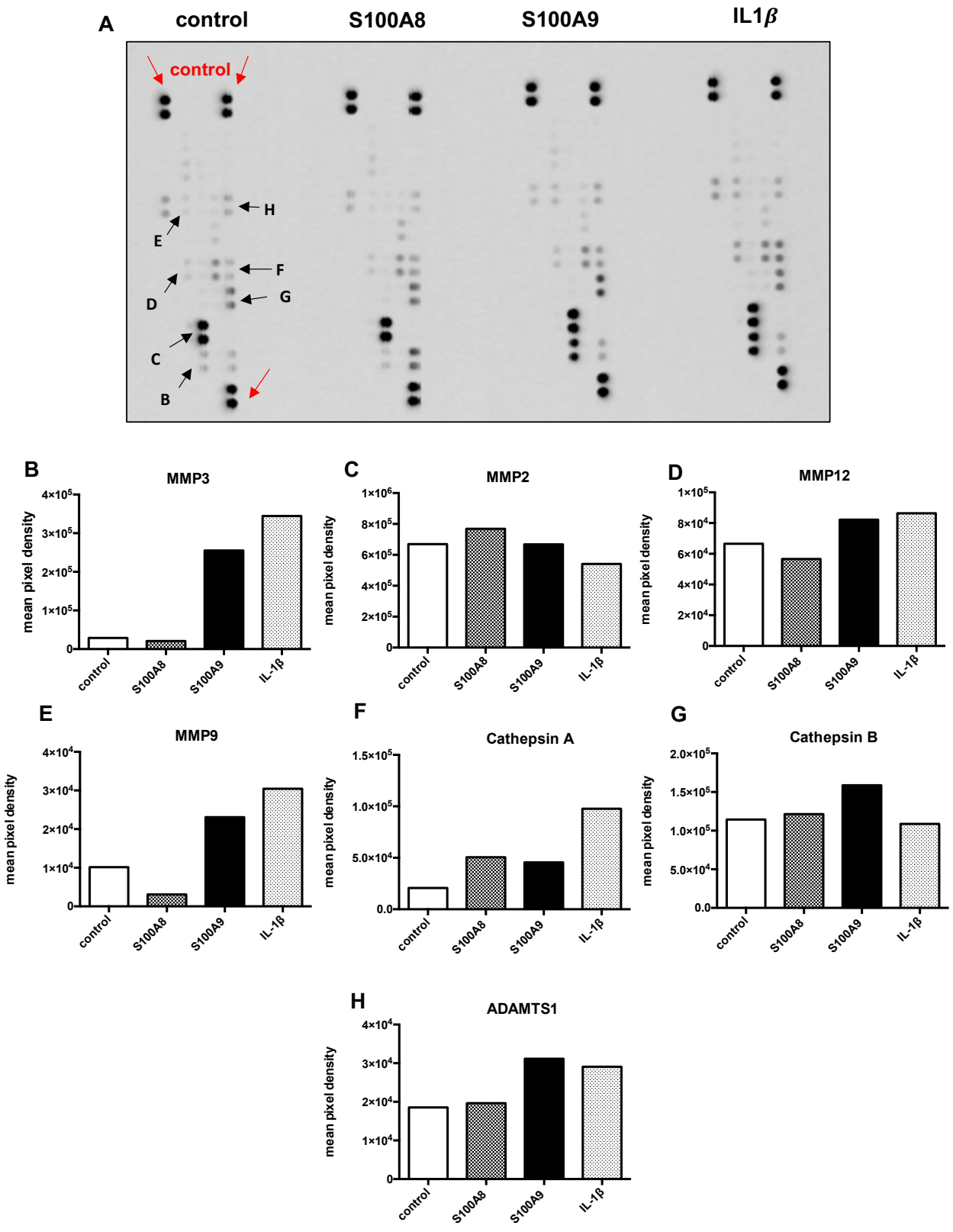

Figure 3.6 Single sample protease array using pooled supernatants (n=3) from tenocytes stimulated with 1µg/ml S100A8, 1µg/ml S100A9 or 10ng/ml IL-1β for 24 hours with unstimulated control. **(B-H)** Signal pixel density of proteases plotted relative to control. Statistical analysis was not performed.

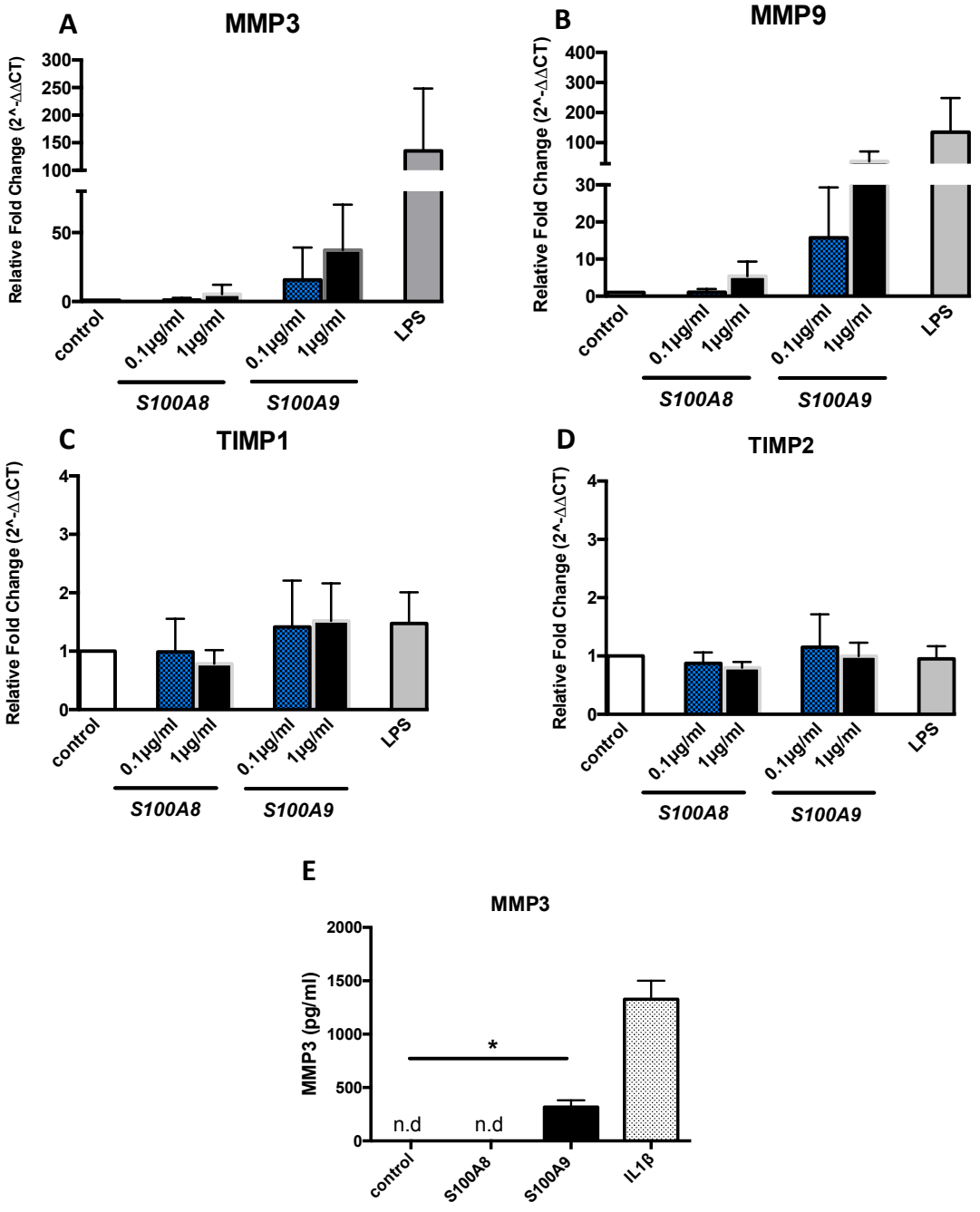

Figure 3.7 The effect of S100 proteins on MMPs and TIMPs in tenocytes

(A) MMP3, **(B)** MMP9, **(C)** TIMP1, **(D)** TIMP2 expression in tenocytes stimulated 0.1 or 1µg/ml recombinant S100A8, 0.1 or 1µg/ml recombinant S100A9 or 1ng/ml LPS for 24 hours was determined by real time PCR. Data represent mean ± SEM of duplicate samples expressed as relative fold change normalised to control (unstimulated) samples, n= 3. **(E)** Concentration of MMP3 in cell culture supernatants (expressed as pg/ml) from tenocytes stimulated with 1ug/ml S100A8, 1ug/ml S100A9 or 10ng/ml IL-1β for 24 hours with unstimulated control. Data represent mean ± SEM, n=3, * $p<0.05$, (paired *t*-test).

3.3.4 Assessing the effect of S100A8 and S100A9 on the inflammatory microenvironment in tendinopathy

We next explored the extent to which S100 proteins may influence the inflammatory microenvironment within the tendon post-injury. Recombinant S100A9 at a concentration of 1ug/ml significantly increased release of IL-6 ($p<0.01$), IL-8 ($p<0.05$) and CCL20 ($p<0.01$) from tenocytes (**Fig 3.8A, B & D**). Furthermore, S100A9 stimulation induced a 40 fold increase in CXCL10 (**Fig 3.8C**) expression compared to control (not detected). S100A8 stimulation displayed the same trend of significant IL-8 ($p<0.05$) and CCL2 ($p<0.001$) release from tenocytes; however, increases in IL-6 and CCL20 release were not significant following 24 hours' stimulation (**Fig. 3.8A-E**). Taken together, these data suggest S100A8 and S100A9 play a role in the activation of resident tenocytes and initiate a cascade of inflammatory processes.

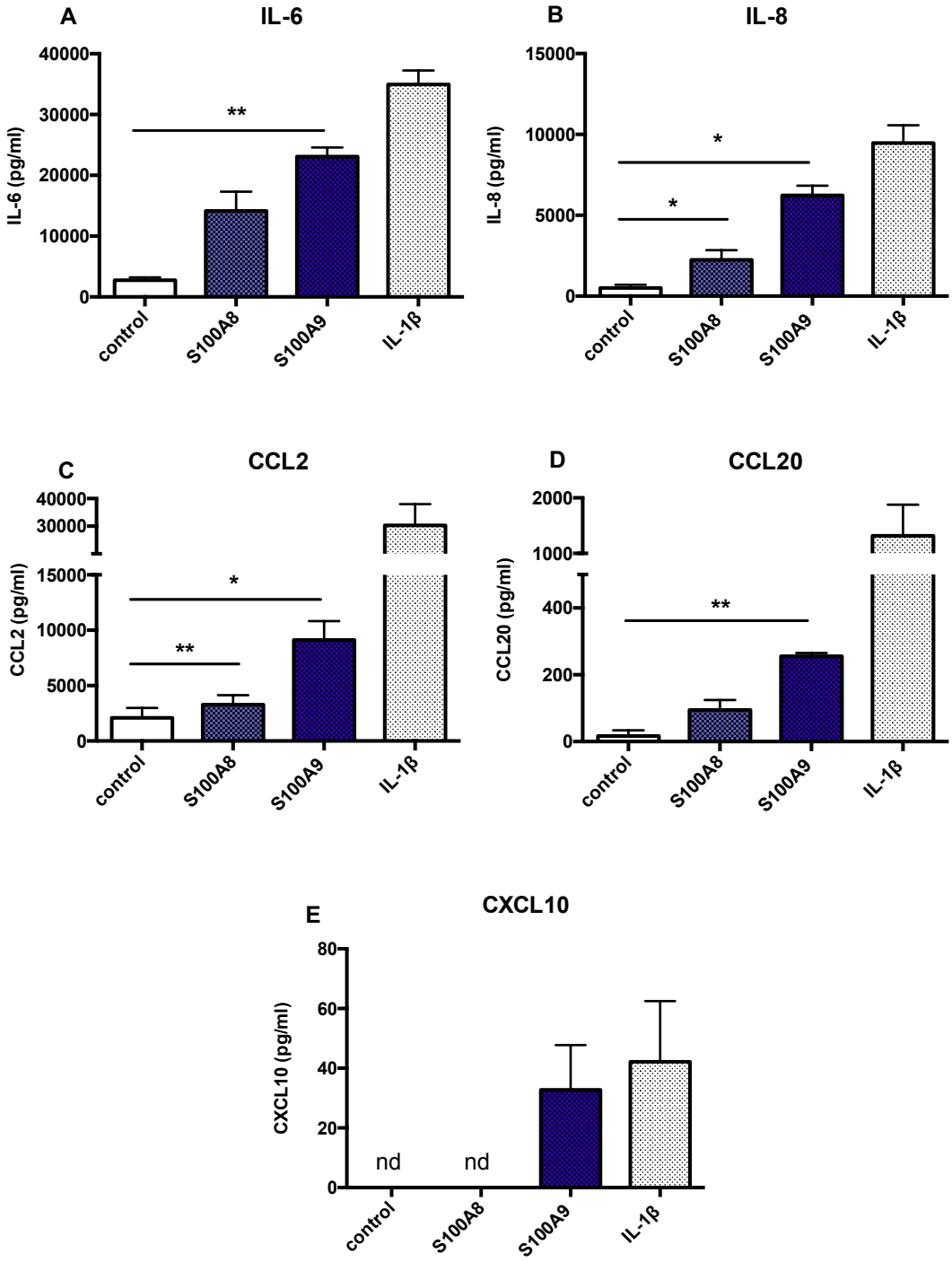

Figure 3.8 S100A8 and S100A9 induce cytokine and chemokine release from human tenocytes
Tenocytes stimulated with 1ug/ml S100A8, 1ug/ml S100A9 or 10ng/ml IL-1β .Concentrations of IL-6 **(A)** IL-8 **(B)** CCL2 **(C)** CCL20 **(D)** or CXCL10 **(E)** in cell culture supernatants measured following 24 hours stimulation. Values expressed as pg/ml. All data represent mean ± SEM, n= 3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to control (unstimulated) samples **(**Paired *t*-test **)**

3.4 Discussion and Conclusions

This chapter has established the presence of S100A8 and S100A9 in a human model of tendinopathy, most notably in the early stages of disease. Coupled with confirmation of passive release of CCL2 in response to acute injury our data supports the concept of S100A8 & A9 acting as functional tissue alarmins in tendinopathy by promoting immune cell recruitment. We have identified that S100A8 & A9 play an important immunomodulatory role in tendinopathy through activation of the innate immune system and manipulation of the stromal microenvironment.

Increasingly, alarmins are becoming recognized as key regulators in musculoskeletal pathologies.[400,124] S100A8 and S100A9 are considered surrogate markers of disease activity in RA where serum concentrations directly reflect levels of active inflammation.[409,150] In addition, S100A8 & A9 promote recruitment of inflammatory monocytes to the synovium in a murine model of osteoarthritis and blockade of S100 signalling ameliorates inflammatory processes.[410] Previous investigations in human models of tendinopathy have consistently established the presence of alarmins in diseased tendon and revealed functional roles *in vitro*.[197,162,163] Most recently we have demonstrated the alarmin HMGB1 regulates expression of inflammatory cytokines and matrix changes in tenocytes in a TLR4 dependent manner.[162] Observations taken from the present study indicate S100A8 and S100A9 regulate expression of inflammatory mediators *in vitro* which corroborates previous findings and suggests S100A8 & A9 are likely acting through DAMP receptors to influence downstream transcription and release of CCL2, CCL20, CXCL10, IL-6 and IL-8.

Hallmark features of tendinopathy include dysregulated collagen synthesis with a detrimental transition from type 1 to an inherently weaker type 3.[125] In addition, ECM turnover is regulated by non-structural matricellular proteins such as decorin and tenascin C that are thought to be upregulated under inflammatory conditions and in response to mechanical strain.[411] Interestingly, the present study did not find any changes in collagen, decorin or tenascin C in tenocytes in response to S100A8 & A9 stimulation. This may reflect differences in endogenous activity of various DAMPs. In addition to passive secretion from necrotic cells, under pathological conditions intracellular alarmins reserve the potential to be secreted

from activated immune cells. In contrast to the relatively slow translocation dependent secretion of nuclear HMGB1[165], active secretion of S100A8 & A9 from monocytes is rapid and energy dependent[412] suggesting they are inherently programmed to facilitate early inflammatory responses rather than directly alter extracellular matrix production.

Protease screening confirmed induction of MMPs in response to S100 stimulation. MMPs are generally considered to be fibrinolytic modulators of extracellular matrix turnover and have been associated with ongoing tissue damage and development of chronic disease.[413,414] Evidence suggests they are also involved in regulation of inflammatory processes including cytokine processing and activation[415,416] and leukocyte migration.[417] We did not observe any changes in other matrix proteins or tissue inhibitors of metalloproteinases (TIMP1 and TIMP2) therefore, in the context of tendinopathy, MMPs may regulate inflammation in addition to influencing matrix remodelling.

Recent evidence has revealed that stromal fibroblast activation markers are persistently upregulated in diseased tendon.[418] In response to recurrent injurious or inflammatory stimuli stromal cells are subject to phenotypic transformations that alter their functional properties; such adaptations reflect the activation state of the cell population. Typically, stromal cell activation is characterised by rapid induction of cytokines, chemokines and extracellular matrix components.[419] We observed an induction of CCL2, CCL20, CXCL10, IL-6 and IL-8 release from tenocytes in response to S100A8 & A9 stimulation suggesting their primary action may be activation of the resident tenocyte population. This, in turn, will promote immune cell recruitment and influence the nature of stromal microenvironmental cues.

The CCL2/CCR2 axis is primarily associated with the initial recruitment of classical inflammatory monocytes to sites of inflammation or tissue damage.[247,420] Within the tendon a portion of the recruited monocytes may continue development into macrophages following egress into the stromal microenvironment. It is thought classical monocytes recruited by CCL2 are programmed to differentiate more readily into inflammatory macrophages and may promote excessive inflammation.[421] Conversely, synergistic activities of IL-6 and CCL2 have been

shown to induce alternative activation of myeloid monocytes.[422] As such, the recruitment and development of monocytes and macrophages is highly niche specific and undifferentiated monocytes/macrophages retain the potential to differentiate according to their environment. Given the complex nature of the stromal microenvironment is it likely that both 'classical' inflammatory macrophages and 'alternatively activated' macrophages exist as a dichotomy that drives a state of chronic inflammation.

Based on our observations using a previously explored *in vitro* injury model we propose that acute injury induces CCL2 mediated monocyte recruitment.[423] Here, soluble factors or biologically active ECM fragments induce subsequent release of S100A8 and S100A9 from monocytes that will, in turn, bind receptors on the tenocyte surface and stimulate release of further inflammatory factors (**Fig.3.9**). Within the tendon matrix monocytes continue development to mature macrophages and differentiate according to environmental cues. Histologically the presence of mononuclear cells is associated with a state of chronic inflammation.[424] Excessive immune cell infiltration and the presence of macrophages may contribute to persistence of inflammation and promote immune cell-matrix crosstalk that drives inflammatory healing characterised by aberrant and inferior matrix repair. It may be postulated in the context of this study that CCL2, CCL20 and CXCL10 mediate initial inflammatory monocyte recruitment while IL-6 and IL-8 participate as potent inflammatory factors in the acute phase response. This will subsequently act to promote a transitory state towards established chronic inflammation. In addition to monocyte recruitment these chemokines are capable of recruiting T cells, mast cells and natural killer cells that will likely contribute to the development of a complex and dynamic inflammatory milieu.

A recent study detailing the expression of alarmins in the fibrotic disorder adhesive capsulitis demonstrated S100A8/S100A9 expression is localised to immune cells; specifically, $CD68^{+}$ macrophages.[425] Our data describing S100A8 & A9 release from monocytes highlights the importance of activated immune cells as a source of alarmins in diseased tendon. Under inflammatory conditions S100A8 and S100A9 are known to regulate positive feedback mechanisms thus it is plausible tenocytes and activated monocytes/macrophages mutually amplify

levels of extracellular alarmins and promote phagocyte recruitment.[426,427] Furthermore, S100A8/S100A9 are involved in myeloid cell differentiation and induce secretion of pro-inflammatory cytokines including TNF- α and IL-1ß from monocytes[139]; it is likely these mediators will also act to exaggerate and sustain inflammatory conditions.

Our data confirms the presence of S100A8 and S100A9 in tendinopathy and suggests they actively contribute to pathological proceedings in the early stages of disease. We propose that by modulating the stromal microenvironment S100A8 and S100A9 promote recruitment of inflammatory cells to the site of injury and support a detrimental transition from acute to chronic inflammation.

Selectively targeting DAMP signalling in early disease provides scope for novel translational strategies in the management of tendon disorders.

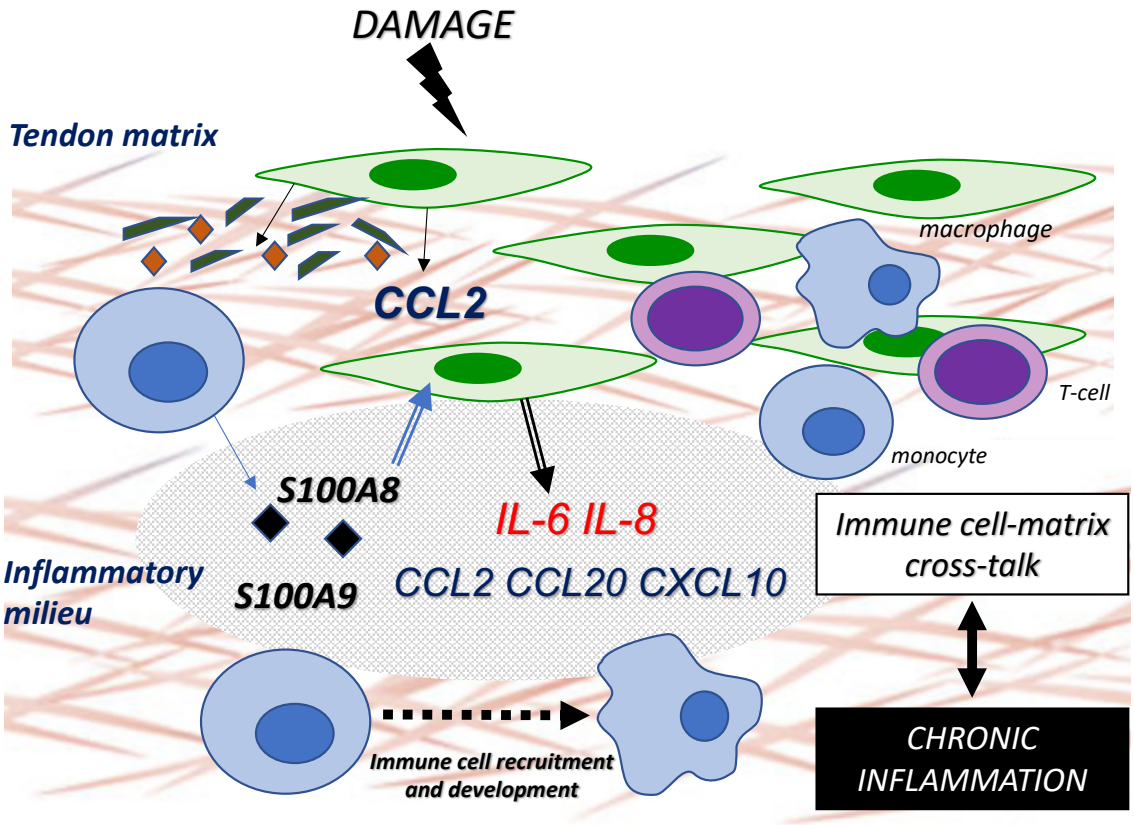

Figure 3.9 S100A8 and S100A9 promote immune cell recruitment and development in tendinopathy

Proposed mechanism whereby damage induces release of CCL2 from tenocytes recruiting monocytes to the site of injury. Soluble factors stimulate release of S100A8 and S100A9 from monocytes recruited to the tendon matrix. S100A8 and S100A9 bind receptors on the tenocyte surface to induce release of cytokines and chemokines promoting further recruitment and development of immune cells. Formation of an inflammatory microenvironment facilitates immune cell-matrix cross talk and promotes a state of chronic inflammation.

Chapter 4: Phenotyping of tendon stromal cells

4.1 Introduction

Historically, the stroma was considered to be immunologically inert and exist purely as a tissue specific scaffold that participates in wound healing responses.[24] Until recently, tenocytes were perceived as a biochemically active cell population merely responding to biomechanical force. Excessive loading of tendons was considered the main pathological stimulus for degeneration, conferred by the mechanosensing properties of tenocytes. It was deemed that the initial response to an injurious stimulus was recruitment and proliferation of tenocytes in addition to synthesis and release of ECM components (including matrix degrading proteins such as MMPs). [428,429]

This theory has subsequently been supplanted by increasing evidence of damage induced inflammation being crucial to disease initiation and progression.[111,29] More recently it has been postulated that recruitment, influx and retention of immune cells is controlled by cytokine and chemokine gradients created by resident stromal cells.[419] Fibroblasts are highly specialised to the tissue in which they reside and vary phenotypically according to their anatomical location or so called 'stromal address code'.[430] It is thought stromal cell properties are defined according to tissue structure at an individual site and the external stimuli they receive. For example, tenocytes are interspersed uniformly throughout the tendon matrix and are adapted to respond to mechanical force and DAMPs through pattern recognition receptors.[162] Recent studies have suggested that repeated exposure to pathological stimuli induce epigenetic changes that drive a persistent state of activation.[431,432] This is characterised by phenotypic alterations including constitutive expression of cell surface receptors termed 'fibroblast activation markers'.[418]

Traditionally, fibroblasts were perceived as a homogenous population with a relatively limited functional capacity. However, emerging evidence suggests that fibroblasts display a degree of plasticity and can exist as functionally distinct subsets analogous to subsets of leukocytes.[433] Recent studies in cancer immunobiology and autoimmune disease have proposed that phenotypically distinct stromal cell populations drive pathology.[434,315,435,319] Single cell sequencing of rheumatoid synovium has described distinct subsets of fibroblasts that are expanded in RA. It is proposed these fibroblasts (characterised by varying

proinflammatory cytokine expression phenotypes) may be pathogenic based on their roles in matrix invasion, immune cell recruitment and osteoclastogenesis.[315]

In addition to extensive characterization of fibroblasts in RA synovium, recent studies in adhesive capsulitis have identified the expression of stromal cell surface markers *in vitro* and *ex vivo*.[436] In order to explore the concept of tenocyte heterogeneity in tendinopathy the aims of this chapter are:

1. To characterise the expression of disease relevant surface markers expressed in whole tendon tissue and tenocytes

2. Profile fibroblast subsets in fresh human tendon tissue (healthy and diseased)

4.2 Results

4.2.1 Differential gene expression in normal and diseased tendon

The heatmaps in Figure 4.1 show preliminary datasets from our lab group generated from human BeadChip Array which allows high throughput differential gene expression analysis. Figure 4.1A illustrates a heatmap generated from a normalized expression matrix that depicts differences between groups. 5 control (healthy subscapularis tendon) and 5 torn supraspinatus tendons (representative of late tendinopathy) were used. These are further subdivided into 4 clusters based on differences in gene expression between control and tendinopathic groups. Clusters 1 and 3 show upregulated genes and clusters 2 and 4 show genes that are downregulated in tendinopathy. Based on recently published datasets we observed several genes of interest that were upregulated between control and torn tendons. Cluster 1 (**Fig 4.1B & Table 4.1**) shows increased expression of CD90 (Thy1), podoplanin (PDPN) and cadherin 11 (CAD11) which have recently been identified as key markers of fibroblast activation and heterogeneity in RA.[437,315] **Table 4.1** summarises key matrix associated genes upregulated in supraspinatus samples.

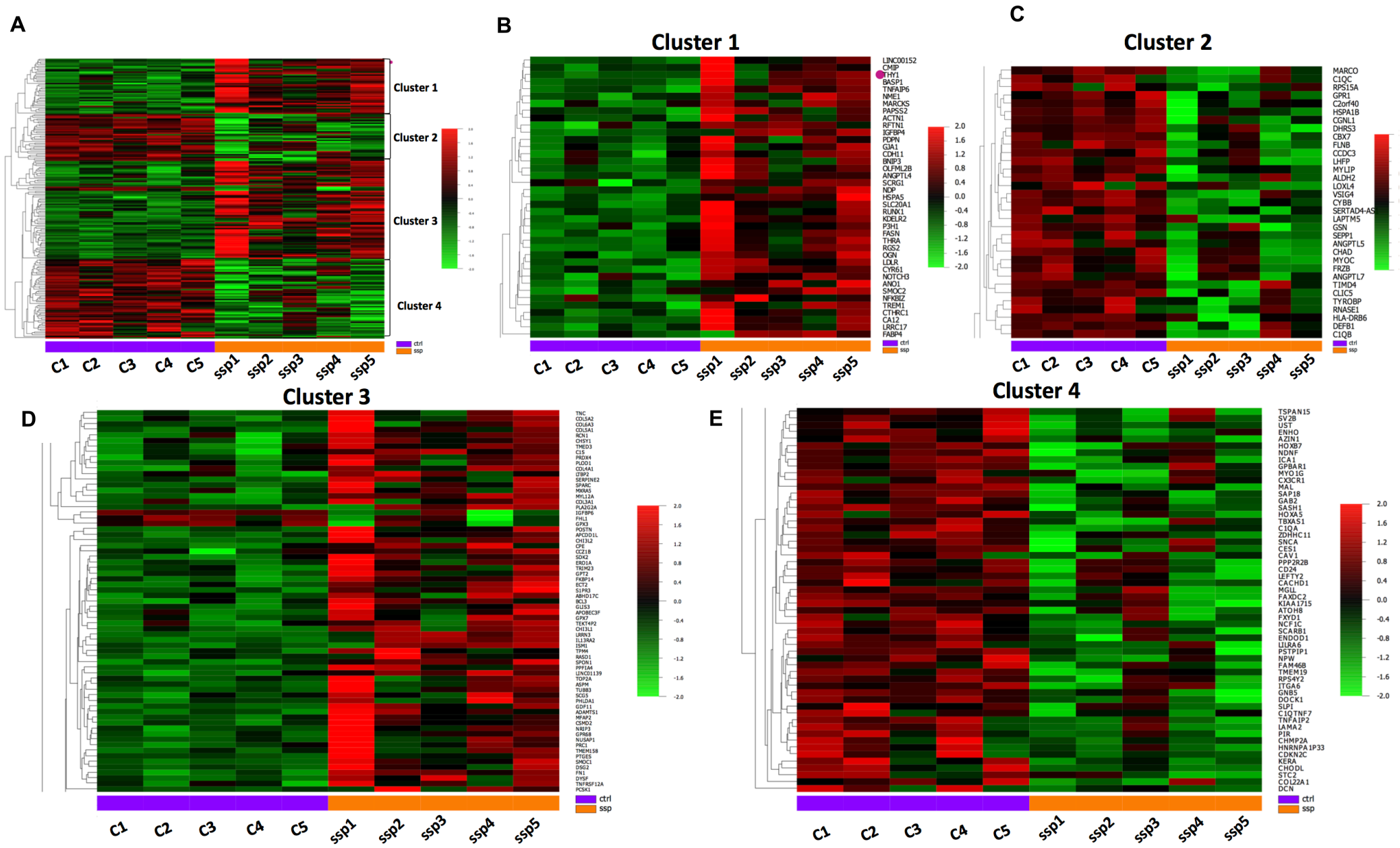

Figure 4.1 Heatmap of differential gene expression in normal and tendinopathic tendon

Euclidian clustering analysis of differential gene expression ($p<0.05$) performed using GenomeStudio. C1-C5 represent control subscapularis tendon, n=5. ssp 1-5 represent torn supraspinatus tendon representative of late pathology, n=5.

Cluster	Gene upregulated in tendinopathy
Cluster 1 *(fibroblast markers)*	○ Thy-1 (CD90) ○ PDPN ○ CDH11 (cadherin 11)
Cluster 3 *(matrix associated genes)*	• TNC (tenascin C) • COL3A1 • COL5A1 • COL5A2 • COL6A3 • SPARC (osteonectin) • POSTN (periostin) • ADAMTS1 (metalloproteinase)

Table 4.1 Summary of matrix associated genes upregulated in tendinopathy
Matrix associated genes identified as differentially expressed by clustering analysis (Fig. 4.1, control vs late tendinopathy).

4.2.2 Stromal surface marker expression is increased in tendinopathy

Having established a pattern of upregulation of various fibroblast surface markers in tendinopathy we sought to further characterize this expression using a panel of candidate markers based on results from gene expression studies and current literature. Immunohistochemical staining of tendinopathic sections showed strong positive staining of CD34 which appears to be localised to blood vessels indicating the presence of this marker in the intimal and subintimal layers (**Fig. 4.2A**). A similar staining pattern was observed for CD146 (**Fig. 4.2D**) which is in accordance with literature indicating CD34 and CD146 are lineage markers of endothelial cells. In addition to the presence of vascular staining, Figure 2B, 2C, and 2E show diffuse staining of podoplanin, CD248 and CD90, respectively with this staining pattern likely corresponding to expression in tendon stromal cells. CD10 expression (**Fig. 4.2F**) was observed around vessels, in the stroma and also in infiltrating immune cells. As positive staining was observed in tendinopathic tissue sections we sought to confirm this at a molecular level using a more extensive panel. RT-PCR of whole tendon tissue showed a significant increase in podoplanin and VCAM1 expression between control and late tendinopathy (**Fig. 4.3A & B**, $p<0.01$). Significant upregulation of CD44 and FAP expression was observed between control and both early and late pathology groups (**Fig. 4.3C & F**). Less consistent increases in expression of CD90 (**Fig. 4.3D**), CD248 and CD146 were observed between control and late tendinopathy. Similarly, CD34 (**Fig. 4.3E**), CD29 (**Fig. 4.3G**) and CD47 (**Fig. 4.3J**) showed a trend towards increased expression in both early and late tendinopathy; however, this was not significant in all analyses. Interestingly, a reduction of CD10 expression was observed between control and early/late tendinopathy groups.

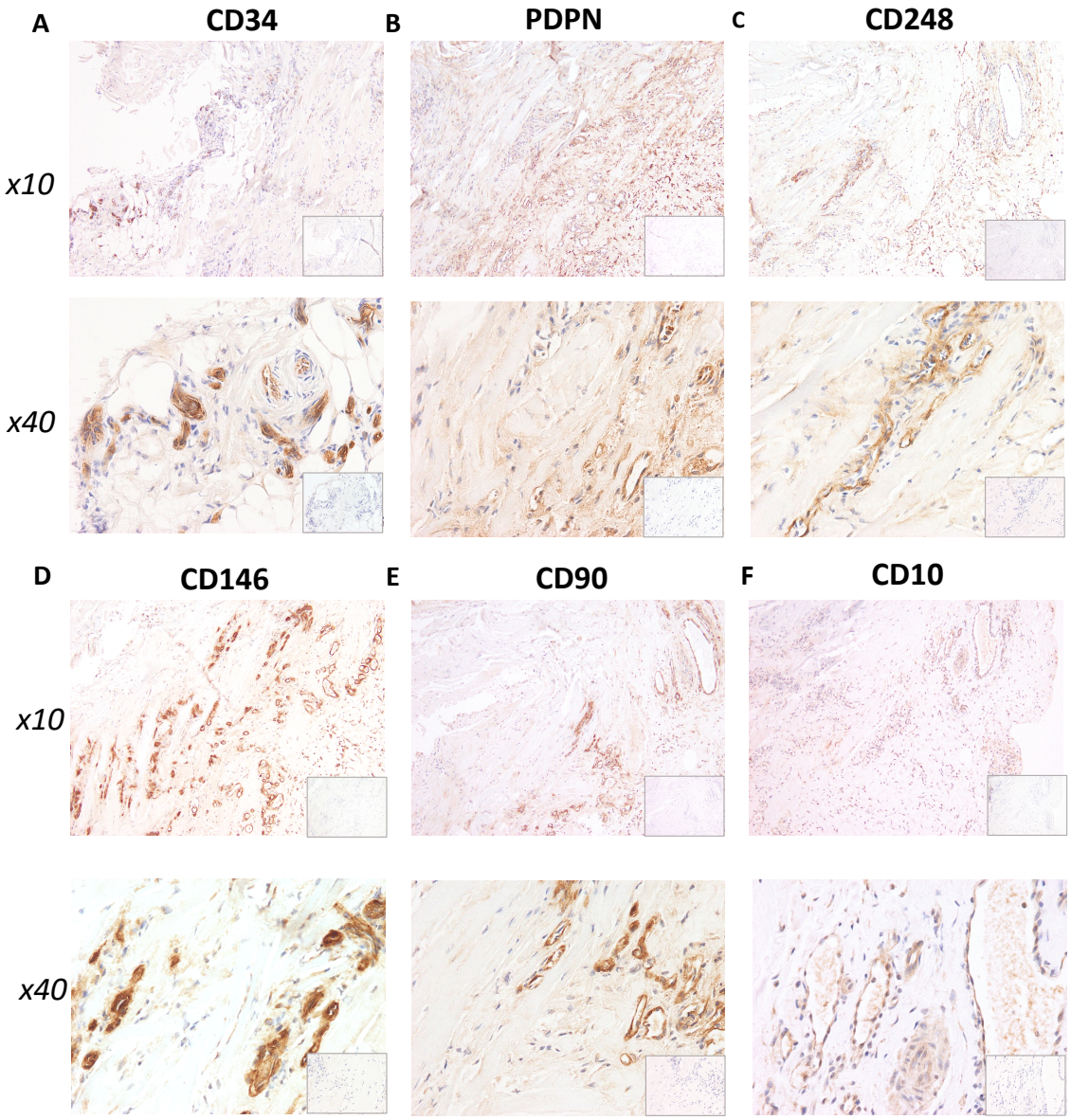

Figure 4.2 Immunostaining of surface markers in tendinopathic tissue
Supraspinatus tendon sections with stained with antibodies directed against **(A)** CD34 **(B)** PDPN **(C)** CD248 **(D)** CD146 **(E)** CD90 **(F)** CD10. All images shown at 10x and 40x magnification. Isotype controls shown in bottom right corner.

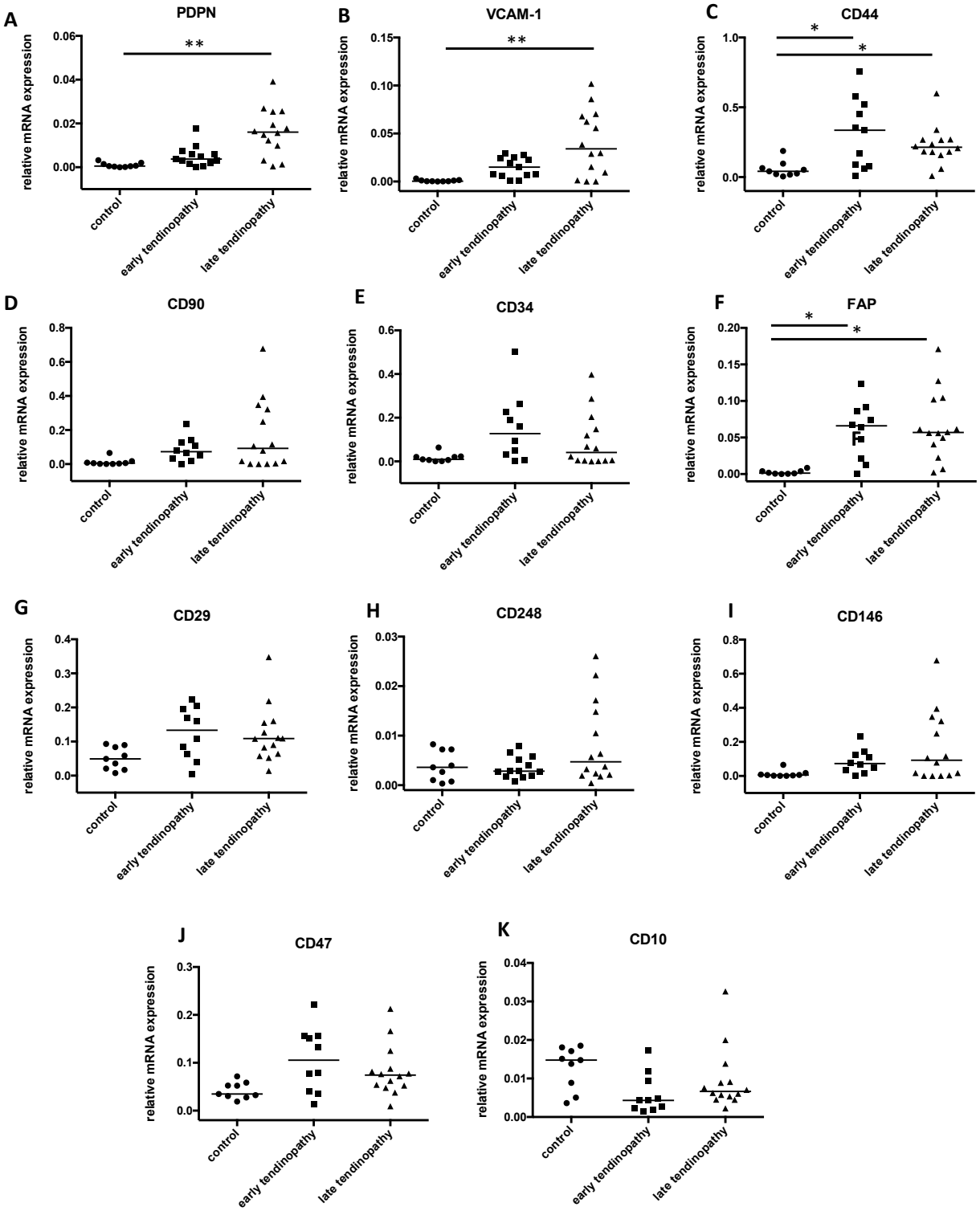

Figure 4.3 Stromal cell surface marker gene expression in tendinopathic tissue
(A-K) Relative mRNA expression (2^-**Δ**CT) of surface marker expression in control (hamstring tendon, n=9), early tendinopathy (intact subscapularis biopsy, n=10) and late tendinopathy (torn supraspinatus tendon, n=14). Data represent mean ± SEM relative to housekeeping gene GAPDH (mean of duplicate analysis). * p<0.05, **p<0.01, (Kruskal-Wallis test) versus control.

4.2.3 Inflammatory mediators increase surface marker expression in tenocytes

Having observed upregulation of various markers in whole tendinopathic tissue we next sought to characterize their expression profile at a cellular level. As staining was observed in various areas of the tissue section we sought to confirm the expression of these markers in human tenocytes and examine their response to inflammatory stimuli *in vitro to* mimic the tendinopathic environment. Upon stimulation with LPS (1ng/ml) a significant induction of VCAM1 expression (**Fig. 4.4B**, $p<0.05$) was observed and a 20-fold increase in expression was noted in response to IL1β stimulation (10ng/ml). Podoplanin (**Fig. 4.4A**) and CD34 (**Fig. 4.4E**) expression was also increased in response to both IL1β and LPS; however, this was not statistically significant.

4.2.4 Characterising surface marker expression in normal and diseased whole tendon tissue

In order to fully characterize surface marker expression in human tendon we next performed screening of 11 markers known to be expressed in stromal cells using flow cytometric analysis of disaggregated tendon tissue. Due to the large number of markers and availability of fluorophores these were divided into two panels for phenotyping studies.

4.2.4.1 Panel 1

In both normal and tendinopathic tendons all markers were found to be positive (to a varying degrees) between patients and the normal and tendinopathic groups (**Fig. 4.5B & C**). A significant increase in surface expression of CD44 (**Fig. 4.5D**, $p<0.01$), podoplanin (**Fig. 4.5E**, $p<0.05$) and CD90 (**Fig. 4.5F**, $p<0.05$) was observed between normal and tendinopathic samples. There did not appear to be any discernible change in cadherin 11 (**Fig. 4.5G**) or CD146 (**Fig. 4.5H**) surface expression. Interestingly, CD34 (**Fig.4.5I**) showed a greater spread of percentage positive cells and the expression pattern appears to be divided into highly and lowly expressed groups.

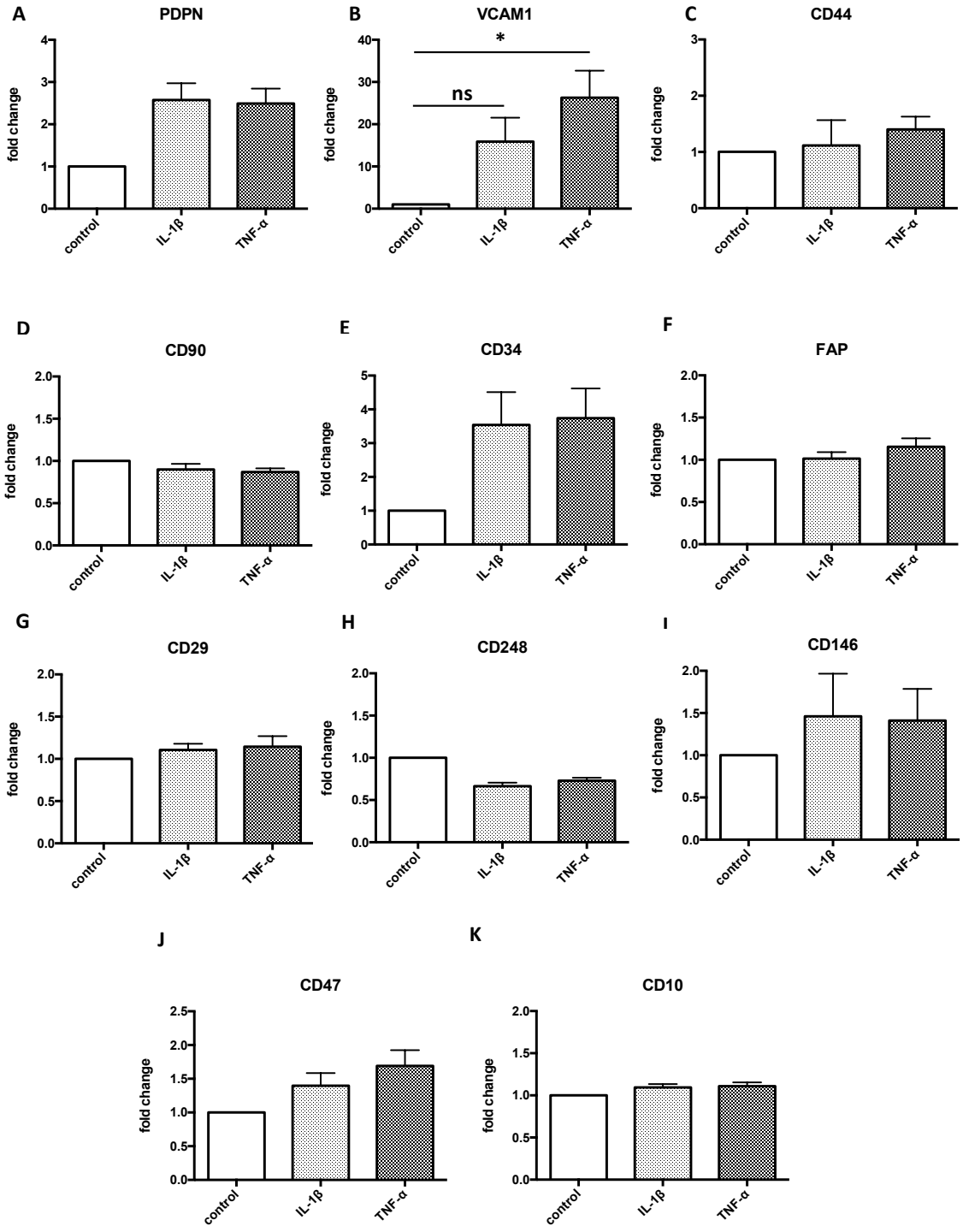

Figure 4. 4 The effect of inflammatory stimuli on surface marker expression in tenocytes
(A-K) Fold change in mRNA ($2^{-\Delta\Delta}CT$) of surface marker expression in tenocytes stimulated with 10ng/ml IL-1β (n=3) or 1ng/ml LPS (n=3) for 24 hours. Data represent mean ± SEM relative to unstimulated control (mean of duplicate analysis). * $p<0.05$ (Friedman test) versus control.

We next sought to identify any consistent co-expression profiles of cells within the tendon tissue samples based on comprehensive analysis of flow cytometry data. Subpopulations based on CD34 and CD146 expression were identified in both normal and tendinopathic samples (**Fig. 4.6A-E**). The highest percentage of singlets was observed for the $CD146^{-}CD34^{+}$ population; accounting for approximately half of all cells. A discreet population of double positive $CD146^{+}CD34^{+}$ cells was also observed and distribution of this population appears to be consistent between normal and tendinopathic donors.[425]

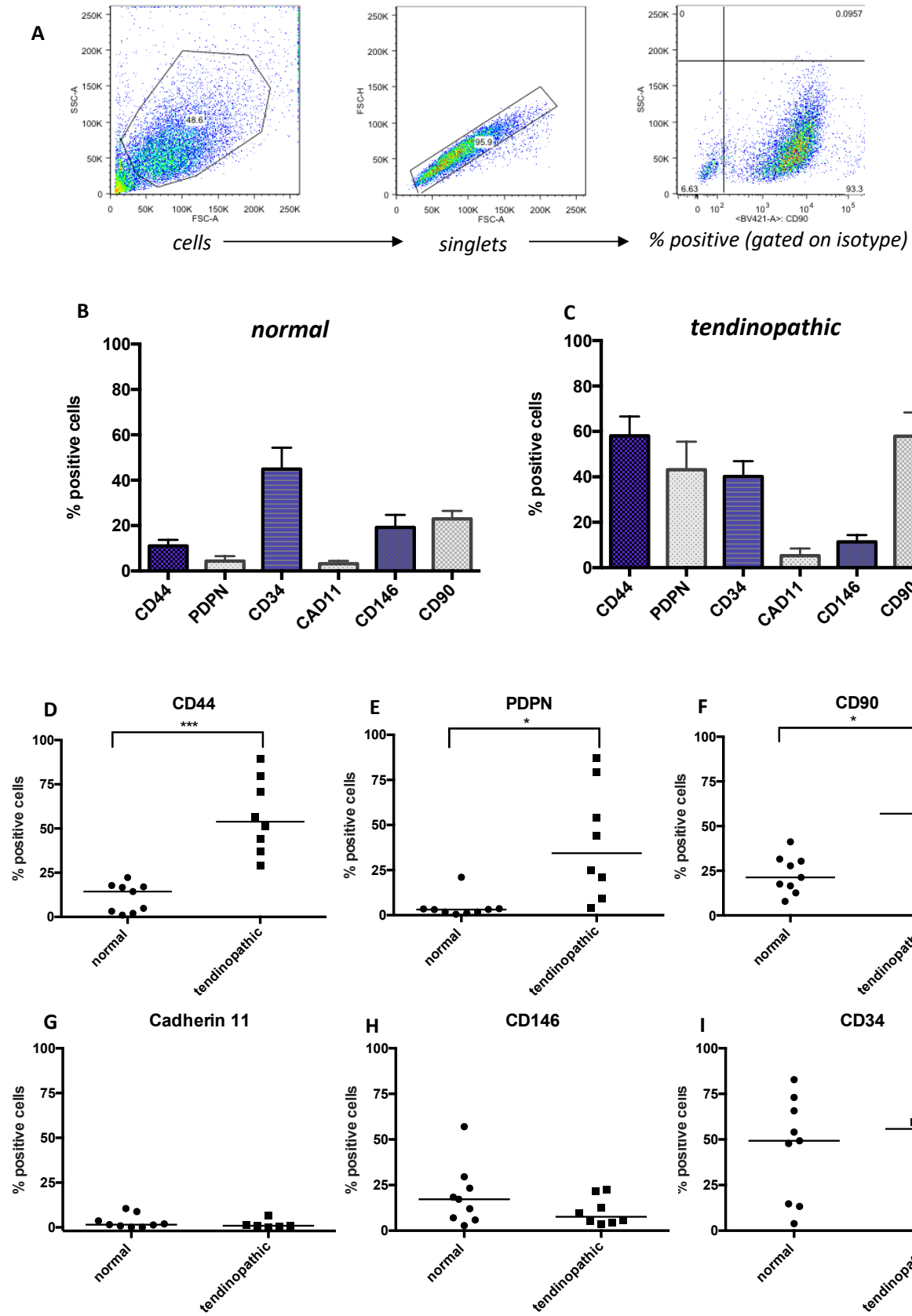

Figure 4.5 Panel 1 surface marker expression in normal and tendinopathic tendon
(A) Gating strategy for quantification of surface marker expression in disaggregated tendon tissue **(B)** & **(C)** Percentatge of cells positive for CD44, PDPN, CD34, cadherin 11, CD146 and CD90. Data expressed as mean ±SEM. **(D)** Percentage of cells positive for CD44 **(E)** podoplanin **(F)** CD90 **(G)** cadherin 11 **(H)** CD146 in normal hamstring (n=9) or torn supraspinatus tendon (n=8). Data expressed a median, * p<0.05, **p<0.01, (paired t-test).

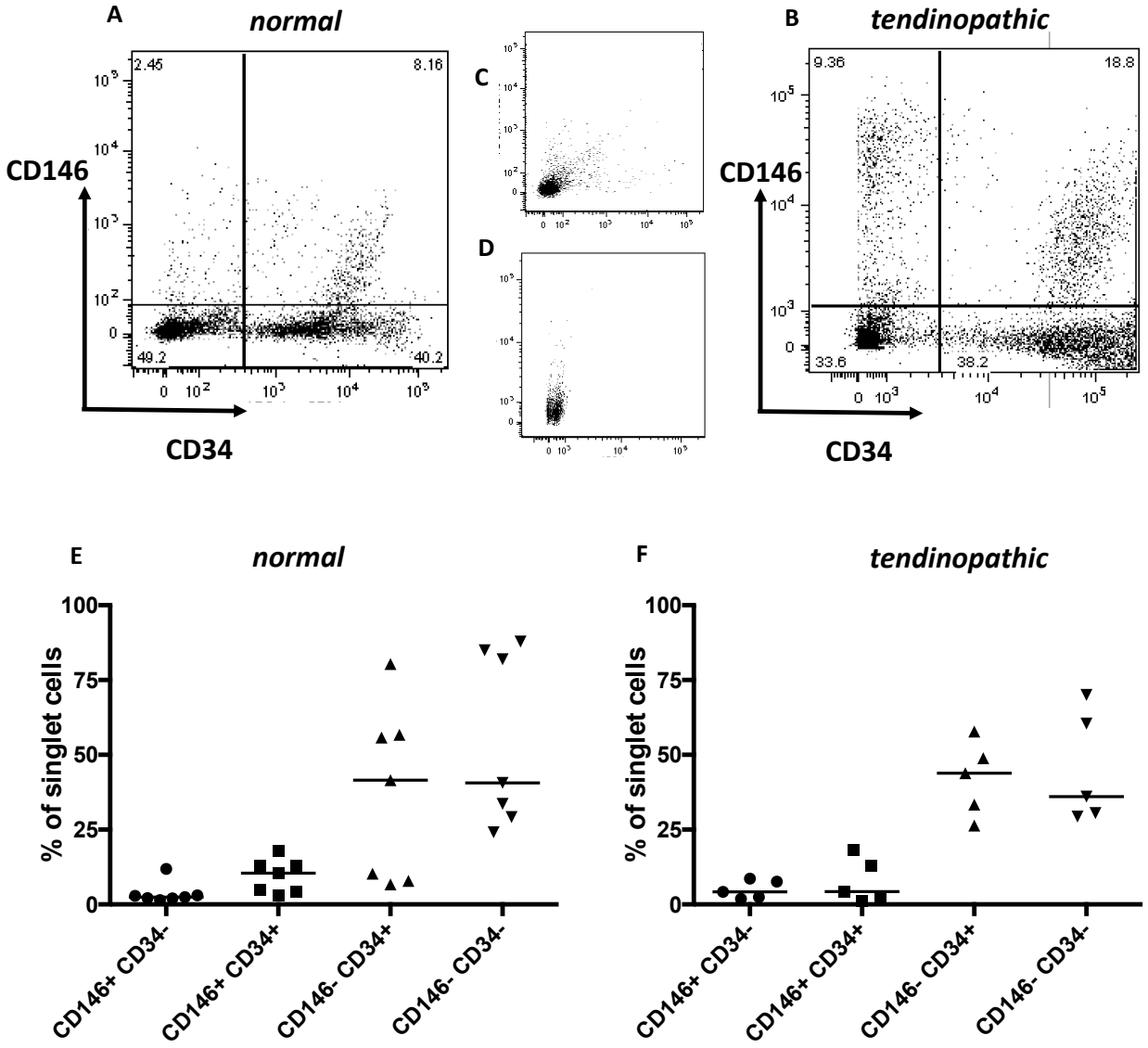

Figure 4.6 Panel 1 surface marker expression in normal and tendinopathic tendon
Representative pseudocolour plot of populations gated on CD34 and CD146 expression in **(A)** normal hamstring and **(B)** torn supraspinatus tendon. Negatives gated on isotype controls **(C & D)**.

4.2.4.2 Panel 2

As with Panel 1, all surface markers were found to exhibit varying degrees of expression in normal and tendinopathic tendons (**Fig. 4.7B & C**). Median CD47 surface expression was the highest of all markers tested with the median value largely unchanged between normal and tendinopathic groups (**Fig. 4.7D**). Similarly, CD10 expression did not appear to vary between groups (**Fig. 7E**). Although not significant, a notable increase in CD29 expression (**Fig. 4.7F**) was observed between control and tendinopathic samples. CD81 (**Fig. 4.7G**) and CD91 (**Fig.4.7H**) showed the least percentage of positive cells with no expression in some donors in the control group and low levels of expression in tendinopathic samples.

Distinct cell populations were observed when gated on CD10 and CD29 (**Fig. 4.8A-E**) with a prominent $CD10^{-}CD29^{+}$ population present in both healthy and diseased groups. Quantitative analysis showed this population was consistently elevated in the tendinopathic group (**Fig. 4.8E**). Another small double positive ($CD29^{+}CD10^{+}$) population was consistently present in both groups. The total number of $CD29^{-}CD10^{+}$ cells was negligible and varied expression of $CD29^{-}CD10^{-}$ cells was observed in both normal and tendinopathic samples.

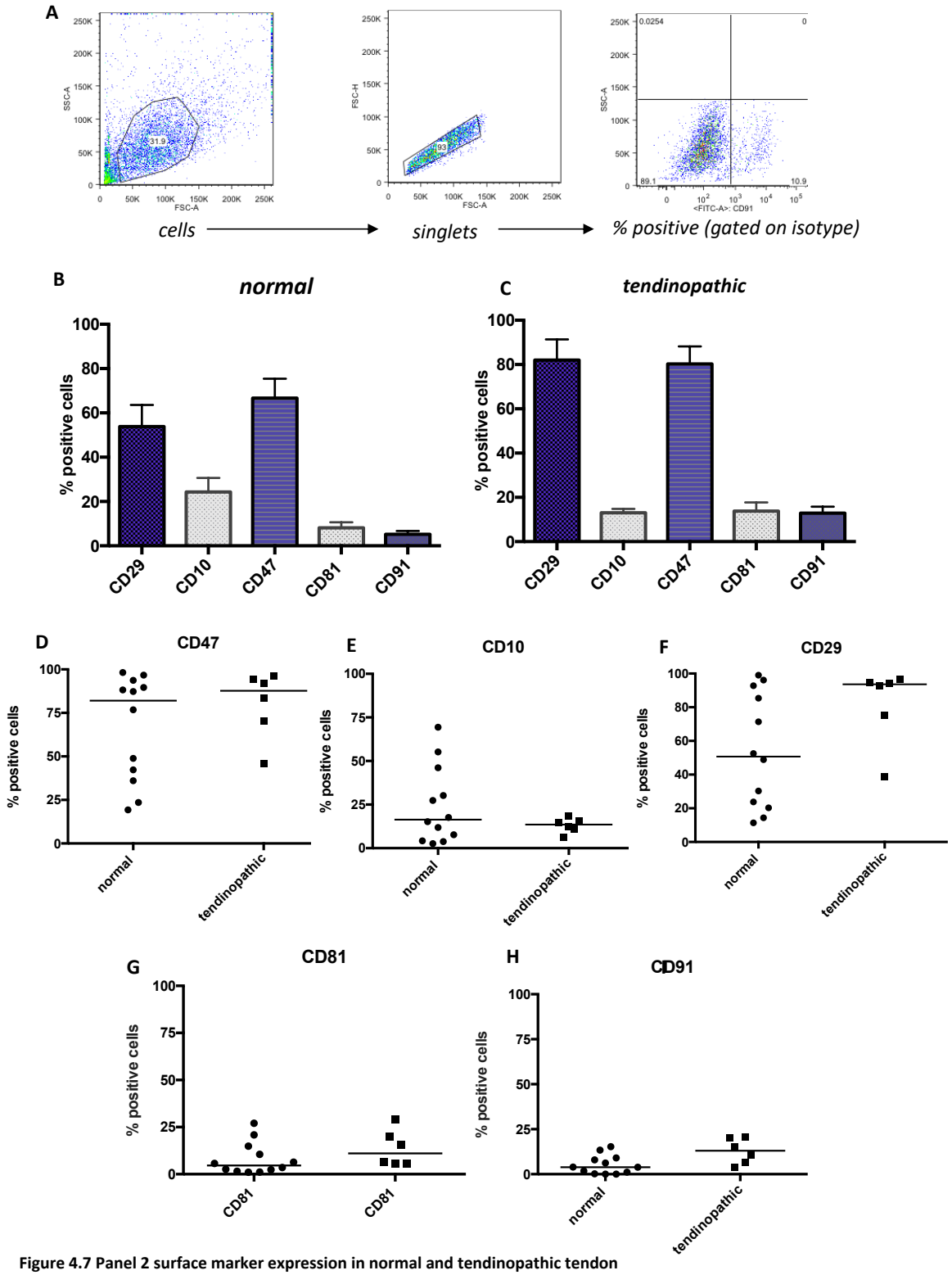

Figure 4.7 Panel 2 surface marker expression in normal and tendinopathic tendon
(A) Gating strategy for quantification of surface marker expression in disaggregated tendon tissue **(B)** & **(C)** Percentatge of cells positive for CD29, CD10, CD47, CD81 and CD91 in normal hamstring (n=12) or torn supraspinatus tendon (n=6). Data expressed as mean ±SEM. **(D)** Percentage of cells positive for CD29 **(E)** CD10 **(F)** CD47 **(G)** CD81 **(H)** CD91 in normal hamstring (n=12) or torn supraspinatus tendon (n=6). Data expressed a median, * p<0.05, **p<0.01, (paired t- test)

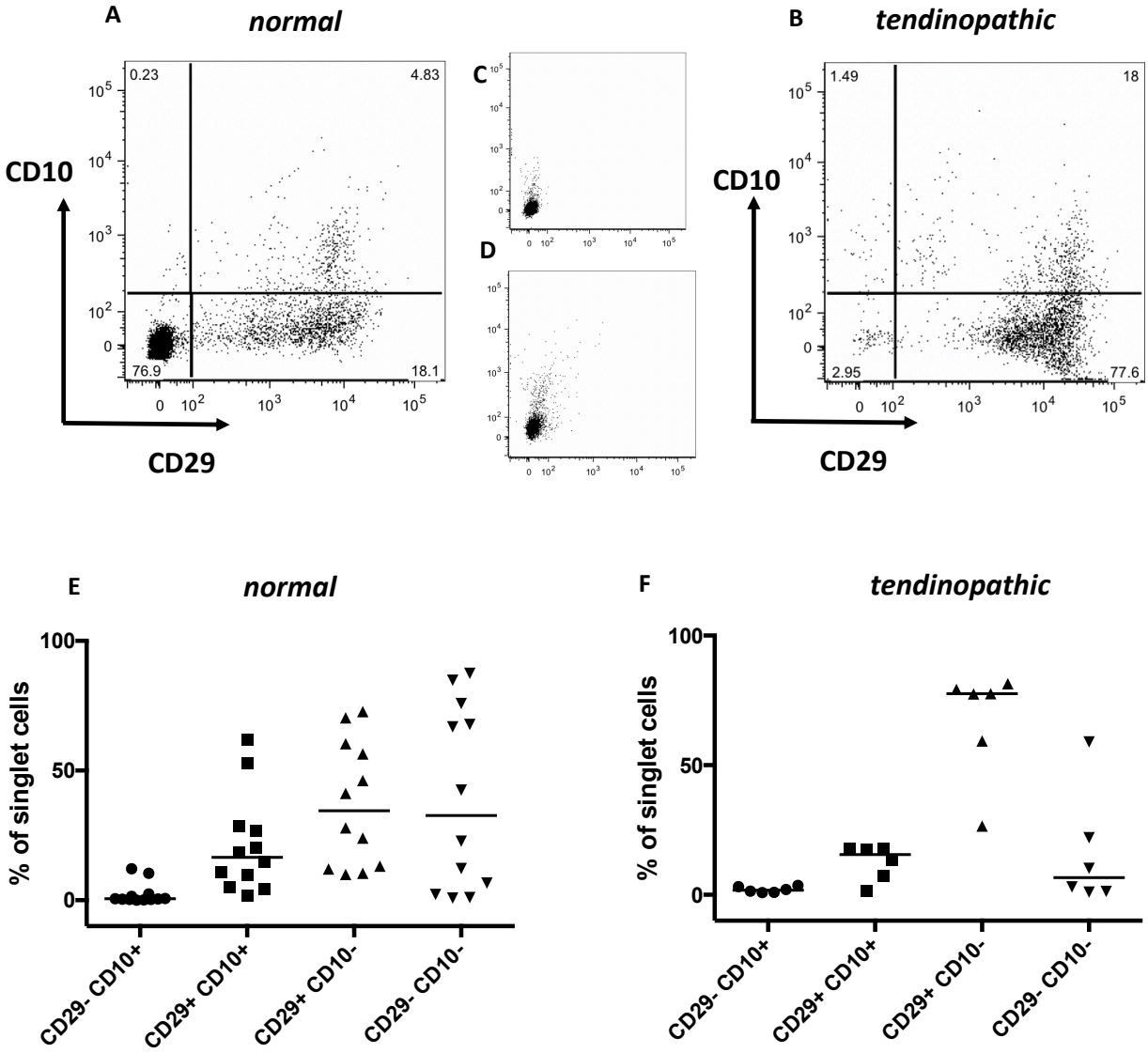

Figure 4.8 Panel 1 surface marker expression in normal and tendinopathic tendon
Representative pseudocolour plot of populations gated on CD29 and CD10 expression in **(A)** normal hamstring (n=12) and **(B)** torn supraspinatus tendon (n=6). Gating based on isotype controls **(C & D)**.

4.2.3 Characterising surface marker expression *in vitro*

As digested tendon tissue does not yield a homogenous population of cells we next sought to confirm the expression of these markers in human tenocytes *in vitro*. Positive expression of all 11 markers was observed (**Fig 4.9A & B**) with almost all tenocytes screened positive for CD44, CD90, CD29, CD10, CD47 and CD91 across 4 donors. Podoplanin and CD81 were also found to be highly expressed (approximately 60% of singlets positive). Less than half of all cells were positive for CD34, CD146 and cadherin 11 with these figures consistent between biological replicates.

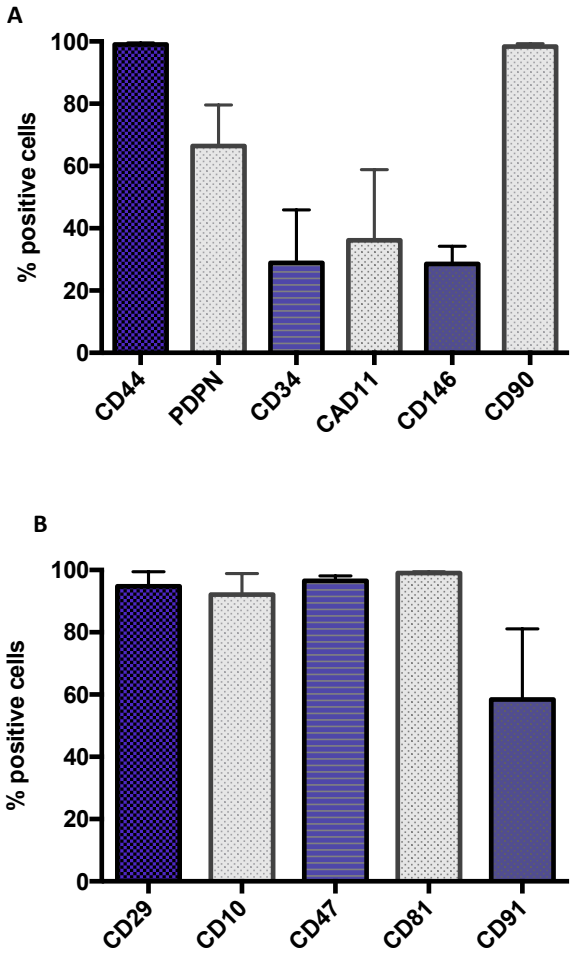

Figure 4.9 Surface marker expression in normal tenocytes
Percentage of cells positive for **(A)** CD44, podoplanin, CD34, cadherin 11, CD146 and CD90 **(B)** CD29, CD10, CD47, CD81 and CD91 in normal tenocytes (n=4). Data expressed as mean ±SEM.

4.2.4 Spanning-tree Progression Analysis of Density Normalised Events (SPADE) of tenocyte subpopulations

In order to gain a more comprehensive insight into the phenotype of tenocytes extracted from whole tendon tissue we next employed the use of bioinformatic tools that allow greater visualization of high dimensional data. SPADE is an algorithm that clusters phenotypically similar cells into hierarchy and allows multidimensional analysis of heterogenous samples. The algorithm was applied to flow cytometry data from four normal and four tendinopathic tissue digests.

Panel 1

Figure 4.10 and 4.11 show four individual SPADE trees representing data obtained from four normal and four tendinopathic tendons. Each node represents a cluster of cells (where size corresponds with the number of cells in each cluster). Gates or 'bubbles' were drawn around populations identified of interest. All markers from Panel 1 including CD44, CD146, CD34, podoplanin, CD90 and cadherin 11 displayed positive populations using the SPADE algorithm. Cadherin 11 positive populations were not identified in three normal donors and CD146 populations were not identified in one tendinopathic donor (data summarised in **Table 4.2**). A $CD146^{+}CD90^{+}$ population was observed in 75% of all samples (three normal and three tendinopathic) whereas a $CD34^{+}CD90^{+}$ positive population was only identified in one normal and one tendinopathic sample. As observed previously, CD90 and podoplanin show different patterns of expression between normal and tendinopathic samples. $CAD11^{+}PDPN^{+}$ and $CD90^{+}PDPN^{+}$ double positive populations were identified in one normal and three out of four tendinopathic samples or no normal and three tendinopathic samples, respectively. Similarly, $CAD11^{+}PDPN^{+}CD90^{+}CD44^{+}$ positive populations were only identified in tendinopathic tissues.

In order to validate populations generated by the SPADE algorithm we undertook back-analysis of flow cytometry data and identified these by manual gating based on isotype controls. **Fig. 4.12** shows a representative example of a SPADE tree and corresponding pseudocolour plots of populations identified by the algorithm.

Panel 1- normal

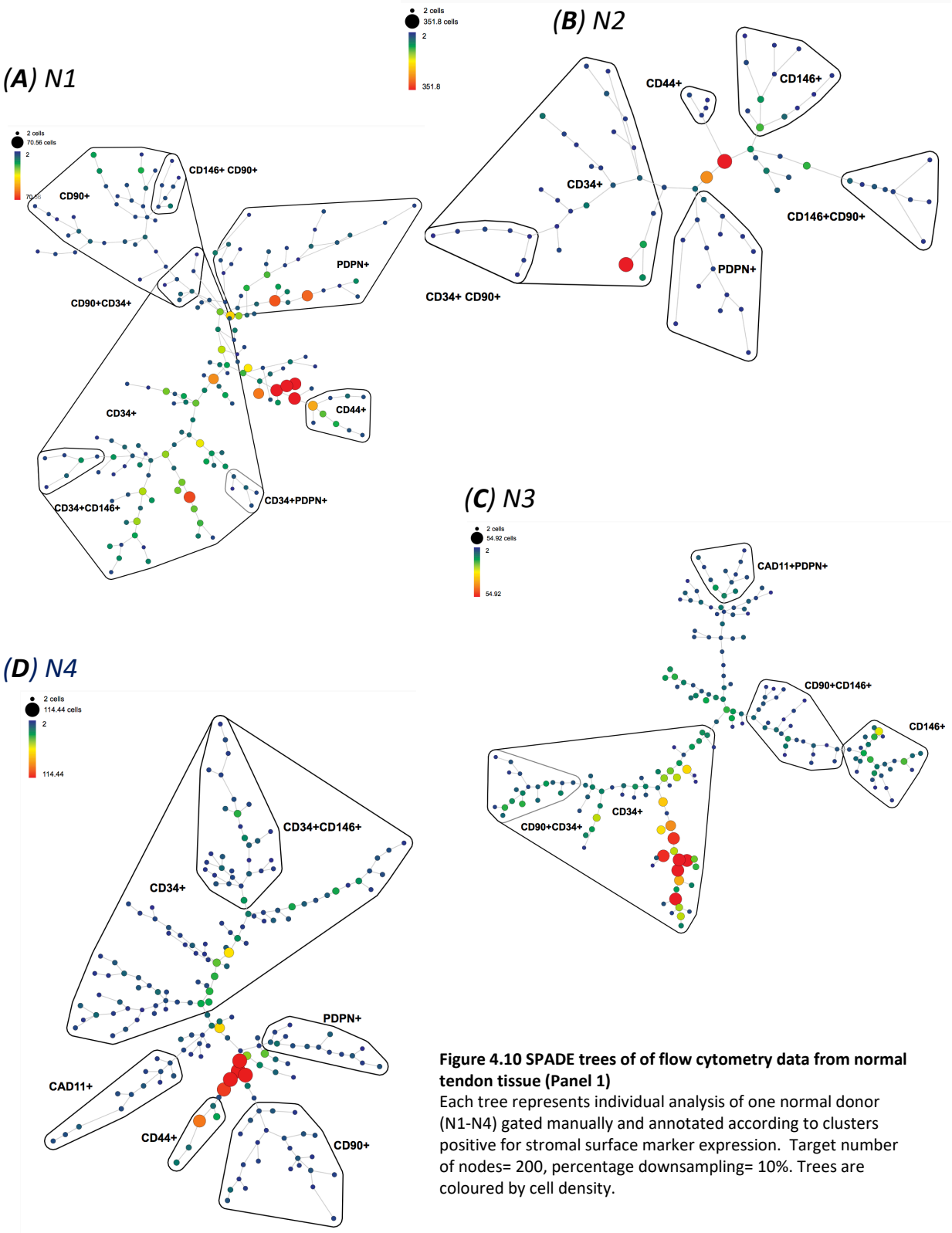

Figure 4.10 SPADE trees of of flow cytometry data from normal tendon tissue (Panel 1)
Each tree represents individual analysis of one normal donor (N1-N4) gated manually and annotated according to clusters positive for stromal surface marker expression. Target number of nodes= 200, percentage downsampling= 10%. Trees are coloured by cell density.

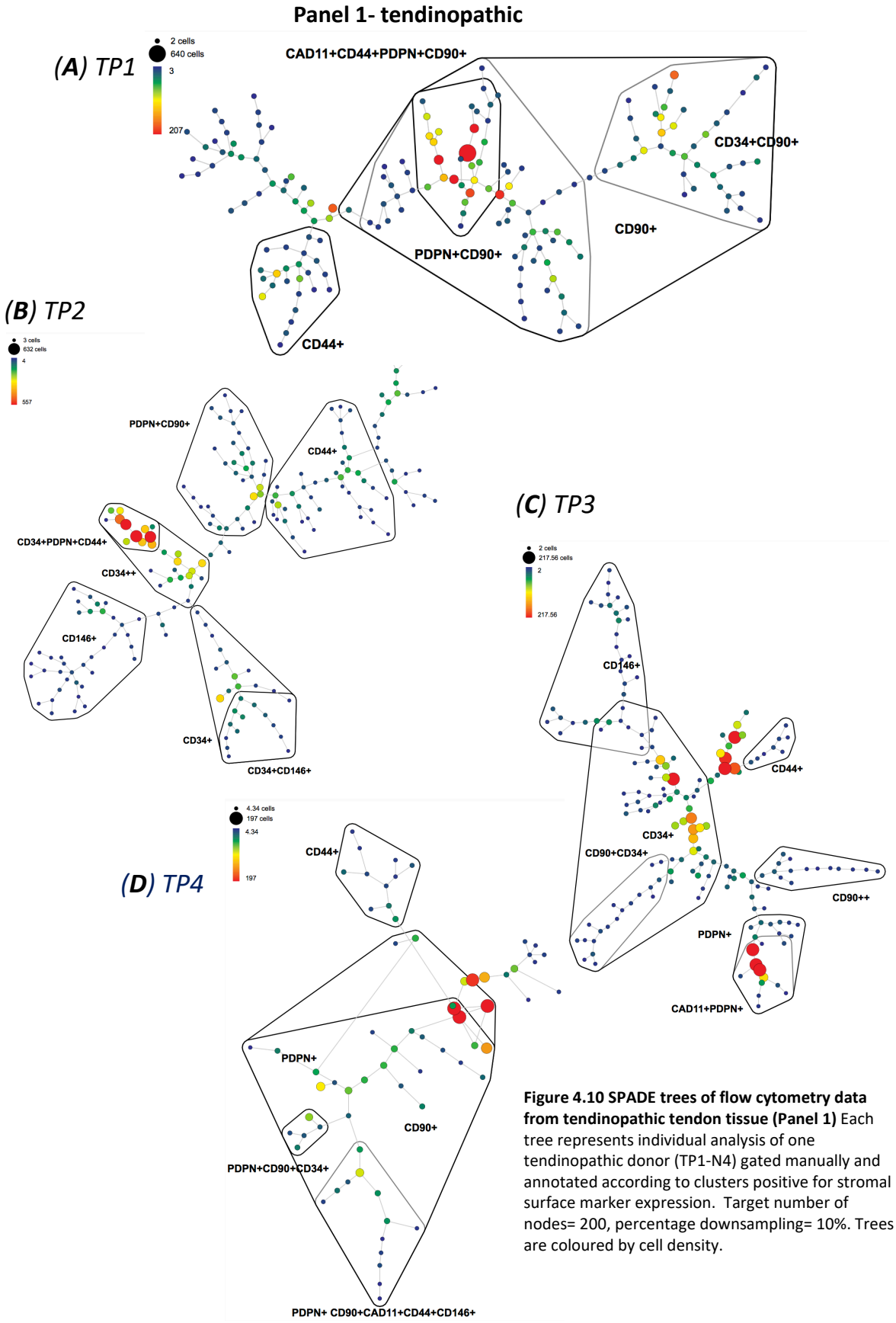

Figure 4.10 SPADE trees of flow cytometry data from tendinopathic tendon tissue (Panel 1) Each tree represents individual analysis of one tendinopathic donor (TP1-N4) gated manually and annotated according to clusters positive for stromal surface marker expression. Target number of nodes= 200, percentage downsampling= 10%. Trees are coloured by cell density.

	CD44+	CD146+	CD34+	PDPN+	CD90+	CAD11+
N1	+	+	+	+	+	-
N2	+	+	+	+	+	-
N3	+	+	+	+	+	+
N4	+	+	+	+	+	-
TP1	+	-	+	+	+	+
TP2	+	+	+	+	+	-
TP3	+	+	+	+	+	+
TP4	+	+	+	+	+	+

	CD34+CD90+	CD146+CD90+	CD34+CD146+	CAD11+PDPN+	CD90+PDPN+	CD34+PDPN+	CAD11+PDPN+ CD90+CD44+
N1	+	+	-	-	-	-	-
N2	+	-	+	-	-	+	-
N3	+	+	-	+	-	-	-
N4	-	-	+	-	-	-	-
TP1	-	-	+	+	+	-	+
TP2	+	-	-	-	+	+	-
TP3	+	-	-	+	-		-
TP4	+	+	-	+	+	+	+

Table 4.2 Tabular comparison of populations identified by SPADE analysis (Panel 1)
n=4 normal hamstring tendon, n=4 tendinopathic (torn) supraspinatus tendon

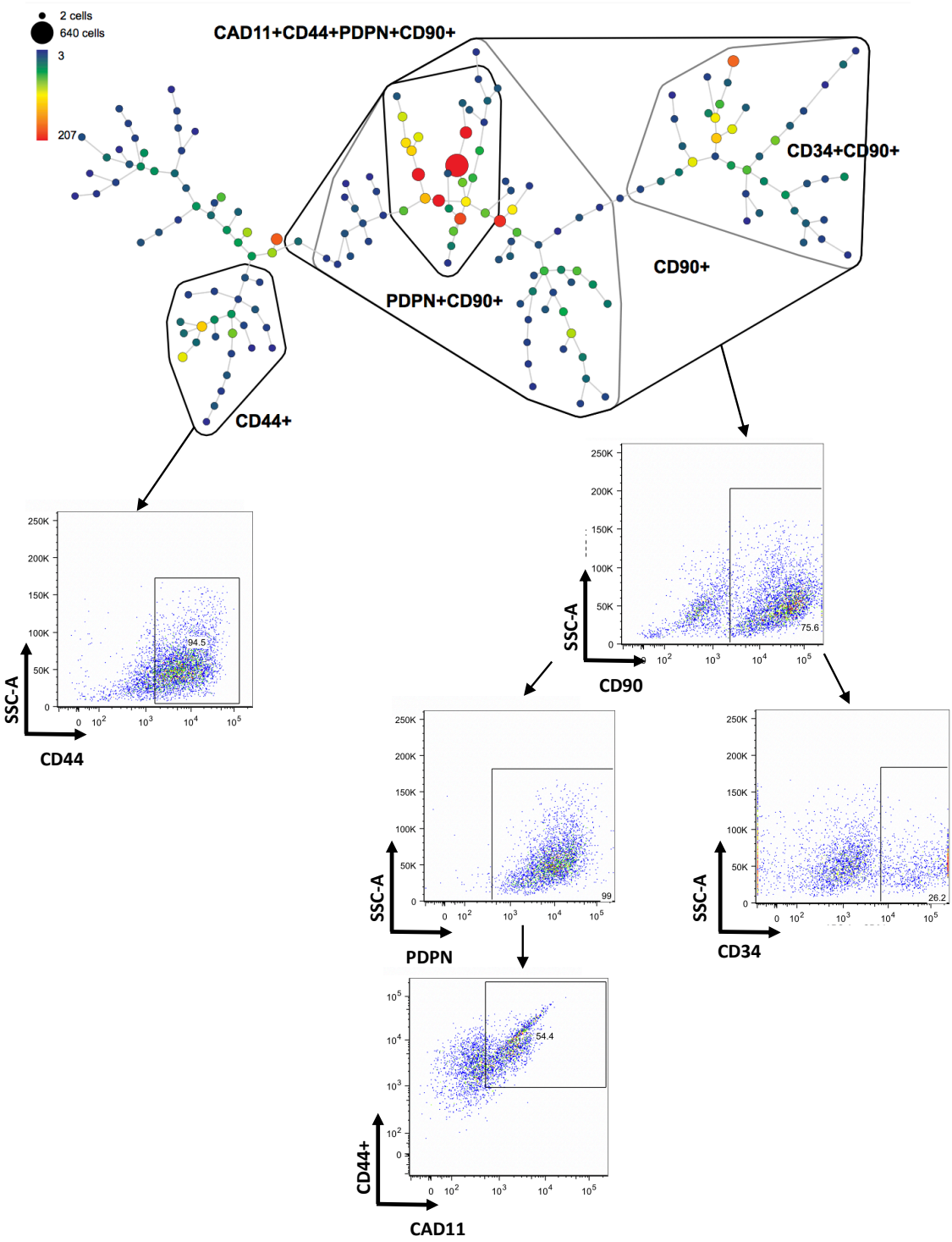

Figure 4.12 Validation of SPADE analysis using manual gating (Panel 1)
Representative comparison of populations derived from SPADE analysis and manual gating using FlowJo. SPADE tree represents individual analysis of flow cytometry data from one donor gated and annotated according to clusters positive for stromal surface marker expression. Pseudocolour plots represent data from the same experiment file analysed on FlowJo. Gating shown is of singlet cells, positive populations were gated relative to isotype controls.

Panel 2

As reported previously, SPADE trees for Panel 2 expression were generated from four normal and four tendinopathic samples (**Fig. 4.13 and 4.14**). Positive populations were identified in all samples for CD29, CD10 and CD47 with only one normal donor lacking CD10 clusters (**Table 4.3**). CD91 populations were identified in two samples and CD81 was only present in one tendinopathic donor. With the exception of $CD29^{+}CD47^{+}$ double positive populations (present in three normal and three tendinopathic samples) clustering observed was generally less well defined for this panel of markers. $CD29^{+}CD10^{+}CD47^{+}$ populations were identified in three samples and one $CD47^{+}CD10^{+}CD29^{+}CD91^{+}$ population was observed in a tendinopathic donor. As with the previous panel we were able to validate the SPADE clustering analysis using manual gating (**Fig. 4.15**).

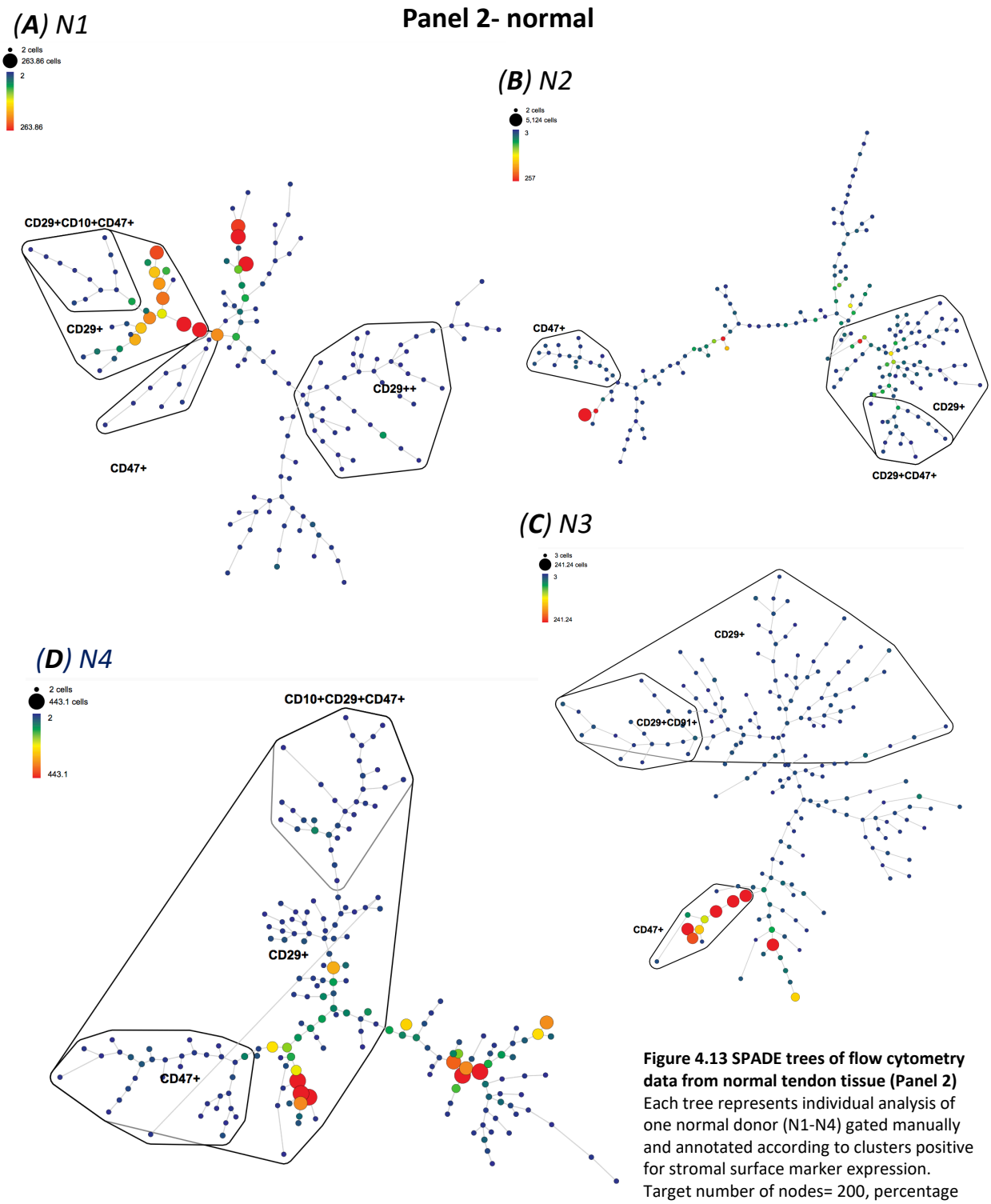

Figure 4.13 SPADE trees of flow cytometry data from normal tendon tissue (Panel 2) Each tree represents individual analysis of one normal donor (N1-N4) gated manually and annotated according to clusters positive for stromal surface marker expression. Target number of nodes= 200, percentage downsampling= 10%. Trees are coloured by cell density.

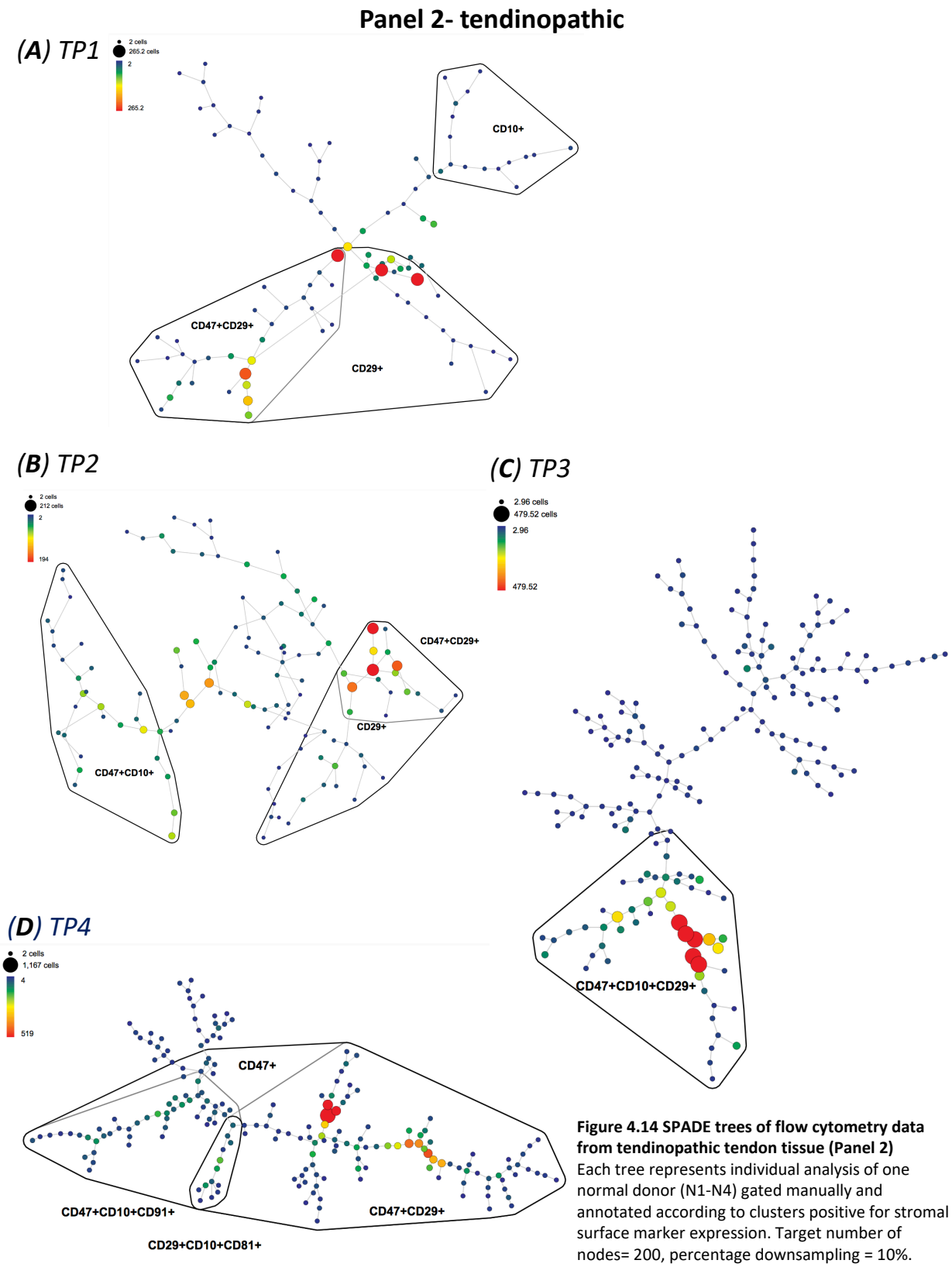

Figure 4.14 SPADE trees of flow cytometry data from tendinopathic tendon tissue (Panel 2) Each tree represents individual analysis of one normal donor (N1-N4) gated manually and annotated according to clusters positive for stromal surface marker expression. Target number of nodes= 200, percentage downsampling = 10%. Trees are coloured by relative cell density.

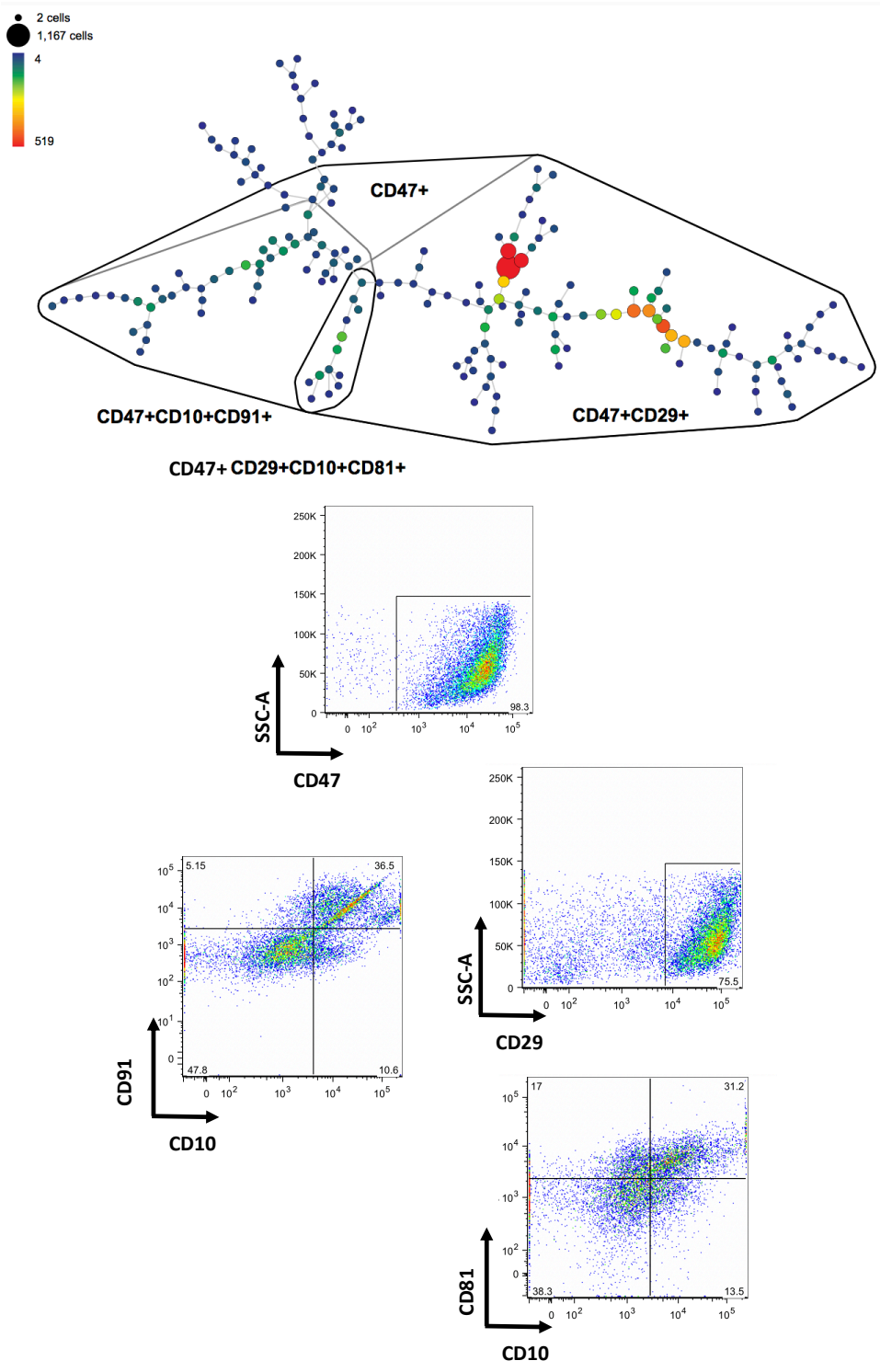

Figure 4.15 Validation of SPADE analysis using manual gating (Panel 2)
Representative comparison of populations derived from SPADE analysis and manual gating using FlowJo. SPADE tree represents individual analysis of flow cytometry data from one donor gated and annotated according to clusters positive for stromal surface marker expression. Pseudocolour plots represent data from the same experiment file analysed on FlowJo. Gating shown is of singlet cells, positive populations were gated relative to isotype controls.

	CD29+	CD10+	CD47+	CD81+	CD91+
N1	+	+	+	-	-
N2	+	+	+	-	-
N3	+	-	+	-	+
N4	+	+	+	-	-
TP1	+	+	+	-	-
TP2	+	+	+	-	-
TP3	+	+	+	-	-
TP4	+	+	+	+	+

	CD29+CD47+	CD10+CD47+	CD29+CD91+	CD29+CD47+CD10+	CD29+CD10+CD81+	CD47+CD29+ CD10+CD91+
N1	+	-	-	+	-	-
N2	+	-	-	-	-	-
N3	-	-	+	-	-	-
N4	+	-	-	+	-	-
TP1	+	-	-	-	-	-
TP2	+	+	-	-	-	-
TP3	-	-	-	+	-	-
TP4	+	-	-	-	+	+

Table 4.3 Tabular comparison of populations identified by SPADE analysis (Panel 2)
n=4 normal hamstring tendon, n=4 tendinopathic (torn) supraspinatus tendon

4.2.5 viSNE analysis of normal and tendinopathic tissue samples

viSNE is a tool that maps high dimensional cytometry data onto 2D while conserving its high dimensional structure. viSNE highlights structure in the heterogeneity of surface phenotype expression.[438] It differs from other tools, such as SPADE, as SPADE clusters cells and determines the average of each cluster while viSNE maintains single cell resolution. Our SPADE analysis identified several distinct clusters of cells in our first panel of markers (Panel 1) and a relative lack of clustering in Panel 2 thus we applied to viSNE algorithm to normal and tendinopathic samples stained for Panel 1. **Figure 4.16/17 A & B** show overlay plots of normal and tendinopathic tissue samples gated for positive staining with CD90, CD44, CD34, CD146, cadherin 11 and podoplanin. Overall, distinct clustering patterns are observed between normal and tendinopathic samples. Most notable differences appear with CD44 clustering (**Fig. 4.16E & F**) where two clusters are observed in normal but absent in tendinopathic samples. A similar pattern is seen with CD90 clustering in the same area although this is not as distinct as observed with CD44. As per our FlowJo and SPADE analysis, a relative lack of cadherin 11 expression in normal donors is evident (**Fig.4.17E**). Visually, the most apparent difference between the normal and tendinopathic samples is the large cluster observed in the bottom right hand of the overlay plots (**Fig. 4.16/17 A & B)**. In the tendinopathic donors this cluster is positive for CD90, CD44, CD34, CD146, cadherin 11 and podoplanin. This co-expression profile may indicate the presence of phenotypically similar activated populations in disease.

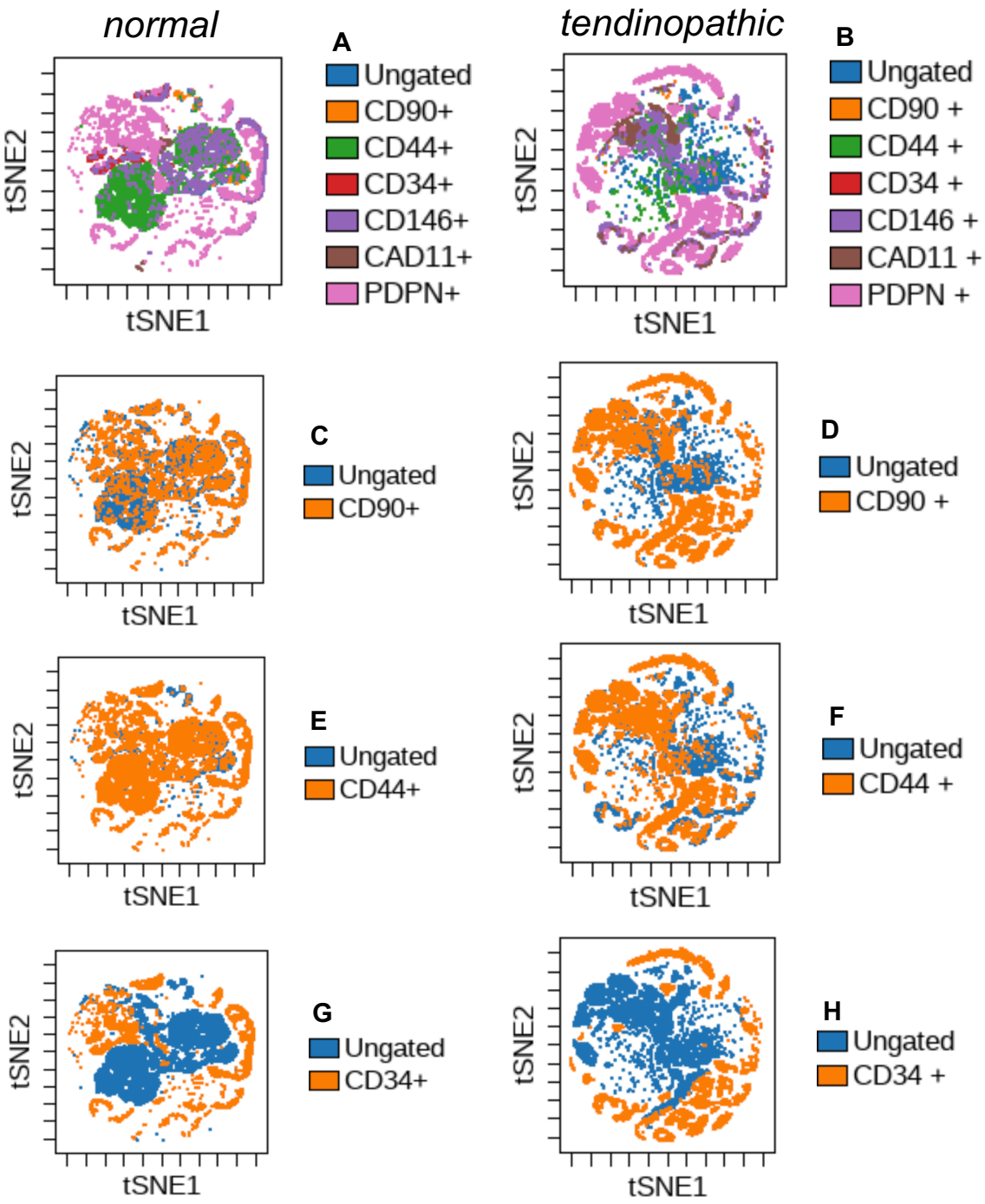

Figure 4.16 viSNE visulisation of flow cytometry data from normal and tendinopathic tissue samples
viSNE algorithm run on concatenated flow cytometry data from 3 normal and 3 tendinopathic donors. (**A & B**) overlay plots of populations manually gated on positive expression of CD90, CD44, CD34, CD146, CAD11 & PDPN. (**C -H**) viSNE plots showing only ungated and CD90, CD44 & CD34 positive populations in normal and tendinopathic donors, n=3. Plots represent equal sampling of normal and tendinopathic donors (18033 events per file, 36066 events total).

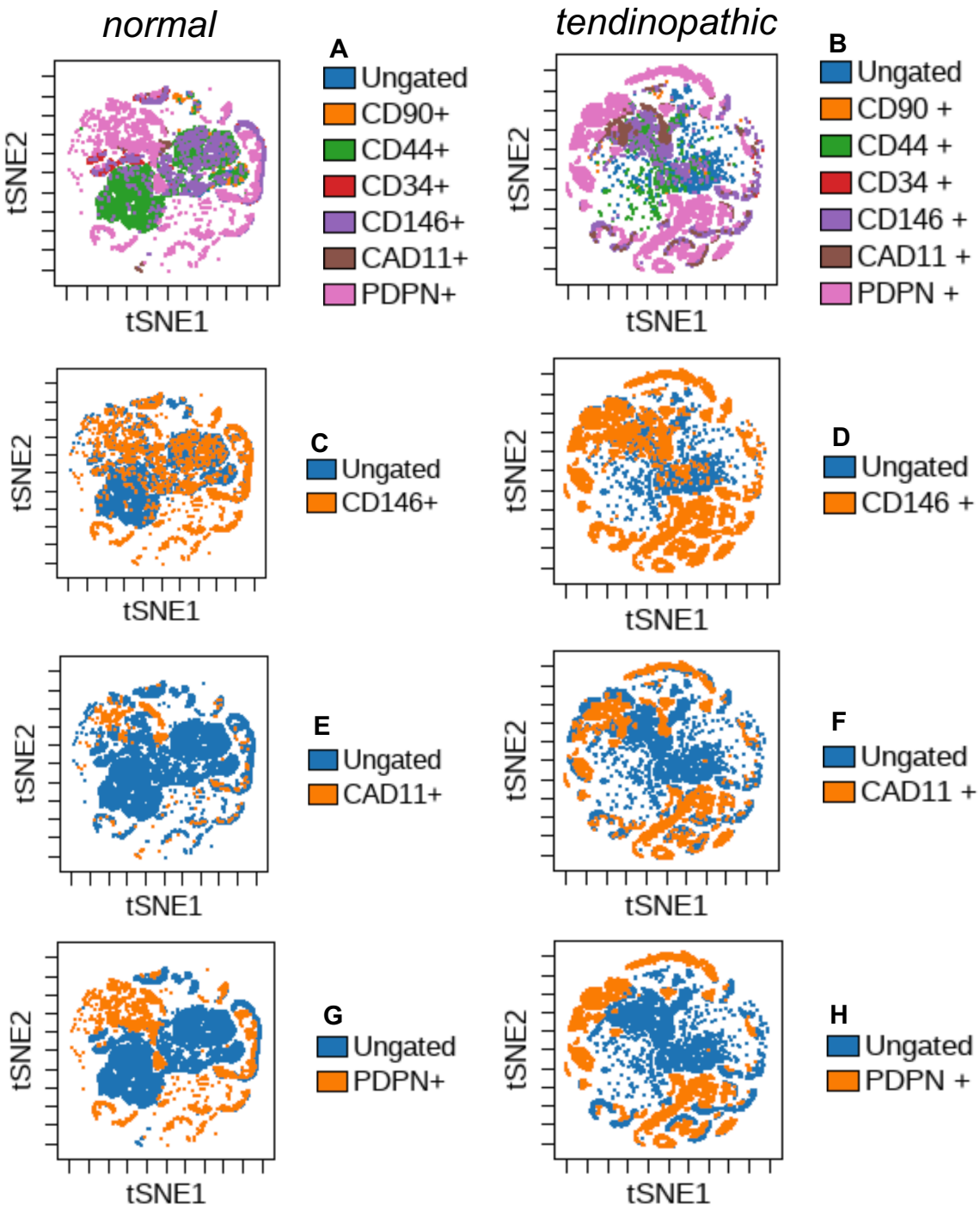

Figure 4.17 viSNE visulisation of flow cytometry data from normal and tendinopathic tissue samples
viSNE algorithm run on concatenated flow cytometry data from 3 normal and 3 tendinopathic donors. (**A & B**) overlay plots of populations manually gated on positive expression of CD90, CD44, CD34, CD146, CAD11 & PDPN. (**C -H**) viSNE plots showing only ungated and CD146, CAD11 and PDPN positive populations in normal and tendinopathic donors, n=3. Plots represent equal sampling of normal and tendinopathic donors (18033 events per file, 36066 events total).

4.3 Discussion and conclusions

In the context of the tendinopathy little is known about the properties of stromal cells and whether any phenotypic discrepancies may relate to pathology. Within the literature there are no globally accepted markers of tenocyte phenotype and their distinction from other fibroblast populations is not well understood. Previous studies have addressed the concept of stromal 'activation markers' in tendinopathy; however, the aim of this work was to explore the concept of multidimensional subpopulations in health and disease. This chapter has identified the presence of stromal surface markers in human tendon and found their expression is upregulated in disease. Furthermore, we have identified the existence of distinct subpopulations present under both homeostatic and pathological conditions.

Our initial screening confirmed the presence of several markers at both protein and transcript level. Several previously reported 'activation' markers including podoplanin, FAP, VCAM-1 and CD248 were found to be upregulated in tendinopathic tissue. In addition, podoplanin and VCAM1 expression in tenocytes was increased in response to IL1β and LPS simulation *in vitro*. 'Activation' was not as pronounced *in vitro* which suggests that upregulation of surface protein expression is dependent on a number of local factors present in the tendon microenvironment rather than a single inflammatory stimulus. Interestingly, we found most significant differences in surface protein and transcript expression between control and late disease indicating a sustained stimulus is required to induce phenotypic modifications.

A recent study has highlighted the presence of a $CD90^{+}CAD11^{+}PDPN^{+}$ synovial fibroblast subpopulation in RA.[315] Although not identified by initial analysis, our hierarchical clustering data showed the presence of such populations in two tendinopathic biopsies. In addition, $CD90^{+}PDPN^{+}$ and $CAD11^{+}CD90^{+}$ populations were observed in three of four tendinopathic donors. Podoplanin expression has been associated with an inflammatory phenotype in RA and tumour invasion in metastatic disease.[439,440] Furthermore, $CD90^{+}$ fibroblasts are the major source of IL-6 that supports the expansion of cancer stem cells in inflammation.[441] Coupled with the absence of these populations in normal tendon biopsy samples, this

suggests a potentially pathological tenocyte subpopulation may exist in disease. CD90 and podoplanin mediate adhesion of leukocytes and platelets to stromal cells[442,443] and cadherin 11 has been shown to modulate adhesion between fibroblasts.[444] This indicates these surface proteins may also act to direct cellular behaviour in the stromal microenvironment. Expression of CD90, cadherin 11 and podoplanin was low in normal donors compared with diseased samples; however, we still observed distinct clustering of these markers. It is plausible these small tenocyte subpopulations in healthy tendon may be expanded in under pathological conditions.

Interestingly, we observed a small yet distinct population of exclusively $CD44^+$ cells in almost all SPADE analyses. Although CD44 expression was upregulated in tendinopathic tissue this population appeared to be consistent between normal and diseased samples. viSNE analysis identified distinct CD44 clustering most prominent in normal samples which suggests these $CD44^+$ populations may play a role in tendon homeostasis. Our hierarchical clustering and viSNE analysis showed the expanded $CD44^+$ population in tendinopathic samples was present alongside podoplanin, cadherin 11 and CD90 which suggests that CD44 may be expressed in phenotypically distinct tenocyte subsets in health and disease. CD44 and podoplanin are co-ordinately upregulated in aggressive cancer cell lines; they directly bind to each other and such interaction is thought to promote directional cell migration.[445] This provides further evidence to suggest the possibility of 'pathogenic' tenocyte subpopulations in tendinopathy.

We did not observe any significant alterations in surface protein expression in our second panel of markers. CD29 and CD47 showed a trend towards increased expression in tendinopathic samples; however, these populations appear to display a bimodal distribution that indicates inherent differences in gene expression between donors. These differences in gene expression were also apparent in our sub analysis based on CD29/CD10 gating. The clustering generated by the SPADE algorithm showed the majority of cells displayed concomitant expression of CD29 and CD47. CD29 and CD47 are also known as integrin $\beta 1$ and integrin associated protein, respectively. CD47 is able to modulate the behaviour of $\beta 1$ integrin complexes thus it possible this subpopulation is active in mediating cell adhesion.[446]

We additionally observed the expression profiles of tenocytes *in vitro* varies significantly to those freshly isolated from tendon tissue. Differential expression profiles are likely highly dependent on the tissue niche and local microenvironmental factors that are absent under culture conditions. Initial analysis of flow cytometry data showed an interesting $CD34^{+}CD146^{+}$ double positive population consistent between normal and tendinopathic samples. Although CD34 and CD146 are both expressed on fibroblasts, endothelial cells are known to express these proteins concomitantly.[447] As disaggregated tendon is not a homogenous population of cells it is possible there is a small population of endothelial cells present that originate from the small vessels within the tendon. In future studies it would therefore be prudent to add an endothelial cell exclusion channel to omit these cells from analysis. Similarly, it is likely that a small population of immune cells is present in these digest samples (existing as a small resident compartment in normal tendon with additional inflammatory infiltrate in the tendinopathic samples). For this reason, a CD45 haematopoietic exclusion channel would also serve to preserve the purity of the tenocyte populations obtained from tissue digests.

We acknowledge that immune cell subsets may express some of the surface markers tested; however, $CD45^{+}$ cells typically account for 2% of total cells obtained from normal digests and 5% of cells in tendinopathic samples thus it is unlikely there is significant contamination. When considering the concept of tissue heterogeneity the effect of enzymatic digestion on cell subsets must also be taken into consideration due to its possible effect on the expression profile of different cells. However, all samples were subject to the same protocol and surface marker expression profiles appear to be consistent between transcript data and cells isolated from fresh tendon.

It is evident that complex tenocyte phenotypic variations exist within the live tendon stroma. Discerning these differences *ex vivo* and translating them to *in vitro* functional studies presents a host of challenges that should be met with caution to preserve the organic nature of this differential gene expression.

On the basis of these results we propose that tenocytes are a heterogenous population of cells that exist as phenotypically distinct subpopulations in health and disease. Further characterization of tenocyte subsets may aid the development of strategies to target pathogenic stroma in diseases associated with abberant matrix remodelling.

Chapter 5: Assessing the effect of surface protein knockdown on stromal-immune cell interactions in tendinopathy

5.1 Introduction

As discussed in the previous chapter, several studies have emerged highlighting the phenomenon of 'fibroblast activation' in various soft tissue pathologies.[418,436,448] Stromal biology is a relatively new area of pharmacologic interest that is commonly considered a branch of immunology. Fibroblasts, the main cellular component of the stroma, regulate the structure and function of healthy tissues and participate in tissue repair following periods of acute inflammation.[419] Under certain conditions, such as chronic inflammation, they can acquire specific properties that include expression of surface proteins and aberrant stimulatory properties.[435]

The previous chapter demonstrated upregulation of several surface proteins including podoplanin and VCAM1. Having identified myeloid cells as a source of DAMPs within the tendon we next sought to directly assess the interactions of immune and stromal cells. It is well cited within the literature that podoplanin and VCAM1 modulate cell interactions thus we hypothesised tenocytes may be express these proteins to facilitate immune cell contact.[362,449]

Podoplanin is a small cell-surface mucin-like glycoprotein expressed on a number of cells including fibroblasts and macrophages. Its expression is upregulated under high levels of inflammation associated with chronic conditions such as RA, psoriasis and multiple sclerosis (MS).[450] CLEC-2 (C-type lectin 2) is the only known ligand for podoplanin and is most abundantly expressed on platelets.[363] Recently it has been identified on myeloid cells, specifically monocytes and dendritic cells.[451] Binding of podoplanin with its ligand is thought to modulate signalling pathways that regulate cell proliferation, migration and ECM remodelling.[450] Little is known about the physiological properties of podoplanin; however, recent studies have highlighted its role in regulating the inflammatory reaction and immune cell infiltration during sepsis.[452]

VCAM1 (vascular cell adhesion molecule-1) expression is associated with the progression of several immunological disorders including RA, asthma and cancer.[449] It is primarily expressed on the surface of endothelial cells; however, its expression is upregulated in other cell types including fibroblasts and

macrophages under inflammatory conditions.[436,453] It primarily functions as an adhesion molecule and is involved in adhesion and transmigration of monocytes across blood vessel walls. Its binds to integrin $\alpha 4\beta 1$ (VLA-4) and this interaction is thought to play an important role in leukocyte recruitment during inflammation.[454]

To assess the physiological significance of stromal activation markers in tendinopathy the aims of this chapter are:

1. To assess the effect of podoplanin and VCAM1 knockdown on tenocyte behaviour
2. Characterize the effect of tenocyte-monocyte co-culture on monocyte phenotype and development
3. Assess the effect of podoplanin and VCAM1 knockdown on monocyte phenotype in our monocyte-tenocyte co-culture system

5.2 Results

5.2.2 Podoplanin knockdown does not directly alter tenocyte behaviour

We first tested the effect of podoplanin knockdown in tenocytes using commercially available siRNA. **Fig. 5.1A and 5.1C** show podoplanin protein knockdown measured by flow cytometry. On average, we obtained an 80% knockdown in protein expression and a 92% knockdown in podoplanin transcript by RT-PCR (**Fig. 5.1B**).

We next sought to assess the effect of podoplanin knockdown on the intrinsic behaviour of tenocytes by measuring cytokine release and production of matrix proteins. We found no significant effect of podoplanin knockdown on release of IL-6 (**Fig 5.2 A &B**), IL-8 (**Fig. 5.2 C & D**) or CCL2 (**Fig. 5.2E & F**) in normal or tendinopathic tenocytes compared with untransfected tenocytes and scramble transfection control. Additionally, the transfection procedure alone (represented by scramble negative control) did not appear to have any discernible effect on cytokine release.

Under the same conditions, tenocytes were stimulated with 10ng/ml IL-1β to test the effect of transfection and podoplanin knockdown on responsiveness to an inflammatory stimulus. Tenocytes responded by producing significantly greater levels of cytokines including IL-6 in normal (**Fig. 5.2A,** all conditions $p<0.05$) and tendinopathic (**Fig 5.2B** siPDPN vs siPDPN + IL-1β $p<0.05$) cultures. Similarly, IL-8 expression was significantly upregulated in normal (**Fig. 5.2C** siPDPN vs siPDPN + IL-1β $p<0.01$) and tendinopathic tenocytes (**Fig. 5.2D** scramble vs scramble + IL-1β and siPDPN vs siPDPN + IL-1β $p<0.05$). CCL2 release was most significantly increased in response to IL-1β stimulation in normal tenocytes (**Fig. 5.2E** control vs control + IL-1β $p<0.001$, scramble vs scramble + IL-1β and siPDPN vs siPDPN + IL-1β) and also in tendinopathic tenocytes (**Fig. 5.2F** control vs control + IL-1β $p<0.05$, scramble vs scramble + IL-1β $p<0.05$). Podoplanin knockdown did not appear to affect the response of tenocytes to IL-1β under any of the conditions tested. It should be noted that IL-1β stimulation produced a great magnitude of response under every stimulatory condition despite some not displaying statistical significance.

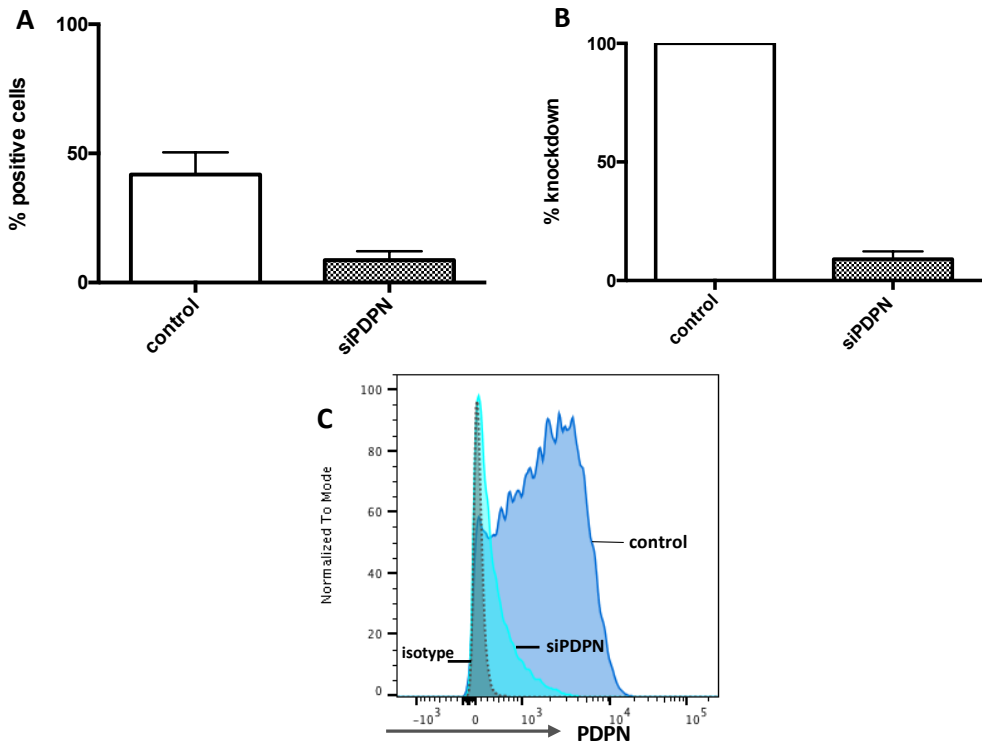

Figure 5.1 Podoplanin knockdown in tenocytes
siRNA knockdown of podoplanin in scramble control and transfected tenocytes (siPDPN) **(A)** expressed as % positive singlet cells based on isotype control gating, n=3 **(B)** percentage knockdown of podoplanin measured by RT-PCR (relative to 18S endogenous control), n=4 **(C)** representative histogram of podoplanin surface expression in transfected tenocytes relative to scramble control. All data represent mean ± SEM.

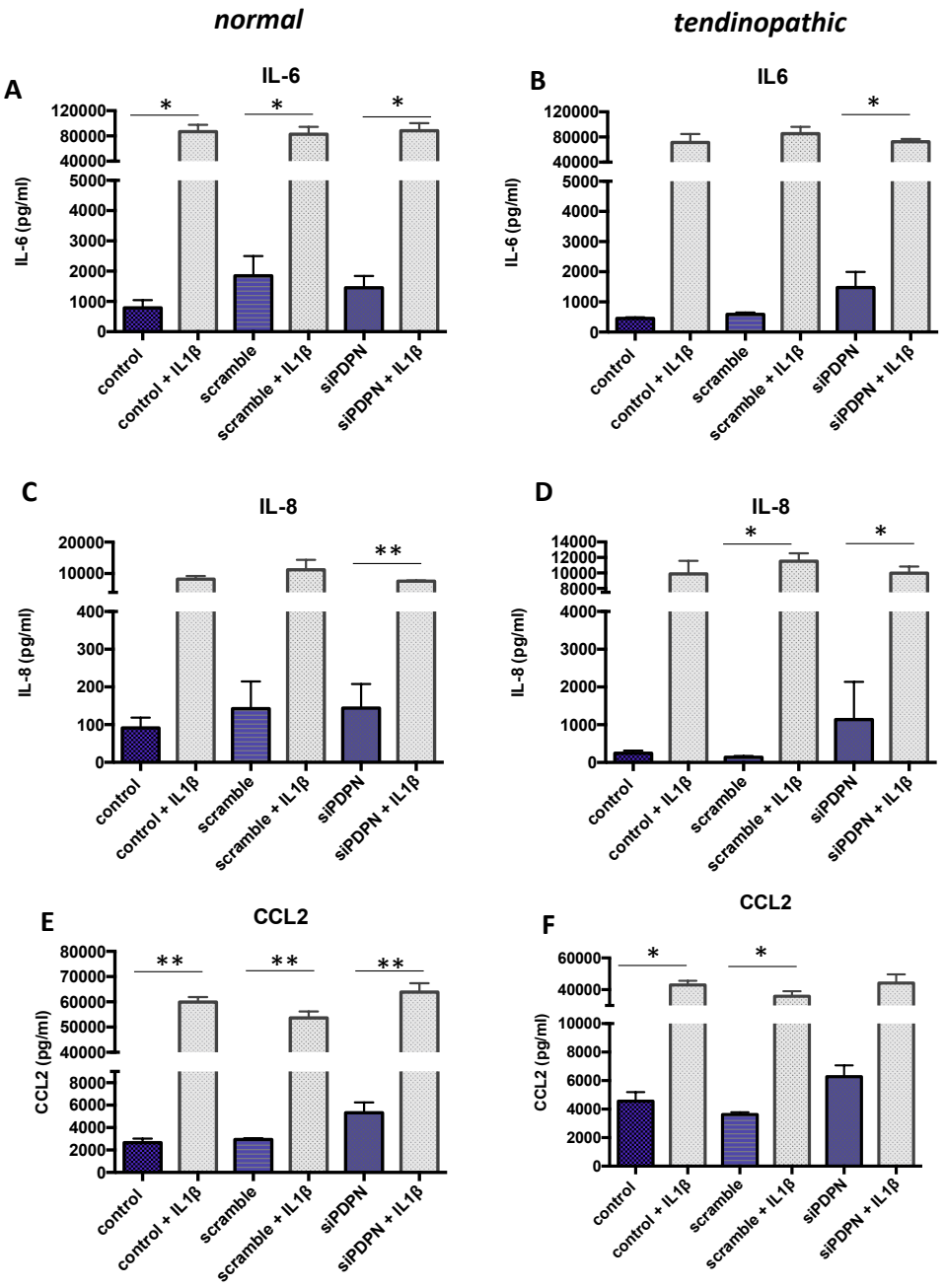

Figure 5.2 Cytokine release from tenocytes with podoplanin knockdown
IL-6, IL-8 and CCL2 release from **(A, C, E)** normal tenocytes **(B, D, F)** tendinopathic tenocytes left untransfected (control), transfected with scramble control and transfected with podoplanin siRNA in the presence or absence of 10ng/ml IL-β stimulation. $p<0.05$, $**p<0.01$ (One way ANOVA with Tukey's multiple comparisons test) All data represent mean ± SEM, n=4.

The effect of podoplanin knockdown and simultaneous stimulation with IL-1β on matrix production was measured by RT-qPCR using a panel of genes associated with matrix regulation by tenocytes. Neither podoplanin knockdown or IL-1β stimulation appeared to have any effect on production of collagen 1 (Col1α1) in normal and tendinopathic tenocytes (**Fig. 5.3A & B**). There was a trend towards increased expression of collagen 3 (Col3α1) (**Fig. 5.3C & D**), tenascin C (**Fig. 5.3 E & F**) and decorin (**Fig. 5.3 I & J**) expression in response to IL-1β stimulation in normal and tendinopathic tenocytes; however, podoplanin knockdown did not appear to have any demonstrable effect. In normal tenocytes periostin expression did not change in response to podoplanin knockdown or IL-1β stimulation **(Fig. 5.3G)**. There appeared to be a small (0.75 fold) deviation in periostin expression in tendinopathic tenocytes in response to podoplanin knockdown and IL-1β stimulation; however, this was not significant (**Fig. 5.3H**).

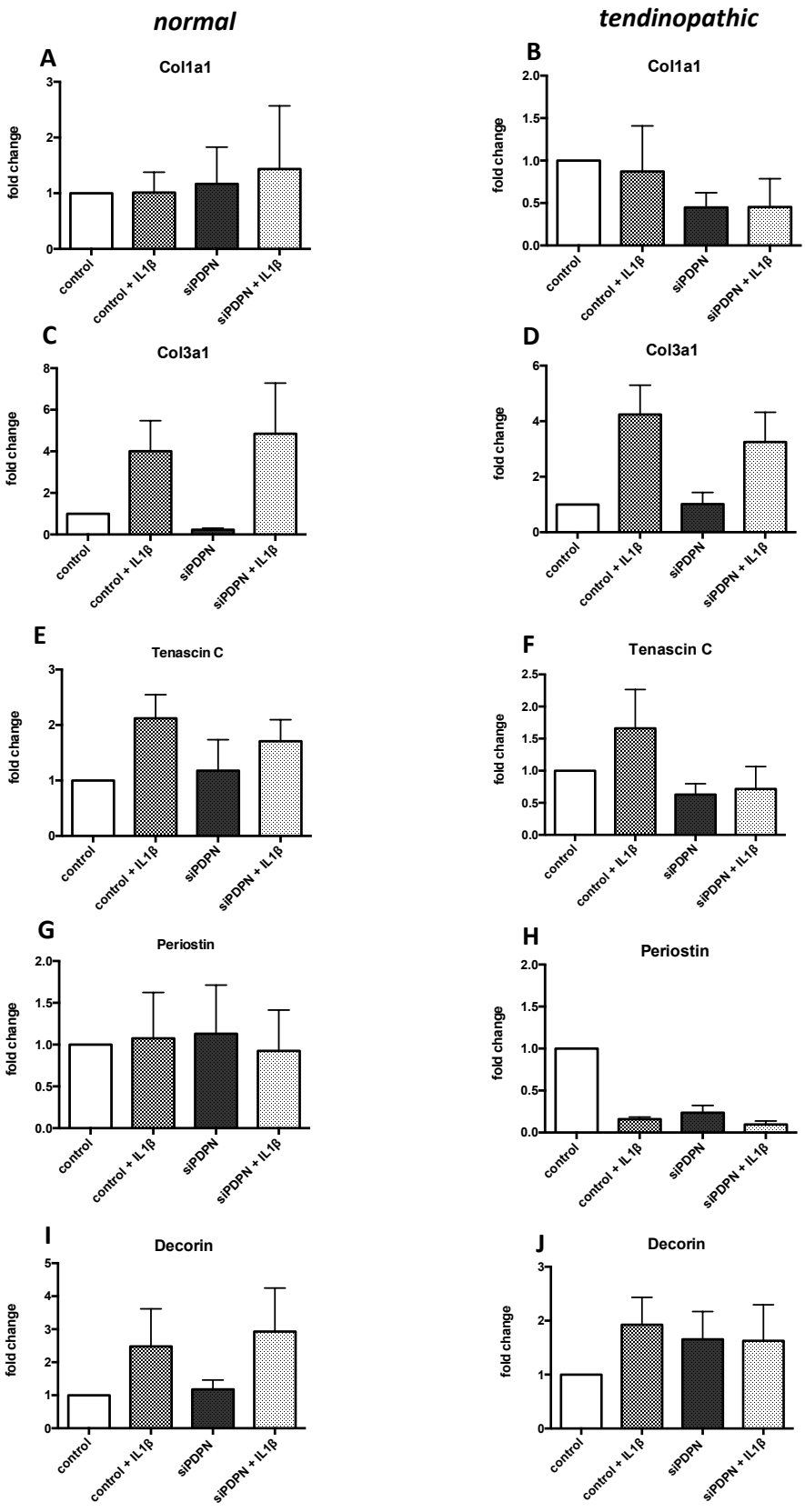

Figure 5.3 Podoplanin knockdown does not directly alter matrix regulation by tenocytes
(A & B) Col1α1 **(C & D)** Col3α1 **(E & F)** tenascin C **(G & H)** periostin **(I & J)** decorin expression in normal or tendinopathic tenocytes transfected with scramble control or podoplanin siRNA in the presence or absence of 10ng/ml IL-1β stimulation. All data are normalized to 18S housekeeping gene and expressed as fold change relative to scramble control, data represent mean ± SEM, normal n=4, tendinopathic n=3.

5.2.2 VCAM1 knockdown does not directly alter tenocyte behaviour

As reported previously, we tested the effect of VCAM1 knockdown *in vitro* using commercially available siRNA and achieved 90% knockdown by RT-qPCR and a 71% knockdown in protein expression (**Fig.5.4A & B**).

Our cytokine release experiments yielded similar results to podoplanin knockdown with the only significant differences being observed with IL-1β stimulation. We found significant upregulation of IL-6 (**Fig. 5.5A &B** $p<0.05$, 0.01 or 0.001**)** and CCL2 (**Fig. 5.5E & F** $p<0.05$ or 0.01) expression in both normal and tendinopathic tenocytes under all stimulatory conditions and significant induction of IL-8 expression in normal and tendinopathic tenocytes (**Fig. 5.5 C** control vs control + IL-1β $p<0.05$, , siVCAM1 vs siVCAM1 + IL-1β $p<0.05$, **Fig 5.5D** scramble vs scramble + IL-1β $p<0.01$, siVCAM1 vs siVCAM1 + IL-1β $p<0.05$).

VCAM1 knockdown did not appear to have any effect on expression on matrix proteins in normal or tendinopathic tenocytes (**Fig. 5.6A-J**). As observed previously there was a trend of increased expression of collagen 3 (**Fig. 5.6C & D**) and tenascin C (**Fig. 5.6G & H**) in normal and tendinopathic tenocytes in response to IL-1β stimulation. There appeared to be no change in expression of collagen 1 (**Fig. 5.6 A & B**), periostin (**Fig. 5.6E & F**) or decorin (**Fig. 5.6I & J**).

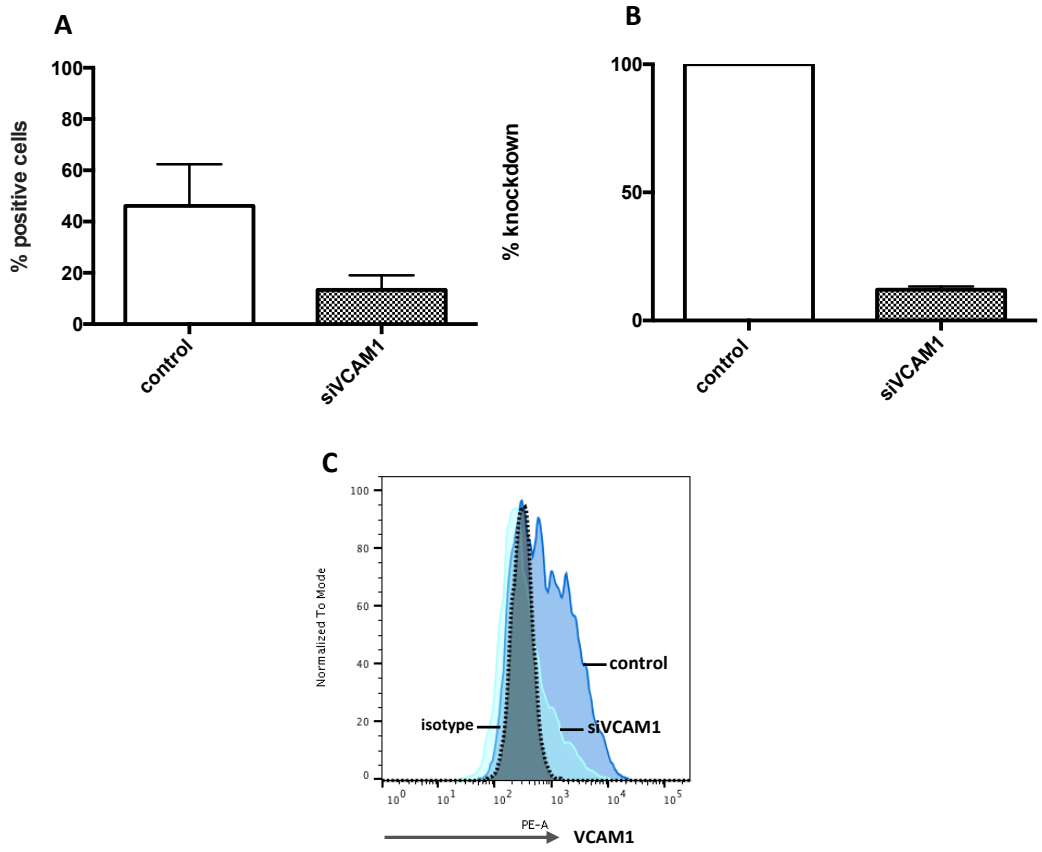

Figure 5.4 VCAM1 knockdown in tenocytes
siRNA knockdown of VCAM1 in scramble control and transfected tenocytes (siPDPN) **(A)** expressed as % positive singlet cells based on isotype control gating, n=3 **(B)** percentage knockdown of VCAM1 measured by RT-PCR (relative to 18S endogenous control), n=3 **(C)** representative histogram of VCAM1 surface expression in transfected tenocytes relative to scramble control.

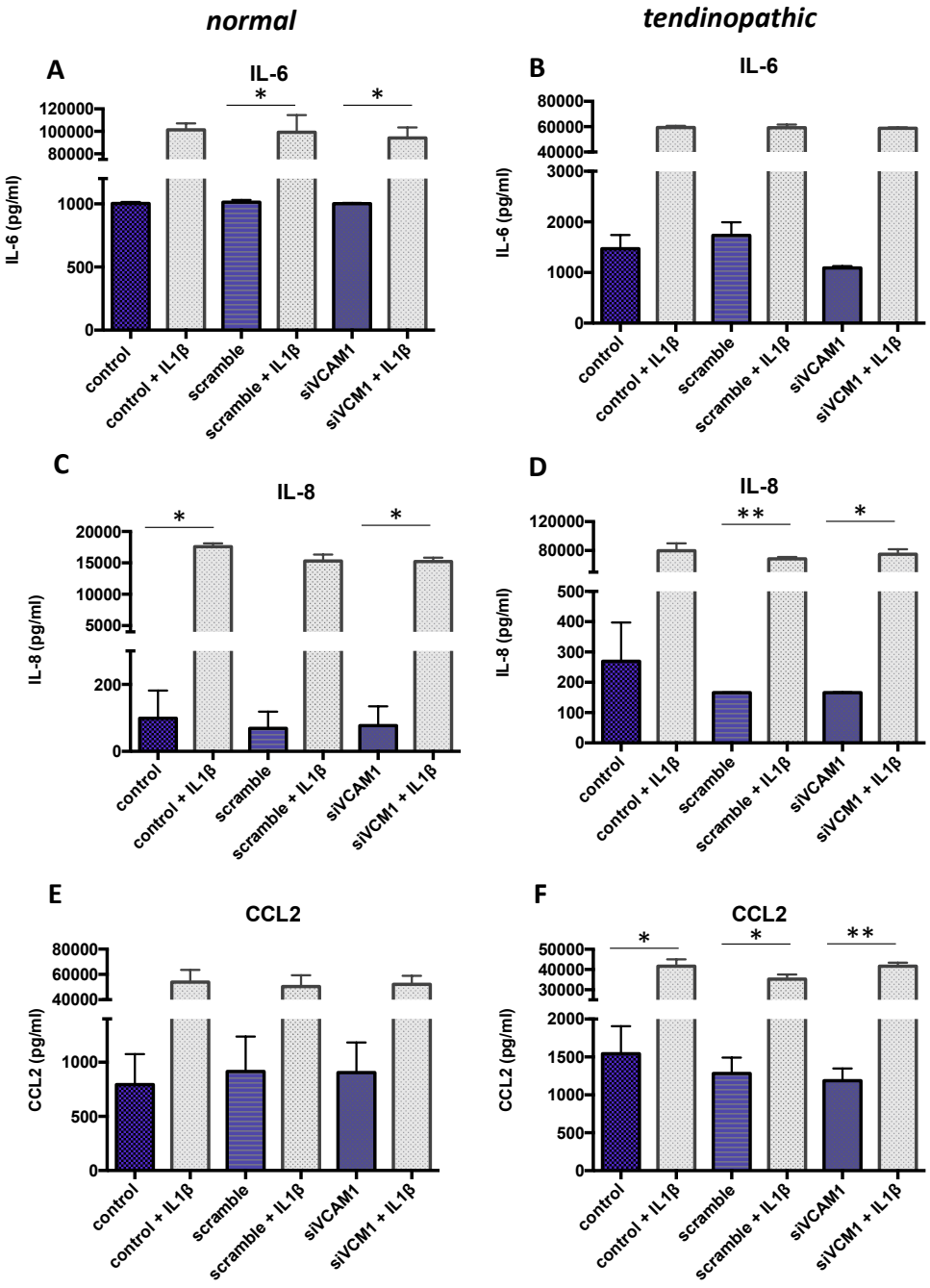

Figure 5.5 Cytokine release from tenocytes with VCAM1 knockdown
IL-6, IL-8 and CCL2 release from **(A, C, E)** normal tenocytes **(B, D, F)** tendinopathic tenocytes left untransfected (control), transfected with scramble control and transfected with VCAM1 siRNA in the presence or absence of 10ng/ml IL-β stimulation. * p<0.05, **p<0.01, ***p<0.001 (One way ANOVA with Tukey's multiple comparisons test) All data represent mean ± SEM, n=4.

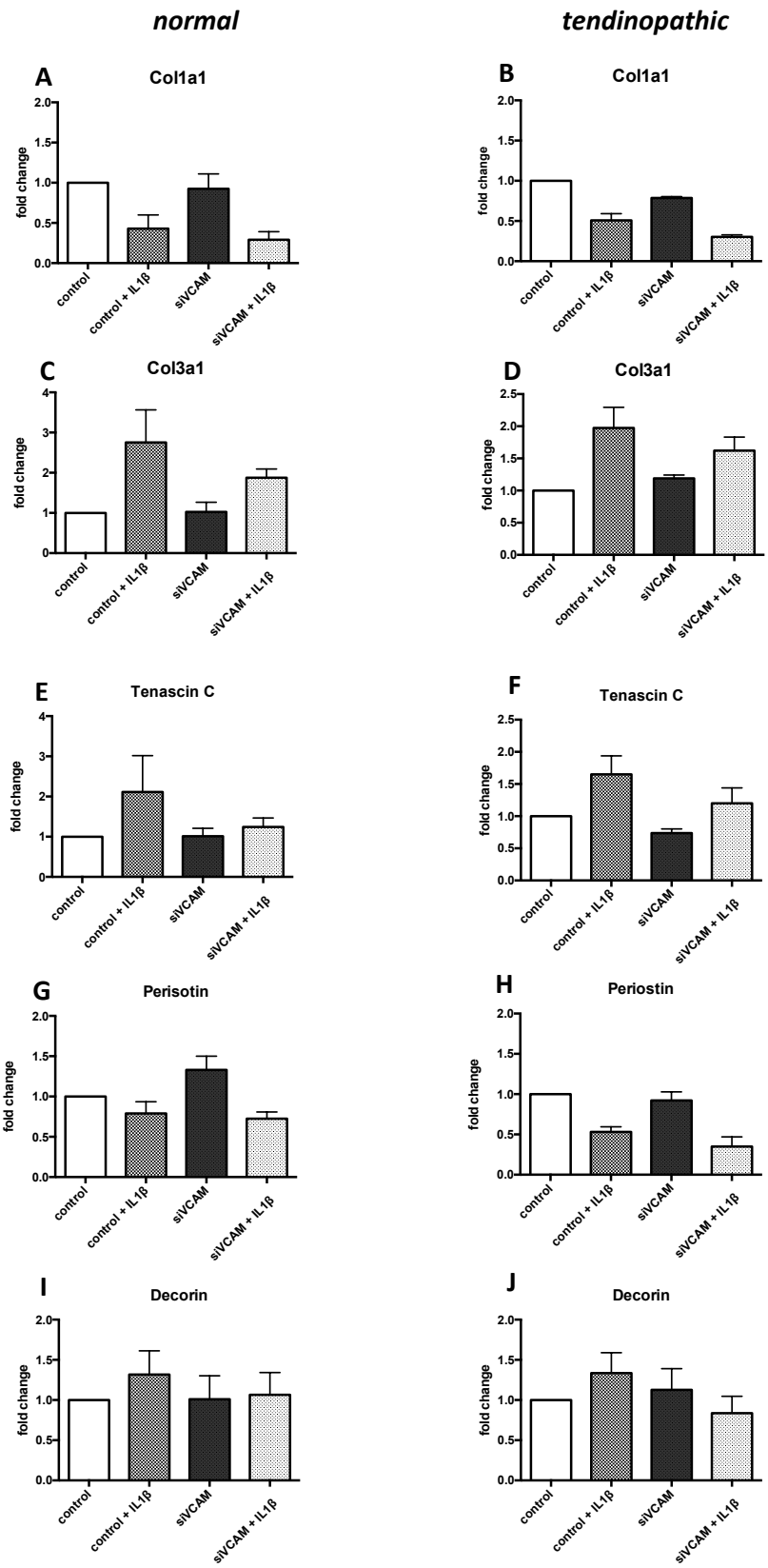

Fig. 5.6 VCAM1 knockdown does not directly alter matrix regulation by tenocytes
(A & B) Col1α1 **(C & D)** Col3α1 **(E & F)** tenascin C **(G & H)** periostin **(I & J)** decorin expression in normal or tendinopathic tenocytes transfected with scramble control or VCAM1 siRNA in the presence or absence of 10ng/ml IL-1β stimulation. All data are normalised to 18S housekeeping gene and expressed as fold change relative to scramble control, data represent mean ± SEM, n=3.

5.2.3 Macrophages derived from tendinopathic tissue express markers associated with a pro-resolving phenotype

To fulfil our aim to assess the effect of tenocyte-monocyte co-culture on monocyte phenotype and differentiation into mature macrophages we next undertook phenotyping studies of macrophages extracted from tendinopathic tendon tissue by enzymatic digestion. We chose a panel of markers associated with a pro-resolving or 'M2' like phenotype based on the current literature. **Figure 5.7A** shows the percentage of $CD45^+$ and $CD64^+$ cells isolated from tendon tissue measured by flow cytometry. We found approximately 6% of singlets were $CD45^+$ (haematopoietic marker representing immune cells) and of these, 4% were also $CD64^+$ indicating the presence of mature macrophages. We stained cells for the presence of CD206, CD163, MERTK and HLA-DR (MHC-II). **Figure 5.7B** shows the expression of these markers expressed as percentage of macrophages ($CD64^+$). We observed high levels of expression of CD206 in all donors ranging from 70% to around 100% (illustrated in **Fig.5.7C**). Similarly, MHC-II expression was maximal with all donors displaying almost 100% positive expression. Conversely, CD163 and MERTK show a different pattern of expression with expression not detected in three out of four donors in each case (illustrated in **Fig 5.7 D & E**). The remaining donor exhibited high levels of expression of CD163 and MERTK (approximately 60% positive) indicating the expression profile may be donor specific. We postulate this may be attributed to the stage at which the samples were taken and may be relative to the progression of pathology. As such, we decided to proceed with all markers tested in our *in vitro* co-culture model.

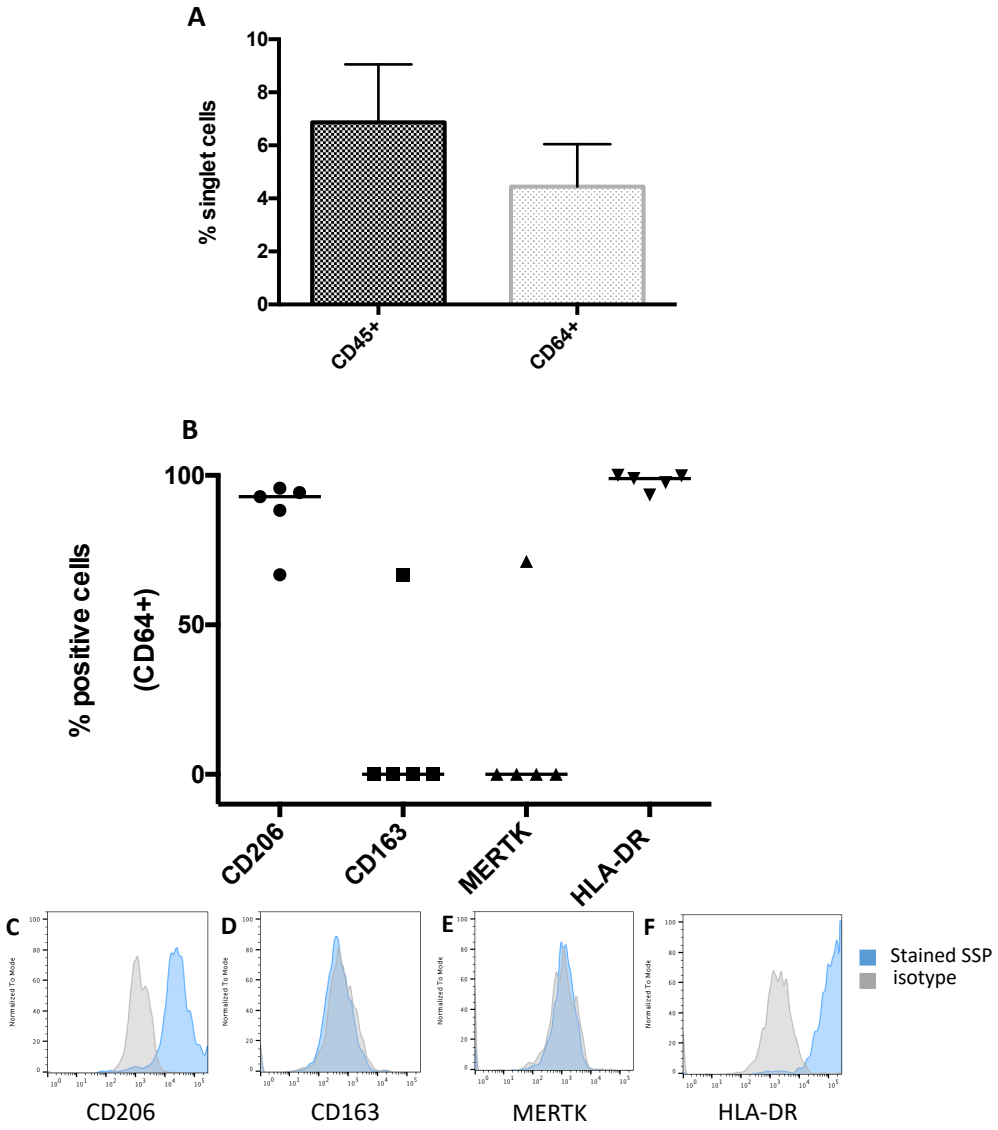

Figure 5.7 Macrophage marker expression in tendinopathic tissue

(A) Percentage of $CD45^+$ and $CD64^+$ cells in torn supraspinatus (late tendinopathy) tendon tissue expressed and percentage of singlet cells. Data represent mean ± SEM, n=4. **(B)** Expression of CD206, CD163, MERTK and HLA-DR in tendinopathic tendon (expressed as percentage positive of $CD64^+$ cells). Data expressed as median, n=4. **(C-F)** Representative histograms of CD206, CD163, MERTK and HLA-DR expression in macrophages from tendinopathic tendon.

5.2.4 Direct monocyte-tenocyte co-culture induces monocyte development and a pro-resolving phenotype

To assess the effect of the tenocyte-monocyte interactions on monocyte phenotype we first performed direct co-cultures to allow for contact dependent signalling. We performed parallel cultures using soluble factors IL-6 and CCL2 (identified in Chapter 3 as possible drivers of monocyte development) and tenocyte conditioned supernatant. M-CSF and GM-CSF were used as controls for *in vitro* monocyte differentiation.

We found direct co-culture resulted in an approximate 40% increase in CD206 expression. CD206 expression also increased in response to tenocyte conditioned supernatant and IL-6, CCL2, and IL-6 and CCL2 together; however, the response was slightly less marked than the co-culture (**Fig. 5.8B & C**). Basal CD163 expression was the lowest of all surface markers at 10% and increased to approximately 60% in the direct co-culture. The effect of IL-6, CCL2 and tenocyte conditioned supernatant was not as marked although slight increases in expression were still observed (Fig. 5.8D & E). Similarly, MERTK expression was relatively low in the monocyte only control and induced in the co-cultures to around 80% positive expression. More discreet increases were observed in response to tenocyte conditioned supernatant, IL-6 and CCL2 (**Fig. 5.8F & G**). In contrast, HLA-DR expression was high under control conditions and increased very slightly to almost 100% positive in all other conditions tested (**Fig. 5.8H & I**).

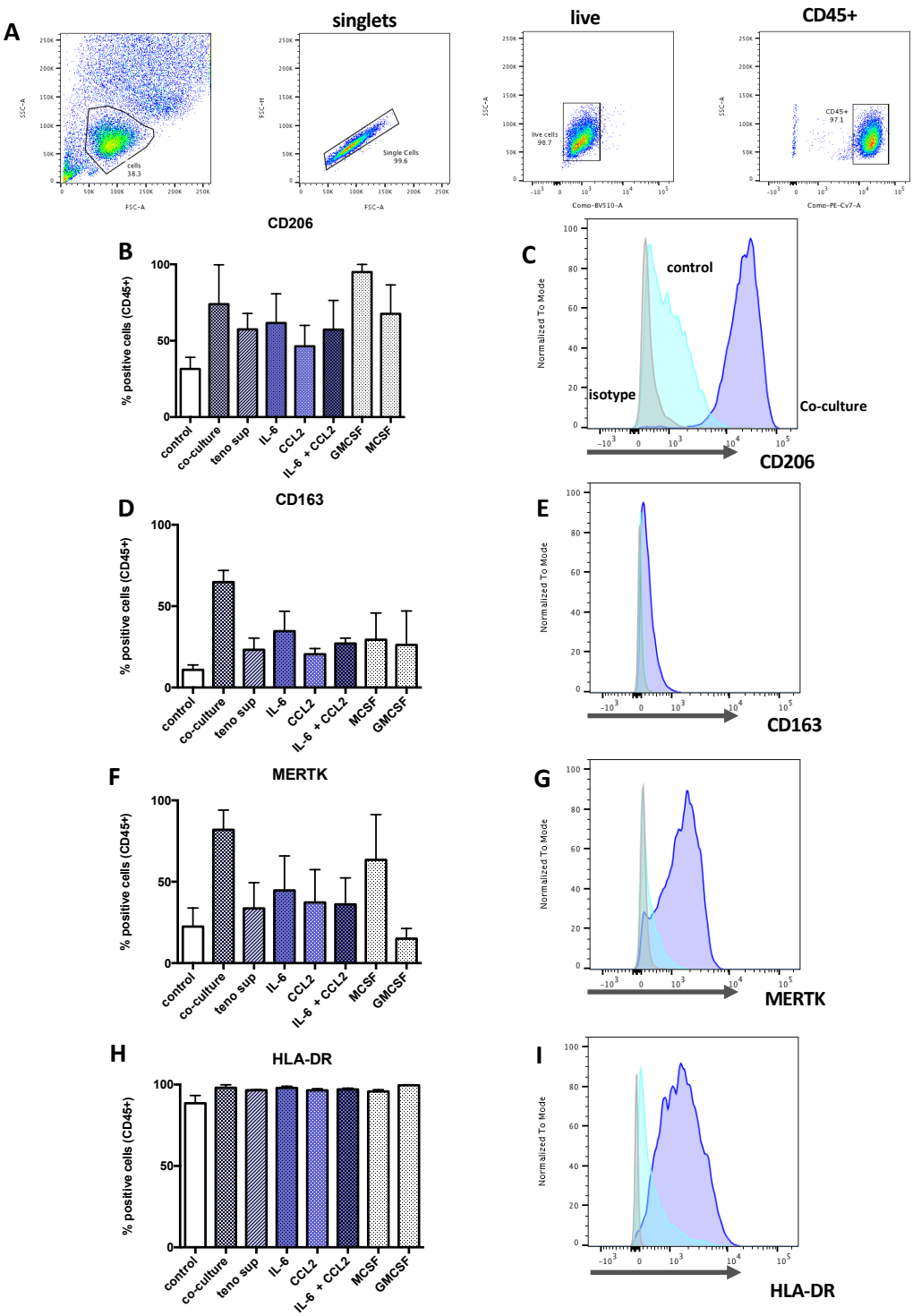

Figure 5.8 Tenocyte-monocyte co-culture influences monocyte phenotype
(A) Gating strategy for isolating monocytes from co-culture. **(B)** CD206 **(D)** CD164 **(F)** MERTK **(H)** HLA-DR expression in control monocytes, monocytes from direct tenocyte-monocyte co-culture, monocytes stimulated with 50% tenocyte conditioned supernatant, 100ng/ml IL-6, 100ng/ml CCL2, 100ng/ml IL6 + CCL2, 25ng/ml MCSF or 100 ng/ml GMCSF. All data represent mean ± SEM, n=4. **(C)** Representative histogram of CD206 **(E)** CD163 **(G)** MERTK **(I)** HLA-DR surface expression in control $CD14^+$ monocytes and monocytes co-cultured with tenocytes relative to isotype control.

5.2.5 Induction of MMP3 expression is contact dependent

Having observed upregulation of expression of CD206, CD163 and MERTK on monocytes in response to direct tenocyte-monocyte co-culture we tested the hypothesis that this response is contact dependent. We performed parallel direct and transwell co-cultures to exclude contact and retain soluble factors in the culture system. We previously observed that unstimulated tenocytes to do not express MMP3 protein (Chapter 3: **Fig. 3.7**) and studies have shown that monocytes/macrophages express MMP3 in response to stimulation[455] thus we used this as a measure of tenocyte-monocyte interaction. As anticipated, we found expression was absent in both tenocyte and monocyte controls as well as the transwell co-culture. Conversely, we found induction of MMP3 expression in the direct tenocyte-monocyte co-culture. This suggests there is essential surface receptor-ligand engagement taking place in this co-culture model.

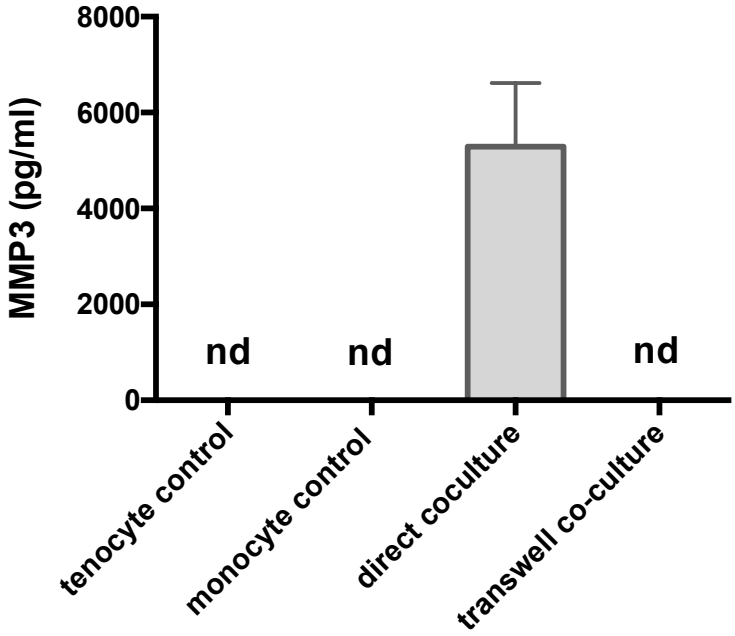

Figure 5.9 MMP3 expression is induced by monocyte-tenocyte contact
MMP3 release from tenocytes and monocytes in direct or transwell co-culture system. Data represent mean ± SEM, n=3.

5.2.6 Podoplanin and VCAM1 knockdown in tenocyte-monocyte co-culture model does not affect monocyte phenotype

Having established a contact dependent tenocyte-monocyte co-culture model that induces the expression of macrophage associated genes we next sought to determine the effect of podoplanin and VCAM1 knockdown on monocyte phenotype.

Podoplanin was knocked down in normal and tendinopathic tenocytes and the expression of surface proteins on monocytes was measured by flow cytometry. We observed no alterations in CD206 (**Fig. 5.10A & B**), CD163 (**Fig. 5.10C & D**) or HLA-DR (**Fig. 5.10G & H**) expression in monocytes subject to direct co-culture with normal or tendinopathic tenocytes with podoplanin knockdown. We observed a slight decrease in MERTK expression in the tendinopathic co-culture compared with normal tenocytes. However, there were no differences between control and podoplanin knockdown cultures (**Fig. 5.10E & F**).

Similarly, we found no apparent change in expression of CD206 (**Fig. 5.11A & B**), CD163 (**Fig. 5.11C & D**), MERTK (Fig. 5.11E & F) or HLA-DR (**Fig. 5.11G & H**) in tenocyte-monocyte co-cultures subject to VCAM1 knockdown in both normal and tendinopathic tenocytes.

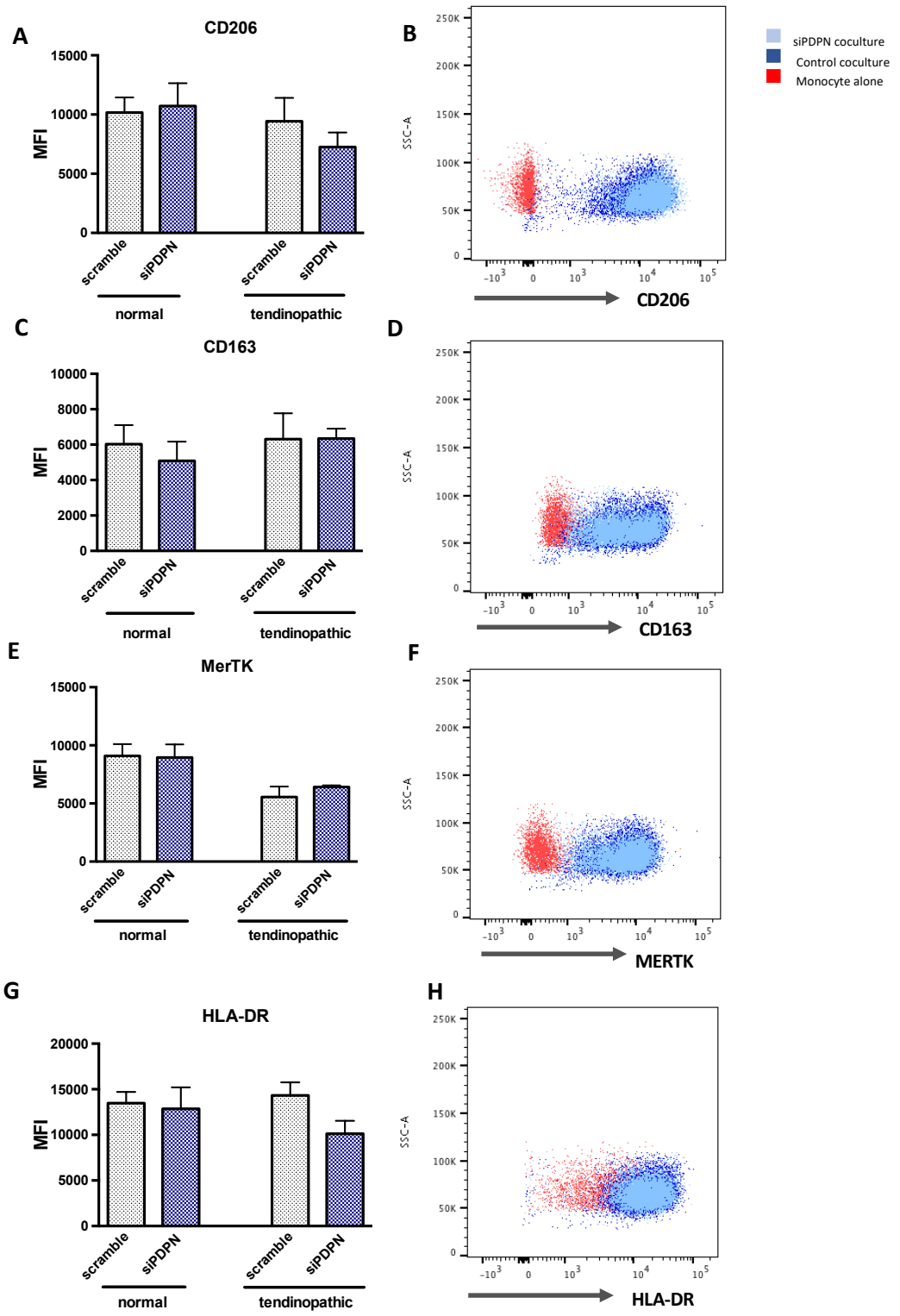

Figure 5.10 Podoplanin knockdown in tenocyte-monocyte co-culture model does not affect monocyte phenotype
(A) CD206 **(C)** CD163 **(E)** MERTK **(G)** HLA-DR expression in monocytes from direct monocyte-tenocyte co-culture expressed as median fluorescence intensity (MFI). All data represent mean ± SEM, n=4. Representative overlay dot plots of **(B)** CD206 **(D)** CD163 **(F)** MERTK **(H)** HLA-DR expression in monocytes from monocyte-tenocyte co-culture.

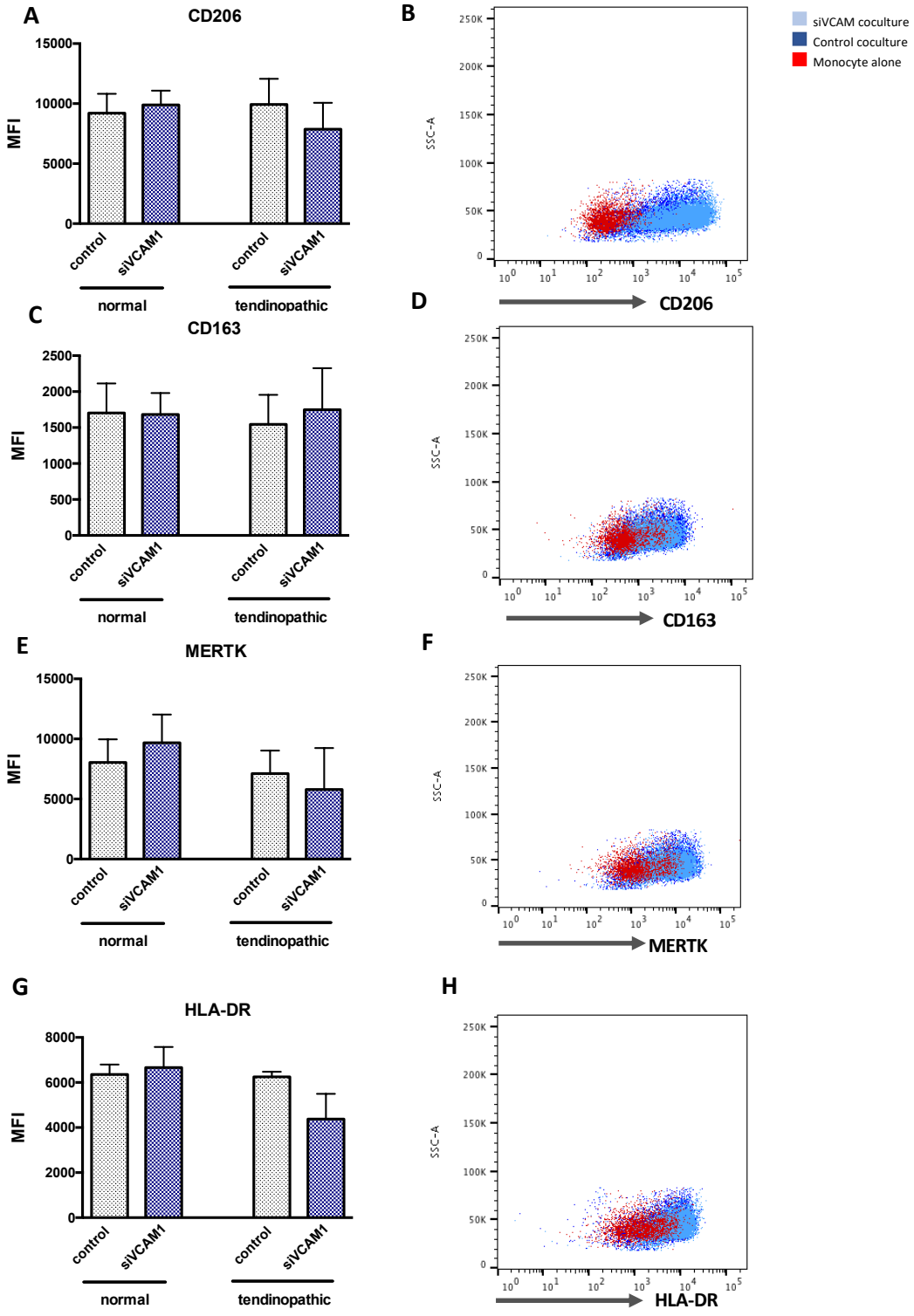

Figure 5.11 VCAM1 knockdown in tenocyte-monocyte co-culture model does not affect monocyte phenotype
(A) CD206 **(C)** CD163 **(E)** MERTK **(G)** HLA-DR expression in monocytes from direct monocyte-tenocyte co-culture expressed as median fluorescence intensity (MFI). All data represent mean ± SEM, n=4. Representative overlay dot plots of **(B)** CD206 **(D)** CD163 **(F)** MERTK **(H)** HLA-DR expression in monocytes from monocyte-tenocyte co-culture.

5.3 Discussion

This chapter sought to assess the functional properties of stromal activation markers in a human model of tendinopathy and explore mechanisms through which stromal and immune cells interact. Our findings suggest that increased expression of stromal activation markers does not direct any discernible phenotypical changes in tenocytes or alter their intrinsic properties. We observed expression of several 'pro-resolving' macrophage markers in macrophages extracted from tendinopathic tissue and found naïve monocytes respond to stimuli generated by tenocytes in a contact dependent manner. We did not, however, observe any alterations in myeloid cell phenotype in response to knockdown of stromal surface markers podoplanin and VCAM1 suggesting alternate communication mechanisms may be implicated in these tenocyte-monocyte interactions.

Phenotyping studies of macrophages isolated from fresh supraspinatus (tendinopathic) tendon revealed expression of several genes associated with a pro-resolving or 'M2' like phenotype. CD206 is the prototypic marker of this phenotype and is described in the literature alongside many combinations of markers that represent a spectrum of 'activation' or 'differentiation' states.[404] We found expression of CD206 and HLA-DR (MHC-II) was ubiquitous in all of our samples; however MERTK AND CD163 were concomitantly expressed in only one donor. CD163 expression is exclusive to monocyte/macrophage lineage and is commonly linked with systemic inflammatory disorders.[456] MERTK is associated with increased phagocytic capacity of macrophages, reduced inflammation and transition towards a homeostatic environment in a number of pathologies.[457] It has been postulated that macrophages expressing CD206, MERTK and CD163 represent a distinct sub-population of 'M2' macrophages that exhibit enhanced phagocytic capacity and 'anti-inflammatory' properties.[458] In the context of tendinopathy, co-expression of these surface markers may represent an advanced state of activation exclusive to late stage pathology.

In addition to 'alternatively activated' macrophages, CD206 and HLA-DR are expressed on tissue macrophages. These may represent an expanded pool of macrophages of tissue origin that cannot be segregated from infiltrating cells based on our gating on macrophage marker CD64. To further explore macrophage

phenotype in tendinopathic tissue, future studies would benefit from increased sample size along with multiple patient cohorts based on stage of disease progression.

We established an *in vitro* model of direct tenocyte-monocyte interaction and found induction of contact dependent expression of surface markers associated with a 'pro-resolving' macrophage phenotype (**Fig. 5.12A**). This indicates that, within the tendon microenvironment, it is likely tenocytes that are driving monocyte differentiation and maturation. *In vitro* we found expression of CD206, CD163, MERTK and HLA-DR in all samples indicating tenocytes retain the capacity to induce expression of these genes under certain conditions. As postulated in Chapter 3, we found that IL-6 and CCL2 are capable of driving monocyte differentiation into macrophages and also influence their phenotype. The response of monocytes to soluble factors—including tenocyte conditioned supernatant—was more modest and less consistent than direct co-culture indicating a contact dependent mechanism or a combination of stimuli is necessary to illicit a complete response. We performed parallel transwell co-cultures to remove cell surface contact from the system and used MMP3 expression as a measure of cell activation as neither tenocytes or monocytes/macrophages express MMP3 under homeostatic conditions. MMP3 was only detected in the supernatant of the direct co-cultures indicating there is surface receptor engagement acting as an activating stimulus. However; as this is a heterogeneous culture it is not possible to discern which cell type is the source of this protein or if both populations are active in secretion.

Our phenotyping studies in the previous chapter highlighted the expression of several 'activation markers' in tenocytes and tendinopathic tissue thus we sought to explore their function in relation to tendon pathology. Previous studies have defined the activation state of tenocytes based on surface protein expression including podoplanin and VCAM1 and have proposed they are primed to respond to inflammatory stimuli.[418] However, based on previous published works by ourselves and others we believe that the activation state of tenocytes is more appropriately reflected by relative expression of inflammatory mediators and matrix proteins.[162,219,459,197,220] siRNA mediated knockdown of podoplanin or VCAM1 did not have any marked effect on release of cytokines or expression of matrix

proteins in normal or diseased tenocytes. Furthermore, tenocytes subject to knockdown did not display any altered response to IL-1β stimulation *in vitro*. This conflicts with the notion that tenocytes expressing specific surface markers are primed to respond to inflammation as tenocytes lacking expression of these surface markers would, in theory, be less responsive.

We applied podoplanin and VCAM1 knockdown to our co-culture model to test the hypothesis that knockdown of surface proteins upregulated in tendinopathy would diminish cell surface interactions and inhibit monocyte differentiation. However, reduced expression of podoplanin and VCAM1 in tenocytes did not have a deleterious effect on expression of macrophage associated genes indicating alternative signalling mechanisms are responsible for inducing these phenotypic changes. Based on our observations, it is evident there are some theoretical and practical challenges to consider when drawing conclusions from our data. In addition to podoplanin and VCAM1, the previous chapter identified upregulation of CD44 and CD90 in tenocytes from tendinopathic tissue and found co-expression of these proteins in tenocyte subpopulations. It may be plausible that knockdown of one surface protein is insufficient to evoke a response; therefore, simultaneous expression of multiple proteins may be required to alter the properties or responsiveness of the cell (**Fig. 5.12B**).

The theory surrounding stromal activation in musculoskeletal disease has been reviewed several times[419,460,461] and differential expression of surface proteins has been described in RA, tendinopathy and adhesive capsulitis.[364,448,212,418,436,462] However, to date, there has been a lack of experimental evidence to convey its biological significance. The phenomenon of 'fibroblast activation' was first described in cancer associated fibroblasts (CAFs) and culminated in the designation of a cell surface glycoprotein as Fibroblast Activation Protein (FAP).[320,321] FAP and other 'activation' proteins are generally absent from the stroma of normal tissues which suggests they play a role in disease pathology.[322] For example, in solid cancers CAFs expressing activation markers establish a strong relationship with cancer cells to support growth, motility and invasion.[463] However, the role of stromal cells in soft tissue disease is less well defined and there is still ambiguity regarding the role they play in immune cell recruitment, inflammation and matrix remodelling. Consequently, in the present context, the

term ‘stromal activation’ should be used with caution to avoid overrepresenting a lesser understood phenomenon.

It has been postulated that populations of fibroblasts displaying increased expression of certain surface proteins may be a result of an expansion of an existing fibroblast subpopulation.[464] Our data from Chapter 4 supports this theory as we reported increased frequency of some tenocyte subpopulations in diseased tissue. These populations were largely defined by their expression of podoplanin, cadherin 11 and CD90, all of which are considered markers of pathogenic stroma or fibroblast activation. Insight from transcriptomic studies in RA synovial fibroblasts has revealed some of the functional properties of these subsets. Mizgouchi *et al* found enrichment of genes associated with fibroblast migration, osteoclastogenesis and an enhanced secretory phenotype characterized by expression of IL-6, CXCL12 and CCL2.[315] Subsequent studies utilizing single cell transcriptomics and mass cytometry identified four fibroblast subpopulations with differential expression in leukocyte rich RA, leukocyte poor RA and OA. In leukocyte rich RA (representative a highly inflammatory environment) sub-lining specific subsets showed enriched expression of genes associated with regulation of leukocyte migration, regulation of the inflammatory response, NFκB signalling and IL-6 production.[318]. IL-6 expression in tendinopathy is well characterised[191,192] and NFκB signalling has recently been identified as a potential therapeutic target in tendon disease indicating that gene expression profiles of tenocyte subpopulations may be key to understanding mechanisms that underlie pathology.[392]

To date, studies involving fibroblast subpopulations have been largely descriptive and it has been acknowledged that further studies are required to explore molecular mechanisms that regulate expansion of fibroblast populations.[318] In the context of the present study it is evident the use of transcriptomics would advance our understanding of pathways that may regulate surface marker expression and potential expansion of tenocyte subpopulations in disease. However, it is pivotal that these studies are succeeded by molecular characterization of signalling pathways involved to inform therapeutic translation.

Based on our observations we propose that stromal-immune cell interactions rely on a combination of juxtacrine and paracrine signalling mechanisms to regulate the inflammatory microenvironment in tendinopathy. Furthermore, we believe that increased expression of stromal cell surface markers in tendinopathy may reflect expansion of existing tenocyte subpopulations that retain potential to modulate the inflammatory response under pathological conditions.

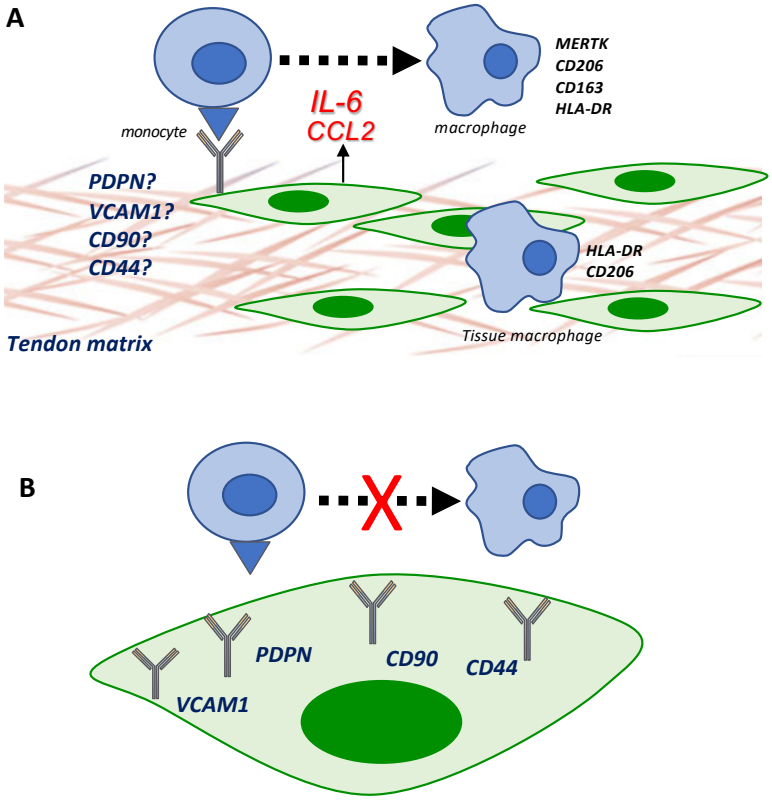

Figure 5.12 Tenocyte monocyte interactions within the tendon matrix
(A) Surface interactions between monocytes and tenocytes and soluble factors including IL-6 and CCL2 induce the expression of MERTK, CD206, CD163 and HLA-DR in monocyte-derived macrophages **(B)** Illustrates the hypothesis that knockdown of multiple surface proteins may abrogate contact dependent signaling required for monocyte maturation.

Chapter 6: Discussion and future directions

The immune landscape in tendinopathy is diverse and host to several functionally and phenotypically distinct populations of cells. Interactions between these cells are mediated by complex signalling pathways that are likely activated sequentially to co-ordinate the immune response. Resident cells within the stroma (tenocytes and tissue macrophages) perform homeostatic functions whilst also acting as immune sentinels capable of responding to infection or injury. As tendinopathy is a damage-associated pathology the latter represents the predominant precipitating factor in the development of tendinopathy. This thesis identified a novel mechanism through which tenocytes respond to injury by inducing release of myeloid-associated alarmins S100A8 and S100A9 from monocytes. In the extracellular space these alarmin molecules promote the release of cytokines and chemokines from tenocytes but do not appear to affect matrix production. Of particular interest in the context of tendinopathy is the repertoire of cytokines expressed by tenocytes. It is well documented that IL-8, IL-6 & CCL2 are factors responsible for mediating the influx of immune cells following activation of the innate immune system and sequential accumulation of immune cells has been described in tendinopathy.[465,187,239] IL-8 induces the recruitment of neutrophils within minutes of injury while, over a period of hours and days IL-6 and CCL2 expression promote recruitment of mononuclear cells. True chronic inflammation occurs when physiological inflammation fails to resolve. It is unclear the point at which this occurs; however, it may be postulated that sustained activation of tenocytes and excessive production of IL-6 and CCL2 perpetuates inflammation (outlined in **Fig.6.1**).

Targeting cytokines—such as IL-6—that mediate the transition from acute to chronic inflammation represents a viable and logical therapeutic approach in tendinopathy. Cytokine blockade has been highly successful in RA and has recently been approved for treatment of spondyloarthropathy, psoriasis and inflammatory bowel disease.[466,467,468] Tendinopathy presents as local inflammation confined to connective tissue which may limit the translation of agents designed to treat systemic inflammation. However, thus far pilot studies have utilised techniques of local administration which may be sufficient to avoid unwarranted side effects disproportionate to the burden of disease associated with tendinopathy.

Dampening the 'activation' of tenocytes in this context refers to the heightened expression of inflammatory mediators and matrix components; however, the term 'activation' is also associated with phenotypic alterations observed in fibroblasts under chronic inflammatory conditions. I investigated this phenomenon and identified upregulation of several surface proteins in tendinopathic tissue, most notably CD90, CD44, VCAM1 and prototypic 'activation markers' podoplanin and FAP. The concept of fibroblast activation is not new, originating in the late 1990s.[302] However, recent interest in the role of stromal cells in chronic inflammatory pathologies have focused largely on this fibroblast 'activation' phenotype with few accompanying functional studies. To address this, I undertook siRNA mediated knockdown of podoplanin and VCAM1 and assessed the effect on production of inflammatory mediators and matrix components. These experiments did not identify any alterations in either which does not align with my previous findings of 'activated' tenocytes releasing increased levels of cytokines and chemokines.

The first observation of activation of fibroblasts and their participation in the innate immune response was described in the rheumatoid synovium where engagement of TLR ligands on the cell surface resulted in the release of chemokines.[302,301] This is in line with my observation of tenocytes taking on an 'activated' phenotype upon stimulation with S100A8 & A9. It should be noted that this form of activation occurs under acute inflammatory conditions upon initiation of the innate immune response. This is in contrast with observations of activation in late-stage pathology in tendinopathy described in this thesis and by others.[418] It may be postulated one state represents 'acute activation' while the latter describes a more chronic form of activation. It has been suggested that epigenetic alterations are responsible for the persistence of stromal activation once the inflammatory stimulus has been removed which leads to the development of the concept of 'stromal memory'.[431,469,432] It is plausible that sustained 'acute activation' induces these epigenetic changes that are responsible for the imprinting of the phenotype associated with 'chronic activation'.

Phenotyping studies illustrated variations in surface marker expression in healthy and diseased tendon and we identified distinct 'subpopulations' of tenocytes expanded under pathological conditions. The concepts of fibroblast activation and

heterogeneity are inextricably linked, and it is possible they represent a 'cause and effect' phenomenon whereby fibroblasts activated by inflammatory/immune stimuli in the early stages of disease give rise to distinct sub-populations present in established pathology. To gain further insight into the properties of tenocyte subsets identified in normal and tendinopathic tissue, cell sorting based on their surface protein expression (e.g. $CD90^{+}PDPN^{+}CAD11^{+}$) and subsequent *in vitro* functional studies would address questions relating to functional differences and pathogenic potential of fibroblast subsets. Functional studies would include cytokine/chemokine profiling, measuring matrix expression and leukocyte migration studies. However, it should be noted that removing cells from their tissue environment may result in acquisition or loss of crucial characteristics that could potentially skew results.

Recent studies have employed the use of transcriptomics and mass cytometry to gain insight into the properties of fibroblasts displaying different phenotypes. One study identified a $CD34^{+}$ fibroblast subset characterized by increased expression of IL-6, CXCL12 and CCL2 and enhanced leukocyte recruitment while another mapped IL-6 expression to a $CD90^{+}$HLA-DRA^{hi} population of sublining fibroblasts expanded in RA.[315,318] Going forward, a bioinformatic approach utilising an overlay analysis of bulk RNA-seq on sorted populations and unbiased single cell RNA-seq on tenocytes would provide detailed information on differential gene expression in health and disease. Furthermore, gene clustering analysis would act to clarify tenocyte sub-populations identified in this thesis and functional studies to validate transcriptomic data would be required as a critical step in the correct utilisation of these technologies.

The primary aim of this PhD project was to assess the effect of interactions between stromal cells, namely tenocytes, and immune cells. Chapter 3 addressed this by identifying interactions occurring as a result of soluble factors and receptor mediated signalling in the tendon microenvironment. Conversely, Chapter 5 addressed contact dependent interactions between tenocytes and monocytes in the form of direct co-culture experiments. These interactions had a notable effect on monocyte phenotype by inducing expression of several macrophage associated markers. CD206, CD163, MerTK and HLA-DR are associated with a 'pro-resolving' phenotype which indicates these interactions may be beneficial to healing.

However, the presence of these macrophages has also been noted in chronic inflammatory pathologies and fibrotic disorders suggesting tenocyte-monocyte interactions may become dysregulated somewhere in the transition from acute to chronic inflammation. It would also be interesting to assess the reciprocal effect of this interaction on tenocyte phenotype by measuring expression of our panel of surface markers.

To assess the effect of 'activation markers' podoplanin and VCAM1 on tenocyte-monocyte interactions I performed co-culture experiments with siRNA mediated knockdown of these proteins. I did not observe any discernible changes in our panel of macrophage associated markers. The heterogenous nature of the culture system limits the number of experiments that can be performed (with focus on a single cell type) thus I intend to sort the $CD14^{+}$ monocytes from the tenocytes and perform bulk RNA-seq on all experimental conditions. As the literature surrounding monocyte/macrophage phenotype and activation is extremely convoluted this will allow us to concentrate on interrogating relevant signalling pathways rather than focus on potentially arbitrary phenotypic characteristics.

In summary, this thesis has identified novel stromal-immune cell interactions in tendinopathy and highlighted the importance of the interface between acute and chronic inflammation in disease progression. Defining mechanisms through which stromal cells with varying expression profiles contribute to inflammation may be key to identifying the molecular switch that drives disease chronicity. On the basis of these results it is evident that stromal cells and immune cells exist in a diverse spectrum of activation states determined by niche specific environmental cues. Further characterization of discreet subpopulations of cells that predominate under pathological conditions may aid in stratifying treatment in musculoskeletal disease and other pathologies associated with chronic inflammation.

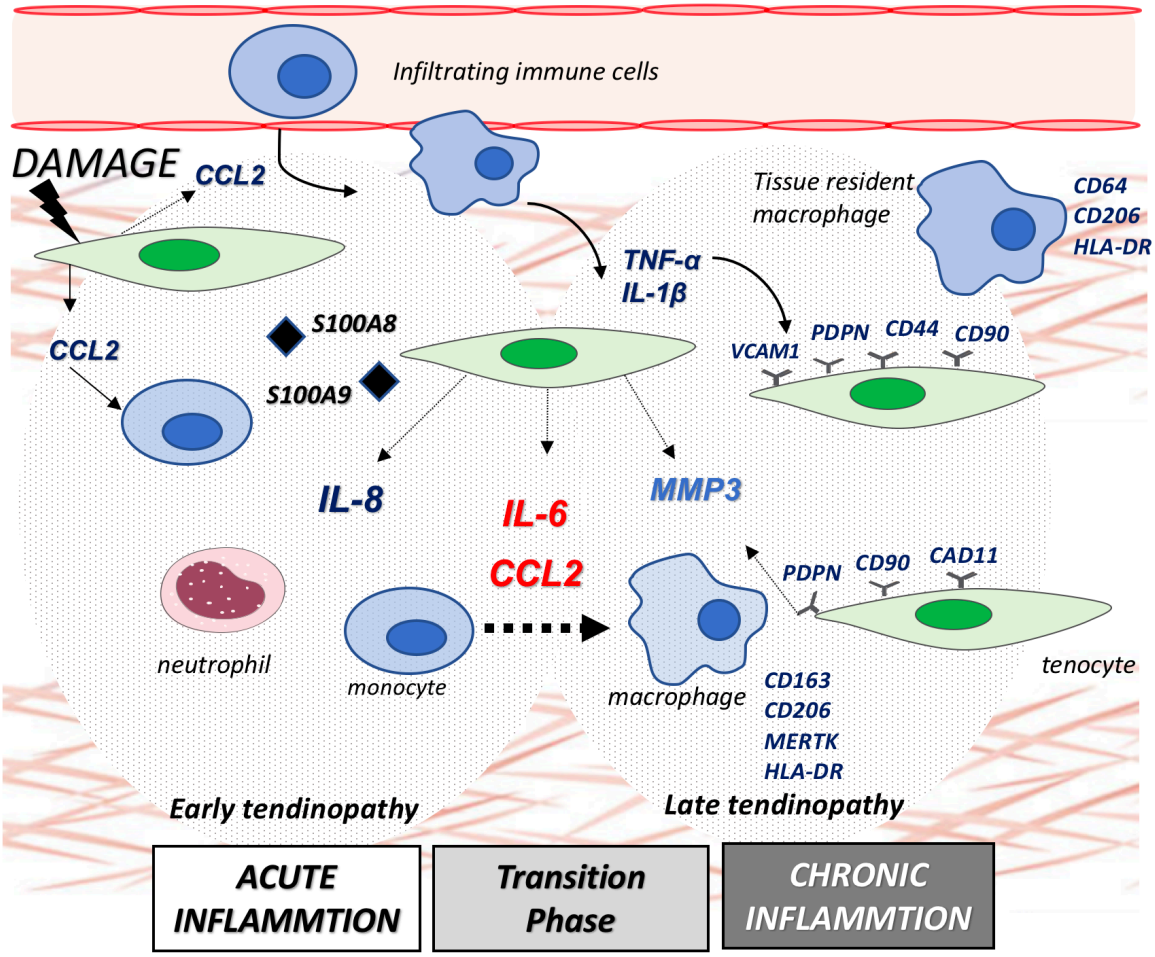

Figure 6.1 Summary of immune-cell matrix cross talk in tendinopathy

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